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Microbial and botanical strategies to support human digestion and gut microbiota.

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INDEX

1. Abstract

The interaction between diet, digestion, and the gut microbiota has become a central topic in nutrition and health research. Growing scientific and commercial interest has focused on functional foods and supplements that aim to support digestion, influence microbial communities, and provide health benefits without disrupting ecological balance. Developing products that are both effective and microbiome-compatible requires experimental models capable of capturing physiologically relevant conditions.

This PhD project examined three categories of microbiome-related interventions: (1) spore-forming probiotics, (2) fungal enzyme supplements, and (3) botanical extracts. A central methodological approach was the application of standardized in vitro models, in particular the INFOGEST 2.0 protocol, which simulates the human gastrointestinal environment and provides reproducible digestion studies. These experiments were complemented by fecal microbiota incubations and targeted microbial cultures to assess viability, metabolic activity, and community responses. The overarching aim was to evaluate how specific products, including *Heyndrickxia coagulans* LMG S-24828, two fungal enzyme blends, and three botanical extracts, interact with gut-related processes. Emphasis was placed on safety, functional performance during digestion, and effects on selected commensal or probiotic strains. The findings contribute to the development of evidence-based strategies for improving digestive efficiency and maintaining microbial balance through microbiome-compatible supplements.

2. Introduction

2.1. The human gut microbiota: structure, diversity, and function

The gut microbiota is the most diverse and densely colonized microbial ecosystem in the human body, and its influence on health and disease is profound (Karkman et al., 2017). Its composition is shaped by birth mode, early-life feeding, lifestyle, medication, and host genetics, as demonstrated by epidemiological, physiological, and multi-omics studies (Fan & Pedersen, 2021). The community is dominated by Bacillota (formerly Firmicutes) and Bacteroidota (formerly Bacteroidetes), but also includes archaea, viruses (including bacteriophages), and eukaryotes (e.g., fungi, protozoa) (Hou et al., 2022). These microorganisms interact with each other and with the host, and while some associations may be neutral or detrimental, most are commensal or mutualistic (Culp & Goodman, 2023). Collectively, the microbiome encodes a genetic repertoire much larger than that of the host, enabling functions such as immune system development, digestion, endocrine regulation, neuro-signaling, xenobiotic metabolism, detoxification, and production of bioactive molecules (Fan & Pedersen, 2021).

Large metagenomic initiatives, such as the Human Microbiome Project and the European MetaHIT consortium, have expanded knowledge of the gut microbial diversity, demonstrating marked inter-individual variation across geography, lifestyle, and health status (Meyer et al., 2008; Qin et al., 2012; Carroll et al., 2011; Zeller et al., 2014, Dethlefsen et al., 2007). Recurring configurations, known as enterotypes, were proposed as broad stratifiers. These clusters, often enriched in *Bacteroides* or *Prevotella*, show distinct functional and ecological features (Costea et al., 2017). However, subsequent large-scale studies indicated that enterotypes may not represent discrete entities but rather gradients, with the Bacteroides-to-Prevotella distribution forming a continuum (Cheng & Ning, 2019). Although the concept simplifies microbial diversity, it remains useful for study design, disease stratification, and the development of personalized nutrition strategies.

2.2. Rise of microbiome-targeted interventions

The most established approach to modulating the gut microbiota is through probiotics ("live microorganisms that, when administered in adequate amounts, confer a health benefit on the host"; Hill et al., 2014) and prebiotics, administered either as functional foods, dietary supplements or live biotherapeutics (Cordaillat-Simmons et al., 2020). Their efficacy, however, varies considerably, is strain dependent, and effects are often transient, persisting only during administration (Ben-Moshe et al., 2018).

Most probiotics strains are not able to colonize the gut or restructure microbial communities, although such traits may be advantageous or desirable in certain therapeutic or preventive settings, but this does not infer that they cannot exert health-promoting effects. There is a great diversity of probiotic function and formulations, and their market is rapidly expanding globally. This has made it difficult for end users to distinguish high- and poor-quality products. In turn, this might threaten consumers' and healthcare provider's trust in probiotic products (Jackson et al., 2019). For example, spore-forming probiotics can survive processing and digestion, but their ability to germinate in the human gut is not well established (Perotti et al., 2024).

Beyond probiotics, environmental factors also shape the microbiota throughout adulthood, with diet emerging as the most consistent modulator of microbial taxa and functions. The impact of diet is not generalized but varies between individuals, reflecting the interaction of host physiology with unique microbial communities (Kolodziejczyk et al., 2019).

The fermentation of fibers, including β-glucans, pectins, and resistant starches, illustrates the importance of microbiota in health. These substrates are inaccessible to human enzymes and instead metabolized by microorganisms into short-chain fatty acids (SCFAs), which regulate metabolism, immunity, and gut integrity. However, differences in microbial composition mean that the same fiber can have distinct effects across individuals, highlighting the need for personalized nutrition (Deehan et al, 2017, Tannock & Liu, 2019). Large initiatives such as HMP, MetaHIT, and ELDERMET have linked microbial diversity with diet, aging, and disease, laying the foundation for targeted microbiome interventions (Yang et al., 2025; Cusack et al., 2013).

In recent years, plant- and fungi-derived compounds have attracted attention as microbiota-directed strategies. Preclinical evidence indicates that polysaccharides, flavonoids, alkaloids, and polyphenols can enrich beneficial taxa such as *Lactobacillus* (*sensu lato*) and *Bifidobacterium* while reducing pro-inflammatory species (Cheung et al., 2020). Compounds such as berberine, curcumin, and *Ganoderma lucidum* polysaccharides have been shown to restore microbial balance and modulate inflammatory pathways. Polyphenols are of particular interest because they serve as microbial substrates while exerting prebiotic-like effects and inhibiting pathogens (Hervert-Hernández & Goñi, 2011). Their microbial catabolism produces phenolic acids that contribute to systemic antioxidant, anti-inflammatory, and cardioprotective effects. Synergistic effects have been observed between polyphenols and fibers, with implications for SCFA production, barrier integrity, and chronic disease prevention (Loo et al., 2020). Studies on extracts such as cranberry and *Ganoderma lucidum*, and others confirm their potential to beneficially modulate the gut microbiota (O'Connor et al., 2019; Yao et al., 2021; Martinez-Carrera et al., 2023). Nevertheless, robust clinical validation and standardization remain essential for translation (Veiga, 2018).

2.3. Need for microbiome-compatible nutraceuticals

Although widely used, some plant-based supplements and botanical preparations still lack comprehensive safety and efficacy data. A systematic review documented adverse reactions ranging from gastrointestinal discomfort to hepatotoxicity, cardiotoxicity, and herb–drug interactions (Di Lorenzo et al., 2015). Risks often result from misidentification, contamination, adulteration, excessive dosage, or pharmacological interactions. While some effects are linked to specific bioactive compounds, such as pyrrolizidine or Ephedra alkaloids, causality often remains unclear due to incomplete data. These uncertainties highlight the need for pharmacovigilance, standardized characterization, and improved monitoring (Di Lorenzo et al., 2014).

Polyphenols represent both promise and complexity. A significant fraction escapes small intestinal absorption and undergoes microbial catabolism in the colon, generating metabolites with antioxidant, anti-inflammatory, and cardioprotective effects (Cardona et al., 2013; Ozdal et al., 2016). At the same time, polyphenols modulate microbiota composition, stimulating beneficial taxa while inhibiting pathogens. This dual role makes them key candidates for personalized nutrition, although inter-individual variation continues to complicate predictions.

Polyphenols and other bioactives also exert antimicrobial effects by disrupting microbial membranes, inhibiting nucleic acid synthesis, modulating oxidative stress, and interfering with quorum sensing (Mandal et al., 2024; Cowan, 1999). Some compounds, including catechins, have found applications beyond nutrition, such as in cosmetics and oral care, where they reduce biofilm formation and pathogenic species (Mita et al., 2025; Visan et al., 2024). However, antimicrobial activity is often not selective, and evidence suggests that such compounds can also reduce beneficial taxa like *Bifidobacterium* and *Faecalibacterium prausnitzii* (Plamada and Vodnar, 2021; Cao et al., 2024), thereby promoting dysbiosis associated with obesity, type 2 diabetes, inflammatory bowel disease, and metabolic dysfunction-associated steatotic liver disease (MASLD) (Zhang et al., 2024; Dahal et al., 2023). Despite these risks, the microbiome-related impact of botanicals is rarely considered in safety assessments. Moreover, defining what constitutes a “beneficial” or “adverse” microbiota response remains challenging, as microbial composition varies widely among individuals, and the functional implications of many taxonomic shifts are still poorly understood. Also, negative changes in microbiota composition may not simply reflect an increase in pathogens but can also include a reduction in beneficial taxa, the proliferation of pathobionts, or a decrease in microbial diversity. Designing appropriate studies to assess microbiome compatibility also requires careful consideration of the study population, whether healthy subjects or target consumer groups, and the balance between in vitro and in vivo approaches, given their respective advantages and limitations. Addressing these questions offers an opportunity to develop nutraceuticals that are not only effective and standardized but also compatible with the gut microbiome.

2.4. Scientific and Technological Challenges

Understanding how foods, nutraceuticals, and bioactives influence human health requires models that capture the complexity of digestion and gut microbial metabolism. Traditional *in vivo* studies in humans and animals provide essential physiological context, yet they are expensive, time consuming, ethically constrained, and often limited in mechanistic resolution for early-stage screening. These constraints have driven the development and wide adoption of *in vitro* digestion and fermentation systems as complementary approaches (Payne et al., 2012; Veintimilla-Gozalbo et al., 2021; Nissen et al., 2020, Brodkorb et al., 2019).

Static digestion models remain popular because they are accessible, reproducible, and relatively low cost. They use fixed conditions for pH, enzymes, and electrolytes, and they have generated decades of valuable data on disintegration and hydrolysis across diverse food matrices. Their main limitation is reduced physiological fidelity, since they do not reproduce time-dependent gastric emptying, peristalsis, secretion kinetics, or gradual pH shifts (Payne et al., 2012; Nissen et al., 2020). To improve comparability across laboratories, the INFOGEST consortium introduced, in 2014 and then updated in 2019, a harmonized static protocol that simulates oral, gastric, and small intestinal phases with defined enzyme activities, salts, and timings. INFOGEST has become a cornerstone for assessing bioaccessibility and matrix effects in food and nutrition research (Minekus et al., 2014; Brodkorb et al., 2019; Zhou et al., 2023). Despite these strengths, INFOGEST does not include host absorption, immune signaling, endocrine feedback, or direct host–microbe interactions, which limits prediction of *in vivo* outcomes for compounds that undergo extensive colonic transformation (Brodkorb et al., 2019; Zhou et al., 2025).

Because many dietary constituents escape small intestinal digestion, colonic fermentation is central to their metabolic fate. Microorganisms transform fibers, resistant starches, and polyphenols into short-chain fatty acids and a wide range of smaller phenolic and nitrogenous metabolites that influence epithelial function, immunity, and systemic metabolism (Payne et al., 2012; Veintimilla-Gozalbo et al., 2021). In vitro fermentation models range from straightforward batch cultures to complex, dynamic multicompartment reactors. Batch systems are well suited to high-throughput screening, require limited infrastructure, and can incorporate fecal inocula from multiple donors to explore interindividual variability. However, they typically lack active pH control (although systems capable of regulating pH, such as the MicroMatrix, are becoming more common), accumulate waste products, and do not maintain long-term stability of complex consortia, which limits their physiological relevance to short time frames (Mathur et al., 2023; Pérez-Burillo et al., 2021; Macfarlane et al., 1998). Standardized batch workflows that integrate INFOGEST digestion with fecal fermentation help bridge this gap by providing physiologically digested substrates to microbial communities, enabling a practical mouth-to-colon evaluation of foods and bioactives (Pérez-Burillo et al., 2021; Refael et al., 2022).

Dynamic continuous models overcome many of these constraints by introducing controlled flow, peristaltic mixing, region-specific pH, nutrient dosing, and, in some systems, removal of fermentation products. SHIME reproduces stomach, small intestine, and three colon regions, supports long-term cultivation, and allows region-specific readouts of microbial activity and composition (Molly et al., 1993; Van den Abbeele et al., 2010). TIM-2 focuses on the colon and uniquely removes metabolites at physiological rates, which preserves community activity and enables steady-state experiments over weeks (Minekus et al., 1999; Venema et al., 2000). SIMGI integrates a peristaltic stomach, a stirred small intestine, and three colonic reactors with region-specific pH control, allowing investigations of digestion and fermentation either jointly or separately (Barroso et al., 2015). These advanced systems have generated robust data on prebiotics, polyphenols, probiotics, and mixed diets, and they have provided insights into cross-feeding and spatial ecology that are not accessible in short batch formats (Payne et al., 2012; Van den Abbeele et al., 2010; Barroso et al., 2015). Their limitations include higher costs, technical complexity, and the lack of host tissues and immune components, although coupling effluents with epithelial or immune co-cultures can partially account for host responses (Lamers et al., 2003; Payne et al., 2012).

Polyphenols illustrate several of these challenges. Small intestinal absorption is limited, a substantial fraction reaches the colon, and the resulting microbial catabolism produces diverse phenolic acids and other metabolites with context-dependent bioactivity. Interindividual differences in microbiota composition result in distinct metabolic outputs and variable physiological effects, complicating predictions based on average *in vitro* responses (Bohn et al., 2017; Pérez-Jiménez & Saura-Calixto, 2016). Standardized digestion coupled to well-controlled fermentations improves comparability, yet translation still requires integration with pharmacokinetics, host cell responses, and, where possible, human validation.

Recent advances in metagenomics, transcriptomics, metabolomics, and stable isotope probing have increased the mechanistic resolution of *in vitro* models by linking taxa to functions and tracking flux through metabolic pathways. Computational approaches now assist with study design, data integration, and hypothesis generation from complex datasets. No single platform replicates human physiology in full. Continued progress will rely on combining standardized digestion such as INFOGEST with advanced fermentation models, donor-stratified inocula, and targeted host co-culture assays, followed by careful triangulation with human studies to bridge the gap between mechanistic insight and clinically relevant outcomes (Brodkorb et al., 2019; Payne et al., 2012; Veintimilla-Gozalbo et al., 2021; Zhou et al., 2023)

This thesis responds to the need for microbiome-compatible interventions that perform under physiologically relevant conditions. It evaluates a spore-forming probiotic, two fungal enzyme blends, and three botanical extracts using a standardized mouth-to-colon workflow that combines INFOGEST digestion with fecal microbiota fermentations and targeted culture assays. The approach prioritizes reproducibility, safety, and functional readouts, while acknowledging the constraints of *in vitro* systems.

2.5 Bibliography

Barroso, E., Cueva, C., Peláez, C., Martínez-Cuesta, M. C., & Requena, T. (2015). Development of human colonic microbiota in the computer-controlled dynamic SIMulator of the GastroIntestinal tract (SIMGI). *LWT-Food Science and Technology, 61*(2), 283–289. https://doi.org/10.1016/j.lwt.2014.11.032

Barroso, E., Cueva, C., Peláez, C., Martínez-Cuesta, M. C., & Requena, T. (2015). The Computer-Controlled Multicompartmental Dynamic Model of the Gastrointestinal System SIMGI. In K. Verhoeckx (Eds.) et. al., *The Impact of Food Bioactives on Health: in vitro and ex vivo models*. (pp. 319–327). Springer. https://doi.org/10.1007/978-3-319-16104-4_28

Benes, V., Kloor, M., Ulrich, C. M., von Knebel Doeberitz, M., Sobhani, I., & Bork, P. (2014). Potential of fecal microbiota for early-stage detection of colorectal cancer. *Molecular Systems Biology, 10*(11), 766. https://doi.org/10.15252/msb.20145645

Bohn, T., Desmarchelier, C., Dragsted, L. O., Nielsen, C. S., Stahl, W., Rühl, R., Keijer, J., Borel, P., Castillo, M. D., Fogelholm, M., Galasso, R., Gunnlaugsdottir, H., Hermoso, M., Heseker, H., Knutsen, S. F., Matusheski, N., Riso, P., Roodenburg, A., Schloetter, M., Thurnham, D., Waśkiewicz, A., & Vinceti, M. (2017). Host-related factors explaining interindividual variability of carotenoid bioavailability and tissue concentrations in humans. *Molecular Nutrition & Food Research, 61*(6), 1600685. https://doi.org/10.1002/mnfr.201600685

Carroll, I. M., Ringel-Kulka, T., Siddle, J. P., Klaenhammer, T. R., & Ringel, Y. (2011). Molecular analysis of the luminal- and mucosal-associated intestinal microbiota in diarrhea-predominant irritable bowel syndrome. *American Journal of Physiology-Gastrointestinal and Liver Physiology, 301*(5), G799–G807. https://doi.org/10.1152/ajpgi.00154.2011

Cheng, M., & Ning, K. (2019). Stereotypes about enterotype: The old and new ideas. *Genomics, Proteomics & Bioinformatics, 17*(1), 4–12. https://doi.org/10.1016/j.gpb.2018.02.004

Cheung, M. K., Yue, G. G. L., Chiu, P. W. Y., & Lau, C. B. S. (2020). A review of the effects of natural compounds, medicinal plants, and mushrooms on the gut microbiota in colitis and cancer. *Frontiers in Pharmacology, 11,* 744. https://doi.org/10.3389/fphar.2020.00744

Cordaillat-Simmons, M., Rouanet, A., & Pot, B. (2020). Live biotherapeutic products: The importance of a defined regulatory framework. *Experimental & Molecular Medicine, 52*, 1397–1406. https://doi.org/10.1038/s12276-020-0437-6

Costea, P., Zeller, G., Sunagawa, S., Bork, P., & others. (2017). Towards standards for human fecal sample processing in metagenomic studies. *Nature Biotechnology, 35*(11), 1069–1076. https://doi.org/10.1038/nbt.3960

Cowan, M. M. (1999). Plant products as antimicrobial agents. *Clinical Microbiology Reviews, 12*(4), 564–582. https://doi.org/10.1128/cmr.12.4.564

Culp, E. J., & Goodman, A. L. (2023). Cross-feeding in the gut microbiome: Ecology and mechanisms. *Cell Host & Microbe, 31*(4), 485–499. https://doi.org/10.1016/j.chom.2023.03.016

Cusack, S., & O'Toole, P. W.; ELDERMET Consortium. (2013). Challenges and implications for biomedical research and intervention studies in older populations: Insights from the ELDERMET study. *Gerontology, 59*(2), 114–121. https://doi.org/10.1159/000343158

Dahal, R. H., Kim, S., Kim, Y. K., Kim, E. S., & Kim, J. (2023). Insight into gut dysbiosis of patients with inflammatory bowel disease and ischemic colitis. *Frontiers in Microbiology, 14,* 1174832. https://doi.org/10.3389/fmicb.2023.1174832

Deehan, E. C., Duar, R. M., Armet, A. M., Perez-Muñoz, M. E., Jin, M., & Walter, J. (2017). Modulation of the gastrointestinal microbiome with nondigestible fermentable carbohydrates to improve human health. *Microbiology Spectrum, 5*(5), 10.1128/microbiolspec.BAD-0019-2017. https://doi.org/10.1128/microbiolspec.BAD-0019-2017

Dethlefsen, L., McFall-Ngai, M., & Relman, D. A. (2007). An ecological and evolutionary perspective on human-microbe mutualism and disease. *Nature, 449*(7164), 811–818. https://doi.org/10.1038/nature06245

Di Lorenzo, C., Ceschi, A., Kupferschmidt, H., Lüde, S., de Souza Nascimento, E., Dos Santos, A., Colombo, F., Frigerio, G., Nørby, K., Plumb, J., Finglas, P., & Restani, P. (2015). Adverse effects of plant food supplements and botanical preparations: A systematic review with critical evaluation of causality. *British Journal of Clinical Pharmacology, 79*(4), 578–592. https://doi.org/10.1111/bcp.12519

Fan, Y., & Pedersen, O. (2021). Gut microbiota in human metabolic health and disease. *Nature Reviews Microbiology, 19,* 55–71. https://doi.org/10.1038/s41579-020-0433-9

Hervert-Hernández, D., & Goñi, I. (2011). Dietary polyphenols and human gut microbiota: A review. *Food Reviews International,* *27*(2), 154–169. https://doi.org/10.1080/87559129.2010.535233

Hill, C., Guarner, F., Reid, G., Gibson, G. R., Merenstein, D. J., Pot, B., Morelli, L., Canani, R. B., Flint, H. J., Salminen, S., Calder, P. C., & Sanders, M. E. (2014). Expert consensus document. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nature reviews. Gastroenterology & hepatology*, *11*(8), 506–514. https://doi.org/10.1038/nrgastro.2014.66

Hou, K., Wu, Z. X., Chen, X. Y., Wang, J. Q., Zhang, D., Xiao, C., Zhu, D., Wang, H. Y., Zhang, Y. H., Hu, C. Y., Li, X. Y., Zhu, X., Wang, L., He, J. Y., Zheng, Y. Y., Li, C. Y., Xu, X., Zhang, X. L., Xu, F., Liu, J. Q., Zhang, J., & Zhou, C. L. (2022). Microbiota in health and diseases. *Signal Transduction and Targeted Therapy, 7,* 135. https://doi.org/10.1038/s41392-022-00974-4

Jackson, S. A., Schoeni, J. L., Vegge, C., Pane, M., Stahl, B., Bradley, M., Goldman, V. S., Burguière, P., Atwater, J. B., & Sanders, M. E .(2019). Improving end-user trust in the quality of commercial probiotic products. *Frontiers in Microbiology, 10,* 739. https://doi.org/10.3389/fmicb.2019.00739

Karkman, A., Lehtimäki, J., & Ruokolainen, L. (2017). The ecology of human microbiota: Dynamics and diversity in health and disease. *Annals of the New York Academy of Sciences, 1399*(1), 78–92. https://doi.org/10.1111/nyas.13326

Kolodziejczyk, A. A., Zheng, D., & Elinav, E. (2019). Diet–microbiota interactions and personalized nutrition. *Nature Reviews Microbiology, 17,* 742–753. https://doi.org/10.1038/s41579-019-0256-8

Lammers, K. M., Brigidi, P., Vitali, B., Gionchetti, P., Rizzello, F., Caramelli, E., Matteuzzi, D., & Campieri, M. (2003). Immunomodulatory effects of probiotic bacteria DNA: IL-1 and IL-10 response in human peripheral blood mononuclear cells. *FEMS immunology and medical microbiology*, *38*(2), 165–172. https://doi.org/10.1016/S0928-8244(03)00144-5

Macfarlane, G. T., Macfarlane, S., & Gibson, G. R. (1998). Validation of a three-stage compound continuous culture system for investigating the effect of retention time on the ecology and metabolism of bacteria in the human colon. *Microbial Ecology, 35*(2), 180–187. https://doi.org/10.1007/s002489900072

Meyer, F., Paarmann, D., D’Souza, M., Olson, R., Glass, E. M., Kubal, M., Paczian, T., Rodriguez, A., Stevens, R., Wilke, A., Wilkening, J., & Edwards, R. A. (2008). The metagenomics RAST server—A public resource for the automatic phylogenetic and functional analysis of metagenomes. *BMC Bioinformatics, 9,* 386. https://doi.org/10.1186/1471-2105-9-386

Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., Carrière, F., Boutrou, R., Corredig, M., Dupont, D., Dufour, C., Egger, L., Golding, M., Karakaya, S., Kirkhus, B., Le Feunteun, S., Lesmes, U., Macierzanka, A., Mackie, A., Marze, S., McClements, D. J., Ménard, O., Recio, I., Santos, C. N., Singh, R. P., Vegarud, G. E., Wickham, M. S. J., Weitschies, W., & Brodkorb, A. (2014). A standardised static in vitro digestion method suitable for food: An international consensus. *Food & Function, 5*(6), 1113–1124. https://doi.org/10.1039/C3FO60702J

Minekus, M., Smeets-Peeters, M., Bernalier, A., Marol-Bonnin, S., Havenaar, R., Marteau, P., Alric, M., Fonty, G., & Huis in't Veld, J. H. (1999). A computer-controlled system to simulate conditions of the large intestine with peristaltic mixing, water absorption and absorption of fermentation products. *Applied microbiology and biotechnology*, *53*(1), 108–114. https://doi.org/10.1007/s002530051622

Molly, K., Vande Woestyne, M., & Verstraete, W. (1993). Development of a 5-step multi-chamber reactor as a simulation of the human intestinal microbial ecosystem. *Applied microbiology and biotechnology*, *39*(2), 254–258. https://doi.org/10.1007/BF00228615

Nissen, L., Casciano, F., & Gianotti, A. (2020). Intestinal fermentation in vitro models to study food-induced gut microbiota shift: An updated review. *FEMS Microbiology Letters, 367*(12), fnaa097. https://doi.org/10.1093/femsle/fnaa097

Payne, A. N., Zihler, A., Chassard, C., & Lacroix, C. (2012). Advances and perspectives in in vitro human gut fermentation modeling. *Trends in biotechnology*, *30*(1), 17-25. https://doi.org/10.1016/j.tibtech.2011.06.011

Pérez-Jiménez, J., & Saura-Calixto, F. (2018). Fruit peels as sources of non-extractable polyphenols or macromolecular antioxidants: Analysis and nutritional implications. *Food research international (Ottawa, Ont.)*, *111*, 148–152. https://doi.org/10.1016/j.foodres.2018.05.023

Qin, J., Li, Y., Cai, Z., Li, S., Zhu, J., Zhang, F., Liang, S., Zhang, W., Guan, Y., Shen, D., Peng, Y., Zhang, D., Jie, Z., Wu, W., Qin, Y., Xue, W., Li, J., Han, L., Lu, D., Wu, P., Dai, Y., Sun, X., Li, Z., Tang, A., Zhong, S., Li, X., Chen, W., Xu, R., Wang, M., Feng, Q., Gong, M., Yu, J., Zhang, Y., Zhang, M., Hansen, T., Sanchez, G., Raes, J., Falony, G., Okuda, S., Almeida, M., LeChatelier, E., Renault, P., Pons, N., Batto, J.-M., Zhang, Z., Chen, H., Yang, R., Zheng, W., Li, S., Yang, H., Wang, J., Ehrlich, S. D., Nielsen, R., Pedersen, O., Kristiansen, K., & Wang, J. (2012). A metagenome-wide association study of gut microbiota in type 2 diabetes. *Nature, 490*(7418), 55–60. https://doi.org/10.1038/nature11450

Refael, G., Tarazi Riess, H., Shani Levi, C., Magzal, F., Tamir, S., Koren, O., & Lesmes, U. (2022). Responses of the human gut microbiota to physiologically digested insect powders or isolated chitin thereof. *Future Foods, 6,* 100197. https://doi.org/10.1016/j.fufo.2022.100197

Tannock, G. W., & Liu, Y. (2019). Guided dietary fibre intake as a means of directing short-chain fatty acid production by the gut microbiota. *Journal of the Royal Society of New Zealand, 50*(3), 434–455. https://doi.org/10.1080/03036758.2019.1657471

Van den Abbeele, P., Grootaert, C., Marzorati, M., Possemiers, S., Verstraete, W., Gérard, P., Rabot, S., Bruneau, A., El Aidy, S., Derrien, M., Zoetendal, E., Kleerebezem, M., Smidt, H., & Van de Wiele, T. (2010). Microbial community development in a dynamic gut model is reproducible, colon region specific, and selective for Bacteroidetes and Clostridium cluster IX. *Applied and environmental microbiology*, *76*(15), 5237–5246. https://doi.org/10.1128/AEM.00759-10

Veintimilla-Gozalbo, E., Asensio-Grau, A., Calvo-Lerma, J., Heredia, A., & Andrés, A. (2021). In Vitro Simulation of Human Colonic Fermentation: A Practical Approach towards Models' Design and Analytical Tools. *Applied Sciences*, *11*(17), 8135. https://doi.org/10.3390/app11178135

Venema, K., van Nuenen, H. M. C., Smeets-Peeters, M., Minekus, M., & Havenaar, R. (2000). TNO's in vitro large intestinal model: An excellent screening tool for functional food and pharmaceutical research. *Ernährung/Nutrition, 24*(12), 558–564.

Vinderola, G., Sanders, M. E., & Salminen, S. (2022). The concept of postbiotics. *Foods, 11*(8), 1077. https://doi.org/10.3390/foods11081077

Vișan, A. I., & Neguț, I. (2024). Coatings based on essential oils for combating antibiotic resistance. *Antibiotics, 13*(7), 625. https://doi.org/10.3390/antibiotics13070625

Zmora, N., Zilberman-Schapira, G., Suez, J., Mor, U., Dori-Bachash, M., Bashiardes, S., Kotler, E., Zur, M., Regev-Lehavi, D., Brik, R. B.-Z., Federici, S., Cohen, Y., Linevsky, R., Rothschild, D., Moor, A. E., Ben-Moshe, S., Harmelin, A., Itzkovitz, S., Maharshak, N., Shibolet, O., Shapiro, H., Pevsner-Fischer, M., Sharon, I., Halpern, Z., Segal, E., & Elinav, E. (2018). Personalized gut mucosal colonization resistance to empiric probiotics is associated with unique host and microbiome features. *Cell, 174*(6), 1388–1405.e21. https://doi.org/10.1016/j.cell.2018.08.041

Zeller, G., Tap, J., Voigt, A. Y., Sunagawa, S., Kultima, J. R., Costea, P. I., Amiot, A., Böhm, J., Brunetti, F., Habermann, N., Hercog, R., Koch, M., Luciani, A., Mende, D. R., Schneider, M. A., Schrotz-King, P., Tournigand, C., Tran Van Nhieu, J., Yamada, T., Zimmermann, J.,

Zhou, H., Tan, Y., & McClements, D. J. (2023). Applications of the INFOGEST in vitro digestion model to foods: A review. *Annual Review of Food Science and Technology, 14*, 135-156. https://doi.org/10.1146/annurev-food-060721-012235

3. Objectives of the work

The main objective of this PhD research was to evaluate the microbiome compatibility, functional performance, and safety profile of selected dietary supplements and ingredients through a physiologically relevant i*n vitro* workflow.

Specifically, the study investigated three categories of microbiome-related interventions: (i) a spore-forming probiotic strain (*Heyndrickxia coagulans* LMG S-24828), (ii) two commercial fungal enzyme formulations, and (iii) three botanical extracts of nutritional or therapeutic interest.

To achieve this, the work employed standardized *in vitro* digestion protocols (INFOGEST 2.0), fecal fermentation models, and targeted microbial cultures, enabling a comprehensive analysis of digestion dynamics, metabolic activity, and community responses. Among the characteristics examined were microbial viability, substrate utilization, enzymatic activity, and the effects on representative commensal and probiotic taxa. Through this approach, the research aimed to contribute to the development of evidence-based, microbiome-compatible nutraceuticals that support digestive health while preserving gut microbial balance.

4. *Heyndrickxia coagulans* LMG S-24828 Is a Safe Probiotic Strain Capable of Germinating in the Human Gut.

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4.1 Abstract

Ensuring the viability and efficacy of probiotic microorganisms during manufacturing and gastrointestinal transit remains challenging, particularly for sensitive strains such as certain lactic acid bacteria and bifidobacteria. This has led to increased interest in spore-forming bacteria, such as *Heyndrickxia coagulans* (formerly *Bacillus coagulans*), which can endure environmental stresses through their endospore forms. This study presents a comprehensive analysis of the probiotic potential of strain LMG S-24828, originally isolated from the feces of a healthy human. The genomic analysis confirmed the strain's taxonomic placement within the species *H. coagulans* and revealed no extrachromosomal plasmid DNA, suggesting genetic stability. Safety assessments demonstrated that LMG S-24828 does not produce D-lactate, deconjugate bile salts, or exhibit hemolytic activity, and it lacks transmissible antibiotic resistances. Phenotypic tests showed the strain's metabolic versatility, including its ability to hydrolyze complex carbohydrates and adhere to intestinal epithelial cells. Moreover, LMG S-24828 exhibited robust survival and germination during *in vitro* and *in vivo* gastrointestinal simulations, with evidence of significant spore germination in the human gut. These findings suggest that *H. coagulans* LMG S-24828 possesses several advantageous traits for probiotic applications, warranting further clinical evaluation to confirm its health benefits.

4.2 Introduction

As explicitly stated in its definition ("live microorganisms that, when administered in adequate amounts, confer a health benefit on the host"; Hill et al., 2014), probiotic microorganisms must be consumed alive. Additionally, according to the guidelines of the Italian Ministry of Health, probiotic microorganisms must be "active in the intestines in such a quantity as to be able to multiply there" (Ministero della Salute, 2018). These requirements, however, are often challenging to meet due to the sensitivity of most probiotic microorganisms, such as lactic acid bacteria and bifidobacteria, to the stresses encountered during manufacturing, like spray- and freeze-drying, as well as during gastrointestinal transit (Terpou et al., 2019). In particular, the sensitivity of many probiotic strains to these stresses limits their use in various food formulations. For this reason, there is increasing interest in the potential use of spore-forming bacteria as probiotics (Soares et al., 2023). These bacteria can be added to probiotic formulations as endospores, which are cryptobiotic forms of resistance that allow the microorganism to withstand numerous chemical, enzymatic, and physical stresses. Among the spore-forming bacteria with potential probiotic properties, the species most extensively studied and used industrially for human consumption is *Heyndrickxia coagulans* (formerly *Bacillus coagulans*) (Liang et al., 2024). *H. coagulans* has been isolated from soil (Xu et al., 2023), hot spring water (Alkan et al., 2007), milk and dairy products (Chopra et al., 1984), and various canned and fermented food and vegetables (Abdhul et al., 2015; Aulitto et al., 2017; Yu et al., 2021; Konuray et al., 2021). Members of this species can also be isolated from the human intestine (Perotti et al., 2024; Zhu et al., 2024) and human breast milk (Choi et al., 2024). Due to its long history of safe use in humans, the species *H. coagulans* has been granted the "qualified presumption of safety" (QPS) status by the European Food Safety Authority (EFSA; (Hazards et al., 2023), see appendixes B and C) and several strains of the species has been approved as "generally recognized as safe" (GRAS) for several applications by the U.S. Food and Drug Administration [FDA; e.g., GRAS Notice (GRN) No. 597 and GRN No. 660]. In addition, *H. coagulans* is now considered novel in food for example as an ingredient of leafy green preparations, but not novel in food supplements (EFSA, 2025).

An increasing number of human intervention trials are demonstrating the positive physiological effects of various strains belonging to the *H. coagulans* species. For instance, supplementation with 4×10^8 spores of *H. coagulans* MTCC 5856 along with zinc and an oral rehydration solution for 5 days reduced the duration of diarrhea in non-hospitalized children (Majeed et al., 2023). In another trial with the same strain, 90 days of administration of 2 billion CFUs per day of *H. coagulans* MTCC 5856 resulted in significant decreases in bloating, vomiting, diarrhea, abdominal pain, and stool frequency compared to a placebo in patients with diarrhea-predominant IBS (IBS-D; Majeed et al., 2016). Similarly, 8 weeks of supplementation with 2 billion CFUs per day of *H. coagulans* LMG S-31876 significantly reduced abdominal pain, bloating, diarrhea, and stool frequency in IBS-D patients compared to the placebo (Kallur et al., 2023). Other *H. coagulans* strains have been employed in various human trials, showing promising health-promoting activities in nonalcoholic fatty liver (strain TCI711, Hsieh et al., 2024), *Helicobacter pylori* infection (strain BCF-01, Chen et al., 2024), intestinal function after gynecological laparoscopic surgery (strain TBC169, Li et al., 2022), and functional constipation (strain Unique IS2; Venkataraman et al., 2023; and strain SNZ 1969; Kang et al., 2021).

It is well established that many bacterial properties, both beneficial (such as immunomodulatory capabilities) and potentially detrimental (such as transmissible antibiotic resistances), can vary from strain to strain. Therefore, it is not possible to extrapolate information obtained from studies conducted on one specific strain to another strain, even if they belong to the same species (Hill et al., 2014; Britton et al., 2021). Consequently, when intending to use a new strain as a probiotic, it is necessary to conduct targeted studies on that specific microorganism to determine its genetic and physiological characteristics, safety profile, and potential probiotic properties.

In the context described above, this study presents a comprehensive analysis of the *H. coagulans* strain LMG S-24828, encompassing genetic, physiological, and functional characterizations. Specifically, we performed whole-genome analysis along with phenotypic and functional characterization, which includes a precise assessment of the antibiotic resistance profile, *in vitro* safety assessment, substrate utilization properties, *in vitro* adhesion properties, and *in vitro* and *in vivo* germination capabilities.

4.3 Material and Methods

4.3.1 Bacterial Strain Under Investigation

This study was carried out on a *Heyndrickxia* (formerly *Bacillus*) *coagulans* strain, deposited at the Belgian Coordinated Collections of Microorganisms (BCCM) under the code LMG S-24828. *H. coagulans* LMG S-24828 is marketed as Weizy® by Giellepi S.p.A. (Milan, Italy). *H. coagulans* LMG S-24828 was cultivated in various media and under different conditions, as specified for each experiment detailed below.

