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Lactic fermentation for the valorisation of plant by-products: new substrates and novel applications.

Coordinatore:
Chiar.mo Prof. Massimiliano Rinaldi

Tutore:
Chiar.ma Prof.ssa Camilla Lazzi

Dottorando: Martina Marrella

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Abstract

Economic development requires a large amount of raw materials, which are quickly consumed. In the agro-food industry, a significant quantity of by-products and/or waste is also produced, resulting in approximately one-third of food production being wasted. Agroindustrial biomasses are often characterised by high water content and chemical composition in fibers, proteins and bioactive compounds, making them matrices suitable for numerous applications. The nutrients present in waste matrices can be an excellent growth substrate for microorganisms. Through the fermentation process, these microorganisms can convert or produce molecules of industrial interest, which is why fermentation has attracted increasing interest. These final products could include bioactives, like antimicrobial agents, antioxidants, volatile compounds, organic acids and deglycosylated polyphenols. Fermentation offers several advantages, including low costs, minimal energy and water consumption, and the ability to convert by-products and waste into high-value products.

Lactic fermentation, in particular, is carried out by lactic acid bacteria, microorganisms which are considered GRAS (Generally Recognised As Safe) by Food and Drug Administration (FDA) or with the Qualified Presumption of Safety (QPS) status by European Food Safety Authority.

Lactic acid bacteria have a long history of use in food production. Due to their simple metabolism, characterised by a high nutrient intake and resistance to stress, such as acidic environments and high temperatures typically encountered in industrial process, their potential as cell factories for the chemical industry is being increasingly explored. With this knowledge, the thesis has been focused on the valorisation of different plant

matrices through the application of lactic acid fermentation followed two lines. The first one was to evaluate the changes on molecular profile of two by-products which may be relevant in our agro-industrial sector, like Okara and *Opuntia Ficus Indica* Cladodes. The second line involved the possible application of an extract with antimicrobial activity, obtained from the lactic fermentation of tomato peels and seeds, in a production process.

Introduction

The issue of food waste is both a global and localized challenge with significant environmental, economic, and social implications. Food waste is a multifaceted issue that demands immediate attention to achieve global sustainability goals (Forbes 2021). However, it is essential to distinguish between food loss, food waste and by-products (Cattaneo et al. 2021). Indeed, *food loss* refers to reductions caused by inefficiencies within the supply chain, such as during production, storage, or transport (Cattaneo et al. 2021). *Food waste* originates from retailers, food services, and consumers at the end of the supply chain (Cattaneo et al. 2021). Additionally, *by-products* refer to materials generated as secondary outputs during the primary production of another good (Saparbekova et al. 2023).

The United Nations 2030 Agenda highlights the urgency to halve per capita food waste at the retail and consumer levels and reduce losses throughout production and supply chains, including post-harvest stages (UN 2015; Lipinski 2017). this can be achieved by improving resource efficiency and promoting sustainable production and consumption practices. Efforts to reduce food waste should focus on several points, such as awareness-raising campaigns to educate consumers about the environmental and social impact of food waste, policies to encourage sustainable practices along the supply chain, innovation and technology to support research on waste reduction technologies, and global cooperation to strengthen international partnerships to share best practices and align efforts with the United Nations 2030 Agenda (Leal Filho et al. 2024; Khajuria et al. 2022; Ciccullo et al. 2021; Martin-Rios et al. 2021).

According to the World Wildlife Fund (2021), food waste accounts for up to 40% of global

food production, equivalent to approximately 2.5 billion tons annually. This staggering volume highlights the inefficiencies within global food systems, contributing significantly to environmental degradation and resource depletion.

In the European Union, 153 million tons of food waste were generated in 2021, surpassing annual food imports of 138 million tons (Vera et al. 2022). This waste corresponds to 6% of the EU's total greenhouse gas emissions, with an estimated economic cost of 143 billion euros (Vera et al. 2022). Such figures underscore the urgent need for coordinated efforts to reduce food waste and its associated impacts. Adopting circular economy principles is a promising strategy to address food waste. The moral and ethical dimensions of food waste are particularly significant given the coexistence of wasteful practices and widespread hunger. Despite global food production being sufficient to meet demand, excessive wastage leaves one in nine people malnourished (Roe et al. 2020). Addressing food waste is therefore not only an environmental and economic issue but also a moral imperative to ensure equitable access to resources (D'Adamo et al. 2023).

To address the environmental and economic challenges posed by food waste, the Food Waste Management Hierarchy-based on the 3R principle: Reduce, Reuse, and Recycle-has been adopted in various countries (Dri et al. 2018). This approach promotes sustainable food waste management, aiming to minimise losses and repurpose discarded materials as valuable resources, thereby reducing the environmental impacts, and creating economic opportunities.

To align with these objectives, various initiatives are embracing circular economy frameworks for managing plant-based waste. These initiatives include energy recovery through the use of food waste to produce biogas and biofuels, composting with the

conversion of organic waste that enriches the soil with nutrients, or the production of bioplastics and value-added molecules for use as food ingredients (Hadj Saadoun et al. 2021; Bibi et al. 2023a).

The processing of fruits and vegetables generates significant amounts of biological residues or by-products, including seeds, core, rag, peels, rind, vine, shell, skin, pomace, stones, and pods (Saparbekova et al. 2023; Rodríguez García and Raghavan 2022). These by-products represent a substantial portion of food waste, with losses throughout the supply chain reaching as high as 50% for fruits and vegetables (FAO 2018).

Notably, fruit and vegetable by-products are rich in valuable nutrients, which present significant potential for recovery and reuse (Gulsunoglu et al. 2019). Indeed, fruit and vegetable losses occur at both harvest and processing stages, exceeding the waste measured for cereals and pulses (FAO. 2019). These losses can be attributed to multiple factors, including overproduction driven by changing eating habits and population growth (Coman et al. 2020), as well as the significant waste generated by industries processing these products. The disposal of such by-product poses environmental and food safety concerns, as its decomposition contributes to greenhouse gas emissions and potential hazards. Processing fruit and vegetable waste and by-products, therefore, represents an opportunity to mitigate these impacts while adding value.

Plants are composed of two major classes of compounds: primary metabolites and secondary metabolites. Primary metabolites, such as carbohydrates, amino acids, proteins, and lipids, are essential for basic cellular functions and growth. Secondary metabolites, on the other hand, play a critical role in a plant's ability to survive and adapt, providing defence against external stressors and enabling interactions with the environment. In addition, most of these compounds protect fruits and vegetables from

being consumed too early due to their unpleasant taste, bitterness and astringency (Padayachee et al. 2017). These metabolites contribute to the nutritional and therapeutic properties of fruits and vegetables, which are recognized as essential components of a balanced diet. Regular consumption of fruits and vegetables is linked to the prevention of major cardiovascular conditions and diet-related chronic diseases (Rodríguez García and Raghavan 2022).

Similarly, the by-products or waste from fruits and vegetables also harbor valuable bioactive compounds, which hold immense potential for various applications. Bioactive compounds include phenols, flavonoids, carotenoids, phytosterols, glucosinolates, saponins, alkaloids, and essential oils (Trigo et al. 2020). The specific bioactive composition of fruit and vegetable waste depends on factors such as plant variety, stage of ripening, and extraction methods, phenols and carotenoids are among the most prevalent bioactive compounds identified in these by-products (Trigo et al. 2020). These compounds support optimal cellular health by modulating metabolic processes and enhancing the action of other nutrients. Additionally, they exhibit diverse biological properties, including antioxidant, antimicrobial, anti-inflammatory, and anti-allergic activities (Santos et al. 2019). Recognizing this untapped potential has spurred interest in developing innovative techniques to extract and utilize bioactive compounds in industries such as food, pharmaceuticals, and cosmetics. The recovery of these compounds not only offers a sustainable approach to reducing waste but also represent a valuable resource for creating functional ingredients that can address health and environmental challenges.

These bioactive compounds are extensively utilized to enhance antioxidant activity, antimicrobial effects, and total phenolic content, while also improving specific attributes

of final food products, such as color (Marranzano et al. 2018). For instance, carotenoids and anthocyanins are commonly employed as natural colorants (Rodríguez García and Raghavan 2022).

Among bioactive compounds, phenolic compounds are particularly noteworthy due to their diverse chemical structures and biological functions. These compounds are synthesized via the shikimic acid, pentose phosphate, and phenylpropanoid pathways (Trigo et al. 2020). Predominantly water-soluble, phenolic compounds comprise a vast class of molecules, with over 8000 distinct structures identified. They are characterized by at least one aromatic ring with one or more hydroxyl substituents and often exist in nature as more complex molecules, referred to as polyphenols. Phenolic compounds are broadly classified into flavonoids, phenolic acids, and tannins, all of which exhibit significant antioxidant and antimicrobial properties (Babbar and Oberoi 2014).

Flavonoids, for example, exert their antimicrobial activity through their ability to form complexes with extracellular and soluble proteins, as well as bacterial cell walls (Guil-Guerrero et al. 2016). Phenolic acids exhibit antimicrobial effects by diffusing through the bacterial membrane, causing cytoplasmic acidification and, sometimes, leading to cell death (Sanchez-Maldonado 2014). Tannins, on the other hand, utilize multiple mechanisms to exert their antimicrobial activity, including inhibition of enzymatic activity, depletion of essential metal ions, and precipitation of membrane proteins (Akhtar et al. 2015; Ismail et al. 2016).

The antioxidant activity of phenolic compounds largely depends on their chemical structure, particularly the number of double bonds and hydroxyl groups present. For instance, the substitution of hydroxyl groups through glycosylation often reduces antioxidant activity.

Given the significant bioactive properties of these compounds, their extraction from by-products and waste presents novel opportunities for their application in the food industry to produce enhanced food products.

One step that can precede traditional or greener extraction methods is fermentation. Through fermentation, it is not only possible to extract known compounds present in the matrix but also to generate new ones. During microbial growth, microorganisms can secrete metabolites or induce the expression of enzymes with antioxidant activity. For instance, enzymes such as glycosyl hydrolases, esterases, decarboxylases, and phenolic acid reductases, when secreted, can deglycosylate phenolic compounds, thereby increasing their biological activity (Tonolo et al. 2023).

Agri-food waste and by-products are rich in minerals, sugars, and proteins, making them an excellent substrate for microbial growth, as it provides the necessary carbon, nutrients, and moisture for their development (Bibi et al. 2023b). Fermentation not only promotes the production of bioactive compounds but also prevents the growth of contaminants during storage, enhancing the stability of the product a critical aspect of food safety (Tonolo et al. 2023).

Fermentation has been used for centuries by ancient civilizations to produce various food products. Today, there is growing interest in leveraging fermentation to improve fermented foods' health, nutritional, technological, and sensory qualities. Thanks to the biodiversity of microorganisms, fermentation can yield products with unique and desirable aromatic profiles while converting various precursors into fine biochemicals, such as aromatic compounds and fragrances (Hadj Saadoun et al. 2021; Ray et al. 2024). Indeed, fermentation is widely employed to modify the aromatic profile of products. Through bacterial metabolism, aromatic notes can be enhanced, or undesirable off-

flavor components reduced (Martelli et al. 2021; Maria Carpena et al. 2021; Ricci et al. 2020). As a result, the metabolites generated during fermentation can include both aromatic compounds and aroma precursors. For instance, esters, often characterized by sweet odors, or aldehydes, associated with floral or fruity notes, may be produced (Hadj Saadoun et al. 2021).

In dairy production, lactic acid bacteria (LAB) play a crucial role in generating complex aromatic molecules that contribute to the characteristic flavors of fermented dairy products. LAB have a long history of use in fermentation processesand are considered GRAS (Generally Recognized as Safe) by the U.S. Food and Drug Administration (FDA) and hold Qualified Presumption of Safety (QPS) status granted by the European Food Safety Authority (EFSA). Their simple metabolism, characterized by high nutrient utilization, allows LAB to grow on diverse carbon sources. Additionally, they exhibit robust resistance to environmental stressors, such as acidic conditions and high temperatures, often encountered in industrial processes (Sauer et al. 2017). Despite its simplicity and great potential, fermentation remains a low-cost, energy-efficient biological process that can be exploited to create value-added products (Tlais et al. 2020).

LAB are also extensively studied for their ability to produce various high-value products. These include lactic acid, plastic polymers, ethanol, exopolysaccharides (which function as thickeners and prebiotics), antimicrobial compounds, food flavorings, and sweeteners such as sorbitol, mannitol, and l-alanine (HadjSaadoun et al. 2021). Their versatility and adaptability make LAB a cornerstone of biotechnological innovation, with applications spanning the food, pharmaceutical, and chemical industries.

In addition, the ability of LABs to enhance sensory properties, improve food stability and provide health benefits underlines their importance in sustainable and functional food

production. Their ability to simultaneously contribute to flavour enhancement and functional benefits reinforces their role in modern fermentation technologies.

Overall, fermentation plays a crucial role in producing fermented biomasses and extracts that serve as additives to enhance or modify the properties of final products across a wide range of industrial applications. Since these additives are derived from a biological process, they can be labeled as "natural," aligning with environmentally sustainable practices and consumer preferences. Given the versatility of fermentation, which can be adapted to various substrates and fine-tuned using different strains and conditions, it stands as a cornerstone for sustainable innovation in multiple industries.

References

Akhtar, Saeed, Tariq Ismail, Daniele Fraternale, and Piero Sestili. 2015. “Pomegranate Peel and Peel Extracts: Chemistry and Food Features.” Food Chemistry. Elsevier Ltd. https://doi.org/10.1016/j.foodchem.2014.11.035.

Babbar, Neha, and Harinder Singh Oberoi. 2014. “Potential of Agro-Residues as Sources of Bioactive Compounds.” In Biotransformation of Waste Biomass into High Value Biochemicals, 9781461480051:261–95. Springer New York. https://doi.org/10.1007/978-1-4614-8005-1_11.

Bibi, Fatima, Noshin Ilyas, Maimona Saeed, Sumera Shabir, Ali A. Shati, Mohammad Y. Alfaifi, Kassian T.T. Amesho, Subrata Chowdhury, and Riyazali Zafarali Sayyed. 2023a. “Innovative Production of Value-Added Products Using Agro-Industrial Wastes via Solid-State Fermentation.” Environmental Science and Pollution Research. Springer. https://doi.org/10.1007/s11356-023-28765-6.

Brian Lipinski. 2017. “How the World Can Cut Food Loss and Waste in Half.” World Resources Institute. September 20, 2017.

Carpena, Maria, Maria Fraga-Corral, Paz Otero, Raquel A. Nogueira, Paula Garcia-Oliveira, Miguel A. Prieto, and Jesus Simal-Gandara. 2021. “Secondary Aroma: Influence of Wine Microorganisms in Their Aroma Profile.” Foods. MDPI AG. https://doi.org/10.3390/foods10010051.

Cattaneo, Andrea, Giovanni Federighi, and Sara Vaz. 2021. “The Environmental Impact of Reducing Food Loss and Waste: A Critical Assessment.” Food Policy 98 (January). https://doi.org/10.1016/j.foodpol.2020.101890.

Ciccullo, Federica, Raffaella Cagliano, Giulia Bartezzaghi, and Alessandro Perego. 2021. “Implementing the Circular Economy Paradigm in the Agri-Food Supply Chain: The Role of Food Waste Prevention Technologies.” Resources, Conservation and Recycling 164 (January). https://doi.org/10.1016/j.resconrec.2020.105114.

Coman, Vasile, Bernadette Emőke Teleky, Laura Mitrea, Gheorghe Adrian Martău, Katalin Szabo, Lavinia Florina Călinoiu, and Dan Cristian Vodnar. 2020. “Bioactive Potential of Fruit and Vegetable Wastes.” In Advances in Food and Nutrition Research, 91:157–225. Academic Press Inc. https://doi.org/10.1016/bs.afnr.2019.07.001.

D'Adamo, Idiano, Simona Desideri, Massimo Gastaldi, and Konstantinos P. Tsagarakis. 2023. "Sustainable Food Waste Management in Supermarkets." Sustainable Production and Consumption 43 (December):204–16. https://doi.org/10.1016/j.spc.2023.11.005.

Dri M., Canfora P., Antonopoulos I. S., and Gaudillat P. 2018. "Best Environmental Management Practice for the Waste Management Sector Learning from Frontrunners." In . JRC Science for Policy Report. https://doi.org/10.2760/50247.

FAO. 2018. "The Future of Food and Agriculture: Alternative Pathways to 2050." 2018.

FAO. 2019. STATE OF FOOD AND AGRICULTURE 2019 : MOVING FORWARD ON FOOD LOSS AND WASTE REDUCTION. FOOD & AGRICULTURE ORG.

Forbes, H. 2021. "Food Waste Index Report 2021." United Nations Environment Programme (UNEP). 2021.

Guil-Guerrero, J. L., L. Ramos, C. Moreno, J. C. Zúñiga-Paredes, M. Carlosama-Yepez, and P. Ruales. 2016. "Antimicrobial Activity of Plant-Food by-Products: A Review Focusing on the Tropics." Livestock Science. Elsevier B.V. https://doi.org/10.1016/j.livsci.2016.04.021.

Gulsunoglu, Zehra, Funda Karbancioglu-Guler, Katleen Raes, and Meral Kilic-Akyilmaz. 2019. "Soluble and Insoluble-Bound Phenolics and Antioxidant Activity of Various Industrial Plant Wastes." International Journal of Food Properties 22 (1): 1501–10. https://doi.org/10.1080/10942912.2019.1656233.

Hadj Saadoun, Jasmine, Gaia Bertani, Alessia Levante, Fabio Vezzosi, Annalisa Ricci, Valentina Bernini, Camilla Lazzi, Silvia Grassi, and Maria Paciulli. 2021. "Fermentation of Agri-Food Waste: A Promising Route for the Production of Aroma Compounds." Foods. https://doi.org/10.3390/foods.

Hadj Saadoun, Jasmine, Luca Calani, Martina Cirlini, Valentina Bernini, Erasmo Neviani, Daniele Del Rio, Gianni Galaverna, and Camilla Lazzi. 2021. "Effect of Fermentation with Single and Co-Culture of Lactic Acid Bacteria on Okara: Evaluation of Bioactive Compounds and Volatile Profiles." Food and Function 12 (7): 3033–43. https://doi.org/10.1039/d0fo02916e.

Ismail, Tariq, Saeed Akhtar, Muhammad Riaz, Aneela Hameed, Khurram Afzal, and Ahsan Sattar Sheikh. 2016. "Oxidative and Microbial Stability of Pomegranate Peel Extracts

and Bagasse Supplemented Cookies." Journal of Food Quality 39 (6): 658–68. https://doi.org/10.1111/jfq.12231.

Khajuria, Anupam, Vella A. Atienza, Suchana Chavanich, Wilts Henning, Ishrat Islam, Ulrich Kral, Meng Liu, et al. 2022. "Accelerating Circular Economy Solutions to Achieve the 2030 Agenda for Sustainable Development Goals." Circular Economy. Elsevier B.V. https://doi.org/10.1016/j.cec.2022.100001.

Leal Filho, Walter, Thais Dibbern, Maria Alzira Pimenta Dinis, Evandro Coggo Cristofoletti, Marcellus Forh Mbah, Ashish Mishra, Amelia Clarke, et al. 2024. "The Added Value of Partnerships in Implementing the UN Sustainable Development Goals." Journal of Cleaner Production 438 (January). https://doi.org/10.1016/j.jclepro.2024.140794.

Marranzano, M., R. L. Rosa, M. Malaguarnera, R. Palmeri, M. Tessitori, and A. C. Barbera. 2018. "Polyphenols: Plant Sources and Food Industry Applications." Current Pharmaceutical Design.

Martelli, Francesco, Martina Cirlini, Camilla Lazzi, Erasmo Neviani, and Valentina Bernini. 2021. "Solid-State Fermentation of Arthrospira Platensis to Implement New Food Products: Evaluation of Stabilization Treatments and Bacterial Growth on the Volatile Fraction." Foods 10 (1). https://doi.org/10.3390/foods10010067.

Martin-Rios, Carlos, Anastasia Hofmann, and Naomi Mackenzie. 2021. "Sustainability-Oriented Innovations in Food Waste Management Technology." Sustainability (Switzerland) 13 (1): 1–12. https://doi.org/10.3390/su13010210.

Padayachee, A., L. Day, K. Howell, and M. J. Gidley. 2017. "Complexity and Health Functionality of Plant Cell Wall Fibers from Fruits and Vegetables." Critical Reviews in Food Science and Nutrition 57 (1): 59–81. https://doi.org/10.1080/10408398.2013.850652.

Ray, R. C., S. Paramithiotis, A. Thekkangil, V. Nethravathy, A. K. Rai, and J. G. P. Martin. 2024. "Food Fermentation and Its Relevance in the Human History." Trending Topics on Fermented Foods.

Ricci, Annalisa, Martina Marrella, Jasmine Hadj Saadoun, Valentina Bernini, Francesco Godani, Franco Dameno, Erasmo Neviani, and Camilla Lazzi. 2020. "Development of Lactic Acid-Fermented Tomato Products." Microorganisms 8 (8): 1–13. https://doi.org/10.3390/microorganisms8081192.

Rodríguez García S. L., and Raghavan V. 2022. “Green Extraction Techniques from Fruit and Vegetable Waste to Obtain Bioactive Compounds—A Review.” Critical Reviews in Food Science and Nutrition. Taylor and Francis Ltd. https://doi.org/10.1080/10408398.2021.1901651.

Roe, Brian E., Danyi Qi, and Kathryn E. Bender. 2020. “Some Issues in the Ethics of Food Waste.” Physiology and Behavior 219 (May). https://doi.org/10.1016/j.physbeh.2020.112860.

Sanchez-Maldonado, A. F. 2014. “Mode of Action, Interaction and Recovery of Plant Secondary Metabolites for Potential Applications as Food Preservatives.” University of Alberta.

Santos, Diana I., Jorge Manuel Alexandre Saraiva, António A. Vicente, and Margarida Moldão-Martins. 2019. “Methods for Determining Bioavailability and Bioaccessibility of Bioactive Compounds and Nutrients.” In Innovative Thermal and Non-Thermal Processing, Bioaccessibility and Bioavailability of Nutrients and Bioactive Compounds, 23–54. Elsevier. https://doi.org/10.1016/B978-0-12-814174-8.00002-0.

Saparbekova, A. A., G. O. Kantureyeva, D. E. Kudasova, Z. K. Konarbayeva, and A. S. Latif. 2023. “Potential of Phenolic Compounds from Pomegranate (Punica Granatum L.) by-Product with Significant Antioxidant and Therapeutic Effects: A Narrative Review.” Saudi Journal of Biological Sciences. Elsevier B.V. https://doi.org/10.1016/j.sjbs.2022.103553.

Sauer, Michael, Hannes Russmayer, Reingard Grabherr, Clemens K. Peterbauer, and Hans Marx. 2017. “The Efficient Clade: Lactic Acid Bacteria for Industrial Chemical Production.” Trends in Biotechnology. Elsevier Ltd. https://doi.org/10.1016/j.tibtech.2017.05.002.

Tlais, Ali Zein Alabiden, Giuseppina Maria Fiorino, Andrea Polo, Pasquale Filannino, and Raffaella Di Cagno. 2020. “High-Value Compounds in Fruit, Vegetable and Cereal Byproducts: An Overview of Potential Sustainable Reuse and Exploitation.” Molecules. MDPI AG. https://doi.org/10.3390/molecules25132987.

Tonolo, Federica, Alessandra Folda, Stefania Ferro, Roberta Seraglia, Angiolella Lombardi, Christian Andrighetto, Alessia Giannoni, Oriano Marin, and Maria Pia Rigobello. 2023. “Fermentation of Corn By-Products: From Agrifood Waste to Higher

Value Antioxidant Products." Fermentation 9 (4). https://doi.org/10.3390/fermentation9040373.

Trigo, João P., Elisabete M.C. Alexandre, Jorge A. Saraiva, and Manuela E. Pintado. 2020. "High Value-Added Compounds from Fruit and Vegetable by-Products–Characterization, Bioactivities, and Application in the Development of Novel Food Products." Critical Reviews in Food Science and Nutrition. Taylor and Francis Inc. https://doi.org/10.1080/10408398.2019.1572588.

UN. 2015. "Goal 2: Zero Hunger." UNITED NATIONS. 2015.

Vera I., Bowman M., and Mechielsen F. 2022. "No Time to Waste: Why the EU Needs to Adopt Ambitious Legally Binding Food Waste Reduction Targets." Feedback EU. 2022.

World Wildlife Fund. 2021. "Driven to Waste: The Global Impact of Food Loss and Waste on Farms." Retrieved from WWF. 2021.

Aim of the thesis

This doctoral thesis project aims to enhance the value of different plant matrices through the application of lactic acid fermentation. Specifically, the study focuses on agro-industrial by-products which may be relevant from an economic and climate change perspective.

The first chapter of this PhD thesis examines okara, a by-product of tofu and soy milk production whose accumulation is increasing due to the growing demand for soy-based foods. The research investigates the fermentation of okara using different LAB species to enhance its polyphenol content for potential food applications.

