CATÓLICA
FACULTY OF BIOTECHNOLOGY
PORTO

ASSESSING BANANA AND RICE WASTE FOR POSTBIOTICS PRODUCTION

by

Joana Maria Santiago Jales Carvalhas

October, 2025

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Thesis presented to Escola Superior de Biotecnologia of the Universidade Católica Portuguesa to fulfil the requirements of Master of Science degree in Biotechnology and Innovation

by

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October, 2025

“*Every great advance in science has issued from a new audacity of imagination.*”

\- John Dewey

RESUMO

A crescente procura por soluções sustentáveis na biotecnologia tem incentivado o aproveitamento de resíduos agroindustriais como matérias-primas alternativas para a produção de compostos de valor acrescentado. Neste contexto, o presente estudo teve como objetivo propor uma estratégia biotecnológica sustentável para a valorização de resíduos, com foco na casca de banana e no farelo de arroz, através da produção microbiana de compostos com potencial efeito benéfico para a saúde. Em conformidade com os princípios da economia circular, pretendeu-se demonstrar que subprodutos alimentares podem ser convertidos em bioativos, conciliando assim a responsabilidade ambiental com aplicações relevantes para o setor da saúde.

Numa fase inicial, ambos os resíduos foram caracterizados para avaliar a sua adequação ao bioprocessamento. Embora tenha sido realizada uma tentativa de extração de ácido elágico da casca de banana, através de extração assistida por ultrassons (UAE), o composto não foi detetado. Consequentemente, a investigação direcionou-se para a produção microbiana de ácido γ-aminobutírico (GABA). Para tal, os resíduos foram submetidos a hidrólise enzimática com duas proteases, alcalase e P24, com o intuito de aumentar a disponibilidade de ácido glutâmico (Glu). Posteriormente, os hidrolisados resultantes foram fermentados com co-culturas de *Saccharomyces cerevisiae* em associação com *Lactobacillus brevis* ou *Lactobacillus plantarum*. As amostras foram recolhidas aos 0, 48 e 72 horas para quantificação de GABA e Glu.

Os resultados evidenciaram que o farelo de arroz constituiu um substrato mais adequado do que a casca de banana para a produção de GABA, devido sobretudo ao seu maior teor proteico e à maior libertação de Glu após a hidrólise com P24. Além disso, a co-cultura com *L. brevis* apresentou níveis mais elevados de GABA nas fases iniciais da fermentação, ao passo que *L. plantarum* revelou um perfil mais gradual e lento. Este contraste demonstra dinâmicas fermentativas distintas e aponta para diferentes estratégias de otimização, dependendo do consórcio microbiano e dos objetivos de produção.

Em síntese, o estudo demonstra o potencial das abordagens fermentativas na valorização de resíduos agroindustriais. Não só se confirmou a eficácia do processo na produção de GABA a partir do farelo de arroz, como também se identificaram possíveis estratégias para melhorar o aproveitamento da casca de banana. Assim, trabalhos futuros deverão incidir na otimização das condições de fermentação, de forma a aumentar a eficiência e a aplicabilidade do processo.

Palavras-chave: Resíduos Agroalimentares; Casca de Banana; Farelo de Arroz; Economia Circular; Posbióticos; GABA

ABSTRACT

The growing demand for sustainable solutions in biotechnology has encouraged the use of agri-food residues as alternative raw materials for producing value-added compounds. Accordingly, this study aimed to propose a sustainable biotechnological strategy for valorizing residues, focusing on banana peel and rice bran, through the microbial production of health-promoting compounds. In line with the principles of the circular economy, the work sought to demonstrate that food by-products can be converted into bioactive compounds, thereby combining environmental responsibility with applications relevant to the health sector.

Initially, both residues were characterized to evaluate their suitability for bioprocessing. Although an attempt to extract ellagic acid from banana peel using ultrasound-assisted extraction (UAE) was performed, the compound was not detected. Consequently, the investigation shifted towards the microbial production of γ-aminobutyric acid (GABA). To this end, the residues were subjected to enzymatic hydrolysis with two proteases, alcalase and P24, to increase the availability of glutamic acid (Glu). Subsequently, the resulting hydrolysates were fermented with co-cultures of *Saccharomyces cerevisiae* and either *Lactobacillus brevis* or *Lactobacillus plantarum*. Samples were collected at 0, 48 and 72 hours for GABA and Glu quantification.

The results indicated that rice bran was a more suitable substrate than banana peel for GABA production, primarily because of its higher protein content and greater release of Glu after hydrolysis with P24. Moreover, the co-culture with *L. brevis* yielded higher GABA levels at earlier fermentation stages, whereas *L. plantarum* displayed a slower and more gradual profile. This contrast highlights distinct fermentation dynamics and suggests different strategies for process optimization depending on the microbial consortium and production goals.

Overall, this study demonstrates the potential of fermentation-based approaches for the valorization of agro-industrial residues. Not only did the process prove effective in generating GABA from rice bran, but it also pointed to possible strategies for improving the use of banana peel. Therefore, future studies should focus on optimizing fermentation conditions to enhance both efficiency and applicability.

Keywords: Agri-food Waste; Banana Peel; Rice Bran; Circular Economy; Postbiotics; GABA

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LIST OF ABBREVIATIONS

BP	Banana Peel
CFU	Colony-Forming Units
EPS	Exopolysaccharides
FAO	Food and Agriculture Organization of the United Nations
GABA	Acid γ-aminobutyric
GHG	Global Greenhouse Gases
GM	Gut Microbiome
GRAS	Generally Recognized As Safe
IBS	Irritable Bowel Syndrome
ISAPP	International Scientific Association of Probiotics and Prebiotics
LAB	Lactic Acid Bacteria
lcFOS	long-chain FructoOligoSaccharides
OD	Optical Density
RB	Rice Bran
SCFA	Short Chain Fatty Acids
scGOS	short-chain GalactoOligoSacharides
TLR	Toll-Like Receptor
UNEP	United Nations Environment Programme
uroA	urolithin A
WHO	World Health Organization

INTRODUCTION

1. Biowaste

Biowaste remain a pressing sustainability issue. According to the United Nations Environment Programme (UNEP), approximately 570 million tonnes of food are wasted globally every year, corresponding to an average of 74 kg per person (United Nations Environment Programme, 2021). This widespread food loss contributes significantly to environmental degradation, accounting for an estimated 8 to 10 % of total Global Greenhouse Gas (GHG) emissions. Such emissions exacerbate climate instability and place additional pressure on environmental systems (United Nations Framework Convention on Climate Change, 2024).

While preventing food waste at its source is the most effective approach, it is equally essential to develop efficient strategies for managing the unavoidable surplus. Handling this residual material effectively is therefore essential. Biowaste alone represents nearly 37 % of global waste, ranging from fruit and vegetable remnants to cereal by-products and processing residues from the food sector (Manfredi, et al., 2015). Considering the environmental, economic and social impacts, it becomes clear that approaches should not only limit disposal but also repurpose these residues, turning what would otherwise be waste into valuable resources.

1.1. Hierarchy of Biowaste

The sustainable management of biowaste is guided by a hierarchy of actions established by the European Union in the Waste Framework Directive (2008/98/EC). This hierarchy focuses on prevention, reduction, and reuse over recycling, recovery, and disposal (**Figure 1**). Accordingly, the valorization of residues and by-products is considered a key component of recycling and recovery. This strategy involves the transformation of agri-food residues into products or ingredients of higher added value, instead of treating them as mere waste. By recovering these materials into new production chains, valorization not only mitigates the environmental impacts associated with disposal but also aligns with the principles of the circular economy, creating secondary streams of valuable materials applicable in the food, nutraceutical, pharmaceutical and cosmetic industries.

Figure 1*. Hierarchy of residues.*

In practice, valorization strategies seek to transform low-value residues into functional and economically relevant products through methods such as composting, anaerobic digestion, enzymatic or chemical extraction of bioactive compounds and microbial fermentation.

1.1.1. Biowaste as a Source of Bioactive Compounds

Biowaste, specifically agri-food residues, are increasingly recognized as valuable resources due to their content in bioactive compounds, which include polyphenols, flavonoids, vitamins, organic acids, fibers and other secondary metabolites. The presence of such compounds transforms low-value residues into promising substrates for bioprocesses, providing opportunities that support human health (Shirahigue & Ceccato-Antonini, 2020); (Messinese, et al., 2024).

The isolation and valorization of bioactive molecules involve complementary physical, chemical and biological methods, each tailored to the nature of the compounds and the intended application. Physical methods include drying, milling and mechanical fractionation to concentrate specific fractions of interest. Chemical and enzymatic extractions allow for targeted isolation of compounds such as polyphenols or bioactive peptides, while biotechnological processes (including microbial fermentation) can enhance the bioavailability of these molecules, transform them into more active derivatives or even generate novel metabolites (Bejenaru, et al., 2024).

This approach reduces environmental burden, minimizes resource inefficiencies and integrates agro-industrial residues into sustainable production cycles, ultimately bridging waste management and functional ingredient development (Agência Portuguesa do Ambiente, 2020).

1.1.2. Bioactive compounds in human health

Beyond their environmental relevance, agri-food residues represent a valuable source of bioactive compounds with demonstrated benefits for human health. Their bioactivity extends beyond basic nutrition, as they can modulate physiological processes, protect against chronic diseases and support overall well-being.

Among them, polyphenols, abundant in fruit peels and seeds, constitute one of the most studied groups due to their strong antioxidant activity, which protects cellular components from oxidative stress by scavenging reactive oxygen species (Arias, Feijoo, & Moreira, 2022). In addition, polyphenols influence signaling pathways associated with inflammation, lipid metabolism and endothelial function, thereby contributing to cardiovascular protection, improved metabolic regulation and neuroprotection (de Araújo, de Paulo Farias, Neri-Numa, & Pastore, 2021). Flavonoids are a subclass of polyphenols commonly found in citrus peels, berries and tea residues, that have demonstrated antiplatelet, antihypertensive and vasodilatory effects, reinforcing their importance in cardiovascular health (Mahmoud, Hernandez Bautista, Sandhu, & Hussein, 2019). Carotenoids and vitamins, often present in colored fruit and vegetable peels, act synergistically with polyphenols to mitigate oxidative damage while also supporting immune function and cellular homeostasis (Muscolo, Mariateresa, Giulio, & Mariateresa, 2024).

Dietary fibers and non-digestible oligosaccharides exert equally relevant functions as they act as prebiotic substrates that selectively stimulate the growth of beneficial gut microbiota. Their microbial fermentation leads to the production of short-chain fatty acids (SCFAs), which exert systemic effects

such as maintaining intestinal barrier integrity, modulating immune responses and regulating energy metabolism (Makki, Deehan, Walter J., & Bäckhed, 2018).

Furthermore, bioactive peptides obtained from plant proteins or generated during microbial fermentation exhibit diverse biological activities, including antioxidant, antihypertensive, antimicrobial and immunomodulatory properties. These peptides often act through specific molecular targets, such as enzyme inhibition or receptor interaction, influencing key physiological pathways (Alzaydi, Barbhuiya, Routray, Elsayed, & Singh, 2023).

The wide spectrum of activities attributed to bioactive compounds underscores their relevance as functional ingredients with potential applications in disease prevention and health promotion. According to sustainable resource management, agri-food residues emerge as valuable sources of such molecules, which can be recovered and redirected into high-value applications. Their valorization not only reduces environmental impacts but also fosters innovative biotechnological processes. So, this perspective introduces the following chapter, which examines the potential of agri-food residues as substrates for microbial fermentation.

2. Agri-food residues as substrates for microbial fermentation

Agri-food residues are increasingly recognized as sources of fermentable substrates with high biotechnological potential. Their diverse composition, often including carbohydrates, fibers, proteins and micronutrients, provides a suitable basis for microbial conversion into value-added compounds (Monteiro, Battisti, Valencia, & de Andrade, 2023). Given their diversity in composition, these residues emerge as functional feedstocks capable of supporting biotechnological innovation.

2.1. Potential of agri-food residues for fermentation

The industrial-scale implementation of microbial fermentation often faces economic limitations, largely due to the costs of conventional substrates, energy consumption and infrastructure. Accordingly, agri-food residues present a valuable alternative, offering both sustainability and cost-effectiveness while providing the essential nutrients for microbial growth (Thorakkattu P. , et al., 2022).

Agro-industrial residues include a wide variety of by-products generated during food processing, such as fruit peels, seeds, vegetable pomace and cereal by-products. These materials are typically rich in fermentable sugars, fibers, proteins and micronutrients. Their nutrient content makes them suitable for supporting microbial metabolism, providing carbohydrates, pectins and polyphenolic compounds. These components serve as energy sources and metabolic precursors during microbial fermentation, enhancing the synthesis of valuable metabolites (Dhiman, et al., 2025). While these substrates offer significant potential due to their nutrient content, they also present challenges such as high moisture content and rapid microbial spoilage. For example, cereal by-products such as rice bran and wheat bran are rich in proteins, fibers, vitamins and minerals that provide a balanced nutrient profile for microbial growth. These by-products are generated in large quantities during milling, making them cost-effective and widely available for fermentation applications (Food and Agriculture Organization of the United Nations, 2024).

The specific chemical composition of agro-industrial residues plays a crucial role in determining fermentation outcomes. Carbohydrates serve as primary energy sources. Proteins and peptides act as

metabolic precursors for microbial pathways. Minerals and vitamins support enzymatic activity and growth. Residual bioactive compounds, such as polyphenols and organic acids, may be transformed into metabolites with additional functional properties during fermentation (Bejenaru, et al., 2024).

The selection of residues for microbial fermentation should consider availability, nutritional composition, stability and fermentability. Moist residues may require stabilization to prevent spoilage, while solid residues like cereal brans may benefit from particle size reduction or enzymatic treatment to improve substrate accessibility (Monteiro, Battisti, Valencia, & de Andrade, 2023). This ensures that the by-products can support efficient microbial fermentation, transforming materials that would otherwise be discarded into substrates capable of generating valuable compounds. Understanding the composition and fermentability of these residues is essential to design targeted fermentation strategies that maximize the production of valuable metabolites (Dhiman, et al., 2025); (European Commission, 2024).

2.1.1. Banana peel

Banana (*Musa* sp.) is one of the most widely consumed fruits globally, ranking third in national consumption (IMVF, 2017). Its peel, which constitutes approximately 30 to 40 % of the total fresh fruit weight, represents a highly abundant by-product. Although technically edible, banana peel (BP) is typically discarded due to its astringent taste and fibrous texture (Waraczewski & Solowiej, 2024).