4.3.2 Genome Determination and Analysis

The draft genome of *H. coagulans* LMG S-24828 was obtained by DNA sequencing with an Illumina HiSeq 2500 system with paired-end and shotgun libraries. Sequencing data have been deposited as FASTQ files in the European Nucleotide Archive (ENA) of the European Bioinformatics Institute under accession code PRJEB80685. Read lengths of 151 nucleotides were achieved for both R1 and R2. A total of 6,830,204 high-quality paired-end reads were generated, each with a Phred quality score exceeding 30. The reads were assembled into contigs and scaffolds using the SPAdes algorithm (version 3.14.1). The success of the assembly was assessed using Bandage (version 0.8), which visualizes the assembly graph. Genome annotation was performed using the Rapid Annotation using Subsystems Technology (RAST) automated pipeline. The presence of antibiotic resistance genes (ARGs) was assessed *in silico* within the draft genome of the LMG S-24828 strain using the CARD bioinformatic tool (Alcock et al., 2023).

The complete sequence of the 16S rRNA gene was used to perform the phylogenetic analysis of strain LMG S-24828 and the type strains of the species belonging to the *Heyndrickxia* genus, using the type strain of the species *E. coli* and *B. subtilis* as outgroups. The obtained phylogram was shown as a midpoint-rooted tree obtained after a ClustalW alignment of the DNA sequences considered. The multi-locus-sequence typing (MLST) analysis of strain LMG S-24828 was conducted as reported by Zhang et al. (2024) by constructing a phylogram based on the concatenated sequences of six housekeeping genes obtained from 9 *H. coagulans* strains. The selected genes were as follows: DNA gyrase subunit B gene, *gyrB* [locus tag in the genome of the type strain DMS 1 = ATCC 7050 (accession number NZ_CP009709): BF29_RS11095]; dihydroxy-acid dehydratase *ilvD* (BF29_RS07495), l-lactate dehydrogenase, *ldh-1* (BF29_RS02275); L-LDH-NAD l-lactate dehydrogenase, *ldh-2* (BF29_RS14745); phosphate acetyltransferase, *pta* (BF29_RS10325); DNA-directed RNA polymerase, *rpoB* (BF29_RS11715).

The presence of plasmid molecules in LMG S-24828 was assessed by extracting DNA by means of alkaline lysis followed by second-dimension electrophoresis for the analysis of circular covalently closed (CCC) and open circle plasmid forms as previously described (Guglielmetti et al., 2007).

4.3.3 Assessment of Substrate Utilization and Enzymatic Activities

The metabolic and enzymatic capabilities of strain LMG S-24828 were evaluated using the Biolog GEN III system (Biolog Inc., Hayward, CA, USA) and the API ZYM system (bioMérieux, Marcy-l'Étoile, France). Both analyses were conducted following the manufacturers' instructions. Briefly, for the Biolog GEN III analysis, the strain was cultured on tryptic soy agar (TSA; Lioilchem, Roseto degli Abruzzi, Italy) plates for 48 h at 37 °C. After aerobic incubation, colonies were suspended in Inoculating Fluid 3 (IF3, Biolog) to achieve the recommended cell density. The cell suspension was then transferred to the GEN III MicroPlate, with 100 µl added per well. Wells that exhibited a distinct color change to purple were recorded as positive. For the API ZYM test, the strain was initially cultured in tryptic soy broth for 48 h at 37 °C. The culture was centrifuged, and the pellet was washed with phosphate buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4) before resuspension in API Suspension Medium to turbidity of 5–6 McFarland units. The procedure continued according to the kit's protocol, and the metabolic end products were identified through colorimetric reactions.

4.3.4 Determination of Growth Curves with Different Carbon Sources

Growth curves for strain LMG S-24828 were determined in sugar-free MRS broth supplemented with various carbohydrates. In preparation for the experiments, the following stock media and sugar solutions were prepared: a sugar-free de Man Rogosa Sharpe (MRS) broth (Difco Laboratories Inc., Detroit, MI, USA) at twice the standard concentration, and 40% (w/v) solutions of dextrose, sucrose, raffinose, lactose, and lactulose, all of which were filter sterilized with a 0.22 um filter. Prior to the experiment, the bacterial strain was cultivated in MRS broth for 48 h and quantified using the forward and side scatter obtained (FSC and SSC) with a BD Accuri™ C6 Plus Flow Cytometer (BD Biosciences, Milan, Italy). The sugar solutions were serially diluted with sterile deionized water. The double-concentration, dextrose-free MRS broth was inoculated with the quantified culture, and 100 µl of this inoculated broth was dispensed into each well to achieve the appropriate final concentration of MRS, carbon source and a cell density of 1×10^5 events per ml. For assessing lactose in the presence of 0.2% glucose, the sterile deionized water used for sugar dilution was pre-adjusted to contain this glucose concentration. Growth curves were monitored using a Powerwave XS2 automated spectrophotometer (Biotek, Milan, Italy), which measured the optical density (OD) at 600 nm every 10 min at 37 °C over a 48-h period. All analyses were performed in triplicate.

4.3.5 Mucin Utilization Assessment

The ability of strain LMG S-24828 to metabolize mucin was assessed following the principles outlined in the experiment described above. In specific, dextrose-free MRS broth was prepared in 5-ml vials, each supplemented with varying concentrations of porcine stomach mucin (2%, 1%, 0.5%, 0.25%, 0.125%, 0.0625%, and 0%; Sigma-Aldrich, St. Louis, MO, USA). A standard MRS broth containing 2% glucose was also prepared as a control. After cultivating the strain in MRS broth for 48 h and quantifying it using a BD Accuri™ C6 Plus Flow Cytometer (BD Biosciences), the cultures were used to inoculate the vials at a concentration of 1 × 10^5 events per ml. *Lacticaseibacillus rhamnosus* GG was used as a negative control. The vials were then incubated under anaerobic conditions in an anaerobic chamber (Bugbox Plus, Baker Ruskin, Sanford, ME, USA) at 37 °C. Samples were collected at three time points (0, 24, and 48 h), diluted, and plated onto MRS agar with phosphate-buffered saline (PBS). The plates were incubated at 37 °C, and viable plate counts were performed to assess bacterial growth. All analyses were conducted in triplicate.

4.3.6 D-Lactate Production

The D-Lactic Acid/L-Lactic Acid kit (R-Biopharm AG, Darmstadt, Germany) was used to analyze lactic acid production, following the manufacturer's instructions. *Weissella cibaria* FB16 was used as a positive control because members of the *Weissella* genus are known to produce both D- and L-lactate (Fusco et al., 2015). *Lacticaseibacillus paracasei* DG served as a negative control, as it does not produce D-lactate (Vitetta et al., 2017). All strains were cultured in MRS broth at 37 °C anaerobically for 48 h prior to analysis to ensure optimal growth conditions.

4.3.7 Detection of Bile Salt Hydrolase Activity

A qualitative assay was performed to detect bile salt hydrolase (BSH) activity by preparing MRS agar medium supplemented with 0.5% Oxgall (Sigma-Aldrich) as described in Begley et al., 2006. Cultures grown in MRS broth for 48 h were streaked onto the agar and incubated anaerobically at 37 °C for 48 h. The presence of white precipitates around the colonies and the clearing of the medium indicated deconjugating activity. *Lactobacillus acidophilus* LA 14 and *Lacticaseibacillus casei* LMG 6904 were used as positive and negative controls for BSH activity, respectively (O'Flaherty et al., 2018).

4.3.8 Hemolytic Activity Evaluation

Hemolytic activity was assessed using Columbia blood agar (Millipore, Burlington, MA, USA) after 48 h of incubation at 37 °C under anaerobic conditions according to the American Society of Microbiology Protocol 28 (https://asm.org/getattachment/7ec0de2b-bb16-4f6e-ba07-2aea25a43e76/protocol-28). *Staphylococcus epidermidis* ATCC 12228, *Escherichia coli* DH5α, and *Streptococcus pyogenes* C11 were used as controls to demonstrate γ-hemolytic (non-hemolytic), α-hemolytic (partial hemolysis), and β-hemolytic (complete hemolysis) activity, respectively. All strains tested were cultured in tryptic soy (TS) broth before testing.

4.3.9 Determination of the Antibiotic Resistance Profile

We assessed the antibiotic sensitivity of strain LMG S-24828 using a panel of nine antibiotics, following the ISO 10932 IDF223:2010 protocol recommended by the European Food Safety Authority (EFSA) (EFSA PoA, 2012; EPo Additives et al., 2018).

The antibiotics used were purchased from Sigma-Aldrich. Briefly, minimum inhibitory concentrations (MICs) were determined using a micro-dilution method. The testing medium consisted of 90% IsoSensitest Broth (Oxoid, Fisher Scientific Italia, Rodano, Italy) and 10% MRS Broth (Difco) (ISOMRS). The strain was tested in duplicate against each antimicrobial concentration, starting from overnight cultures grown in ISOMRS. Bacterial cells were quantified by flow cytometry and inoculated at a concentration of 1×10^5 events per ml. *L. paracasei* LMG12586 was employed as the reference strain, as specified in ISO 10932. The plates were incubated anaerobically at 37 °C for 48 h, and bacterial growth was assessed visually and by measuring optical density at 600 nm using a spectrophotometer (MicroWave RS2, Biotek, USA). The MIC was defined as the lowest concentration of antibiotic that inhibited visible bacterial growth. Results were interpreted according to the EFSA Guidance on the assessment of bacterial antimicrobial susceptibility (Guglielmetti et al., 2007).

4.3.10 In Vitro Survival and Germination in the Gastrointestinal Tract

The survival of LMG S-24828 during transit through the orogastrointestinal tract was assessed in vitro using the INFOGEST static simulation of gastrointestinal digestion protocol developed by Brodkorb et al., 2019. Specifically, 1 g of industrially prepared LMG S-24828 spores was mixed with 1 ml of simulated salivary fluid containing 15.1 mM KCl, 3.7 mM KH_2PO_4, 13.6 mM $NaHCO_3$, 0.15 mM $MgCl_2(H_2O)_6$, 0.06 mM $(NH_4)_2CO_3$, 1.1 mM HCl, and 1.5 mM $CaCl_2(H_2O)_2$. The mixture was homogenized using a Stomacher 3500 peristaltic homogenizer. α-Amylase from *Aspergillus oryzae* (Merck, Milan, Italy) was added to achieve a final concentration of 75 U/ml, and the suspension was diluted to 2 ml with deionized water. The sample was then incubated at 37 °C for 2 min with continuous shaking. To simulate gastric conditions, 4 ml of simulated gastric fluid was prepared with 6.9 mM KCl, 0.9 mM KH_2PO_4, 25 mM $NaHCO_3$, 47.2 mM NaCl, 0.12 mM $MgCl_2(H_2O)_6$, 0.5 mM $(NH_4)_2CO_3$, 15.6 mM HCl, and 0.15 mM $CaCl_2(H_2O)_2$. The pH of the mixture was adjusted to 3.0 using 5 M HCl. Porcine pepsin (Merck, Milan, Italy) was introduced at a final concentration of 2000 U/ml, and rabbit gastric extract (RGE; Lipolytech, Marseille, France) was added to reach a final lipase concentration of 60 U/ml. The samples were then diluted to 4 ml with deionized water and incubated at 37 °C for 2 h under continuous shaking. For the intestinal simulation, 4 ml of simulated intestinal fluid containing 6.8 mM KCl, 0.8 mM KH_2PO_4, 85 mM $NaHCO_3$, 38.4 mM NaCl, 0.33 mM $MgCl_2(H_2O)_6$, 8.4 mM HCl, and 0.6 mM $CaCl_2(H_2O)_2$ was added. The pH was adjusted to 7.0 using 5 M NaOH. Porcine pancreatin and bovine bile salts (Merck, Milan, Italy) were incorporated at final concentrations of 100 U/ml of trypsin and 10 mM bile salts, respectively. The total volume was adjusted to 8 ml, and the samples were incubated at 37 °C with continuous shaking for 2 h. Finally, the total volume was adjusted to 10 ml with phosphate-buffered saline (PBS). The samples were then diluted and plated on GYEA agar medium (composition per liter: glycerol 5 g, yeast extract 2 g, K_2HPO_4 1 g, bromocresol green 0.05 g, agar 15 g, pH 5.5). The plates were incubated at 55 °C for 48 h, after which the colonies were enumerated. *H. coagulans* is a bacterium with minimal nutritional requirements and is capable of growth across a broad temperature range (15–60 °C). These traits facilitated the development of a cultivation protocol that is highly specific and selective for the probiotic strain under investigation, employing glycerol as the carbon source and an incubation temperature of 55 °C (Perotti et al., 2025). To determine the spore counts, the same protocol was followed with one modification: the samples were diluted 1:10 in maximum recovery diluent and incubated at 90 °C for 10 min in a laboratory water bath. The number of vegetative cells was calculated by subtracting the spore count obtained after pasteurization from the total colony-forming units (CFUs), which include both spores and vegetative cells.

The survival and germination abilities of strain LMG S-24828 were also assessed in a human fecal sample. To this aim, a fresh fecal sample was collected from a healthy donor and immediately processed to create a fecal slurry. This was achieved by adding 1 ml of phosphate buffer and 15% w/v glycerol per gram of feces. Ten grams of the fecal sample were spiked with an industrial LMG S-24828 spore preparation to achieve a concentration of 1×10^9 CFU/g. The sample was incubated under anaerobic conditions. At various time points (0, 0.25, 3, and 18 h), 1 ml of the slurry was collected, diluted, and plated onto GYEA, with or without pasteurization as described previously. The same techniques were employed to determine spore counts.

4.3.11 In Vivo Viable Recovery Trial

A pilot viable recovery study was conducted to assess the viable and spore counts of LMG S-24828 in fecal samples from five healthy adult volunteers after ingestion of an industrial probiotic formulation. Demographic characteristics and dietary habits of the volunteers are reported in Supplementary Table S1. The study received approval from the Research Ethics Committee of the University of Milan (opinion nr. 89/22). The administered product consisted of a hydroxypropyl methylcellulose (HPMC) capsule filled with industrial biomass of *H. coagulans* LMG S-24828 (Weizy®), without any excipients. Each capsule contained 6 billion spores and was prepared within 1 week prior to the start of the trial. Each volunteer consumed one capsule daily for 3 days. After the final ingestion, the first fecal sample produced by each volunteer was collected, immediately frozen at home, and delivered frozen for analysis. The samples were analyzed on the same day they were collected. The analysis followed a protocol previously described (Perotti et al., 2024). Briefly, 1 g of each fecal sample was diluted 1:10 in

Maximum Recovery Diluent (MRD; Scharlab, Lodi, Italy) and placed into a stomacher bag. The contents were manually manipulated for 1 min and then homogenized for an additional minute using a stomacher. The resulting mixture was transferred to a sterile glass tube, serially diluted in MRD, and plated on GYEA agar medium. The plates were incubated at 55 °C for 48 h, after which colonies were enumerated. To determine spore counts, the same protocol was followed with a modification: the fecal sample was resuspended in a stomacher bag with MRD pre-warmed to 90 °C and then incubated at 90 °C for 10 min in a laboratory water bath. The number of vegetative cells was calculated by subtracting the spore count obtained after pasteurization from the total CFUs, which included both spores and vegetative cells.

4.3.12 Experiments with Caco-2 Cells

The Caco-2 intestinal epithelial cell layer was used to assess the adhesion ability of and the pro-inflammatory response induced by *H. coagulans* LMG S-24828. Caco-2 cells were cultured at 37 °C in 95% air and 5% carbon dioxide in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% (v/v) heat-inactivated fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.1 mM non-essential amino acids, and 2 mM l-glutamine.

Adhesion experiments were conducted with Caco-2 cells 15 days after confluence, as previously described (Guglielmetti et al., 2008; Guglielmetti et al., 2010). Briefly, bacterial spores and vegetative forms were quantified microscopically using a Neubauer-improved counting chamber (Marienfeld GmbH, Lauda-Königshofen, Germany). Approximately 2×10^8 spores (from industrial preparation) or vegetative forms (from an overnight culture in MRS) were incubated with a monolayer of approximately 1×10^6 Caco-2 cells for 1 h at 37 °C. Following incubation, the monolayers were washed three times with PBS, treated with 3 ml of methanol for 8 min at room temperature, and stained with 3 ml of Giemsa stain solution (1:20; Carlo Erba, Milan, Italy) for 30 min at room temperature in the dark. Finally, the monolayers were washed three times with PBS, dried in an incubator for 1 h, and examined microscopically. The adhesion experiments were performed in duplicate.

The potential pro-inflammatory/pro-apoptotic activity of strain LMG S-24828 was assessed by measuring NF-κB activation in Caco-2 cells, as previously described (Brunelli et al., 2022; Taverniti et al., 2013). Briefly, a recombinant Caco-2 cell line stably transfected with the alkaline phosphatase (SEAP) reporter vector pNiFty2-Seap (InvivoGen, Labogen, Rho, Italy) was used. Approximately 2.5×10^5 cells per well of recombinant Caco-2 cells were incubated with 2.5×10^7 spores (from industrial preparation) or vegetative forms (from an overnight culture in TSB), resulting in a multiplicity of infection (MOI) of approximately 100. Two nanograms per milliliter of interleukin (IL)-1β were used to stimulate the Caco-2 cells. SEAP activity was quantified in the supernatant after 24 h of incubation at 37 °C using the Quanti-Blue reagent

(InvivoGen). Data were reported as relative units of NF-κB activation (RUNFA), calculated as normalized SEAP activity. Two independent experiments were conducted.

4.3.13 Statistical Analysis

Data were analyzed using Prism 8 (GraphPad Software, CA, USA). Specifically, mucin utilization by *H. coagulans* LMG S-24828 (six independent experiments; $n = 6$) and *L. rhamnosus* GG ($n = 3$) was assessed checking normality with the Shapiro-Wilk test, then using the Kruskal–Wallis nonparametric ANOVA, followed by Dunn's multiple comparisons tests. The activation of the transcriptional regulator NF-κB was measured in two independent experiments, each conducted in duplicate, and analyzed using an unpaired Student's *t*-test to assess significant differences.

4.3.14 Declaration of Generative AI in the Writing Process

During the preparation of this work, the authors used the AI-powered language model ChatGPT-4 (https://chat.openai.com/) in order to improve readability and language. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

4.4 Results

4.4.1 H. coagulans LMG S-24828 Genome Analysis

We generated a draft genome sequence of *H. coagulans* LMG S-24828 consisting of 3.67 Mbp. The guanine and cytosine (G/C) content was calculated to be 46.2%, which is consistent with that of other *H. coagulans* genomes (Aulitto et al., 2022). RASTtk annotation identified 4121 putative coding sequences (CDSs), including the CDSs of 10 complete ribosomal operons. We did not find CDSs that could be unequivocally assigned to plasmid replicons. Alkaline lysis and bidimensional agarose gel electrophoresis of the obtained DNA confirmed the absence of extrachromosomal plasmid DNA (Supplementary Figure S1), indicating that the genome of strain LMG S-24828 consists of a single chromosomal molecule. In addition, RASTtk annotation revealed several putative genes for the utilization of numerous carbohydrates (see Biolog and APIzyme results for more) and the gene clusters for the synthesis of vitamins such as thiamin (vitamin B1), biotin (vitamin B8), folic acid (vitamin B9), and menaquinone (vitamin K2).

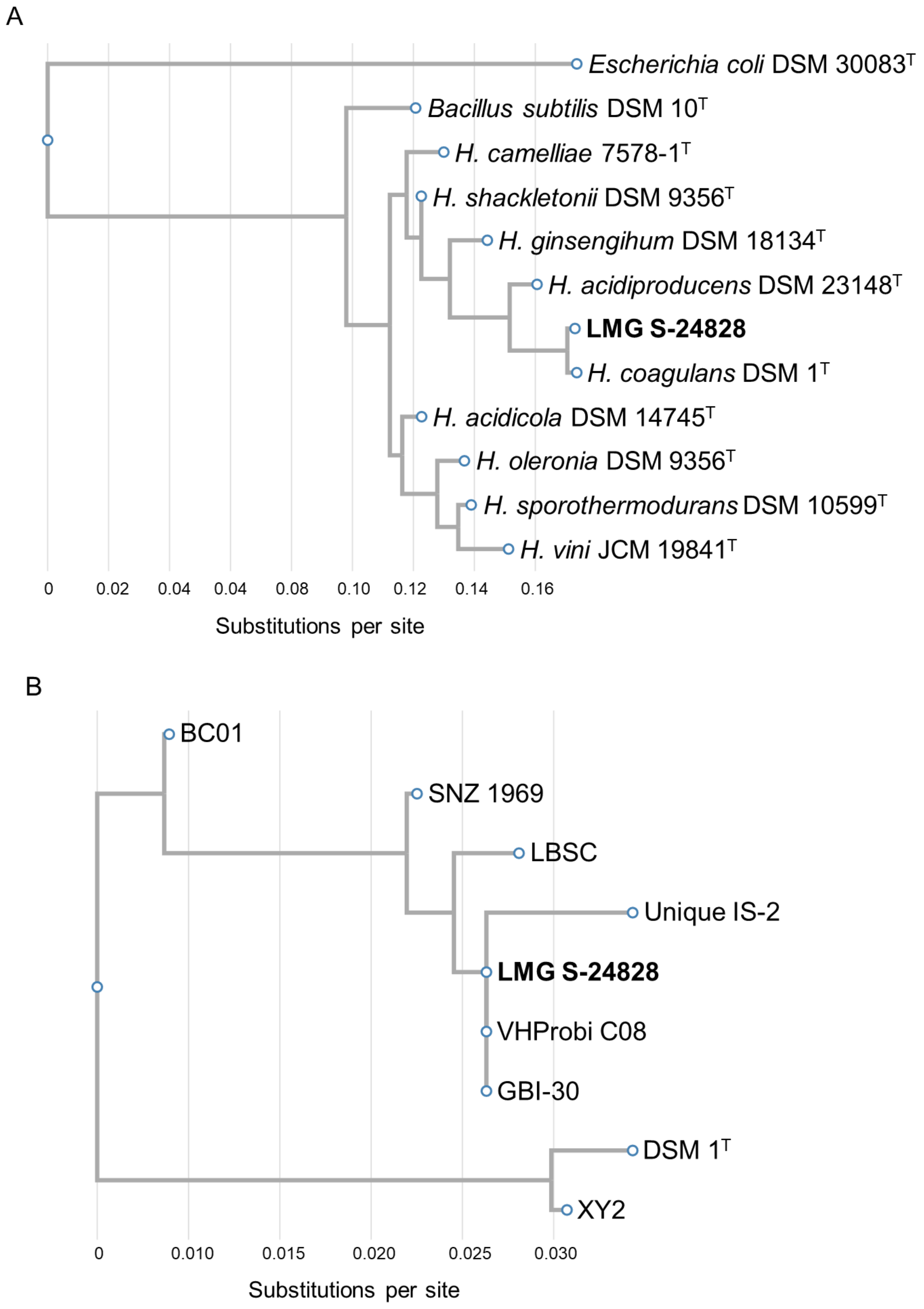


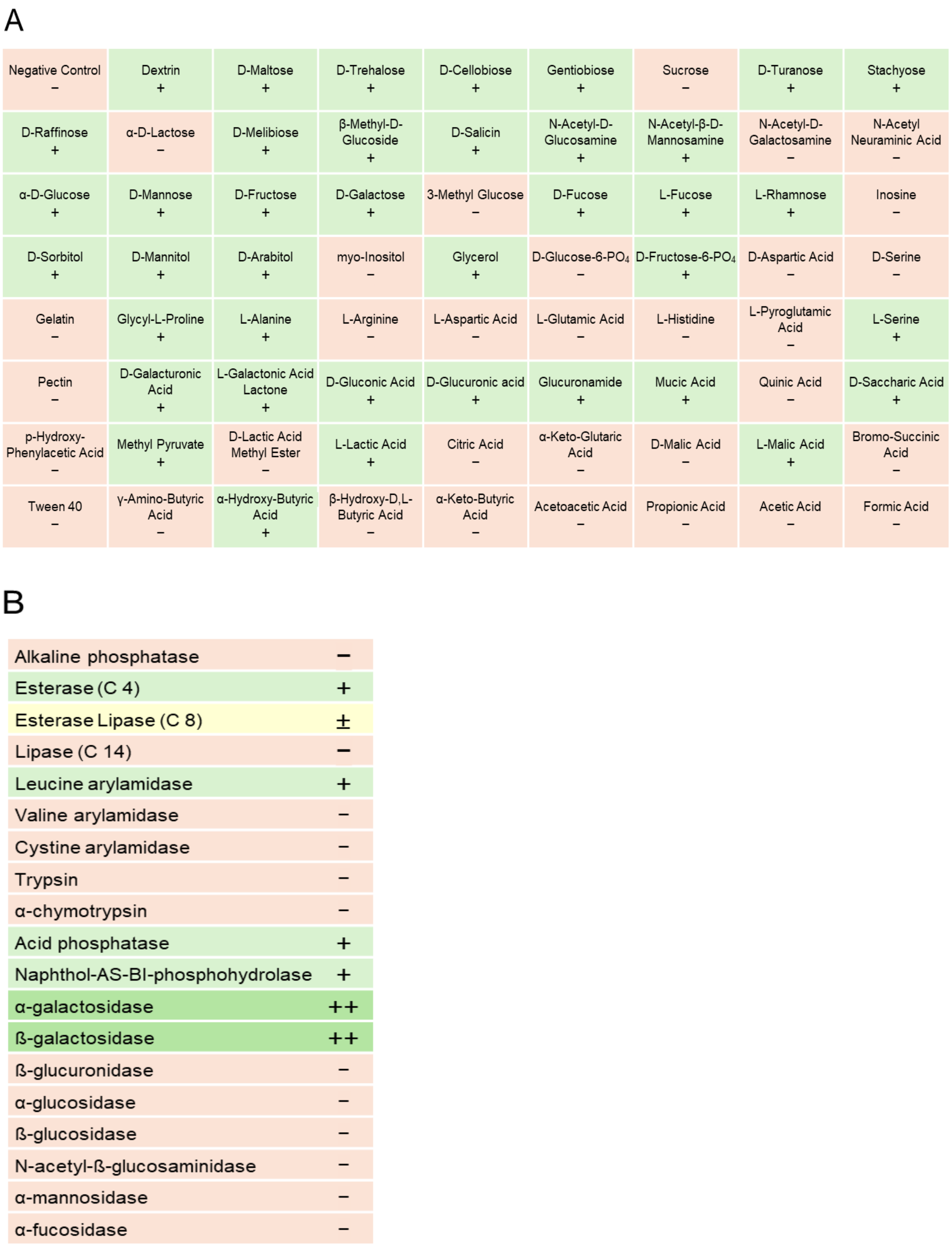
Figure 1 Phylogenetic analysis of Heyndrickxya coagulans LMG S-24828. ***A*** *Phylogram (midpoint rooted tree) based on 16S rRNA gene sequences from strain LMG S-24828 and from the type strains of the species belonging to the Heyndrickxia genus. E. coli and B. subtilis type strains were introduced in the analysis as outgroups.* ***B*** *Phylogram based on the concatenated sequences of the six conserved genes from 9 H. coagulans strains (see "Material and Methods" for details)*

A BLASTN search revealed that the 16S rRNA gene of strain LMG S-24828 shares over 99% sequence identity with DSM 1^T, the type strain of the species *H. coagulans*. A phylogenetic tree based on 16S rRNA gene sequences from the *Heyndrickxia* genus confirmed the assignment of the strain LMG S-24828 to the species *H. coagulans* (Fig. 1A).

Subsequently, we performed multi-locus sequence typing (MLST) using sequences of six conserved genes (*gyrB*, *ilvD*, *ldh-1*, *ldh-2*, *pta*, and *rpoB*) to elucidate the genetic relationship between LMG S-24828 and other *H. coagulans* strains employed as probiotics. This analysis revealed that all nine *H. coagulans* strains (including the type strain) are all closely related (no less than 95% sequence identity) and demonstrated that strain LMG S-24828 is most closely related to strains VHProbiC08 and GBI-30 (Fig. 1B).

4.4.2 Metabolic and Physiological Properties

The ability of *H. coagulans* LMG S-24828 to utilize different carbon sources was assessed using the Biolog Gen III Microplate test. Strain LMG S-24828 metabolized 39 out of 71 substrates, including 7 monosaccharides (α-d-glucose, d-mannose, d-fructose, d-galactose, d-fucose, l-fucose, l-rhamnose), 6 disaccharides (d-maltose, d-trehalose, d-cellobiose, gentiobiose, d-turanose, d-melibiose), and 3 oligosaccharides (dextrin, d-raffinose, stachyose), as well as 4 polyalcohols (d-sorbitol, d-mannitol, d-arabitol, glycerol). In contrast, the disaccharides sucrose and lactose were not metabolized by strain LMG S-24828 in the Biolog Gen III Microplate (Fig. 2A). The ability of *H. coagulans* LMG S-24828 to utilize sucrose and lactose was also tested in experiments where increasing concentrations of these disaccharides were used as the sole carbon source in sugar-free MRS broth (Fig. 3). These experiments showed that strain LMG S-24828 utilized both sucrose and lactose, but only slowly. To explore the hypothesis that the observed limited ability to metabolize lactose might be due to insufficient ATP for the active import of the disaccharide, we cultivated *H. coagulans* LMG S-24828 with lactose in the presence of 0.2% glucose. However, the growth of strain LMG S-24828 was still slow under these conditions, showing a diauxic growth curve (Fig. 3). In contrast, *H. coagulans* LMG S-24828 demonstrated efficient growth when lactulose (4-O-β-d-galactosyl-d-fructose) was provided as the sole carbon source (Fig. 3), highlighting its capability to hydrolyze β(1 → 4) galactosidic bonds. Accordingly, LMG S-24828's genome contains a CDS putatively coding for a protein that shares 96% identity (641/665) and 98% positivity (656/665) with an experimentally validated β-galactosidase enzyme known to hydrolyze lactose (Liu et al., 2019). Furthermore, β-galactosidase activity in this strain was confirmed using the API ZYM kit (Fig. 2B), which assesses hydrolysis of the substrate 2-naphthyl-ßd-galactopyranoside.



A

Negative Control −	Dextrin +	D-Maltose +	D-Trehalose +	D-Cellobiose +	Gentiobiose +	Sucrose −	D-Turanose +	Stachyose +
D-Raffinose +	α-D-Lactose −	D-Melibiose +	β-Methyl-D-Glucoside +	D-Salicin +	N-Acetyl-D-Glucosamine +	N-Acetyl-β-D-Mannosamine +	N-Acetyl-D-Galactosamine −	N-Acetyl Neuraminic Acid −
α-D-Glucose +	D-Mannose +	D-Fructose +	D-Galactose +	3-Methyl Glucose −	D-Fucose +	L-Fucose +	L-Rhamnose +	Inosine −
D-Sorbitol +	D-Mannitol +	D-Arabitol +	myo-Inositol −	Glycerol +	D-Glucose-6-PO_4 −	D-Fructose-6-PO_4 +	D-Aspartic Acid −	D-Serine −
Gelatin −	Glycyl-L-Proline +	L-Alanine +	L-Arginine −	L-Aspartic Acid −	L-Glutamic Acid −	L-Histidine −	L-Pyroglutamic Acid −	L-Serine +
Pectin −	D-Galacturonic Acid +	L-Galactonic Acid Lactone +	D-Gluconic Acid +	D-Glucuronic acid +	Glucuronamide +	Mucic Acid +	Quinic Acid −	D-Saccharic Acid +
p-Hydroxy-Phenylacetic Acid −	Methyl Pyruvate +	D-Lactic Acid Methyl Ester −	L-Lactic Acid +	Citric Acid −	α-Keto-Glutaric Acid −	D-Malic Acid −	L-Malic Acid +	Bromo-Succinic Acid −
Tween 40 −	γ-Amino-Butyric Acid −	α-Hydroxy-Butyric Acid +	β-Hydroxy-D,L-Butyric Acid −	α-Keto-Butyric Acid −	Acetoacetic Acid −	Propionic Acid −	Acetic Acid −	Formic Acid −

B

Alkaline phosphatase	−
Esterase (C 4)	+
Esterase Lipase (C 8)	±
Lipase (C 14)	−
Leucine arylamidase	+
Valine arylamidase	−
Cystine arylamidase	−
Trypsin	−
α-chymotrypsin	−
Acid phosphatase	+
Naphthol-AS-BI-phosphohydrolase	+
α-galactosidase	++
ß-galactosidase	++
ß-glucuronidase	−
α-glucosidase	−
ß-glucosidase	−
N-acetyl-ß-glucosaminidase	−
α-mannosidase	−
α-fucosidase	−

Figure 2. Phenotypic characterization of Heyndrickxya coagulans LMG S-24828. A, results of the biochemical tests performed through the Biolog GEN III Microplate; the metabolized substrates are indicated on a green background. B, enzymatic activity assessment determined through the API ZYM system; -, negative (the activity was not detected); ±, weak activity; +, positive; ++, strongly positive.

H. coagulans LMG S-24828 was also characterized using the API ZYM kit, which assays 19 enzymatic activities simultaneously. This analysis revealed the expression of 6 enzymes by LMG S-24828: esterase (C4 specificity), leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, and ß-galactosidase (Fig. 2B). The observed α-galactosidase activity plausibly supports the ability of LMG S-24828 to metabolize raffinose (Fig. 3). Additionally, a mild esterase/lipase activity was detected when tested with an ester featuring an eight-carbon acyl chain (C8). Importantly, strain LMG S-24828 did not exhibit ß-glucuronidase activity (Fig. 2B).

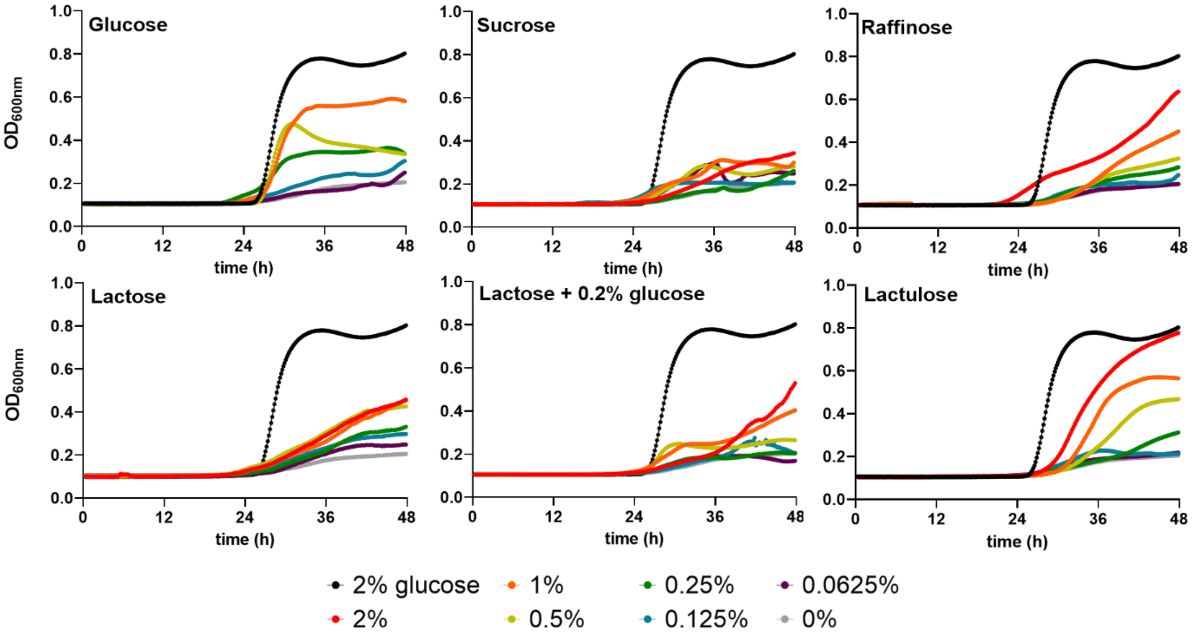


Figure 3 Growth curves of Heyndrickxya coagulans LMG S-24828 in sugar-free MRS supplemented with increasing amounts of different carbohydrates. Growth was monitored over 48 h in 96-well microtiter plates through measuring turbidity spectrophotometrically at 600 nm. In order to maintain readability only the means are shown, but each result is the result of 6 replicates.

In a subsequent experiment, we assessed the ability of *H. coagulans* LMG S-24828 to utilize mucin. For this purpose, we cultivated strain LMG S-24828 in glucose-free MRS supplemented with increasing concentrations of porcine gastric mucin. After 48 h of static incubation under anaerobic conditions with 2% mucin, *H. coagulans* LMG S-24828 significantly increased its growth by 0.9 ± 0.3 (mean ± standard deviation) $\log_{10}$ CFU/ml (on average from 7.0 to 7.9 $\log_{10}$ CFU/ml), which was lower but not statistically significantly different from the increase in cell count obtained in the presence of 2% glucose (+ 1.4 ± 0.4 $\log_{10}$ CFU/ml) (Fig. 4A). In contrast, the probiotic *L. rhamnosus* GG, which was used as a negative control, did not increase its growth when mucin was added to the same culture medium (Fig. 4B).