The second chapter of this PhD thesis explores the potential of prickly pear (*Opuntia Ficus Indica* L.), a plant native to the Americas and cultivated in Italy. The plant's ability to thrive in dry conditions and high temperatures makes it a promising crop for agriculture in areas most affected by climate change and moving towards desertification. While its fruits are widely consumed, the discarded cladodes pose disposal challenges for farmers. The research investigates the use of lactic acid fermentation to enhance the polyphenol and volatile compound content of the cladodes or to produce antimicrobial extracts. One part of this study was conducted in collaboration with iBET (Instituto de Biologia Experimental e Tecnológica, Portugal), where the chemical characterisation analyses of the fermented products were made (Figure 1).

The third chapter of PhD thesis was related to a potential application as water sanitizers of an antimicrobial of natural origin (NA), recently patented by the University of Parma, derived from a fermentation and extraction process of peels and seeds from the tomato processing industry. This study was carried out in collaboration with a partner company

in this PhD project, interested in the possible reuse of the antimicrobial in processing plants, in line with the theme of sustainability and circular economy.

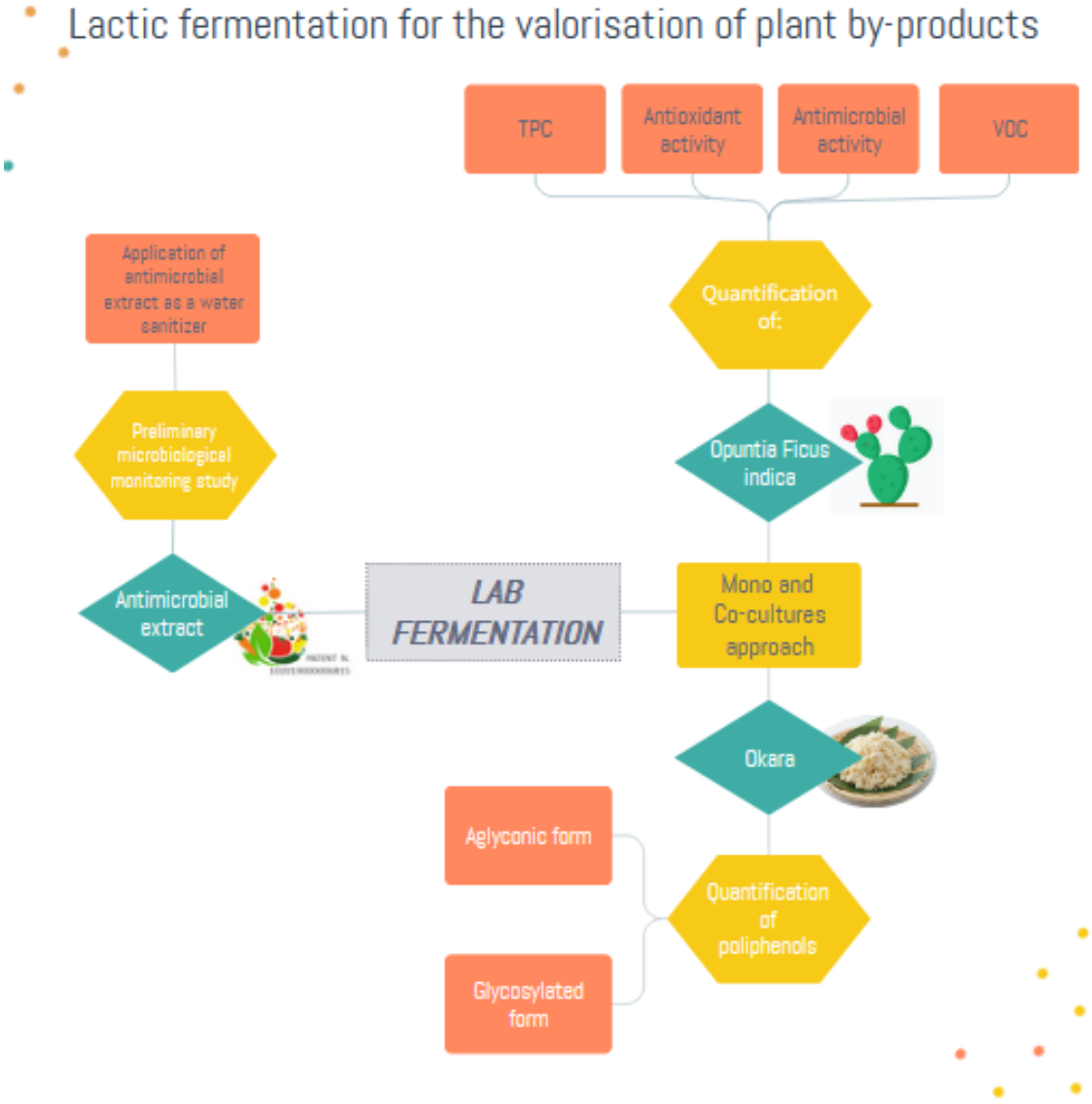

Figure 1. Chart describing the plant by-product valorisation work carried out in this dissertation

Chapter 1

Evaluation and predictive modelling of okara (poly)phenol metabolite production via lactic acid bacteria fermentation

Vicente Agullo Garcia, Martina Marrella, Lorena Sanchez Martinez, Sonia Figuccia, Luca Fontechiari, Jasmine Hadj Saadoun, Camilla Lazzi, Annalisa Ricci, Letizia Bresciani

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Introduction

Soybean (Glycine max) is one of the most widely consumed legumes in the world. It has been part of the oriental diet for centuries and is one of the most popular foods in this population, forming part of traditional dishes such as soups, sauces and fermented products such as tempeh (Liu et al. 2022). However, as a result of globalisation, soya and its derivatives have gradually been introduced into the Western diet. Nowadays, it is estimated to be the fourth most important crop worldwide with an annual production of around 370 million tonnes of soybeans/year, and Brazil, USA, Argentina, China, India, Paraguay and Canada are the main soybean-producing countries ("Soybeans | USDA Foreign Agricultural Service" 2024). Consumption of soya and soya products continues to grow, in part because of their nutritional and functional properties that contribute to the improvement of human health. In fact, epidemiological studies have revealed a positive association between the consumption of soy-rich foods and the prevention of non-communicable diseases such as cancer, neurodegenerative diseases and cardiovascular diseases (Kim 2022; Wang et al. 2021; Wei et al. 2020). This is mainly due to its high content of bioactive compounds such as (poly)phenols, including daidzein, genistein and glycitein, β-carotene and essential fatty acids, mainly linoleic acid, palmitic acid, stearic acid, oleic acid and linoleic acid (Kim et al. 2021). However, the production of soy products such as tofu and soy-based beverages generates a large amount of insoluble waste material from the extraction of the aqueous phase of soybean crushing, which is commonly known as "okara", but may also be known as "douzha" and "biji" (Vong and Liu 2016). It is estimated that the production of vegetable soy beverage from dried soybeans generates between 1.4 and 1.8 kg of wet okara per kilo of dried soybeans, while the production of tofu produces around 1.2 kg of fresh okara per kilo of

dried soybeans (Guimarães et al. 2018). China (2,800,000 tonnes/year), Japan (800,000 tonnes/year) and Korea (310,000 tonnes/year) are the main okara producers (Mok et al. 2019). Okara has a high moisture content, an unpleasant taste and spoils easily. Consequently, the vast majority of okara produced by the food industry is discarded as industrial waste, with a small proportion used as animal feed and/or fertiliser (Wang et al. 2024). This means that okara not only contributes to food waste, but it also represents an environmental issue. However, okara could be reintroduced into the food chain as a functional ingredient, due to its soy-like nutritional composition, being high in crude fibre, protein and minerals, and its high content of bioactive compounds, such as isoflavones (daidzein, genistein and glycitein), which are known for their antioxidant, immunomodulatory, anticarcinogenic and estrogenic activities (Feng et al. 2021; Khosravi and Razavi 2021). In fact, several studies have shown that the consumption of isoflavone-rich products is associated with an improvement in the incidence of chronic diseases, including cancer, osteoporosis, cardiovascular diseases, diabetes and/or obesity, and microbial infections (Jannah et al. 2020; Guevara-Cruz et al. 2020; Kim 2022). Therefore, the fortification of common foods such as yoghurt, milk or biscuits with okara could be a good nutritional strategy to improve human health, due to its high isoflavone content, and would also contribute to the reduction of the environmental impact caused by the production of soy derivatives. In this context, solid-state fermentation (SSF) of okara could be a potential strategy to reintroduce okara into the food chain as a functional ingredient: it could improve the sensory profile of okara (taste and texture) and its functional properties, by contributing to an increase in soluble fibre and protein content, as well as by promoting the biotransformation of β-isoflavone glycosides into potentially more bioactive isoflavone aglycones, for which a better

absorption compared to their glycoside forms has been reported (Asghar et al. 2023; Hadj Saadoun et al. 2021). Furthermore, it should be noted that SSF is a low-cost, environmentally friendly technique with a simple methodology based on microbial growth and product formation on the surface of solid materials in the absence of liquid, which means that it can be easily implemented by the food industry (Mok et al. 2019). Lactic acid bacteria (LAB) are the most commonly used microorganisms for this type of fermentation, due to their ability to produce several metabolites with biological functions that enhance the functional properties of okara, also imparting a pleasant flavours and aromas to this food matrix (Hadj Saadoun et al. 2021). However, there are very few studies evaluating the impact of SSF in both monoculture and coculture on the qualitative and quantitative profile of phenolic metabolites derived from the metabolism of the native compounds present in okara, nor optimising the fermentation process to obtain a potentially bioavailable phenolic metabolite profile. In humans, isoflavones are metabolised by colonic microbiota leading to the production of peculiar catabolites, namely *O*-desmethylangolensin and equol (Rafii 2015). The present work aimed to evaluate the effect of mono- and co-culture LAB fermentation on okara (poly)phenols, focusing on the ability of selected microorganisms to produce new (poly)phenol-derived metabolites. Moreover, based on the obtained data on growth parameters and (poly)phenol biotransformation, predictive models were applied to determine the most important factors able to affect the okara polyphenols and the optimal condition for achieving the highest production of specific (poly)phenols.

Materials and methods

Chemicals

Analytical standards of 3-(4'-hydroxyphenyl)lactic, 3-(phenyl)lactic acid, ferulic acid, and naringenin were purchased from Sigma-Aldrich (Merck KGaA, DA, Germany). Daidzein, dihydrodaidzein, glycitein, dihydrogenistein, genistein, and *O*-desmethylangolesin were purchased from Toronto Research Chemicals (Toronto, ON, Canada). Vanillic acid was purchased from Alfa Aesar Chemicals and *p*-coumaric acid from Honeywell Fluka™ Chemicals (both Thermo Scientific Chemicals, Thermo Fisher Scientific Inc., MA, USA). All chemicals and HPLC-grade solvents (water, methanol, formic acid, and acetonitrile) and reagents were purchased from VWR International (Radnor, PA, USA).

Raw material and bacterial strains

Okara was provided by Sojasun company, from the plant located in Fidenza (Parma, Italy). It was stored at -20°C and finally sterilised at 121°C for 20 minutes before fermentation.

Based on the reported microbial enzymatic activity involved in isoflavone metabolism (Lee et al. 2018), different species were selected. Four strains belonging to *Lacticaseibacillus rhamnosus* (UPCC 1473) isolated from Parmigiano Reggiano cheese, *Lacticaseibacillus paracasei* (UPCC 6227) isolated from Pecorino Toscano cheese, *Pediococcus pentosaceus* (UPCC 6268) isolated from tomato and *Bacillus subtilis* (UPCC 5002) isolated from rice, were used to carry out the fermentation process.

All the strains belonged to the University of Parma Culture Collection (UPCC). The bacterial cultures were maintained in frozen stocks at −80 °C in De Man Rogosa and

Sharpe (MRS) broth (Oxoid, Basingstoke, UK) (*L. rhamnosus*, *L. paracasei*, *P. pentosaceus*) or Nutrient broth (VWR, Radnor, Pennsylvania, USA) (*B. subtilis)* and added with 12.5% glycerol (v/v) until their use.

Okara Fermentation

Before fermentation process all the strains were revitalized twice in MRS broth (Oxoid, Basingstoke, UK) (*L. rhamnosus*, *L. paracasei*, *P. pentosaceus*) or Nutrient broth (VWR, Radnor, Pennsylvania, USA) (*B. subtilis)* and let grow overnight at the optimal growth temperature, i.e. 37°C for *L. rhamnosus* and *L. paracasei,* 30° C for *P. pentosaceus* and 25° C for *B. subtili*s. The cells were harvested by centrifugation (10000 g for 10 min at 4 °C) and resuspended in Ringer's solution (Oxoid, Milan, Italy) to obtain the microbial load of 9 Log CFU/mL.

Each strain was initially inoculated as monoculture into okara to reach a concentration of 3 or 7 Log CFU/g and incubated at 25 °C or 42 °C for 48 or 96 h, as defined in the Design of Experiment (DoE). After fermentation, the fermented okara samples were stored at -20 °C until they were analysed to evaluate the (poly)phenolic profile.

To investigate the synergic effect of several strains on the (poly)phenolic profile of okara, five co-cultures were prepared by mixing three strains at a time (*B. subtilis* 5002 + *L. rhamnosus* 1473 + *L. paracasei* 6227, *B. subtilis* 5002 + *L. rhamnosus* 1473 + *P. pentosaceus* 6268, *L. rhamnosus* 1473 + *L. paracasei* 6227 + *P. pentosaceus* 6268, *B. subtilis* 5002 + *L. paracasei* 6227 + *P. pentosaceus* 6268) in a ratio of 1:1:1 and all the four strains together at a time (*B. subtilis* 5002 + *L. rhamnosus* 1473 + *L. paracasei* 6227 + *P. pentosaceus* 6268) in a ratio of 1:1:1:1. The fermentations with the five co-cultures were

conducted by adding an inoculum of 3 Log CFU/g, at 25°C for 96 hours.

All experiments were performed in duplicate. The initial and final microbial concentrations were verified by plate counts of viable cells on the optimal agar medium for each strain and incubated for 48 hours at optimal temperature. The pH variation after fermentation, starting from okara, was determined using a pH meter (Mettler Toledo, Columbus, Ohio, US).

Design of Experiment

An experimental design (MODDE Pro 13.1 software, MKS Umetrics, Sweden) was set up to determine the factors influencing the growth of the strains in monoculture, as well as to determine the factors influencing the catabolism of the characteristic (poly)phenols of okara.

The model was developed around four factors, one qualitative (multilevel), the strains used as starters and three quantitative, among the factors affecting cell density is the concentration of the inoculum (ranging between 3 and 7 Log CFU/g), the incubation temperature (ranging between 25° and 42° C) and the incubation time (ranging between 48 and 96 hours). The responses measured were strain/s growth, expressed as delta (Δ) of growth for each fermentation condition and concentration of characteristic (poly)phenols of okara after fermentation. A D-optimal linear design was set up to evaluate the effect of each factor. The model with the highest G-efficiency (74.50) and the lowest condition number (2.97) was selected. A list of 11 experiments (Table 1) was generated and each experiment was replicated twice. The data were expressed as mean ± standard deviation and the raw data were analysed using MODDE 13.1 software.

Table 1. Fermentation conditions

Strains	Initial inoculum (Log CFU/g)	Fermentation temperature (°C)	Fermentation time (h)
L. rhamnosus 1473	7	25	48
L. rhamnosus 1473	3	42	48
L. rhamnosus 1473	3	25	96
L. rhamnosus 1473	7	42	96
P. pentosaceus 6268	7	42	48
P. pentosaceus 6268	3	25	96
L. paracasei 6227	3	25	48
L. paracasei 6227	7	25	96
L. paracasei 6227	3	42	96
B. subtilis 5002	7	42	48
B. subtilis 5002	3	25	96

Qualitative and Quantitative Analysis of Phenolic Compounds

For the extraction of phenolic compounds from okara, 300 mg of the sample were accurately weighed and added with 2 mL of a methanol, water, and formic acid solution (79.9/20/0.1; v/v/v). The mixture was vortexed for 1 minute, followed by sonication for 25 minutes while carefully controlling the temperature to prevent overheating. After sonication, the mixture was vortexed again for 30 seconds and then centrifuged at 4000 rpm for 15 minutes (Centrisart A-14C Refrigerated Micro-Centrifuge and Rotor YCSR-A1C, Sartorius Lab Instruments GmbH and Co. KG, Göttingen, Germany). The supernatant was collected, and 1 mL of the solvent mixture was added to the remaining pellet. The extraction procedure was repeated twice to ensure maximum extraction of phenolic compounds and supernatants pooled. The combined supernatant was finally centrifuged at 12000 rpm for 10 minutes, transferred to a vial, and stored at -18°C until further analysis. Samples were analyzed through UHPLC DIONEX Ultimate 3000 fitted

with a TSQ Vantage triple quadrupole mass spectrometer, equipped with a heated-electrospray ionization (H-ESI-II) source (Thermo Fisher Scientifc Inc., San Jose, CA, USA). Chromatographic and ionization parameters were set following the method previously described by Brindani et al., with some modifications (Brindani et al. 2017). Briefly, separations were performed with a Kinetex EVO C18 (100 × 2.1 mm), 2.6 µm particle size (Phenomenex, Torrance, CA, USA), installed with a pre-column (Phenomenex). For UHPLC, mobile phase A was 0.01% formic acid in water and mobile phase B was acetonitrile containing 0.01% formic acid. The gradient started with 5% B, keeping isocratic conditions for 0.5 min, reaching 95% B at 7 min, followed by 1 min at 95% B and then 4 min at the start conditions to re-equilibrate the column. The flow rate was set at 0.4 mL/min, the injection volume was 5 µL, and the column temperature was 40 °C. The MS worked in negative ionization mode with capillary temperature at 270 °C, while the source was at 300 °C. The sheath gas flow was 60 units, while auxiliary gas pressure was set to 10 units. The source voltage was 3 kV. Ultra-high-purity argon gas was used for collision-induced dissociation (CID).

Statistical analysis

All samples were extracted in triplicate and the LC-MS analyses were performed for each sample extraction. Quantitative data are reported as mean ± standard deviation (SD). An analysis of variance (ANOVA) and Duncan's multiple range tests were carried out to compare samples. Statistical analysis was carried out using the IBM SPSS Statistics 23.0 software package (IBM, Chicago, IL, USA). The level of statistical significance was set at $p < 0.05$.

Predictive model

Using the data obtained from the growth of microorganisms in monocultures and (poly)phenol biotransformation in monoculture fermented okara, predictive models will be employed to identify the key factors influencing fermentation and (poly)phenols in fermented okara.

The model's accuracy was evaluated using four statistical parameters: R^2, Q^2, model validity, and reproducibility. R^2 represents how well the model fits the data, with values below 0.5 indicating a poor fit. Q^2 pertains to predictive precision and reflects the quality of the prediction, where values above 0.5 signify a good model and values exceeding 0.9 indicate an excellent model. Model validity measures the overall appropriateness of the model, with values greater than 0.25 suggesting a useful model. Model validity measures the overall appropriateness of the model, with values greater than 0.25 suggesting a useful model. Outliers or issues with data transformation often cause low model validity, but a negative value can sometimes arise from extremely low replicate errors due to high reproducibility (John et al. 2020; Eriksson et al. 2013; Moldovan et al. 2016). Reproducibility reflects the degree to which replicate error is minimal compared to the overall variability in the design. A high reproducibility value (> 0.5) indicates results that are both biologically and technically consistent (Eriksson et al. 2013).

Results

Monoculture fermentation

To identify the optimal fermentation condition of okara, eleven experiments using monocultures were carried out as reported in Table 1. The bacterial growth and the pH reached after fermentation for each sample are listed in Table 2.

Table 2. Fermentation condition, microbial growth and pH values. The growth is expressed as Δ Log CFU/g.

Mean and standard deviation were evaluated on two biological replicates.

Fermentation parameters				**Fermentation process**			
LAB	**Fermentation temperature (°C)**	**Incubation time (h)**	**Inoculum (Log CFU/g)**	**T_0 (Log CFU/g)**	**T_{48}/T_{96} (Log CFU/g)**	**LAB growth (Δ Log CFU/g)**	**pH value after incubation**
L. rhamnosus 1473	25°C	48h	*7*	6.95 ± 0.02	9.34 ± 0.01	2.40 ± 0.01	4.68 ± 0.05
L. rhamnosus 1473	42°C	48h	*3*	2.67 ± 0.14	7.76 ± 0.40	5.08 ± 0.54	6.00 ± 0.15
L. rhamnosus 1473	25°C	96h	*3*	3.00 ± 0.00	9.08 ± 0.05	6.08 ± 0.05	4.64 ± 0.02
L. rhamnosus 1473	42°C	96h	*7*	7.26 ± 0.00	5.46 ± 0.65	-1.80 ± 0.65	4.97 ± 0.20
P. pentosaceus 6268	42°C	48h	*7*	7.52 ± 0.01	7.77 ± 0.22	0.26 ± 0.24	4.56 ± 0.04
P. pentosaceus 6268	25°C	96h	*3*	3.30 ± 0.17	9.01 ± 0.03	5.71 ± 0.15	4.62 ± 0.02
L. paracasei 6227	25°C	48h	*3*	3.62 ± 0.13	7.58 ± 0.17	3.96 ± 0.30	5.82 ± 0.13
L. paracasei 6227	25°C	96h	*7*	7.45 ± 0.03	8.81 ± 0.02	1.36 ± 0.05	4.39 ± 0.01
L. paracasei 6227	42°C	96h	*3*	3.62 ± 0.09	1.99 ± 0.00	-1.63 ± 0.09	6.28 ± 0.01
B. subtilis 5002	42°C	48h	*7*	6.85 ± 0.14	6.22 ± 0.06	-0.63 ± 0.08	5.84 ± 0.00
B. subtilis 5002	25°C	96h	*3*	2.38 ± 0.07	6.69 ± 0.21	4.30 ± 0.13	5.93 ± 0.02

Various factors were evaluated to determine their impact on okara's fermentation process. However, not all of these factors significantly affect microbial growth. Indeed, the factors influencing the growth were the ones whose error bars did not cross the zero line (Figure 1). The increase in cell density (expressed as Δ Log CFU/mL) is influenced by the initial cell quantity inoculated in the raw okara, the fermentation temperature, and the microorganisms employed in the fermentation process (Figure 1), but not by the incubation time. Notably, a higher inoculum and higher fermentation temperature led to a lower Δ Log. In fact, all samples incubated at 25°C showed an increase in microbial load, with the Δ Log between 1.36 and 6.08 Log CFU/g. On the other hand, all the samples incubated at 42°C showed a microbial load with a negative or nearly to 0 Δ Log CFU/g, except in the case of *L. rhamnosus* 1473 (initial inoculum 3 Log CFU/g) incubated at 42°C for 48 hours (Δ Log CFU/g equal to 5.08 ± 0.54) (Table 2). The different strains exhibit varying growth capacities in okara, with *L. rhamnosus* demonstrating the highest adaptability to this matrix.

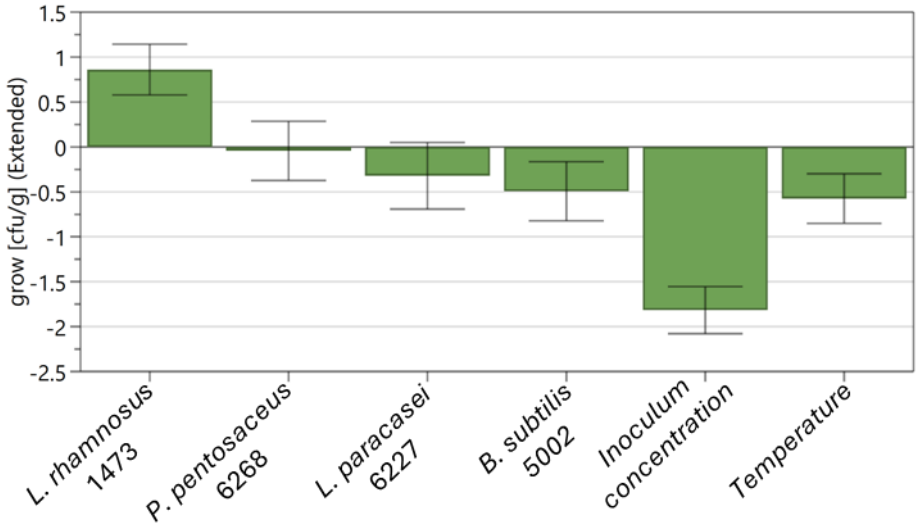


Figure 1. Factors significantly affecting the growth of microorganisms in okara

The goodness of the model is supported by high R^2 (0.98), Q^2 (0.93), model validity (0.74), and reproducibility (0.97) (Figure 2).

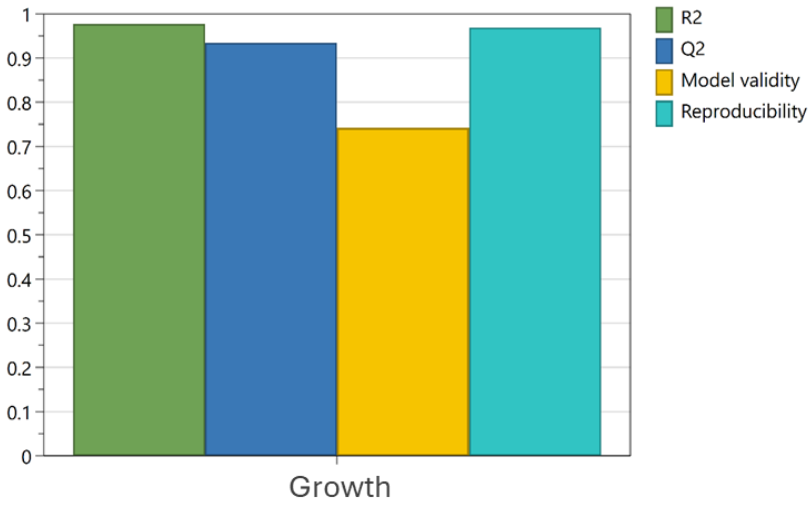


Figure 2. Summary of Fit growth

This model can reliably predict okara fermentability within the studied range of conditions and strains employed (Figure 3).