From a compositional standpoint, BP content consists of ash (9-11 g/100 g dry weight), moisture between 75 % and 90 %, protein from 5.5 to 7.87 g/100 g, fat from 2.24 to 11.6 g/100 g and carbohydrates from 59.51 to 76.58 g/100 g, all on a dry weight basis (Zaini, et al., 2022). It is also primarily composed of structural biopolymers such as cellulose, hemicellulose, lignin, pectin and chlorophyll, which contribute to its rigidity and nutritional potential. In addition, it contains a diverse profile of macronutrients and micronutrients, including lipids, carbohydrates, proteins, minerals and simple sugars (Bishnoi, Sharma, & Agrawal, 2023).

BP has been the focus of numerous studies due to its rich bioactive composition and abundant availability. In recent years, various strategies have been developed to value this by-product. One prominent example is the incorporation of BP flour into bakery products, aiming to increase their fiber and antioxidant content (Zaini, et al., 2022). Beyond the food sector, BP has also been employed in a wide range of applications, including the development of cosmetic formulations with antioxidant and antimicrobial activity, water treatment processes using the peel as a natural adsorbent for heavy metal removal and the production of bioplastics by exploiting its high polysaccharide content (Zaini, et al., 2022).

In addition to these applications, BP may contain ellagitannins – polyphenolic compounds with notable bioactivity – which can be converted into urolithins by the action of specific intestinal microorganisms or through targeted biotechnological processes. This conversion pathway adds further value to BP as a substrate to produce high value metabolites (Aboul-Enein, et al., 2016); (He, et al., 2024).

Urolithin A (uroA) is a secondary metabolite resulting from the microbial conversion of phenolic compounds of the ellagitannin family, naturally present in foods such as pomegranate, walnuts, raspberries and other wild fruits. Though no information on ellagitannins content in banana peels exists so far, a study showed that out of samples of different parts of six vegetables and six fruits, banana peel

contained the highest soluble and hydrolysable polyphenol contents and there is a considerable possibility that part of these are ellagitannins (Faller & Fialho, 2010).

This conversion occurs in the human gut and is highly dependent on the composition of the gut microbiota, as only certain microorganisms have the enzymatic capacity needed to convert ellagic acid from ellagitannins into bioactive urolithins. Due to the proven benefits of uroA, namely its antioxidant, anti-inflammatory, mitophagic and neuroprotective activity, there has been an intensified interest in developing strategies for extracellular and fermentation-controlled production, using selected microorganisms (Andreux, et al., 2019); (García-Villalba, et al., 2022); (D'Amico, et al., 2021).

The precursors of uroA are complex phenolic compounds, in particular ellagitannins, which are initially hydrolyzed by microbial enzymes, leading to the formation of ellagic acid (Mantzourani, Kalouri, Palikaras, Tarantilis, & Kokotou, 2024). This compound is the main intermediate in the conversion pathway leading to urolithins production. This bioconversion is carried out by anaerobic, facultative or strict intestinal bacteria, mostly belonging to the phyla Actinobacteria and Firmicutes (He, et al., 2024). Among the species most recognized for their urolithinogenic capacity are *Gordonibacter urolithinfaciens*, *Gordonibacter pamelaeae*, *Ellagibacter isourolithinifaciens*, as well as certain strains of *Bifidobacterium pseudocatenulatum*, *Lactococcus garvieae* and *Enterocloster bolteae*. These bacteria perform successive reactions of decarboxylation, dihydroxylation and reduction on ellagic acid originating a series of intermediate urolithins (urolithin D, C, A and B), with uroA being the main accumulated final product (**Figure 2**) (Selma, Beltrán, Garcá-Villalba, Espín, & Tomás-Barberán, 2014).

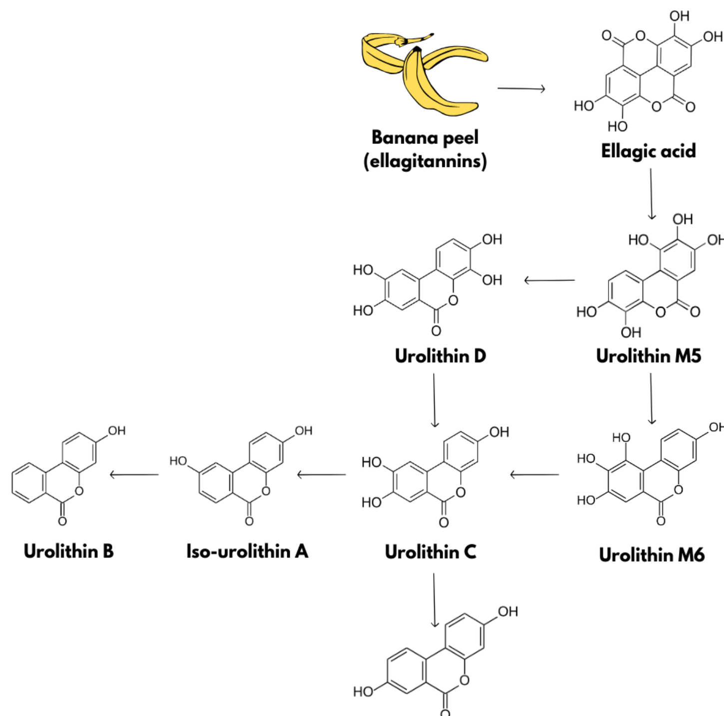


***Figure 2**. Urolithin A pathway from BP.*

The production of uroA can be reproduced under in vitro conditions through controlled fermentations with purified bacterial strains, using plant substrates rich in ellagitannins (El Barnossi, Moussaid, & Housseini, 2021). Through fermentation, ellagic acid is converted sequentially into the various forms of urolithins. This is, therefore, a sustainable and scalable alternative to its direct extraction from plant

matrices or chemical synthesis, both often limited by the low yield and structural complexity of the compost. In addition, fermentation with specialized microorganisms allows the valorization of agro-industrial residues rich in ellagitannins, contributing to the promotion of an approach based on the circular economy (García-Villalba, et al., 2022). The uroA produced can be applied in several sectors, with special emphasis on the nutraceutical, food and pharmaceutical industries, being the subject of research in clinical trials due to its potential to combat pathologies such as sarcopenia, neurodegenerative diseases and metabolic dysfunctions (Chiu, Venkatakrishnan, & Wang, 2020).

2.1.2. Rice bran

Rice (Oryza sativa L.) is a dietary staple food for more than 60 % of the global population, which explains its remarkable agriculture and economic importance (Fukagawa & Ziska, 2019). The rice grain (**Figure 3a**) is composed of a white endosperm surrounded by a bran layer, which is further protected by a fibrous husk (Sharif, Butt, Anjum, & Khan S. H., 2014). During milling, the husk is removed and the grain undergoes polishing and whitening, resulting in the production of white rice (**Figure 3b**). While widely cosnumed, this process strips away the bran germ, leading to a loss of valuable nutrients (Sharif, Butt, Anjum, & Khan S. H., 2014).

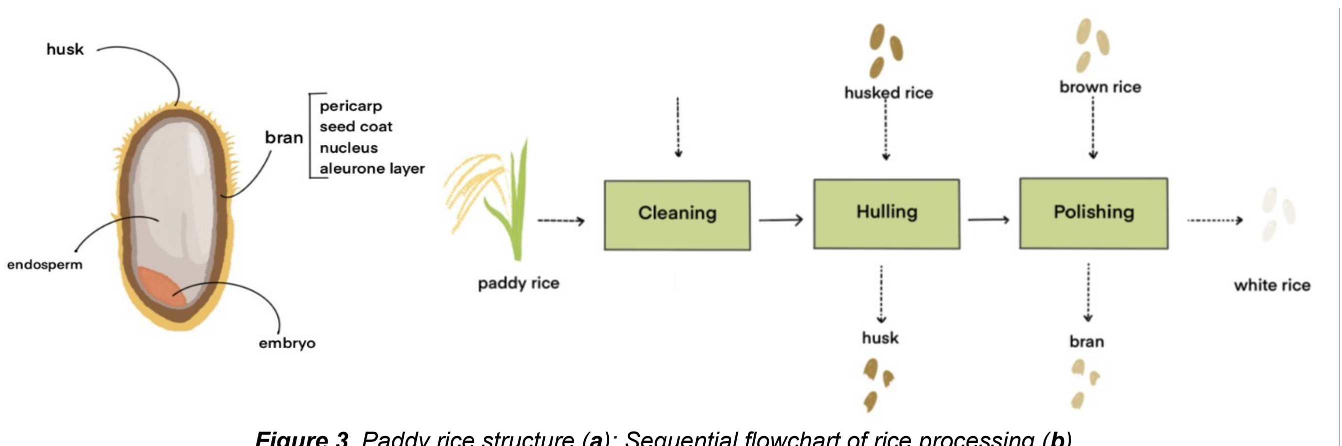


***Figure 3**. Paddy rice structure (**a**); Sequential flowchart of rice processing (**b**).*

Rice bran (RB) is therefore generated as a plentiful by-product of rice processing. However, its direct use is limited because, once exposed to oxygen, endogenous lipases hydrolyze its oil fraction, causing rancidity and reducing shelf life (Manzoor, et al., 2023). Consequently, stabilization is essential to enable its safe and effective use, particularly in the food industry. This process aims to inactivate lipase, thereby enhancing shelf life and preserving the bran's nutritional profile (Yilmaz Tuncel, 2023). Several methods have been developed for RB stabilization, including various thermal treatments (e.g. dry and hot air heating, dry heat, steaming, extrusion, ohmic heating, infrared heating and microwave heating), as well as non-thermal approaches such as acid treatment and cold storage at 0 ºC (Das, et al., 2025).

Nutritionally, RB is notable for its high protein content (11-17 %), fat (11-18 %) and carbohydrates (up to 60 %), as well as its richness in vitamins, minerals and bioactive compounds (Chakraborty, Budhwar, & Pooja, 2018). Among these are polyphenols, tocopherols, tocotrienols and carotenoids, which contribute to strong antioxidant capacity and make RB attractive not only for food

and cosmetic applications but also due to its potential benefits for human health and overall well-being (Colombo, Moretto, Barberis, Frosi, & Papetti, 2023).

In recent years, RB valorization has received growing attention. For instance, Punia et al. investigated the production of functional oils from RB oil, which is rich in oleic acid, linoleic acid and γ-oryzanol, all of which known for their antioxidant and cholesterol-lowering properties (Punia, Kumar, Sandhu, & Whiteside, 2021). Additionally, RB oil has been incorporated into cosmetic formulations due to its moisturizing properties and its ability to protect against damage caused by ultraviolet radiation (Bispo-dos-Santos, Trevisan, Rapôso, Velho, & Ricci Leonardi, 2025).

RB is also known to have a high content of glutamic acid, a precursor of GABA, making it a potential substrate to produce its production (Iorizzo, Paventi, & Di Martino, 2023). **Figure 4** illustrates the biosynthetic pathway of GABA from the Glu present in RB. In fact, GABA has been widely recognized for its beneficial effects on human health, particularly in the nervous system regulation. It functions as a key inhibitory neurotransmitter, contributing to the reduction of neuronal excitability and promoting relaxation (Nidhishree, Menezes, Venkatachalam, & Bhat, 2024). Moreover, GABA intake has been associated with improved sleep quality, reduced anxiety, and enhanced stress tolerance (Almutairi, Sivadas, & Kwakowsky, 2024). Its potential role in lowering blood pressure and supporting cardiovascular health has also been increasingly studied (Barakat & Aljutaily, 2025). Due to these multifaceted benefits, GABA is gaining attention as a functional compound in the development of nutraceuticals and functional foods (Diez-Gutiérrez, San Vicent, Barrón, del Carmen Villarán, & Chávarri, 2020).

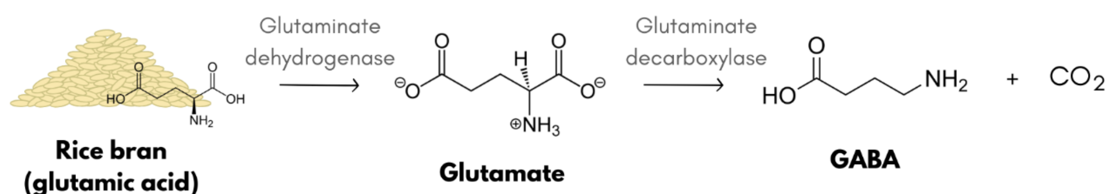
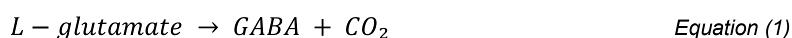


***Figure 4**. GABA pathway from RB.*

The ability of some specific bacteria strains to synthesize GABA relies on a conserved enzymatic system centered on glutamate decarboxylase (GAD). This enzyme catalyzes the decarboxylation of L-glutamate into GABA, releasing carbon dioxide in the process. Its activity depends on the presence of pyridoxal-5'-phosphate (PLP), a co-enzyme derived from vitamin B6, essential for its catalytic activity (Iorizzo, Paventi, & Di Martino, 2023). The reaction occurs according to the equation:



$$L - glutamate \rightarrow GABA + CO_2 \qquad \text{Equation (1)}$$

Beyond its role in GABA biosynthesis, this pathway contributes to bacterial survival under acidic conditions. The conversion of glutamate intro GABA consumes a proton, helping cells maintain intracellular pH and ensuring homeostasis (Kabała & Janicka, 2024).

Several factors affect the efficiency of GABA synthesis. Among them, pH is particularly decisive because GAD exhibits optimal activity between pH 4.0 and 5.5. In very acidic or neutral environments, enzyme performance tends to decrease, so do GABA yield (Cui, Miao, Niyaphorn, & Qu, 2020); (Dhakal, Bajpai, & Baek, 2012). Another key factor is substrate availability. Glu can be supplied in its free form

(glutamic acid) or as monosodium Glu (MSG), often used as a supplement in fermentation media. Cofactors, such as PLP or precursors that support its biosynthesis, also play a direct role on sustaining GAD's activity (Cui, Miao, Niyaphorn, & Qu, 2020).

Also, the use of genetically selected or adapted strains and the design of microbial co-cultures that favor the fermentation environment promote a more efficient production (Milon, Hu, Zhang, Hu, & Ren, 2024).

2.2. Main Used Microorganisms

Microorganisms play a central role in residue valorization through fermentation. Thanks to their metabolic diversity, they can convert organic substrates into a wide spectrum of compounds of nutritional, functional and industrial interest. A solid understanding of their physiology and ecological traits is therefore essential to optimize fermentation and improve the conversion of agri-food residues into high-value products.