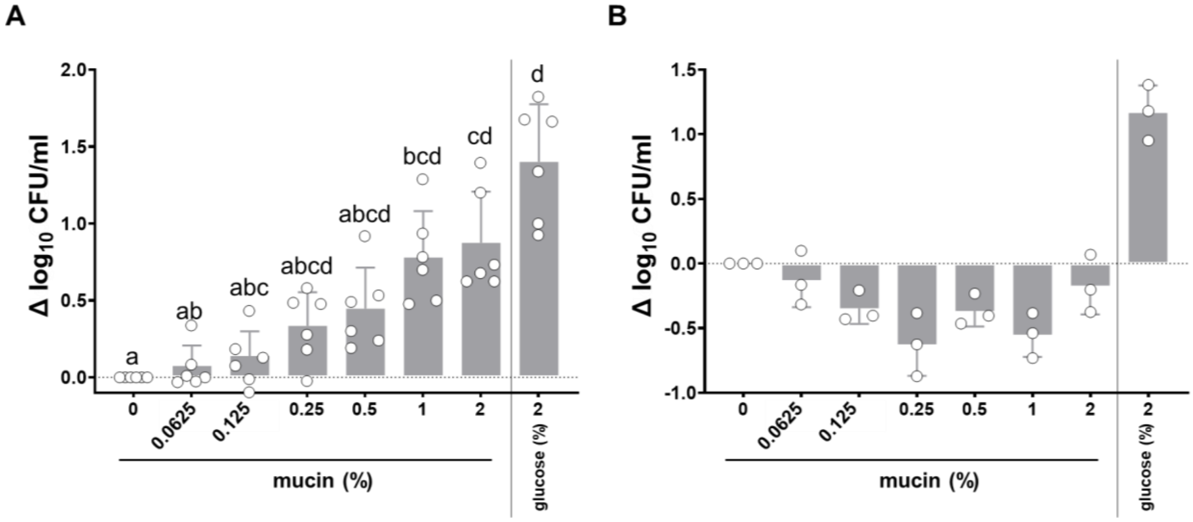


Figure 4 Modification of viable counts of Heyndrickxia coagulans LMG S-24828 (panel A) and Lacticaseibacillus rhamnsosus GG (panel B) after 48 h incubation in MRS broth without glucose, supplemented with porcine gastric mucin. MRS with 2% glucose was used as a positive control. Histograms represent the mean ± standard deviation of data collected from six (panel A) or three (panel B) independent experiments. Each dot in the graph indicates the result of a different experiment. Different lowercase letters indicate significant differences at $p < 0.05$ *according to the Kruskal-Wallis nonparametric ANOVA with Dunn's multiple comparisons test.*

4.4.3 In Vitro Safety Assessment

Several *in vitro* tests were carried out in order to assess the safety of *H. coagulans* LMG S-24828, including antibiotic resistance profile, D-lactate production, bile-salt hydrolysis, hemolytic activity, and toxicity toward human epithelial cells.

The presence of antibiotic resistance genes (ARGs) was initially assessed *in silico* within the draft genome of the LMG S-24828 strain using the CARD bioinformatic tool. This analysis did not identify any "perfect" hits but revealed the presence of four "strict" hits associated with ARGs. Specifically, three hits corresponded to the genes *vanT* (35% Identity of Matching Region, IMR), *vanY*, and *vanH* (40% IMR), which confer resistance to vancomycin by altering the antibiotic target. Additionally, one hit corresponded to the gene *qacJ* (40% IMR), which encodes a small multidrug resistance efflux pump conferring resistance to quaternary ammonium compounds (Supplementary Table S2). A subsequent BLAST search confirmed the presence of these four potential ARGs in the genomes of several other *H. coagulans* strains (not shown).

Subsequently, the presence of acquired (potentially transmissible) antibiotic resistances was investigated using the ISO 10932 IDF223:210 protocol recommended by the EFSA. None of the minimum inhibitory concentrations (MICs) determined for strain LMG S-24828 exceeded the EFSA breakpoints for the nine antibiotics recommended in the EFSA guidelines (EFSA PoA, 2012), including vancomycin (Table 1).

Subsequent experiments showed that *H. coagulans* LMG S-24828 does not produce D-lactate (Fig. 5A), is unable to de-conjugate primary bile salts from oxgall (Fig. 5B), and did not exhibit α- or β-hemolytic activity (Fig. 5C). Furthermore, the safety of *H. coagulans* LMG S-24828 was assessed by incubating the industrial spore preparation and the vegetative forms of this bacterial strain with fully differentiated Caco-2 cells, in which inflammatory stress had been induced by IL-1β. After 24 h of incubation, neither the spores nor the vegetative forms of LMG S-24828 altered the activation of the inflammatory transcription factor NF-κB (Fig. 5D) and did not cause damage to the stressed enterocyte cell layer in vitro.

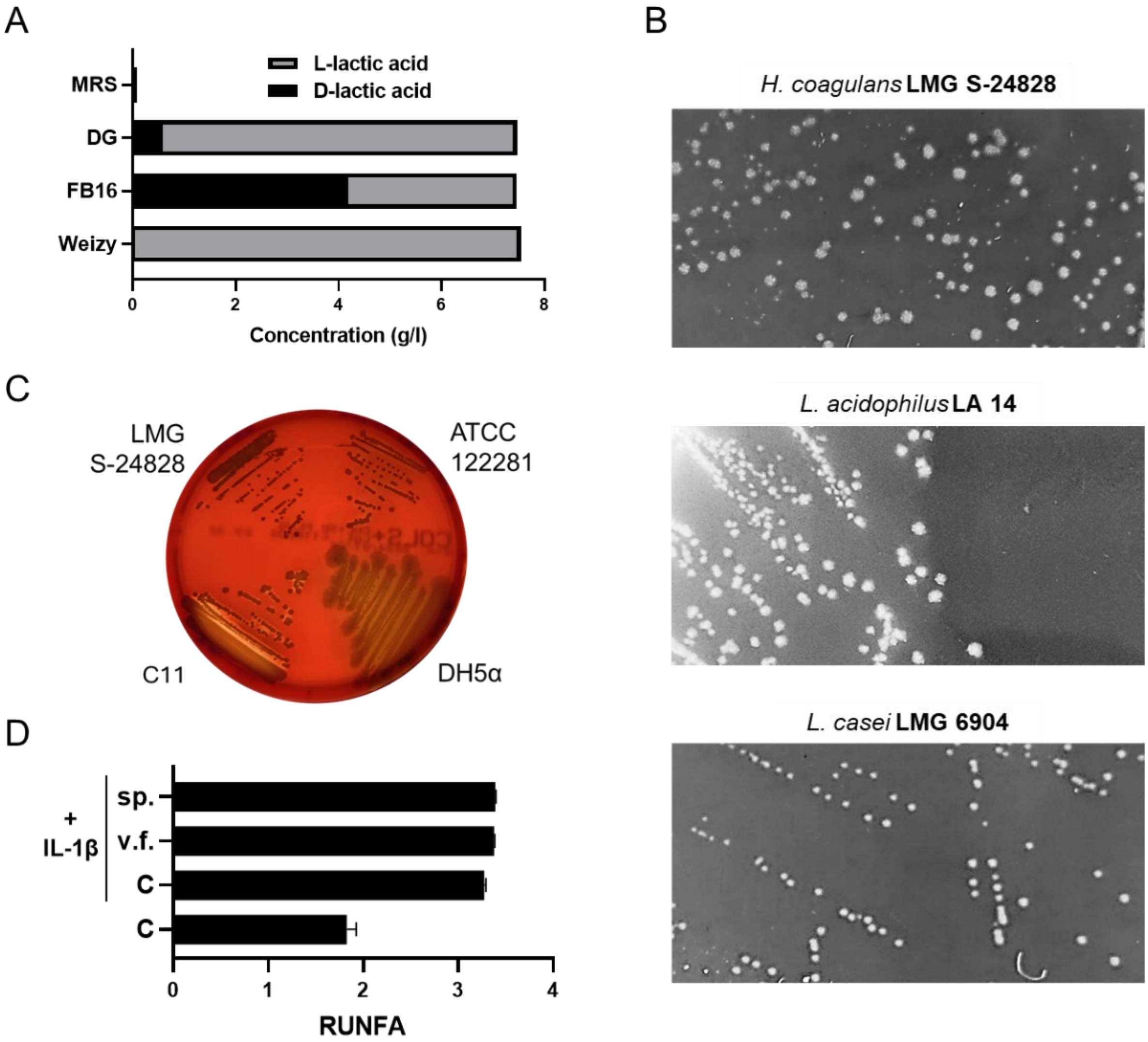


Figure 5 In vitro assessment of Heyndrickxia coagulans *LMG S-24828 phenotypes associated witrh safety. A, enzymatic-colorimetric quantification of lactate enantiomers in MRS broth;* Weissella cibari*a FB16 and* Lacticaseibacillus paracasei *DG were used as positive and negative controls for D-lactate production, respectively. B, detection of bile salt hydrolase (BSH) activity in MRS agar supplemented with 0.5 % (wt/vol) oxgall after 48 h of incubation at 37 °C under anaerobic conditions. The precipitates around colonies are indicative of BSH activity*; Lactobacillus acidophilus *LA 14 and* Lacticaseibacillus *casei LMG 6904 were used as positive and negative controls for BSH activity, respectively. C, hemolytic activity assessed on Columbia blood agar after 48 h of incubation at 37°C in anaerobic conditions;* Staphylococcus epidermidis *ATCC 122281,* Escherichia coli *DH5α and* Streptococcus pyogenes *C11 were used as controls for γ, α and β-hemolytic activity, respectively. D, activation of the transcriptional regulator NF-κB in a fully differentiated Caco-2 cell layer transfected with an alkaline phosphatase (SEAP) reporter vector; SEAP activity was measured after incubation of the Caco-2 cell layer with IL-1 β stimulation (2 ng/ml). RUNFA, Relative Units of NF-κB Activation, calculated as normalized SEAP*

4.4.4 Survival, Adhesion, and Germination in the Gastrointestinal Tract

The capacity to survive gastrointestinal transit was initially assessed using the INFOGEST static *in vitro* simulated gastrointestinal digestion with an industrial freeze-dried preparation of *H. coagulans* LMG S-24828. The viable count of this preparation remained the same before and after pasteurization, indicating that the only viable microbes in this preparation were exclusively endospores. The results of the INFOGEST experiment evidenced a marked capacity for survival of the LMG S-24828 strain since the viable count did not change significantly (from 11.18 to 11.15 $\log_{10}$ CFU/g). In addition, the viable count calculated after pasteurization of the samples at 90 °C for 10 minutes revealed that pasteurization-sensitive CFUs increased from 3 to 17%, suggesting that part of the spores germinated during the INFOGEST experiment (Fig. 6A).

The germination ability of spores of LMG S-24828's was also assessed by incubating the industrial freeze-dried preparation in a human fecal sample, and subsequently performing the viable count with pasteurization (to quantify spores) and without pasteurization at different time points of incubation at 37 °C under anaerobic conditions. The results showed that already after 15 min the amount of pasteurization-sensitive CFUs increased from 3% to 86%, indicating that most of the cells had germinated (Fig. 6B). After 3 h, this quantification increased to 91% and remained at 87% after 18 h of incubation (Fig. 6B).

Subsequently, the ability of *H. coagulans* LMG S-24828 to survive and germinate during gastrointestinal transit was studied in a pilot human *in vivo* trial. This trial involved analyzing fecal samples collected from five healthy adult volunteers after 3-day ingestion of one capsule per day containing 6 billion CFUs of LMG S-24828 spores and no vegetative forms. Viable LMG S-24828 (i.e., microbial forms able to generate colony) was detected in all five fecal samples (mean ± standard deviation, 6.3 ± 0.2 $\log_{10}$ CFU/g of feces) demonstrating the ability of this bacterial strain to survive gastrointestinal transit. Furthermore, pasteurization-sensitive forms were present in all samples (from 10% to 78% of total CFUs), suggesting that LMG S-24828's spores had germinated in the gut (Fig. 6C).

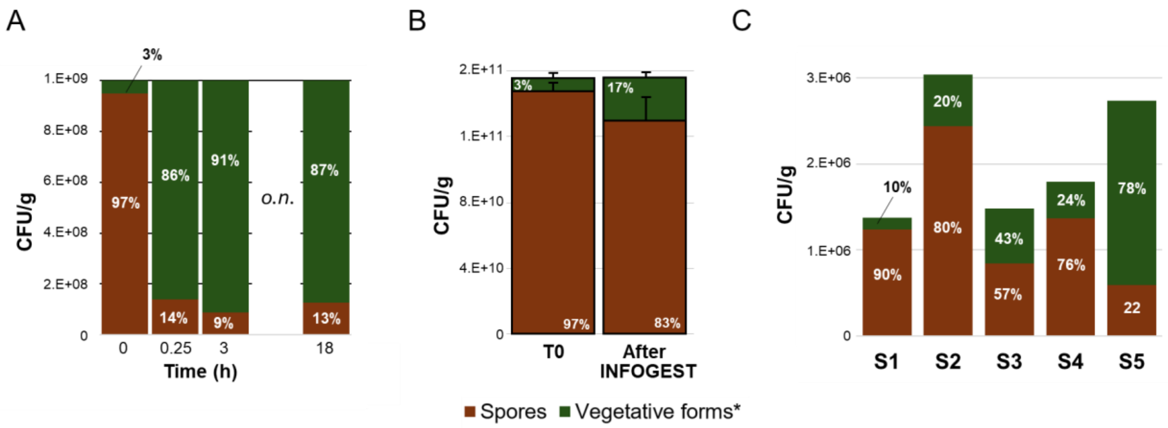


Figure 6 Survival and germination of Heyndrickxia coagulans *LMG S-24828 spores in the gastrointestinal tract. A, spores and vegetative forms before and after the INFOGEST simulated gastrointestinal transit. B, spores and vegetative forms determined during incubation at 37°C in a human fecal sample. C, spores and vegetative forms recovered from the feces of five healthy volunteers after three days of LMG S-24828 intake. *, vegetative forms have been determined as pasteurization-sensitive units.*

Finally, we employed differentiated Caco-2 cell layers to qualitatively assess the adhesion ability of *H. coagulans* LMG S-24828. The experiment was performed with the bacterial endospores from the industrial freeze-dried preparation and with freshly prepared vegetative forms of the bacterium. Both spores and vegetative forms adhered to Caco-2 cells. Nonetheless, the adhesive phenotype was particularly evident for LMG S-24828's endospores (Fig. 7). The adhesion ability of *L. rhamnosus* GG, introduced as a control, did not appear dissimilar to that of LMG S-24828's cells (not shown).

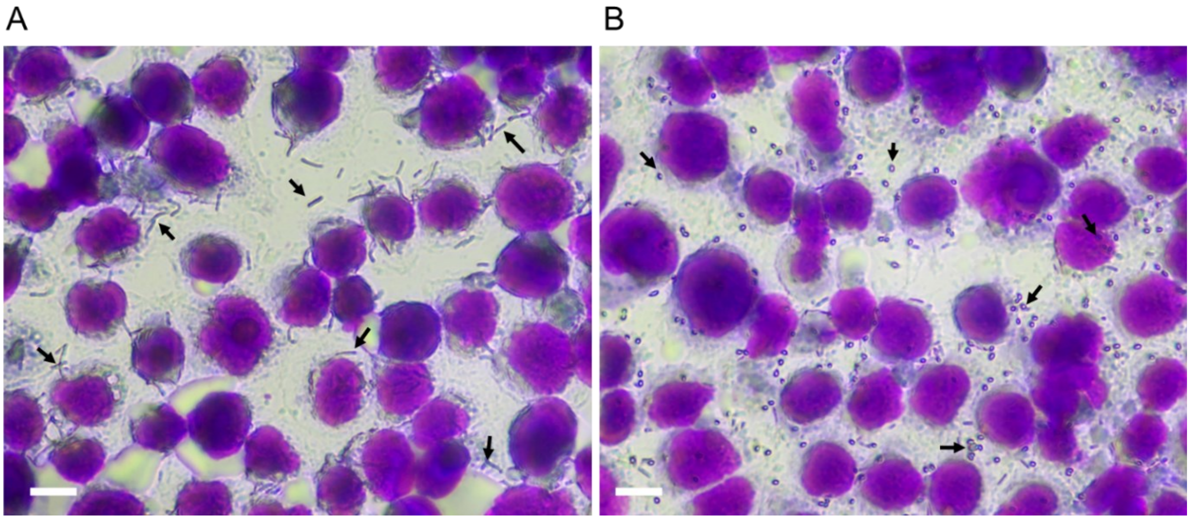


Figure 7 Adhesion of Heyndrickxia coagulans *LMG S-24828 to a Caco-2 cell monolayer as observed with Giemsa staining under a light microscope. A, adhesion of LMG S-24828 vegetative forms after cultivation overnight in MRS broth at 37 °C. B, adhesion of LMG S-24828 endospores in an industrial freeze-dired preparation. White magnification bars, 5 μm. Black arrows indicate vegetative forms in panel A and endoposres in panel B.*

4.5 Discussion

The identification of a new bacterial strain for probiotic use requires an initial assessment that includes (i) its taxonomic classification, (ii) its safety profile, and (iii) its ability to survive gastrointestinal transit (Binda et al., 2020). Obtaining the genome of the bacterium under study has become an essential prerequisite for acquiring this information. Therefore, in our study, to determine its probiotic potential, we first sequenced the draft genome of LMG S-24828, a bacterial strain initially isolated from healthy human feces.

The genomic analysis of strain LMG S-24828 unambiguously confirmed its taxonomic placement within the *H. coagulans* species. Furthermore, molecular typing revealed a close genetic relationship with other probiotic strains, specifically VHProbiC08 and GBI-30. Notably, GBI-30, 6068 (BC30), is one of the most studied *H. coagulans* strains. It has been shown to reduce the average number of bowel movements per day in IBS-D patients (Dolin, 2009), decrease upper respiratory and gastrointestinal tract symptoms in school-aged children (Anaya-Loyola et al., 2019), improve amino acid absorption from plant protein in older women (Walden et al., 2024), and ameliorate clinical symptoms in rheumatoid arthritis patients (Mandel et al., 2010). Given the high genetic affinity, we can speculate that LMG S-24828 may share similar beneficial properties. However, as emphasized in the introduction, probiotic characteristics can vary significantly between strains, necessitating specific evaluations of each strain's properties. Genomic analysis also revealed that LMG S-24828 does not possess plasmid molecules. The absence of extrachromosomal DNA can ensure greater genetic stability and reduce the risk of genetic drift (Sanders et al., 2014).

Various qualified sources, including FAO guidelines (FAO/WHO, 2002), emphasize that even if a probiotic strain belongs to a species with a long history of safe use, it is necessary to test at the strain level other aspects of potential harm to human health. The most important of these is the potential presence of transmissible antibiotic resistances, which could be acquired by other bacteria in the human gastrointestinal tract, leading to an increase in the intestinal and environmental resistome (Fredriksen et al., 2023). In this context, the European Food Safety Authority has drafted a guidance document for assessing the resistance profile toward selected antibiotics of human and veterinary importance. This is based on defining microbiological cut-off values to distinguish resistant from susceptible strains (EFSA PoA, 2012; EPo Additives et al., 2018). Following the protocol indicated in these guidelines, we verified that *H. coagulans* LMG S-24828 is sensitive to antibiotic concentrations below the breakpoints for all tested antibiotics, including vancomycin, although three CDS in the genome showed partial similarity to three genes known to encode enzymes that confer resistance to this antibiotic by modifying its target. Nonetheless, these CDS, along with a CDS showing similarity to a putative small multidrug resistance efflux pump, have a sequence identity of 35–40% with the corresponding known genes and are present also in the genomes of several other *H.*

coagulans strains, including the probiotic strain GBI-30. In general, our data suggest that *H. coagulans* LMG S-24828 is a safe strain regarding transmissible antibiotic resistances.

According to FAO/WHO guidelines, the safety of a probiotic strain should also be determined through the assessment of certain metabolic activities, such as D-lactate production, bile salt deconjugation, and hemolytic activity (FAO/WHO, 2002). D-Lactate production by probiotics has been suggested to promote D-lactic acidosis, potentially leading to brain fogginess (Rao et al., 2018). Although this hypothesis is not convincingly supported by experimental data for healthy individuals (both adults and children; (Quigley et al., 2018; Papagaroufalis et al., 2014), it is potentially substantiated in susceptible individuals with conditions such as short bowel syndrome (Munakata et al., 2010). However, *H. coagulans* LMG S-24828 was found to be incapable of producing D-lactate. LMG S-24828 was also unable to deconjugate primary bile salts. Bile salt deconjugation is commonly present in several bacteria used as probiotics that express the bile salt hydrolase (BSH) enzyme (Hernández-Gómez et al., 2021). The ability to deconjugate bile salts by probiotics has been proposed as a strategy to reduce cholesterol absorption in hypercholesterolemic subjects (Jones et al., 2012; Malpeli et al., 2015). Nonetheless, it has been proposed that deconjugation can lead to reduced absorption of unconjugated bile salts, with various negative consequences for host health (Gadaleta et al., 2022; Bourgin et al., 2021). Additionally, as reported for other members of the *H. coagulans* species, LMG S-24828 did not exhibit α- and β-hemolytic activity. The safety of *H. coagulans* LMG S-24828 was also evidenced by the absence of β-glucuronidase, a bacterial enzymatic activity associated with the reactivation of potentially harmful glucuronidated compounds in the gut, which has been proposed to be involved in the onset of colorectal cancer (Kim et al., 2001). In contrast, *H. coagulans* LMG S-24828 expresses enzymes such as α-galactosidase and β-galactosidase, supporting the strain's capability to hydrolyze complex carbohydrates, enhancing its functional role in the gut. These enzymatic capabilities could potentially be involved in the observed ability of LMG S-24828 to metabolize mucin, which can be important for promoting an active metabolism during gastrointestinal transit (Raba & Luis, 2023). In support of the ability to metabolize mucin, we found in the genome of LMG S-24828 four CDS putatively coding for enzymes belonging to the glycosyl hydrolase families GH31 (3 CDS, α-*N*-acetylgalactosaminidase) and GH42 (1 CDS, galactosidase), which are involved in the degradation of the glycan chains of mucin (Labourel et al., 2023).

Finally, the safety of *H. coagulans* LMG S-24828 was assessed using a differentiated Caco-2 cell layer, which serves as a widely accepted model for the intestinal epithelium. The experiments conducted further demonstrated the safety of this bacterial strain, as both spores and vegetative cells did not cause any damage to the Caco-2 cell layer, even in the presence of a potent pro-inflammatory stimulus such as IL-1β. This is evidenced by the absence of increased NF-κB activation, which is a key mediator of cellular responses to inflammation and stress in enterocytes (Lea, 2015).

An essential characteristic of a probiotic is the ability to survive transit and be metabolically active at the intestinal level. Studies of viable recovery in feces have been conducted for only a limited number of known probiotic strains, such as *Bifidobacterium animalis* subsp. *lactis* BB12, *L. rhamnosus* GG, and *Lacticaseibacillus paracasei* Shirota, and *L. paracasei* DG (Granata et al., 2013; Poutsiaka et al., 2017; Wang et al., 2015; Arioli et al., 2018). As regards spore-forming probiotics, since these bacteria are generally ingested as spores, there is a question as to whether the ingested spores can actually germinate in the intestine. This ability was demonstrated in a recent viable recovery study for the *H. coagulans* strain SNZ1969, ingested by 24 healthy adult subjects through gummy candies, where the germination of spores was verified in terms of pasteurization-sensitive CFUs (Perotti et al., 2024). Using the same experimental approach, we verified both *in vitro* and *in vivo* the ability of *H. coagulans* LMG S-24828 to survive gastrointestinal transit and significantly germinate. In the *in vivo* human trial, pasteurization-sensitive CFUs varied considerably among subjects but were present in all subjects, as previously reported (Perotti et al., 2024). It should also be considered that this data might be underestimated because some spores could result from the re-sporulation of vegetative forms derived from the initial germination. The ability of *H. coagulans* LMG S-24828 spores to germinate in the intestine may also be favored by their capacity to adhere to the intestinal epithelium, as verified in this study using a differentiated Caco-2 cell layer. A good adhesion capacity of *H. coagulans* spores was also reported for strain MTCC 5856 on HT-29 cells (Shinde et al., 21049). Notably, in our study, we also observed that the spores of this bacterium demonstrated an ability to germinate in human feces within a few minutes (approximately 85% of the spores had germinated in 15 min), which is in line with other studies, reporting that bacilli spores can germinate within minutes (Latorre et al., 2014; Setlow, 2014).

In conclusion, the findings of this study contribute to the growing body of knowledge on *H. coagulan*s as a potential probiotic, specifically focusing on the LMG S-24828 strain. Through genomic, physiological, and functional characterization, our results highlight several key attributes that underscore the probiotic potential of *H. coagulans* LMG S-24828. Specifically, *H. coagulans* LMG S-24828 exhibits several desirable characteristics for a probiotic strain, including metabolic versatility, safety, resistance to gastrointestinal conditions, germination ability, and adhesion to intestinal cells. These properties indicate that LMG S-24828 has significant potential for use in probiotic formulations, warranting further investigation in clinical trials to fully elucidate its health benefits.

4.6 Data Availability

The datasets generated and/or analyzed in this study are available from the corresponding author upon reasonable request.

4.6 References

Abdhul K, Ganesh M, Shanmughapriya S, Vanithamani S, Kanagavel M, Anbarasu K, et al. (2015) Bacteriocinogenic potential of a probiotic strain Bacillus coagulans [BDU3] from Ngari. *Int J Biol Macromol* 79:800–806. https://doi.org/10.1016/j.ijbiomac.2015.06.005

Alcock BP, Huynh W, Chalil R, Smith KW, Raphenya AR, Wlodarski MA, et al. (2023) CARD 2023: expanded curation, support for machine learning, and resistome prediction at the Comprehensive Antibiotic Resistance Database. *Nucleic Acids Res* 51(D1):D690–D699. https://doi.org/10.1093/nar/gkac920

Alkan H, Baysal Z, Uyar F, Dogru M (2007) Production of lipase by a newly isolated Bacillus coagulans under solid-state fermentation using melon wastes. *Appl Biochem Biotechnol* 136(2):183–192. https://doi.org/10.1007/bf02686016

Anaya-Loyola MA, Enciso-Moreno JA, López-Ramos JE, García-Marín G, Orozco Álvarez MY, Vega-García AM, et al. (2019) Bacillus coagulans GBI-30, 6068 decreases upper respiratory and gastrointestinal tract symptoms in healthy Mexican scholar-aged children. *Food Res Int* 125:108567. https://doi.org/10.1016/j.foodres.2019.108567

Arioli S, Koirala R, Taverniti V, Fiore W, Guglielmetti S (2018) Quantitative recovery of viable Lactobacillus paracasei CNCM I-1572 (L. casei DG®) after gastrointestinal passage in healthy adults. *Frontiers in microbiology* 9:1720. https://doi.org/10.3389/fmicb.2018.01720

Aulitto M, Fusco S, Bartolucci S, Franzén CJ, Contursi P (2017) Bacillus coagulans MA-13: a promising thermophilic and cellulolytic strain for the production of lactic acid from lignocellulosic hydrolysate. *Biotechnol Biofuels* 10:210. https://doi.org/10.3390/ijms23063135

Aulitto M, Martinez-Alvarez L, Fiorentino G, Limauro D, Peng X, Contursi P (2022) A comparative analysis of Weizmannia coagulans genomes unravels the genetic potential for biotechnological applications. *Int J Mol Sci* 23(6).

Begley M, Hill C, Gahan CG (2006) Bile salt hydrolase activity in probiotics. *Appl Environ Microbiol* 72(3):1729–1738. https://doi.org/10.1128/aem.72.3.1729-1738.2006

Binda S, Hill C, Johansen E, Obis D, Pot B, Sanders ME, Tremblay A, Ouwehand AC (2020) Criteria to qualify microorganisms as “probiotic” in foods. *Front Microbiol* 11:1662. https://doi.org/10.3389/fmicb.2020.01662

Bourgin M, Kriaa A, Mkaouar H, Mariaule V, Jablaoui A, Maguin E, et al. (2021) Bile salt hydrolases: at the crossroads of microbiota and human health. *Microorganisms* 9(6):1122. https://doi.org/10.3390/microorganisms9061122

Britton RA, Hoffmann DE, Khoruts A (2021) Probiotics and the microbiome: how can we help patients make sense of probiotics? *Gastroenterology* 160(2):614–623. https://doi.org/10.1053/j.gastro.2020.11.047

Brodkorb A, Egger L, Alminger M, Alvito P, Assunção R, Ballance S, et al. (2019) INFOGEST static in vitro simulation of gastrointestinal food digestion. *Nat Protoc* 14(4):991–1014. https://doi.org/10.1038/s41596-018-0119-1

Brunelli L, De Vitis V, Ferrari R, Minuzzo M, Fiore W, Jäger R, et al. (2022) In vitro assessment of the probiotic properties of an industrial preparation containing Lacticaseibacillus paracasei in the context of athlete health. *Front Pharmacol* 13:857987. https://doi.org/10.3389/fphar.2022.857987

Chen Z, Tang Z, Li W, Deng X, Yu L, Yang J, et al. (2024) Weizmannia coagulans BCF-01: a novel gastrogenic probiotic for Helicobacter pylori infection control. *Gut Microbes* 16(1):2313770. https://doi.org/10.1080/19490976.2024.2313770

Choi H, Mun D, Ryu S, Kwak M-J, Kim B-K, Park D-J, et al. (2024) Spore-forming probiotics Weizmannia coagulans isolated from human breast milk improves on the flavor development using micro-cheese platform. *Food Biosci* 57:103550. https://doi.org/10.1016/j.fbio.2023.103550

Chopra AK, Mathur DK (1984) Isolation, screening and characterization of thermophilic Bacillus species isolated from dairy products. *J Appl Bacteriol* 57(2):263–271. https://doi.org/10.1111/j.1365-2672.1984.tb01390.x

Dolin BJ (2009) Effects of a proprietary Bacillus coagulans preparation on symptoms of diarrhea-predominant irritable bowel syndrome. *Methods Find Exp Clin Pharmacol* 31(10):655–659. https://doi.org/10.1358/mf.2009.31.10.1441078

EFSA PoA, Products or substances used in animal feed (2012) Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance. *EFSA Journal* 10(6):2740. https://doi.org/10.2903/j.efsa.2012.2740

EFSA (2025) Consultation request for the determination of novel food status pursuant to Article 4(2) of the Regulation (EU) 2015/2283 of the European Parliament and of the Council of 25 November 2015 on novel foods https://doi.org/10.2903/j.efsa.2012.2740

EPo Additives, Feed PoSuiA, Rychen G, Aquilina G, Azimonti G, Bampidis V, et al. (2018) Guidance on the characterisation of microorganisms used as feed additives or as production organisms. *EFSA Journal* 16(3):e05206. https://doi.org/10.2903/j.efsa.2018.5206d

FAO/WHO (2002) Report of a joint FAO/WHO expert consultation on guidelines for the evaluation of probiotics in food. World Health Organization and Food and Agriculture Organization of the United Nations, London Ontario, Canada.

Fredriksen S, de Warle S, van Baarlen P, Boekhorst J, Wells JM (2023) Resistome expansion in disease-associated human gut microbiomes. *Microbiome* 11(1):166. https://doi.org/10.1186/s40168-023-01610-1

Fusco V, Quero GM, Cho GS, Kabisch J, Meske D, Neve H, et al. (2015) The genus Weissella: taxonomy, ecology and biotechnological potential. *Front Microbiol* 6:155. https://doi.org/10.3389/fmicb.2015.00155

Gadaleta RM, Cariello M, Crudele L, Moschetta A (2022) Bile salt hydrolase-competent probiotics in the management of IBD: unlocking the “Bile Acid Code”. *Nutrients* 14(15). https://doi.org/10.3390/nu14153212.

Granata M, Brandi G, Borsari A, Gasbarri R, Gioia DD (2013) Synbiotic yogurt consumption by healthy adults and the elderly: the fate of bifidobacteria and LGG probiotic strain. Int J Food Sci Nutr 64(2):162–168. https://doi.org/10.3109/09637486.2012.718742

Guglielmetti S, Karp M, Mora D, Tamagnini I, Parini C (2007) Molecular characterization of Bifidobacterium longum biovar longum NAL8 plasmids and construction of a novel replicon screening system. *Appl Microbiol Biotechnol* 74(5):1053–1061. https://doi.org/10.1007/s00253-006-0755-1

Guglielmetti S, Tamagnini I, Mora D, Minuzzo M, Scarafoni A, Arioli S, et al. (2008) Implication of an outer surface lipoprotein in adhesion of Bifidobacterium bifidum to Caco-2 cells. *Appl Environ Microbiol* 74(15):4695–4702. https://doi.org/10.1128/aem.00124-08

Guglielmetti S, Taverniti V, Minuzzo M, Arioli S, Stuknyte M, Karp M, et al. (2010) Oral bacteria as potential probiotics for the pharyngeal mucosa. *Appl Environ Microbiol* 76(12):3948–3958. https://doi.org/10.1128/aem.00109-10

Hazards EPOB, Koutsoumanis K, Allende A, Alvarez-Ordóñez A, Bolton D, Bover-Cid S, et al. (2024) Update of the list of qualified presumption of safety (QPS) recommended microbiological agents intentionally added to food or feed as notified to EFSA 19. *EFSA Journal* 22(1):e8517. https://doi.org/10.2903/j.efsa.2024.8517

Hernández-Gómez JG, López-Bonilla A, Trejo-Tapia G, Ávila-Reyes SV, Jiménez-Aparicio AR, Hernández-Sánchez H (2021) In vitro bile salt hydrolase (BSH) activity screening of different probiotic microorganisms. *Foods* 10(3). https://doi.org/10.3390/foods10030674

Hill C, Guarner F, Reid G, Gibson GR, Merenstein DJ, Pot B, et al. (2014) The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nat Rev Gastroenterol Hepatol* 11(8):506–514. https://doi.org/10.1038/nrgastro.2014.66

Hsieh RH, Chien YJ, Lan WY, Lin YK, Lin YH, Chiang CF, et al. (2024) Bacillus coagulans TCI711 supplementation improved nonalcoholic fatty liver by modulating gut microbiota: a randomized, placebo-controlled clinical trial. *Curr Dev Nutr* 8(3):102083. https://doi.org/10.1016/j.cdnut.2024.102083

Jones ML, Martoni CJ, Parent M, Prakash S (2012) Cholesterol-lowering efficacy of a microencapsulated bile salt hydrolase-active Lactobacillus reuteri NCIMB 30242 yoghurt formulation in hypercholesterolaemic adults. *Br J Nutr* 107(10):1505–1513. https://doi.org/10.1017/s0007114511004703

Kallur RK, Madapati S, Mathur A, Bhattacharya S (2023) The role of Weizmannia (Bacillus) coagulans LMG S-31876 in treating IBS-diarrhea. *Front Nutr* 10:1310462. https://doi.org/10.3389/fnut.2023.1310462

Kang S, Park MY, Brooks I, Lee J, Kim SH, Kim JY, et al. (2021) Spore-forming Bacillus coagulans SNZ 1969 improved intestinal motility and constipation perception mediated by microbial alterations in healthy adults: a randomized controlled trial. *Food Res Int* 146:110428. https://doi.org/10.1016/j.foodres.2021.110428

Kim DH, Jin YH (2001) Intestinal bacterial beta-glucuronidase activity of patients with colon cancer. *Arch Pharm Res* 24(6):564–567. https://doi.org/10.1007/bf02975166

Konuray Altun G, Erginkaya Z (2021) Identification and characterization of Bacillus coagulans strains for probiotic activity and safety. *LWT* 151:112233. https://doi.org/10.1016/j.lwt.2021.112233

Latorre JD, Hernandez-Velasco X, Kallapura G, Menconi A, Pumford NR, Morgan MJ et al (2014) Evaluation of germination, distribution, and persistence of Bacillus subtilis spores through the gastrointestinal tract of chickens. *Poult Sci* 93(7):1793–1800. https://doi.org/10.3382/ps.2013-03809

Labourel A, Parrou JL, Deraison C, Mercier-Bonin M, Lajus S, Potocki-Veronese G (2023) O-Mucin-degrading carbohydrate-active enzymes and their possible implication in inflammatory bowel diseases. *Essays Biochem* 67(3):331–344. https://doi.org/10.1042/ebc20220153

Lea T (2015) Caco-2 cell line. In: Verhoeckx K, Cotter P, López-Expósito I, Kleiveland C, Lea T, Mackie A, et al. (eds) *The impact of food bioactives on health: in vitro and ex vivo models*. Springer International Publishing, Cham, pp 103-111

Li Z, Guan Z, Bai N, Yan Y, Niu Z, Xu J, et al. (2022) Bacillus coagulans TBC169 probiotics for the recovery of intestinal function after gynecological laparoscopic surgery: a randomized, placebo-controlled trial. *Int J Clin Pharm* 44(6):1287–1295. https://doi.org/10.1007/s11096-022-01451-2

Liang J, Li C, Chen Z, Guo F, Dou J, Wang T, et al. (2024) Progress of research and application of Heyndrickxia coagulans (Bacillus coagulans) as probiotic bacteria. *Front Cell Infect Microbiol* 14:1415790. https://doi.org/10.3389/fcimb.2024.1415790

Liu P, Xie J, Liu J, Ouyang J (2019) A novel thermostable β-galactosidase from Bacillus coagulans with excellent hydrolysis ability for lactose in whey. *J Dairy Sci* 102(11):9740–9748. https://doi.org/10.3168/jds.2019-16654

Majeed M, Nagabhushanam K, Arumugam S, Chadalavada N, Seepana J, Annamalai T, et al. (2023) Probiotic Weizmannia coagulans MTCC 5856 as adjunct therapy in children's acute diarrhea: a randomized, double-blind, placebo-controlled study. *Front Pediatr* 11:1338126. https://doi.org/10.3389/fped.2023.1338126