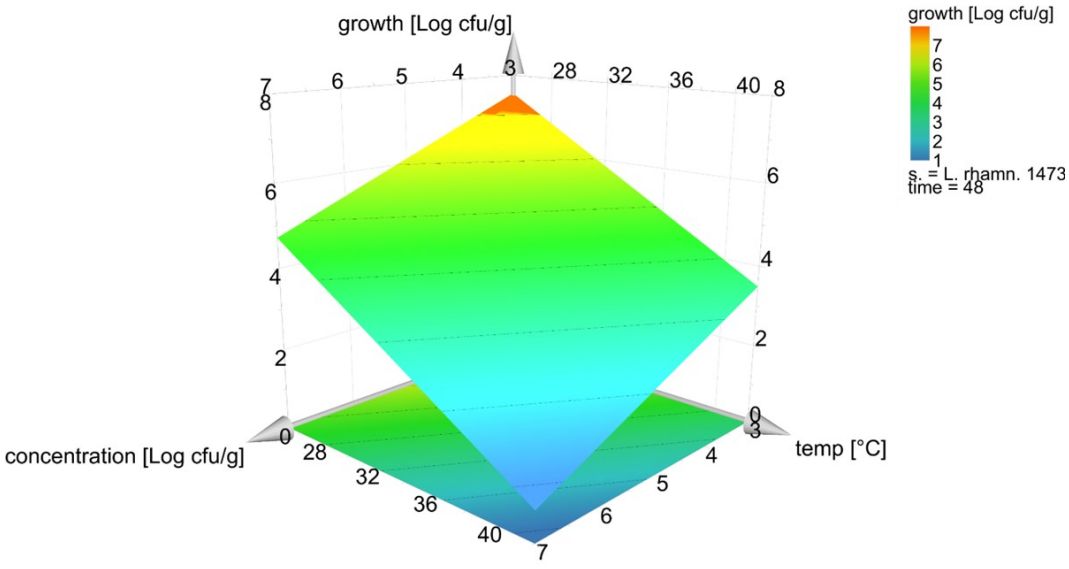


Figure 3. Growth surface of L. rhamnosus

The analysis of the response surface (Figure 3), predicted the more appropriate condition for okara fermentation. In summary, according to the model prediction, the matrix supports the growth of all the strains evaluated but the best condition, that better support the growth, was reached with the lowest initial inoculum (3 Log CFU/g) and the lowest temperature of incubation (25°C).

Phenolic characterization of okara material

As the bacteria were selected on the basis of enzyme expression similarities reported for bacteria capable of isoflavone degradation, a total of 37 *m/z* were monitored, (Table S1 of the Supplementary Material) representing a total of 63 possible metabolites. The analysis of the fermented and non-fermented okara material allowed the identification of 32 (poly)phenol compounds (Table 3), mainly belonging to isoflavones and, to a lesser extent, to phenolic acids.

Table 3. Chromatographic and mass spectrometry characteristics of (poly)phenol compounds detected in okara samples.

Compound	Retention time (min)	Parent ion $[M-H]^-$ (*m/z*)	Quantifier Product ion (*m/z*)	Qualifier Product ion (*m/z*)
3-(4'-Hydroxyphenyl)lactic acid	1.84	181	119	137, 121, 113
4-Hydroxybenzoic acid	2.35	137	93	92
4-Hydroxybenzaldehyde	3.30	121	92	91
4-Hydroxy-3-methoxybenzoic acid (Vanillic acid)	3.45	167	152	108, 123
Equol methyl	4.27	255	240	
3-(Phenyl)lactic acid	4.38	165	119	121, 150
Genistein-hexoside (Genistin) isomer I	4.44	431	269	133, 159, 135
4'-Hydroxycinnamic acid (p-Coumaric acid)	4.48	163	119	93
Daidzein-hexoside (Daidzin) isomer I	4.50	415	253	223, 91

Glycitein-hexoside (Glycitin)	4.58	445	283	268
3'-Hydroxycinnamic acid (m-Coumaric acid)	4.60	163	119	93
2'Hydroxygenistein-7-O-glucoside	4.62	447	285	133, 151
Hydroxy-methoxycinnamic acid ((Iso)Ferulic acid)	4.72	193	134	
Daidzein-hexoside (Daidzin) isomer II	4.73	415	253	223, 91
Genistein-hexoside (Genistin) isomer II	4.86	431	269	133, 159, 135
Genistein-acetylhexoside isomer I	4.88	473	269	133, 159, 135
Daidzein-acetylhexoside isomer I	4.97	457	253	223, 91
Genistein-acetylhexoside isomer II	5.02	473	269	133, 159, 135
Daidzein-acetylhexoside isomer II	5.11	457	253	223, 91
Glycitein-acetylhexoside isomer I	5.15	487	283	268, 113
Daidzein-acetylhexoside isomer III	5.22	457	253	223, 91
Genistein-acetylhexoside isomer III	5.31	473	269	133, 159, 135
Genistein-acetylhexoside isomer IV	5.42	473	269	133, 159, 135
Daidzein	5.44	253	223	91
Glycitein-acetylhexoside isomer II	5.47	487	283	268, 113
Glycitein	5.52	283	268	240
Dihydrogenistein	5.76	271	165	137
Naringenin	5.85	271	165	137
Genistein	5.90	269	133	159, 135

Compounds in bold were identified and quantified using authentic standards. Genistein derivates were quantified using genistein-glucuronide. Daidzein derivates were quantified using daidzdein-glucuronide. Glycitein derivates were quantified using glycitein. Equol methyl was quantified with equol-glucuronide. 4-Hydroxybenzoic acid. 4-Hydroxybenzaldehyde. 3'-Hydroxycinnamic acid (m-Coumaric acid).

The most representative compounds were daidzein (52-64% of the total concentration), glycitein (11-24% of the total concentration) and genistein (9-12% of the total concentration) independently of the bacteria and the fermentation conditions, in terms of concentration.

Phenolic profile comparison of non-fermented and monoculture fermented okara

The phenolic profile of okara fermented under different conditions with various monocultures showed significant variations in the presence and concentration of specific phenolic metabolites, particularly isoflavones such as genistein, daidzein, and glycitein derivatives (Table S2 of the Supplementary Material).

The results show a consistent trend across the different fermentation treatments, where the fermentation process leads to the degradation of isoflavone glycosides and an increase in aglycone concentrations compared to the control. This pattern is observed across most microbial cultures, except for *B. subtilis*, which not only degrades the glycosylated forms but also leads to a marked reduction in the aglycones (Table 4).

Table 4. Concentration of single isoflavone's derivatives and their aglycones after fermentations.

Mean and standard deviation were evaluated on two biological replicates.

Culture	Sum of Derivates (mg/100g)			Aglycones (mg/100g)		
	Genistein	**Daidzein**	**Glycitein**	**Genistein**	**Daidzein**	**Glycitein**
Control	11.02 ± 1.64^{a}	1.42 ± 0.30^{a}	1.68 ± 0.57^{a}	9.17 ± 1.80^{e}	56.99 ± 10.46^{c}	24.07 ± 6.22^{b}
42°C/48h - *P. pentosaceus*	9.34 ± 1.76^{b}	1.20 ± 0.28^{ab}	1.57 ± 0.39^{a}	10.85 ± 1.90^{cde}	67.11 ± 11.61^{bc}	28.33 ± 6.49^{ab}
25°C/96h - *P. pentosaceus*	3.90 ± 1.08^{d}	0.47 ± 0.14	0.97 ± 0.37^{b}	15.03 ± 2.87^{a}	88.14 ± 20.75^{a}	34.42 ± 10.58^{a}
25°C/48h - *P. paracasei*	9.06 ± 1.16^{b}	1.12 ± 0.20^{bc}	1.44 ± 0.24^{a}	10.24 ± 1.31^{de}	64.70 ± 8.22^{bc}	29.15 ± 5.25^{ab}
25°C/96h - *P. paracasei*	6.98 ± 1.70^{c}	0.96 ± 0.26^{cd}	1.51 ± 0.40^{a}	14.05 ± 3.11^{ab}	76.91 ± 15.72^{ab}	35.28 ± 11.02^{a}
42°C/96h - *P. paracasei*	10.27 ± 1.55^{ab}	1.35 ± 0.24^{ab}	1.66 ± 0.44^{a}	11.78 ± 1.50^{bcd}	70.99 ± 11.36^{bc}	32.69 ± 5.91^{ab}
42°C/48h - *B. subtilis*	6.09 ± 0.61^{c}	0.55 ± 0.08^{ef}	0.80 ± 0.19^{bc}	5.78 ± 0.62^{f}	29.43 ± 4.12^{d}	5.18 ± 0.60^{c}
25°C/96h - *B. subtilis*	4.45 ± 0.46^{d}	0.43 ± 0.10^{f}	0.64 ± 0.24^{bc}	6.50 ± 1.00^{f}	35.57 ± 4.20^{d}	7.50 ± 1.66^{c}
25°C/48h - *L. rhamnosus*	3.65 ± 1.41^{d}	0.34 ± 0.10^{f}	0.45 ± 0.13^{c}	11.57 ± 1.24^{cde}	62.70 ± 5.69^{bc}	27.29 ± 4.20^{ab}
42°C/48h - *L. rhamnosus*	6.80 ± 1.39^{c}	0.80 ± 0.20^{d}	1.00 ± 0.31^{a}	11.96 ± 1.01^{bcd}	67.60 ± 4.00^{bc}	27.91 ± 2.64^{ab}
25°C/96h - *L. rhamnosus*	4.47 ± 0.50^{d}	0.47 ± 0.05^{f}	0.78 ± 0.14^{bc}	13.20 ± 0.60^{abc}	77.10 ± 4.12^{ab}	32.41 ± 1.87^{ab}
42°C/96h - *L. rhamnosus*	6.35 ± 1.66^{c}	0.73 ± 0.13^{de}	0.91 ± 0.23^{b}	11.00 ± 1.27^{cde}	64.92 ± 9.27^{bc}	24.84 ± 5.00^{b}

The most efficient microorganisms in converting glycosides into aglycones were *P. pentosaceus* and *L. paracasei*. Notably, the fermentations at 25°C for 96 hours with these strains showed the greatest reduction in glycosylated forms of genistein, daidzein, and glycitein, accompanied by a significant increase in the respective aglycones.

Specifically, *P. pentosaceus* achieved a total glycoside concentration of 5.34 mg/100g, representing a 62.17% decrease compared to the control, while the total aglycone concentration reached 137.59 mg/100g, corresponding to a 52.48% increase. Similarly, *L. rhamnosus* under the same conditions exhibited a significant conversion, yielding 122.71 mg/100g of aglycones, which is a 50.69% increase compared to the control. Additionally, *L. paracasei* at 25°C for 96 hours demonstrated effective glycoside degradation, resulting in an aglycone yield of 126.24 mg/100g, indicating a 46.64% increase relative to the control. In contrast, *B. subtilis* displayed an atypical degradation pattern, showing a reduction in both glycosylated derivatives and aglycones. Under the conditions of 25°C for 96 hours, glycosides decreased to 5.52 mg/100g, reflecting a 60.91% decrease, while aglycones were reduced to 49.57 mg/100g, marking a 45.07% decrease compared to the control.

Moreover, regarding the phenolic profiles of fermented okara, *P. pentosaceus* and *L. rhamnosus* enhanced the production of phenolic acids such as ferulic acid and *p*-hydroxybenzoic acid compared to the non-fermented control, demonstrating a higher efficacy in increasing phenolic acid content. In the case of *L. paracasei,* this strain also promoted the accumulation of phenyl lactic acid. However, although *B. subtilis* led to a significant reduction in most phenolic compounds, this microorganism produced a unique set of metabolites such as *p*-coumaric acid and hydroxybenzoic acid, which may

result from the degradation of isoflavones.

The total phenolic content (TPC) for each fermentation condition is illustrated in Figure 4. The sterilized control sample presented a baseline TPC of 106.55 ± 20.89 mg/100g.

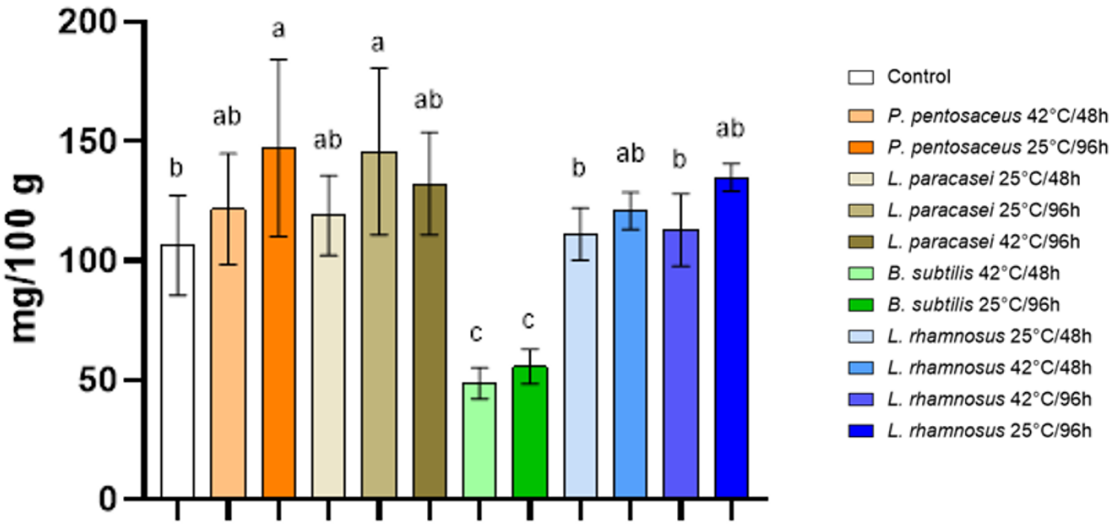


Figure 4. Comparison of total phenolic content from control and the different cultures and conditions. Mean and standard deviation were evaluated on two biological replicates.

P. pentosaceus also significantly enhanced TPC, particularly at 25ºC for 96 hours, where a 38% increase over the control was observed, being the highest among all tested conditions. When fermented at 42ºC for 48 hours, TPC increased by approximately 14%. Similarly, *L. paracasei* led to significant TPC enhancements, with the most substantial increase of around 37% occurring at 25ºC for 96 hours. This strain also caused a 24% rise at 42ºC for 96 hours, while a shorter fermentation at 25ºC for 48 hours yielded a smaller 12% increase.

In contrast, *B. subtilis* led to a significant decrease in TPC. For example, fermentation at 42ºC for 48 hours led to a 54% decrease, and at 25ºC for 96 hours, TPC dropped by 48%, indicating that this microorganism may either degrade phenolic compounds or inhibit

their synthesis during okara fermentation.

L. rhamnosus showed moderate but significant effects on TPC, with the most notable increase, around 27%, observed at 25ºC for 96 hours. Shorter fermentation periods and higher temperatures generally resulted in smaller increases, with the lowest, around 14%, occurring at 42ºC for 48 hours.

In the non-fermented okara, no 3-(phenyl)lactic and 3-(4'-hydroxyphenyl)lactic acids were detected. The highest production of these compounds was observed with *L. paracasei* at 25ºC for 96 hours, yielding 4.51% of the total phenolic compounds. Similarly, *L. rhamnosus* at 25ºC resulted in a 3% increase, on average for both times, and at 42ºC in 2% on average for both times. In contrast, *B. subtilis* showed minimal production of these acids across all conditions.

These findings demonstrate that both the microorganism used, and the specific fermentation conditions significantly influence the TPC of fermented okara. Specifically, *P. pentosaceus* and *L. paracasei* at 25ºC for extended periods showed the greatest potential for enhancing the phenolic content, while *Bacillus subtilis* was associated with a marked reduction in these bioactive compounds.

Influence of fermentation parameters on (poly)phenol biotransformation

Various factors were evaluated to study the overall influence of the fermentation parameters on the (poly)phenol biotransformation in monocultures fermented okara. The model's accuracy was evaluated using four statistical parameters: R2, Q2, model validity, and reproducibility and the results are shown in Supplementary material, Figure S1.

However, not all of these factors significantly affect fermented okara's (poly)phenol profile. Significant factors in the model can be identified from the coefficient plot, where error bars that do not cross zero correspond to a p-value of less than 0.05 (Eriksson et al. 2013).

The production of phenyllactic acids (3-(4'-hydroxyphenyl)lactic and 3-(phenyl)lactic acids) was particularly observed after lactic acid fermentation. All the variables studied influenced 3-(4'-hydroxyphenyl)lactic acid production, whereas only incubation temperature and the microorganisms affected 3-(phenyl)lactic acid production. Lower fermentation temperatures resulted in higher 3-(phenyl)lactic acid production, due to microorganism metabolism while a higher inoculum and longer fermentation time enhanced 3-(4'-hydroxyphenyl)lactic acid production. Among the strains, *L. rhamnosus* displayed the greatest capacity to produce these compounds (Figure 5 A and B).

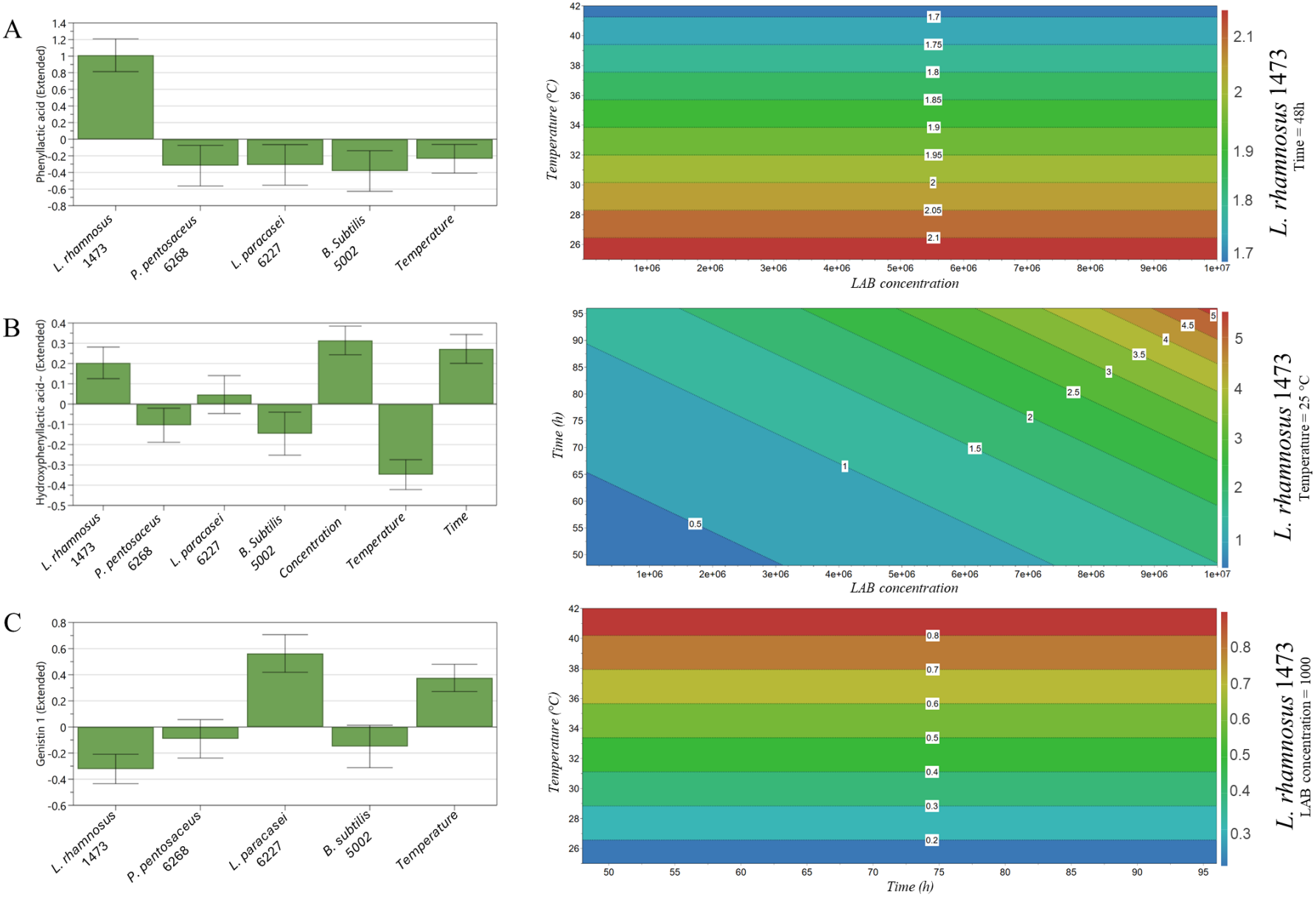
The concentrations of genistin and daidzin were influenced by the microorganisms used, with *L. rhamnosus* leading to the highest conversions, likely due to β-glucosidase activity. Fermentation temperature also played a crucial role, with lower temperatures leading to the decrease of genistin and daidzin levels in fermented okara (Figure 5 C, D, K and H). In contrast, depending on the specific isomer studied, daidzein-acetylhexoside and genistein-acetylhexoside concentrations were impacted by the starter microorganisms, initial inoculum load, fermentation temperature, and fermentation time (Figure 5 E, F, G, I and L). Biotransformation of genistin and daidzin leads to the release of their corresponding aglycones (genistein and daidzein). Their concentrations in fermented okara are influenced by the microorganisms employed in the fermentation and the incubation time. Extended fermentation periods resulted in higher genistein and daidzein levels, likely due to the enzymatic activity of LAB strains (Figure 5 J and M).

Glycitin concentration was affected by the microorganisms employed, particularly *L. rhamnosus* and *B. subtilis*, which achieved the highest conversion rates. Fermentation temperature and time also contributed to glycitin reduction (Figure 5 N). Conversely, glycitein-acetylhexoside catabolism was influenced by the initial inoculum load. Glycitein, the aglycone form of glycitin, was present in the fermented matrix as a result of microbial metabolic activity. However, fermentation with *B. subtilis* yields the lowest glycitein concentration, suggesting further catabolism by this strain.

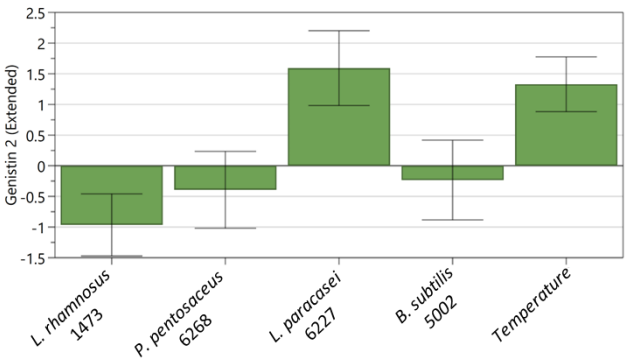
Similarly, the concentration of 4-hydroxybenzoic acid in fermented okara was affected by the strains employed and the fermentation time (Figure 5 O).

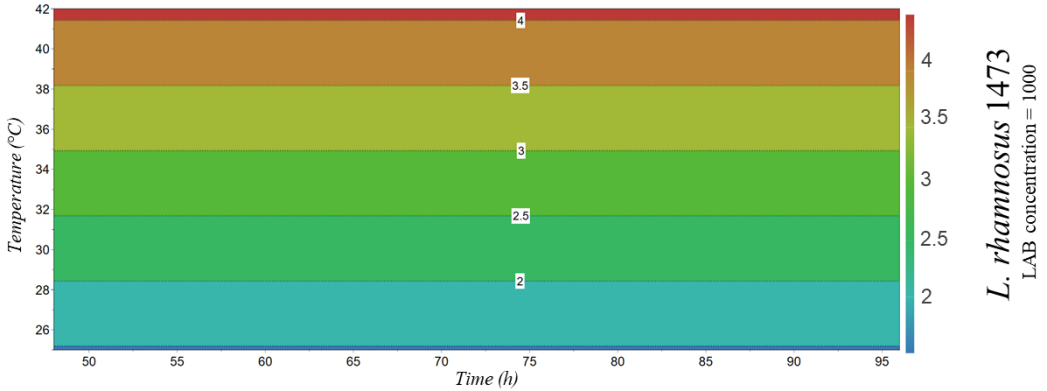
Also, the total (poly)phenolic content was influenced by the strains employed and the fermentation time. In particular, the higher amount was related to the fermentation with LAB strains while the lower to the presence of *B. subtilis*. Instead, longer fermentation periods correspond to a higher total (poly)phenolic content (Figure 5 P).

Overall various factors involved in the fermentation process have an impact on the (poly)phenols biotransformation. However, not all (poly)phenolic compounds are affected by the same fermentation conditions, indicating that by adjusting the fermentation parameters different amounts of specific (poly)phenols in the fermented okara could be obtained.