2.1.2. Lactic Acid Bacteria (LAB)

Lactic acid bacteria (LAB) comprise a phylogenetically diverse group of Gram-positive, non-sporulated microorganisms whose main metabolic pathway is the fermentation of sugars into lactic acid. This group includes genera such as *Lactobacillus*, *Bifidobacterium*, *Lactococcus*, *Pediococcus*, *Leuconostoc* and *Streptococcus*, all of which are recognized for their metabolic versatility and microbiological safety (GRAS status) and have a long history of use in food biotechnology (Florou-Paneri, Christaki, & Bonos, 2013).

During fermentation, LAB act as versatile biocatalysts. Depending on the strain, the substrate and environmental factors such as pH, temperature, nutrient supply and oxygen levels, they can produce not only lactic acid but also peptides, organic acids and a variety of secondary metabolites (Prajapati, et al., 2023). Their ability to thrive on agro-industrial residues makes them particularly relevant for sustainable processes that align with the principles of the circular economy. Beyond sustainability, their long-standing use in fermented foods and their well-documented health benefits explain the continued interest in this group of microorganisms. (Wegh, Geerlings, Knol, Roeselers, & Belzer, 2019).

2.2.2. *Saccharomyces cerevisiae*

Saccharomyces cerevisiae is one of the most studied and widely used yeasts in food and beverage industries, known for bread, wine and beer production, but also increasingly valued for its biotechnological versatility (Franco, 2024). Like LAB, its metabolic activity intensifies during the stationary growth phase, when the synthesis of secondary metabolites becomes predominant. Its robustness, safety and adaptability make it a model organism in fermentation-based valorization processes (de Azevedo Seara, 2023). Additionally, the ability of *S. cerevisiae* to grow in agro-industrial substrates reinforces its role in sustainable fermentation strategies that contribute to circular economy approaches.

The use of genetically selected or adapted strains and the design of microbial co-cultures that favor the fermentation environment promote a more efficient process (Milon, Hu, Zhang, Hu, & Ren, 2024). Building on the discussion of GABA production from rice bran, combinations such as *S. cerevisiae* with Lactobacillus have shown synergistic effects. Yeasts create anaerobic conditions favorable to the activation of the GAD system in Lactobacillus by consuming oxygen and producing secondary

metabolites, in addition to contributing to the stabilization of the environment and nutritional enrichment (Vilijoen, 2006). So, the use of co-cultures has been pointed out as a promising approach to increase the efficiency of GABA production, while simulating more natural and complex environments, similar to those found in traditional fermented foods (Zhang, et al., 2020).

Overall, in addition to converting food residues into value-added products, microbial fermentation enables the production of bioactive compounds that interact with the human gut, modulating microbiota and supporting intestinal health, thus paving the way for microbiome-targeted innovation.

3. Gut health

The healthcare sector has been undergoing a phase of transformation driven by the growing demand for more effective solutions. The increasing prevalence of chronic diseases, coupled with an aging population and growing pressure on healthcare systems, has emphasized the need for more integrated approaches that not only treat diseases but also highlight prevention and overall well-being (Junaid, et al., 2022). As people become more health-conscious and recognize the importance of holistic well-being, they are turning to natural alternatives that promote physical and mental balance (Vignesh, Amal, Sarvalingam, & Vasanth, 2024). As a result, this shift opens new opportunities for innovation, particularly in the field of gut health, now recognized as a key factor in maintaining and enhancing multiples bodily functions (Rowland, et al., 2018).

Accordingly, the demand for preventive strategies combined with a heightened interest in clean label products and sustainability has driven the increased adoption of dietary supplements, functional foods and natural products aimed at enhancing overall well-being. Nevertheless, despite the growing supply, the available solutions exhibit certain limitations, particularly in terms of effectiveness and sustainability, thereby fostering the development of innovative products in this regard (Hilton, 2017).

The study of the human microbiome is currently one of the most prominent fields in food science, particularly the gut microbiome which constitutes a complex ecosystem that plays a crucial role in human health. Consequently, the study of gut microbiome has shown that the diversity, composition and interactions of microbial communities are closely linked to an individual's general health (Bianchetti, et al., 2023).

3.1. Gut microbiome

The gut microbiome (GM) is composed of a wide variety of microorganisms with bacteria predominating, representing about 90 % of the microbial population. The main bacterial phyla in the gut are Firmicutes and Bacteroidetes, while Actinobacteria and Proteobacteria, though play essential roles in gut health, are less prevalent (Rinninella, et al., 2019); (Stojanov, Berlec, & Štrukelj, 2020). Among the Firmicutes and Actinobacteria phyla, bacteria from the genera Lactobacillus and Bifidobacterium, respectively, which are particularly known for their probiotic properties. These microorganisms are fundamental in production of beneficial metabolites and the promotion of balance in the gut microbiota (Stojanov, Berlec, & Štrukelj, 2020). In fact, microorganisms are distributed throughout the entire gastrointestinal tract based on factors like pH, oxygen levels and nutrient availability (Mackie et al. 1999). Therefore, the colon hosts a vast range of bacteria, as the environment is highly conducive to the growth

of anaerobic microorganisms. In contrast, the small intestine has a lower microbial diversity due to its acidic pH and the presence of bile (Stojanov, Berlec, & Štrukelj, 2020).

The diversity and balanced structure of the GM are essential to ensure the overall health of the organism. As such, maintaining a healthy microbiome relies on a balanced diet rich in fiber and nutrients, as well as a lifestyle that minimizes factors contributing to dysbiosis such as stress and excessive antibiotic use (Conlon & Bird, 2014). This imbalance is often associated with intestinal diseases such as inflammatory bowel diseases including Crohn's disease, ulcerative colitis, type 2 diabetes, irritable bowel syndrome (IBS) and mental health disorders like depression and anxiety. Furthermore, studies show that greater microbial diversity in the gut is associated with a lower prevalence of metabolic, inflammatory and autoimmune diseases (DeGruttola, Low, Mizoguchi, & Mizoguchi, 2016); (Gieryńska, Szulc-Dabrowska, Struzik, Mielcarska, & Gregorczyk-Zboroch, 2022). The integrity of the intestinal barrier, composed of epithelial cells and mucus is critical in protecting the body from toxins and pathogens (Gieryńska, Szulc-Dabrowska, Struzik, Mielcarska, & Gregorczyk-Zboroch, 2022); (Takiishi, Fenero, & Câmara, 2017). Research also demonstrates that the balance of the gut microbiota can directly influence mental health, immune function, physical performance and even promote longevity and healthy aging. These findings have driven increased interest in functional foods, particularly prebiotics, probiotics and postbiotics, as strategies to promote microbiota balance and overall health (De Vos, Tilg, Van Hul, & Cani, 2022).

3.2. Biotics – preventive and personalized approaches

The rising interest in probiotics and prebiotics among consumers and healthcare professionals reflects the increasing focus on interventions that aim to modulate gut microbiota. However, despite significant advances, there are important gaps in this field, notably about the effectiveness, stability, individuality and sustainability of current interventions (Schupack, Mars, Voelker, Abeykoon, & Kashyap, 2022); (Hou, et al., 2022).

According to the definition established by World Health Organization (WHO) and the Food and Agriculture Organization of the United Nations (FAO), probiotics are "live microorganisms that, when administered in adequate amounts, confer a health benefit to the host" (FAO, 2006). This concept involves a wide diversity of microorganisms, essentially bacteria among which species of the genera Lactobacillus, Bifidobacterium predominate, which contribute to intestinal balance. Despite their recognized benefits, probiotics also present relevant limitations. Many strains do not survive the harsh conditions of the gastrointestinal tract, such as gastric acidity and digestive enzymes, which can compromise their effectiveness (Peruzzolo, et al., 2025). In addition, the effects of probiotics vary considerably from person to person, depending on the individual composition of the gut microbiota. This variability limits the predictability of results and underlines the need for more consistent solutions. In parallel, the increasing demand for sustainable solutions highlights the importance of exploring underutilized raw materials as substrates for novel biotic products (Oudat & Okour, 2025).

Prebiotics have received significant recognition for their ability to promote gut health. Unlike probiotics, which are live microorganisms, prebiotics are non-digestible fibers that selectively stimulate the growth and activity of beneficial bacteria for the gut. For this reason, prebiotics represent a resource of interest when it comes to improving metabolic and immune health (Rauch, Mika, McCubbin,

Huschtscha, & Costa, 2022). The International Scientific Association of Probiotics and Prebiotics (ISAPP) defines them as "a non-viable food component that confers a host health benefit associated with microbiota modulation" (International Scientific Association for Probiotics and Prebiotics, 2020). The most commonly used prebiotics include inulin derivatives, lactulose and oligosaccharides present in breast milk (HMO – *human milk oligossacharides*). Among oligosaccharides, short-chain GalactoOligoSaccharides (scGOS) and long-chain FructoOliSaccharides (lcFOS) are currently of greatest scientific and clinical relevance (Bode, 2009). Although prebiotics represent a promising strategy for enhancing gut health, they may offer less precise or immediate outcomes, highlighting the need for further research into their specific mechanisms and clinical applications.

Postbiotics are emerging as a promising alternative to traditional probiotics and prebiotics. Because they do not depend on live microorganisms, they have greater stability during storage and in the gastrointestinal tract (Rafique, et al., 2023). However, research on postbiotics is still relatively recent and, therefore, there is a lack of studies that explore their production from sustainable sources, as well as their effectiveness in different areas of health.

Postbiotics contain bioactive metabolites such as SCFA, EPS, enzymes and peptidoglycans that act directly on the host without the need for colonization of the intestine (Gurunathan, Thangaraj, & Kim, 2023)

While the link between gut health and other areas is already recognized, the available solutions focus primarily on general interventions, without addressing specific needs. In mental health field, for example, current treatments rarely consider the modulation of the microbiota as a complementary component. In the context of sports performance, the relationship between the gut microbiota and physical performance is starting to be explored, but the available solutions do not yet integrate postbiotics (Xiong, et al., 2023).

Current solutions for gut health, while promising, have several limitations in terms of both effectiveness and sustainability. Reliance on conventional treatments, the scarcity of sustainable alternatives and the need for a more personalized approach to the gut microbiota are just some of the issues that remain a challenge. The search for more accessible, effective and sustainable solutions remains a key objective for the development of new treatments and therapies, highlighting the importance of an innovative approach that can fill these gaps (Schupack, Mars, Voelker, Abeykoon, & Kashyap, 2022).

4. Postbiotics

Postbiotics (**Figure 5**) have emerged as a promising field in the study of the gut microbiota and its effects on human health. Interest in these compounds has increased after the realization that some of the benefits provided by probiotics derive not only from live microorganisms, but also from their metabolites and cellular components, the importance of postbiotics has been discovered with the development of studies on the microbiota, in which it has been observed that even after the death of some microorganisms, certain beneficial effects still persisted (Prajapati, et al., 2023). This knowledge has encouraged further research into inert microbial products that, even in the absence of live bacteria, could confer health benefits, especially for individuals with fragile immune systems or sensitivity to live probiotics (Mazziotta, Tognon, Martini, Torreggiani, & Rotondo, 2023). ISAPP defined postbiotics as “a

preparation of inanimate microorganisms and/or their components that confer a health benefit to the host". (International Scientific Association for Probiotics and Prebiotics, 2020)

4.1. Types of postbiotics and mechanisms of action

Postbiotics comprise a wide array of biologically active compounds produced during microbial metabolism, particularly using LAB. These compounds can exert significant effects on the host's physiology, modulating immune responses, reinforcing intestinal barrier function and even influencing metabolic and neurological processes. Their diversity reflects the multitude of roles they play in gut and systemic health (Hijová, 2024).

To provide a clearer overview, the main classes of postbiotics, along with their biological functions, are summarized in **Table 1**.

4.2. Advantages over other biotics

Unlike probiotics, which contain live microorganisms that are sensitive to temperature, pH and humidity, postbiotics are more stable. In addition, they have greater resistance to industrial processing, allowing for a longer shelf-life and their storage is simpler since they do not require refrigeration (Prajapati, et al., 2023). Regarding their safety, postbiotics do not contain live microorganisms, so the risk of infection in immunocompromised individuals is nil. Also, the transfer of antibiotic resistance genes is a minimized problem (Zhao, et al., 2023).

Postbiotics contain bioactive metabolites that act directly on the host without the need for colonization of the intestine. In addition, their action is predictable, unlike probiotics, whose effect depends on adaptation to the individual's microbiome. Postbiotics can be incorporated into functional foods, supplements, cosmetics and nutraceuticals without compromising the viability of the microorganisms. They are also compatible with thermal processes such as pasteurization that would inactivate live probiotics (Liu, et al., 2023).

Building on the advantages of postbiotics as stable and safe, it becomes clear that the sustainable use of agri-food residues offers a broader opportunity. Their growing awareness as rich sources of bioactive compounds highlights the close relationship between human health, nutrition and the sustainable use of bioresources. As research continues to reveal the potential of these natural by-products, developing innovative strategies to transform and apply them could benefit human health while also supporting more sustainable and resilient food systems.

5. Objectives of the project

The present work aims to evaluate the feasibility of using agri-food residues as raw materials to produce bioactive compounds with potential health benefits, within a framework of circular economy and sustainable bioprocessing. Specifically, the work aimed to assess the potential of rice bran and banana peel, two abundant and underutilized by-products, as substrates for the microbial production of uroA and GABA. The goal was to develop an approach that not only valorizes food waste but also contributes to the creation of functional ingredients that could support gut health and overall well-being.

***Table 1**. Categories of postbiotics and its functions.*

Category	Functions	Exemples of Postbiotics	References
SCFA	- Gut microbiome regulation and energy metabolism - Maintenance of the intestinal barrier - Anti-inflammatory properties - Benefits against tumors and metabolic diseases - Regulation of intestinal pH	Acetate, Propionate, Butyrate	(Canani, et al., 2011); (Den Besten, et al., 2013)
Bacteriocins	- Inhibition of pathogenic bacteria - Promoting efficient digestion - Stimulation of the immune system (cytokine production and activation of immune cells) - Alternative to antibiotics	Nisine, pediocin, enterocin	(Ali, et al., 2023); (Prajapati, et al., 2023); (Rafique, et al., 2023)
EPS	- Protection of the intestinal mucosa (reduction of pathogen adhesion) - Immunoregulatory effect (cytokine production) - Reduction of chronic inflammation - Therapeutic potential for diseases such as ulcerative colitis and IBS	EPS from Bifidobacterium and Lactobacillus	(Angelin & Kavitha, 2020); (Kaur & Dey, 2023); (Sadeghi, Haghshenas, & Nami, 2024)
Cell Wall Components	- Stimulating the production of anti-inflammatory cytokines - Strengthening of the innate immune response - Induction of immune tolerance - Maintenance of intestinal balance and defense against infections	Peptidoglycan, lipopolysaccharides, lipoteichoic acids	(Belkaid & Hand, 2014); (Yin, et al., 2023)
SLC	- Anti-inflammatory effect - Anticancer properties - Obesity control (improvement of thermogenesis and insulin sensitivity) - Reduction of inflammatory factors associated with tumor growth	SLC from *Lactobacillus paracasei* and *Bifidobacterium bifidum*	(Cho, Han, Xu, & Moon, 2024); (Rafique, et al., 2023)
Vitamins	- Vitamin supplementation (e.g. vitamin B12) - Increased bioavailability and effectiveness of vitamins at the site of absorption (gut)	Vitamins B12, B2 and folic acid	(Abdul Hakim, Xuan, & Oslan, 2023)

MATERIALS & METHODS

1. Materials

For the execution of this project, agro-industrial residues were used, specifically BPs and RB. The BPs were sourced from consumed bananas that were purchased at a local supermarket in Portugal, originating from Costa Rica and Colombia, and were stored at -18 ^{o}C until use. The RB was generously provided by Qualiriso, a Portuguese company specialized in the production of flour and stabilized RB. Prior to use, the RB underwent enzymatic denaturation through a thermal stabilization process, followed by grinding.