Majeed M, Nagabhushanam K, Natarajan S, Sivakumar A, Ali F, Pande A, et al. (2016) Bacillus coagulans MTCC 5856 supplementation in the management of diarrhea predominant irritable bowel syndrome: a double blind randomized placebo controlled pilot clinical study. *Nutr J* 15:21. https://doi.org/10.1186/s12937-016-0140-6

Malpeli A, Taranto M, Cravero R, Tavella M, Fasano V, Vicentin D, et al. (2015) Effect of daily consumption of Lactobacillus reuteri CRL 1098 on cholesterol reduction in hypercholesterolemic subjects. *Food Nutr Sci* 6:1583–1590. https://doi.org/10.4236/fns.2015.617163

Mandel DR, Eichas K, Holmes J (2010) Bacillus coagulans: a viable adjunct therapy for relieving symptoms of rheumatoid arthritis according to a randomized, controlled trial. *BMC Complement Altern Med* 10:1. https://doi.org/10.1186/1472-6882-10-1

Ministero della Salute (2018) Direzione generale per l'igiene e la sicurezza degli alimenti e la nutrizione - Ufficio 4. Guidelines on probiotics and prebiotics, revised March 2018. https://www.salute.gov.it/imgs/C_17_pubblicazioni_1016_ulterioriallegati_ulterioreallegato_0_alleg.pdf

Munakata S, Arakawa C, Kohira R, Fujita Y, Fuchigami T, Mugishima H (2010) A case of D-lactic acid encephalopathy associated with use of probiotics. *Brain Dev* 32(8):691–694. https://doi.org/10.1016/j.braindev.2009.09.024

O'Flaherty S, Briner Crawley A, Theriot CM, Barrangou R (2018) The Lactobacillus bile salt hydrolase repertoire reveals niche-specific adaptation. *mSphere* 3(3). https://doi.org/10.1128/mSphere.00140-18

Papagaroufalis K, Fotiou A, Egli D, Tran LA, Steenhout P (2014) A randomized double blind controlled safety trial evaluating D-lactic acid production in healthy infants fed a Lactobacillus reuteri-containing formula. *Nutr Metab Insights* 7:19–27. https://doi.org/10.4137/nmi.s14113

Perotti S, Mantegazza G, Pierallini E, Kirika N, Duncan R, Telesca N, et al. (2024) Human in vivo assessment of the survival and germination of Heyndrickxia coagulans SNZ1969 spores delivered via gummy candies. *Curr Res Food Sci* 9:100793. https://doi.org/10.1016/j.crfs.2024.100793
Poutsiaka DD, Mahoney IJ, McDermott LA, Stern LL, Thorpe CM, Kane AV et al (2017) Selective method for identification and quantification of Bifidobacterium animalis subspecies lactis BB-12 (BB-12) from the gastrointestinal tract of healthy volunteers ingesting a combination probiotic of BB-12 and Lactobacillus rhamnosus GG. *J Appl Microbiol* 122(5):1321–1332. https://doi.org/10.1111/jam.13436

Quigley EMM, Pot B, Sanders ME (2018) ‘Brain fogginess’ and D-lactic acidosis: probiotics are not the cause. *Clin Transl Gastroenterol* 9(9):187. https://doi.org/10.1038/s41424-018-0057-9

Raba G, Luis AS (2023) Mucin utilization by gut microbiota: recent advances on characterization of key enzymes. *Essays Biochem* 67(3):345–353. https://doi.org/10.1042/ebc20220121

Rao SSC, Rehman A, Yu S, Andino NM (2018) Brain fogginess, gas and bloating: a link between SIBO, probiotics and metabolic acidosis. *Clin Transl Gastroenterol* 9(6):162. https://doi.org/10.1038/s41424-018-0030-7

Sanders ME, Klaenhammer TR, Ouwehand AC, Pot B, Johansen E, Heimbach JT, et al. (2014) Effects of genetic, processing, or product formulation changes on efficacy and safety of probiotics. *Ann N Y Acad Sci* 1309:1–18. https://doi.org/10.1111/nyas.12363

Setlow P (2014) Germination of spores of Bacillus species: what we know and do not know. J Bacteriol 196(7):1297–1305. https://doi.org/10.1128/jb.01455-13

Shinde T, Vemuri R, Shastri MD, Perera AP, Tristram S, Stanley R et al (2019) Probiotic Bacillus coagulans MTCC 5856 spores exhibit excellent in-vitro functional efficacy in simulated gastric survival, mucosal adhesion and immunomodulation. *Journal of Functional Foods* 52:100–108. https://doi.org/10.1016/j.jff.2018.10.031

Soares MB, Almada CN, Pereira EPR, Ferreira BM, Balthazar CF, Khorshidian N, et al. (2023) Sporeforming probiotic bacteria: characteristics, health benefits, and technological aspects for their applications in foods and beverages. *Trends Food Sci Technol* 138:453–469. https://doi.org/10.1016/j.tifs.2023.06.029

Taverniti V, Stuknyte M, Minuzzo M, Arioli S, De Noni I, Scabiosi C, et al. (2013) S-layer protein mediates the stimulatory effect of Lactobacillus helveticus MIMLh5 on innate immunity. *Appl Environ Microbiol* 79(4):1221–1231. https://doi.org/10.1128/aem.03056-12

Terpou A, Papadaki A, Lappa IK, Kachrimanidou V, Bosnea LA, Kopsahelis N (2019) Probiotics in food systems: significance and emerging strategies towards improved viability and delivery of enhanced beneficial value. *Nutrients* 11(7):1591. https://doi.org/10.3390/nu11071591

Venkataraman R, Shenoy R, Ahire JJ, Neelamraju J, Madempudi RS (2023) Effect of Bacillus coagulans Unique IS2 with lactulose on functional constipation in adults: a double-blind placebo-controlled study. *Probiotics Antimicrob Proteins* 15(2):379–386. https://doi.org/10.1007/s12602-021-09855-8

Vitetta L, Coulson S, Thomsen M, Nguyen T, Hall S (2017) Probiotics, D-lactic acidosis, oxidative stress and strain specificity. *Gut Microbes* 8(4):311–322. https://doi.org/10.1080/19490976.2017.1279379

Walden KE, Hagele AM, Orr LS, Gross KN, Krieger JM, Jäger R, et al. (2024) Probiotic BC30 improves amino acid absorption from plant protein concentrate in older women. *Probiotics Antimicrob Proteins* 16(1):125–137. https://doi.org/10.1007/s12602-022-10028-4

Wang R, Chen S, Jin J, Ren F, Li Y, Qiao Z et al (2015) Survival of Lactobacillus casei strain Shirota in the intestines of healthy Chinese adults. *Microbiol Immunol* 59(5):268–276. https://doi.org/10.1111/1348-0421.12249

Xu L, Zhan ZC, Du S, Wang S, Zhang Q, Wang C, et al. (2023) Isolation, functional evaluation, and fermentation process optimization of probiotic Bacillus coagulans. *PLoS ONE* 18(11):e0286944. https://doi.org/10.1371/journal.pone.0286944

Yu Y, Yu X, Ouyang J, Ma X (2021) Complete genome sequence of Bacillus coagulans BC01, a promising human probiotic strain isolated from thick broad bean sauce. *Microbiol Resour Announc* 10(19). https://doi.org/10.1128/mra.00392-21.

Zhang Y, Overbeck TJ, Skebba VLP, Gandhi NN (2024) Genomic and phenotypic safety assessment of probiotic Bacillus coagulans Strain JBI-YZ6.3. *Probiotics Antimicrob Proteins*. https://doi.org/10.1007/s12602-024-10305-4.

Zhu M, Zhu J, Fang S, Zhao B (2024) Complete genome sequence of Heyndrickxia (Bacillus) coagulans BC99 isolated from a fecal sample of a healthy infant. *Microbiol Resour Announc* 13(1):e0044923. https://doi.org/10.1128/mra.00449-23

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4.9 Ethics declarations

Competing Interests

R.R. is employed by Giellepi S.p.A. He was not involved in study design, data analysis and interpretation of results.

4.10 Additional information

Publisher's Note

Declaration of AI and AI-Assisted Technologies in the Writing Process

In the preparation of this manuscript, the authors utilized AI-based tools, including ChatGPT-3.5 (https://chat.openai.com/) and Grammarly (https://app.grammarly.com/) for grammar and style enhancement. After using these tools, the authors reviewed and edited all content and take full responsibility for the publication's content.

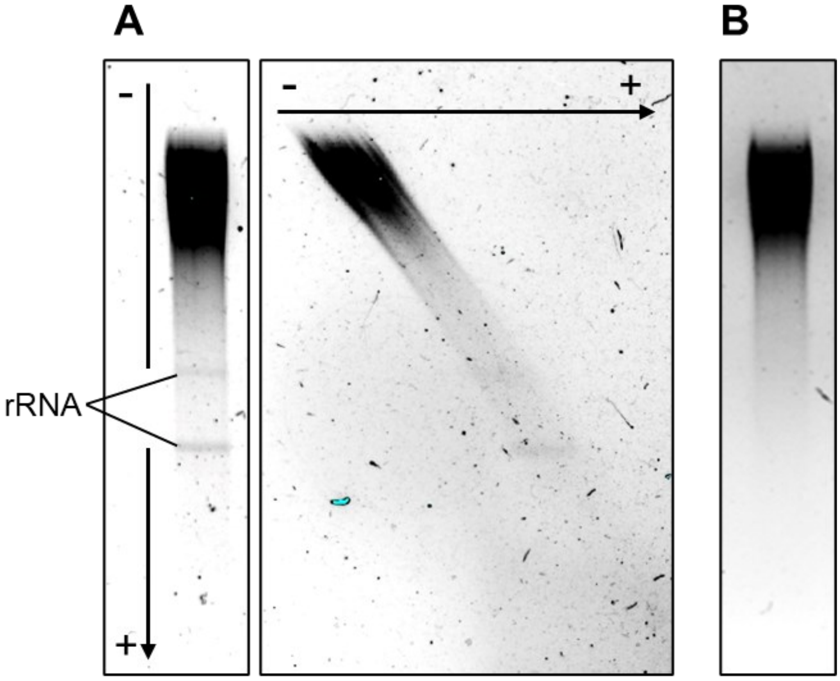
4.11 Supplementary Information

Table S1. Putative antibiotic resistance genes of *Heyndrickxia coagulans* LMG S-24828 detected through the CARD's Resistance Gene Identifier software. RGI, resistance gene identifier. AMR, antimicrobial resistance. ARO, antibiotic resistance ontology.

# Perfect Hits	**# Strict Hits**	**# Loose Hits**
0	4	0

RGI Criteria	**ARO Term**	**Detection Criteria**	**AMRGene Family**	**Drug Class**	**Resistance Mechanism**	**% Identity of Matching Region**	**% Length of Reference Sequence**
Strict	*vanT* gene in vanG cluster	protein homolog model	glycopeptide resistance gene cluster, vanT	glycopeptide antibiotic	antibiotic target alteration	34,6	53,79
Strict	*vanY* gene in vanM cluster	protein homolog model	vanY, glycopeptide resistance gene cluster	glycopeptide antibiotic	antibiotic target alteration	37,97	113,3
Strict	*qacJ*	protein homolog model	small multidrug resistance (SMR) antibiotic efflux pump	disinfecting agents and antiseptics	antibiotic efflux	39,81	114,02
Strict	*vanH* gene in vanO cluster	protein homolog model	vanH, glycopeptide resistance gene cluster	glycopeptide antibiotic	antibiotic target alteration	40,38	94

Figure S1. Detection of plasmid extrachromosomal DNA in *Heyndrickxia coagulans* LMG S-24828. The extract obtained from alkaline lysis was observed using two-dimensional agarose gel electrophoresis (panel A). The horizontal run was conducted after exposing the agarose gel to UV light to generate nicks in potential covalently closed circular (CCC) DNA. Subsequently, the same sample was analyzed using agarose gel electrophoresis after treatment with 100 µg/ml RNase (panel B).



5. Simulated gastrointestinal digestion of two convenience meals using fungal enzyme formulations,

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5.1 Abstract

Dietary supplements containing microbial enzymes are widely used to support digestion and optimize nutrient absorption, particularly in individuals with functional dyspepsia characterized by impaired gastric emptying and endogenous enzyme secretion. This study aimed to evaluate the *in vitro* digestive performance of two fungal-derived enzyme blends, Poolzyme® MULTI and Poolzyme® DAIRY, on representative convenience meals using the standardized INFOGEST 2.0 static digestion protocol. Simulated oral, gastric, and intestinal phases of digestion were conducted according to the INFOGEST model. Two food matrices, a fast-food hamburger with fries and a multi-cheese frozen pizza, were incubated under enzyme-free control conditions or with Poolzyme® MULTI or Poolzyme® DAIRY at manufacturer-recommended doses. At the end of the intestinal phase, digesta were collected and analyzed for total free amino acids, branched-chain amino acids (BCAAs), residual lactose, glucose release, and free fatty acids (FFAs) using validated analytical assays. Both enzyme blends significantly enhanced proteolysis compared to controls, as evidenced by increased total free amino acids and BCAA liberation. Poolzyme® DAIRY yielded a dose-dependent reduction in residual lactose in the cheese-based matrix. Poolzyme® MULTI's amylolytic and cellulolytic activities augmented glucose release from carbohydrate-rich foods, while lipolytic activity in both formulations markedly increased FFA liberation. Kinetic profiles indicated accelerated substrate hydrolysis and elevated nutrient bioaccessibility across both meal types. Fungal-derived enzyme blends effectively complement endogenous digestive enzymes, improving macronutrient hydrolysis and bioaccessibility in diverse dietary contexts. These mechanistic insights support clinical observations of symptom alleviation in functional dyspepsia and underscore the potential of targeted enzyme supplementation to optimize digestion of processed convenience foods.

5.2 Introduction

The use of dietary supplements containing exogenous enzymes to complement the body's digestive enzyme activities has gained increasing interest, particularly for addressing digestive inefficiencies and optimizing nutrient absorption (Ianiro et al., 2016). These dietary supplements utilize enzymes sourced from diverse origins, including: (i) animals (where permitted by local regulations (Precup et al., 2024); e.g., pancreatin from porcine pancreas), (ii) plants (e.g., bromelain from pineapple stem) (iii) and microorganisms (e.g., neutral proteases from *Bacillus* spp. and acid proteases from *Aspergillus* spp.) (Singh et al., 2018)., Among these sources, microbial-derived enzymes have emerged as a preferred choice due to their broad pH activity range, superior stability, and enhanced effectiveness (Roxas, 2008). Additionally, most microbial enzymes can be efficiently produced through cost-effective fermentation and downstream processing from the biomass of wild-type microorganisms (Garvey et al., 2022; Nakamura et al., 1998; Tran Do et al., 2016). For instance, neutral proteases from bacterial genera such as *Bacillus* have been shown to facilitate protein hydrolysis, thereby improving the bioavailability of essential amino acids (Song et al., 2023). Similarly, yeasts, including *Candida* and *Kluyveromyces* spp., are known for their ability to produce lipases, lactase and other enzymes that assist in lipid and carbohydrate metabolism (Chandra et al., 2020; Roxas, 2008). Nevertheless, filamentous fungi represent the primary microbial source of enzymes with nutritional applications. These fungal enzymes have been successfully employed to enhance food processing, improve nutrient bioavailability, and support digestive efficiency. For example, enzymes from *Aspergillus* spp. contribute to protein digestion, making plant-based proteins more bioavailable (Kumitch et al., 2020; Rathi et al., 2024). Additionally, fungal enzymes are widely used in food production to improve flavor, texture, and nutritional quality (Pouris et al., 2024). Another example is fungal phytases, which release bound minerals from plant-based foods, increasing their bioavailability (Singh & Kumar, 2019). Notably, filamentous fungi such as *Aspergillus* spp. and *Trichoderma* spp. are extensively employed as sources of proteases, lipases, cellulases, and lactases, which exhibit optimal activity under physiological conditions in the gastrointestinal tract (Singh & Kumar, 2019; Singh et al., 2019; Gautam & Naraian, 2020).

Functional dyspepsia, characterized by postprandial bloating, early satiety, and upper abdominal discomfort without identifiable causes on endoscopy, is common and affects up to 30 % of the global population (Singh & Kumar, 2019). Given the absence of effective pharmacological treatments for many cases, microbial enzymes have been investigated as potential therapeutic adjuncts for functional dyspepsia. For example, a study demonstrated that supplementation with an acid-resistant microbial lipase significantly reduced postprandial fullness in healthy individuals consuming a high-fat meal, with no significant effects on gastric myoelectrical activity or other upper gastrointestinal symptoms (Levine et al., 2015).

Furthermore, a randomized, double-blind, placebo-controlled trial found that a multienzyme complex containing bacterial protease and lipase, as well as fungal amylase, cellulase, lactase, and lipase, significantly improved functional dyspepsia symptoms, including bloating and postprandial distress, compared to placebo (Majeed et al., 2018). Similarly, Ullah et al. (2023) published the results of a recent randomized, double-blind, placebo-controlled clinical trial that assessed the efficacy of a commercially available multi-enzyme blend derived from filamentous fungi. This formulation, containing protease, amylase, lipase, cellulase, and lactase, was tested in patients with functional dyspepsia. Results showed that two months of supplementation significantly improved quality of life, reduced pain severity, and enhanced sleep quality, with no reported side effects (Ullah et al., 2023).

The study by Ullah et al. served as the starting point for the present investigation, which aimed to evaluate the same enzyme blend alongside an alternative formulation containing the same enzymatic components but optimized for dairy product digestion. Specifically, we sought to gain a mechanistic understanding of the efficacy of these enzymes by assessing whether the fungal-derived enzymes collectively enhance the hydrolysis of their respective substrates under physiologically relevant conditions, without specifically testing the activity of each enzyme in isolation. Additionally, we aimed to assess whether these microbial enzymes act additively in combination with endogenous human digestive enzymes.

To achieve these objectives and simulate human digestion under standardized and physiologically relevant conditions, we employed the INFOGEST static *in vitro* digestion model (Brodkorb et al., 2019), which simulates the sequential oral, gastric, and intestinal phases of human digestion under physiologically relevant conditions. Its reproducibility and adaptability to diverse food matrices make it a valuable tool for evaluating nutrient bioaccessibility and the efficacy of functional ingredients.

The enzyme blends were tested using two realistic, multi-nutrient meals (a fast-food hamburger with fries and a multi-cheese frozen pizza), selected based on macronutrient composition and relevance to real-world dietary habits. These matrices, rich in proteins, fats, complex carbohydrates, and lactose, allowed us to investigate enzymatic activity across all major macronutrient classes. Quantitative analyses focused on protein hydrolysis, lactose breakdown, and the release of free fatty acids and glucose, assessing the ability of fungal enzymes to function throughout digestion and complement endogenous enzymatic activity.

5.3 Material and methods

5.3.1. Composition of food supplements based on fungal enzymes

The products employed in this investigation are from Poolzyme®, a series of fungal origin enzyme blends by Giellepi S.p.A. (Milan, Italy). Specifically, Poolzyme® MULTI contains five enzymes to facilitate the digestion of proteins, carbohydrates, fats, lactose, and fibers. In contrast, Poolzyme® DAIRY is a 3-enzyme blend (lactase, protease, lipase) designed to aid those experiencing minor discomfort after consuming dairy (Table 1).

Enzyme	**Source**	**Activity**		**Unit of activity**
		DAIRY	**MULTI**	
Amylase	*Aspergillus oryzae*	-	13000	SKB
Cellulase	*Trichoderma reesei*	-	500	CU
Protease	*Aspergillus oryzae*	83000	67000	HUT
Lactase	*Aspergillus oryzae*	17000	13000	ALU
Lipase	*Rhizopus oryzae*	1000	1000	FIP

Table 1. Enzymatic composition of the two Poolzyme® formulations used in the study and relative activity (expressed per g of lyophilized ingredient with an uncertainty of ±5 %). CU, cellulase unit defined as the amount of activity that produces a relative fluidity change of 1 in 5 min in a defined carboxymethylcellulose substrate under the conditions of the assay (pH 4.5 and 40 °C). SKB, α-amylase dextrinizing unit, defined as the quantity of α-amylase that dextrinizes soluble starch in the presence of an excess of β-amylase at the rate of 1 g per h at 30 °C. HUT, unit of proteolytic (protease) activity, defined as that amount of enzyme that produces in 1 min a hydrolysate whose absorbance at 275 nm is the same as that of a solution containing 1.10 g per l of tyrosine in 0.006 N hydrochloric acid. ALU, acid lactase unit, defined as the quantity of enzyme that liberates 1 µmol of o-nitrophenol per minute at 37 °C and a pH of 4.5 (based on a 15-min hydrolysis of an O-nitrophenyl-β-D-galactopyranoside substrate). FIP, standardized unit of measurement for lipase enzyme activity as defined by the Fédération Internationale Pharmaceutique; one FIP unit is defined as that quantity of a standard lipase preparation (Fungi Lipase-International FIP Standard) that liberates the equivalent of 1 µmol of fatty acid per minute from the substrate emulsion under the described assay conditions.

5.3.2. Test meals

We tested two food items: first, a frozen “white” cheese pizza made with multiple cheeses, including Provolone Valpadana D.O.P. (9.2 %), Asiago D.O.P. (6.6 %), Gorgonzola D.O.P. (6.6 %), Stracchino (3.5 %), Mascarpone (3.5 %), and Parmigiano Reggiano D.O.P. (2.9 %), without tomato. D.O.P. stands for “*Denominazione di Origine Protetta*”, an Italian quality label that guarantees a food product is entirely produced, processed, and prepared in a specific region using traditional methods. The pizza “quattro formaggi” was purchased from a local (Milan, Italy) supermarket of the chain Esselunga S.p.A.and cooked in an electric oven set to 200 °C for 12 min. The second item was a fast-food meal consisting of a hamburger and fries, obtained as part of a “BigMac meal” from a local (Milan, Italy) fast-food restaurant of the chain McDonald's Italia Srl.

5.3.3. In vitro digestion protocol

Both meals described above underwent simulated digestion following the INFOGEST protocol as described by Brodkorb et al. (2019), consisting of an oral, gastric and intestinal phase.

Oral phase. One hundred grams of each meal, sampled to maintain the proportion of ingredients, were homogenized by adding simulated salivary fluid with the following composition: 15.1 mM KCl, 3.7 mM KH_2PO4, 13.6 mM $NaHCO_3$, 0.15 mM $MgCl_2(H_2O)_6$, 0.06 mM $(NH_4)_2CO_3$, 1.1 mM HCl, and 1.5 mM $CaCl_2(H_2O)_2$. The mixture was blended for 30 s at medium speed using a Bimby blender (Vorwerk, Wuppertal, Germany). The suspension was diluted to 200 ml minus the volume needed to add α-amylase from *Aspergillus oryzae* (Merck, Milan, Italy) to achieve a final concentration of 75 U/ml. The pH was adjusted to 7 using 5 M NaOH or HCl. Before initiating the gastric phase, the corresponding capsule containing the specific enzyme blend was added to an aliquot of 5 ml of oral suspension. For samples not treated with enzyme blends, an empty capsule was added.

Enzyme blend addition. Before initiating the gastric phase, the enzyme blends were added to aliquots of the food homogenized in salivary fluid in the form of powder encapsulated in hydroxypropyl methylcellulose (HPMC) capsules, without any addition of excipients. The weights of the enzyme blends in the capsules were adjusted to achieve doses per meal. Specifically, the fast-food meal (hamburger and fries) weighed 410 g, and the enzyme doses were 200 and 400 mg of Poolzyme® MULTI for the whole meal. The frozen cheese pizza meal weighed 380 g, and the enzyme doses were 300 and 600 mg of Poolzyme® DAIRY for the entire portion. Digested samples were also prepared without enzyme blends, along with non-digested samples that were simply diluted 1:8 with deionized water, matching the dilution occurring in the INFOGEST protocol. Empty capsules were added to samples not treated with the enzyme blends. The samples were then incubated at 37 °C for 2 min with continuous shaking.

Gastric phase. To simulate gastric conditions, 4 ml of simulated gastric fluid with 6.9 mM KCl, 0.9 mM KH_2PO_4, 25 mM $NaHCO_3$, 47.2 mM NaCl, 0.12 mM $MgCl_2(H_2O)_6$, 0.5 mM $(NH_4)_2CO_3$, 15.6 mM HCl, and 0.15 mM $CaCl_2(H_2O)_2$ was added. The pH of the mixture was adjusted to 3.0 using 6 M HCl. Rabbit gastric extract (RGE15; Lipolytech, Marseille, France) was added to reach a final lipase concentration of 60 U/ml. RGE15 also contains pepsin and with porcine pepsin (Merck, Milan, Italy) was used to obtain a final concentration of 2000 U/ml of pepsin units in the gastric phase. The samples were then diluted to 10 ml with deionized water and incubated at 37 °C for 2 h under continuous shaking.

Intestinal phase. Then, 4.25 ml of simulated intestinal fluid (6.8 mM KCl, 0.8 mM KH_2PO_4, 85 mM $NaHCO_3$, 38.4 mM NaCl, 0.33 mM $MgCl_2(H_2O)_6$, 8.4 mM HCl, and 0.6 mM $CaCl_2(H_2O)_2$) were added to the 10 ml of gastric suspension. The pH was adjusted to 7.0 using 6 M NaOH. Porcine pancreatin and bovine bile salts (Merck, Milan, Italy) were incorporated at final concentrations of 100 U/ml of trypsin and 10 mM bile salts, respectively. The total volume was adjusted to 20 ml, and the samples were incubated at 37 °C with continuous shaking for 2 h.

After *in vitro* digestion, the enzymes were inactivated by heating the samples to 90 °C for 10 min.

During a mock INFOGEST assay, under agitation, the capsules were found to open in less than 2 min and were fully dissolved within 5 min.

The INFOGEST experiments were conducted on three different aliquots of the same meal for each condition.

5.3.4. Protein hydrolysis measurement

The degree of protein hydrolysis in the samples was assessed through the quantification of the free terminal nitrogen (FTN) groups using the Primary Amino Nitrogen (PANOPA) Assay kit from Megazyme (Bray, Ireland), following the manufacturer's instructions for microplate analysis. Briefly, the samples were serially diluted 1:2 with deionized water to obtain absorbance readings within the kit's linear range. A calibration curve was generated using the standard solution provided with the kit. All measurements were performed in triplicate. The FTN groups in the samples reacted with N-acetyl-L-cysteine and o-phthaldialdehyde, resulting in the formation of isoindole derivatives (amino nitrogen + N-acetyl-L-cysteine + o-phthaldialdehyde → isoindole derivative). The amount of isoindole derivative produced in this reaction is stoichiometrically proportional to the quantity of FTN present. The isoindole derivatives were quantified by monitoring the absorbance increase at 340 nm using a Powerwave XS2 automated spectrophotometer (Biotek, Milan, Italy). Data were expressed as mg of reactive nitrogen per g of food sample.

5.3.5. Lactose quantification

Following the centrifugation of samples at 13000 *g* for 10 min at room temperature, the analysis of lactose degradation was conducted using the Lactose & D-Galactose (Rapid) Assay kit by Megazyme (Bray, Ireland). This protocol employs spectrophotometry to quantify lactose and galactose in the samples. The underlying principle of this method involves the hydrolysis of lactose to D-galactose and D-glucose by *Aspergillus niger* β-galactosidase at pH 5.0, as outlined in a modified version of Association of Official Agricultural Chemists (AOAC) Official Method 984.15 for lactose in milk. The resulting D-galactose undergoes interconversion of α- and β-anomeric forms catalyzed by galactose mutarotase (GalM). Subsequently, β-D-galactose is oxidized to D-galactonic acid in the presence of β-galactose dehydrogenase (β-GalDH) at pH 8.6. The stoichiometric amount of NADH formed in this reaction correlates with the quantity of lactose; finally, the produced NADH is measured through the increase in absorbance at 340 nm. All lactose and galactose concentrations are expressed on a wet-weight (as-is) basis, *i.e.*, mg per 100 g of the initial food prior to digestion and without moisture correction.

5.3.6. Amino-acid profile determination

One g of sample was vortexed in 20 ml of water, centrifuged at 6000×*g* for 10 min at 4 °C, and the supernatant was transferred into a 50 ml volumetric flask. The residue was extracted with 20 ml of water, treated as described above, and the final volume was adjusted to 50 ml with water. Amino acid derivatization using AccQ·Tag reagents (Waters S.p.A., Sesto San Giovanni, Italy) was conducted according to the manufacturer's protocol and as previously reported (Gardana et al., 2018). Liquid chromatographic separation was performed on an Acquity UPLC system (Waters) coupled with an eLambda DAD (Waters). The injection volume was 2 µl. The derivatives were separated on an AccQ·Tag Ultra column (1.7 µm BEH, 100 mm × 2.1 mm) maintained at 50 °C. The flow rate was 0.7 ml/min. Data were acquired in the range of 200–450 nm, and the chromatogram was integrated at 260 nm. The stock solution contained a 2.5 mM concentration of each amino acid, except for cysteine at 1.25 mM. Calibration curves were prepared in the range of 2.2–51 µg/ml.

5.3.7. Quantification of sugars

Glucose, fructose, and sucrose standards were from Sigma-Aldrich (St. Louis, MO, USA). Two hundred mg of the sample were dispersed in 100 ml of sterile deionized water, and the suspension was sonicated for 10 min, centrifuged at 1000×*g* for 5 min at room temperature, and the supernatant was recovered. The residue was extracted with 50 ml of water and treated as described above. The supernatants were combined, and the final volume was adjusted to 200 ml with acetonitrile (Merck, Darmstadt, Germany). The sugar content was assessed using a UHPLC Vanquish model Flex (Thermo Fisher Scientific S.p.A., Rodano, Italy) coupled to a

Q-Exactive Focus Orbitrap (Thermo Fisher Scientific) equipped with a HESI-II probe for ESI. The column used was a BEH amide C18 (150 mm × 2.1 mm i.d., 1.7 µm; Waters), and the flow rate was 0.2 ml/min. One µl was injected. The column and sample were maintained at 25 °C and 20 °C, respectively. The eluents were 0.02 % NH_4OH in water and 0.02 % NH_4OH in acetonitrile (75:25, v/v). The operative conditions were as follows: spray voltage 3.0 kV, sheath gas flow rate 40 (arbitrary units), auxiliary gas flow rate 10 (arbitrary units), capillary temperature 275 °C, capillary voltage −95 V, S lens −50 V, and heater temperature 275 °C. The analytes were identified in negative ESI mode by fullscan acquisition (m/z 100–1500 u), using an isolation window of ±2 ppm. The AGC target, injection time, and mass resolution were 1 × 106, 100 ms, and 70 K, respectively. The MS data were processed using Xcalibur software (Thermo Fisher Scientific). Peak identity was ascertained by evaluating both the accurate mass and retention time. Calibration curves were prepared in the range of 2–50 µg/ml.

5.3.8. Free fatty acids quantification

Acetonitrile, ammonium formate, tertbutyl methyl ether (TBME), formic acid, lauric acid, myristic acid, palmitic acid, stearic acid, oleic acid, 1,2-dioleoyl-glycerol, and 1,2-dipalmitoyl-glycerol were obtained from Sigma-Aldrich. 2-Propanol and ethanol were purchased from VWR International (Bruchsal, Germany). The lipid extraction was performed by adding 10 ml of a chloroform:methanol solution (3:1, v/v) to 2 g of the sample. The mixture was vortexed for 1 min and centrifuged at 1650×*g* for 5 min at room temperature. The chloroform phase was recovered, and the residue was reextracted twice using the same chloroform:methanol solution. The combined chloroform phases were evaporated to dryness under a nitrogen stream. The dried extract was then resuspended in 1 ml of an ethanol:tetrahydrofuran solution (1:1, v/v), followed by centrifugation at 6000×*g* for 2 min at room temperature. The resulting supernatant was transferred to a vial for analysis by UHPLC-HR-MS. The UHPLC-MS system consisted of a Vanquish Flex (Thermo Fisher Scientific) connected to an HR-MS Orbitrap model Q-Exactive Focus (Thermo Fisher Scientific), equipped with a HESI probe. Chromatography was performed on a 1.8 µm HSS T3 C18 column (150 mm × 2.1 mm) with ACN containing 0.1 % HCOOH (A) and 1 mM NH_4HCOO in 2-propanol (B). The gradient was as follows: 0 % B (0–5 min), 5–20 min at 20 % B, 20–42 min to 75 % B, 42–55 min at 75 % B, increased to 100 % A at 55–60 min, and kept constant at 100 % A for 5 min. The flow rate was 0.4 ml/min, and the column and sample temperatures were 45 °C and 20 °C, respectively. The injection volume was 5 µl.

Detection was performed in the ESI− mode from 0 to 9.1 min for the analysis of FA and MG, and in the ESI + mode from 9.1 to 55 min for the analysis of DG and TG. The total ion current chromatogram was recorded in scan mode in the range of m/z 100–1500 u. The operative conditions were as follows: spray voltage 3.5 kV, sheath gas flow rate 50 (arbitrary units), auxiliary gas flow rate 20 (arbitrary units), capillary temperature 350 °C, capillary voltage −95 V, S lens −100 V, and heater temperature 320 °C. The analytes were identified in negative ESI mode by fullscan acquisition (m/z 100–1500 u), using an isolation window of ±2 ppm. The AGC target, injection time, and mass resolution were 1 × 106, 100 ms, and 70 K, respectively. The MS data were processed using Xcalibur software (Thermo Fisher Scientific). Peak identity was ascertained by evaluating the accurate mass and retention time. Calibration curves were prepared in the range of 0.5–20 µg/ml.

5.3.9. Statistical analysis

All experiments were conducted in triplicate. Data were analyzed using Prism 10 (GraphPad Software, CA, USA). Statistical significance was assessed using one-way ANOVA followed by Tukey's multiple comparisons test. Post-hoc Compact Lettering Display (CLD) was assigned with a significance threshold of $p < 0.05$.

2.10. Declaration of generative AI in the writing process

During the preparation of this work the authors used the AI-powered language model ChatGPT-4 (https://chat.openai.com/) in order to improve readability and language. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

5.4 Results

5.4.1. Protein hydrolysis in tested meals after simulated digestion

Protein hydrolysis was assessed by quantifying free terminal nitrogen (FTN) groups in the food matrices, serving as an indicator of proteolytic activity from the fungal enzymes contained in the Poolzyme® blends. In undigested samples, FTN levels were 0.19 mg/g in the multi-cheese pizza and 0.068 mg/g for the fast-food meal. After *in vitro* digestion using the standardized INFOGEST protocol (control condition: pepsin, gastric lipase, and pancreatin), FTN values increased significantly, reaching 2.43 mg/g for the pizza and 1.60 mg/g for the fast-food meal. The addition of Poolzyme® enzyme blends to the control digestion system resulted in a further significant increase in FTN levels, supporting the hypothesis that these formulations enhance proteolytic activity beyond endogenous enzymes alone. For Poolzyme® DAIRY, FTN concentrations increased to 3.37 mg/g at the lower dose and to 3.49 mg/g at the higher dose. However, the difference between the two concentrations was not statistically significant, although both values were significantly higher than the control. In contrast, Poolzyme® MULTI showed a dose-dependent effect, with FTN values rising from 1.94 mg/g at the lower concentration to 2.21 mg/g at the higher concentration, with statistically significant differences observed between all tested conditions (Fig. 1).

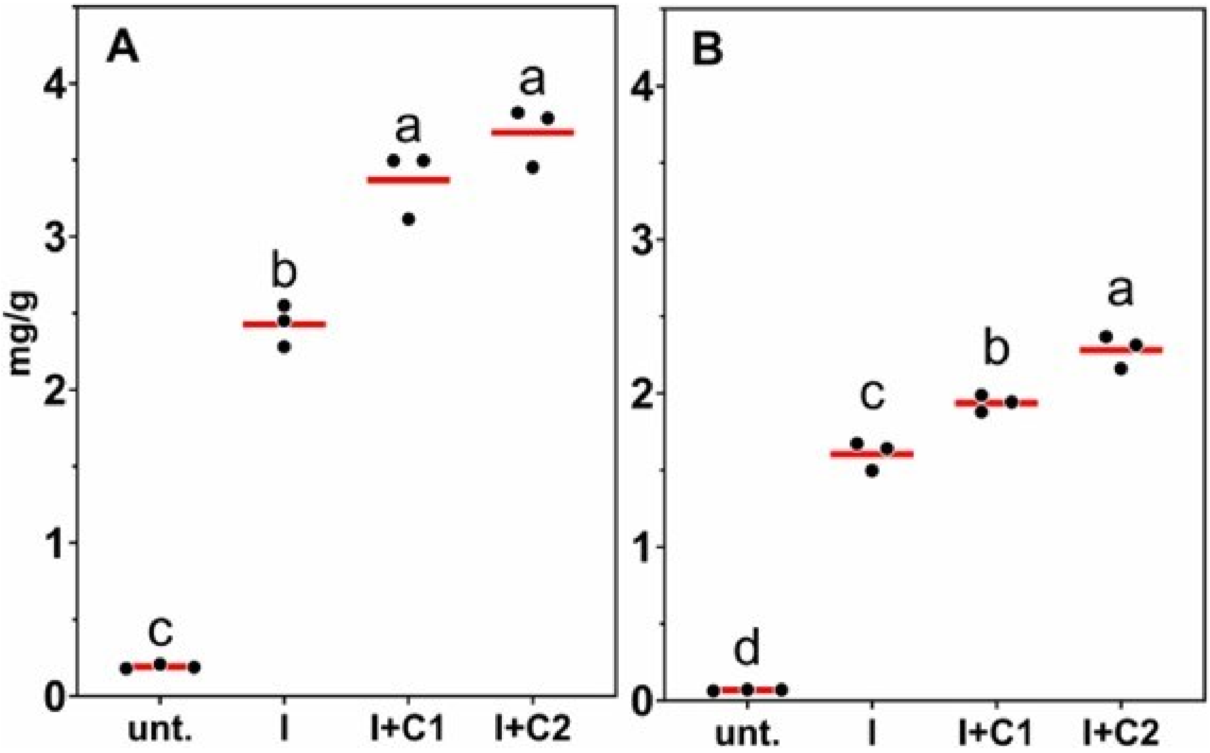


Figure 1. Fig. 1. Overall protein hydrolysis assessed using Primary Amino Nitrogen (PANOPA) Assay, expressed as mg of free terminal nitrogen groups per gram of test meal. The data are shown for the untreated (unt.) meal, INFOGEST (I) with no addition of enzyme blend, and INFOGEST with enzyme blend-treated samples at the low dosage (I + C1) and at the high dosage (I + C2). Panel A presents data for the pizza test meal with the Poolzyme® DAIRY enzyme blend, while panel B presents data for the hamburger meal with the Poolzyme® MULTI enzyme blend. For all conditions, three independent replicates were performed, and the median is shown as a red bar. Statistical significance was assessed using one-way ANOVA followed by Tukey's multiple comparisons test. Post-hoc Compact Lettering Display (CLD) was assigned with a significance threshold of $p < 0.05$. Significant differences between samples are indicated by different lower-case letters.