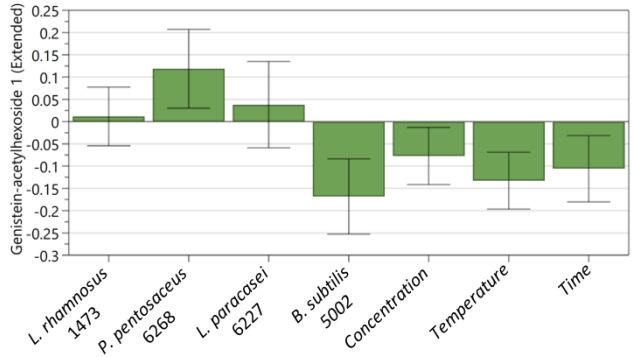

A
Phenyllactic acid (Extended)
L. rhamnosus 1473
P. pentosaceus 6268
L. paracasei 6227
B. Subtilis 5002
Temperature
Temperature (°C)
LAB concentration
L. rhamnosus 1473
Time = 48h
B
Hydroxyphenyllactic acid~ (Extended)
L. rhamnosus 1473
P. pentosaceus 6268
L. paracasei 6227
B. Subtilis 5002
Concentration
Temperature
Time
Time (h)
LAB concentration
L. rhamnosus 1473
Temperature = 25 °C
C
Genistin 1 (Extended)
L. rhamnosus 1473
P. pentosaceus 6268
L. paracasei 6227
B. subtilis 5002
Temperature
Temperature (°C)
Time (h)
L. rhamnosus 1473
LAB concentration = 1000

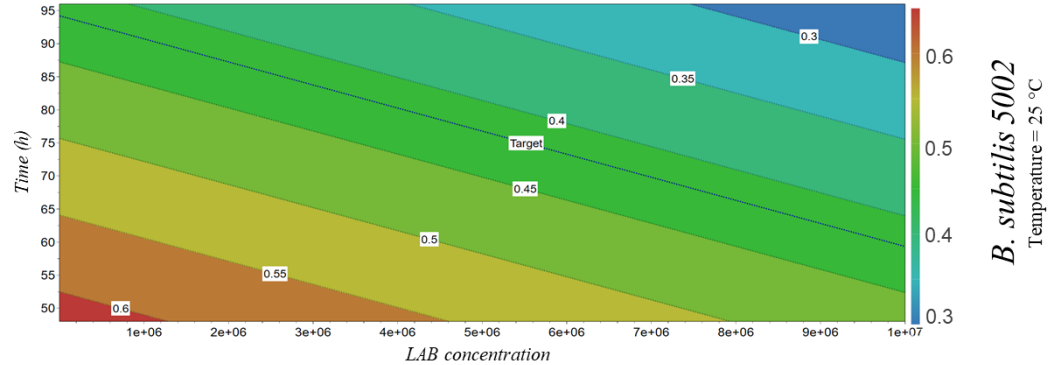
D


Genistin 2 (Extended)
2.5
2
1.5
1
0.5
0
-0.5
-1
-1.5
L. rhamnosus 1473
P. pentosaceus 6268
L. paracasei 6227
B. subtilis 5002
Temperature

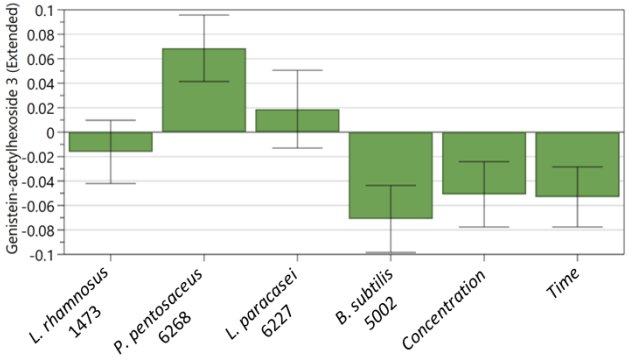

Temperature (°C)
42
40
38
36
34
32
30
28
26
4
3.5
3
2.5
2
50
55
60
65
70
75
80
85
90
95
Time (h)
4
3.5
3
2.5
2
L. rhamnosus 1473
LAB concentration = 1000

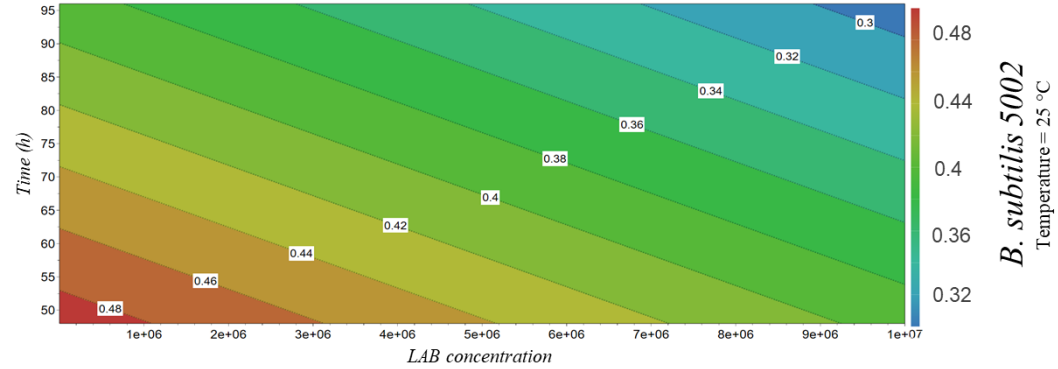
E


Genistein-acetylhexoside 1 (Extended)
0.25
0.2
0.15
0.1
0.05
0
-0.05
-0.1
-0.15
-0.2
-0.25
-0.3
L. rhamnosus 1473
P. pentosaceus 6268
L. paracasei 6227
B. subtilis 5002
Concentration
Temperature
Time

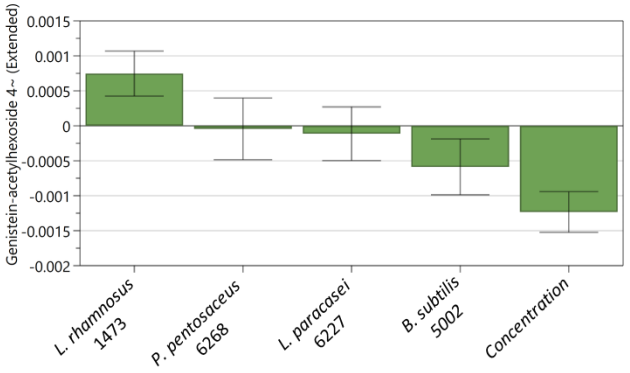

Time (h)
95
90
85
80
75
70
65
60
55
50
0.3
0.35
0.4
Target
0.45
0.5
0.55
0.6
1e+06
2e+06
3e+06
4e+06
5e+06
6e+06
7e+06
8e+06
9e+06
1e+07
LAB concentration
0.6
0.5
0.4
0.3
B. subtilis 5002
Temperature = 25 °C

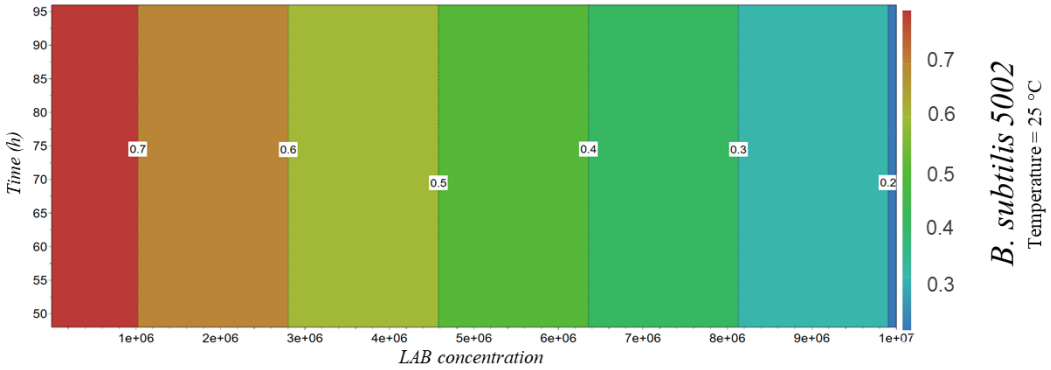
F


Genistein-acetylhexoside 3 (Extended)
0.1
0.08
0.06
0.04
0.02
0
-0.02
-0.04
-0.06
-0.08
-0.1
L. rhamnosus 1473
P. pentosaceus 6268
L. paracasei 6227
B. subtilis 5002
Concentration
Time

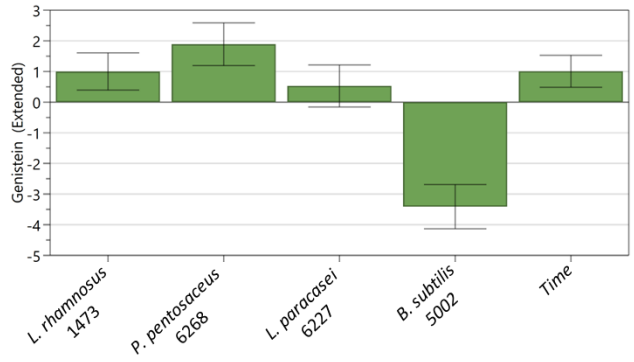

Time (h)
95
90
85
80
75
70
65
60
55
50
0.3
0.32
0.34
0.36
0.38
0.4
0.42
0.44
0.46
0.48
1e+06
2e+06
3e+06
4e+06
5e+06
6e+06
7e+06
8e+06
9e+06
1e+07
LAB concentration
0.48
0.44
0.4
0.36
0.32
B. subtilis 5002
Temperature = 25 °C

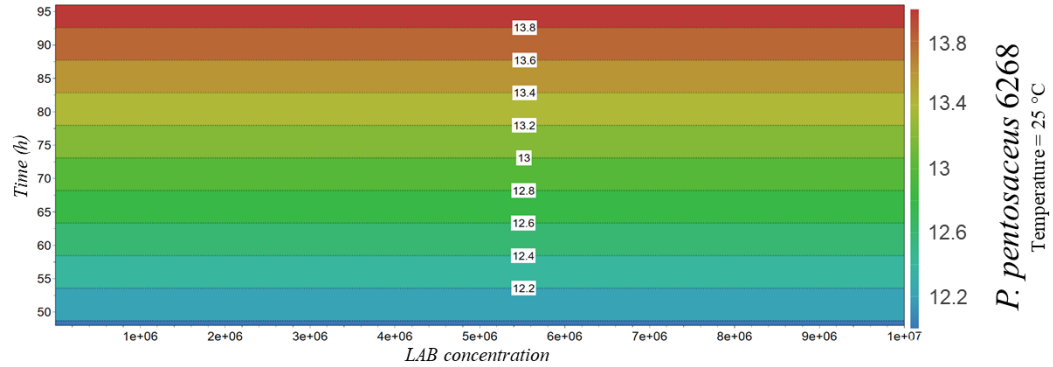
G


Genistein-acetylhexoside 4~ (Extended)
0.0015
0.001
0.0005
0
-0.0005
-0.001
-0.0015
-0.002
L. rhamnosus 1473
P. pentosaceus 6268
L. paracasei 6227
B. subtilis 5002
Concentration

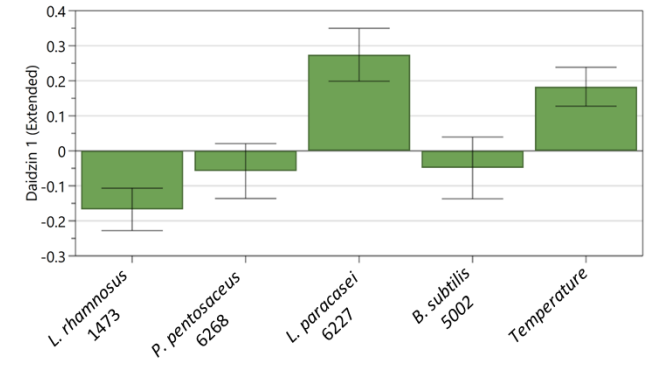

Time (h)
95
90
85
80
75
70
65
60
55
50
0.7
0.6
0.5
0.4
0.3
0.2
1e+06
2e+06
3e+06
4e+06
5e+06
6e+06
7e+06
8e+06
9e+06
1e+07
LAB concentration
0.7
0.6
0.5
0.4
0.3
B. subtilis 5002
Temperature = 25 °C

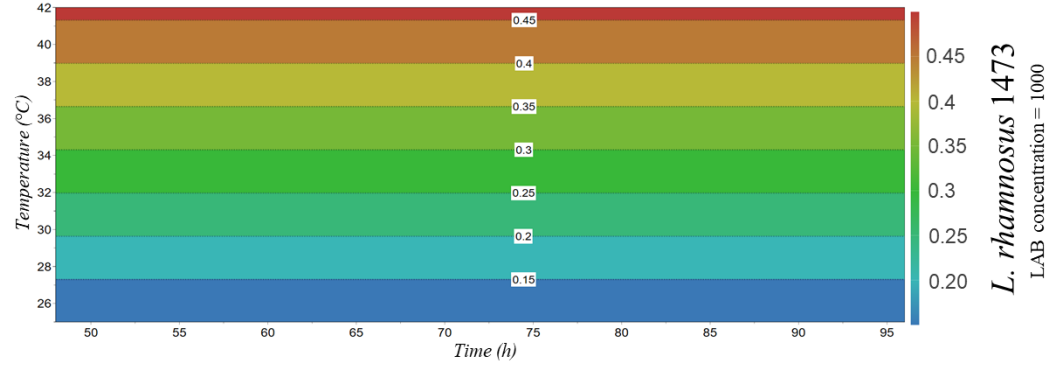
J


Genistein (Extended)
3
2
1
0
-1
-2
-3
-4
-5
L. rhamnosus 1473
P. pentosaceus 6268
L. paracasei 6227
B. subtilis 5002
Time

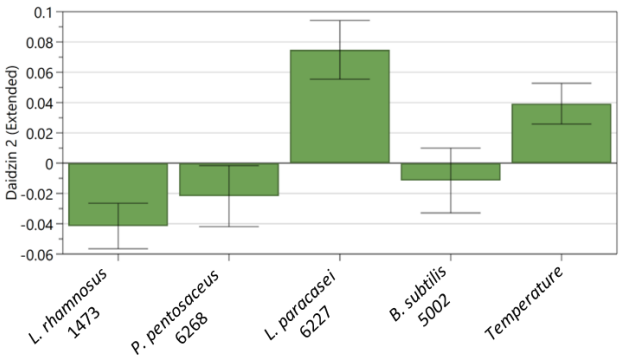

Time (h)
95
90
85
80
75
70
65
60
55
50
13.8
13.6
13.4
13.2
13
12.8
12.6
12.4
12.2
1e+06
2e+06
3e+06
4e+06
5e+06
6e+06
7e+06
8e+06
9e+06
1e+07
LAB concentration
13.8
13.4
13
12.6
12.2
P. pentosaceus 6268
Temperature = 25 °C

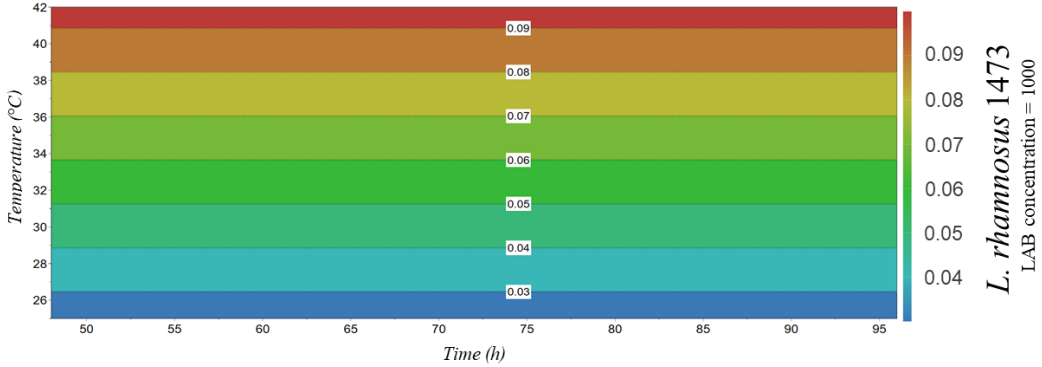
K


Daidzin 1 (Extended)
0.4
0.3
0.2
0.1
0
-0.1
-0.2
-0.3
L. rhamnosus 1473
P. pentosaceus 6268
L. paracasei 6227
B. subtilis 5002
Temperature

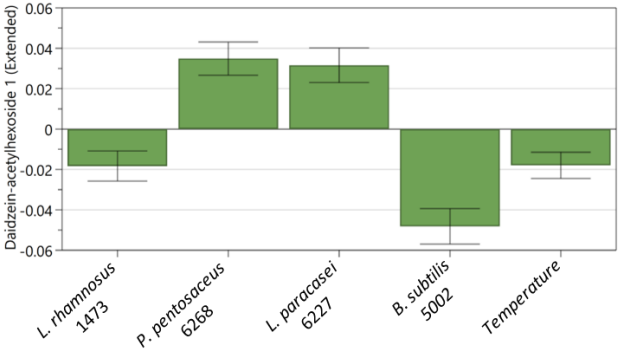

Temperature (°C)
42
40
38
36
34
32
30
28
26
0.45
0.4
0.35
0.3
0.25
0.2
0.15
50
55
60
65
70
75
80
85
90
95
Time (h)
0.45
0.4
0.35
0.3
0.25
0.20
L. rhamnosus 1473
LAB concentration = 1000

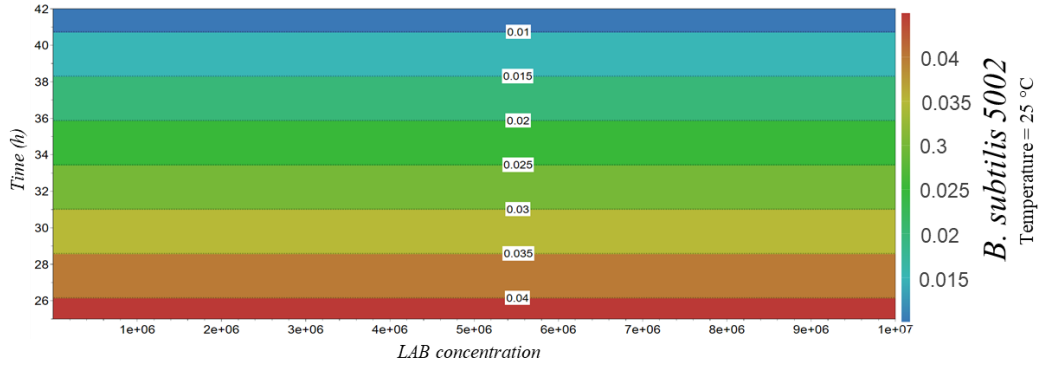
H


Daidzin 2 (Extended)
0.1
0.08
0.06
0.04
0.02
0
-0.02
-0.04
-0.06
L. rhamnosus 1473
P. pentosaceus 6268
L. paracasei 6227
B. subtilis 5002
Temperature

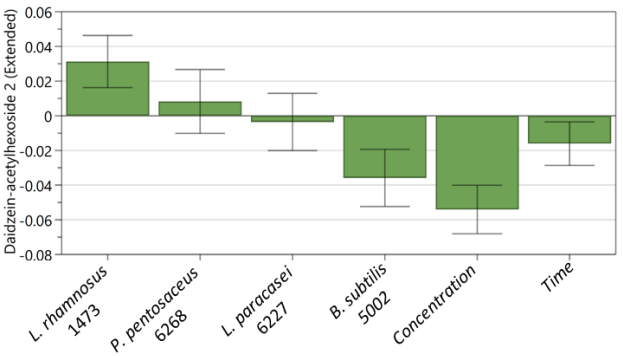

Temperature (°C)
42
40
38
36
34
32
30
28
26
50
55
60
65
70
75
80
85
90
95
Time (h)
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.09
0.08
0.07
0.06
0.05
0.04
L. rhamnosus 1473
LAB concentration = 1000

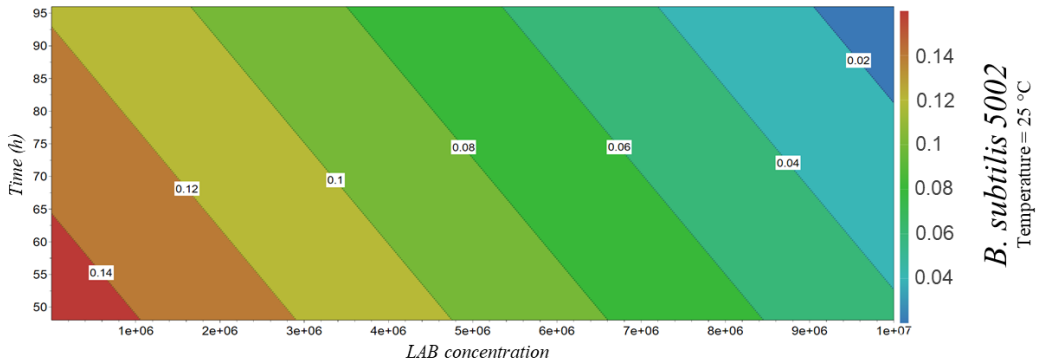
I


Daidzein-acetylhexoside 1 (Extended)
0.06
0.04
0.02
0
-0.02
-0.04
-0.06
L. rhamnosus 1473
P. pentosaceus 6268
L. paracasei 6227
B. subtilis 5002
Temperature

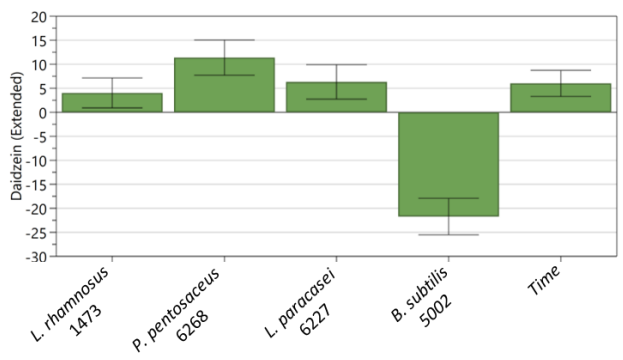

Time (h)
42
40
38
36
34
32
30
28
26
1e+06
2e+06
3e+06
4e+06
5e+06
6e+06
7e+06
8e+06
9e+06
1e+07
LAB concentration
0.01
0.015
0.02
0.025
0.03
0.035
0.04
0.04
0.035
0.3
0.025
0.02
0.015
B. subtilis 5002
Temperature = 25 °C

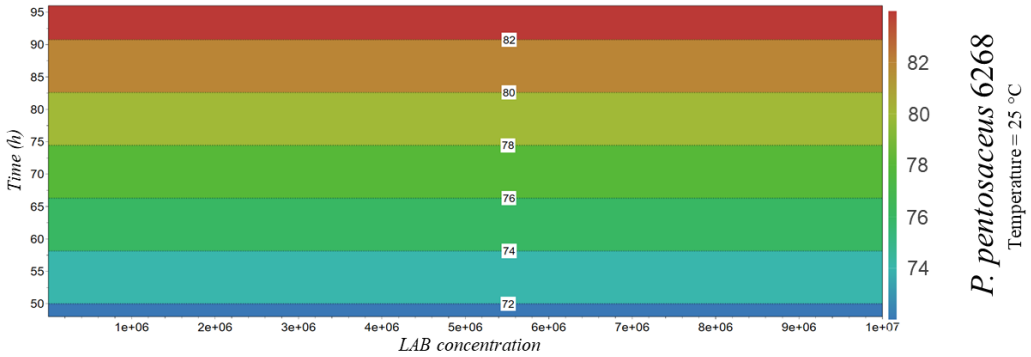
L


Daidzein-acetylhexoside 2 (Extended)
0.06
0.04
0.02
0
-0.02
-0.04
-0.06
-0.08
L. rhamnosus 1473
P. pentosaceus 6268
L. paracasei 6227
B. subtilis 5002
Concentration
Time


Time (h)
95
90
85
80
75
70
65
60
55
50
1e+06
2e+06
3e+06
4e+06
5e+06
6e+06
7e+06
8e+06
9e+06
1e+07
LAB concentration
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0.14
0.12
0.1
0.08
0.06
0.04
B. subtilis 5002
Temperature = 25 °C