This study also involved the use of microorganisms, namely bacteria of the Lactobacillus genus and yeasts. Both bacterial strains – *Lactobacillus plantarum* and *Lactobacillus brevis* – as well as the yeast *Saccharomyces cerevisiae,* were kindly supplied by the Center for Biotechnology and Fine Chemistry at the Catholic University of Porto.

2. Procedures

2.1. Banana Peel and Rice Bran characterization

Banana peel was characterized by determining its moisture, ash, total protein, total lipid, sugars, polyphenols and fiber content. Samples were oven-dried at 105 ^{o}C until a stable weight was obtained and then ground.

Rice bran characterization was previously performed by Qualiriso team.

Moisture content was calculated from the mass loss after drying the samples to constant weight. Additionally, ash content was determined by incinerating the sample in a muffle furnace at 550 ^{o}C until constant weight was reached and calculating the residue percentage. Total protein and lipid content were determined using the Dumas combustion method (FOSS, Dumatec^{TM} 8000), with a conversion factor of 6.25 and Soxhlet method (FOSS, Soxtec^{TM} 8000), respectfully. Furthermore, total sugars were determined using the Phenol-Sulfuric Acid method, while total polyphenols were quantified using the Folin-Ciocalteau method.

2.2. Precursors

Following the characterization of the samples, the presence of uroA and GABA precursors was assessed. For BP, it was expected to identify ellagic acid and ellagitannins, whereas glutamic acid was the target compound in RB.

Different extraction conditions were tested for BP, specifically varying the solvent and drying time. Four distinct assays were performed, each in duplicate. In the first two assays, samples were oven-dried at 50 ^{o}C for 48 hours, then ground and sieved using a mesh screen. Subsequently, 1 g of the dried and ground samples was dissolved in 22.5 mL of ethanol (70 %) and an equal amount of sample was dissolved in the same volume of methanol (70 %). In the remaining two assays, the samples were oven-dried at 70 ^{o}C for 10 hours, then ground, sieved and dissolved following the same procedure as above. The samples were then subjected to ultrasound-assisted extraction (UAE) at 75 % amplitude, 4 ^{o}C for 45 minutes, followed by centrifugation at 5000 rpm for 15 minutes (Thermo Scientific, HERAEUS

Megafuge 16R Centrifuge). The supernatant was collected, filtered using a 0.22 μm membrane filter and centrifuged again under the same conditions. Finally, the samples were analyzed using an Impact II qTOF mass spectrometer (Bruker, Germany).

For RB, two enzymatic hydrolysis were tested: one using alcalase and another using P24. The procedure used for hydrolysis with alcalase was based on Silpradit et al. (2010). Initially, 50 g of bran was dissolved in 400 mL of water and the pH of the mixture was adjusted to 8.5 with 1M NaOH. The final volume was adjusted to 500 mL using deionized water. The mixture was then subjected to a stirred bath (100 rpm) at 55 °C for 10 minutes (Julabo, SW22) and then 2 % alcalase per gram of protein was added to the sample, which means 141.3 μL for RB and 75.7 μL for BP. The solution was subjected to stirring again under the same conditions, this time for 4 hours. Enzyme inactivation was performed by heating the sample at 90 °C for 10 minutes. The solution was centrifuged (8000 rpm) for 10 minutes at 4 °C and at the end the supernatant was collected and filtered with a porous membrane filter (0.45 μm) which was later analyzed by qTOF.

For P24, 500 mg of the enzyme was diluted in 50 mL of water and 50 g of RB was dissolved in 400 mL of deionized water. The pH of the mixture was adjusted with the sample at 3 with HCl 1M and only then was the solution with enzyme added, completing a final volume of 500 mL. The mixture was subjected to a stirred bath (120 rpm) at 37 °C for 2 hours. To inactivate the enzyme, the pH of the mixture was adjusted to 8 with NaOH 1M. The mixture was centrifuged (5000 rpm, 10 minutes, 4 °C) and the resulting supernatant was filtered, diluted and subsequently analyzed by qTOF.

2.3. Optimization of postbiotics' production process

2.3.1. Substrates

Banana peel and rice bran hydrolysates were used as substrates to produce GABA. The hydrolysates were prepared as described previously, their pH was adjusted between 5.0 and 6.0 using NaOH 1M and subsequently filtered with a 0.22 μm vacuum filter to remove suspended solids.

2.3.2. Microorganisms

The microbial strains used in this study were *Saccharomyces cerevisiae* (commercial strain, Phibro), *Lactobacillus brevis* G31 and *Lactobacillus plantarum* DSM 9843.

2.3.3. Co-culture Preparation

Fermentations were carried out in co-cultures combining *S. cerevisiae* + *L. plantarum* and *S. cerevisiae* + *L. brevis*, for each hydrolysate. The microorganisms were grown overnight in their specific medium and growth conditions. Then were inoculated at 1 % (v/v) from an inoculum standardized with an 0.5 McFarland standard (ca. 10^8 CFU/mL) and then adjusted to obtain a final inoculum concentration of 10^5 CFU/mL in the testing solutions.

2.3.4. Fermentation Process

The fermentation process was conducted in two stages. Initially, *S. cerevisiae* was inoculated under aerobic conditions, using flasks with 50 % headspace and incubated at 30 °C with the pH adjusted to between 5.0 and 5.5, in an orbital shaking at 150 rpm for 4 hours. Following this period, cultures of *L. brevis* or *L. plantarum* were inoculated with a concentration of approximately 10^7 CFU/mL. The co-

cultures were then incubated under anaerobic conditions in flasks with approximately 30 % headspace, at 35 ^{o}C and with the pH adjusted to between 5.5 and 6.0. The fermentation was monitored over periods of 48 and 72 h, with sacrificial flasks used for each sampling time point.

3. Analytical methods

3.1. Phenol-sulfuric acid method for total sugar content determination

In the phenol-sulfuric acid assay, a 5 % (m/v) phenol solution was prepared by dissolving 5 g of phenol in 100 mL of deionized water. Sample solutions were diluted in deionized water to achieve suitable absorbance readings. Then, 80 µL of each diluted sample were transferred into glass tubes in duplicate, followed by the addition of 150 µL of the 5 % phenol solution and 1 mL of 95 % sulfuric acid. The mixtures were vortexed and incubated at 100 ^{o}C for 10 minutes. After heating, the tubes were cooled at room temperature for about 10 minutes. Absorbance was then recorded at 490 nm using a UV-Vis spectrophotometer (UV-1900, Shimadzu, Japan). Carbohydrate concentrations were determined by interpolation from a glucose standard curve ranging from 0.031 to 0.250 mg/mL and expressed in mg/mL.

3.2. Folin-Ciocalteau method for total phenolics content determination

For the Folin-Ciocalteu assay, a 7.5 % (m/v) sodium carbonate solution was prepared by dissolving 15 g of Na_2CO_3 in 200 mL of deionized water. Sample solutions were made in deionized water at a concentration of 25 mg/mL. In triplicate, 50 µL of each sample – or deionized water in the case of the blank – were pipetted into glass tubes. This was followed, in order, by the addition of 50 µL of Folin-Ciocalteu reagent, 1000 µL of the prepared sodium carbonate solution and 1400 µL of deionized water. Each tube was vortexed to ensure proper mixing, then left to incubate for 1 hour at room temperature, protected from light. After incubation, absorbance was measured at 750 nm using a UV-1900 UV-Vis spectrophotometer (Shimadzu, Japan). Total phenolic content was determined by interpolating the absorbance values from a gallic acid calibration curve (0.062 – 0.493 mg/mL), with results expressed as milligrams of gallic acid equivalents per gram of dry sample (mg GAE/g).

3.3. Dumas method for total protein content determination

The Dumas method determines nitrogen content through rapid combustion of the sample in a pure oxygen environment. As the resulting gases pass through the system, changes in thermal conductivity are detected by a Thermal Conductivity Detector (TCD), producing a signal proportional to the nitrogen released during combustion (FOSS, n.d.).

For this analysis, a Dumatec™ 8000 (FOSS Analytical A/S, Hillerød, Denmark) was used. Approximately 100 mg of dried sample were precisely weighed, wrapped in aluminum foil, compacted into a pellet and placed into the analyzer. EDTA (ethylenediaminetetraacetic acid) served as the calibration standard, within a range of 10 – 150 mg. Between five and eight standards were prepared to ensure proper calibration. During the measurements, the oxygen flow was maintained at 300 mL/min, with an oxygen factor of 1.4 L/mg. The combustion, reduction and degassing units operated at 980 ^{o}C, 650 ^{o}C and 300 ^{o}C, respectively. Protein content was calculated using a nitrogen-to-protein conversion

factor of 6.25. For the calibration phase, the oxygen flow rate was adjusted to 400 mL/min, with an oxygen factor of 1 mL/mg.

3.4. Ellagic acid identification by qTOF

Phenolic compounds were identified and quantified using LC-ESI-UHR-QqTOF-MS, as described by (Oliveira, Barros, Silva Ferreira, & Silva, 2015). Dried extracts were dissolved in 50 % ethanol, filtered (0.45 µm) and analyzed using a Bruker Elute system with an Impact II mass spectrometer and a C18 column (100x2.1 mm, 2.2 µm). Separation used a 0.25 mL/min flow rate with a gradient of solvent A (0.1 % formic acid in water) and solvent B (0.1 % formic acid in acetonitrile), running from 0 % to 100 % B and back over 26 minutes. Detection was in negative ion mode with optimized parameters (e.g: 3.0 kV capillary voltage, 200 °C drying temperature). Sodium formate clusters served for internal calibration. Compounds were identified by accurate mass ($[M-H]^-$ / $[M+H]^+$) and isotope pattern (mSigma), using Bruker Compass DataAnalysis (v5.1). Quantification was expressed in µg/g of sample.

3.5. Glutamic acid identification by qTOF

Samples were purified for further quantification of free amino acids by using the method optimized by (Oliveira, Campos, Vidigal, Pintado, & Oliveira, 2024). This method consists of mixing 100 µL of the sample with 20 µL of homoserine IS-working solution (200 mg/L) and 400 µL of cold methanol (0.1 % formic acid). Then the samples were centrifuged at 15,000xg for 10 minutes at 4 °C. An additional extraction was then performed by mixing 150 µL of the methanolic extract with 150 µL of FMOC reagent and 150 µL of carbonate buffer. The mixture was incubated at 25 °C for 10 minutes without agitation using a thermomixer (ThermoMixer C, Eppendorf, Germany) and subsequently centrifuged again at 15,000xg for 10 minutes at 4 °C. Finally, a 300 µL aliquot of the supernatant was transferred to a glass vial with a 400 µL insert for derivatization.

3.6. Physicochemical Analysis

The pH of the fermentation medium was measured at the beginning and at the end of the process using the Seven Compact pH meter with an InLab Expert Pro-ISM pH electrode (Mettler Toledo, USA). Microbial growth was estimated by measuring the optical density at 600 nm (OD_{600}) using a UV-Vis spectrophotometer (UV-1900, Shimadzu, Japan). GABA and Glu quantification were carried out by qTOF. Following fermentation, the samples were processed according to the protocol outlined in subchapter **3.5**.

3.7. Microbiological Analysis

All microbiological and physicochemical analyses were conducted under sterile conditions using previously autoclaved materials. Decimal serial dilutions were performed in sterile peptone water (1 g/L) previously prepared. For the enumeration of *Lactobacillus* species, 0.20 µL of each dilution was drop-plated onto MRS agar supplemented with fluconazole at a final concentration of 0.2 g/L to inhibit yeast growth. For the enumeration of *S. cerevisiae*, 0.20 µL was spread-plated onto PDA agar. Then, MRS+Fluconazole plates were incubated at 37 °C for 24 h and PDA plates were incubated at 30 °C for

48 h. After the incubation period, colony-forming units (CFU) per mL were calculated using the following formula:

$$CFU/mL = Counts \times \frac{1}{volume} \times \frac{1}{Dilution\ factor}$$

RESULTS AND DISCUSSION

1. Waste Characterization

Table 2 presents the proximate analysis of the two matrices used as substrates: BP and BP.

***Table 2**. Banana Peel (BP) and Rice Bran (RB) composition.*

Component	BP (%)	RB (%)
Moisture	86.89 ± 0.1	6.75
Ashes	18.25 ± 0.1	6.88
Protein	7.67 ± 0.25	14.13
Lipids	6.12 ± 0.25	18.30
Carbohydrates	54.9 ± 0.24	28.0
Phenols	1.89 ± 0.3	-

Banana peel is characterized by its high moisture content (approximately 87 %), a typical feature of fruit-based residues, which may pose logistical challenges, particularly regarding transport and storage, as it promotes microbial spoilage and accelerates degradation. However, the substantial carbohydrate content of BP represents a significant advantage as a fermentable substrate, offering an accessible energy source for microorganisms. The protein content is relatively modest yet not negligible, especially considering its status as an underutilized residue. Notably, previous studies have reported the presence of free glutamic acid in BP – a key amino acid involved in the metabolic pathway of GABA biosynthesis (Bishnoi, Sharma, & Agrawal, 2023). The high ash content (18.25 %) suggests a rich mineral composition, some of which could contribute enzymatic cofactors in specific fermentation pathways, although others might exert inhibitory effects if present in excess.