These results demonstrate that both fungal-derived enzyme blends improve protein digestion in realistic multi-nutrient meals, with Poolzyme® MULTI exhibiting a more evident dose-dependent effect.

5.4.2. Lactose reduction

The activity of the β-galactosidase enzyme in the Poolzyme® enzymatic blends was tested by measuring residual lactose in food after simulated digestion. Lactose was below the detection limit in all fast-food meal samples (data not shown). In contrast, multi-cheese pizza samples contained measurable amounts of lactose. In the undigested pizza sample, lactose content was 458 mg per 100 g of food, which unexpectedly decreased to 360 mg/100 g following digestion with the standard INFOGEST protocol (21 % reduction; Fig. 2(a)). Since lactose quantification with this method relies on measuring free galactose before and after hydrolysis by *A. niger* β-galactosidase, galactose quantification was analyzed to clarify the observed lactose reduction. Galactose content (Fig. 2(b)) did not decrease post-INFOGEST digestion, indicating that lactose reduction was not attributable to lactase/β-galactosidase activity within the INFOGEST reagents. Instead, we hypothesize that interference by the INFOGEST digestion matrix, possibly due to bile salts or other components, might have occurred. Supporting this hypothesis, a similar lactose reduction was observed when pure lactose was incubated solely in the intestinal phase of INFOGEST digestion, again showing an apparent reduction in quantifiable lactose while free galactose was not detected (data not shown).

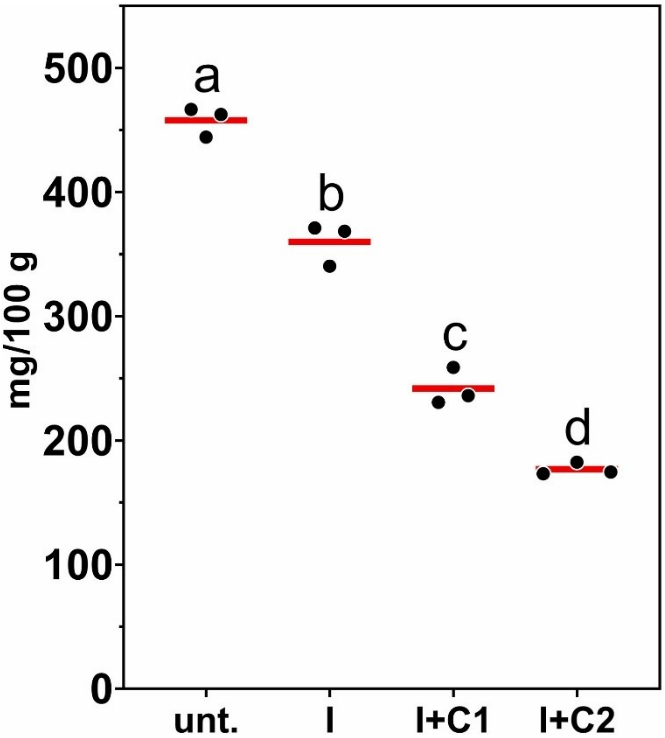


Figure 2 Lactose (panel a) and galactose (panel b) quantification in "white" pizza samples. All concentrations are on a wet-weight basis (mg per 100 g of initial food). Un, undigested food (no INFOGEST); I, food after in vitro digestion without Poolzyme® DAIRY; I + C1, food after in vitro digestion in the presence of the lowest amount of Poolzyme® DAIRY (one capsule corresponding to 300 mg of Poolzyme® DAIRY); I + C2, food after in vitro digestion in the presence of the highest amount of Poolzyme® (two capsules corresponding to 600 mg of Poolzyme® DAIRY). Each condition has been tested in three independent experiments using aliquots of the same meal. Statistical significance was assessed using one-way ANOVA followed by Tukey's multiple comparisons test. Post-hoc Compact Lettering Display (CLD) was assigned with a significance threshold of $p < 0.05$. Significant differences between samples are indicated by different lower-case letters.

Supplementation with Poolzyme® DAIRY significantly reduced lactose concentration in a dose-dependent manner, decreasing to 242 mg/100 g and 177 mg/100 g at lower and higher enzyme concentrations, respectively (Fig. 2(a)). This was accompanied by a corresponding dose-dependent increase in galactose concentration (from 38 mg/100 g to 161 mg/100 g and 204 mg/100 g at lower and higher enzyme concentrations, respectively; Fig. 2(b)).

These findings confirm the effective dose-dependent enhancement of lactose hydrolysis by the fungal-derived enzyme blend, suggesting its potential utility in complementing endogenous digestive activity and improving carbohydrate digestion in dairy-rich foods.

5.4.3. Modification of branched-chain amino acid (BCAA) concentration

The proteolytic activity of the Poolzyme® enzymatic blends was further demonstrated by an increase in free branched-chain amino acids (BCAAs) after simulated digestion, compared to the control condition. The BCAA data presented here are based on the sum of the amino acids leucine, isoleucine, and valine, as determined by UHPLC amino acid profile analysis. The baseline concentration of free BCAAs was 1.5 mg/g in the fast-food meal and 1.2 mg/g in the multi-cheese pizza, respectively (Fig. 3). *In vitro* digestion using the INFOGEST protocol induced a substantial increase in free BCAAs, particularly in the fast-food meal, where levels rose up to 9.4 mg/g while they did not increase significantly for the pizza meal. Supplementation with the enzyme blends further elevated BCAA concentrations in both meals. For Poolzyme® DAIRY, levels increased significantly to 5.2 mg/g and 5.3 mg/g at the lower and higher doses, respectively. In the case of Poolzyme® MULTI, BCAA levels were significantly increased over the control only at the highest tested dose, reaching 11.5 mg/g (Fig. 3).

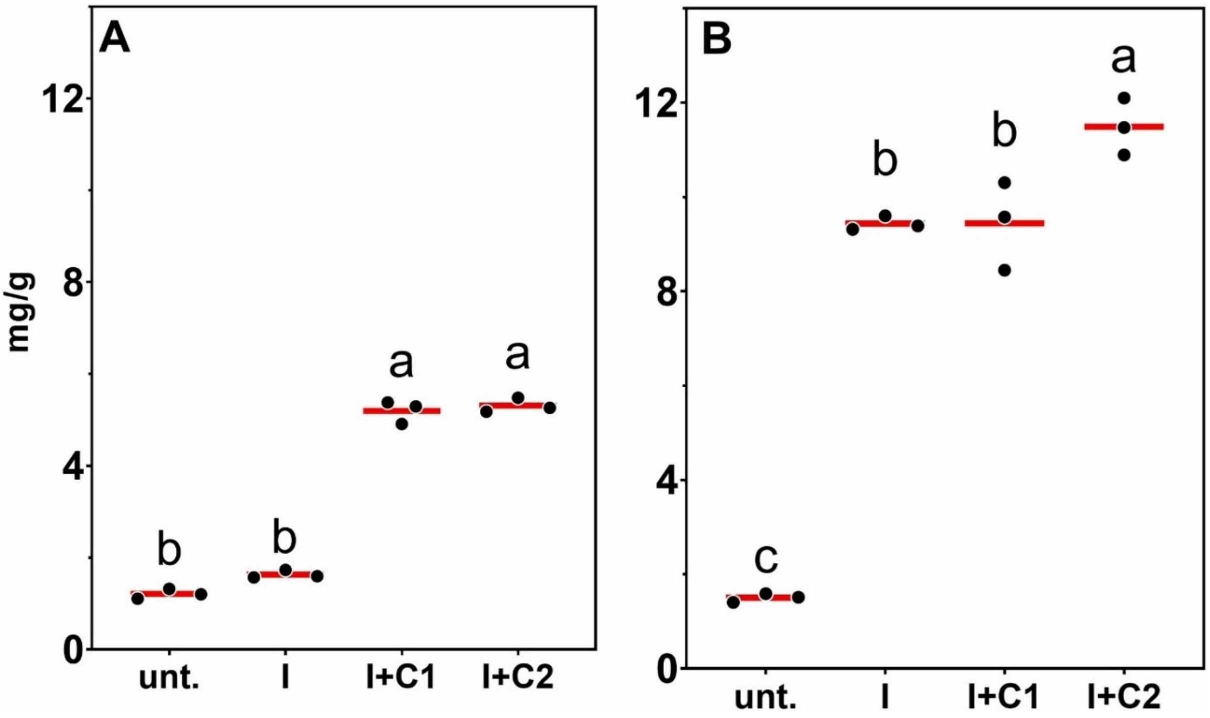


Figure 3 Free branched chain amino acids (BCAA) in tested meals before and after INFOGEST simulated digestion. Data are expressed as mg of BCAA per gram of test meal. The data are shown for the untreated (unt.) meal, INFOGEST (I) with no addition of enzyme blend, and INFOGEST with enzyme blend-treated samples at the low dosage (I + C1) and at the high dosage (I + C2). Panel A presents data for the pizza test meal with the Poolzyme® DAIRY enzyme blend, while panel B presents data for the hamburger meal with the Poolzyme® MULTI enzyme blend. For all conditions, three independent replicates were performed, and the median is shown as a red bar. Statistical significance was assessed using one-way ANOVA followed by Tukey's multiple comparisons test. Post-hoc Compact Lettering Display (CLD) was assigned with a significance threshold of $p < 0.05$. Significant differences between samples are indicated by different lower-case letters.

5.4.4. Changes in free essential amino acid levels

The proteolytic activity of the Poolzyme® enzymatic blends was further confirmed by quantifying the release of free essential amino acids (EAAs). These were calculated as the sum of the following amino acids (phenylalanine, valine, tryptophan, threonine, isoleucine, methionine, histidine, leucine, and lysine) identified and quantified by UHPLC after simulated digestion. Baseline concentrations of free EAAs were 1.3 mg/g in the multi-cheese pizza and 1.6 mg/g in the fast-food meal. In the INFOGEST control condition, a modest increase was observed for the pizza, reaching 3.3 mg/g, while a substantial increase to 17.7 mg/g was detected in the fast-food meal. Supplementation with the enzyme blends further elevated free EAA concentrations. Specifically, Poolzyme® DAIRY increased EAA levels to 9.2 mg/g, while Poolzyme® MULTI reached 18.3 mg/g. At the higher concentration of Poolzyme® MULTI, a slight but statistically significant increase to 21.3 mg/g was observed, whereas Poolzyme® DAIRY reached 9.6 mg/g (Fig. 4). Notably, several individual EAAs were significantly increased following digestion with Poolzyme® DAIRY (Supplementary Fig. S1).

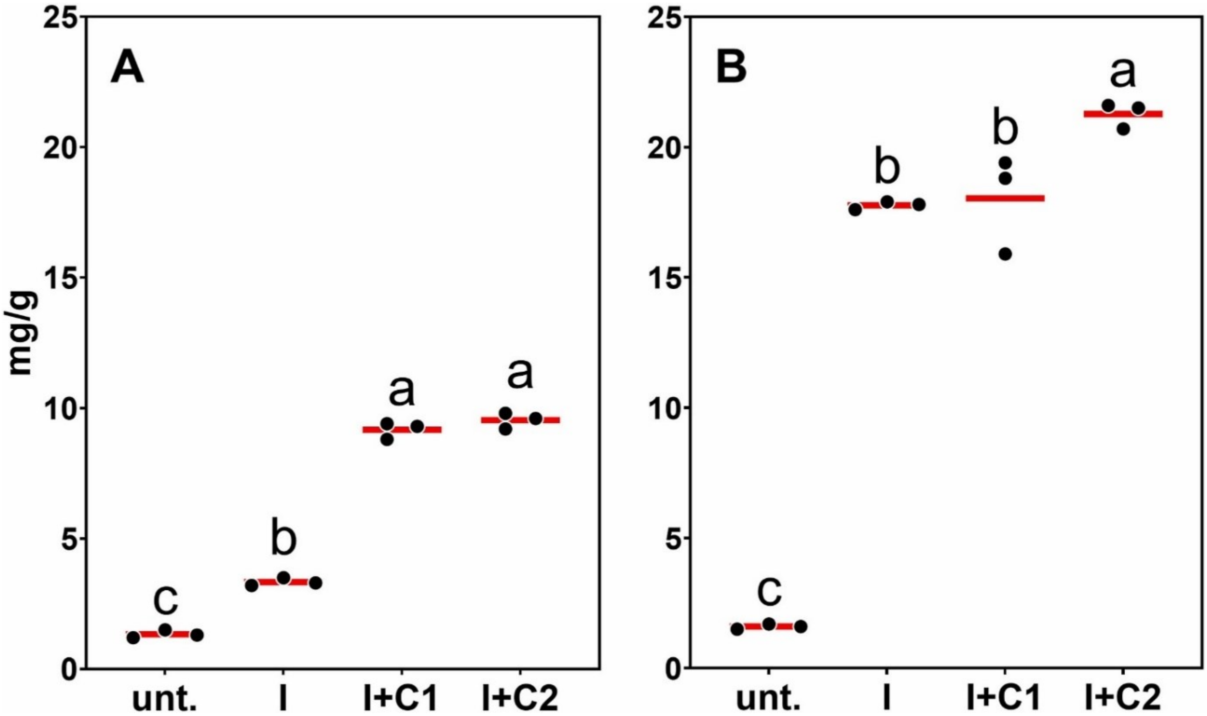


Figure 4 Sum of the concentration of free essential amino acids (histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine) in tested meals before and after INFOGEST simulated digestion. Data are expressed as mg of EAA per gram of test meal. The data are shown for the untreated (unt.) meal, INFOGEST (I) with no addition of enzyme blend, and INFOGEST with enzyme blend-treated samples at the low dosage (I + C1) and at the high dosage (I + C2). Panel A presents data for the pizza test meal with the Poolzyme® DAIRY enzyme blend, while panel B presents data for the hamburger meal with the Poolzyme® MULTI-enzyme blend. For all conditions, three independent replicates were performed, and the median is shown as a red bar. Statistical significance was assessed using one-way ANOVA followed by Tukey's multiple comparisons test. Post-hoc Compact Lettering Display (CLD) was assigned with a significance threshold of p < 0.05. Significant differences between samples are indicated by different lower-case letters.

These results confirm that the fungal-derived enzyme blends enhanced protein hydrolysis and promoted the release of EAAs during simulated digestion of the model meals.

5.4.5. Release of sugars from carbohydrate hydrolysis

To assess the amylase and cellulase activity of Poolzyme® MULTI, mono-, di-, tri-, and tetrasaccharides were quantified in the fast-food meal before and after simulated digestion. The initial glucose concentration was 10.4 mg/g, which increased to 24.7 mg/g following standard INFOGEST digestion. Supplementation with Poolzyme® MULTI led to a further increase in glucose levels: 27.6 mg/g at the lower enzyme concentration and 28.7 mg/g at the higher concentration. All changes were statistically significant compared to the control, except for the difference between the two enzyme doses. Fructose levels remained unchanged across all digestion conditions. In contrast, significant increases in di-, tri-, and tetrasaccharide concentrations were observed after digestion compared to the undigested sample, whereas no significant differences emerged among the enzyme-treated conditions. With the exception of fructose, all sugars showed statistically significant differences between undigested and digested samples (Fig. 5).

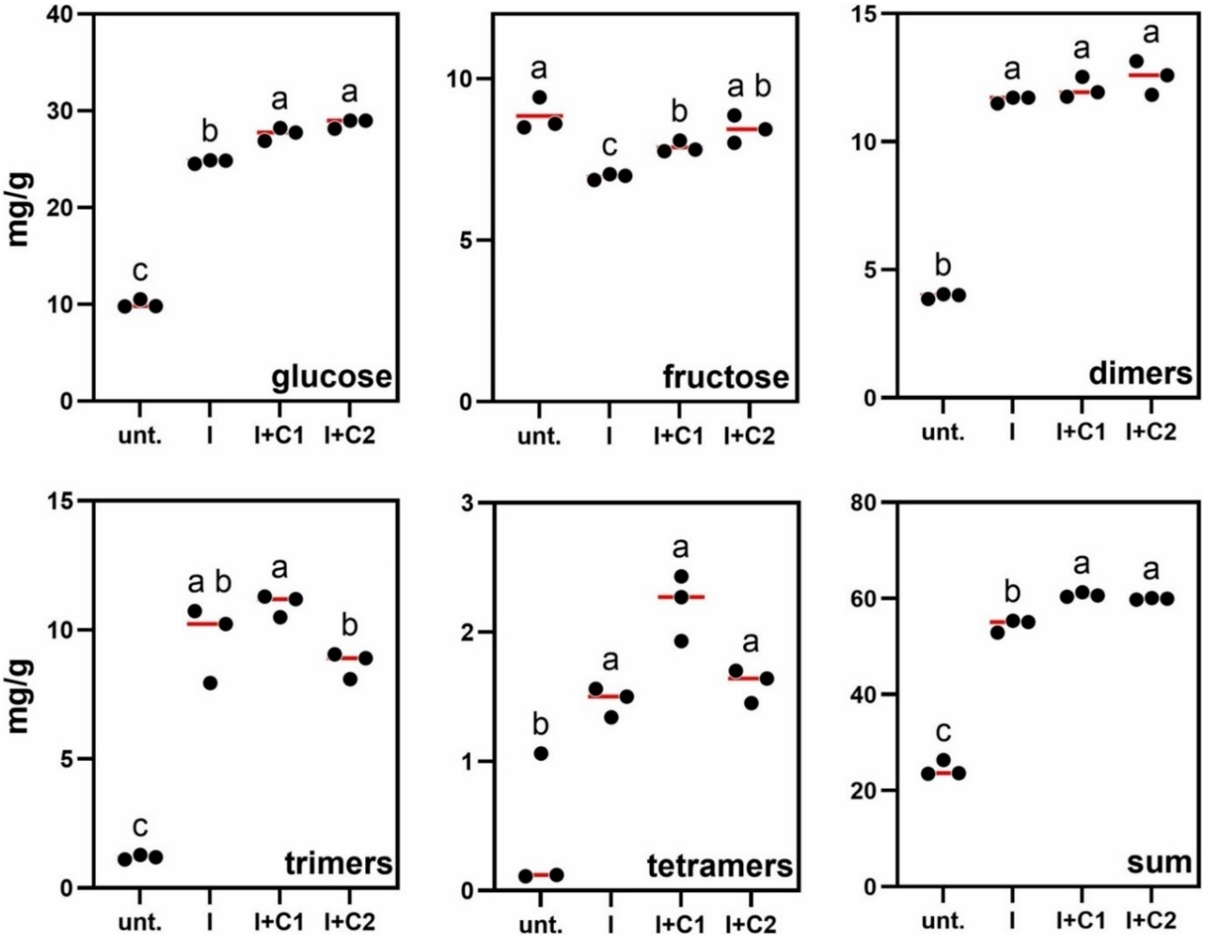


Figure 5 Sugars quantified in the fast-food meal before and after the INFOGEST simulated digestion. Data are expressed on a wet-weight basis, as mg of sugar per gram of test meal. The data are shown for the untreated (unt.) meal, INFOGEST (I) with no addition of enzyme blend, and INFOGEST with enzyme blend-treated samples at the low dosage (I + C1) and at the high dosage (I + C2). For all conditions, three independent replicates were performed, and the median is shown as a red bar. Statistical significance was assessed using one-way ANOVA followed by Tukey's multiple comparisons test. Post-hoc Compact Lettering Display (CLD) was assigned with a significance threshold of $p < 0.05$. Significant differences between samples are indicated by different lower-case letters.

These findings indicate that Poolzyme® MULTI effectively enhanced carbohydrate hydrolysis, as evidenced by the increased release of free glucose from the food matrix.

5.4.6. Increase in free fatty acids

Free fatty acid (FFA) levels were measured before and after simulated digestion to assess lipase activity. In undigested samples, baseline FFA concentrations were low, with 1.6 mg/g in the multi-cheese pizza and 2.10 mg/g in the fast-food meal. Following digestion with the standard INFOGEST protocol, FFA levels increased significantly, reaching 5.8 mg/g and 8.2 mg/g in the pizza and fast-food meal, respectively. The addition of enzyme blends resulted in a substantial further increase in FFAs. For Poolzyme® DAIRY, FFA concentrations rose to 21.4 mg/g at the lower dose and to 22.5 mg/g at the higher dose, although the difference between these two values was not statistically significant. In contrast, Poolzyme® MULTI induced a more pronounced, dose-dependent response, with FFA levels increasing to 24.1 mg/g at the lower concentration and to 26.1 mg/g at the higher dose. This difference was statistically significant (Fig. 6).

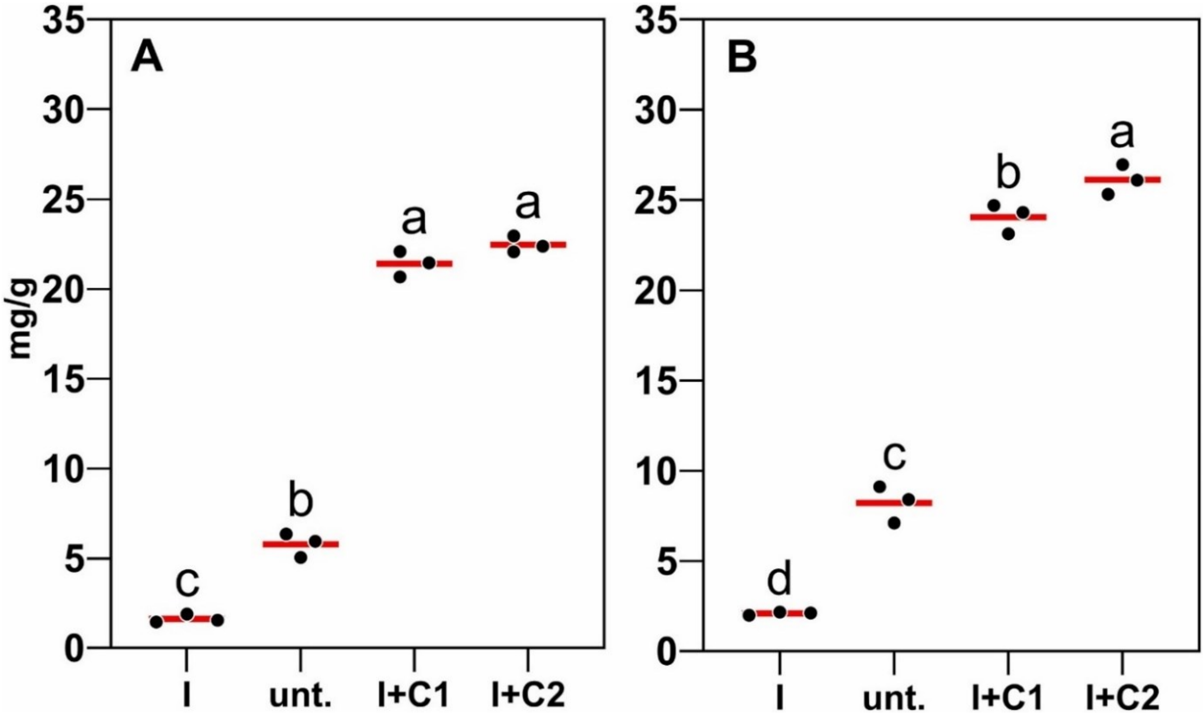


Figure 6 Free fatty acids in tested meals before and after INFOGEST simulated digestion. Data are expressed as mg of fatty acid per gram of test meal. The data are shown for the untreated (unt.) meal, INFOGEST (I) with no addition of enzyme blend, and INFOGEST with enzyme blend-treated samples at the low dosage (I + C1) and at the high dosage (I + C2). Panel A presents data for the pizza test meal with the Poolzyme® DAIRY enzyme blend, while panel B presents data for the hamburger meal with the Poolzyme® MULTI enzyme blend. For all conditions, three independent replicates were performed, and the median is shown as a bar. Statistical significance was assessed using one-way ANOVA followed by Tukey's multiple comparisons test. Post-hoc Compact Lettering Display (CLD) was assigned with a significance threshold of $p < 0.05$. Significant differences between samples are indicated by different lower-case letters.

Overall, both enzyme blends significantly enhanced lipolysis, as evidenced by the increased release of free fatty acids, confirming their efficacy in promoting triglyceride digestion during simulated gastrointestinal conditions.

5.5 Discussion

This study evaluated the *in vitro* digestive activity of two fungal-derived enzyme blends, Poolzyme® MULTI and Poolzyme® DAIRY, using the INFOGEST protocol to simulate the human digestive process. This approach provides a mechanistic basis for interpreting the results of a previous clinical study showing improvements in quality of life and a reduction in digestive symptoms in individuals with functional dyspepsia consuming Poolzyme® MULTI (Ullah et al., 2023). Our study aimed to support these findings by providing a detailed analysis of the enzymatic properties of the formulations and their impact on macronutrient digestion.

The selection of the two test meals (hamburger with fries and frozen white pizza) was driven by the need to evaluate enzymatic activity on a food matrix containing a diverse combination of macronutrients. The hamburger with fries represents a meal rich in proteins, fats, and complex carbohydrates, whereas the frozen white pizza was chosen for its lactose and casein protein content, making it ideal for testing the specificity of the Poolzyme® DAIRY formulation. These convenience meals are commonly consumed in Western countries, including Italy (Scarsi et al., 2024), and their nutritional profile may influence digestion, particularly in individuals with functional dyspepsia (Amerikanou et al., 2023).

The INFOGEST static *in vitro* digestion model was chosen for its reproducibility and physiological relevance. It has been validated through inter-laboratory studies demonstrating uniform digestion profiles, particularly for proteins (Egger et al., 2019). Despite its advantages, INFOGEST has limitations. Being a static system, it does not simulate gradual enzyme secretion, gastric emptying, or peristalsis, factors that influence digestion kinetics. Unlike *in vivo* digestion, where enzymes and bile salts act dynamically, static models may underestimate nutrient release rates (Rathi et al., 2024). Studies comparing INFOGEST with dynamic models, such as DIDGI®, indicate similar endpoint digestion but distinct kinetic profiles, with dynamic systems more accurately replicating amino acid release patterns (Egger et al., 2019). In this study, we focused on endpoint digestion while acknowledging the limitation of not capturing intermediate kinetics. Another drawback is the lack of absorption and post-absorptive metabolism, meaning the model assesses bioaccessibility rather than bioavailability. Another potential limitation is that, although pancreatin supplies essential digestive enzymes, it lacks certain human-specific cofactors (such as colipase) that are required for optimal lipase activity and efficient lipid digestion (Lowe, 2002). However, the presence of fungal lipases in Poolzyme® likely compensated for this limitation. The incorporation of brush-border enzymes in recent INFOGEST adaptations has improved physiological relevance (Egger et al., 2017), but the model remains a simplification that does not account for hormonal regulation or microbiome interactions. Nonetheless, INFOGEST has demonstrated strong qualitative agreement with *in vivo* findings, making it a valuable screening tool before committing to costly *in vivo* studies (Egger et al., 2019). While its static nature imposes constraints, it remains a reliable model for controlled digestive studies.

The beneficial effects of Poolzyme® MULTI and Poolzyme® DAIRY in breaking down complex meals align with a growing body of evidence supporting the efficacy of exogenous digestive enzymes in enhancing nutrient digestion. Our findings indicate that supplementing the standard digestive mixture with fungal-derived enzymes significantly improved protein proteolysis, starch hydrolysis, and triglyceride lipolysis compared to endogenous enzymes alone. These enhancements are consistent with the results of Rathi et al. (2024), who assessed a multi-component enzyme blend (DigeSEB Super, containing amylase, protease, lipase, cellulase, lactase, and hemicellulase) on a diskette-shaped complex food model that contains proteins, carbohydrates, fats, and other nutrients in a proportion that nearly matches the specifications of nutritional requirements suggested by the US FDA. Their *in vitro* digestion study demonstrated that enzyme supplementation accelerated food disintegration and increased nutrient release under INFOGEST conditions. Notably, they reported a 2.75-fold faster reduction in gastric digesta viscosity, indicating a more rapid breakdown of the food matrix structure (Rathi et al., 2024). In addition, Rathi et al. observed that enzymatic supplementation led to a substantial increase in reducing sugars (indicative of enhanced starch hydrolysis), as well as higher levels of free amino acids, peptides, and free fatty acids, confirming improved protein and fat digestion (2024b). These results closely mirror our data, where Poolzyme® MULTI promoted the release of BCAAs and EAAs from the hamburger meal, while Poolzyme® DAIRY increased free fatty acid levels and reduced the residual lactose in the pizza meal. Additional studies further corroborate the advantages of enzyme supplementation. For instance, Calvo-Lerma et al. (2019) investigated the optimal pancreatin dosage for various meals, demonstrating that increasing exogenous lipase significantly enhanced fat digestion, as evidenced by greater free fatty acid release. Similarly, proteolytic enzymes from diverse sources have been shown to enhance protein digestion in mixed meals. Furthermore, their findings emphasize that enzyme efficacy depends on factors such as meal composition.

Although supplementation increased free BCAAs and EAAs, these analytes accounted for <20 % of total meal nitrogen, indicating partial hydrolysis comparable to that occurring physiologically in the proximal small intestine (Trommelen et al., 2021), confirming that the enzyme blends promoted bioaccessibility without inducing over-hydrolysis.

Freitas et al. (2022) reported that fungal protease supplementation improved the bioaccessibility of essential amino acids from gluten digestion, though their study focused on individual food items rather than complete meals, as in our case. Likewise, Garvey et al. (2022) demonstrated that adding microbial proteases to an *in vitro* digestion significantly increased free amino acid release. Their study, which applied the INFOGEST static model to a meal composed of chicken, green peas, and mashed potatoes with unsalted butter, reported enhanced macronutrient hydrolysis, paralleling our observations. The concordance between our findings and those of Garvey et al. is particularly noteworthy, as both studies employed real-world mixed meals, underscoring the ability of multi-enzyme supplements to effectively process complex macronutrient matrices.

Our findings also revealed improved starch conversion to sugars, consistent with literature on multi-enzyme supplements containing amylase. Regarding lactose digestion, Poolzyme® DAIRY effectively compensated for the absence of lactase in the pancreatin mix, fully hydrolyzing lactose in a dairy-containing meal, an effect well-documented in lactase supplementation research (Mazhar et al., 2024).

Finally, *in vivo* studies reinforce the relevance of enzyme supplementation. A clinical trial on DigeZyme® (a multi-enzyme complex) using a standardized test meal demonstrated improved protein digestibility and reduced gastrointestinal symptoms over 60 days (Majeed et al., 2018). While healthy individuals typically exhibit sufficient digestive capacity, enzyme supplementation appears particularly advantageous in cases of functional dyspepsia, pancreatic enzyme insufficiency, or after consuming large, nutrient-dense meals. This aligns with Ullah et al. (2023), who reported that Poolzyme® MULTI significantly improved gastrointestinal symptoms and quality of life in individuals with functional dyspepsia. Our study provides a mechanistic foundation for these observations, illustrating how exogenous enzymes enhance nutrient release during digestion.

5.6 Conclusions

This study strengthens the scientific foundation supporting microbial enzyme supplementation for digestive enhancement. Both Poolzyme® MULTI and Poolzyme® DAIRY significantly improved macronutrient digestion under simulated gastrointestinal conditions. Notably, these enhancements were achieved without modifying the digestion model parameters, emphasizing the intrinsic efficacy of the enzyme blends.

Future research should explore enzyme supplementation in diverse dietary contexts, including plant-based diets with high fiber or antinutritional factor content, to extend the applicability of this approach beyond the tested meal types. Controlled clinical trials remain essential to translate *in vitro* findings into tangible gastrointestinal health benefits.

5.7 CRediT authorship contribution statement

Robin Duncan: Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation, Conceptualization. **Giacomo Mantegazza:** Methodology, Investigation, Conceptualization. **Claudio Gardana:** Methodology, Investigation, Formal analysis, Data curation. **Fabio Angelini:** Writing – review & editing, Formal analysis, Conceptualization. **Rosario Russo:** Writing – review & editing, Resources, Funding acquisition, Conceptualization. **Simone Guglielmetti:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Formal analysis, Data curation, Conceptualization.

5.8 Data availability statement

Data are available upon reasonable request from the corresponding author.

5.9 Funding

This research was funded by Giellepi S.p.A.

5.10 Declaration of competing interest

R.R. is employed by Giellepi S.p.A. and was not involved in the data analysis or interpretation of results. R.D.'s doctoral fellowship is co-funded by Giellepi S.p.A.

5.11 Data availability

Data will be made available on request.

5.12 References

Amerikanou C, Kleftaki S-A, Valsamidou E, Chroni E, Biagki T, Sigala D, Koutoulogenis K, Anapliotis P, Gioxari A, Kaliora ACF (2023) Dietary patterns, or is eating behavior to blame? Analyzing the nutritional aspects of functional dyspepsia. *Nutrients* 15:1544. https://doi.org/10.3390/nu15061544

Brodkorb A, Egger L, Alminger M, Alvito P, Assunção R, Ballance S, Bohn T, Bourlieu-Lacanal C, Boutrou R, Carrière F, et al. (2019) INFOGEST static in vitro simulation of gastrointestinal food digestion. *Nat Protoc* 14:991–1014. https://doi.org/10.1038/s41596-018-0119-1

Calvo-Lerma J, Fornés-Ferrer V, Peinado I, Heredia A, Ribes-Koninckx C, Andrés A (2019) A first approach for an evidence-based in vitro digestion method to adjust pancreatic enzyme replacement therapy in cystic fibrosis. *PLoS ONE* 14:e0212459. https://doi.org/10.1371/journal.pone.0212459

Chandra P, Enespa, Singh R, Arora PK (2020) Microbial lipases and their industrial applications: a comprehensive review. *Microb Cell Fact* 19:169. https://doi.org/10.1186/s12934-020-01428-8

Egger L, Ménard O, Baumann C, Duerr D, Schlegel P, Stoll P, Vergères G, Dupont D, Portmann R (2019) Digestion of milk proteins: comparing static and dynamic in vitro digestion systems with in vivo data. *Food Res Int* 118:32–39. https://doi.org/10.1016/j.foodres.2017.12.049

Egger L, Schlegel P, Baumann C, Stoffers H, Guggisberg D, Brügger C, Dürr D, Stoll P, Vergères G, Portmann R (2017) Physiological comparability of the harmonized INFOGEST in vitro digestion method to in vivo pig digestion. *Food Res Int* 102:567–574. https://doi.org/10.1016/j.foodres.2017.09.047

Freitas D, Gómez-Mascaraque LG, Brodkorb A (2022) Digestion of protein and toxic gluten peptides in wheat bread, pasta and cereal and the effect of a supplemental enzyme mix. *Front Nutr* 9:986272. 10.3389/fnut.2022.986272

Gardana C, Del Bo' C, Quicazán MC, Corrrea AR, Simonetti P (2018) Nutrients, phytochemicals and botanical origin of commercial bee pollen from different geographical areas. *J Food Compos Anal* 73:29–38. https://doi.org/10.1016/j.jfca.2018.07.009

Garvey SM, Guice JL, Hollins MD, Best CH, Tinker KM (2022) Fungal digestive enzymes promote macronutrient hydrolysis in the INFOGEST static in vitro simulation of digestion. *Food Chem* 386:132777. https://doi.org/10.1016/j.foodchem.2022.132777

Gautam RL, Naraian RT (2020) A factory of multipurpose enzymes: cloning of enzymatic genes. In: Hesham A-E, Upadhyay RS, Sharma GD, Manoharachary C, Gupta VK (eds) *Fungal biotechnology and bioengineering*. Springer International Publishing, Cham, pp 137–162.