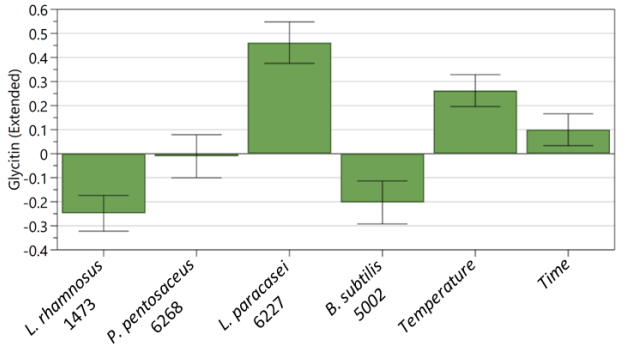
M

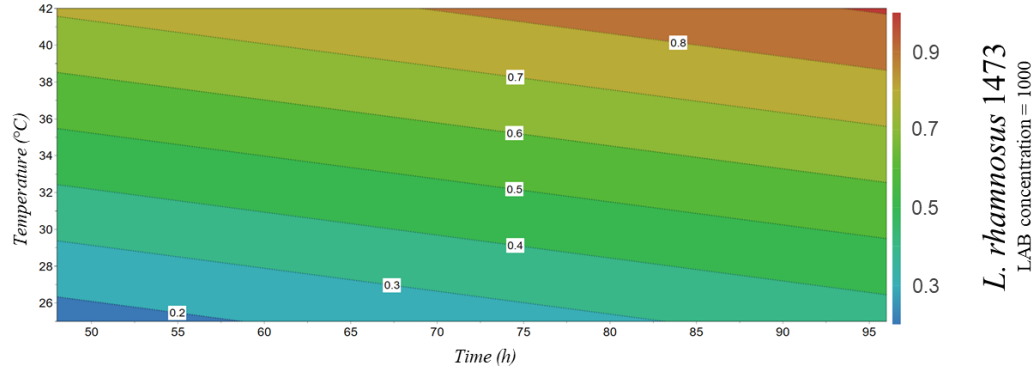




P. pentosaceus 6268
Temperature = 25 °C

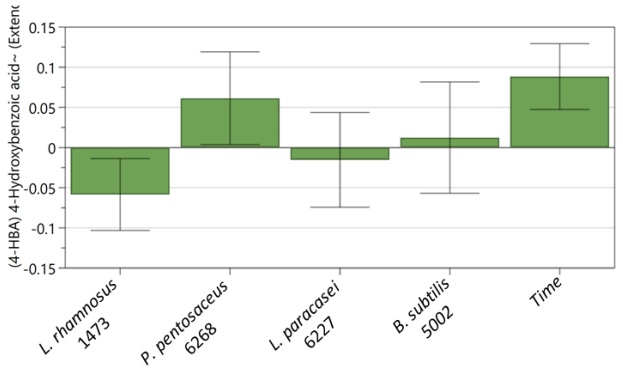
N

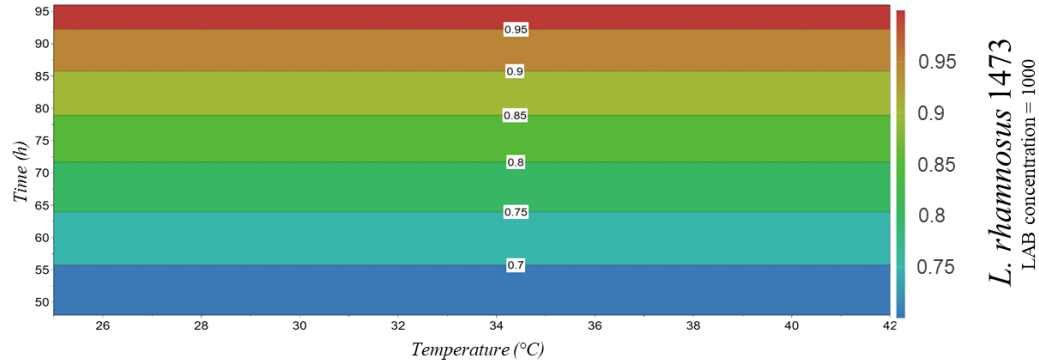




L. rhamnosus 1473
LAB concentration = 1000

O





L. rhamnosus 1473
LAB concentration = 1000

P

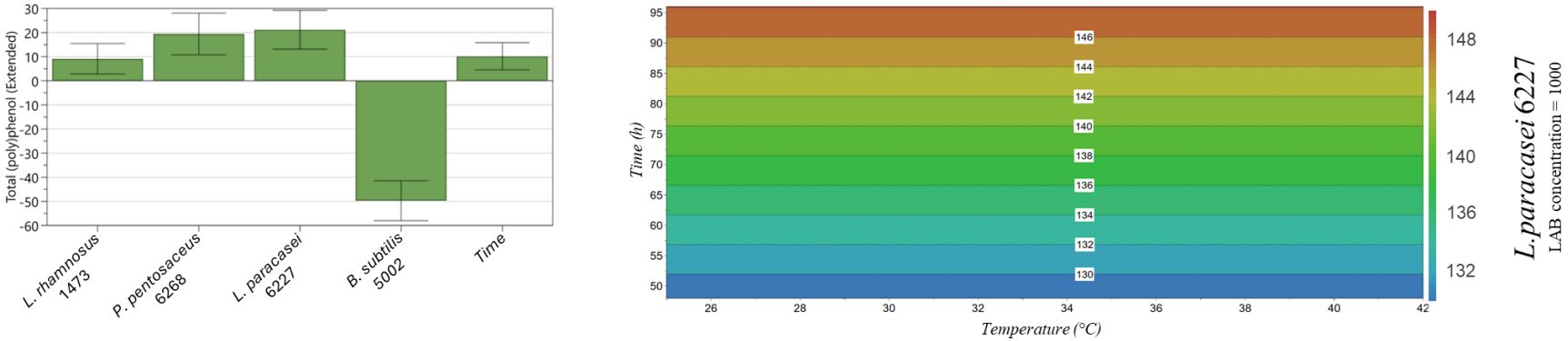


Figure 5. Coefficient plot and response surface related to the (poly)phenol biotransformation after okara fermentation. A) phenyllactic acid, B) Hydroxyphenyllactic acid, C) Genistin 1, D) Genistin 2, E) Genistein acetylhexoside 1, F) Genistein acetylhexoside 3, G) Genistein acetylhexoside 4, J) Genistein, K) Daidzin 1, H) Daidzin 2, I) Daidzein acetylhexoside 1, L) Daidzein acetylhexoside 2, M) Daidzein, N) Glycitin, O) 4-Hydroxybenzoic acid, P) Total (poly)phenol content.

Co-culture fermentation

Based on the results obtained from the DoE, to explore the potential synergic effect of strains growing together, it was decided to carry out fermentations with co-culture. Five experiments were performed: four of them were realised combining the bacterial strains three at a time while in last one all of the bacteria were co-cultured together. The fermentation conditions used were 3 Log CFU/g as the initial inoculum and 25°C as the incubation temperature, resulted significantly influencing the growth in monoculture, while the incubation time chosen was 96 hours.

As for the monocultures, the microbial growth was evaluated by microbial plate counts, on MRS agar for the LAB strains and on Nutrient agar for *B. subtilis*. Results are reported in Table 5.

All the bacteria combinations demonstrated to be able to ferment the matrix, increasing their concentration with a Δ Log of ~6 CFU/g for the lactic acid bacteria and Δ Log of ~3 CFU/g for *B. subtilis* 5002 in four out of five co-cultures (Table 5, co-cultures X2, X3, X4 and X5). The combination with *B. subtilis* 5002, *L. rhamnosus* 1473 and *L. paracasei* 6227 growing together (Table 5, co-culture X1), resulted instead in a different growth, with LAB strains reaching a lower Δ Log of ~4 Log CFU/g and *B. subtilis* 5002 reaching a higher Δ Log of ~7 Log CFU/g. This last condition was the only one in which *B. subtilis* 5002 and *P. pentosaceus* 6268 were not coexisting, suggesting *P. pentosaceus* 6268 as a possible *B. subtilis* 5002 competitor for matrix resources. In fact, the growth of *B. subtilis* 5002 is lower in all the conditions in which *P. pentosaceus* 6268 is present, specifically co-cultures X2, X3, X4 and X5 in Table 5. Moreover, in the absence of *P. pentosaceus* 6268 (Table 5, co-culture X1), *B. subtilis* 5002 reached a higher concentration and seemed to

interfere with the growth of *L. rhamnosus* 1473 and *L. paracasei* 6227, since they grew less only in this particular condition.

At the same time, the initial pH of okara, corresponding to 6.35 ± 0.06, became particularly low (4.85 ± 0.05) in samples where *P. pentosaceus* 6268 was present (Table 5, co-cultures X2, X3, X4 and X5), while it was higher (8.05 ± 0.21) in the fermentation with the highest *B. subtilis* 5002 growth (Table 5, co-culture X1).

Table 5. Microbial growth results of co-cultures. All the samples were incubated at 25°C for 96 hours and the growth is reported as Δ Log CFU/g. Mean and standard deviation were evaluated on two biological replicates.

		Fermentation process																					
		LAB									***B. subtilis***												
Sample	**Co-cultures**	**T_0 (Log CFU/g)**			**T_{48}/T_{96} (Log CFU/g)**			**LAB growth (Δ Log CFU/g)**			**T_0 (Log CFU/g)**			**T_{96} (Log CFU/g)**			***B. subtilis* growth (Δ Log CFU/g)**			**pH value after incubation**		
X1	*B. subtilis* 5002 + *L. rhamnosus* 1473 + *L. paracasei* 6227	3.02	±	0.08	7.29	±	0.24	4.27	±	0.16	2.15	±	0.12	9.28	±	0.21	7.13	±	0.33	8.05	±	0.21
X2	*B. subtilis* 5002 + *L. rhamnosus* 1473 + *P. pentosaceus* 6268	2.92	±	0.02	9.08	±	0.02	6.16	±	0.00	1.53	±	0.38	4.99	±	0.00	3.46	±	0.38	4.84	±	0.04
X3	*L. rhamnosus* 1473 + *L. paracasei* 6227 + *P. pentosaceus* 6268	2.89	±	0.07	9.23	±	0.27	6.34	±	0.34		/			/			/		4.91	±	0.00
X4	*B. subtilis* 5002 + *L. paracasei* 6227 + *P. pentosaceus* 6268	3.05	±	0.05	9.18	±	0.02	6.12	±	0.07	1.69	±	0.06	4.99	±	0.00	3.30	±	0.06	4.85	±	0.05
X5	*B. subtilis* 5002 + *L. rhamnosus* 1473 + *L. paracasei* 6227 + *P. pentosaceus* 6268	2.97	±	0.04	9.16	±	0.11	6.19	±	0.07	1.65	±	0.07	4.99	±	0.00	3.34	±	0.07	4.82	±	0.07

Phenolic profile comparison of non-fermented and co-culture fermented okara

Based on the results obtained with the mono-culture fermentation, co-cultures were evaluated at 25ºC for 96 h to assess their effect on phenolic compounds (Table S3 of the Supplementary Material). The co-culture fermentations showed a similar general trend to the mono-culture fermentations, with degradation of isoflavone glycosides and an increase in aglycones compared to the control (Table 6). However, the conversion efficiency and the final concentration of TPC varied between the co-cultures.

Table 6. Concentration of single isoflavone's derivatives and their aglycones after co-culture fermentation. Mean and standard deviation were evaluated on two biological replicates.

Culture	Sum of Derivates (mg/100g)			Aglycones (mg/100g)		
	Genistein	**Daidzein**	**Glycitein**	**Genistein**	**Daidzein**	**Glycitein**
Control	11.02 ± 1.64^{a}	1.42 ± 0.30^{a}	1.68 ± 0.57^{a}	9.17 ± 1.80^{b}	56.99 ± 10.46^{b}	24.07 ± 6.22^{a}
Co-culture X1	2.26 ± 0.45^{c}	0.57 ± 0.21^{b}	0.37 ± 0.04^{c}	0.35 ± 0.11^{c}	3.34 ± 0.52^{c}	0.32 ± 0.14^{b}
Co-culture X2	4.17 ± 1.02^{b}	0.45 ± 0.10^{b}	0.64 ± 0.18^{bc}	11.66 ± 0.81^{a}	67.12 ± 4.75^{a}	26.17 ± 2.71^{a}
Co-culture X3	3.99 ± 0.72^{b}	0.40 ± 0.09^{b}	0.71 ± 0.21^{b}	10.89 ± 0.45^{a}	65.58 ± 4.78^{a}	25.68 ± 2.33^{a}
Co-culture X4	4.58 ± 0.68^{b}	0.50 ± 0.06^{b}	0.86 ± 0.10^{b}	11.79 ± 0.19^{a}	68.32 ± 1.93^{a}	25.36 ± 1.14^{a}
Co-culture X5	3.96 ± 0.70^{b}	0.42 ± 0.06^{b}	0.63 ± 0.12^{bc}	11.66 ± 0.43^{a}	69.29 ± 1.65^{a}	27.19 ± 1.57^{a}

The TPC for each co-fermentation is illustrated in Figure 6.

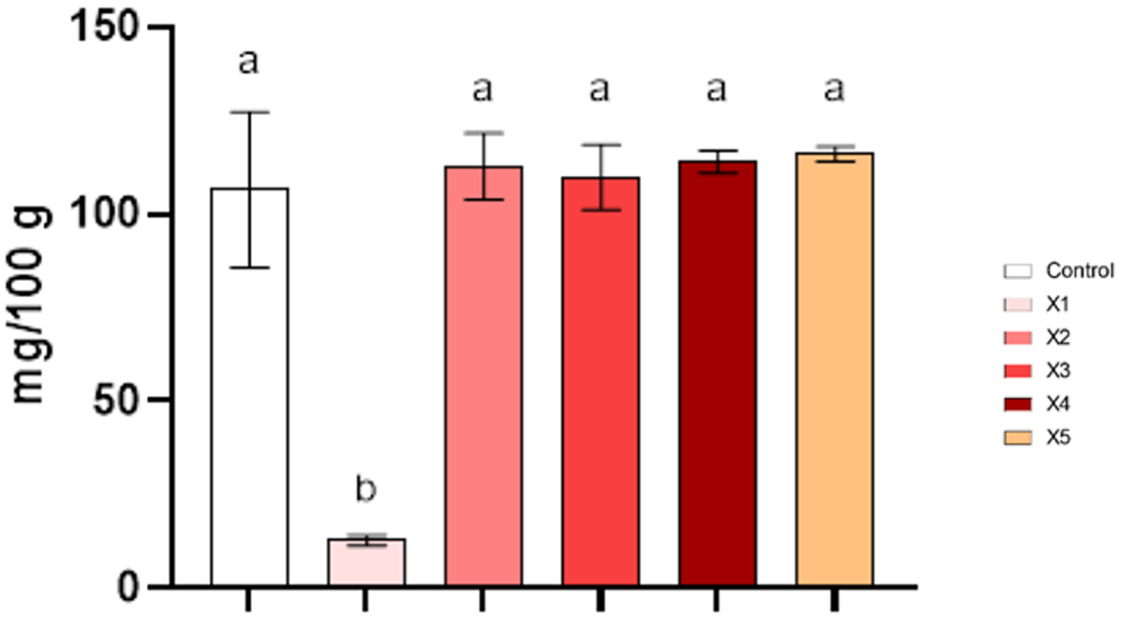


Figure 6. Comparison of total phenolic content from control and the different co-cultures at 25ºC for 96 hours fermentation. Mean and standard deviation were evaluated on two biological replicates.

The X1 co-culture of *B. subtilis*, *L. rhamnosus* and *L. paracasei* resulted in a dramatic reduction in TPC of approximately 88% compared to the control. This significant reduction suggests a strong negative interaction, probably due to the presence of *B. subtilis*. Conversely, the X2 co-culture containing *B. subtilis*, *L. rhamnosus* and *P. pentosaceus* maintained TPC close to the control level, with a slight increase of 6%. This suggests a balancing effect between the micro-organisms, counteracting the usual reduction in TPC associated with *B. subtilis*. Co-culture X3, composed of *L. rhamnosus*, *L. paracasei* and *P. pentosaceus*, resulted in a slight non-significant increase in TPC of about 3% compared to the control, suggesting a cooperative interaction between these lactic acid bacteria that helps to preserve phenolic compounds. The X4 co-culture, which included *B. subtilis*, *L. paracasei* and *P. pentosaceus*, showed a modest non-significant increase in TPC of around 7%. This result suggests that in this combination the detrimental effects of *B. subtilis* were mitigated by the other microorganisms. Finally, the X5 co-culture of *B. subtilis*, *L. rhamnosus*, *L. paracasei* and *P.*

pentosaceus confirmed the results obtained with the 3 strains-co-coltures, with a non-significant increase of 9% in TPC compared to the control. This suggests a potential synergistic effect that mitigated the negative impact of *B. subtilis,* maintaining the amount of phenolic compounds.

3-(4'-Hydroxyphenyl)lactic and 3-(phenyl)lactic acid were produced in all the tested co-cultures, reaching about 1% of TPC in all combinations, with the exception of co-culture X1, in which the percentage of these metabolites reached 33%, whereas most of percentage of the native compounds were degraded. Overall, these results highlight the significant influence that specific combinations of microorganisms can have on the TPC of fermented okara. While some co-cultures, particularly those with B. subtilis, resulted in drastic reductions in TPC, others helped to maintain or slightly increase phenolic content compared to the control.

Discussion

Currently, the search for a circular economy in the food industry has become a daily challenge. Several strategies have been applied over the years to transform food waste and by-products into value-added products. In fact, food waste and by-products are still rich in nutrients and bioactive compounds of human interest, such as (poly)phenols, carotenoids, and other phytochemicals, vitamins, aromatic compounds, organic acids, pectin, cellulose and other dietary fibres. The development of the processes applied in the recovery of these molecules are various including drying, SSF, ultrasound, solvent extraction, pressurised liquid extraction, supercritical CO_2 extractions, enzyme and microwave assisted extraction, composting and aerobic digestion (Sarker et al. 2024).

The present work aimed to evaluate the effect of mono- and co-culture fermentation on okara (poly)phenols and the possible ability of selected microorganisms to produce new (poly)phenol-derived metabolites generally detected after isoflavone consumption in humans (Rafii 2015). The ability of four microbial strains (*B. subtilis*, *L. rhamnosus*, *P. pentosaceus* and *L. paracasei*) to modify the (poly)phenolic profile of okara, a by-product derived from the soybean during the production of tofu and soy-based beverages, was analysed, first in mono-culture and then in co-culture fermentation. All the strains were able to ferment the matrix to different extents. Indeed, the variation in the growth observed between the microbial strains could be attributed to their specific adaptability in okara.

The best fermentation ability was achieved with *L. rhamnosus*, confirming previous results (Hadj Saadoun et al. 2021; Voss et al. 2018). In addition to fermentation capabilities, these previous studies reported the contribution of the metabolism of this species to the modification of the volatile profile of okara, highlighting a reduction in those molecules responsible for the unpleasant odour

associated with soy-based products, e.g. hexanal, as well as an increase in ketones, associated with sweet and creamy notes (Hadj Saadoun et al. 2021; Zhu, Wang, and Zhang 2019; Schindler et al. 2011; Wang and Cha 2018).

B. subtilis was able to ferment okara, achieving in the best conditions, 7 Log CFU/mL in mono-culture and about 9 Log CFU/mL in co-culture. The fermentation of okara by *B. subtilis* has previously been studied and increased antioxidant activity after fermentation has been demonstrated: the proteinases produced by *B. subtilis* are indeed suitable for the hydrolysis of soybean proteins and can give rise to several molecules with antioxidant activity, such as ϒ-polyglutamic acid and bioactive peptides (Zhu et al. 2008; Oh et al. 2007). However, in the present work *B. subtilis* caused a significant reduction of TPC by up to 54%, indicating its unsuitability for maximizing phenolic content, although further investigations should be undertaken to elucidate the reasons for this reduction and to evaluate whether *B. subtilis* would be able to utilize (poly)phenols as a growth substrate. Recently, some authors reported the ability of *B. subtilis* to phosphorylate specific isoflavones such as genistein and daidzein through a novel phosphotransferase, flavonoid phosphate synthetase, which is involved in ATP-dependent phosphorylation in *B. subtilis* (Wang et al. 2024; Ray et al. 2024). These authors also reported that genistein phosphorylation serves as a mechanism to limit its growth inhibition and increase its water solubility.

L. paracasei was also able to grow on okara, although to a lesser extent than the other microbial strains. Okara fermentation with this LAB was previously associated with a reduction in the amount of soluble and insoluble dietary fibre, an increase in the concentration of amino acids due to microbial degradation of proteins and peptides, an increase in ferulic acid and a change in the volatile profile, with fermented okara being rich in acetic acid, giving a vinegar-like odour (Vong and Liu 2019). In addition, the production of β-glucosidase by *L. paracasei* during fermentation gives this

strain the capability to reduce some isoflavone glycosides, namely daidzin, genistin and glycitin, to their aglycone forms, namely daidzein, genistein and glycitein, respectively (Ali et al. 2005; Donkor and Shah 2008; Vong and Liu 2019).

Okara fermentation with *P. pentosaceus* has never been investigated before, but this strain has been used to ferment soybean germs, resulting in an increase in dihydrogenistein, probably from the transformation of genistein, an equol precursor (Kwon et al. 2018; Lee et al. 2018).

Apart from the results obtained from the monocultures, a better understanding of the cooperation between different bacterial strains in the fermentation of the matrix was obtained from the co-culture experiments. The observed growth suggested an antagonistic role of *P. pentosaceus* against *B. subtilis* for the matrix resources while *B. subtilis* seemed to interfere with the growth of *L. rhamnosus* and *L. paracasei*. Despite the competition, almost all the combinations of microbial strains used demonstrated to maintain their fermentation ability when employed in co-culture. The same synergistic effect was observed by Hadj Saadoun et al. (2021), who suggested that the fusion of metabolic pathways of different bacteria was more effective in modifying the matrix (Hadj Saadoun et al. 2021).

Interestingly, in the present work co-cultures with *B. subtilis* generally moderated the extreme TPC reductions observed in its monocultures. Co-culturing *B. subtilis* with *L. rhamnosus*, *L. paracasei*, and *P. pentosaceus*, resulted in a modest non-significant increase in TPC of 9%, a marked improvement compared to the substantial reduction observed in *B. subtilis* monocultures. This suggests that the presence of multiple microorganisms may buffer the negative effects of *B. subtilis* on phenolic compounds. On the contrary, co-culture with *B. subtilis*, *L. rhamnosus*, and *L. paracasei* resulted in an 88% decrease in TPC, worse than any individual fermentation. This highlights the potential for antagonistic interactions in certain microorganism combinations, where the presence of *B. subtilis*

dominated, leading to severe phenolic degradation. Therefore, the exclusion or careful balancing of *B. subtilis* may provide a valuable approach to optimising the phenolic content of fermented okara.

The LAB fermentation of soy products and by-products, such as okara, has garnered substantial interest due to its impact on phenolic compound profiles, particularly through bioconversion processes that could enhance the bioavailability of bioactive compounds. This transformation is largely attributed to the β-glucosidase activity exhibited by various strains of LAB, which catalyze the hydrolysis of glycosidic bonds in glycosylated isoflavones, releasing their aglyconic forms. Since the aglycones would be more bioavailable, the fermentation process not only modifies the isoflavone profile but also potentially increases the nutritional and functional properties of the products (Moraes Filho, Busanello, and Garcia 2016; Vong, Hua, and Liu 2018; Hadj Saadoun et al. 2021; Queiroz Santos et al. 2018).

In addition to isoflavone bioconversion, LAB fermentation has been shown to increase the TPC in soy and okara products, especially under SSF conditions (Queiroz Santos et al. 2018; Shi et al. 2020; Rui et al. 2017). This increase in TPC is thought to be due to microbial release of bound phenolics or the synthesis of novel phenolic compounds during fermentation, associated with LAB enzymatic activity. In this work, *P. pentosaceus* and *L. paracasei* have been shown to possess robust glucosidase activity, facilitating the release of phenolic compounds from their glycoside precursors and leading to a significant increase in TPC of 38% and 37%, respectively, establishing them as the most effective strains for enhancing phenolic content when utilized individually. This increase in phenolic content enhances the possible bioactive properties of fermented products, suggesting that such processes may be strategically employed to improve the nutritional and functional quality of food products made from okara and soy.

A notable result of the LAB fermentation of okara is the formation of new phenolic acids, including

3-(phenyl)lactic and 3-(4’-hydroxyphenyl)lactic acids, and the increase of some low molecular weight phenolics, such as hydroxybenzoic acid and hydroxybenzaldehyde derivative (Suppl Table S2 and S3). On the contrary, neither *O*-desmethylangolensin nor equol, nor their derivatives were detected after okara fermentation, highlighting the peculiar ability of a few colonic microorganisms and the specific environmental characteristics required to produce these isoflavone bioactive metabolites. Phenyllactic acid compounds, which are not typically present in non-fermented soy products, are produced by specific LAB strains through metabolic pathways activated during fermentation (Hadj Saadoun et al. 2021). The results of this work highlight *L. paracasei* and *L. rhamnosus* as the more efficient strains in their production. Although these acids are primarily derived from amino acid pathways, there is growing interest in whether phenolic precursors, such as isoflavones or their metabolites, may also contribute to their formation during fermentation (Wu et al. 2023). While direct evidence linking isoflavones to 3-(phenyl)lactic or 3-(4’-hydroxyphenyl)lactic acid production is still limited, the structural similarities suggest a potential role of deglycosylated phenolics, such as aglycones formed by LAB β-glucosidase activity, in similar microbial metabolic pathways. Further research is needed to elucidate whether isoflavones may act as substrates or precursors for these phenolic acids during fermentation, especially in systems where both amino acid and phenolic acid metabolism are activated by specific LAB strains. In addition, phenyllactic acids possess antimicrobial properties, while their phenolic structure confers additional antioxidant activity, potentially amplifying the bioactive profile and health-promoting properties of the fermented product (Rajanikar et al. 2021; Wu et al. 2023). Finally, employing LAB species in fermented products might introduce probiotic strains, which have been associated with numerous health benefits, such as improved gut health, enhanced immune function, and reduced risk of gastrointestinal infections (Gul and Durante-Mangoni 2024). In the case of okara and soy fermentation, the presence of probiotic

strains might facilitate the bioconversion of isoflavones introducing beneficial bacterial populations to the product. Thereby, regular consumption of probiotic-rich fermented okara may be associated with several health benefits, particularly relevant to chronic disease prevention.