In contrast, RB exhibited a low moisture content (6.75 %), which enhances stability during storage and handling, making it more suitable for industrial processing. Its protein (14.13 %) and lipid (18.30 %) contents are significantly higher than those of BP, indicating a macronutrient-rich matrix with substantial biotechnological potential. The high protein level suggests a greater availability of pepitdes and amino acids upon hydrolysis, including Glu, the direct precursor of GABA. Additionally, although often overlooked, the lipid content may positively influence microbial membrane fluidity or modulate secondary metabolism, depending on the specific fatty acid profile (Wu, Baumeister, & Heimbucher, 2023).

Overall, the chemical composition of both matrices underscores their relevance as fermentable substrates. BP, despite its logistical limitations, presents a suitable profile for sugar-rich fermentations, whereas RB offers a more robust protein base for processes relying on free amino acids release.

2. Precursors Quantification

Quantification of the precursors of uroA, specifically ellagic acid and ellagitannins, as well as GABA precursors, in BP and RB was performed using qTOF LC-MS analysis. One of the initial objectives of this study was to assess the potential of BP as a natural source of these uroA precursors. UAE was applied under varying conditions, including extraction time, solvent type and temperature, followed by qTOF analysis. Despite the use of sensitive and validated analytical techniques, neither ellagic acid nor ellagitannins were detected in BP. This may be since these compounds might be present in concentrations below the detection limit or in forms not extractable under the applied conditions. Also, the ripening stage of BP might have contributed to the absence of ellagic acid, as maturation could affect the presence and stability of certain phenolic compounds.

The lack of positive results in this stage led to experimental redirection. Considering that BP has been reported in the literature as a viable source of Glu, it was decided to repurpose this matrix to produce this bioactive compound. This shift aligned the valorization of BP with the main goal of the project: the transformation of agro-industrial residues into value-added products, namely, postbiotics.

Nonetheless, it may be worthwhile exploring alternative sources of ellagitannins and ellagic acid. Pomegranate, although seasonal, is widely consumed in Portugal between September and December and is known to be rich in these compounds. Additionally, nuts such as walnuts and almonds, which are commonly consumed and whose shells are frequently discarded, may represent valuable and underexplored sources.

3. Enzymatic hydrolysis

Prior to enzymatic hydrolysis, the raw matrices were analyzed for their natural content of free Glu and GABA. RB showed Glu levels ranging from 0.0765 to 0.113 mg/g and GABA levels from 0.0175 to 0.030 mg/g, while BP displayed considerably lower Glu concentrations (0.0015 to 0.002 mg/g) and GABA ranging from 0.0150 to 0.026 mg/g. These initial values reflect the intrinsic composition of each substrate and served as a reference for evaluating the impact of enzymatic hydrolysis.

Following hydrolysis, significant increases were observed in both compounds, particularly in RB. The Glu content was markedly higher in RB hydrolysates (6.93 mg/g with alcalase and 7.03 mg/g with P24) compared to BP (0.08 mg/g and 0.11 mg/g, respectively). This confirms RB's higher protein content and greater potential for Glu release upon enzymatic treatment.

Regarding GABA levels, RB hydrolysates again exhibited superior values (1.17 mg/g with alcalase, 1.15 mg/g with P24) relative to BP (0.22 mg/g with alcalase, 0.66 mg/g with P24). These differences suggest that not only the protein richness of the substrate, but also enzyme specificity and the accessibility of Glu to decarboxylation pathways, influence the final GABA content. **Figure 5** illustrates Glu and GABA concentrations (mg/g) across substrates and enzymatic treatments, highlighting P24's slightly better performance in releasing both compounds, particularly in the RB matrix.

These results validate the enzymatic hydrolysis approach as an effective strategy for enhancing the bioactive potential of agri-food residues. RB proved to be a more favorable substrate than BP, both in terms of Glu avalability and GABA yield.

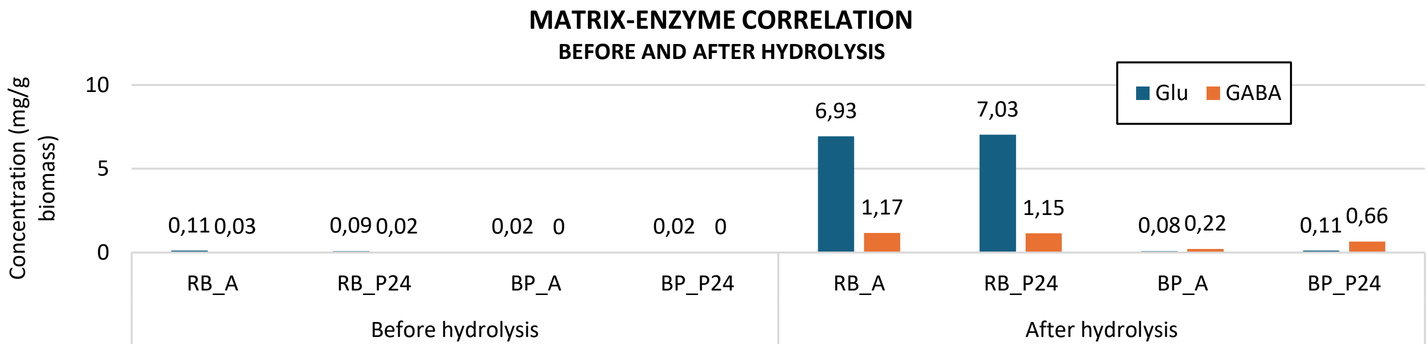


***Figure 5**. Graphical representation of the concentration of Glu and GABA (mg/g), comparing the matrix (RB or BP) and the enzyme (A or P) before and after hydrolysis.*

4. GABA Production

4.1. Quantification of GABA and Glutamic Acid

To evaluate the effectiveness of the fermentation process and the substrates' suitability for GABA biosynthesis, the levels of GABA and its precursor Glu were quantified in all samples. These data provide the basis for assessing the efficiency of Glu-to-GABA conversion and identifying the influence of the pre-treatment, fermentation parameters and matrix characteristics on GABA biosynthesis. **Figure 6** presents the concentrations of GABA and Glu measured at time points T0, T48, and T72, comparing the effects of alcalase and P24 hydrolysis on both RB and BP substrates during fermentation.

Overall, GABA levels increased notably in RB hydrolysates treated with P24, particularly when co-cultured with *L. plantarum*, suggesting a synergistic effect between high Glu availability, efficient enzymatic hydrolysis and microbial decarboxylation capacity. In contrast, BP fermentations resulted in lower or even declining GABA concentrations, indicating that limited Glu availability is a majot limiting factor. In some BP samples, it is plausible that the small amounts of GABA initially present were later metabolized by microorganisms as a nitrogen source under nutrient-limited conditions.

An interesting pattern was observed at T0, where GABA levels were already higher than expected in some samples. This may reflect early activation of GAD immediately after inoculation or even during sample preparation. Additionally, the addition of microbial biomass likely diluted the initial metabolite concentration per gram of total dry mass, now including cells and some rapid adsorption or initial metabolism of free Glu and GABA by the microbes may also have occurred.

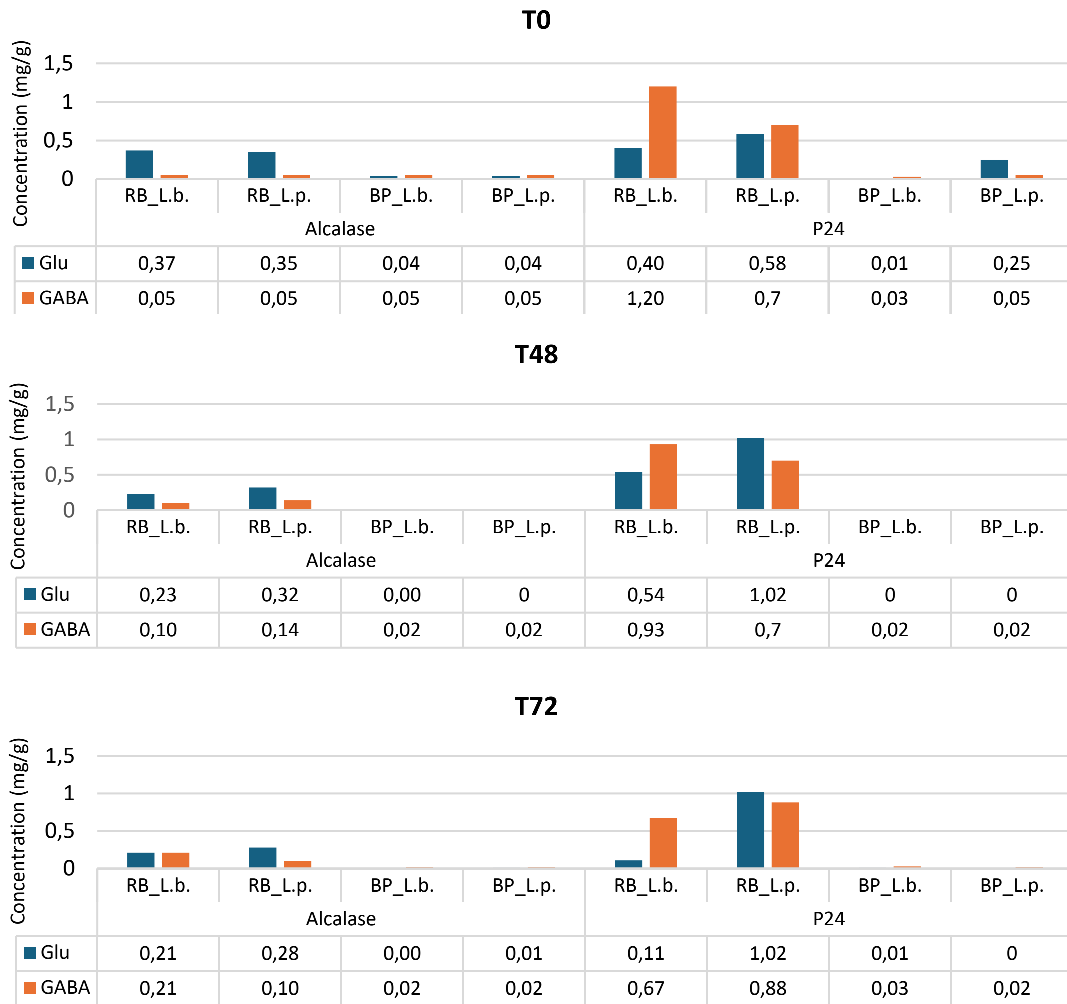


***Figure 6**. GABA and Glu (mg/g dry biomass) at T0, T48, and T72 with alcalase or P24 pretreatment, using RB and BP substrates and co-cultures S. cerevisiae + L. brevis or L. plantarum.*

In later stages (T48 to T72), the decline or stagnation of GABA levels could be attributed to pH shifts from acid production, which may have inhibited GAD activity and Glu may have been redirected towards other metabolic pathways such as protein or amino acid biosynthesis. These dynamics reflect microbial adaptation to the fermentation environment and highlight the importance of maintaining favorable conditions, including optimal pH and cofactor availability (e.g. PLP), to ensure sustained GABA production.

To further enhance GABA production, several optimization strategies should be considered. As has been said, maintaining the pH within the optimal range for GAD activity (approximately 4.0 – 5.0) is crucial for efficient Glu decarboxylation. The addition of PLP could also stimulate enzymatic activity. Moreover, adapting microbial cultures to the specific characteristics of each hydrolysate could improve growth and metabolic performance. In upstream processing, combining P24 with other proteases might increase the release of Glu and facilitate its conversion. In addition to the enzymatic hydrolysis methods applied in this study, it would have been interesting to explore saccharification either as an alternative or in combination with hydrolysis. Saccharification enables the conversion of polysaccharides into fermentable sugars, which could provide an additional carbon source for the microbial cultures used in

GABA production (Jahangeer, et al., 2024). Therefore, testing saccharification alone, hydrolysis alone and the combined application of both could reveal potential synergistic effects, highlighting substrate availability and ultimately improving GABA biosynthesis efficiency. This approach would allow better exploitation of the residues' potential, especially those rich in carbohydrates such as BP and optimize valorization within circular economy principles.

The data clearly demonstrate that enzymatic hydrolysis with P24, particularly in RB, leads to significantly higher GABA production. Although BP remains a viable substrate, it requires optimization, especially due to its lower Glu content. The presence of GABA in hydrolysates before fermentation highlights the importance of the enzymatic treatment, not only as a preparatory step but also as a direct contributor to bioactive compound release. The quantification of GABA and Glu in the fermented samples allowed a deeper understanding of the influence of each process variable.

4.1.1. Influence of Matrix: Banana Peel vs Rice Bran

Substrate selection proved to be a critical determinant of fermentation success. RB samples consistently exhibited higher Glu and GABA levels throughout the process. As has been seen, RB_P24 samples particularly showed high GABA levels even at T0, suggesting rapid decarboxylation or precursor accumulation during pre-treatment. In contrast, BP samples showed GABA values mostly between 0.02 and 0.05 mg/g across all timepoints, reflecting their limited Glu availability and possible presence of inhibitory compounds such as phenolics and tannins. These compounds can negatively affect microbial growth and enzymatic activity by binding to proteins or disrupting membranes. To mitigate this, strategies such as tannase application, activated charcoal treatment or mild washing steps could enhance the suitability of BP for fermentation (Cosme, et al., 2025); (Stamogiannou, et al., 2021).

Despite reports of bioactive compounds in BP, its low free Glu content and potential antimicrobial compounds limited GABA biosynthesis. Further matrix-specific optimization is necessary to unlock its full potential.

4.1.2. Influence of Enzymatic Hydrolysis: Alcalase vs P24

Hydrolysis with P24 consistently outperformed alcalase in supporting GABA biosynthesis, particularly in RB fermentations. In RB_P24 with *L. brevis*, GABA reached 1.20 mg/g at T0 – slightly above the concentration found in the hydrolysate – indicating rapid microbial conversion. This suggests that P24 not only released higher amounts of Glu but possibly generated peptides or compounds that stimulated early GAD activation.

On the other hand, alcalase-treated hydrolysates led to much lower GABA production. For instance, RB_A only reached 0.21 mg/g at T72. This could be due to less efficient Glu release or the presence of peptides that are less favorable for microbial metabolism or GAD induction. Although BP-based fermentations yielded low GABA levels overall, P24-treated BP samples still outperformed those treated with alcalase, reinforcing the enzyme's superior ability to unlock the limited protein content of this matrix.