Ianiro G, Pecere S, Giorgio V, Gasbarrini A, Cammarota G (2016) Digestive enzyme supplementation in gastrointestinal diseases. *Curr Drug Metab* 17:187–193. http://dx.doi.org/10.2174/138920021702160114150137

Kumitch HM, Stone A, Nosworthy MG, Nickerson MT, House JD, Korber DR, Tanaka T (2020) Effect of fermentation time on the nutritional properties of pea protein-enriched flour fermented by *Aspergillus oryzae* and *Aspergillus niger*. *Cereal Chem* 97:104–113. https://doi.org/10.1002/cche.10234

Levine ME, Koch SY, Koch KL (2015) Lipase supplementation before a high-fat meal reduces perceptions of fullness in healthy subjects. *Gut Liver* 9:464–469. https://doi.org/10.5009/gnl14005

Lowe ME (2002) The triglyceride lipases of the pancreas. *J Lipid Res* 43:2007–2016. https://doi.org/10.1194/jlr.R200012-JLR200

Majeed M, Majeed S, Nagabhushanam K, Arumugam S, Pande A, Paschapur M, Ali F (2018) Evaluation of the safety and efficacy of a multienzyme complex in patients with functional dyspepsia: a randomized, double-blind, placebo-controlled study. *J Med Food* 21:1120–1128. https://doi.org/10.1089/jmf.2017.4

Mazhar S, Simon A, Colom J, Khokhlova E, Buckley M, Phipps C, Deaton J, Rea K (2024) Acute physiological effects on macromolecule digestion following oral ingestion of the enzyme blend Elevase® in ileostomy subjects: a randomized, double-blind, placebo-controlled exploratory study. *Front Nutr* 11:1357803. https://doi.org/10.3389/fnut.2024.1357803

Nakamura T, Takeuchi T, Tando Y (1998) Pancreatic dysfunction and treatment options. *Pancreas* 16.

Pouris J, Kolyva F, Bratakou S, Vogiatzi CA, Chaniotis D, Beloukas A (2024) The role of fungi in food production and processing. *Appl Sci* 14:5046. https://doi.org/10.3390/app14125046

Precup G, Marini E, Zakidou P, Beneventi E, Consuelo C, Fernández-Fraguas C, Garcia Ruiz E, Laganaro M, Magani M, Mech A, et al. (2024) Novel foods, food enzymes, and food additives derived from by-products of plant or animal origin: principles and overview of the EFSA safety assessment. *Front Nutr* 11:1390734. https://doi.org/10.3389/fnut.2024.1390734

Rathi A, Gaonkar T, Dhar D, Kallapura G, Jadhav S (2024) Study of amino acids absorption and gut microbiome on consumption of pea protein blended with enzymes-probiotics supplement. *Front Nutr* 11:1307734. https://doi.org/10.3389/fnut.2024.1307734

Rathi A, Potale S, Vaze R, Muley AB, Jadhav S (2024) In vitro simulated study of macronutrient digestion in complex food using digestive enzyme supplement. *Heliyon* 10:e30250.
Roxas M (2008) The role of enzyme supplementation in digestive disorders. *Altern Med Rev* 13:307–314. : 10.1016/j.heliyon.2024.e30250

Scarsi N, Pastorino R, Savoia C, Raspolini GM, Pezzullo AM, Boccia S (2024) Dietary patterns of adults in Italy: results from the third Italian national food consumption survey, INRAN-SCAI. *medRxiv*. https://doi.org/10.1101/2024.10.18.24315728

Singh P, Kumar S (2019a) Microbial enzyme in food biotechnology. In: Kuddus M (ed) *Enzymes in food biotechnology*. Academic Press, pp 19–28.

Singh RS, Singh T, Pandey A (2019b) Microbial enzymes—an overview. In: Singh RS, Singhania RR, Pandey A, Larroche C (eds) *Advances in enzyme technology*. Elsevier, pp 1–40.

Singh R, Singh A, Sachan S (2018) Enzymes used in the food industry: friends or foes? In: Kuddus M (ed) *Enzymes in food biotechnology: production, applications, and future prospects*. Elsevier, pp 827–843.

Song P, Zhang X, Wang S, Xu W, Wang F, Fu R, Wei F (2023) Microbial proteases and their applications. *Front Microbiol* 14:1236368. https://doi.org/10.3389/fmicb.2023.1236368

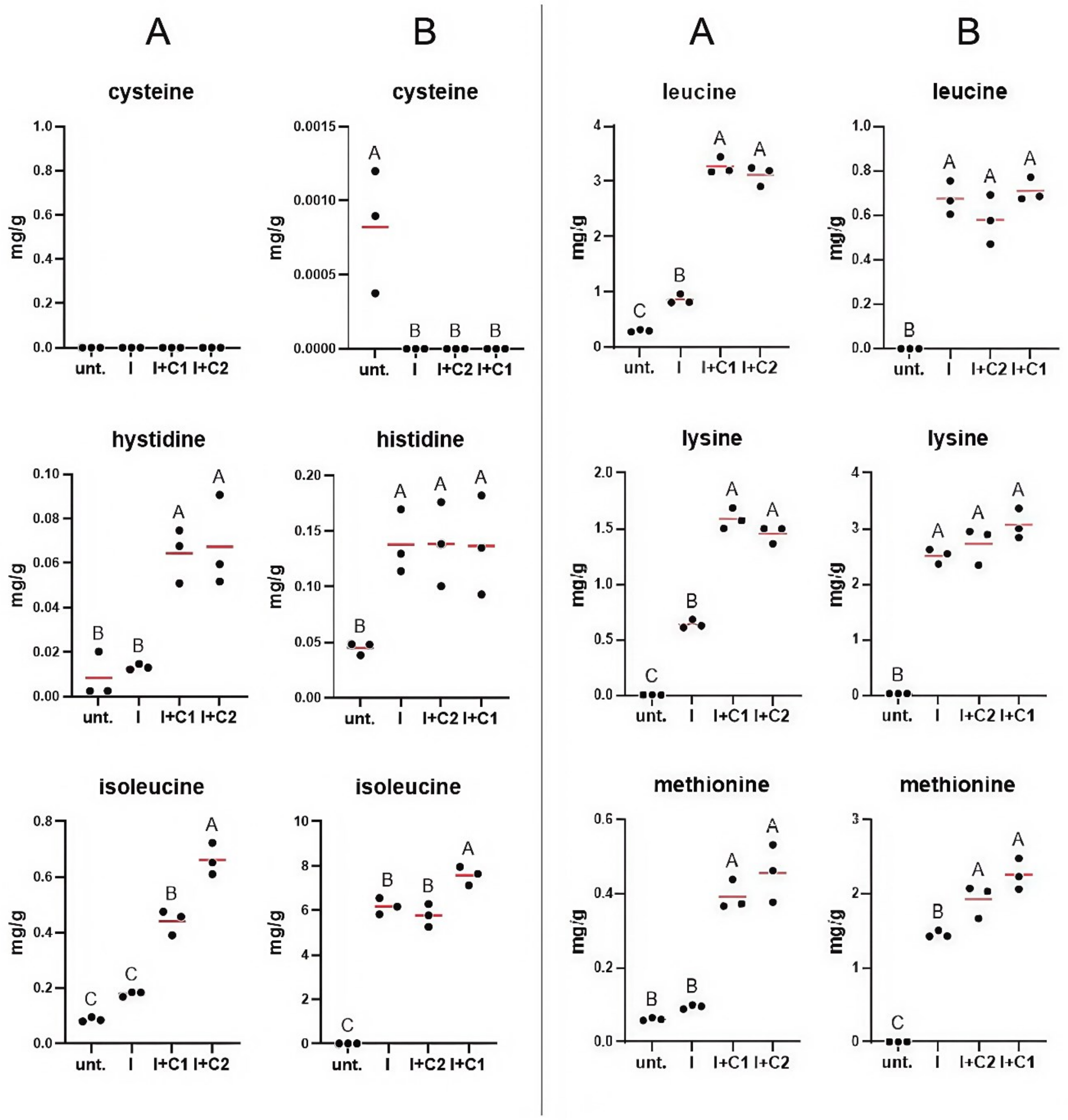
Tran Do DH, Kong F, Penet C, Winetzky D, Gregory K (2016) Using a dynamic stomach model to study efficacy of supplemental enzymes during simulated digestion. *LWT* 65:580–588. https://doi.org/10.1016/j.lwt.2015.08.054

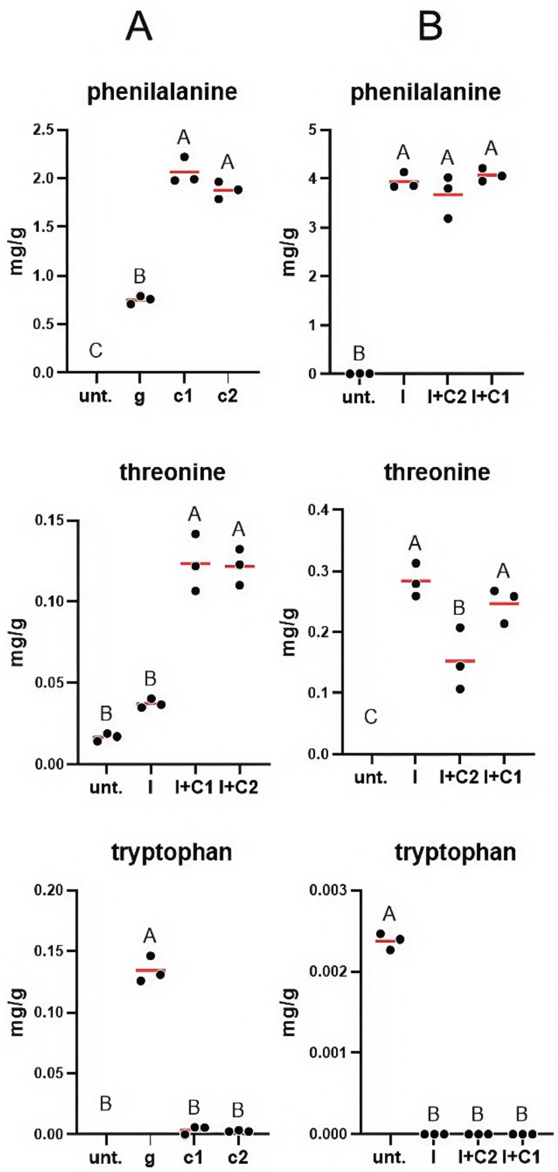
Trommelen J, Tomé D, van Loon L (2021) Gut amino acid absorption in humans: concepts and relevance for postprandial metabolism. *Clin Nutr Open Sci* 36:43–55. https://doi.org/10.1016/j.nutos.2020.12.006

Ullah H, Di Minno A, Piccinocchi R, Buccato DG, De Lellis LF, Baldi A, Daglia M (2023) Efficacy of digestive enzyme supplementation in functional dyspepsia: a monocentric, randomized, double-blind, placebo-controlled clinical trial. *Biomed Pharmacother* 169:115858. https://doi.org/10.1016/j.biopha.2023.115858

5.13 Supplementary materials

Supplementary Figure S1. Amino acid concentration in tested meals before and afterINFOGEST simulated digestion. Data are expressed as mg per gram of test meal. The data areshown for the untreated (unt.) meal, INFOGEST (I) with no addition of enzyme blend, andINFOGEST with enzyme blend-treated samples at the low dosage (I+C1) and at the high dosage(I+C2). Graphs A present data for the pizza test meal with the Poolzyme® DAIRY enzyme blend,while graphs B present data for the hamburger meal with the Poolzyme® MULTI-enzyme blend.For all conditions, three independent replicates were performed, and the median is shown as ared bar. Significant differences between samples (p ≤ 0.05; one-way ANOVA) are indicated bydifferent lower-case letters.c, d, e, f).

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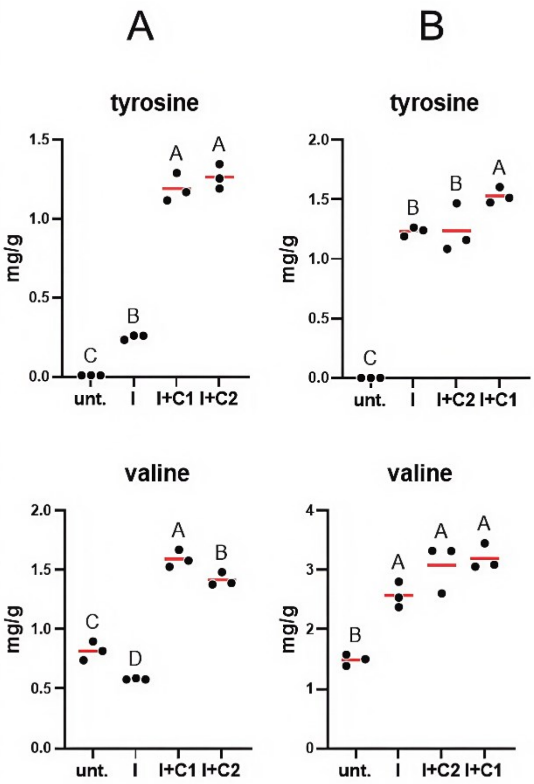
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6. In Vitro Assessment of the Impact of Astragalus, Pineapple Stem and Bergamot Extracts on the Human Fecal Microbiota.

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6.1. Abstract

Botanical extracts are increasingly used in functional foods and supplements, yet their impact on gut microbial ecosystems remains poorly understood. In this study, an *Astragalus membranaceus* root extract, a pineapple stem extract, and a bergamot extract were evaluated both in native form and after simulated gastrointestinal digestion (INFOGEST protocol) for their effects on human gut bacteria. Antimicrobial activity was first assessed in monocultures of nine commensal bacterial strains, including *Lactobacillus acidophilus LA14, Lacticaseibacillus paracasei DG, Hafnia alvei HA4597, Bifidobacterium longum subsp. longum BB536, Bifidobacterium animalis subsp. lactis BL-04, Odoribacter splanchnicus S57, Bacteroides fragilis NCTC 9343, Akkermansia muciniphila DSM 22959, Collinsella aerofaciens DSM 3979*. Only minimal, strain-specific inhibition was observed at the highest non-physiological concentration of 100 mg/mL, primarily affecting *Akkermansia muciniphila*, *Bacteroides fragilis*, and *Collinsella aerofaciens*. Subsequently, a stabilized fecal suspension (SFS) model was employed to investigate community-level responses over a 48 h anaerobic incubation. 16S rRNA gene profiling revealed that α-diversity and β-diversity were not significantly altered by any botanical treatment or digestion status. Minor shifts in specific taxa (most notably a decrease in *Collinsella* spp. with digested *Astragalus* extract) were detected, while keystone species such as *Faecalibacterium prausnitzii* and *A. muciniphila* remained stable. These findings indicate that, at physiologically relevant doses, the tested botanicals do not compromise overall microbial diversity or deplete essential taxa, supporting their classification as “*microbiome-friendly*” functional ingredients.

Keywords: botanical extracts; gut microbiota; Shannon index; in vitro digestion

6.2. Introduction

Botanical extracts have emerged as major components of the functional food and nutraceutical markets. Their widespread use extends beyond foods and food supplements to encompass cosmetic and pharmaceutical applications, often as highly processed and standardized ingredients (EFSA, 2009). These products are typically marketed based on their bioactive content and associated health benefits, which may include antioxidant, anti-inflammatory, immunomodulatory, and metabolic regulatory effects (Chandra et al., 2020; Dietz et al., 2016). However, accumulating evidence indicates that botanical ingredients can also influence the composition and functionality of the intestinal microbiota, a key endogenous ecosystem contributing to host homeostasis (Cardona et al., 2013; Ozdal et al., 2016).

Historically, research on plant–microbiota interactions focused predominantly on the prebiotic properties of dietary fibers and indigestible carbohydrates found in plant material (Gibson & Roberfroid, 2015). These substrates are known to selectively stimulate the growth of beneficial gut bacteria, such as *Bifidobacterium* spp. and *Lactobacillus* spp. (Gibson et al., 1995; Blaut, 2002). However, botanical extracts are chemically complex mixtures that go well beyond carbohydrate content, delivering diverse phytochemicals (such as polyphenols, catechins, flavonoids, saponins, tannins, and terpenes) that can modulate the intestinal microbial ecosystem through distinct and sometimes antimicrobial mechanisms (Ashraf et al., 2023; Tomas-Barberan et al., 2013). These compounds are typically poorly absorbed in the upper gastrointestinal tract, resulting in low systemic bioavailability but prolonged exposure in the colon. This pharmacokinetic profile enables extended interaction with resident microbial communities and supports the hypothesis that these compounds can substantially shape the gut microbiota community structure.

In this context, multiple plant-derived compounds have demonstrated the capacity to inhibit enteric pathogens, including *Salmonella* spp. and *Escherichia coli*, by disrupting bacterial membrane integrity, altering cell wall synthesis, and interfering with critical metabolic and signaling pathways (Mandal et al., 2024; Mita et al., 2025; Visan et al., 2024; Cowan et al., 1999). However, such antimicrobial activities are not inherently selective. Indeed, the same antimicrobial pressure may unintentionally decrease the abundance of commensal keystone taxa such as *Bifidobacterium* spp. and *Faecalibacterium prausnitzii*, and reduce overall microbial diversity (Plamada & Vodnar, 2021; De Rossi et al., 2025; Cao et al., 2024), potentially triggering dysbiosis, an ecologically disrupted microbial configuration mechanistically linked to a variety of chronic pathologies such as obesity, type 2 diabetes, inflammatory bowel disease, and metabolic dysfunction-associated steatotic liver disease (MASLD) (Zhang et al., 2024; Dahal et al., 2023). Despite these potential consequences, the impact of botanical extracts on gut microbial ecosystems is rarely considered in their safety and efficacy evaluations. This knowledge gap highlights the need for studies that systematically investigate how plant-derived bioactives influence the gut microbiome, especially when these ingredients are used in functional foods and supplements.

The present study evaluates the effects on fecal microbiota composition of three commercially available botanical ingredients: *Astragalus membranaceus* root extract, pineapple stem extract, and bergamot extract. In particular, the *A. membranaceus* extract is rich in typical saponins and polysaccharides and has been reported to attenuate inflammation and mitigate joints disorders (Maresca et al., 2017). The pineapple extract supplies bromelain, a cysteine protease with documented anti-inflammatory and immunomodulatory activities able to down regulate the activity of COX-2 (Bottega et al., 2021). The bergamot extract delivers flavonoids intended to attenuate inflammatory responses and improve plasma lipid profiles, offering promise for the dietary management of hyperlipidaemia and other metabolic disorders (Fogacci et al., 2023). Each botanical extract, both in its native form and after simulated *in vitro* digestion, was evaluated using two complementary systems: (i) mono-culture growth assays with selected commensal strains and (ii) a complex human gut model based on a stabilized fecal suspension. This dual strategy allowed us to assess microbial responses at both targeted and community levels, with the primary aim of determining whether these extracts can modulate microbiota composition without impairing overall diversity or depleting essential keystone taxa.

6.3. Materials and Methods

6.3.1 Botanicals Under Investigation

The three botanical preparations examined in this study, named Axtragyl® (AX), Bromeyal® (BR), and Kalita® (KA), are commercially available branded ingredients used in food supplements. AX is a standardized hydroalcoholic extract from the root of *Astragalus membranaceus* Bunge, sourced from plants cultivated in the Sichuan and Gansu provinces of China. BR is a proteolytic bromelain enzyme-rich preparation derived from the stem of *Ananas comosus* (pineapple). KA is obtained through spray-drying of secondary juices extracted from *Citrus bergamia* Risso (bergamot) fruits, following the procedure described by Della Vedova et al. (2023). All three preparations were kindly provided by Giellepi S.p.A. (Milan, Italy).

6.3.2. Cultivation of Human Intestinal Bacterial Strains in the Presence of Botanical Ingredients

The following bacterial strains, selected for their human intestinal origin and relevance to human health, were used to assess the potential modulatory effects of the three botanical preparations: *Lactobacillus acidophilus* LA14, *Lacticaseibacillus paracasei* DG, *Hafnia alvei* HA4597, *Bifidobacterium longum* subsp. *longum* BB536, *Bifidobacterium animalis* subsp. *lactis* BL-04, *Odoribacter splanchnicus* S57, *Bacteroides fragilis* NCTC 9343, *Akkermansia muciniphila* DSM 22959, *Collinsella aerofaciens* DSM 3979.

L. acidophilus, *L. paracasei*, and *H. alvei* were isolated from commercial food supplements. The two *Bifidobacterium* strains were obtained from Giellepi S.p.A. (Milan, Italy), while *O. splanchnicus* S57 (Hiippala et al., 2020) was sourced from the microbial collection of the Department of Biotechnology and Biosciences (BtBs), University of Milano-Bicocca. *A. muciniphila* DSM 22959 and *C. aerofaciens* DSM 3979 were acquired from the Leibniz Institute DSMZ (Braunschweig, Germany). Finally, the *B. fragilis* strain originated from the culture collection of the Department of Food, Environmental and Nutritional Sciences, University of Milan.

All bacterial strains were cultivated at 37°C and under anaerobic conditions using a Baker Ruskinn Bugbox Plus anaerobic chamber (Carli Biotec S.r.l., Frascati, Italy) maintained with a gas mixture of 90% N_2, 5% CO_2, and 5% H_2. Strict anaerobes were manipulated exclusively with pre-reduced media and buffers inside the anaerobic environment. Cultivation conditions were as follows: *Lb. acidophilus* and *Lb. paracasei* were grown in de Man, Rogosa, and Sharpe (MRS) medium (Difco, Franklin Lakes, NJ, USA). *Hafnia alvei* was cultured in tryptic soy broth (TSB), whereas both *Bifidobacterium* strains were cultured in MRS supplemented with 0.5 g/L cysteine hydrochloride (Sigma, St. Louis, MO, USA). *O. splanchnicus* and *B. fragilis* were grown in anaerobe basal broth (ABB) (Oxoid, Basingstoke, UK), while *A. muciniphila* was cultured in brain heart infusion (BHI) medium supplemented with 2 g/L bovine mucin (Sigma).

C. aerofaciens was cultivated in Modified Reinforced Clostridial Medium (MRCM), composed of: tryptose (10 g/L), beef extract (10 g/L), yeast extract (3 g/L), dextrose (5 g/L), NaCl (5 g/L), soluble starch (1 g/L), L-cysteine hydrochloride (0.5 g/L), sodium acetate (3 g/L), and resazurin (4 mL of a 0.025% stock solution per liter).

To evaluate the impact of the botanical preparations on bacterial growth, the basal, strain-specific broth was first prepared at 1.25 × concentration. Each botanical product was dissolved in sterile Milli-Q water to yield a 500 mg mL^{-1} stock solution (corresponding to 5-fold the highest target test level).

Working media containing the botanicals at 1, 10, and 100 mg mL^{-1} were obtained by mixing appropriate volumes of the 1.25 × broth stock, the 500 mg mL^{-1} botanical stock, and sterile Milli-Q water. Before mixing, the pH of both the concentrated broth and each botanical stock was individually adjusted to the standard medium pH (7.0 ± 0.1). After dilution to the final 1 × strength, the pH of every preparation was re-checked; in > 90 % of the cases the value remained within ± 0.05 pH units, confirming that the additional Milli-Q water did not measurably alter medium acidity.

Aliquots of 5 mL were distributed into sterile culture tubes sealed with cotton plugs and pre-reduced overnight under anaerobic conditions prior to inoculation. Pre-inocula were prepared 24 h in advance by transferring 50 µL of frozen glycerol stock into the appropriate medium. Prior to inoculation, the bacterial density of the pre-inoculum was determined using a counting chamber (Marienfeld, Lauda-Königshofen, Germany) and microscopy, and each tube was inoculated to achieve a final concentration of approximately 10^6 cells/mL. The following controls were included: (i) inoculated media without botanical ingredients, (ii) uninoculated media, and (iii) uninoculated media containing the highest concentration of each botanical product. Samples were collected at 48 h, serially diluted, and plated on strain-specific agar to determine viable counts. All experiments were conducted in triplicate.

6.3.3. In Vitro Digestion of Botanical Ingredients

Gastrointestinal digestion was simulated *in vitro* using the standardized INFOGEST protocol (Brodkorb et al., 2019), a widely accepted method for mimicking human digestive processes. In brief, each of the three botanical powders underwent sequential digestion through oral, gastric, and intestinal phases. Initially, 1 g of each product was homogenized with simulated salivary fluid using a Stomacher 3500 peristaltic homogenizer (Seward, Worthing, UK) to replicate the oral phase. The homogenate was adjusted to a volume of 2 mL and agitated for 2 min at 37°C. Subsequently, simulated gastric fluid was added along with rabbit gastric extract containing pepsin (RGE15, Lipolytech, Marseille, France), and the mixture was incubated at 37°C for 2 h to simulate gastric digestion. For the intestinal phase, simulated intestinal fluid was introduced with appropriate pH adjustments, followed by the addition of porcine pancreatin

(8× USP, Sigma-Aldrich, St. Louis, USA) and bovine bile salts. The final volume of each digested sample was adjusted to 8 mL. The samples were then incubated under agitation at 37°C for another 2 h. Enzymatic activity was terminated by heating the digesta at 90°C for 10 min in a water bath. All digestion experiments were performed in triplicate, and the resulting samples were stored at −80°C until further analysis.

6.3.4. Stabilized Fecal Suspension Preparation

The fecal samples used in this study were obtained from a single healthy adult volunteer (permission from the Research Ethics Committee of the University of Milan; opinion nr. 89/22). The subject had not consumed antibiotics or probiotics in the previous month. The collection was performed on-site using sterile fecal collection containers (F.L. Medical, Padova, Italy), and the samples were processed (start to finish) within two hours of collection. Processing involved the weighing of the samples, followed by the insufflation of nitrogen gas into a Whirl-Pak bag (Nasco, Pleasant Prairie, Wisconsin, USA). Subsequently, an equal volume of pre-reduced phosphate buffer solution (PBS) at pH 7 and 15% glycerol, equivalent to the weight of the fecal matter, was added under anaerobic conditions within the Baker Ruskinn Bugbox Plus anaerobic chamber. The fecal sample was then meticulously mixed by hand manipulation through the bag under these controlled conditions, aliquoted into 10 mL samples under the anaerobic chamber, and promptly stored at -80°C. To assess the overall cell viability and integrity over time, multiple sample tubes were periodically thawed, diluted, and plated onto Brain Heart Infusion (BHI) agar with 2% (w/v) glucose and 0.3 % (w/v) yeast extract (gyBHI). Additionally, for flow cytometry analysis, samples were diluted and stained with SYTO24 and propidium iodide at a final concentration of 0.1 µM and 0.2 µM respectively as according to ISO 19344:2015 guidelines. The flow cytometry was performed using a BD Accuri™ C6 Plus Flow Cytometer (BD, Milan, Italy) after incubation at 37°C for 15 min in the dark with the staining agents.

6.3.5. Fecal Incubations in the Presence of Botanical Ingredients

The design of the fecal incubation experiment is reported in Figure 1. In detail, on the day of incubation, fecal aliquots were thawed inside the anaerobic cabinet. The three botanical preparations (either in their native form or after INFOGEST in vitro digestion) were added to the respective fecal samples. Tested concentrations were selected based on physiological relevance, considering an estimated average bowel movement weight of 100 g and a recommended daily intake of approximately 500 mg per product. Accordingly, three concentrations were tested: 50 mg, 500 mg, and 5000 mg per 100 g of fecal material. To achieve these dosages, botanical powders were resuspended and diluted in sterile, pre-reduced PBS, and 1 mL of each preparation was added to the corresponding fecal aliquots. Then, the inoculated suspensions were incubated for 48 h at 37°C under anaerobic conditions. Samples (200 μL) were collected at 0, 12, 24, and 48 hours, under strict anaerobic handling, and immediately stored at −80°C until subsequent DNA extraction.

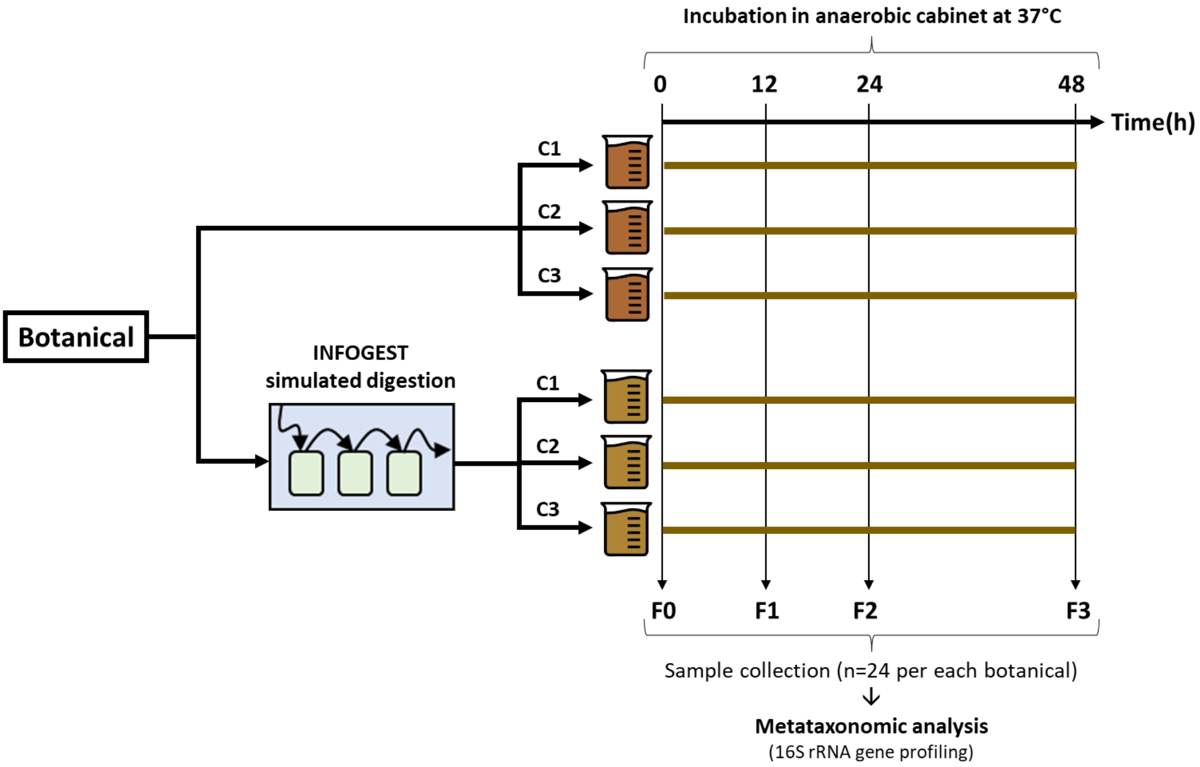


Figure 1 Experimental design of fecal incubations with botanical ingredients. Human fecal samples were incubated with three different botanical preparations, either in their native form or after simulated gastrointestinal digestion (INFOGEST protocol). Each preparation was tested at three concentrations (C1, C2, and C3 = 50, 500, and 5000 mg per 100 g of fecal material) under anaerobic conditions at 37°C for 48 h. Samples were collected at 0, 12, 24, and 48 h for metataxonomic analysis.

6.3.6. DNA Extraction from Fecal Suspensions and 16S rRNA Gene Profiling

Fecal samples were stored at –80 °C until DNA extraction. Upon thawing at 4 °C, the suspensions were homogenized by vortexing. Then, 150 mg of each suspension was weighed and processed using the QIAsymphony PowerFecal Pro Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA concentrations were quantified with the Qubit Broad Range Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA), and the resulting extracts were subjected to metataxonomic analysis via 16S rRNA gene profiling. Amplicon sequencing targeted the V3–V4 hypervariable regions of the 16S rRNA gene and was performed on the Illumina NovaSeq 6000 platform with paired-end 2 × 250 bp reads (NovaSeq 6000 SP Reagent Kit, 500 cycles). PCR amplification employed primers 341F (5′-CCTACGGGNGGCWGCAG-3′) and 805R (5′-GACTACHVGGGTATCTAATCC-3′) (LC Sciences, Houston, TX, USA). Raw sequencing reads were processed in the QIIME 2 (v2022.2) environment, using the DADA2 algorithm (Callahan et al., 2016) for quality filtering, denoising, and chimera removal. Taxonomic assignment of Amplicon Sequence Variants (ASVs) was carried out against the Greengenes (gg_13_8) reference database. To minimize batch effects and technical variability, all samples were sequenced in a single NovaSeq run. After ASV inference, singleton variants (i.e., ASVs represented by only one read across the entire dataset) were filtered out. The resulting ASV table was then rarefied to the minimum library size across samples (9598 reads per sample) to standardize sequencing depth. Read abundances underwent centered log-ratio transformation (CLR). Alpha diversity was estimated by calculating the Shannon index (H') from the rarefied ASV table (9598 reads per sample). Shannon index calculations were performed using the “diversity” plugin in QIIME 2 (v2022.2) on the rarefied ASV feature table. Weighted UniFrac distance matrices were generated with the “diversity” plugin of QIIME 2 (v2022.2) using the rarefied ASV table. Principal Coordinates Analysis (PCoA) was then applied to the distance matrices to visualize sample clustering patterns and assess community dissimilarities. All raw sequencing data have been deposited in the European Nucleotide Archive (ENA) under accession number PRJEB91377, where they are available as FASTQ files.

6.3.7. Statistical Analysis

Colony-forming units were expressed as decimal logarithms ($\log_{10}$ CFU/mL) prior to analysis to stabilize variances and approximate normality. For each bacterial strain, the effect of treatment (four levels: control medium, AX, BR, or KA) and dose (three concentrations: 1, 10 and 100 mg/mL) on bacterial growth was evaluated by a one-way analysis of variance (ANOVA) performed independently for each strain. Normality of the residuals and homoscedasticity were verified with the Shapiro–Wilk and Levene's tests, respectively; all datasets met parametric assumptions. When the global ANOVA was significant ($P < 0.05$), pairwise differences among group means were resolved with Tukey's honestly significant difference (HSD) post hoc test, which controls the family-wise Type I error rate for multiple comparisons. Data are presented as mean ± standard deviation (SD) obtained from three independent biological replicates. All statistical computations were executed with GraphPad Prism v8.4.3 (GraphPad Software, LCC, San Diego, CA, USA), and significance was defined at a two-tailed α level of 0.05.

16S rRNA profiling data lacks the independent observations required to estimate residual variance, because every extract–dose combination was represented by a single biological replicate; consequently, either α-diversity and single taxa relative abundances are reported descriptively and have been interpreted qualitatively.

The analysis of beta-diversity was performed through Pairwise Analysis of Similarities (ANOSIM) to assess differences in microbial community structure between two experimental conditions (between incubation times, and with *vs* without INFOGEST), using sample scores on the first two axes (PC1 and PC2) of a weighted UniFrac PCoA. Euclidean distance matrices were computed from the two-dimensional coordinate matrix using the pdist function in SciPy (v1.7.1). The ANOSIM R statistic was calculated according to Clarke (1993). Statistical significance was assessed by 999 random permutations of group labels, with the permutation-based p-value defined as the proportion of permuted R values that were greater than or equal to the observed R. All computations were implemented in Python (v3.8) with NumPy (v1.20.3) and pandas (v1.3.4).

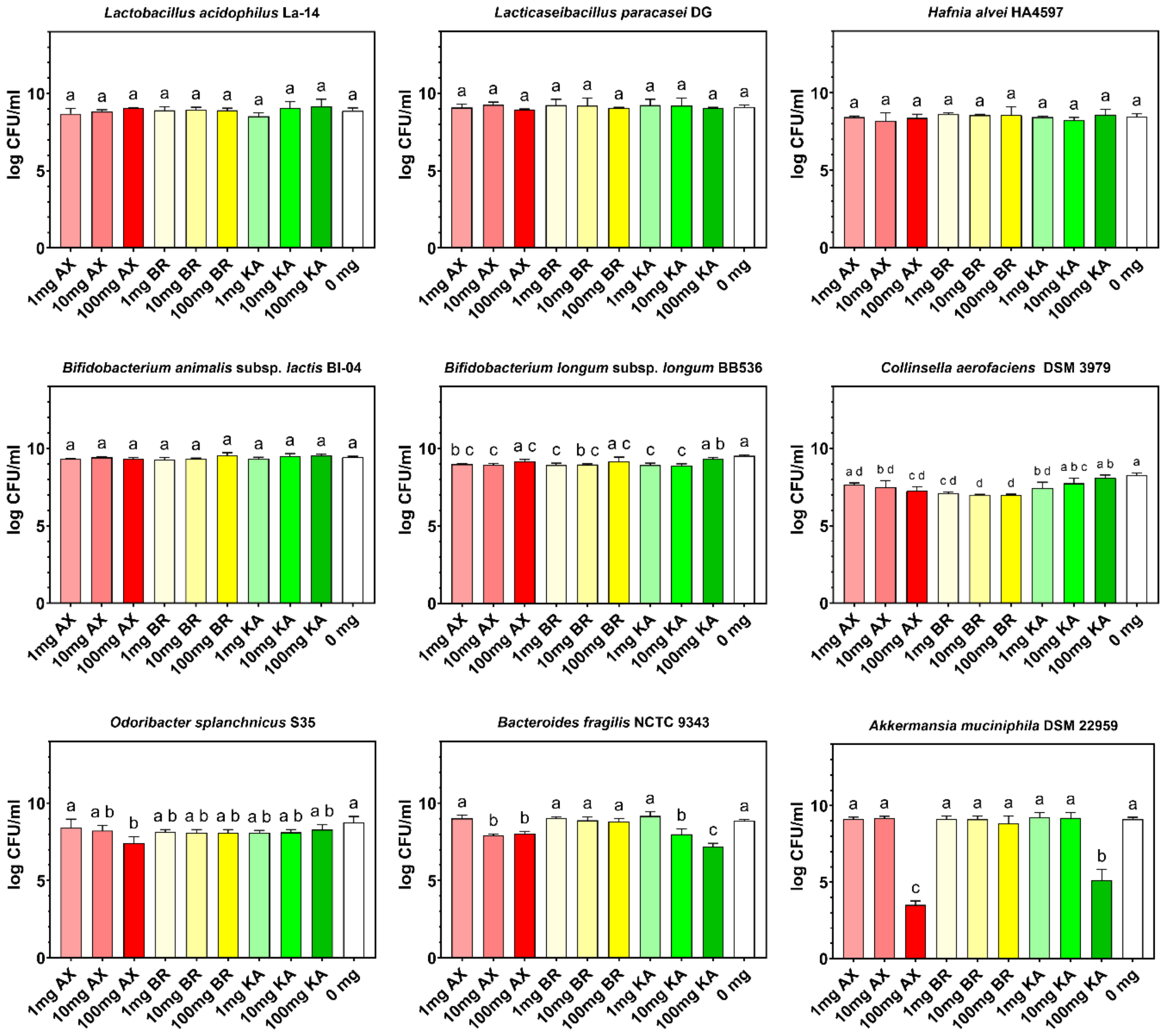
6.4. Results

6.4.1. Impact of Botanical Extracts on the Growth of Individual Gut Microbial Strains

The potential inhibitory effects of the botanical extracts under investigation were assessed on the growth of nine human gut bacterial strains. Each extract was evaluated at three concentrations (1, 10, and 100 mg/mL) to determine any dose-dependent responses. Following 48 h of incubation in strain-specific optimal culture media, the results indicated that AX exerted negligible influence on the growth of bacterial strains. A notable exception was *A. muciniphila*, whose proliferation was markedly suppressed at the highest AX concentration (100 mg/mL) (Figure 2). Additionally, a significant reduction of approximately 1 log CFU was observed for *B. fragilis* at 10 and 100 mg/mL, as well as for *O. splanchnicus*. A milder yet statistically significant decrease (less than 0.5 log CFU) was detected for *C. aerofaciens* and *Bif. longum*.