Moreover, based on the obtained data on growth parameters and (poly)phenol biotransformation, predictive models were applied to predict the most important factors able to influence okara (poly)phenols.

Conclusions

Fermentation of okara with four LAB strains showed to have a marked effect on its phenolic profile, resulting in a significant increase in bioactive compounds. This effect can be attributed to the metabolic activities of the microbial strains used, which vary widely in their enzymatic capabilities and interactions during fermentation, although beside phenyllactic acid derivatives, no human microbial metabolites, such as *O*-desmethylangolensin and equol, were identified. The isoflavone content of fermented okara suggests its potential use as a functional food ingredient with health benefits.

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References

Ali, Ali A., Manuel T. Velasquez, Carl T. Hansen, Ali I. Mohamed, and Sam J. Bhathena. 2005. "Modulation of Carbohydrate Metabolism and Peptide Hormones by Soybean Isoflavones and Probiotics in Obesity and Diabetes." Journal of Nutritional Biochemistry 16 (11): 693–99. https://doi.org/10.1016/j.jnutbio.2005.03.011.

Asghar, Aasma, Muhammad Afzaal, Farhan Saeed, Aftab Ahmed, Huda Ateeq, Yasir Abbas Shah, Fakhar Islam, Muzzamal Hussain, Noor Akram, and Mohd Asif Shah. 2023. "Valorization and Food Applications of Okara (Soybean Residue): A Concurrent Review." Food Science and Nutrition. John Wiley and Sons Inc. https://doi.org/10.1002/fsn3.3363.

Brindani, Nicoletta, Pedro Mena, Luca Calani, Iris Benzie, Siu Wai Choi, Furio Brighenti, Franca Zanardi, Claudio Curti, and Daniele Del Rio. 2017. "Synthetic and Analytical Strategies for the Quantification of Phenyl-γ-Valerolactone Conjugated Metabolites in Human Urine." Molecular Nutrition and Food Research 61 (9). https://doi.org/10.1002/mnfr.201700077.

Donkor, O. N., and N. P. Shah. 2008. "Production of β-Glucosidase and Hydrolysis of Isoflavone Phytoestrogens by Lactobacillus Acidophilus, Bifidobacterium Lactis, and Lactobacillus Casei in Soymilk." Journal of Food Science 73 (1). https://doi.org/10.1111/j.1750-3841.2007.00547.x.

Eriksson, L., T. Byrne, E. Johansson, J. Trygg, and C. Vikström. 2013. "Multi-and Megavariate Data Analysis Basic Principles and Applications (Vol. 1)." Umetrics Academy.

Feng, Jing Yu, Rui Wang, Kiran Thakur, Zhi Jing Ni, Yun Yang Zhu, Fei Hu, Jian Guo Zhang, and Zhao Jun Wei. 2021. "Evolution of Okara from Waste to Value Added Food Ingredient: An Account of Its Bio-Valorization for Improved Nutritional and Functional Effects." Trends in Food Science and Technology. Elsevier Ltd. https://doi.org/10.1016/j.tifs.2021.08.011.

Guevara-Cruz, Martha, Einar T. Godinez-Salas, Monica Sanchez-Tapia, Gonzalo Torres-Villalobos, Edgar Pichardo-Ontiveros, Rocio Guizar-Heredia, Liliana Arteaga-Sanchez, et al. 2020. "Genistein Stimulates Insulin Sensitivity through Gut Microbiota Reshaping and Skeletal Muscle AMPK Activation in Obese Subjects." BMJ Open Diabetes Research and Care 8 (1). https://doi.org/10.1136/bmjdrc-2019-000948.

Guimarães, Rafaiane Macedo, Thamara Evangelista Silva, Ailton Cesar Lemes, Marussa Cássia Favaro Boldrin, Marco Antônio Pereira da Silva, Fabiano Guimarães Silva, and Mariana Buranelo Egea.

2018. “Okara: A Soybean by-Product as an Alternative to Enrich Vegetable Paste.” LWT 92 (June):593–99. https://doi.org/10.1016/j.lwt.2018.02.058.

Gul, Sabiha, and Emanuele Durante-Mangoni. 2024. “Unraveling the Puzzle: Health Benefits of Probiotics—A Comprehensive Review.” Journal of Clinical Medicine. Multidisciplinary Digital Publishing Institute (MDPI). https://doi.org/10.3390/jcm13051436.

Hadj Saadoun, Jasmine, Luca Calani, Martina Cirlini, Valentina Bernini, Erasmo Neviani, Daniele Del Rio, Gianni Galaverna, and Camilla Lazzi. 2021. “Effect of Fermentation with Single and Co-Culture of Lactic Acid Bacteria on Okara: Evaluation of Bioactive Compounds and Volatile Profiles.” Food and Function 12 (7): 3033–43. https://doi.org/10.1039/d0fo02916e.

Jannah, Ayik Rochyatul, Faiza Rahma Ebnudesita, Savira Butsainah Dienanta, and Reny I’tishom. 2020. “The Potential of Soy Isoflavones (Glycine Max) and Magnetic Hydroxyapatite Nanoparticles as Osteoporosis Therapy for Menopausal Women.”

John, Warren A., Nina L. Böttcher, Britta Behrends, Marcello Corno, R. N. D’souza, Nikolai Kuhnert, and Matthias S. Ullrich. 2020. “Experimentally Modelling Cocoa Bean Fermentation Reveals Key Factors and Their Influences.” Food Chemistry 302 (January). https://doi.org/10.1016/j.foodchem.2019.125335.

Khosravi, Azin, and Seyed Hadi Razavi. 2021. “Therapeutic Effects of Polyphenols in Fermented Soybean and Black Soybean Products.” Journal of Functional Foods. Elsevier Ltd. https://doi.org/10.1016/j.jff.2021.104467.

Kim, Il Sup. 2022. “Current Perspectives on the Beneficial Effects of Soybean Isoflavones and Their Metabolites on Plants.” Food Science and Biotechnology. The Korean Society of Food Science and Technology. https://doi.org/10.1007/s10068-022-01070-7.

Kim, Il Sup, Cheorl Ho Kim, and Woong Suk Yang. 2021. “Physiologically Active Molecules and Functional Properties of Soybeans in Human Health—a Current Perspective.” International Journal of Molecular Sciences. MDPI. https://doi.org/10.3390/ijms22084054.

Kwon, Jeong Eun, Jaewon Lim, Inhye Kim, Donghyuk Kim, and Se Chan Kang. 2018. “Isolation and Identification of New Bacterial Stains Producing Equol from Pueraria Lobata Extract Fermentation.” PLoS ONE 13 (2). https://doi.org/10.1371/journal.pone.0192490.

Lee, Pyung Gang, Uk Jae Lee, Hanbit Song, Kwon Young Choi, and Byung Gee Kim. 2018. “Recent Advances in the Microbial Hydroxylation and Reduction of Soy Isoflavones.” FEMS Microbiology Letters. Oxford University Press. https://doi.org/10.1093/femsle/fny195.

Liu, Libo, Xiaoqian Chen, Linlin Hao, Guofang Zhang, Zhao Jin, Chun Li, Yuzhuo Yang, Jiajia Rao, and Bingcan Chen. 2022. “Traditional Fermented Soybean Products: Processing, Flavor Formation, Nutritional and Biological Activities.” Critical Reviews in Food Science and Nutrition. Taylor and Francis Ltd. https://doi.org/10.1080/10408398.2020.1848792.

Mok, Wai Kit, Yong Xing Tan, Jaslyn Lee, Jaejung Kim, and Wei Ning Chen. 2019. “A Metabolomic Approach to Understand the Solid-State Fermentation of Okara Using Bacillus Subtilis WX-17 for Enhanced Nutritional Profile.” AMB Express 9 (1). https://doi.org/10.1186/s13568-019-0786-5.

Moldovan, Radu Cristian, Ede Bodoki, Timea Kacsó, Anne Catherine Servais, Jacques Crommen, Radu Oprean, and Marianne Fillet. 2016. “A Micellar Electrokinetic Chromatography–Mass Spectrometry Approach Using in-Capillary Diastereomeric Derivatization for Fully Automatized Chiral Analysis of Amino Acids.” Journal of Chromatography A 1467 (October):400–408. https://doi.org/10.1016/j.chroma.2016.08.035.

Moraes Filho, M. L., M. Busanello, and S. Garcia. 2016. “Optimization of the Fermentation Parameters for the Growth of Lactobacillus in Soymilk with Okara Flour.” LWT 74 (December):456–64. https://doi.org/10.1016/j.lwt.2016.08.009.

Oh, S. M., E. K. Jang, J. H. Seo, M. J. Ryu, and S. P. Lee. 2007. “Characterization of γ-Polyglutamic Acid Produced from the Solid-State Fermentation of Soybean Milk Cake Using Bacillus Sp.” Food Science and Biotechnology.

Queiroz Santos, Vidiany A., Camila G. Nascimento, Carla A.P. Schimidt, Daniel Mantovani, Robert F.H. Dekker, and Mário Antônio A. da Cunha. 2018. “Solid-State Fermentation of Soybean Okara: Isoflavones Biotransformation, Antioxidant Activity and Enhancement of Nutritional Quality.” LWT 92 (June):509–15. https://doi.org/10.1016/j.lwt.2018.02.067.

Rafii, Fatemeh. 2015. “The Role of Colonic Bacteria in the Metabolism of the Natural Isoflavone Daidzin to Equol.” Metabolites 5 (1): 56–73. https://doi.org/10.3390/metabo5010056.

Rajanikar, R. V., Basavaprabhu Haranahalli Nataraj, Harshita Naithani, Syed Azmal Ali, Narender Raju Panjagari, and Pradip V. Behare. 2021. “Phenyllactic Acid: A Green Compound for Food Biopreservation.” Food Control. Elsevier Ltd. https://doi.org/10.1016/j.foodcont.2021.108184.

Ray, R. C., S. Paramithiotis, A. Thekkangil, V. Nethravathy, A. K. Rai, and J. G. P. Martin. 2024. “Food Fermentation and Its Relevance in the Human History.” Trending Topics on Fermented Foods.

Rui, Xin, Mingjia Wang, Yuqian Zhang, Xiang Chen, Lan Li, Yulin Liu, and Mingsheng Dong. 2017. “Optimization of Soy Solid-State Fermentation with Selected Lactic Acid Bacteria and the Effect

on the Anti-Nutritional Components." Journal of Food Processing and Preservation 41 (6). https://doi.org/10.1111/jfpp.13290.

Sarker, Aniruddha, Raju Ahmmed, S M Ahsan, Juwel Rana, Mithun Kumar Ghosh, and Rakhi Nandi. 2024. "A Comprehensive Review of Food Waste Valorization for the Sustainable Management of Global Food Waste." Sustainable Food Technology. https://doi.org/10.1039/d3fb00156c.

Schindler, Sabrina, Maximilian Wittig, Kateryna Zelena, Ulrich Krings, Jürgen Bez, Peter Eisner, and Ralf G. Berger. 2011. "Lactic Fermentation to Improve the Aroma of Protein Extracts of Sweet Lupin (Lupinus Angustifolius)." Food Chemistry 128 (2): 330–37. https://doi.org/10.1016/j.foodchem.2011.03.024.

Shi, Hui, Min Zhang, Weiqin Wang, and Sakamon Devahastin. 2020. "Solid-State Fermentation with Probiotics and Mixed Yeast on Properties of Okara." Food Bioscience 36 (August). https://doi.org/10.1016/j.fbio.2020.100610.

"Soybeans | USDA Foreign Agricultural Service." 2024. USDA. 2024.

Vong, Weng Chan, Xin Yi Hua, and Shao Quan Liu. 2018. "Solid-State Fermentation with Rhizopus Oligosporus and Yarrowia Lipolytica Improved Nutritional and Flavour Properties of Okara." LWT 90 (April):316–22. https://doi.org/10.1016/j.lwt.2017.12.050.

Vong, Weng Chan, and Shao Quan Liu. 2016. "Biovalorisation of Okara (Soybean Residue) for Food and Nutrition." Trends in Food Science and Technology. Elsevier Ltd. https://doi.org/10.1016/j.tifs.2016.04.011.

Voss, Glenise B., Luísa MP Valente, and Manuela E. Pintado. 2018. "Impact of Fructose and Fructooligosaccharides Supplementation upon the Fermentation of Hydrolyzed Okara and Its Impact upon Bioactive Components." SDRP Journal of Food Science & Technology.

Wang, Che Wei, Hsin Ya Tsai, Chen Hsu, Ching Chun Hsieh, I. Shu Wang, Chi Fon Chang, and Nan Wei Su. 2024. "Structure-Specific Metabolism of Flavonol Molecules by Bacillus Subtilis Var. Natto BCRC 80517." Food Chemistry 430 (January). https://doi.org/10.1016/j.foodchem.2023.136975.

Wang, Huixian, Xingqiao He, Juanni Li, Jintao Wu, Shuaiming Jiang, Hui Xue, Jiachao Zhang, Rajesh Jha, and Ruimin Wang. 2024. "Lactic Acid Bacteria Fermentation Improves Physicochemical Properties, Bioactivity, and Metabolic Profiles of Opuntia Ficus-Indica Fruit Juice." Food Chemistry 453 (September). https://doi.org/10.1016/j.foodchem.2024.139646.

Wang, Wenfeng, and Yong Jun Cha. 2018. “Volatile Compounds in Seasoning Sauce Produced from Soy Sauce Residue by Reaction Flavor Technology.” Preventive Nutrition and Food Science 23 (4): 356–63. https://doi.org/10.3746/pnf.2018.23.4.356.

Wang, Xiaowen, Canqing Yu, Jun Lv, Liming Li, Yonghua Hu, Keyang Liu, Kokoro Shirai, Hiroyasu Iso, and Jia Yi Dong. 2021. “Consumption of Soy Products and Cardiovascular Mortality in People with and without Cardiovascular Disease: A Prospective Cohort Study of 0.5 Million Individuals.” European Journal of Nutrition 60 (8): 4429–38. https://doi.org/10.1007/s00394-021-02602-3.

Wei, Yuxia, Jun Lv, Yu Guo, Zheng Bian, Meng Gao, Huaidong Du, Ling Yang, et al. 2020. “Soy Intake and Breast Cancer Risk: A Prospective Study of 300,000 Chinese Women and a Dose–Response Meta-Analysis.” European Journal of Epidemiology 35 (6): 567–78. https://doi.org/10.1007/s10654-019-00585-4.

Wu, H., C. Guang, W. Zhang, and W. Mu. 2023. “Recent Development of Phenyllactic Acid: Physicochemical Properties, Biotechnological Production Strategies and Applications.” Critical Reviews in Biotechnology.

Zhu, Y. P., J. F. Fan, Y. Q. Cheng, and L. T. Li. 2008. “Improvement of the Antioxidant Activity of Chinese Traditional Fermented Okara (Meitauza) Using Bacillus Subtilis B2.” Food Control 19 (7): 654–61. https://doi.org/10.1016/j.foodcont.2007.07.009.

Zhu, Yan, Zimeng Wang, and Li Zhang. 2019. “Optimization of Lactic Acid Fermentation Conditions for Fermented Tofu Whey Beverage with High-Isoflavone Aglycones.” LWT 111 (August):211–17. https://doi.org/10.1016/j.lwt.2019.05.021.

***Table S1**. Chromatographic and mass spectrometry characteristics of (poly)phenol compounds detected in okara samples.*

Compound	*Retention time (min)*	*Parent ion $[M-H]^-$ (m/z)*	*Quantifier Product ion (m/z)*	*Qualifier Product ion (m/z)*	*Standard (in bold those reference compounds used to quantify the corresponding metabolite)*
Hydroxyphenyllactic acid	*1.84*	*181*	*119*	*137, 121, 113*	*Hydroxyphenyllactic acid*
4-Hydroxybenzoic acid	*2.35*	*137*	*93*	*92*	*4-Hydroxy-3-methoxybenzoic acid*
4-Hydroxybenzaldehyde	*3.30*	*121*	*92*	*91*	*4-Hydroxy-3-methoxybenzoic acid*
4-Hydroxy-3-methoxybenzoic acid (Vanillic acid)	*3.45*	*167*	*152*	*108, 123*	*4-Hydroxy-3-methoxybenzoic acid*
Equol methyl	*4.27*	*255*	*240*		*Equol-7-glucuronide*
Phenyllactic acid	*4.38*	*165*	*119*	*121, 150*	*Phenyllactic acid*
Genistein-hexoside (Genistin) isomer I	*4.44*	*431*	*269*	*133, 159, 135*	*Genistein-7-O-glucuronide*
4'-Hydroxycinnamic acid (p-Coumaric acid)	*4.48*	*163*	*119*	*93*	*4'-Hydroxycinnamic acid*
Daidzein-hexoside (Daidzin) isomer I	*4.50*	*415*	*253*	*223, 91*	*Daidzein-7-glucuronide*
Glycitein-hexoside (Glycitin)	*4.58*	*445*	*283*	*268*	*Glycitein*
3'-Hydroxycinnamic acid (m-Coumaric acid)	*4.60*	*163*	*119*	*93*	*4'-Hydroxycinnamic acid*
2'Hydroxygenistein-7-O-glucoside	*4.62*	*447*	*285*	*133, 151*	*Daidzein-7-glucuronide*
Hydroxy-methoxycinnamic acid ((Iso)Ferulic acid)	*4.72*	*193*	*134*		*4'-Hydroxy-3'-methoxycinnamic acid*
Daidzein-hexoside (Daidzin) isomer II	*4.73*	*415*	*253*	*223, 91*	*Daidzein-7-glucuronide*

Not identified 1	*4.79*	*167*	*123*		*3-(3'-Methoxyphenyl)propanoic acid-4'-glucuronide*
Genistein-hexoside (Genistin) isomer II	*4.86*	*431*	*269*	*133, 159, 135*	*Genistein-7-O-glucuronide*
Genistein-acetylhexoside isomer I	*4.88*	*473*	*269*	*133, 159, 135*	*Genistein-7-O-glucuronide*
Daidzein-acetylhexoside isomer I	*4.97*	*457*	*253*	*223, 91*	*Daidzein-7-glucuronide*
Genistein-acetylhexoside isomer II	*5.02*	*473*	*269*	*133, 159, 135*	*Genistein-7-O-glucuronide*
Daidzein-acetylhexoside isomer II	*5.11*	*457*	*253*	*223, 91*	*Daidzein-7-glucuronide*
Glycitein-acetylhexoside isomer I	*5.15*	*487*	*283*	*268, 113*	*Glycitein*
Daidzein-acetylhexoside isomer III	*5.22*	*457*	*253*	*223, 91*	*Daidzein-7-glucuronide*
Genistein-acetylhexoside isomer III	*5.31*	*473*	*269*	*133, 159, 135*	*Genistein-7-O-glucuronide*
Genistein-acetylhexoside isomer IV	*5.42*	*473*	*269*	*133, 159, 135*	*Genistein-7-O-glucuronide*
Daidzein	*5.44*	*253*	*223*	*91*	*Daidzein*
Dihydrodaidzein	*5.45*	*255*	*149*	*91, 135*	*Dihydrodaidzein*
Glycitein-acetylhexoside isomer II	*5.47*	*487*	*283*	*268, 113*	*Glycitein*
Glycitein	*5.52*	*283*	*268*	*240*	*Glycitein*
Dihydrogenistein	*5.76*	*271*	*165*	*137*	*Dihydrogenistein*
Naringenin	*5.85*	*271*	*165*	*137*	*Dihydrogenistein*
Genistein	*5.9*	*269*	*133*	*159, 135*	*Genistein*
Desmethylangolensin	*6.12*	*257*	*136*	*108, 109*	*Desmethylangolensin*

Table S2. *Quantification of single phenolic compounds for monocultures, Pediococcus pentosaceus, Lacticaseibacillus paracasei, Bacillus subtilis and Lacticaseibacillus rhamnosus. Data are indicated as mean ± SD, were evaluated on two biological replicates, and are expressed as mg/100g.*

	Control	***Pediococcus pentosaceus 6268***		***Lacticaseibacillus paracasei 6227***			***Bacillus subtilis 5002***		***Lacticaseibacillus rhamnosus 1473***			
		42°C/48	25°C/96h	25°C/48h	25°C/96h	42°C/96h	42°C/48h	25°C/96h	25°C/48h	42°C/48h	25°C/96h	42°C/96h
Genistein-hexoside isomer I	1.40 ± 0.40^{ab}	1.33 ± 0.32^{ab}	0.23 ± 0.08^{ef}	1.20 ± 0.20^{b}	1.50 ± 0.46^{ab}	1.62 ± 0.40^{a}	0.44 ± 0.03^{de}	0.24 ± 0.01^{ef}	0.09 ± 0.03^{f}	0.73 ± 0.26^{cd}	0.33 ± 0.03^{ef}	0.87 ± 0.12^{c}
Genistein-hexoside isomer II	6.94 ± 0.68^{a}	5.62 ± 0.97^{bc}	1.05 ± 0.33^{f}	5.54 ± 0.60^{c}	3.71 ± 0.72^{d}	6.59 ± 0.82^{ab}	4.14 ± 0.40^{d}	2.46 ± 0.18^{e}	1.59 ± 1.10^{ef}	3.87 ± 0.90^{d}	1.59 ± 0.27^{ef}	4.07 ± 1.23^{d}
Daidzein-hexoside isomer I	0.83 ± 0.16^{a}	0.69 ± 0.15^{b}	0.10 ± 0.02^{e}	0.65 ± 0.10^{b}	0.63 ± 0.16^{b}	0.84 ± 0.14^{a}	0.31 ± 0.04^{d}	0.16 ± 0.04^{e}	0.11 ± 0.05^{e}	0.44 ± 0.13^{cd}	0.12 ± 0.02^{e}	0.47 ± 0.08^{c}
Daidzein-hexoside isomer II	0.20 ± 0.05^{ab}	0.17 ± 0.04^{bc}	0.02 ± 0.01^{g}	0.17 ± 0.03^{cd}	0.14 ± 0.03^{de}	0.22 ± 0.04^{a}	0.08 ± 0.01^{f}	0.08 ± 0.01^{f}	0.01 ± 0.00^{g}	0.11 ± 0.03^{ef}	0.03 ± 0.01^{g}	0.10 ± 0.02^{ef}
2'Hydroxygenistein-7-O-glucoside	0.07 ± 0.02^{a}	0.04 ± 0.02^{bc}	0.01 ± 0.00^{d}	0.06 ± 0.02^{ab}	0.06 ± 0.02^{ab}	0.01 ± 0.00^{d}	0.04 ± 0.00^{bcd}	0.02 ± 0.01^{cd}	0.03 ± 0.00^{cd}	0.02 ± 0.01^{cd}	0.03 ± 0.01^{cd}	0.04 ± 0.01^{bc}
Glycitein-hexoside	1.29 ± 0.46^{a}	1.22 ± 0.28^{a}	0.55 ± 0.23^{cd}	1.03 ± 0.14^{ab}	1.21 ± 0.32^{a}	1.34 ± 0.33^{a}	0.60 ± 0.14^{cd}	0.39 ± 0.15^{de}	0.10 ± 0.05^{e}	0.67 ± 0.22^{cd}	0.36 ± 0.09^{de}	0.73 ± 0.16^{bc}
Genistein-acetylhexoside isomer I	0.43 ± 0.14^{cdef}	0.52 ± 0.09^{bcde}	0.87 ± 0.31^{a}	0.49 ± 0.07^{cde}	0.69 ± 0.24^{b}	0.46 ± 0.07^{cde}	0.34 ± 0.04^{ef}	0.38 ± 0.04^{def}	0.68 ± 0.11^{b}	0.60 ± 0.02^{bc}	0.55 ± 0.02^{bcd}	0.24 ± 0.06^{f}
Genistein-acetylhexoside isomer II	0.27 ± 0.07^{ab}	0.23 ± 0.07^{bc}	0.22 ± 0.06^{bc}	0.23 ± 0.04^{bc}	0.08 ± 0.02^{e}	0.19 ± 0.04^{cd}	0.09 ± 0.00^{e}	0.14 ± 0.03^{de}	0.14 ± 0.04^{d}	0.16 ± 0.01^{d}	0.32 ± 0.01^{a}	0.16 ± 0.02^{d}
Genistein-acetylhexoside isomer III	0.60 ± 0.15^{a}	0.56 ± 0.10^{ab}	0.57 ± 0.13^{ab}	0.53 ± 0.11^{abc}	0.60 ± 0.15^{a}	0.50 ± 0.09^{abcd}	0.42 ± 0.04^{cd}	0.39 ± 0.06^{d}	0.46 ± 0.05^{bcd}	0.55 ± 0.05^{abc}	0.56 ± 0.05^{ab}	0.37 ± 0.04^{d}
Genistein-acetylhexoside isomer IV	1.32 ± 0.19^{a}	1.04 ± 0.18^{bc}	0.95 ± 0.18^{bcd}	1.01 ± 0.13^{bcd}	0.34 ± 0.08^{g}	0.90 ± 0.13^{cd}	0.63 ± 0.09^{f}	0.83 ± 0.13^{de}	0.68 ± 0.07^{ef}	0.87 ± 0.14^{cde}	1.10 ± 0.10^{b}	0.60 ± 0.18^{f}
Daidzein-acetylhexoside isomer I	0.12 ± 0.04^{abc}	0.12 ± 0.03^{abc}	0.15 ± 0.05^{a}	0.11 ± 0.02^{abcd}	0.14 ± 0.04^{ab}	0.11 ± 0.02^{abcd}	0.09 ± 0.02^{cde}	0.07 ± 0.03^{de}	0.10 ± 0.02^{bcd}	0.11 ± 0.01^{abcd}	0.09 ± 0.01^{bcde}	0.06 ± 0.01^{e}
Daidzein-acetylhexoside isomer II	0.24 ± 0.05^{a}	0.20 ± 0.05^{ab}	0.19 ± 0.05^{bc}	0.19 ± 0.04^{bc}	0.06 ± 0.03^{f}	0.16 ± 0.03^{bc}	0.07 ± 0.01^{ef}	0.10 ± 0.01^{de}	0.12 ± 0.03^{d}	0.14 ± 0.02^{cd}	0.21 ± 0.01^{ab}	0.10 ± 0.02^{de}
Daidzein-acetylhexoside isomer III	0.03 ± 0.01^{a}	0.02 ± 0.00^{b}	0.02 ± 0.00^{bc}	0.01 ± 0.01^{bcd}	0.00 ± 0.00^{fg}	0.01 ± 0.00^{cde}	0.00 ± 0.00^{g}	0.01 ± 0.00^{ef}	0.01 ± 0.00^{e}	0.01 ± 0.00^{de}	0.01 ± 0.00^{de}	0.01 ± 0.00^{de}
Glycitein-acetylhexoside isomer I	0.21 ± 0.05^{ab}	0.16 ± 0.06^{bcd}	0.16 ± 0.04^{bcd}	0.20 ± 0.04^{abc}	0.04 ± 0.01^{h}	0.15 ± 0.02^{cde}	0.05 ± 0.02^{gh}	0.11 ± 0.06^{def}	0.09 ± 0.03^{fg}	0.10 ± 0.04^{efg}	0.24 ± 0.03^{a}	0.09 ± 0.03^{fgh}
Glycitein-acetylhexoside isomer II	0.17 ± 0.05^{bcd}	0.19 ± 0.05^{abcd}	0.26 ± 0.09^{ab}	0.21 ± 0.05^{abcd}	0.26 ± 0.07^{ab}	0.18 ± 0.09^{abcd}	0.15 ± 0.03^{cde}	0.13 ± 0.03^{de}	0.26 ± 0.05^{a}	0.22 ± 0.06^{abc}	0.17 ± 0.02^{bcd}	0.08 ± 0.04^{e}
Genistein	9.17 ± 1.80^{e}	10.85 ± 1.90^{cde}	15.03 ± 2.87^{a}	10.24 ± 1.31^{de}	14.05 ± 3.11^{ab}	11.78 ± 1.50^{bcd}	5.78 ± 0.62^{f}	6.50 ± 1.00^{f}	11.57 ± 1.24^{cde}	11.96 ± 1.01^{bcd}	13.20 ± 0.60^{abc}	11.00 ± 1.27^{cde}
Daidzein	56.99 ± 10.46^{c}	67.11 ± 11.61^{bc}	88.14 ± 20.75^{a}	64.70 ± 8.22^{bc}	76.91 ± 15.72^{ab}	70.99 ± 11.36^{bc}	29.43 ± 4.12^{d}	35.57 ± 4.20^{d}	62.70 ± 5.69^{bc}	67.60 ± 4.00^{bc}	77.10 ± 4.12^{ab}	64.92 ± 9.27^{bc}
Glycitein	24.07 ± 6.22^{b}	28.33 ± 6.49^{ab}	34.42 ± 10.58^{a}	29.15 ± 5.25^{ab}	35.28 ± 11.02^{a}	32.69 ± 5.91^{ab}	5.18 ± 0.60^{c}	7.50 ± 1.66^{c}	27.29 ± 4.20^{ab}	27.91 ± 2.64^{ab}	32.41 ± 1.87^{ab}	24.84 ± 5.00^{b}