4.1.3. Co-culture Performance: *L. brevis* vs *L. plantarum*

Among the LAB strains tested, *L. brevis* exhibited superior performance in most cases. In RB_P24, it produced 1.20 mg/g GABA at T0, while *L. plantarum* reached only 0.70 mg/g under the same conditions. However, *L. brevis* also demonstrated faster kinetics, with GABA levels peaking early (T0 or T48) and subsequently declining or stabilizing. In contrast, *L. plantarum* showed slower but sustained production, increasing from T48 to T72. This suggests a longer adaptation or expression phase for GAD in *L. plantarum*, which may benefit from extended fermentation durations. These observations align with literature describing *L. brevis* as acid-tolerant with constitutive GAD activity, suitable for rapid GABA production (Chen et al., 2020), while *L. plantarum* may require adaptation but can maintain stable GABA levels over prolonged fermentations. Strain selection should thus consider process parameters and desired GABA yield kinetics.

4.1.4. Fermentation time

Temporal analysis revealed a clear connection between Glu consumption and GABA production. For example, in RB_P24 with *L. brevis*, Glu decreased from 0.40 mg/g at T0 to 0.11 mg/g at T72, while GABA dropped from 1.20 to 0.67 mg/g. This suggests early conversion followed by possible degradation due to environmental factor, as already discussed, or utilization of GABA by LAB as a nutrient source. With *L. plantarum*, GABA increased from 0.70 to 0.88 mg/g between T48 and T72, with Glu levels remaining relatively high (1.02 mg/g), indicating ongoing biosynthesis. In BP fermentations, Glu was often undetectable after T48 and this didn’t translate into higher GABA levels, indicating either metabolic redirection, compound degradation or insufficient GAD activation. These observations highlight the need for matrix-specific process tuning, particularly in terms of fermentation time and nutrient availability.

4.1.5. Influence of pH

It is important to note that the pH was not actively controlled during the fermentation process, in order to observe the natural evolution of the system, but it might be regarded as an oversight. The fermentation was carried out over the weekend, with pH values recorded only at the initial (T0) and final sampling points (T48 and T72). The initial pH ranged between 5 and 6, while at the end of the fermentation the pH was significantly higher, between 9.83 and 9.90. This alkaline shift probable inhibited the metabolic activity of the co-cultures, impairing GABA production and possibly promoting degradation of bioactive compounds, thus affecting overall fermentation performance.

4.1.6. Interplay between Glu and GABA: Insights into Decarboxylation Efficiency

Across the tested conditions, samples with higher initial Glu content generally showed greater GABA accumulation, confirming that precursor availability is a limiting factor for decarboxylation. This was particularly evident in RB hydrolysates treated with P24, where GABA production correlated well with Glu concentrations, especially in fermentations with *L. brevis*. In contrast, in BP fermentations, low Glu levels resulted in minimal GABA accumulation, underscoring the need for Glu supplementation or enhanced hydrolysis strategies when using low-protein substrates.

Interestingly, the Glu:GABA ratio over time also revealed differences in microbial decarboxylation dynamics. While *L. brevis* tended to consume Glu rapidly, with early GABA peaks followed by a decline – possibly due to GABA catabolism or conversion into succinate via the GABA shunt – *L. plantarum* maintained higher Glu levels throughout fermentation, with a more gradual increase in GABA. This suggests differing regulation of the GAD system between species and highlights the importance of matching microbial metabolism with substrate composition and fermentation time. Monitoring the interplay between Glu consumption and GABA production provides valuable insights into decarboxylation efficiency and can guide future optimization efforts.

Overall, the results obtained highlight the multifactorial nature of GABA biosynthesis in microbial fermentation systems and underscore the importance of substrate selection, enzymatic pre-treatment, microbial strains and fermentation conditions in modulating productivity. RB hydrolysates, particularly those treated with P24, consistently provided a favorable biochemical environment for GABA production, largely due to their higher Glu content and compatibility with the metabolic capabilities of *L. brevis*. In contrast, BP showed potential but revealed important limitations, namely low precursor availability and possible presence of inhibitory compounds, indicating a need for process refinement. The distinct kinetic profiles observed between the two LAB strains further illustrate the dynamic interplay between microbial metabolism and substrate characteristics. These findings not only demonstrate the feasibility of using agro-industrial residues to produce bioactive compounds but also reinforce the value of integrated optimization strategies – from upstream processing to microbial performance – to unlock the full potential of circular bioprocesses.

4.2. Microbiological Analysis

4.2.1. Colony Forming Units

The microbial growth in the fermentation was monitored through total viable counts on PDA and MRS media. The results are expressed as Log(CFU/mL) and are summarized below (**Table 3**). Full raw data, including counts and dilution factors, are available in **Appendix III**.

Table 3. *Log CFU/mL values at T0, T48 and T72 for samples fermented with S. cerevisiae and L. brevis or L. plantarum. Results are shown as individual values, mean and standard deviation (n = 2).*

Sample	Log (CFU/mL) PDA	Log (CFU/mL) MRS	Average Log (CFU/mL)	St. Dev
T0 A alc	6.41	6.60	6.51	0.0949
T0 C alc	6.01	6.54	6.28	0.2651
T0 E alc	6.37	6.63	6.50	0.1299
T0 G alc	6.01	6.62	6.31	0.3024
T48 A alc	9.01	9.54	9.28	0.2651
T48 C alc	9.26	9.43	9.35	0.0851
T48 E alc	9.67	9.62	9.64	0.0247
T48 G alc	9.28	9.06	9.17	0.1090

T72 B alc	9.02	9.42	9.22	0.1990
T72 D alc	9.37	9.23	9.30	0.0680
T72 F alc	9.34	9.67	9.51	0.1685
T72 H alc	9.22	9.38	9.30	0.0791
T0 A P24	7.71	7.69	7.70	0.0076
T0 C P24	7.65	7.59	7.62	0.0313
T0 E P24	7.43	7.36	7.40	0.0348
T0 G P24	9.01	8.98	8.99	0.0165
T48 A P24	9.41	9.67	9.54	0.1274
T48 C P24	9.56	9.45	9.51	0.0537
T48 E P24	9.32	9.64	9.48	0.1594
T48 G P24	10.38	10.25	10.31	0.0655
T72 B P24	10.16	10.34	10.25	0.0905
T72 D P24	10.54	10.43	10.49	0.0548
T72 F P24	10.58	10.46	10.52	0.0591
T72 H P24	10.28	10.54	10.41	0.1298

The overall results confirmed successful microbial growth in all fermentations, with a substantial increase in CFU/mL observed between T0 and the subsequent time points (T48 and T72).

At the initial time point (T0), average microbial loads ranged between Log 6.28 and log 6.51 CFU/mL in most samples (e.g. T0 A alc = 6.51; T0 C alc = 6.28), indicating the presence of viable cells immediately after inoculation. These initial values suggest that inoculation was effective and that the medium composition already supported microbial survival, even before significant proliferation. From T0 to T48 or T72, a marked increase in cell counts was observed, reaching values between log 9.17 and log 10.52 CFU/mL in most samples. For instance, sample A with alcalase treatment (RB_L.b.) progressed from log 6.51 at T0 to log 9.28 at T48, while sample B (same conditions, T72) reached Log 9.22. These increases of more than 2 to 3 logarithmic units are indicative of robust microbial proliferation under the tested conditions.

Furthermore, a comparison between the two enzymatic pretreatments (alcalase and P24) revealed a trend of slightly higher microbial counts in the samples treated with P24. For example, T48 G alc (BP_L.p.) reached an average of log 9.17, whereas the same condition with P24 (T48 G P24) achieved log 10.31. Similarly, T72 D alc (RB_L.p.) showed log 9.30, while the corresponding P24-treated sample (T72 D P24) exhibited log 10.49. These differences suggest that the hydrolysates generated with P24 may have provided a more favorable nutrient composition or higher peptide availability for microbial growth. This trend aligns with some of the GABA production data, where higher yields were also detected in P24-treated substrates.

The use of two selective media was essential to verify the survival of both microbial strains present in the co-cultures. The presence of colonies on both MRS and PDA confirmed the viability of *Lactobacillus spp*. and *Saccharomyces cerevisiae*, respectively, at all timepoints. Interestingly, when

comparing the CFU evolution across time points for the two different *Lactobacillus* species used, distinct proliferation patterns were observed. Samples inoculated with *L. brevis* (A, B, E, F) showed a sharper increase in microbial load between T0 and T48, with values generally stabilizing or slightly increasing by T72. For example, sample T0 A alc showed log 6.51, which rose to log 9.28 at T48, and then remained at log 9.22 in the corresponding T72 sample (B). This trend aligns with what is described in the literature regarding *L. brevis*, which tends to act more rapidly in terms of GABA synthesis and has a faster initial metabolic response to available Glu. On the other hand, samples containing *L. plantarum* (C, D, G, H) often presented a more gradual increase in CFU over time. For instance, sample G alc showed log 6.31 at T0, increasing to log 9.17 at T48 and reaching log 9.30 by T72 (sample H). This suggests that *L. plantarum* may require more time to reach peak activity, consistent with its known behavior of adapting slowly to environmental stress and low pH, but sustaining metabolic activity for longer periods. This delayed but stable growth pattern may correlate with its gradual contribution to GABA production observed in the biochemical results.

These findings suggest that the choice of *Lactobacillus* strain not only affects the timing and efficiency of GABA production but also influences microbial growth dynamics. The faster onset of activity observed in *L. brevis*-inoculated samples supports its suitability for short-term fermentations aimed at rapid GABA synthesis, whereas *L. plantarum* may be more suited to extended fermentations where prolonged GAD activity can be maintained.

4.2.2. Optical Density Analysis

Optical Density (OD) measurements were used to estimate microbial growth during fermentation at 48 h and 72 h. For samples treated with alcalase, OD values generally increased over time, indicating microbial proliferation. For example, in RB with *Lactobacillus brevis*, OD rose from 0.325 (48 h, diluted) to 0.753 (72 h, diluted), while in BP with *Lactobacillus plantarum*, OD increased from 0.251 (48 h, undiluted) to 0.793 (72 h, undiluted). In contrast, samples treated with P24 protease showed lower and sometimes decreasing OD values between 48 h and 72 h, suggesting less favorable growth conditions.

These OD trends align with the GABA production results, where alcalase-treated substrates generally led to higher GABA yields, reflecting more efficient microbial activity and growth. The higher OD in BP samples, particularly with *L. plantarum*, also supports the substrate's suitability for fermentation. However, the final increase in pH (up to 9.9) likely inhibited microbial growth and GAD enzyme activity, which can explain stagnant or decreasing OD values in some cases. This highlights the importance of pH control in optimizing fermentation performance.

Although OD_{600} provides a rapid estimation of biomass increase over time, it does not distinguish between live and dead cells. In contrast, CFU counts reflect only viable microorganisms capable of forming colonies. The trends observed in OD_{600} measurements generally align with the CFU data, especially in terms of growth dynamics across time points and among different co-cultures and substrates. For instance, samples inoculated with *L. brevis* exhibited higher OD and CFU values at T48, suggesting more active growth in early fermentation stages, while *L. plantarum* co-cultures showed a steadier increase in both OD and CFUs up to T72. These observations reinforce the complementary nature of both methods in characterizing microbial activity throughout fermentation.

This way, OD results confirm the impact of hydrolysis method and substrate type on microbial growth, supporting the observed GABA production patterns and emphasizing the need for controlled fermentation conditions in future work.

CONCLUSION AND FUTURE WORK

This work aimed to develop a sustainable biotechnological route for the valorization of agro-industrial waste, with particular focus on BP and RB by harnessing microbial processes to produce bioactive compounds with potential health-promoting properties. Grounded in the framework of the circular economy, this study intended to highlight the potential of food industry residues as low-cost, nutrient-rich substrates for the generation of functional metabolites, thereby aligning environmental sustainability with innovative solutions for the health and biotechnology sectors.

The results clearly identified RB as a significantly more effective substrate than BP in promoting GABA biosynthesis. This was directly linked to its higher protein content, which, following enzymatic hydrolysis (particularly with enzyme P24), released greater amounts of Glu. RB combined with *L. brevis* strain proved particularly efficient, with high GABA concentrations observed as early as the initial fermentation timepoint, indicating rapid and effective conversion. In contrast, BP presented substantial limitations, including low Glu content and evidence of inhibitory compounds such as phenolics and tannins, which may compromise both enzymatic activity and microbial growth. Hydrolysis with P24 consistently outperformed alcalase, demonstrating greater efficiency in releasing precursors and enhancing GABA production. Furthermore, the choice of microbial strain strongly influenced the kinetics of GABA production: *L. brevis* promoted a rapid but transient response, while *L. plantarum* exhibited a slower, more sustained production profile. The lack of active pH control during fermentation was identified as a critical factor, as the significant increase in alkalinity likely inhibited GAD activity and reduced overall fermentation performance.

Overall, this thesis reinforces the feasibility of valorizing agri-food waste as sources of bioactive compounds and demonstrates that the production of postbiotics serves as a proof of concept for this approach. The valorization of agri-food residues can be effectively achieved by applying integrated strategies that account for the specific characteristics of each matrix, employ suitable enzymatic pre-treatments, select microbial strains thoughtfully and maintain careful control over fermentation conditions. Future work should focus on further optimization – such as pH control, enzyme synergy, microbial culture adaptation and potential integration of saccharification – to fully unlock the potential of carbohydrate-rich residues like BP and enhance process efficiency.

Ultimatetly, this work contributes to a growing body of research supporting circular and sustainable innovation within biotechnology, offering an integrated approach that combines waste reduction, microbial fermentation and the generation of health-relevant compounds – key elements for future bioeconomy-based systems.

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APPENDIX

I. Measurement of pH

The table below summarizes the average pH values measured at the beginning (T0) and end (T48 and T72) of the fermentation process, illustrating the natural pH variations over time.

Table 4. *Average pH values measured at the start (T0) and end (T48, T72) of fermentation.*

	Alcalase				**P24**			
	RB_L.b.	RB_L.p.	BP_L.b.	BP_L.p.	RB_L.b.	RB_L.p.	BP_L.b.	BP_L.p.
T0	5.02	5.03	5.09	5.46	5.11	5.09	5.06	5.03
T48	9.72	9.80	9.47	9.81	9.84	9.87	9.88	9.88
T72	9.88	9.80	9.73	9.80	9.90	9.90	9.90	9.90

II. Measurement of Optical Density (OD)

OD was measured at T48 and T72 to assess microbial growth. Because some samples showed OD values below the spectrophotometer's detection limit, a dilution of 800 µL PBS to 200 µL sample was applied before measurement. For samples with higher cell density, no dilution was used.

Table 5*. Average optical density (OD) values at T48 and T72 for samples after fermentation.*

	Alcalase				**P24**			
	RB_L.b.	RB_L.p.	BP_L.b.	BP_L.p.	RB_L.b.	RB_L.p.	BP_L.b.	BP_L.p.
T48	0.505	0.453	0.246	0.386	0.325	0.270	0.236	0.251
T72	0.351	0.332	0.236	0.393	0.753	0.509	0.661	0.793

OD values were normalized by multiplying the measured readings by the dilution factor (5) to estimate the actual cell density in the original samples.