BR demonstrated a modest inhibitory effect, characterized by an approximate 1 log CFU reduction, exclusively against *C. aerofaciens* across all tested concentrations. A slight decrease (under 0.5 log CFU) was also noted for *Bif. longum* at 1 and 10 mg/mL (Figure 2). Similarly, KA induced a slight but statistically significant reduction in *Bif. longum* counts at 10 and 100 mg/mL. Notably, KA exhibited a more pronounced dose-dependent inhibitory activity against *B. fragilis* and significantly reduced *A. muciniphila* growth only at the highest concentration tested (Figure 2).

Collectively, these findings suggest that the botanical extracts possess limited inhibitory activity, predominantly at the highest concentration (100 mg/mL), with primary effects observed on the Gram-negative bacteria *B. fragilis* and *A. muciniphila*. Importantly, BR did not elicit substantial inhibition of any of the tested human gut bacterial strains.


Lactobacillus acidophilus La-14

Lacticaseibacillus paracasei DG

Hafnia alvei HA4597

Bifidobacterium animalis subsp. *lactis* Bl-04

Bifidobacterium longum subsp. *longum* BB536

Collinsella aerofaciens DSM 3979

Odoribacter splanchnicus S35

Bacteroides fragilis NCTC 9343

Akkermansia muciniphila DSM 22959

Figure 2 Impact of botanical extracts on the growth of selected human gut bacterial strains at varying concentrations (1, 10, and 100 mg/mL). The botanical extracts evaluated include Astragalus membranaceus root extract (AX), pineapple stem extract (BR), and bergamot extract (KA). Bacterial growth was quantified by enumerating colony-forming units (CFU) on agar plates, expressed as the decimal logarithm (log_{10} CFU). The control (C) represents cultures incubated in media devoid of botanical additives. Each bar denotes the mean ± standard deviation derived from three independent experiments. Distinct letters above the bars signify statistically significant differences ($P < 0.05$), as determined by one-way ANOVA followed by Tukey's post hoc test.

6.4.2. Stability and Taxonomic Composition of the Fecal Suspension

To evaluate the effects of botanical extracts on a complex microbial ecosystem, we employed a human fecal sample. Prior to conducting the main experiments, preliminary assessments were performed to determine whether the addition of prereduced PBS and glycerol could preserve bacterial viability during storage at −80 °C.

Cultivation assays on gyBHI agar demonstrated that, in the absence of cryoprotectants, viable bacterial counts decreased by approximately 1.5 log CFU over a 7-day storage period. Conversely, samples supplemented with PBS and glycerol exhibited no significant reduction in viable counts under identical storage conditions (Figure 3(a)). Flow cytometry analyses further corroborated these findings, revealing that the proportion of bacterial cells with intact membranes remained stable in samples treated with PBS and glycerol, whereas a decline was observed in untreated controls (Figure 3(b)). Subsequently, three aliquots of the fecal slurry stored at −80 °C were subjected to 16S rRNA gene profiling to assess the taxonomic composition. The analysis revealed a microbial community dominated by families commonly associated with the human colonic microbiota, including Ruminococcaceae, Lachnospiraceae, Coriobacteriaceae, Bacteroidaceae, and Bifidobacteriaceae (Figure 3(c)).

Collectively, these results indicate that the implemented cryopreservation strategy effectively maintains both the viability and taxonomic integrity of fecal bacterial populations during storage at −80 °C, thereby facilitating subsequent *in vitro* experimentation with preserved fecal slurries.

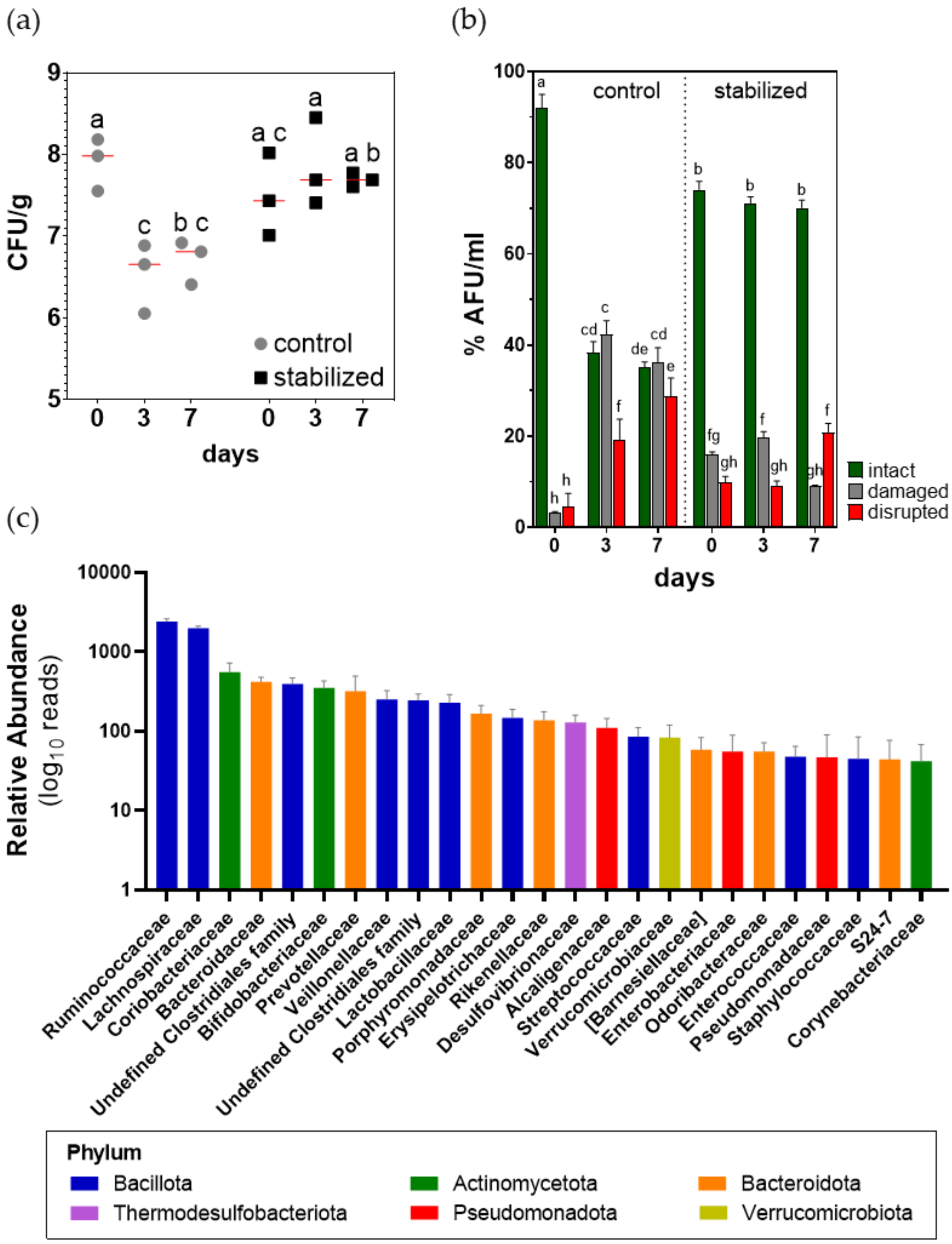


Figure 3 Microbiological characterization of the stabilized fecal suspension utilized in incubation experiments with botanical extracts. The fecal sample was analyzed with (treated) and without (control) the addition of phosphate-buffered saline (PBS) and glycerol. Viable bacterial counts were determined by plating on brain heart infusion (BHI) agar medium before and after 3 and 7 days of storage at −80 °C (panel a). The same samples were subjected to flow cytometry following dual staining with SYTO24 and propidium iodide to assess membrane integrity; cells with intact (green), damaged (grey), and disrupted (red) membranes were differentially quantified (panel b) AFU stand for active fluorescent units. Three aliquots of the stabilized fecal suspension (treated) were analyzed through 16S rRNA gene profiling to determine taxonomic composition (panel c). Different letters in panels (a) and (b) indicate statistically significant differences at $P < 0.05$, as determined by one-way ANOVA followed by Tukey's post hoc test.

6.4.3. Impact of Botanical Extracts on the Bacterial Community Structure of Stabilized Fecal Suspensions

The influence of botanical preparations on the microbial community within stabilized fecal suspensions was evaluated at three concentrations (C1, C2, and C3) over a 48-h incubation period, with sampling at 0, 12, 24, and 48 h. The resulting metataxonomic data were analyzed descriptively and interpreted qualitatively, as detailed in Section 2.7 (Statistical Analysis).

Analysis of α-diversity (assessed using the Shannon index, which accounts for both richness and evenness of bacterial taxa) showed no significant differences between treatment conditions, with Shannon values remaining consistently near 8 throughout the incubation (Figure 4).

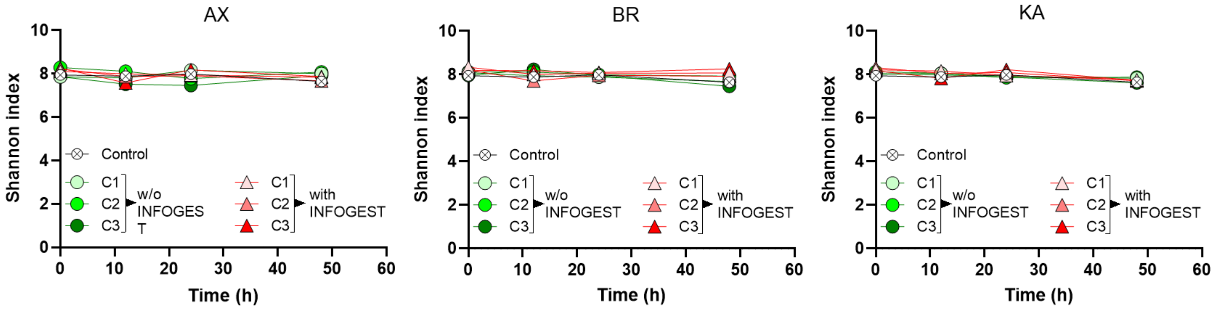


Figure 4 α-diversity analysis using the Shannon index of fecal samples incubated with three botanical extracts (AX, Astragalus membranaceus root extract; BR, pineapple stem extract; KA, bergamot extract), with or without INFOGEST pre-treatment. Extracts were added at concentrations of 50 (C1), 500 (C2), and 5000 (C3) mg per 100 g of fecal material.

The analysis of β-diversity, based on the weighted UniFrac metric, indicated that temporal progression was the primary driver of changes in overall community structure, rather than the specific botanical treatments or their concentrations. In fact, ANOSIM showed significant differences between samples at T0 and subsequent time points: T0 vs. T12 (n=19 per group; R=0.280; P=0.004), T0 vs. T24 (R=0.439; P=0.001), T0 vs. T48 (R=1.147; P=0.001), and T24 vs. T48 (R=0.472; P=0.01). Notably, ANOSIM did not reveal significant differences between samples subjected to INFOGEST pre-treatment and those that were not (n=36 vs. n=40; R=0.008; P=0.319) (Figure 5(b)).

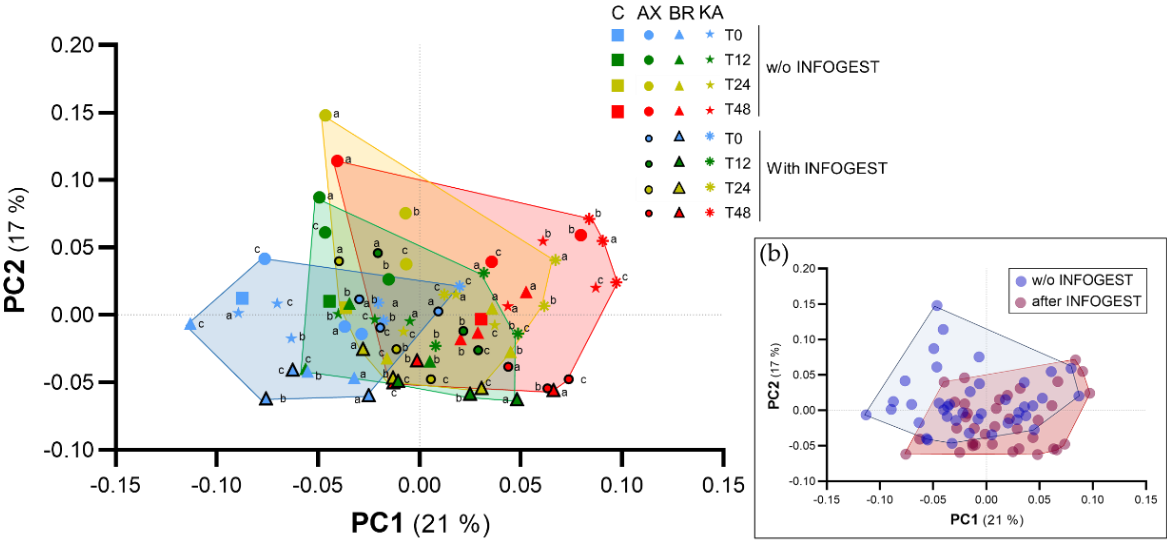


Figure 5 Beta diversity analysis using the weighted UniFrac algorithm of fecal samples incubated with three botanical extracts, with or without (w/o) INFOGEST pre-treatment, at concentrations of (a) 50 mg/100 g, (b) 500 mg/100 g, and (c) 5000 mg/100 g of fecal material. Samples are clustered according to incubation time points: T0 (baseline), T12, T24, and T48 hours. C: control (fecal sample incubated without botanical extracts); AX, Astragalus membranaceus root extract; BR, pineapple stem extract; KA, bergamot extract.

6.4.4. Effect of Botanical Extracts on the Abundance of Specific Bacterial Taxa in the Fecal Suspension

The impact of botanical preparations on the relative abundance of specific bacterial taxa was further assessed by monitoring the top 10 most abundant families and genera in the initial fecal sample over the 48-h incubation period. Overall, none of these taxa exhibited drastic reductions in relative abundance (Supplementary Figure S1). The most notable decreases were observed in the genus *Oscillospira* with AX treatment without INFOGEST pre-treatment at lower concentrations, and in the genus *Collinsella* following treatment with BR (in a dose-dependent manner) and AX, both after simulated INFOGEST digestion. Manual taxonomic analysis of amplicon sequence variants (ASVs) via BLASTn confirmed that sequences assigned to the genus *Collinsella* corresponded to *C. aerofaciens* (Figure 6). Similarly, ASVs corresponding to *A. muciniphila* and *F. prausnitzii*, both recognized for their relevance to human health, were evaluated. The relative abundances of these species remained mostly unaffected across all incubation conditions, with the only exception of an increase in the relative abundance of *A. muciniphila* with KA compared with the control (Figure 6).

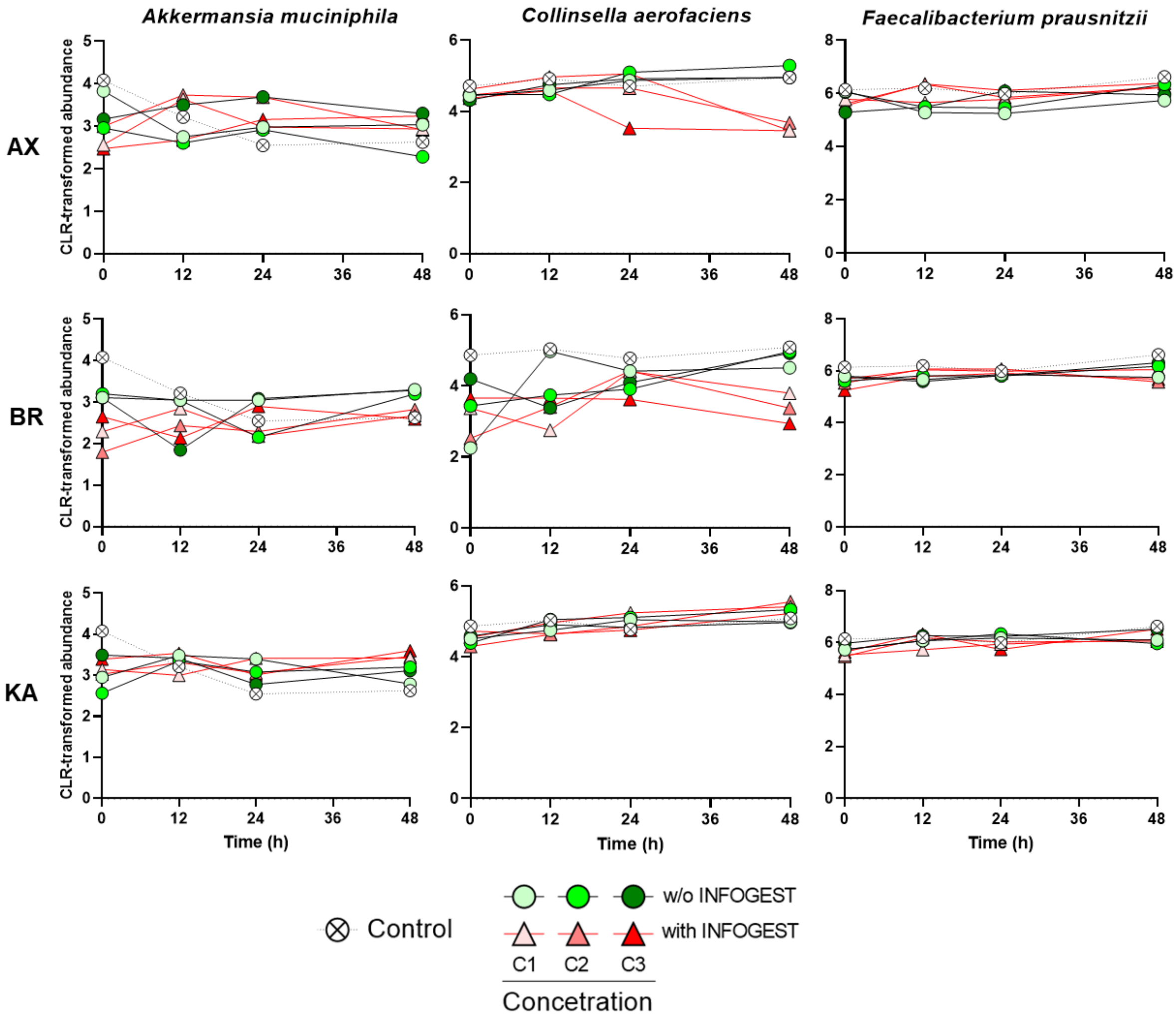


Figure 6 Impact of botanical preparations on the relative abundance of selected bacterial species in the fecal suspension. Extracts were applied at 50 (C1), 500 (C2), and 5000 (C3) mg per 100 g of fecal material. AX, Astragalus membranaceus root extract; BR, pineapple stem extract; KA, bergamot extract Abundances are shown as centered log-ratio (CLR) transformed read counts. Each botanical was tested both in its native form (w/o INFOGEST) and after simulated in vitro digestion using the INFOGEST protocol (w/ INFOGEST).

6.5. Discussion

Plant-derived preparations are promoted for antioxidant, anti-inflammatory and metabolic benefits, yet many of their signature molecules (flavanones, triterpenoid saponins, cysteine proteases, catechins, thiosulfinates or essential-oil terpenes) have been frequently reported to display antibacterial properties (Ankri & Mirelman, 1999; Pérez-Burillo et al., 2021; Jia et al., 2022). Since 90–95% of dietary polyphenols and related phytochemicals reportedly escape absorption in the small intestine and reach the colon (Clifford. 2004), it can be hypothesized that these compounds may also act upon gut-resident microbes, potentially altering community structure and reducing global diversity.

To explore this hypothesis, we conducted *in vitro* testing of three commercial botanical preparations, originally developed for health benefits not primarily related to intestinal function, for their capacity to impact human fecal microbiota. We selected three formulations containing distinct categories of phytochemicals with known broad-spectrum antimicrobial properties. AX contains *Astragalus*-derived saponins (e.g., astragaloside IV) and flavonoids that permeabilize bacterial membranes; ethanolic extracts inhibit a wide array of pathogens and commensals *in vitro* (Cui et al., 2023; Samuel et al.,2021). BR supplies bromelain, a cysteine protease that degrades surface adhesins and peptidoglycan in *E. coli* and other Gram-negative bacteria (Jancic et al., 2021; Chakraborty et al., 2021; Ataide et al., 2017). Finally, Bergamot juice powders such as KA are enriched in glycosylated flavanones (naringin, neohesperidin, neoeriocitrin) that, once deglycosylated, disrupt bacterial membranes and inhibit both Gram-negative and Gram-positive species, including lactic acid bacteria (Mandalari et al., 2017). Notably, pure naringenin suppresses *Escherichia coli*, *Streptococcus* spp. and *Staphylococcus aureus* at micromolar levels (Veiko et al.,2023; Cai et al., 2023).

These compositional features suggest that botanical preparations may pose a risk of unintended antimicrobial activity against beneficial gut microbes. Nevertheless, current regulatory frameworks, such as those of EFSA and FDA, primarily focus on toxicological endpoints (e.g., acute or sub-chronic toxicity, contaminant screening) and do not require evaluation of gut microbiota structure or function (Dusemund et al., 2012). This regulatory gap remains, despite growing evidence linking dysbiosis to multiple chronic diseases (Sasidharan et al.,2024).

To address this issue, we first evaluated the antimicrobial activity of the three botanical preparations against specific bacterial strains cultivated in monoculture in their respective optimal media. Five of the selected strains have intestinal origin and exhibit documented probiotic potential: *L. acidophilus* La-14 (shown to increase lymphocyte counts and protect against liver injury in mice; (Artanti et al., 2021; Lv et al., 2021), *Lacticaseibacillus paracasei* DG *(*enhances antiviral responses, and modulates immune responses and gut microbiome; (Balzaretti et al., 2017; Salaris et al., 2021; Ferrario er al., 2014), *Hafnia alvei* HA4597 (produces the ClpB protein with satiety-mimetic activity; (Lucas et al., 2019; Legrand et al., 2020; Ismael et al.,2023), *Bif. longum* subsp. *longum* BB536 *(*a well-characterized probiotic with gastrointestinal and immune benefits*;* (Takeda et al., 2023; Wong et al., 2019; Xu et al., 2024), and *Bifidobacterium animalis* subsp. *lactis* BL-04 (supports antiviral immunity and reduces upper respiratory infections; (Turner et al., 2022; Zabel et al., 2023). None of these five strains exhibited impaired growth in monoculture upon incubation with the botanical preparations, even at the highest tested concentration (100 mg/mL), suggesting compatibility of AX, BR, and KA with probiotic supplementation under the tested conditions.

In contrast, mild inhibitory effects were observed against four additional gut-associated bacterial strains. Specifically, AX exhibited slight inhibition of *Odoribacter splanchnicus* S57, a butyrate-producing anaerobe that releases anti-inflammatory outer membrane vesicles (Hiippala et al., 2020; Bosch et al., 2023). However, this effect was only significant at 100 mg/mL, far exceeding the expected luminal concentration in the colon, given the recommended daily intake of 480 mg. AX and, especially, KA moderately inhibited *Bacteroides fragilis* NCTC 9343, a non-toxigenic strain producing capsular polysaccharide A (PSA), known to induce regulatory T cells and restore Th1/Th2 balance in germ-free mice (Coyne et al., 2000). In contrast, enterotoxigenic *B. fragilis* (ETBF) strains carrying the *bft* gene are associated with diarrheal disease and colitis (Sears, 2009). All three botanicals, particularly KA, also showed moderate inhibition of *C. aerofaciens* DSM 3979, an anaerobic bacterium involved in bile acid metabolism. Increased *Collinsella* levels have been associated with obesity, NAFLD, IBS, and rheumatoid arthritis, possibly via promotion of low-grade inflammation (Mena-Vazquez et al., 2020; Gargari et al., 2024; Purohit et al., 2024). The only marked inhibition was observed for *A. muciniphila* DSM 22959, a mucin-degrading strain considered a next-generation probiotic with proven metabolic benefits (e.g., increased acetate/propionate, improved insulin sensitivity; (Chiantera et al., 2023; Liu et al., 2022; Li et al., 2024). However, this inhibition occurred only at the unrealistic dose of 100 mg/mL, making *in vivo* suppression unlikely under physiological conditions.

Phytochemicals often exert antimicrobial activity in their aglycone form, whereas they occur predominantly as glycosides in plants (Cushnie & Lamb, 2005; Shamsudin et al., 2022; Xiong et al., 2023). Human colonic microbiota can enzymatically release these aglycones (Selma et al., 2009), thereby uncovering bioactivities that would otherwise remain undetectable in axenic culture. Therefore, we next evaluated the impact of the three preparations on a complex microbial community using a stabilized human fecal suspension. We also assessed the effect of a simulated gastrointestinal digestion step on their bioactivity, as the matrix in which phytochemicals are embedded can influence their accessibility and, consequently, their biological activity.

For this experiment, we adopted specific measures for the preparation of the fecal suspension to preserve microbial viability, including the use of pre-reduced PBS supplemented with glycerol and strict anaerobic handling in a workstation flushed with a reducing gas mixture. This protocol proved highly effective in maintaining microbial viability, even after storage at −80 °C. Consequently, we were able to perform the experiment using aliquots that were compositionally stable and microbiologically consistent throughout the study. The suspension was prepared from a single donor whose fecal taxonomic profile encompassed key constituents of the adult gut microbiota, including families of the phylum Bacillota such as Ruminococcaceae and Lachnospiraceae (major butyrate producers and metabolically active fermenters (Singh et al., 2022; Vacca et al., 2020), families of the phylum Bacteroidota such as Bacteroidaceae and Prevotellaceae (dominant in two of the three canonical human enterotypes; Arumugam et al., 2011), and Gram-positive families Bifidobacteriaceae and Lactobacillaceae, whose strains confer a number of documented health benefits. Nonetheless, it must be emphasized that this study has the clear limitation of relying on feces from a single donor, which restricts the generalizability of the findings across the highly variable human gut microbiota types. Moreover, this constraint precludes proper statistical treatment of the data and limits the interpretation to a predominantly qualitative assessment of the results.

Unexpectedly, we found no evidence that INFOGEST-pretreated botanicals induced more substantial changes in the fecal microbiota than their native (non-digested) forms, despite the simulated digestion reagents having known antimicrobial activity. This result can be explained primarily by the fact that, in accordance with the INFOGEST protocol, samples were subjected to heat treatment to inactivate enzymes prior to their addition to the fecal slurry. Furthermore, the intestinal bacteria present in the fecal suspension are inherently adapted to withstand antimicrobial molecules, such as bile salts, which are naturally abundant in their ecological niche.

The primary finding of this study is that, despite the botanical preparations used containing substantial quantities of antimicrobial molecules, none of them triggered major compositional disruptions or a reduction in overall microbial diversity. Overall, we observed only few taxon-specific changes, the most relevant being the reduction of *Collinsella* spp., and specifically *C. aerofaciens*, following simulated digestion of AX. From a translational standpoint, the selective attenuation of *Collinsella* could be metabolically advantageous, as these taxa have been associated with pathological conditions such as rheumatoid arthritis and non-alcoholic fatty liver disease (Purohit et al., 2024; Ruiz-Limon et al., 2022).

Studies on similar topics have used animal models. For example, rat models using metagenomic, metaproteomic, and metabolomic approaches have shown that diets enriched with walnuts, broccoli, whole grains, or polyphenolic extracts such as green tea and *Ganoderma* can selectively increase *Lactobacillus*, *Bifidobacterium*, and *Akkermansia*, while reducing *Clostridium* and *Bacteroides* spp.(Čoklo et al., 2020).These effects often coincide with changes in microbial diversity: alpha diversity increased following walnut, broccoli, and camellia-oil supplementation, but decreased after long-term green-tea polyphenol intake (.(Čoklo et al., 2020)). This highlights the fact that different botanical supplements cause different outcomes regarding microbiomes, even when the bioactive compounds contained are similar. In our study, neither α-diversity nor β-diversity exhibited significant treatment-dependent alterations, with time being the predominant driver of the observed community shifts.

Several factors may account for this microbial resilience. First, within the gastrointestinal context, 90–95% of dietary polyphenols and related phytochemicals escape absorption in the small intestine and reach the colon embedded in a protein- and fiber-rich matrix. This matrix sequesters amphipathic aglycones via hydrogen bonding and hydrophobic interactions (Duenas et al., 2015), reducing their freely diffusible concentration by one to two orders of magnitude, well below most reported minimum inhibitory concentrations. Second, unabsorbed compounds undergo extensive microbial biotransformation. For example, Bacteroides-derived β-glucosidases cleave naringin into naringenin, which is subsequently reduced, ring-cleaved, and dehydroxylated into phenyl-γ-valerolactones, which are metabolites with attenuated antimicrobial activity and potentially prebiotic effects (Duenas et al., 2015). Saponins follow a similar pathway, in which terminal glucose residues are sequentially removed, generating sapogenins that integrate into micelles rather than bacterial membranes (Li et al., 2023). Findings from studies on garlic, tea, cranberry, oregano, and crucifer-derived isothiocyanates support these observations: although bactericidal in broth-based assays, they elicit only modest shifts in microbial composition in vivo (Ankri & Mirelman, 199; Jia et al., 2022; Khameneh et al., 2021). Our results extend this paradigm to bergamot flavanones, *Astragalus*-derived saponins, and bromelain, reinforcing the notion that predictions based solely on reductionist in vitro assays must be tempered by ecological considerations.

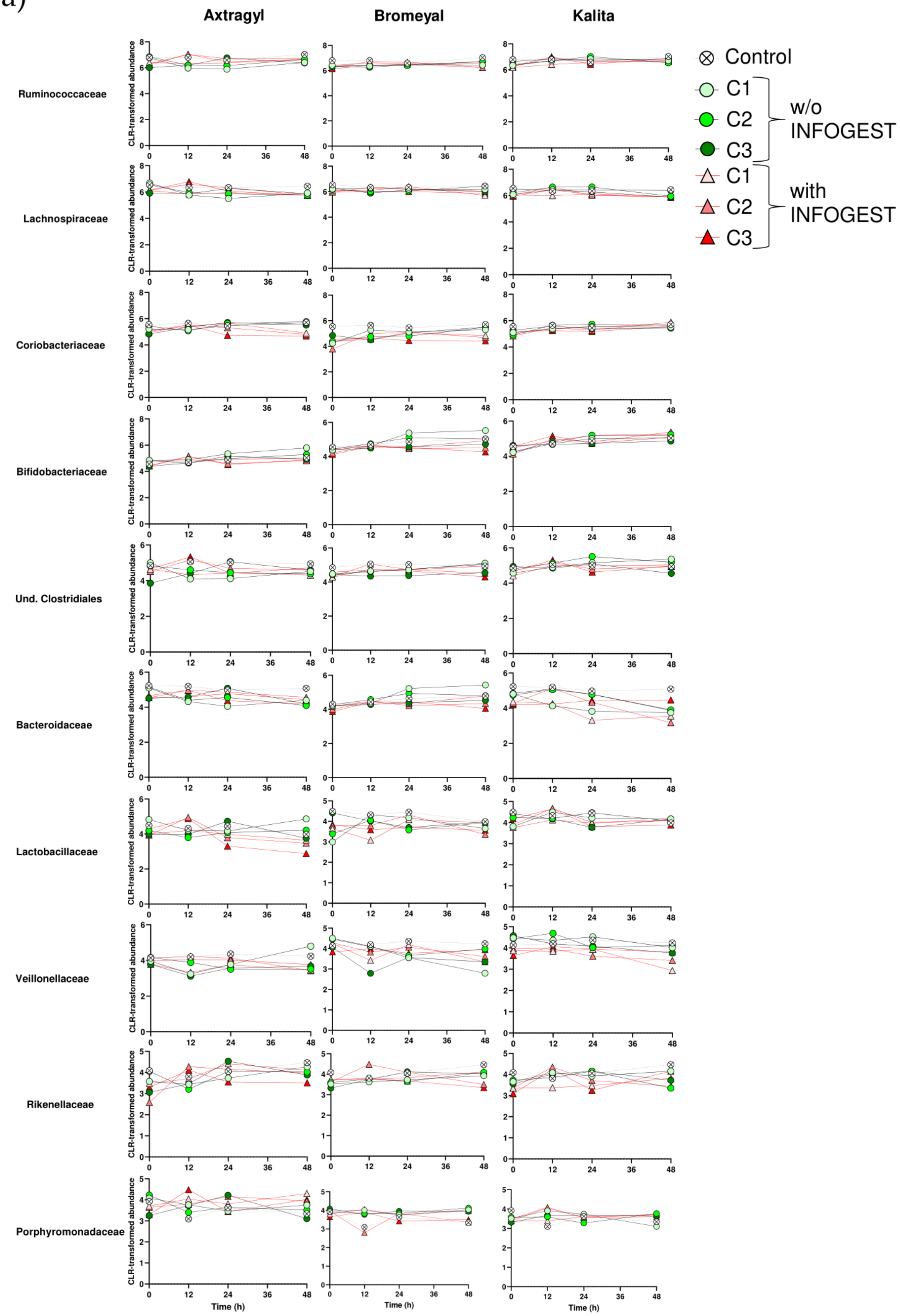
6.6. Conclusions

This study integrated mono-culture assays with a stabilized fecal suspension model, providing a multilayered perspective on microbial responses to botanical exposures. The results indicate that the three tested commercial botanical preparations rich in potentially antimicrobial phytochemicals, did not induce significant reductions in overall microbial diversity or inhibit key members of the human gut microbiota when tested at physiologically relevant concentrations. These findings support the classification of such botanical preparations as microbiome-compatible (“***microbiome-friendly***”) functional ingredients, while highlighting the importance of conducting community-scale monitoring using the whole botanical matrix, rather than relying solely on taxon-specific antimicrobial evaluations with isolated phytochemicals, to accurately predict the intestinal footprint of plant-derived bioactives.