Dihydrogenistein	0.01 ± 0.00^{ab}	0.00 ± 0.00^{ab}	0.01 ± 0.01^{a}	0.01 ± 0.00^{a}	0.01 ± 0.00^{ab}	0.01 ± 0.00^{ab}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{ab}	0.00 ± 0.00^{ab}	0.00 ± 0.00^{ab}	0.00 ± 0.00^{ab}
Equol methyl	0.01 ± 0.00^{a}	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	0.01 ± 0.00^{a}	0.00 ± 0.00^{c}	0.01 ± 0.01^{a}	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	0.01 ± 0.00^{b}	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}
3-(4′-hydroxyphenyl)lactic acid	0.00 ± 0.00^{e}	0.05 ± 0.05^{e}	0.17 ± 0.10^{e}	0.09 ± 0.03^{e}	3.01 ± 1.00^{a}	0.03 ± 0.00^{e}	0.00 ± 0.00^{e}	0.00 ± 0.00^{e}	1.26 ± 0.10^{bc}	1.01 ± 0.39^{cd}	1.55 ± 0.09^{b}	0.73 ± 0.27^{d}
3-(phenyl)lactic acid	0.00 ± 0.00^{e}	0.12 ± 0.04^{e}	0.29 ± 0.11^{e}	0.42 ± 0.12^{e}	3.57 ± 1.09^{a}	0.04 ± 0.01^{e}	0.04 ± 0.01^{e}	0.03 ± 0.02^{e}	2.14 ± 0.29^{bc}	1.69 ± 0.59^{cd}	2.51 ± 0.11^{b}	1.29 ± 0.43^{d}
Hydroxy-methoxycinnamic acid	0.01 ± 0.00^{ab}	0.01 ± 0.00^{a}	0.01 ± 0.00^{ab}	0.00 ± 0.00^{ab}	0.01 ± 0.00^{ab}	0.00 ± 0.00^{b}	0.00 ± 0.00^{ab}	0.00 ± 0.00^{ab}	0.01 ± 0.00^{ab}	0.00 ± 0.00^{ab}	0.01 ± 0.00^{a}	0.01 ± 0.00^{a}
4'-Hydroxycinnamic acid	0.06 ± 0.01^{ab}	0.06 ± 0.01^{ab}	0.07 ± 0.03^{a}	0.05 ± 0.01^{ab}	0.07 ± 0.02^{ab}	0.06 ± 0.01^{ab}	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	0.05 ± 0.01^{b}	0.05 ± 0.01^{b}	0.07 ± 0.01^{ab}	0.06 ± 0.01^{ab}
3'-Hydroxycinnamic acid	0.01 ± 0.00^{b}	0.01 ± 0.01^{ab}	0.01 ± 0.01^{a}	0.01 ± 0.00^{ab}	0.01 ± 0.00^{ab}	0.01 ± 0.00^{ab}	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	0.01 ± 0.00^{ab}	0.01 ± 0.00^{ab}	0.01 ± 0.00^{ab}	0.01 ± 0.00^{ab}
4-Hydroxy-3-methoxybenzoic acid	0.01 ± 0.00^{c}	0.02 ± 0.00^{ab}	0.03 ± 0.02^{a}	0.01 ± 0.00^{c}	0.01 ± 0.00^{c}	0.02 ± 0.01^{ab}	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	0.02 ± 0.00^{b}	0.01 ± 0.01^{b}	0.02 ± 0.01^{ab}	0.02 ± 0.00^{b}
4-Hydroxybenzoic acid	0.61 ± 0.26^{c}	0.89 ± 0.33^{bc}	1.44 ± 0.58^{a}	0.74 ± 0.22^{bc}	1.18 ± 0.48^{ab}	1.13 ± 0.31^{ab}	0.83 ± 0.32^{bc}	0.63 ± 0.28^{c}	0.66 ± 0.11^{c}	0.76 ± 0.10^{bc}	0.85 ± 0.15^{bc}	0.78 ± 0.18^{bc}
4-Hydroxybenzaldehyde	1.48 ± 0.49^{bcd}	2.17 ± 0.79^{ab}	2.36 ± 0.91^{a}	1.85 ± 0.33^{abc}	2.24 ± 0.80^{a}	2.36 ± 0.48^{a}	0.00 ± 0.00^{e}	0.00 ± 0.00^{e}	0.97 ± 0.19^{d}	1.43 ± 0.28^{cd}	1.49 ± 0.22^{bcd}	1.26 ± 0.23^{cd}
Total phenolic content	106.55 ± 20.89^{b}	121.73 ± 23.26^{ab}	147.37 ± 37.04^{a}	118.93 ± 16.74^{ab}	145.81 ± 34.97^{a}	132.41 ± 21.52^{ab}	48.73 ± 6.46^{c}	55.76 ± 7.35^{c}	111.16 ± 10.97^{b}	121.07 ± 7.74^{ab}	135.03 ± 5.74^{ab}	112.93 ± 15.19^{b}

Table S3. *Quantification of single phenolic compounds for co-cocultures X1, X2, X3, X4 and X5.*

Data are indicated as mean ± SD, were evaluated on two biological replicates, and are expressed as mg/100g.

		X1	X2	X3	X4	X5
	Control	**C.C 5002+1473+6227**	**C.C 5002+1473+6268**	**C.C 1473+6227+6268**	**C.C 5002+6227+6268**	**C.C 5002+1473+6227+6268**
				25°C/96h		
Genistein-hexoside isomer I	1.40 ± 0.40^{a}	0.17 ± 0.04^{b}	0.21 ± 0.08^{b}	0.24 ± 0.07^{b}	0.24 ± 0.04^{b}	0.21 ± 0.06^{b}
Genistein-hexoside isomer II	6.94 ± 0.68^{a}	1.56 ± 0.32^{b}	1.45 ± 0.59^{b}	1.60 ± 0.42^{b}	1.84 ± 0.44^{b}	1.48 ± 0.39^{b}
Daidzein-hexoside isomer I	0.83 ± 0.16^{a}	0.17 ± 0.03^{b}	0.11 ± 0.04^{b}	0.12 ± 0.03^{b}	0.13 ± 0.02^{b}	0.12 ± 0.02^{b}
Daidzein-hexoside isomer II	0.20 ± 0.05^{b}	0.35 ± 0.17^{a}	0.04 ± 0.01^{c}	0.03 ± 0.01^{c}	0.03 ± 0.01^{c}	0.04 ± 0.01^{c}
2'Hydroxygenistein-7-O-glucoside	0.07 ± 0.02^{a}	0.00 ± 0.00^{c}	0.01 ± 0.01^{bc}	0.01 ± 0.00^{c}	0.02 ± 0.01^{b}	0.01 ± 0.00^{bc}
Glycitein-hexoside	1.29 ± 0.46^{a}	0.32 ± 0.03^{b}	0.30 ± 0.09^{b}	0.37 ± 0.12^{b}	0.46 ± 0.04^{b}	0.32 ± 0.04^{b}
Genistein-acetylhexoside isomer I	0.43 ± 0.14^{c}	0.28 ± 0.06^{d}	0.78 ± 0.06^{a}	0.71 ± 0.03^{a}	0.58 ± 0.03^{b}	0.72 ± 0.06^{a}
Genistein-acetylhexoside isomer II	0.27 ± 0.07^{a}	0.00 ± 0.00^{c}	0.19 ± 0.05^{b}	0.15 ± 0.03^{b}	0.26 ± 0.02^{a}	0.19 ± 0.01^{b}
Genistein-acetylhexoside isomer III	0.60 ± 0.15^{a}	0.13 ± 0.01^{c}	0.55 ± 0.05^{ab}	0.50 ± 0.06^{b}	0.53 ± 0.03^{ab}	0.51 ± 0.08^{ab}
Genistein-acetylhexoside isomer IV	1.32 ± 0.19^{a}	0.13 ± 0.02^{e}	0.98 ± 0.19^{bc}	0.79 ± 0.10^{d}	1.11 ± 0.10^{b}	0.85 ± 0.10^{cd}
Daidzein-acetylhexoside isomer I	0.12 ± 0.04^{a}	0.05 ± 0.01^{b}	0.12 ± 0.02^{a}	0.10 ± 0.02^{a}	0.11 ± 0.01^{a}	0.10 ± 0.01^{a}
Daidzein-acetylhexoside isomer II	0.24 ± 0.05^{a}	0.00 ± 0.00^{d}	0.17 ± 0.02^{b}	0.13 ± 0.04^{c}	0.21 ± 0.02^{a}	0.15 ± 0.02^{bc}
Daidzein-acetylhexoside isomer III	0.03 ± 0.01^{a}	0.00 ± 0.00^{d}	0.02 ± 0.00^{bc}	0.01 ± 0.01^{c}	0.02 ± 0.00^{ab}	0.01 ± 0.00^{c}
Glycitein-acetylhexoside isomer I	0.21 ± 0.05^{a}	0.00 ± 0.00^{d}	0.15 ± 0.03^{bc}	0.11 ± 0.05^{c}	0.19 ± 0.03^{ab}	0.12 ± 0.04^{c}
Glycitein-acetylhexoside isomer II	0.17 ± 0.05^{a}	0.05 ± 0.02^{b}	0.20 ± 0.06^{a}	0.22 ± 0.05^{a}	0.21 ± 0.03^{a}	0.19 ± 0.04^{a}
Genistein	9.17 ± 1.80^{b}	0.35 ± 0.11^{c}	11.66 ± 0.81^{a}	10.89 ± 0.45^{a}	11.79 ± 0.19^{a}	11.66 ± 0.43^{a}
Daidzein	56.99 ± 10.46^{b}	3.34 ± 0.52^{c}	67.12 ± 4.75^{a}	65.58 ± 4.78^{a}	68.32 ± 1.93^{a}	69.29 ± 1.65^{a}

Glycitein	24.07 ± 6.22[a]	0.32 ± 0.14[b]	26.17 ± 2.71[a]	25.68 ± 2.33[a]	25.36 ± 1.14[a]	27.19 ± 1.57[a]
Dihydrogenistein	0.01 ± 0.00[a]	0.00 ± 0.00[a]	0.00 ± 0.00[a]	0.01 ± 0.01[a]	0.00 ± 0.00[a]	0.01 ± 0.00[a]
Equol methyl	0.01 ± 0.00[b]	0.02 ± 0.01[a]	0.00 ± 0.00[c]	0.00 ± 0.00[c]	0.00 ± 0.00[c]	0.00 ± 0.00[c]
3-(4′-hydroxyphenyl)lactic acid	0.00 ± 0.00[c]	1.95 ± 0.37[a]	0.42 ± 0.06[b]	0.32 ± 0.03[b]	0.34 ± 0.04[b]	0.47 ± 0.02[b]
3-(phenyl)lactic acid	0.00 ± 0.00[c]	2.23 ± 0.28[a]	0.49 ± 0.08[b]	0.38 ± 0.05[b]	0.35 ± 0.02[b]	0.55 ± 0.04[b]
Hydroxy-methoxycinnamic acid	0.01 ± 0.00[a]	0.00 ± 0.00[b]	0.01 ± 0.00[a]	0.01 ± 0.00[ab]	0.01 ± 0.00[a]	0.01 ± 0.00[ab]
4'-Hydroxycinnamic acid	0.06 ± 0.01[a]	0.00 ± 0.00[c]	0.04 ± 0.00[b]	0.05 ± 0.01[b]	0.05 ± 0.00[b]	0.04 ± 0.00[b]
3'-Hydroxycinnamic acid	0.01 ± 0.00[a]	0.00 ± 0.00[b]	0.01 ± 0.00[a]	0.01 ± 0.00[a]	0.01 ± 0.00[a]	0.01 ± 0.00[a]
4-Hydroxy-3-methoxybenzoic acid	0.01 ± 0.00[b]	0.00 ± 0.00[b]	0.03 ± 0.01[a]	0.02 ± 0.01[a]	0.03 ± 0.01[a]	0.02 ± 0.01[a]
4-Hydroxybenzoic acid	0.61 ± 0.26[b]	0.96 ± 0.20[a]	0.69 ± 0.15[b]	0.76 ± 0.16[b]	0.70 ± 0.07[b]	0.73 ± 0.07[b]
4-Hydroxybenzaldehyde	1.48 ± 0.49[a]	0.42 ± 0.07[c]	0.90 ± 0.11[b]	1.03 ± 0.16[b]	1.11 ± 0.03[b]	0.99 ± 0.05[b]
Total phenolic content	106.55 ± 20.89[a]	12.79 ± 1.35[b]	112.85 ± 8.88[a]	109.88 ± 8.67[a]	114.06 ± 3.02[a]	116.04 ± 1.99[a]

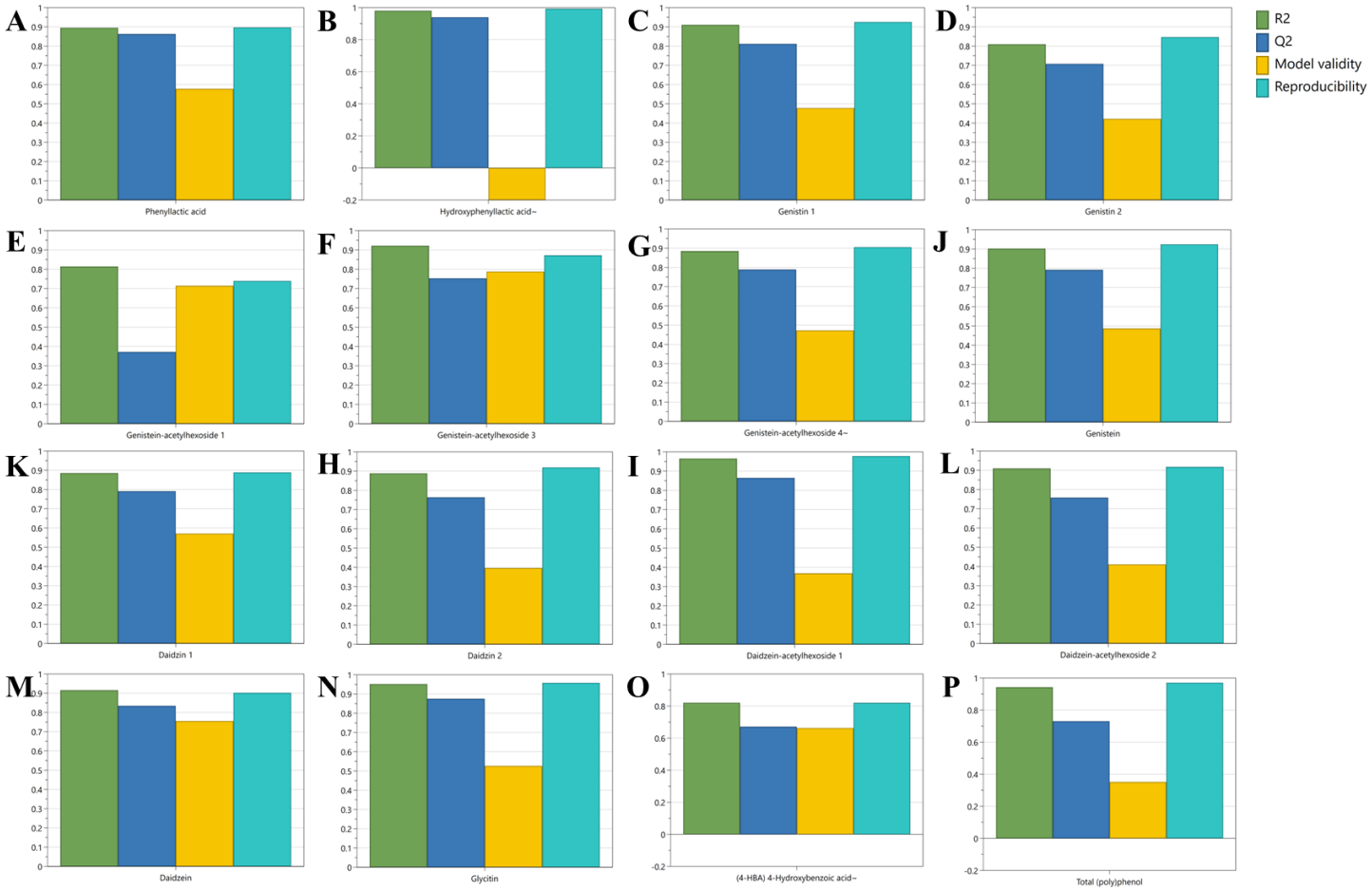

Figure S1. *R^2, Q^2, model validity, and reproducibility representing the (poly)phenol biotransformation after okara fermentation. Capital letters correspond to: A phenyllactic acid, B Hydroxyphenyllactic acid, C Genistin 1, D Genistin 2, E Genistein acetylhexoside 1, F Genistein acetylhexoside 3, G Genistein acetylhexoside 4, J Genistein, K Daidzin 1, H Daidzin 2, I Daidzein acetylhexoside 1, L Daidzein acetylhexoside 2, M Daidzein, N Glycitin, O 4-Hydroxybenzoic acid, P Total (poly)phenol.*

Chapter 2

Modelling the lactic fermentation of *Opuntia* cladodes to maximise bioactive production

Marrella Martina, Hadj Saadoun Jasmine, Ricci Annalisa, Fernández Naiara, Ventura Carolina, Bronze Maria Do Rosário, Lazzi Camilla

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Abstract

Global climate change necessitates the sustainable use of dryland systems, with the prickly pear cactus (*Opuntia Ficus-indica* L.) presenting a valuable resource for mitigating environmental degradation. While the fruits of this plant are widely utilized, its cladodes remain an underutilized biomass with potential for bioactive compound recovery. This study explores the fermentability of cladodes by seven strains of lactic acid bacteria (LAB) and investigates the impact of fermentation on antioxidant activity and polyphenolic content. A design of experiments (DoE) approach was employed to evaluate the effects of incubation time, temperature, and microbial strains on these parameters. Fermentation led to enhanced antioxidant activity and significant changes in polyphenolic content, particularly under specific co-culture conditions. Statistical modeling provided insights into optimal fermentation settings, suggesting that LAB fermentation could unlock the cladodes' potential as a source of functional ingredients. This work supports the valorization of prickly pear cladodes through microbial fermentation, contributing to waste reduction and sustainable agricultural practices.

Introduction

The need for more efficient use of dryland systems is driven by global warming, climate change and increasing human and livestock populations (Jorge et al. 2023). Adapted perennial crops with higher productivity per unit area are needed to protect natural rangeland systems from degradation, and among these, cactus species are of relevant interest (Acharya et al. 2019). *Opuntia* species have been able to withstand prolonged periods of drought, high temperatures and erosion from wind and water (Jorge et al. 2023). These features, together with its wide range of uses as fruit and fodder, make it ideal for the agricultural development in areas affected by desertification and climate change (Nefzaoui et al. 2010). Prickly pear (*Opuntia Ficus Indica L.)* is a native plant of the American continent, that belongs to the Cactaceae family (Fernández-López et al. 2010).

Among all the producer countries, approximately 96% of world production is covered by Mexico, Italy and South African (Stavi 2022; Mondragón-Jacobo et al. 2001). Regarding Italy, Sicily produces 96% of the total Italian cactus pear harvest, covering a production area of 8300 ha and leading to a production of about 87000 tonnes every year (Mondragón-Jacobo et al. 2001; Timpanaro et al. 2015).

The prickly pear fruits, also called “tuna”, are generally consumed fresh or processed as jam, juice or ice cream (Yamina and Dalila 2021). Cladodes, the flattened fleshy stems, commonly called “nopal”, are usually discarded during pruning operations causing high costs for farmers and a problem for disposal (Procacci et al. 2021; De Kock 2001). Although their main end use in livestock feed, in some countries, especially in South America, they are also used for human consumption (Hernández-Becerra et al. 2022).

Like other vegetables, cladodes contain a high-water content (88-95%), carbohydrates (3-7%) and fibres (1%). Some authors reported the presence of starch but relevant is the presence of mucilage

polysaccharide polymers, that helps plant to regulate the water available during dry periods (Ayadi et al. 2009; Medina-Torres et al. 2000; Quinzio et al. 2018).

Comparing to other costituents, protein, lipids and organic acid are present to a lesser extent (Stintzing and Carle 2005; Guevara-Figueroa et al. 2010; Figueroa-Pérez et al. 2018). Moreover, cladodes are also sources of phytochemicals such as vitamins, pigments (carotenoids and betalins), and polyphenolic compounds that could exert antioxidant and anti-inflammatory properties (Mena et al. 2018; Mondragón-Jacobo et al. 2001). Different contents of polyphenols can be found in cladodes depending on the age of plant and the varieties (Mena et al. 2018).

The complex composition of cladode makes these parts, which are generally discarded, attractive for bioactive recovery, making prickly pear a multipurpose plant (Stintzing and Carle 2005).

All parts of the plant should be recovered and valorised and to achieve this purpose, an interesting method the fermentation process to obtain different molecules and products (Filannino et al. 2016; Quines-Lagmay et al. 2020; López-Domínguez et al. 2019). Lactic acid fermentation, driven by lactic acid bacteria (LAB), is an ancient process widely used in fermented foods. Nowadays, fermentation of plant material is a promising tool for improving nutritional and functional features of food or to develop innovative fermented products. The fermentation leads to an intense acidification with a correlated improvement in the release of bioactive compounds, increasing their bioaccessibility and bioavailability (Pontonio et al. 2019).

Although several studies have shown an increase in the polyphenol profile following lactic fermentation of plant matrices, few of these have involved pricky pear, particularly cladodes (De Montijo-Prieto et al. 2023; Filannino et al. 2016; Ozturk et al. 2024).