Table 6*. Average optical density (OD) values at T48 and T72 for samples after fermentation, with corresponding dilution factors applied for normalization.*

	Alcalase				**P24**			
	RB_L.b.	RB_L.p.	BP_L.b.	BP_L.p.	RB_L.b.	RB_L.p.	BP_L.b.	BP_L.p.
T48	2.525	2.265	1.230	1.930	1.625	1.350	0.236	0.251
T72	1.755	1.660	1.180	1.955	0.753	0.509	0.661	0.793

III. CFU

For microbial quantification, CFU/mL values were calculated using the following formula:

$$CFU\ /mL = Counts\ \times \frac{1}{volume} \times \frac{1}{Dilution\ factor}$$ *Equation (2)*

Where:

- ***Counts*** is the number of colonies counted
- ***Volume*** is the volume of the plated aliquot (in mL)
- ***Dilution factor*** is the dilution factor of the sample.

An example calculation is shown below, followed by the complete table of results (**Table 7**).

For example, for sample **T0 A1 alc** with a colony count of 49, a dilution factor of 1×10^{-2} and an inoculated volume of 0.1 mL, the CFU per milliliter is calculated as follows:

$$CFU\ /mL = 49\ \times \frac{1}{0.1} \times \frac{1}{0.01}$$

$$CFU/mL = 2.60\text{E} + 06$$

***Table 7**. Colony Forming Units (CFU/mL) counted in the different samples, including raw values, dilution factors and the mean and standard deviation of the log-transformed values.*

Sample	Counts				Dilution Factor		UFC/mL 1	UFC/mL 2	Log (UFC/mL) 1	Log (UFC/mL) 2	Average Log (UFC/mL)	St. Dev
	PDA		MRS									
T0 A alc	49	55	79	82	1.00E-02	1.00E-02	2.60E+06	4.03E+06	6.41	6.60	6.51	0.0949
T0 C alc	20	21	74	65	1.00E-02	1.00E-02	1.03E+06	3.48E+06	6.01	6.54	6.28	0.2651
T0 E alc	48	46	81	90	1.00E-02	1.00E-02	2.35E+06	4.28E+06	6.37	6.63	6.50	0.1299
T0 G alc	20	21	88	77	1.00E-02	1.00E-02	1.03E+06	4.13E+06	6.01	6.62	6.31	0.3024
T48 A alc	20	24	90	99	1.00E-05	1.00E-05	1.03E+09	3.48E+09	9.01	9.54	9.28	0.2651
T48 C alc	35	38	51	57	1.00E-05	1.00E-05	1.83E+09	2.70E+09	9.26	9.43	9.35	0.0851
T48 E alc	96	90	88	78	1.00E-05	1.00E-05	4.65E+09	4.15E+09	9.67	9.62	9.64	0.0247
T48 G alc	37	39	24	22	1.00E-05	1.00E-05	1.90E+09	1.15E+09	9.28	9.06	9.17	0.1090
T72 B alc	22	20	55	50	1.00E-05	1.00E-05	1.05E+09	2.63E+09	9.02	9.42	9.22	0.1990
T72 D alc	48	45	29	39	1.00E-05	1.00E-05	2.33E+09	1.70E+09	9.37	9.23	9.30	0.0680
T72 F alc	45	42	88	101	1.00E-05	1.00E-05	2.18E+09	4.73E+09	9.34	9.67	9.51	0.1685
T72 H alc	36	30	42	53	1.00E-05	1.00E-05	1.65E+09	2.38E+09	9.22	9.38	9.30	0.0791
T0 A P24	100	103	95	101	1.00E-03	1.00E-03	5.08E+07	4.90E+07	7.71	7.69	7.70	0.0076
T0 C P24	90	89	80	75	1.00E-03	1.00E-03	4.48E+07	3.88E+07	7.65	7.59	7.62	0.0313
T0 E P24	59	49	42	50	1.00E-03	1.00E-03	2.70E+07	2.30E+07	7.43	7.36	7.40	0.0348
T0 G P24	21	20	20	18	1.00E-05	1.00E-05	1.03E+09	9.50E+08	9.01	8.98	8.99	0.0165
T48 A P24	50	54	90	97	1.00E-05	1.00E-05	2.60E+09	4.68E+09	9.41	9.67	9.54	0.1274
T48 C P24	76	70	58	56	1.00E-05	1.00E-05	3.65E+09	2.85E+09	9.56	9.45	9.51	0.0537
T48 E P24	46	38	90	85	1.00E-05	1.00E-05	2.10E+09	4.38E+09	9.32	9.64	9.48	0.1594
T48 G P24	51	45	36	35	1.00E-06	1.00E-06	2.40E+10	1.78E+10	10.38	10.25	10.31	0.0655
T72 B P24	28	30	46	42	1.00E-06	1.00E-06	1.45E+10	2.20E+10	10.16	10.34	10.25	0.0905
T72 D P24	66	73	55	53	1.00E-06	1.00E-06	3.48E+10	2.70E+10	10.54	10.43	10.49	0.0548
T72 F P24	71	80	60	55	1.00E-06	1.00E-06	3.78E+10	2.88E+10	10.58	10.46	10.52	0.0591
T72 H P24	40	37	70	70	1.00E-06	1.00E-06	1.93E+10	3.50E+10	10.28	10.54	10.41	0.1298

IV. Raw Data of GABA and Glu Quantification

This appendix contains the raw concentration values of GABA and Glu measured in all samples at different time points (T0, T48, and T72). The data are expressed as mg per gram of dry biomass and are organized by substrate (RB and BP), enzymatic treatment (alcalase or P24), and co-culture type.

A. Peak Areas

The raw peak areas obtained from the qTOF analysis are presented below (**Table 8**) followed by the normalized areas (**Table 9**) adjusted according to the sample dilution factors to allow accurate compafison between samples.

Table 8*. Integrated peak area values obtained from qTOF analysis.*

	Areas		
Name	**GABA**	**Glu**	**IS (Internal Standard)**
Formula	**C19H19NO4**	**C20H19NO6**	**C19H19NO5**
RT	**7.0**	**5.7**	**5.4**
m/z	**324.12413**	**368.11396**	**340.11905**
St_1ppm	67360	572467	6023694
St_2ppm	238727	1408346	8087465
St_5ppm	708604	2513720	5372384
St_10ppm	1102960	3621787	4502854
St_20ppm	2707369	8086101	4469811
St_50ppm	8914565	22855510	5216876
T0 A1 alc	813597	28360394	6089648
T0 A2 alc	917418	30489940	6396676
T0 C1 alc	792474	29144848	6200886
T0 C2 alc	748606	28211010	6512199
T0 E1 alc	900138	3013416	6954249
T0 E2 alc	1015600	3364108	7221541
T0 G1 alc	1094025	3500584	7733910
T0 G2 alc	975500	3161645	6676007
T48 A1 alc	2423830	20493856	6925674
T48 A2 alc	2466279	21361666	7458754
T48 C1 alc	4842667	32126362	8712958
T48 C2 alc	2250244	23339646	5407703
T48 E1 alc	77272	119827	7165347
T48 E2 alc	87995	113516	8758844
T48 G1 alc	72844	88970	7014955
T48 G2 alc	66655	65737	7624261
T72 B1 alc	4706058	15987964	6882828
T72 B2 alc	4957062	17297394	7720223
T72 D1 alc	2672907	21784878	6813582
T72 D2 alc	1737485	22527240	7273832
T72 F1 alc	84075	100656	6944826
T72 F2 alc	97938	96444	6718843

T72 H1 alc	81418	174482	3590882
T72 H2 alc	113186	656953	4985633
T0 A1 P24	1542628	27200124	6265574
T0 A2 P24	1135310	22561550	6143571
T0 C1 P24	1696561	31898028	6816000
T0 C2 P24	2352080	37582164	5206469
T0 E1 P24	188814	718204	7243359
T0 E2 P24	437123	1210463	8860162
T0 G1 P24	226055	327189	4667728
T0 G2 P24	1248733	30233642	6099249
T48 A1 P24	9835346	58307608	5708200
T48 A2 P24	10635961	59471212	6721111
T48 C1 P24	12405982	58496888	5598188
T48 C2 P24	11915547	57581092	5561804
T48 E1 P24	29437	63082	5987933
T48 E2 P24	29246	49778	5654426
T48 G1 P24	60021	341940	7503942
T48 G2 P24	26179	94532	7539806
T72 B1 P24	10279663	59380176	5643488
T72 B2 P24	11402455	60123532	6067514
T72 D1 P24	12327089	59253760	5325333
T72 D2 P24	12990305	59709468	5054597
T72 F1 P24	90071	324372	8427986
T72 F2 P24	142681	991075	7937854
T72 H1 P24	40695	80061	6302941
T72 H2 P24	47293	116249	7027782
HA 1 alc	1533307	35661272	5824906
HA 2 alc	1662620	37505864	5456216
HA 1 P24	1278462	33451872	4327921
HA 2 P24	1330269	33195444	4440210
HB 1 alc	38826	125718	7030839
HB 2 alc	46485	308853	6739534
HB 1 P24	1658533	984911	5868047
HB 2 P24	1839053	1227491	6089355

***Table 9**. Normalized peak areas of GABA and Glu obtained by qTOF analysis after adjustment for sample dilution.*

	Normalized Area	
	GABA	Glu
T0 A1 alc	0.13	4.66
T0 A2 alc	0.14	4.77
T0 C1 alc	0.13	4.70
T0 C2 alc	0.11	4.33
T0 E1 alc	0.13	0.43
T0 E2 alc	0.14	0.47
T0 G1 alc	0.14	0.45
T0 G2 alc	0.15	0.47
T48 A1 alc	0.35	2.96
T48 A2 alc	0.33	2.86
T48 C1 alc	0.56	3.69
T48 C2 alc	0.42	4.32
T48 E1 alc	0.01	0.02
T48 E2 alc	0.01	0.01

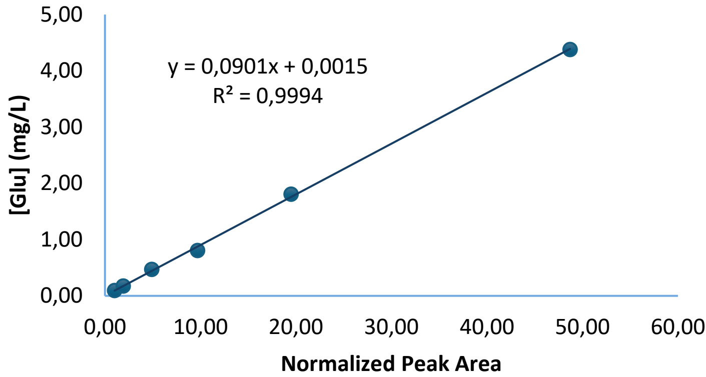
T48 G1 alc	0.01	0.01	**T48 C2 P24**	2.14	10.35
T48 G2 alc	0.01	0.01	**T48 E1 P24**	0.00	0.01
T72 B1 alc	0.68	2.32	**T48 E2 P24**	0.01	0.01
T72 B2 alc	0.64	2.24	**T48 G1 P24**	0.01	0.05
T72 D1 alc	0.39	3.20	**T48 G2 P24**	0.00	0.01
T72 D2 alc	0.24	3.10	**T72 B1 P24**	1.82	10.52
T72 F1 alc	0.01	0.01	**T72 B2 P24**	1.88	9.91
T72 F2 alc	0.01	0.01	**T72 D1 P24**	2.31	11.13
T72 H1 alc	0.02	0.05	**T72 D2 P24**	2.57	11.81
T72 H2 alc	0.02	0.13	**T72 F1 P24**	0.01	0.04
T0 A1 P24	0.25	4.34	**T72 F2 P24**	0.02	0.12
T0 A2 P24	0.18	3.67	**T72 H1 P24**	0.01	0.01
T0 C1 P24	0.25	4.68	**T72 H2 P24**	0.01	0.02
T0 C2 P24	0.45	7.22	**HA 1 alc**	0.26	6.12
T0 E1 P24	0.03	0.10	**HA 2 alc**	0.30	6.87
T0 E2 P24	0.05	0.14	**HA 1 P24**	0.30	7.73
T0 G1 P24	0.05	0.07	**HA 2 P24**	0.30	7.48
T0 G2 P24	0.20	4.96	**HB 1 alc**	0.01	0.02
T48 A1 P24	1.72	10.21	**HB 2 alc**	0.01	0.05
T48 A2 P24	1.58	8.85	**HB 1 P24**	0.28	0.17
T48 C1 P24	2.22	10.45	**HB 2 P24**	0.30	0.20

B. Calibration Curve

The table below (**Table 10**) lists the peak area values corresponding to each standard concentration used to generate the calibration curves for GABA and Glutamic Acid quantification (**Figure 7**).

***Table 10**. Normalized peak area values for standard solutions at different concentrations used in calibration curve construction.*

mg/L		**mg/L**	
[Glu]	**Normalized Area**	**[GABA]**	**Normalized Area**
1.00	0.10	1.20	0.01
1.90	0.17	2.50	0.03
4.90	0.47	6.20	0.13
9.70	0.80	12.50	0.25
19.50	1.81	24.90	0.61
48.70	4.38	62.30	1.71
Slope	*0.0901*	***Slope***	*0.0280*
Int	*0.0015*	***Int***	*-0.0557*



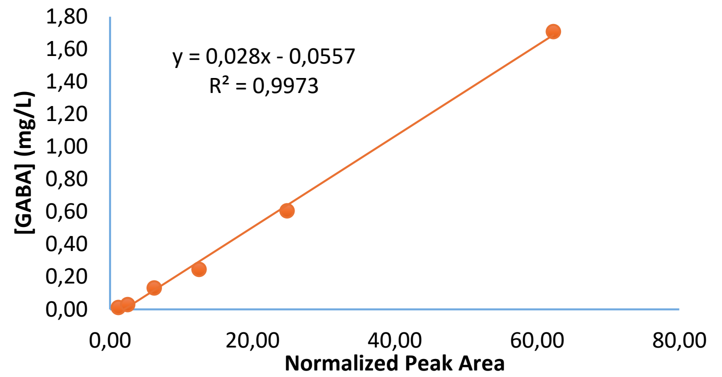


Figure 7. *Calibration curves for GABA and Glutamic Acid showing the relationship between compound concentration (mg/L) and normalized peak area obtained from qTOF analysis.*

C. Glutamic Acid and GABA concentration (mg/L)

The following table (**Table 11**) presents the quantified concentrations of GABA and Glu in mg/L, calculated from the normalized peak areas using the calibration curves.