6.7. Supplementary Materials:

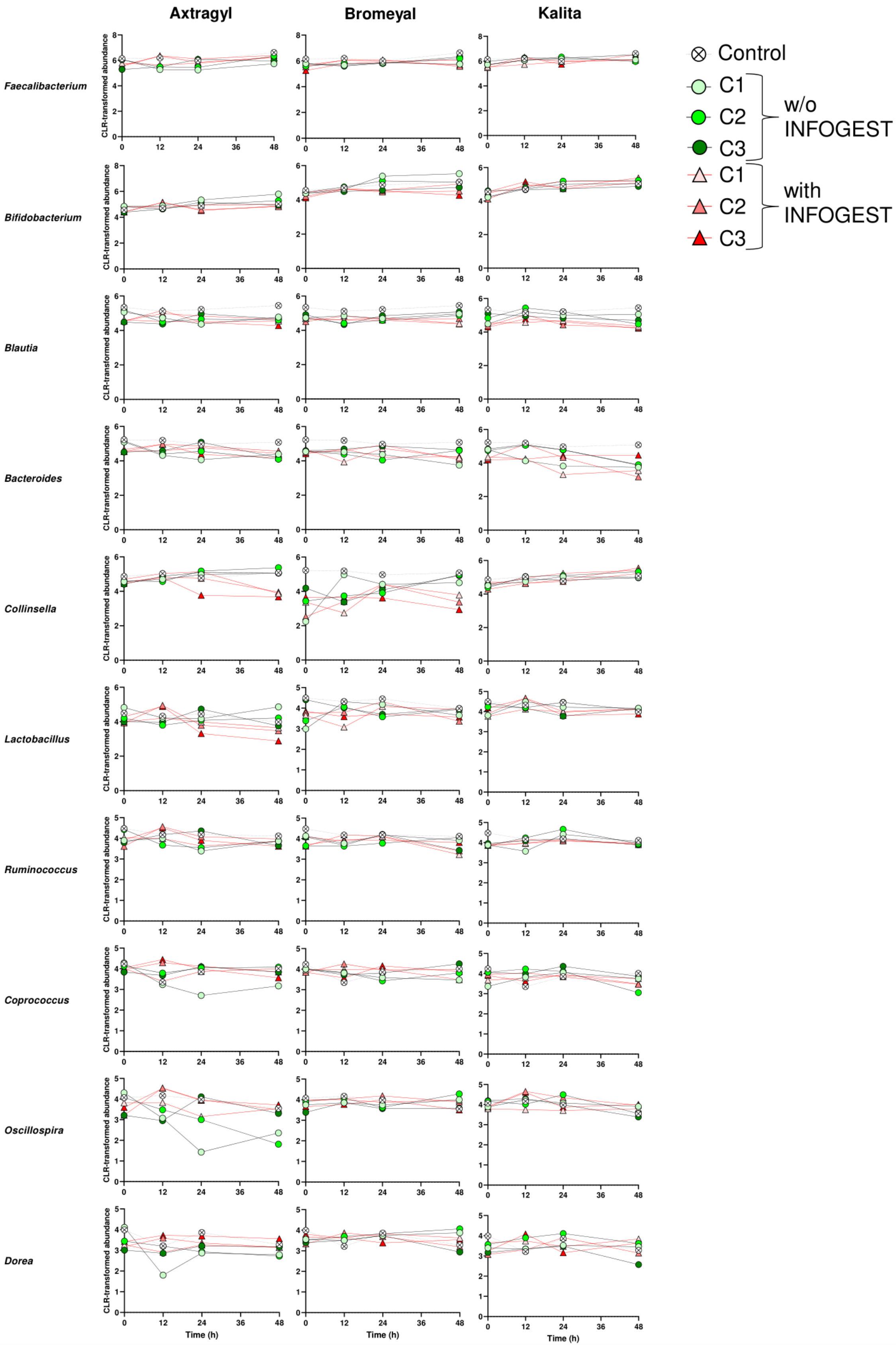
Figure S1. Impact of botanical preparations on the relative abundance of the ten most abundant bacterial families and genera in the fecal suspension. The ten families (panel a) and genera (panel b)were selected based on their mean abundances across all aliquots at the initial timepoint (T0). Extracts were applied at concentrations of 50 (C1), 500 (C2), and 5000 (C3) mg per 100 g of fecal material. Abundances are presented as centered log-ratio (CLR)–transformed read counts. Each botanical was tested both in its native form (without INFOGEST) and after simulated in vitro digestion using the INFOGEST protocol (with INFOGEST).

(a)



Axtragyl
Bromeyal
Kalita
Ruminococcaceae
Lachnospiraceae
Coriobacteriaceae
Bifidobacteriaceae
Und. Clostridiales
Bacteroidaceae
Lactobacillaceae
Veillonellaceae
Rikenellaceae
Porphyromonadaceae
CLR-transformed abundance
Time (h)
0
12
24
36
48
Control
C1
C2
C3
w/o INFOGEST
C1
C2
C3
with INFOGEST

(b)


Axtragyl
Bromeyal
Kalita
Faecalibacterium
Bifidobacterium
Blautia
Bacteroides
Collinsella
Lactobacillus
Ruminococcus
Coprococcus
Oscillospira
Dorea
CLR-transformed abundance
0
12
24
36
48
Time (h)
Control
C1
C2
C3
w/o INFOGEST
C1
C2
C3
with INFOGEST

6.8. Author Contributions:

Conceptualization, R.D., R.R. and S.G.; methodology, R.D., G.M. and G.G.; formal analysis, G.G., and S.G.; investigation, R.D., G.M. and G.G.; resources, R.R.; data curation, R.D., G.G., S.G.; writing—original draft preparation, R.D. and S.G.; writing—review and editing, R.D., R.R. and S.G.; supervision, S.G.; funding acquisition, R.R. and S.G. All authors have read and agreed to the published version of the manuscript.

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6.10. Data Availability Statement:

The raw data supporting the conclusions of this article will be made available by the authors on request.

6.11. Conflicts of Interest:

R.R. is employed by Giellepi S.p.A. and was not involved in the data analysis or interpretation of results. R.D.'s doctoral fellowship is co-funded by Giellepi S.p.A.

6.12. Declaration of AI and AI-Assisted Technologies in the Writing Process:

During the preparation of this manuscript, the authors utilized AI-based tools, including Grammarly (https://app.grammarly.com/) and ChatGPT-3.5 (https://chat.openai.com/), for grammar and style enhancement. Following the use of these tools, the authors thoroughly reviewed and edited all content, assuming full responsibility for the final publication's accuracy and integrity.

6.13. References

Ankri, S., & Mirelman, D. (1999). Antimicrobial properties of allicin from garlic. *Microbes and Infection, 1*(2), 125–129. https://doi.org/10.1016/S1286-4579(99)80003-3

Artanti, D., Sari, Y. E. S., Azizah, F., Puwaningsih, N. V., Rohmayani, V., & Nasrullah, D. (2021). Effect of giving probiotic supplement *Lactobacillus acidophilus* La-14 as an immunomodulator to maintain a respiratory system in *Mus musculus*. *Iranian Journal of Microbiology, 13*(3), 381–388. https://doi.org/10.18502/ijm.v13i3.6401

Arumugam, M., Raes, J., Pelletier, E., Le Paslier, D., Yamada, T., Mende, D. R., Fernandes, G. R., Tap, J., Bruls, T., Batto, J. M., … Dore, J. (2011). Enterotypes of the human gut microbiome. *Nature, 473*(7346), 174–180. https://doi.org/10.1038/nature09944

Ashraf, M. V., Pant, S., Khan, M. A. H., Shah, A. A., Siddiqui, S., Jeridi, M., Alhamdi, H. W. S., & Ahmad, S. (2023). Phytochemicals as antimicrobials: Prospecting Himalayan medicinal plants as source of alternate medicine to combat antimicrobial resistance. *Pharmaceuticals, 16*(6), 881. https://doi.org/10.3390/ph16060881

Ataide, J. A., de Carvalho, N. M., Rebelo, M. A., Chaud, M. V., Grotto, D., Gerenutti, M., Rai, M., Mazzola, P. G., & Jozala, A. F. (2017). Bacterial nanocellulose loaded with bromelain: Assessment of antimicrobial, antioxidant and physical-chemical properties. *Scientific Reports, 7*, 18031. https://doi.org/10.1038/s41598-017-18271-4

Balzaretti, S., Taverniti, V., Guglielmetti, S., Fiore, W., Minuzzo, M., Ngo, H. N., Ngere, J. B., Sadiq, S., Humphreys, P. N., & Laws, A. P. (2017). A novel rhamnose-rich hetero-exopolysaccharide isolated from *Lactobacillus paracasei* DG activates THP-1 human monocytic cells. *Applied and Environmental Microbiology, 83*(9), e02702-16. https://doi.org/10.1128/AEM.02702-16

Blaut, M. (2002). Relationship of prebiotics and food to intestinal microflora. *European Journal of Nutrition, 41*(Suppl. 1), i11–i16. https://doi.org/10.1007/s00394-002-1102-7

Bosch, B., Moutaharrik, S., Gazzaniga, A., Hiippala, K., Santos, H. A., & Maroni, A. (2023). Development of a time-dependent oral colon delivery system of anaerobic *Odoribacter splanchnicus* for bacteriotherapy. *European Journal of Pharmaceutics and Biopharmaceutics, 190*, 73–80. https://doi.org/10.1016/j.ejpb.2023.07.010

Bottega, R., Persico, I., De Seta, F., Romano, F., & Di Lorenzo, G. (2021). Anti-inflammatory properties of a proprietary bromelain extract (Bromeyal™) after in vitro simulated gastrointestinal digestion. *International Journal of Immunopathology and Pharmacology, 35*, 20587384211034686. https://doi.org/10.1177/20587384211034686

Brodkorb, A., Egger, L., Alminger, M., Alvito, P., Assuncao, R., Ballance, S., Bohn, T., Bourlieu-Lacanal, C., Boutrou, R., Carriere, F., … Recio, I. (2019). INFOGEST static in vitro simulation of gastrointestinal food digestion. *Nature Protocols, 14*(4), 991–1014. https://doi.org/10.1038/s41596-018-0119-1

Cai, J., Wen, H., Zhou, H., Zhang, D., Lan, D., Liu, S., Li, C., Dai, X., Song, T., Wang, X., … Xu, G. (2023). Naringenin: A flavanone with anti-inflammatory and anti-infective properties. *Biomedicine & Pharmacotherapy, 164*, 114990. https://doi.org/10.1016/j.biopha.2023.114990

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J., & Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods, 13*(7), 581–583. https://doi.org/10.1038/nmeth.3869

Cao, S., Liu, M., Han, Y., Li, S., Zhu, X., Li, D., Shi, Y., & Liu, B. (2024). Effects of saponins on lipid metabolism: The gut-liver axis plays a key role. *Nutrients, 16*(10), 1514. https://doi.org/10.3390/nu16101514

Cardona, F., Andres-Lacueva, C., Tulipani, S., Tinahones, F. J., & Queipo-Ortuno, M. I. (2013). Benefits of polyphenols on gut microbiota and implications in human health. *Journal of Nutritional Biochemistry, 24*(8), 1415–1422. https://doi.org/10.1016/j.jnutbio.2013.05.001

Chakraborty, A. J., Mitra, S., Tallei, T. E., Tareq, A. M., Nainu, F., Cicia, D., Dhama, K., Emran, T. B., Simal-Gandara, J., & Capasso, R. (2021). Bromelain a potential bioactive compound: A comprehensive overview from a pharmacological perspective. *Life, 11*(4), 317. https://doi.org/10.3390/life11040317

Chandra, G., Mukherjee, D., Ray, A. S., & Chatterjee, S. (2020). Phytoextracts as antibacterials: A review. *Current Drug Discovery Technologies, 17*(5), 523–533. https://doi.org/10.2174/1570163816666191106103730

Chiantera, V., Lagana, A. S., Basciani, S., Nordio, M., & Bizzarri, M. (2023). A critical perspective on the supplementation of *Akkermansia muciniphila*: Benefits and harms. *Life, 13*(6), 1247. https://doi.org/10.3390/life13061247

Čoklo, M., Maslov, D. R., & Kraljević Pavelić, S. (2020). Modulation of gut microbiota in healthy rats after exposure to nutritional supplements. *Gut Microbes*, *12*(1). https://doi.org/10.1080/19490976.2020.1779002

Clarke, K. R. (1993). Non-parametric multivariate analyses of changes in community structure. *Australian Journal of Ecology, 18*(1), 117–143. https://doi.org/10.1111/j.1442-9993.1993.tb00438.x

Clifford, M. N. (2004). Diet-derived phenols in plasma and tissues and their implications for health. *Planta Medica, 70*(12), 1103–1114. https://doi.org/10.1055/s-2004-835835

Cowan, M. M. (1999). Plant products as antimicrobial agents. *Clinical Microbiology Reviews, 12*(4), 564–582. https://doi.org/10.1128/CMR.12.4.564

Coyne, M. J., Kalka-Moll, W., Tzianabos, A. O., Kasper, D. L., & Comstock, L. E. (2000). *Bacteroides fragilis* NCTC9343 produces at least three distinct capsular polysaccharides: Cloning, characterization, and reassignment of polysaccharide B and C biosynthesis loci. *Infection and Immunity, 68*(11), 6176–6181. https://doi.org/10.1128/IAI.68.11.6176-6181.2000

Cui, L., Ma, Z., Li, W., Ma, H., Guo, S., Wang, D., & Niu, Y. (2023). Inhibitory activity of flavonoids fraction from *Astragalus membranaceus* Fisch. ex Bunge stems and leaves on *Bacillus cereus* and its separation and purification. *Frontiers in Pharmacology, 14*, 1183393. https://doi.org/10.3389/fphar.2023.1183393

Cushnie, T. P., & Lamb, A. J. (2005). Antimicrobial activity of flavonoids. *International Journal of Antimicrobial Agents, 26*(5), 343–356. https://doi.org/10.1016/j.ijantimicag.2005.09.002

Dahal, R. H., Kim, S., Kim, Y. K., Kim, E. S., & Kim, J. (2023). Insight into gut dysbiosis of patients with inflammatory bowel disease and ischemic colitis. *Frontiers in Microbiology, 14*, 1174832. https://doi.org/10.3389/fmicb.2023.1174832

De Rossi, L., Rocchetti, G., Lucini, L., & Rebecchi, A. (2025). Antimicrobial potential of polyphenols: Mechanisms of action and microbial responses—A narrative review. *Antioxidants, 14*(2), 200. https://doi.org/10.3390/antiox14020200
Della Vedova, L., Gado, F., Vieira, T. A., Grandini, N. A., Palacio, T. L. N., Siqueira, J. S., Carini, M., Bombardelli, E., Correa, C. R., Aldini, G., et al. (2023). Chemical, nutritional and biological

evaluation of a sustainable and scalable complex of phytochemicals from bergamot by-products. *Molecules, 28*(7), 2964. https://doi.org/10.3390/molecules28072964

Dietz, B. M., Hajirahimkhan, A., Dunlap, T. L., & Bolton, J. L. (2016). Botanicals and their bioactive phytochemicals for women's health. *Pharmacological Reviews, 68*(4), 1026–1073. https://doi.org/10.1124/pr.115.010843

Duenas, M., Munoz-Gonzalez, I., Cueva, C., Jimenez-Giron, A., Sanchez-Patan, F., Santos-Buelga, C., Moreno-Arribas, M. V., & Bartolome, B. (2015). A survey of modulation of gut microbiota by dietary polyphenols. *BioMed Research International, 2015*, 850902. https://doi.org/10.1155/2015/850902

Dusemund, B., Gilbert, J., Gott, D., Kenigswald, H., König, J., Lambré, C., Leblanc, J.-C., Mortensen, A., & Tobback, P. (2012). Food additives and nutrient sources added to food: Developments since the creation of EFSA. *EFSA Journal, 10*(s1006), s1006. https://doi.org/10.2903/j.efsa.2012.s1006

EFSA Scientific Committee. (2009). Guidance on safety assessment of botanicals and botanical preparations intended for use as ingredients in food supplements. *EFSA Journal, 7*(9), 1249. https://doi.org/10.2903/j.efsa.2009.1249

Ferrario, C., Taverniti, V., Milani, C., Fiore, W., Laureati, M., De Noni, I., Stuknyte, M., Chouaia, B., Riso, P., & Guglielmetti, S. (2014). Modulation of fecal *Clostridiales* bacteria and butyrate by probiotic intervention with *Lactobacillus paracasei* DG varies among healthy adults. *Journal of Nutrition, 144*(11), 1787–1796. https://doi.org/10.3945/jn.114.197723

Fogacci, F., Giovannini, M., Imbalzano, E., Grandi, E., Rizzoli, E., D'Addato, S., & Cicero, A. F. G. (2023). Metabolic and vascular effect of a new standardized bergamot phytocomplex: A three-arm, placebo-controlled, double-blind clinical trial. *Archives of Medical Science, 19*(5), 1228–1235. https://doi.org/10.5114/aoms/163368

Gargari, G., Mantegazza, G., Cremon, C., Taverniti, V., Valenza, A., Barbaro, M. R., Marasco, G., Duncan, R., Fiore, W., Ferrari, R., et al. (2024). *Collinsella aerofaciens* as a predictive marker of response to probiotic treatment in non-constipated irritable bowel syndrome. *Gut Microbes, 16*(1), 2298246. https://doi.org/10.1080/19490976.2023.2298246

Gibson, G. R., & Roberfroid, M. B. (1995). Dietary modulation of the human colonic microbiota: Introducing the concept of prebiotics. *Journal of Nutrition, 125*(6), 1401–1412. https://doi.org/10.1093/jn/125.6.1401

Hiippala, K., Barreto, G., Burrello, C., Diaz-Basabe, A., Suutarinen, M., Kainulainen, V., Bowers, J. R., Lemmer, D., Engelthaler, D. M., Eklund, K. K., et al. (2020). Novel *Odoribacter splanchnicus* strain and its outer membrane vesicles exert immunoregulatory effects *in vitro. Frontiers in Microbiology, 11*, 575455. https://doi.org/10.3389/fmicb.2020.575455

Holscher, H. D. (2017). Dietary fiber and prebiotics and the gastrointestinal microbiota. *Gut Microbes, 8*(2), 172–184. https://doi.org/10.1080/19490976.2017.1290756

Ismael, S., Vaz, C., Durao, C., Silvestre, M. P., Calhau, C., Teixeira, D., & Marques, C. (2023). The impact of *Hafnia alvei* HA4597 on weight loss and glycaemic control after bariatric surgery—Study protocol for a triple-blinded, blocked randomized, 12-month, parallel-group, placebo-controlled clinical trial. *Trials, 24*(1), 362. https://doi.org/10.1186/s13063-023-07383-0

Jancic, U., & Gorgieva, S. (2021). Bromelain and nisin: The natural antimicrobials with high potential in biomedicine. *Pharmaceutics, 14*(1), 10076. https://doi.org/10.3390/pharmaceutics14010076

Jia, L., Wu, J., Lei, Y., Kong, F., Zhang, R., Sun, J., Wang, L., Li, Z., Shi, J., Wang, Y., et al. (2022). Oregano essential oils mediated intestinal microbiota and metabolites and improved growth performance and intestinal barrier function in sheep. *Frontiers in Immunology, 13*, 908015. https://doi.org/10.3389/fimmu.2022.908015

Khameneh, B., Eskin, N. A. M., Iranshahy, M., & Fazly Bazzaz, B. S. (2021). Phytochemicals: A promising weapon in the arsenal against antibiotic-resistant bacteria. Antibiotics, 10(9), 1044. https://doi.org/10.3390/antibiotics10091044auth

Legrand, R., Lucas, N., Dominique, M., Azhar, S., Deroissart, C., Le Solliec, M. A., Rondeaux, J., Nobis, S., Guerin, C., Leon, F., et al. (2020). Commensal *Hafnia alvei* strain reduces food intake and fat mass in obese mice—A new potential probiotic for appetite and body weight management. *International Journal of Obesity, 44*(5), 1041–1051. https://doi.org/10.1038/s41366-019-0515-9

Li, L., Li, M., Chen, Y., Yu, Z., Cheng, P., Yu, Z., Cheng, W., Zhang, W., Wang, Z., Gao, X., et al. (2024). Function and therapeutic prospects of next-generation probiotic *Akkermansia muciniphila* in infectious diseases. *Frontiers in Microbiology, 15*, 1354447. https://doi.org/10.3389/fmicb.2024.1354447

Li, Z., Hu, E., Zheng, F., Wang, S., Zhang, W., Luo, J., Tang, T., Huang, Q., & Wang, Y. (2023). The effects of astragaloside IV on gut microbiota and serum metabolism in a mice model of intracerebral hemorrhage. *Phytomedicine, 121*, 155086. https://doi.org/10.1016/j.phymed.2023.155086

Liu, M. J., Yang, J. Y., Yan, Z. H., Hu, S., Li, J. Q., Xu, Z. X., & Jian, Y. P. (2022). Recent findings in *Akkermansia muciniphila*-regulated metabolism and its role in intestinal diseases. *Clinical Nutrition, 41*(11), 2333–2344. https://doi.org/10.1016/j.clnu.2022.08.029

Lucas, N., Legrand, R., Deroissart, C., Dominique, M., Azhar, S., Le Solliec, M. A., Leon, F., do Rego, J. C., Dechelotte, P., Fetissov, S. O., et al. (2019). *Hafnia alvei* HA4597 strain reduces food intake and body weight gain and improves body composition, glucose, and lipid metabolism in a mouse model of hyperphagic obesity. *Microorganisms, 8*(1), 35. https://doi.org/10.3390/microorganisms8010035

Lv, L., Yao, C., Yan, R., Jiang, H., Wang, Q., Wang, K., Ren, S., Jiang, S., Xia, J., Li, S., et al. (2021). *Lactobacillus acidophilus* LA14 alleviates liver injury. *mSystems, 6*(4), e00384-21. https://doi.org/10.1128/mSystems.00384-21

Mandal, M. K., & Domb, A. J. (2024). Antimicrobial activities of natural bioactive polyphenols. *Pharmaceutics, 16*(6), 718. https://doi.org/10.3390/pharmaceutics16060718

Mandalari, G., Bennett, R. N., Bisignano, G., Trombetta, D., Saija, A., Faulds, C. B., Gasson, M. J., & Narbad, A. (2007). Antimicrobial activity of flavonoids extracted from bergamot (*Citrus bergamia* Risso) peel, a by-product of the essential oil industry. *Journal of Applied Microbiology, 103*(6), 2056–2064. https://doi.org/10.1111/j.1365-2672.2007.03456.x

Maresca, M., Micheli, L., Cinci, L., Bilia, A. R., Ghelardini, C., & Di Cesare Mannelli, L. (2017). Pain relieving and protective effects of *Astragalus* hydroalcoholic extract in rat arthritis models. *Journal of Pharmacy and Pharmacology, 69*(12), 1858–1870. https://doi.org/10.1111/jphp.12828

Mena-Vazquez, N., Ruiz-Limon, P., Moreno-Indias, I., Manrique-Arija, S., Tinahones, F. J., & Fernandez-Nebro, A. (2020). Expansion of rare and harmful lineages is associated with established rheumatoid arthritis. *Journal of Clinical Medicine, 9*(4), 1044. https://doi.org/10.3390/jcm9041044

Mita, S. R., Muhtar, N. I., Kusuma, S. A. F., Sriwidodo, S., & Hendrawan, R. P. (2025). Catechins as antimicrobial agents and their contribution to cosmetics. *Cosmetics, 12*(1), 11. https://doi.org/10.3390/cosmetics12010011

Ozdal, T., Sela, D. A., Xiao, J., Boyacioglu, D., Chen, F., & Capanoglu, E. (2016). The reciprocal interactions between polyphenols and gut microbiota and effects on bioaccessibility. *Nutrients, 8*(2), 78. https://doi.org/10.3390/nu8020078

Pérez-Burillo, S., Navajas-Porras, B., Lopez-Maldonado, A., Hinojosa-Nogueira, D., Pastoriza, S., & Rufian-Henares, J. A. (2021). Green tea and its relation to human gut microbiome. *Molecules, 26*(13), 3907. https://doi.org/10.3390/molecules26133907

Plamada, D., & Vodnar, D. C. (2021). Polyphenols–gut microbiota interrelationship: A transition to a new generation of prebiotics. *Nutrients, 14*(1), 137. https://doi.org/10.3390/nu14010137

Purohit, A., Kandiyal, B., Kumar, S., Pragasam, A. K., Kamboj, P., Talukdar, D., Verma, J., Sharma, V., Sarkar, S., Mahajan, D., et al. (2024). *Collinsella aerofaciens* linked with increased ethanol production and liver inflammation contribute to the pathophysiology of NAFLD. *iScience, 27*(1), 108764. https://doi.org/10.1016/j.isci.2023.108764

Ruiz-Limon, P., Mena-Vazquez, N., Moreno-Indias, I., Manrique-Arija, S., Lisbona-Montanez, J. M., Cano-Garcia, L., Tinahones, F. J., & Fernandez-Nebro, A. (2022). *Collinsella* is associated with cumulative inflammatory burden in an established rheumatoid arthritis cohort. *Biomedicine & Pharmacotherapy, 153*, 113518. https://doi.org/10.1016/j.biopha.2022.113518

Salaris, C., Scarpa, M., Elli, M., Bertolini, A., Guglielmetti, S., Pregliasco, F., Brun, P., & Castagliuolo, I. (2021). *Lacticaseibacillus paracasei* DG enhances the lactoferrin anti-SARS-CoV-2 response in Caco-2 cells. *Gut Microbes, 13*(1), 1961970. https://doi.org/10.1080/19490976.2021.1961970

Samuel, A. O., Huang, B. T., Chen, Y., Guo, F. X., Yang, D. D., & Jin, J. Q. (2021). Author correction: Antioxidant and antibacterial insights into the leaves, leaf tea and medicinal roots from *Astragalus membranaceus* (Fisch.) Bge. *Scientific Reports, 11*, 20581. https://doi.org/10.1038/s41598-021-00118-8

Sasidharan Pillai, S., Gagnon, C. A., Foster, C., & Ashraf, A. P. (2024). Exploring the gut microbiota: Key insights into its role in obesity, metabolic syndrome, and type 2 diabetes. *Journal of Clinical Endocrinology & Metabolism, 109*(9), 2709–2719. https://doi.org/10.1210/clinem/dgae499

Sears, C. L. (2009). Enterotoxigenic *Bacteroides fragilis*: A rogue among symbiotes. *Clinical Microbiology Reviews, 22*(3), 349–369. https://doi.org/10.1128/CMR.00053-08

Selma, M. V., Espin, J. C., & Tomas-Barberan, F. A. (2009). Interaction between phenolics and gut microbiota: Role in human health. *Journal of Agricultural and Food Chemistry, 57*(15), 6485–6501. https://doi.org/10.1021/jf902107d

Shamsudin, N. F., Ahmed, Q. U., Mahmood, S., Ali Shah, S. A., Khatib, A., Mukhtar, S., Alsharif, M. A., Parveen, H., & Zakaria, Z. A. (2022). Antibacterial effects of flavonoids and their structure-activity relationship study: A comparative interpretation. *Molecules, 27*(4), 1149. https://doi.org/10.3390/molecules27041149

Singh, V., Lee, G., Son, H., Koh, H., Kim, E. S., Unno, T., & Shin, J. H. (2022). Butyrate producers, “the sentinel of gut”: Their intestinal significance with and beyond butyrate, and prospective use as microbial therapeutics. *Frontiers in Microbiology, 13*, 1103836. https://doi.org/10.3389/fmicb.2022.1103836

Takeda, T., Asaoka, D., Nojiri, S., Yanagisawa, N., Nishizaki, Y., Osada, T., Koido, S., Nagahara, A., Katsumata, N., Odamaki, T., et al. (2023). Usefulness of *Bifidobacterium longum* BB536 in elderly individuals with chronic constipation: A randomized controlled trial. *American Journal of Gastroenterology, 118*(4), 561–568. https://doi.org/10.14309/ajg.0000000000002028

Tomas-Barberan, F. A., Selma, M. V., & Espin, J. C. (2016). Interactions of gut microbiota with dietary polyphenols and consequences to human health. *Current Opinion in Clinical Nutrition & Metabolic Care, 19*(6), 471–476. https://doi.org/10.1097/MCO.0000000000000314

Turner, R. B., Lehtoranta, L., Hibberd, A., Mannikko, S., Zabel, B., Yeung, N., Huttunen, T., Burns, F. R., & Lehtinen, M. J. (2022). Effect of *Bifidobacterium animalis* ssp. *lactis* Bl-04 on rhinovirus-induced colds: A randomized, placebo-controlled, single-center, phase II trial in healthy volunteers. *EClinicalMedicine, 43*, 101224. https://doi.org/10.1016/j.eclinm.2021.101224

Vacca, M., Celano, G., Calabrese, F. M., Portincasa, P., Gobbetti, M., & De Angelis, M. (2020). The controversial role of human gut *Lachnospiraceae*. *Microorganisms, 8*(4), 573. https://doi.org/10.3390/microorganisms8040573

Veiko, A. G., Olchowik-Grabarek, E., Sekowski, S., Roszkowska, A., Lapshina, E. A., Dobrzynska, I., Zamaraeva, M., & Zavodnik, I. B. (2023). Antimicrobial activity of quercetin, naringenin and catechin: Flavonoids inhibit *Staphylococcus aureus*-induced hemolysis and modify membranes of bacteria and erythrocytes. *Molecules, 28*(3), 1252. https://doi.org/10.3390/molecules28031252

Visan, A. I., & Negut, I. (2024). Coatings based on essential oils for combating antibiotic resistance. *Antibiotics, 13*(7), 625. https://doi.org/10.3390/antibiotics13070625

Wong, C. B., Odamaki, T., & Xiao, J.-z. (2019). Beneficial effects of *Bifidobacterium longum* subsp. *longum* BB536 on human health: Modulation of gut microbiome as the principal action. *Journal of Functional Foods, 54*, 506–519. https://doi.org/10.1016/j.jff.2019.02.002

Xiong, H. H., Lin, S. Y., Chen, L. L., Ouyang, K. H., & Wang, W. J. (2023). The interaction between flavonoids and intestinal microbes: A review. *Foods, 12*(2), 317. https://doi.org/10.3390/foods12020320

Xu, C., Hiraku, A., Arai, S., Iwabuchi, N., Tanaka, M., & Nakamura, M. (2024). Probiotic *Bifidobacterium longum* BB536 and its impact on subjective symptoms of physical conditions associated with common cold-like symptoms in healthy adults: A randomized, double-blind, placebo-controlled trial. *Journal of Functional Foods, 115*, 106113. https://doi.org/10.1016/j.jff.2024.106113

Zabel, B., Makela, S. M., Nedveck, D., Hibberd, A. A., Yeung, N., Latvala, S., Lehtoranta, L., Junnila, J., Walters, K. B., Morovic, W., et al. (2023). The effect of *Bifidobacterium animalis* subsp. *lactis* Bl-04 on influenza A virus infection in mice. *Microorganisms, 11*(10), 2582. https://doi.org/10.3390/microorganisms11102582

Zhang, L., Wang, P., Huang, J., Xing, Y., Wong, F. S., Suo, J., & Wen, L. (2024). Gut microbiota and therapy for obesity and type 2 diabetes. *Frontiers in Endocrinology, 15*, 1333778. https://doi.org/10.3389/fendo.2024.1333778

7. Conclusions and future perspectives

This thesis investigated the safety, digestive performance, and microbiome compatibility of selected nutraceuticals through a modular standardized in vitro workflow (static INFOGEST digestion; fecal batch fermentations where relevant; targeted mono-culture assays), applied according to the intervention category.

The study demonstrated that the spore-forming bacterial strain *Heyndrickxia coagulans* LMG S-24828 is, functionally active, and capable of surviving and germinating under simulated gastrointestinal conditions, confirming its suitability as a probiotic candidate. Fungal enzyme blends enhanced proteolysis (increased free amino acids/BCAA), lipolysis (increased free fatty acids), lactose hydrolysis (DAIRY) and amylolysis (MULTI) in static digestion models, increasing nutrient bioaccessibility in realistic meal matrices. The three botanical extracts did not measurably alter α-diversity or community structure in short-term single-donor fecal batch cultures and did not inhibit key commensals at physiologically plausible exposures; inhibitory effects emerged only in monoculture at supraphysiological concentrations. Collectively, these findings support the consideration of these ingredients as microbiome-compatible ("*microbiome-friendly*") under the tested conditions.

Overall, the findings underscore the value of physiologically informed in vitro systems for early-stage screening and mechanistic evaluation of microbiome-targeted interventions. Through the integration of static digestion and batch fermentation models, this research generated reproducible insights into compound bioaccessibility, microbial metabolism, and proxies of host–microbe interaction. Importantly, the results contribute to the development of nutraceutical strategies that are not only effective but also consistent with the principles of microbial ecology, host compatibility, and long-term safety.

Looking forward, future research should focus on:

- Expanding the diversity of donor-derived microbial consortia to better reflect interindividual variability;
- Integrating dynamic digestion-fermentation models with epithelial or immune co-cultures;
- Incorporating metagenomic and metabolomic analyses to deepen functional interpretation;
- Validating promising candidates through clinical trials.

Ultimately, advancing microbiome-compatible nutrition will depend on interdisciplinary approaches that bridge *in vitro* screening, omics technologies, and human validation. This thesis represents a step toward that goal, offering a methodological and conceptual framework for evaluating next-generation functional foods and supplements in the context of digestive health and microbial balance.

8. Declaration of generative AI and AI-assisted technology in the writing

During the preparation of this manuscript, the author utilized AI-based tools, including ChatGPT-4o (https://chat.openai.com/), for grammar and style enhancement. Following the use of these tools, the author thoroughly reviewed and edited all content, assuming full responsibility for the final draft's accuracy and integrity.

9. Appendices

9.1 Publications in journals that have an impact factor

1. Barbaro, M. R., Cremon, C., Marasco, G., Savarino, E., Guglielmetti, S., Bonomini, F., Palombo, M., Fuschi, D., Rotondo, L., Mantegazza, G., **Duncan, R.** *et al.* (2024). Molecular mechanisms underlying loss of vascular and epithelial integrity in irritable bowel syndrome. *Gastroenterology, 167*(6), 1152–1166.
2. **Duncan, R.**, Mantegazza, G., Gardana, C., Angelini, F., Russo, R., & Guglielmetti, S. (2025). Simulated gastrointestinal digestion of two convenience meals using fungal enzyme formulations. *Food Bioscience, 107*, 283.
3. **Duncan, R**., Mantegazza, G., Gargari, G., Pierallini, E., Russo, R., & Guglielmetti, S. (2024). *Heyndrickxia coagulans* LMG S-24828 is a safe probiotic strain capable of germinating in the human gut. *Probiotics and Antimicrobial Proteins*, 1–15.
4. **Duncan, R**., Gargari, G., Mantegazza, G., Russo, R., & Guglielmetti, S. (2025). In vitro assessment of the impact of astragalus, pineapple stem and bergamot extracts on the human fecal microbiota. *Submitted for publication at BMC Complementary Medicine (August 2025)*
5. Gargari, G., Mantegazza, G., Cremon, C., Taverniti, V., Valenza, A., Barbaro, M. R., Marasco, G., **Duncan, R.,** Fiore, W., Ferrari, R., *et al.* (2024). *Collinsella aerofaciens* as a predictive marker of response to probiotic treatment in non-constipated irritable bowel syndrome. *Gut Microbes, 16*(1), 2298246.
6. Mantegazza, G., Dalla Via, A., Licata, A., **Duncan, R.**, Gardana, C., Gargari, G., Alamprese, C., Arioli, S., Taverniti, V., Karp, M., *et al.* (2023). Use of kefir-derived lactic acid bacteria for the preparation of a fermented soy drink with increased estrogenic activity. *Food Research International, 164*, 112322.
7. Mantegazza, G., **Duncan, R.**, Telesca, N., Gargari, G., Perotti, S., Riso, P., & Guglielmetti, S. (2024). Lactic acid bacteria naturally associated with ready-to-eat rocket salad can survive the human gastrointestinal transit. *Food Microbiology, 118*, 104418.
8. Mantegazza, G., Gargari, G., **Duncan, R.**, Consalez, F., Taverniti, V., Riso, P., & Guglielmetti, S. (2023). Ready-to-eat rocket salads as potential reservoir of bacteria for the human microbiome. *Microbiology Spectrum, 11*(1), e02970–22.
9. Perotti, S., Mantegazza, G., Pierallini, E., Kirika, N., **Duncan, R.**, Telesca, N., Sarrica, A., & Guglielmetti, S. (2024). Human in vivo assessment of the survival and germination of *Heyndrickxia coagulans* SNZ1969 spores delivered via gummy candies. *Current Research in Food Science, 9*, 100793.

9.2 National and international conferences

1. Mantegazza, G., *Duncan, R.*, Gargari, G., Telesca, N., Riso, P., & Guglielmetti, S. (2023, Milano, Italy). *Ready-to-eat salad: A potential source of beneficial bacteria for the human gut microbiota* [Poster presentation]. Microbiotami 2023.
2. Mantegazza, G., Dalla Via, A., Licata, A., **Duncan, R**., Gardana, C., Gargari, G., Alamprese, C., Arioli, S., Taverniti, V., Karp, M., & Guglielmetti, S. (2023, Egmond aan Zee, The Netherlands). *Identification and characterization of lactic acid bacterial strains for improved functional properties and increased estrogenic activity in fermented soymilk* [Poster presentation]. 14th LAB Symposium.
3. **Duncan, R**., Gargari, G., Mantegazza, G., Russo, R., Terruzzi, F., & Guglielmetti, S. (2024, Torino, Italy). *In vitro assessment of the effect of commercial botanical products on the human fecal bacterial community structure* [Poster presentation]. Food Systems Microbiomes.
4. **Duncan, R.** (2023, Portici, NA, Italy). *Study on the modulation of the intestinal microbiota induced by nutraceutical preparations designed to improve immune and metabolic function* [Poster presentation]. 27th Workshop on the Developments in the Italian PhD Research on Food Science, Technology and Biotechnology.
5. **Duncan, R**. (2024, Catania, CT, Italy). *Study on the modulation of the intestinal microbiota induced by nutraceutical preparations designed to improve immune and metabolic function* [Poster presentation]. 28th Workshop on the Developments in the Italian PhD Research on Food Science, Technology and Biotechnology.
6. **Duncan, R**. (2025, Teramo, TE, Italy). *Study on the modulation of the intestinal microbiota induced by nutraceutical preparations designed to improve immune and metabolic function* [Oral presentation]. 29th Workshop on the Developments in the Italian PhD Research on Food Science, Technology and Biotechnology.

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