Table 11. *GABA and Glu concentrations (mg/L) obtained from the calibration curves for each sample.*

	mg/L	
	GABA	**Glu**
T0 A1 alc	6.8	51.7
T0 A2 alc	7.1	52.9
T0 C1 alc	6.6	52.1
T0 C2 alc	6.1	48.1
T0 E1 alc	6.6	4.8
T0 E2 alc	7.0	5.2
T0 G1 alc	7.0	5.0
T0 G2 alc	7.2	5.2
T48 A1 alc	14.5	32.8
T48 A2 alc	13.8	31.8
T48 C1 alc	21.9	40.9
T48 C2 alc	16.9	47.9
T48 E1 alc	2.4	0.2
T48 E2 alc	2.3	0.1
T48 G1 alc	2.4	0.1
T48 G2 alc	2.3	0.1
T72 B1 alc	26.4	25.8
T72 B2 alc	24.9	24.8
T72 D1 alc	16.0	35.5
T72 D2 alc	10.5	34.4
T72 F1 alc	2.4	0.1
T72 F2 alc	2.5	0.1
T72 H1 alc	2.8	0.5
T72 H2 alc	2.8	1.4

T0 A1 P24	10.8	48.2
T0 A2 P24	8.6	40.7
T0 C1 P24	10.9	51.9
T0 C2 P24	18.1	80.1
T0 E1 P24	2.9	1.1
T0 E2 P24	3.8	1.5
T0 G1 P24	3.7	0.8
T0 G2 P24	9.3	55.0
T48 A1 P24	63.6	113.3
T48 A2 P24	58.5	98.2
T48 C1 P24	81.2	116.0
T48 C2 P24	78.5	114.9
T48 E1 P24	2.2	0.1
T48 E2 P24	2.2	0.1
T48 G1 P24	2.3	0.5
T48 G2 P24	2.1	0.1
T72 B1 P24	67.1	116.8
T72 B2 P24	69.1	110.0
T72 D1 P24	84.7	123.5
T72 D2 P24	93.8	131.1
T72 F1 P24	2.4	0.4
T72 F2 P24	2.6	1.4
T72 H1 P24	2.2	0.1
T72 H2 P24	2.2	0.2
HA 1 alc	11.4	67.9
HA 2 alc	12.9	76.3
HA 1 P24	12.5	85.8
HA 2 P24	12.7	83.0
HB 1 alc	2.2	0.2
HB 2 alc	2.2	0.5
HB 1 P24	12.1	1.8
HB 2 P24	12.8	2.2

D. Conversion from mg/L to mg/g of dry biomass

To convert the concentration of GABA (or Glu) from mg/L to mg/g, the following formula was applied:

$$[Glu/GABA]_f = \frac{[Glu/GABA]_i * V}{Dry\ weight}$$ *Equation (3)*

Where:

- **$[Glu/GABA]_i$** is the concentration obtained from the calibration curve
- **V** is the volume of the extract (in L)
- **Dry weight** is the dry weight of the biomass (in g) used for the extraction.

For example, for sample **T0 A1 alc** with 6.8 mg/L of GABA, 35 mL of extract and 50.08 g of dry biomass:

$$[GABA]_f = \frac{6.80 * 0.035}{50.08} = 0.05\, mg/g$$

The table below (**Table 12**) shows the concentrations of GABA and Glu expressed in mg per gram of dry biomass, obtained after applying the conversion from mg/L.

***Table 12**. GABA and Glutamic Acid concentrations (mg/g dry biomass) for all samples after conversion from mg/L.*

	mg/g	
	GABA	**Glu**
T0 A1 alc	0.05	0.36
T0 A2 alc	0.05	0.37
T0 C1 alc	0.05	0.36
T0 C2 alc	0.04	0.34
T0 E1 alc	0.05	0.03
T0 E2 alc	0.05	0.04
T0 G1 alc	0.05	0.04
T0 G2 alc	0.05	0.04
T48 A1 alc	0.10	0.23
T48 A2 alc	0.10	0.22
T48 C1 alc	0.15	0.29
T48 C2 alc	0.12	0.33
T48 E1 alc	0.02	0.00
T48 E2 alc	0.02	0.00
T48 G1 alc	0.02	0.00
T48 G2 alc	0.02	0.00
T72 B1 alc	0.21	0.21
T72 B2 alc	0.20	0.20
T72 D1 alc	0.13	0.28
T72 D2 alc	0.08	0.27
T72 F1 alc	0.02	0.00
T72 F2 alc	0.02	0.00
T72 H1 zlc	0.02	0.00
T72 H2 alc	0.02	0.01
T0 A1 P24	0.09	0.42
T0 A2 P24	0.08	0.36
T0 C1 P24	0.10	0.46
T0 C2 P24	0.16	0.70
T0 E1 P24	0.03	0.01
T0 E2 P24	0.03	0.01
T0 G1 P24	0.03	0.01
T0 G2 P24	0.08	0.48
T48 A1 P24	0.56	1.00
T48 A2 P24	0.51	0.86
T48 C1 P24	0.71	1.02
T48 C2 P24	0.69	1.01
T48 E1 P24	0.02	0.00
T48 E2 P24	0.02	0.00
T48 G1 P24	0.02	0.00
T48 G2 P24	0.02	0.00
T72 B1 P24	0.66	1.14
T72 B2 P24	0.68	1.08
T72 D1 P24	0.83	1.21
T72 D2 P24	0.92	1.28
T72 F1 P24	0.02	0.00
T72 F2 P24	0.03	0.01
T72 H1 P24	0.02	0.00
T72 H2 P24	0.02	0.00
HA alc	0.68	4.67
HA alc	0.69	4.52
HA P24	1.10	6.53
HA P24	1.24	7.33
HB alc	0.21	0.02
HB alc	0.22	0.05
HB P24	0.64	0.10
HB P24	0.68	0.12

The following table (**Table 13**) presents the average concentrations of GABA and Glu (mg/g of dry biomass) for each sample, along with the respective standard deviations calculated from biological duplicates (**Table 12**).

***Table 13**. Mean concentrations (mg/g) of GABA and Glu and corresponding standard deviations (n = 2).*

	GABA (mg/g))	**stdGABA**	**Glu (mg/g))**	**stdGlu**
T0 A alc	0.05	0.00123	0.37	0.00424
T0 C alc	0.04	0.00160	0.35	0.01428
T0 E alc	0.05	0.00140	0.03	0.00126
T0 G alc	0.05	0.00058	0.04	0.00081
T48 A alc	0.10	0.00241	0.23	0.00369
T48 C alc	0.14	0.01744	0.31	0.02439
T48 E alc	0.02	9.22437E-05	0.00	0.00015
T48 G alc	0.02	0.00021	0.00	0.00016
T72 B alc	0.20	0.00594	0.20	0.00365
T72 D alc	0.11	0.02187	0.28	0.00444
T72 F alc	0.02	0.00035	0.00	6.2E-06
T72 H alc	0.02	4.12508E-06	0.01	0.00369
T0 A P24	0.09	0.00965	0.39	0.03264
T0 C P24	0.13	0.03187	0.58	0.12389
T0 E P24	0.03	0.00366	0.01	0.00183
T0 G P24	0.06	0.02458	0.25	0.23864
T48 A P24	0.54	0.02225	0.93	0.06698
T48 C P24	0.70	0.01158	1.02	0.0047
T48 E P24	0.02	4.48182E-05	0.00	9.4E-05
T48 G P24	0.02	0.00079	0.00	0.00179
T72 B P24	0.67	0.01010	1.11	0.03328
T72 D P24	0.87	0.04462	1.25	0.03726
T72 F P24	0.02	0.00127	0.01	0.00469
T72 H P24	0.02	4.77481E-05	0.00	0.00021
HA alc	0.69	0.00408	4.59	0.07652
HA P24	1.17	0.07127	6.93	0.40112
HB alc	0.21	0.00236	0.03	0.01491
HB P24	0.66	0.01832	0.11	0.00991

V. Sample Code Mapping

To ensure clarity and consistency between the sample codes used in the analytical data tables and those used throughout the **Results and Discussion** section, the list below provides a correspondence between both designations.

T0 A alc → RB_L.b. (T0, alcalase)

T48 A alc → RB_L.b. (T48, alcalase)

T72 B alc → RB_L.b. (T72, alclase)

T0 C alc → RB_L.p. (T0, alcalase)

T48 C alc → RB_L.p. (T48, alcalase)

T72 D alc → RB_L.p. (T72, alcalase)

T0 E alc → BP_L.b. (T0, alcalase)

T48 E alc → BP_L.b. (T48, alcalase)

T72 F alc → BP_L.b. (T72, alcalase)

T0 G alc → BP_L.p. (T0, alcalase)

T48 G alc → BP_L.p. (T48, alcalase)

T72 H alc → BP_L.p. (T72, alcalase)

T0 A P24 → RB_L.b. (T0, P24)

T48 A P24 → RB_L.b. (T48, P24)

T72 B P24 → RB_L.b. (T72, P24)

T0 C P24 → RB_L.p. (T0, P24)

T48 C P24 → RB_L.p. (T48, P24)

T72 D P24 → RB_L.p. (T72, P24)

T0 E P24 → BP_L.b. (T0, P24)

T48 E P24 → BP_L.b. (T48, P24)

T72 F P24 → BP_L.b. (T72, P24)

T0 G P24 → BP_L.p. (T0, P24)

T48 G P24 → BP_L.p. (T48, P24)

T72 H P24 → BP_L.p. (T72, P24)

Where:

- **L.b.** means *L. brevis*
- **L.p.** means *L. plantarum*

GLOSSARY

Agri-food residues: Residues from food production processes, including plant or animal-derived materials that are not used for direct consumption.

Agro-industrial by-products: Secondary materials generated during agro-industrial processing, potentially rich in proteins, carbohydrates, lipids or bioactive compounds.

Alcalase: Commercial serine endopeptidase from *Bacillus licheniformis*; cleaves peptide bonds in proteins, optimal at alkaline pH and moderate temperature.

Anaerobic conditions: Environment lacking oxygen, where obligate or facultative anaerobes can perform metabolic processes.

Autoclave: Laboratory equipment for sterilization using pressurized saturated steam, typically at 121 °C and 1 atm above atmospheric pressure.

Bacteriocins: Ribosomally synthesized antimicrobial peptides produced by bacteria, active against closely related or other bacterial species.

Bioactive compounds: Molecules capable of modulating biological processes.

Biowaste: Organic waste derived from biological sources, including food scraps, agricultural residues and microbial biomass.

CFU (Colony Forming Unit): Unit used to estimate the number of viable microorganisms capable of forming colonies on solid media.

Co-culture: Cultivation system in which two or more microbial species are grown together under controlled conditions.

Dumas method: Analytical technique to quantify total nitrogen in a sample by combustion in oxygen, followed by detection of nitrogen-containing gases.

Ellagic acid: Polyphenolic compound found in plant materials, with antioxidant properties; often bound in ellagitannins.

Enzymatic hydrolysis: Process of cleaving chemical bonds in macromolecules using enzymes, producing smaller molecules such as peptides or sugars.

Facultative heterofermentative: Refers to lactic acid bacteria that can produce lactic acid via homofermentative or heterofermentative pathways depending on substrate and conditions.

Fermentation: Metabolic process where microorganisms convert organic substrates into simpler compounds, often producing acids, gases or alcohol.

Folin-Ciocalteu method: Colorimetric assay for total phenolic content, based on the reduction of a phosphomolybdate-phosphotungstate complex.

GABA (γ-aminobutyric acid): Non-proteinogenic amino acid derived from decarboxylation of glutamic acid; acts as a neurotransmitter in mammals.

Glutamic acid (Glu): Proteinogenic amino acid with acidic side chain; precursor in the biosynthesis of GABA.

Headspace: Gas-filled volume in a fermentation vessel above the liquid, influencing oxygen availability.

Hydrolysate: Product of enzymatic or chemical hydrolysis, containing solubilized peptides, amino acids or sugars.

Lactobacillus brevis: Gram-positive, facultatively heterofermentative, rod-shaped, non-spore-forming, catalase-negative lactic acid bacterium.

Lactobacillus plantarum: Gram-positive, facultatively heterofermentative, rod-shaped, non-spore-forming, catalase-negative lactic acid bacterium.

MRS agar: Selective growth medium designed for the cultivation of lactic acid bacteria, rich in nutrients and buffering agents.

MRS broth: Liquid form of MRS medium, containing peptone, dextrose, yeast extract, salts, and buffering agents; specifically designed for the growth of lactic acid bacteria.

Optical Density (OD): Measurement of light absorbance by a microbial suspension, used to estimate cell concentration.

Peptone water: Simple liquid culture medium containing peptone and sodium chloride; provides basic nutrients for the recovery and maintenance of microorganisms without promoting rapid growth or selectivity.

Phenol-sulfuric acid method: Colorimetric assay for total carbohydrate content, based on dehydration of sugars by sulfuric acid and reaction with phenol.

PLP (Pyridoxal-5'-phosphate): Active cofactor form of vitamin B6.

Protease: Enzyme that catalyzes the hydrolysis of peptide bonds in proteins.

P24 protease: Commercial protease with broad substrate specificity; hydrolyzes proteins into peptides and amino acids.

qTOF (Quadrupole Time-of-Flight Mass Spectrometry): High-resolution mass spectrometry technique for accurate mass measurement of molecules.

Saccharification: Conversion of polysaccharides into simple sugars via enzymatic or chemical hydrolysis.

Saccharomyces cerevisiae: Gram-positive, oval/round budding yeast; facultative anaerobe widely used in fermentation and biotechnology.

SCFA (Short-chain fatty acids): Fatty acids with ≤ 6 carbon atoms; microbial fermentation products of carbohydrates.

Selective media: Culture media designed to favor growth of certain microorganisms while inhibiting others.

Substrate: Material on which an enzyme or microorganism acts, including proteins, carbohydrates or other nutrients.

UAE (Ultrasound-Assisted Extraction): Extraction technique using ultrasonic waves to improve solubilization of target compounds from solid matrices.

Urolithin A (uroA): Microbial metabolite derived from ellagic acid, with potential bioactive properties.

Vacuum filtration: Filtration method using reduced pressure to speed up liquid passage through a membrane.

Viable count: Estimation of living microorganisms in a sample, typically expressed in CFU per mL.

YPD agar: Nutrient-rich solid medium containing yeast extract, peptone, and dextrose; used for the cultivation of yeasts.

YPD broth: Liquid formulation of YPD medium (yeast extract, peptone, dextrose) used for yeast cultivation in suspension culture.