Considering that conversion abilities are strain- and species-specific, in this study we want to investigate the fermentability of prickly pear by different LAB and the effect of fermentation on the polyphenolic profile and the antioxidant activity. This goal was achieved by building an experimental

design (DoE) in which different factors, such as incubation time, fermentation temperature and microbial strains, were evaluated. The identification of the key factors influencing the process and the possibility of using a prediction tool to find the optimal condition for increasing the polyphenolic profile and antioxidant activity is a useful step to exploit cladodes with a view to developing a new ingredient.

Materials and methods

Strains

To explore the cladodes fermentability seven lactic acid bacteria (LAB) differing in metabolism and belonging to the University of Parma Culture Collection (UPCCO) were used. *Levilactobacillus brevis* 4867 (Lb), *Leuconostoc mesenteroides* 6089 (Lm), *Lacticaseibacillus rhamnosus* 2310 (Lr), *Lactiplantibacillus plantarum* 4932 (Lpl), *Lactobacillus delbruecki* 1865 (Ld), *Streptococcus thermophilus* 4734 (St), *Lactobacillus helveticus* 5519 (Lh). *L. brevis* was isolated from sourdough, while all others were isolated from dairy sources. The bacterial stock cultures were maintained as frozen at −80°C in M17 broth (Merk, Germany) for *S. thermophilus* 4734 and in Man Rogosa and Sharpe (MRS) broth (Oxoid, UK) for the other LAB, added with 12.5% glycerol (v/v). The strains were revitalized three times with 3% (v/v) inoculum in the medium and incubated at their optimal temperature, 30°C for *L. plantarum* 4932, *L. brevis* 4867, *L. mesenteroides* 6089, 37°C for *L. rhamnosus* 2310, *S. thermophilus* 4734, *L. helveticus* 5519 and at 42°C for *L. delbruecki* 1865.

Prickly pear *cladodes and Design of Experiment (DoE)*

The cladodes used in this study were collected in a local farm in Puglia (Italy) and were chopped with a through laboratory blenders (Knife Mill Grindomix GM 200, Retsch GmbH, Haan, Germany). Samples were aliquoted (45 g) into glass jars and sterilised at 121°C for 20 minutes before use.

Experiments were designed according to a mathematical model using MODDE Pro v12.0.1 software (MKS Umetrics, Sweden), and a total of 19 experiments were performed in duplicate for a total of 38 experiments.

The model was designed with the strains as a qualitative factor and two quantitative factors: time

and temperature of incubation. A minimum and maximum value was set for each quantitative factor, with the addition of two center points referred to average values. Fermentation temperature ranged between 25 and 37°C, incubation time from 28 to 72h.

Each strain was used to ferment the substrate under the conditions defined by DoE. Co-cultures were obtained by mixing single revitalized strains in equal volume and each culture/co-culture was inoculated into cladodes puree to reach 6 Log CFU/mL, except for the central points where the inoculum was 3 Log CFU/mL. Samples were incubated at the DoE condition and the concentration of the strains was checked just after inoculum (T_0), and at the end of fermentation (T_1) by plate count on MRS agar. After the fermentation process, all samples were stored at -20°C and dried with a freeze-dryer (Lio 5P, Milano, Italy).

The responses measured were the antioxidant activity (expressed as µmol TEAC/g dw extract) of each sample measured by the ORAC assay (Section Oxygen radical absorbance capacity (ORAC)) and the total polyphenolic compounds (TPC, expressed as mgGAE/g dw of extract) by HPLC-DAD (Section Total phenolic content by HPLC-DAD).

The results expressed as the concentration of Trolox equivalent antioxidant activity at the end of incubation time (µmol TEAC/g dw extract) were elaborated using MODDE Pro v 13.00.1.

A subsequent analysis was added to analyse the data with the prediction tool to obtain the best fermentation condition.

Chemical analysis

Reagent

Ethanol, Methanol, gallic acid, 2',2'-azobis (2-amidinopropane) dihydrochloride (AAPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and phosphate buffer solution (PBS) were

purchased from Sigma–Aldrich (St. Quentin Fallavier, France). Disodium fluorescein (FL) was obtained from TCI Europe (Antwerp, Belgium).

Extract preparation

The extraction of all the samples was carried out in triplicate for one hour in an ultrasonic bath with 1.5 mL of ethanol/water (50:50, v/v) in 100 mg of dry samples (Mazari and Portillo 2021). After the extraction, the supernatant was separated by centrifugation (10,000 rpm for 10 min at room temperature) and evaporated to dryness under vacuum.

The dried extract was resuspended in 2 mL of methanol/water (50:50, v/v) and filtered first with a 25mm PVDF Hydrophilic Syringe Filter 0.45 µm (Labfil, China) and then with 13 mm PTFE Syringe Filter 0.2 µm (Cytiva Whatman, USA).

Oxygen radical absorbance capacity (ORAC)

The antioxidant capacity of the extracts was evaluated through the ORAC assay. It allows to evaluate the ability of the antioxidant compounds to inhibit the oxidation of FL catalysed by peroxyl radicals generated from AAPH.

This assay was carried out following the method described by Serra et al. (2011). The analysis was performed using 96-well microplates, in which 25 µL of extract and 150 µL of FL were placed and incubated for 10 min at 37 °C. Subsequently, 25 µL of AAPH was added and the microplate was shaken for 30 seconds.

PBS was used as a blank and a calibration curve was obtained from 5, 10, 20, 30 and 40 µM Trolox solutions. The extracts were diluted 1:1000 and 1:1500 in PBS and each dilution was analyzed in

triplicate. The fluorescence emitted by the reduced form of FL was recorded for 40 min every 1 minute at 37 °C with the FLx800 microplate fluorescent reader (Bio-Tek, USA). The emission and excitation wavelengths were 530 ± 25 nm and 485 ± 20 nm, respectively.

All data were expressed as micromoles of Trolox Equivalent Antioxidant Capacity per gramme of dry weight of extract (µmol TEAC/g dw extract).

Total phenolic content by HPLC-DAD

The extracts were also quantified with HPLC-DAD for the total phenolic content (mg GAE/g extract). The LC system used was a Vanquish (Thermo Fischer, USA) equipped with an autosampler, a Photodiode Array detector and an electrochemical detector. Chromatographic separation was carried out on a Luna C18 100A column (250 × 4 mm, particle size 5 mm, Phenomenex®). The Photodiode array detector scanned between 190 and 680 nm, the detection was monitored using 280, 320 and 360 nm channels and the auto sampler's temperature was set at 12 °C. The mobile phase consisted of eluent B with acetonitrile–water-formic acid (90%:9.5%:0.5%) and eluent A with water-formic acid (99.5%:0.5%) at a flow rate of 0.60 mL/min and injection volume of 10 µL. The system was run with the following gradient program: 0–15 min from 94.4 to 80% A; 15–32 min from 80 to 60% A; 45-55 min at 0% A; 55-65 min from 0 to 94.4% A.

The data acquisition system was Chromeleon version 7.0 (Waltham, MA, USA).

Statistical analysis

All conditions were performed with two biological replicates and for chemical analysis each sample was extracted and analysed in triplicate. Quantitative data are reported as mean ± standard deviation (SD). The T-test was performed to compare all samples with the unfermented sample

(control). Statistical analysis was carried out using the IBM SPSS Statistics 23.0 software package (IBM, Chicago, IL, USA). The level of statistical significance was set at $p < 0.05$.

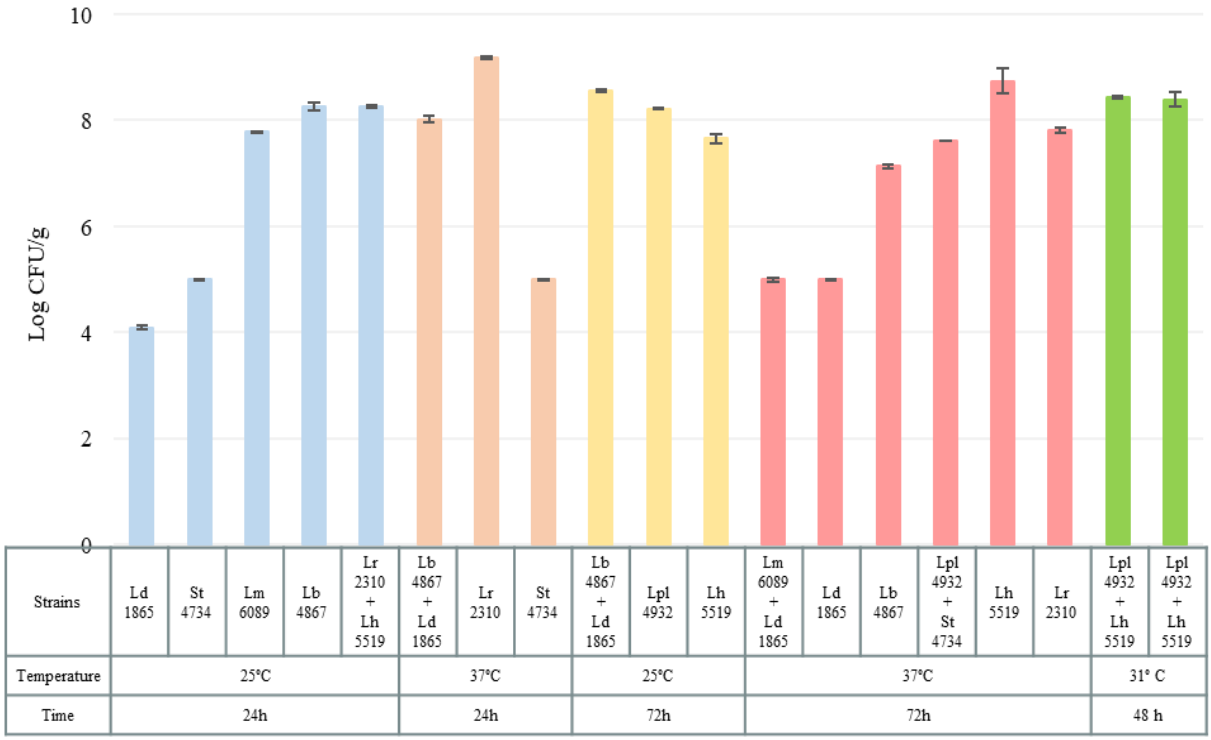
Results and Discussion

Evaluation of microbial growth in Cladodes

An experimental design with 19 different conditions was set up to study the fermentability and chemical changes of the cladodes. A temperature range of 25°C to 37°C, an incubation time range of 24 h to 72 h, and different inoculum of lactic acid bacteria were applied, some in monoculture and some in co-culture. All experiments were performed with biological replicates (Table 1).

The fermentation of prickly pear has been evaluated by other authors who have investigated either the use of different microorganisms such as yeasts or the use of different lactic acid bacteria but isolated from plant matrices (Navarrete-Bolaños et al. 2013; Filannino et al. 2016). M. Carpena et al. (2023), reported that this technique can be an efficient method for the recovery of high-value molecules or for the production of new products from waste or different parts of *Opuntia* plants.

In this study, it observed that the cladodes are a matrix that favours the growth of the lactic acid bacteria isolated from different niches. In fact, starting from an inoculum of 6 log CFU/g, growth is observed in many samples at the end of fermentation (Figure 1). Samples inoculated with Lr 2310, Lb 4867, Lh 5519 and Lpl 4932, both mono and coculture, showed a higher microbial load after incubation regardless of process conditions. Conversely, there are strains that are not able to grow in this substrate in specific condition. This is the case of Ld 1865 and St 4734, which did not show an increase in microbial load when inoculated in monoculture, even within their optimal temperature range. However, the samples inoculated with their coculture exhibited a high microbial load.



Strains	Ld 1865	St 4734	Lm 6089	Lb 4867	Lr 2310 + Lh 5519	Lb 4867 + Ld 1865	Lr 2310	St 4734	Lb 4867 + Ld 1865	Lpl 4932	Lh 5519	Lm 6089 + Ld 1865	Ld 1865	Lb 4867	Lpl 4932 + St 4734	Lh 5519	Lr 2310	Lpl 4932 + Lh 5519	Lpl 4932 + Lh 5519
Temperature	25°C					37°C			25°C			37°C						31° C	
Time	24h					24h			72h			72h						48 h	

Figure 1. Growth ability of all conditions used to ferment prickly pear cladodes.

The results were reported as mean value ± standard deviation and were evaluated on two biological replicates.

The ability of *L. brevis* and *L. plantarum* to grow in plant matrices and also in cladodes has been reported (Filannino et al. 2016), while it is the first time that other strains and cocultures have been used. It has been observed that some strains grow better in coculture than when inoculated individually. This ability could be due to the synergistic action of the metabolic pathways of the strains involved, which leads for example to greater degradation of the substrates (Hadj Saadoun et al. 2021).

Table 1. Results of growth, pH, antioxidant activity (ORAC) and total phenolic compounds (HPLC-DAD) of all conditions.

Mean and standard deviation were evaluated on two biological replicates for antioxidant activity (ORAC) and HPLC – DAD analysis each sample was extracted and analysed in triplicate.

Incubation		*Strains*	Samples	Abb.	LAB growth (Δ Log CFU/g)	pH	ORAC (µmol TEAC/g extract)	HPLC - DAD (mgGAE/g extract)
Time	Temp.							
			C	Unfermented	0.00 ± 0.00	4.52 ± 0.01	571.50 ± 121.07	15.19 ± 0.42
		Lactobacillus delbruecki 1865	9 & 32	Ld 1865	4.09 ± 0.06	4.63 ± 0.04	560.10 ± 73.48	12.49 ± 1.43
		Streptococcus thermophilus 4734	11 & 34	St 4734	4.99 ± 0.00	4.66 ± 0.02	766.50 ± 99.87	14.35 ± 1.27
24h	25°C	*Leuconostoc mesenteroides 6089*	12 & 35	Lm 6089	7.78 ± 0.31	4.45 ± 0.01	858.32 ± 74.72	13.90 ± 1.08
		Levilactobacillus brevis 4867	16 & 39	Lb 4867	8.27 ± 0.16	4.49 ± 0.07	779.22 ± 96.17	17.60 ± 1.47
		Lacticaseibacillus rhamnosus 2310 + Lactobacillus helveticus 5519	20 & 43	Lr 2310 + Lh 5519	8.26 ± 0.20	4.37 ± 0.03	726.82 ± 60.00	13.38 ± 0.55
		Levilactobacillus brevis 4867 + Lactobacillus delbruecki 1865	3 & 26	Lb 4867 + Ld 1865	8.02 ± 0.20	4.55 ± 0.06	684.85 ± 61.86	10.99 ± 0.72
24h	37°C	*Lacticaseibacillus rhamnosus 2310*	6 & 29	Lr 2310	9.18 ± 0.79	4.21 ± 0.03	770.16 ± 130.45	14.79 ± 0.78
		Streptococcus thermophilus 4734	13 & 36	St 4734	4.99 ± 0.00	4.65 ± 0.02	645.08 ± 93.38	12.11 ± 0.71
		Levilactobacillus brevis 4867 + Lactobacillus delbruecki 1865	2 & 25	Lb 4867 + Ld 1865	8.56 ± 0.05	3.95 ± 0.03	635.34 ± 83.92	11.44 ± 0.18
72h	25°C	*Lactiplantibacillus plantarum 4932*	8 & 31	Lp 4932	8.22 ± 0.08	3.76 ± 0.01	734.67 ± 83.12	12.28 ± 2.20
		Lactobacillus helveticus 5519	18 & 41	Lh 5519	7.66 ± 0.78	4.00 ± 0.08	577.47 ± 39.01	16.74 ± 1.52
		Leuconostoc mesenteroides 6089 + Lactobacillus delbruecki 1865	5 & 28	Lm 6089 + Ld 1865	4.99 ± 0.00	3.96 ± 0.03	639.33 ± 34.51	13.82 ± 0.54
		Lactobacillus delbruecki 1865	7 & 30	Ld 1865	4.99 ± 0.00	4.53 ± 0.01	585.92 ± 41.43	10.79 ± 0.99
72h	37°C	*Levilactobacillus brevis 4867*	10 & 33	Lb 4867	7.14 ± 0.07	3.98 ± 0.04	638.36 ± 67.92	15.21 ± 1.20
		Lactiplantibacillus plantarum 4932 + Streptococcus thermophilus 4734	15 & 38	Lp 4932 + St 4734	7.62 ± 0.06	3.45 ± 0.00	804.05 ± 103.58	16.72 ± 1.85
		Lactobacillus helveticus 5519	17 & 40	Lh 5519	8.74 ± 0.39	3.71 ± 0.23	616.46 ± 52.66	17.33 ± 1.52
		Lacticaseibacillus rhamnosus 2310	19 & 42	Lr 2310	7.82 ± 0.15	3.95 ± 0.05	689.14 ± 67.55	16.77 ± 1.17
48h	31°C	*Lactiplantibacillus plantarum 4932 + Lactobacillus helveticus 5519*	22 & 45	Lp 4932 +Lh 5519	8.43 ± 0.03	4.58 ± 0.04	788.65 ± 81.70	14.33 ± 1.65
			23 & 46		8.39 ± 0.02	4.55 ± 0.13	798.78 ± 66.25	13.33 ± 0.74

The pH parameter shows a different trend as it is depicted in Figure 2. In general, in cases where the strains have not been able to grow, the values do not decrease. The values only decrease during certain fermentations and depend on the conditions under which the strains were able to grow. The fermentation with Lpl 4932 in monoculture, which showed an increase of 2 Log CFU/g, resulted in a lower pH compared to the unfermented sample. Conversely, the same strains, when in coculture with Lh 5519, showed a growth ability but no decrease of pH was observed. The different pH variations could be related to the metabolites produced during fermentation under different conditions, which could modulate the pH of the substrates differently.

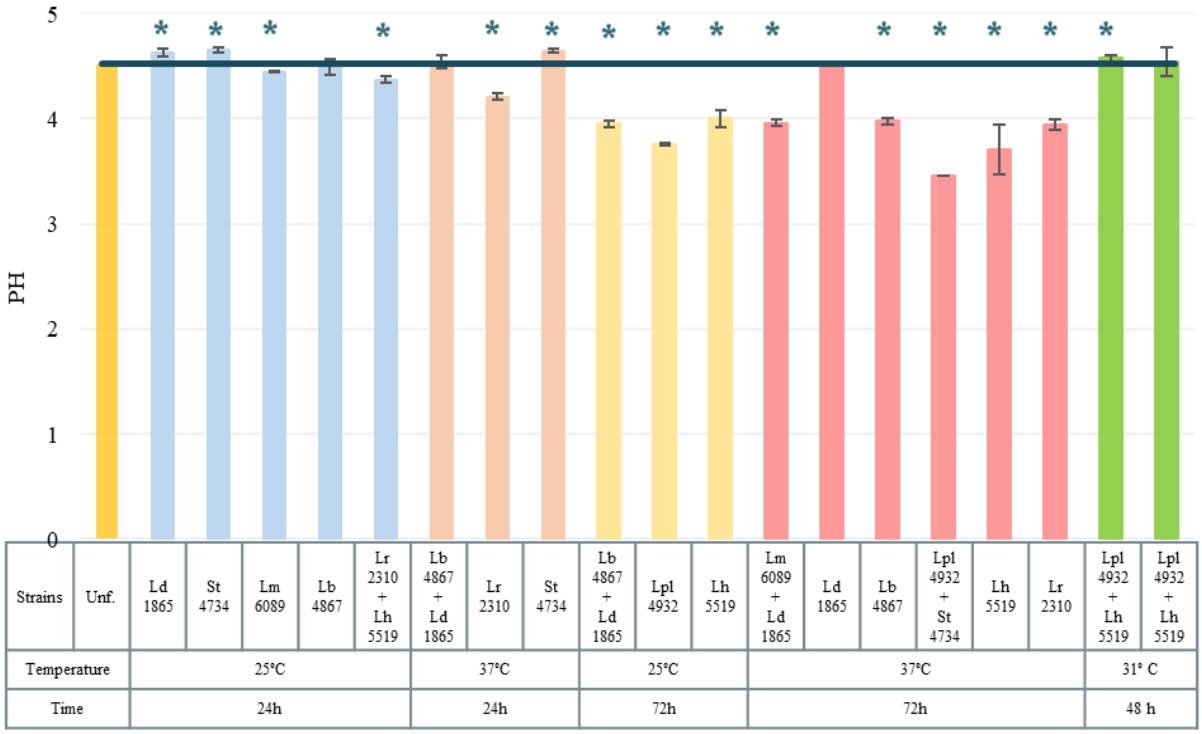


Figure 2. pH trend for all conditions used to ferment prickly pear cladodes. Results were reported as mean ± standard deviation of two biological replicates and measured in triplicate. The stars indicate the conditions with a significant difference (P<0.05) compared to the unfermented sample, highlighted with a T-test.

Maximizing Antioxidant Activity and Polyphenol Content through predictive model

Fermentation has a significant impact on the antioxidant capacity of products, increasing the release of flavonoids from plant foods, inducing the synthesis of various bioactive compounds or increasing total phenolic compounds (Hur et al. 2014). In this study antioxidant activity was evaluated using DoE to understand which factors of fermentation process could improve the release of bioactive compounds. The antioxidant activity was evaluated by the ORAC assay. Figure 3 shows the mean values of the biological replicates for each DoE condition and the first bar, in yellow, shows the value of the starting matrix (unfermented).

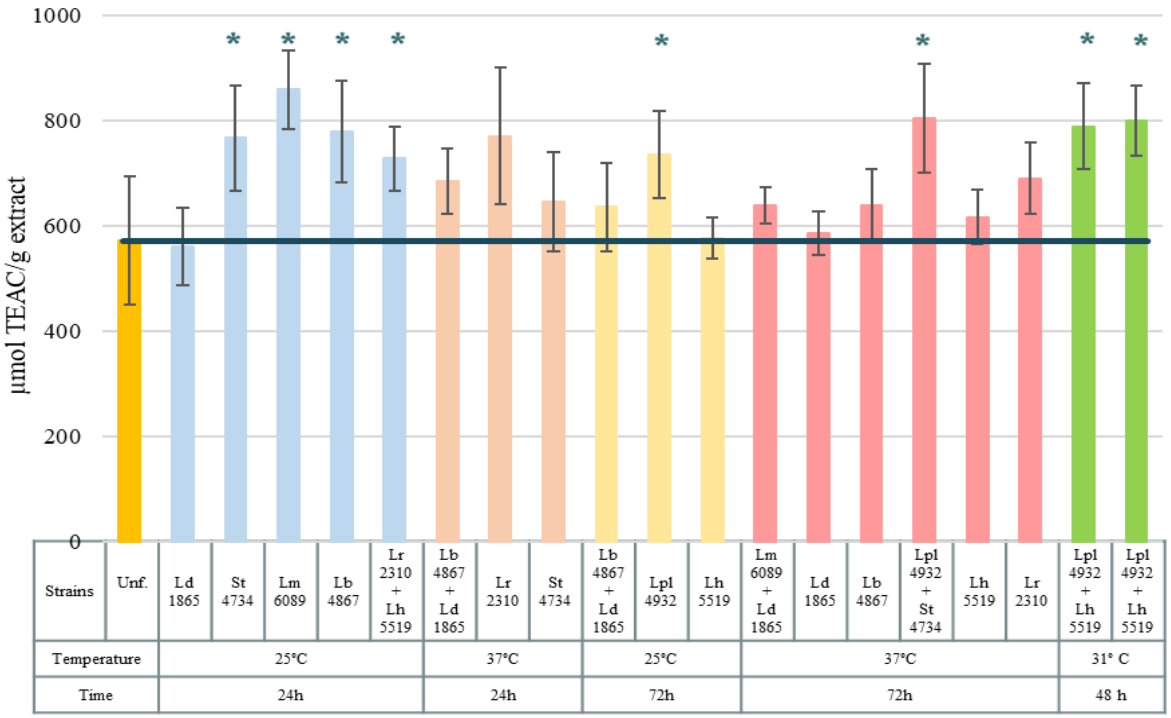


Figure 3. The antioxidant activity was measured for all conditions obtained after fermentation of prickly pear cladodes. Results were reported as mean ± standard deviation of two biological replicates each extracted and analysed in triplicate. The stars indicate the conditions with a significant difference (P<0.05) compared to the unfermented sample, highlighted with a T-test.

An increase in antioxidant activity was detected in different samples in which bacterial grow. In detail, samples that showed significantly different values compared to the unfermented control, were Lm, Lb and Lr + Lh incubated at 25° C for 24 h and Lpl + Lh incubated at 31° C for 48 h, Lpl incubated at 25°C for 72h and Lpl + St incubated at 37°C for 72h.

On the other hand, only replicates inoculated with St, incubated at 25°C for 24 h show a high antioxidant activity among the samples without growth. The increase in antioxidant activity may be due to the enzymatic activity of the microorganisms, such as β-galactosidase activity, which degrades the high molecular weight phenolic compounds and releases the free phenolics from the bound sugars (Lee et al. 2008). This activity can also be provided by enzymes released into the matrix as cells lyse, as in the case of St, where a decrease of almost 2 Log CFU/g from the initial inoculum was observed (Liu et al. 2021).

Data of the antioxidant activity were collected, and the data analysis was performed using MODDE to verify which factors affected the response (Figure 4). The coefficient plot (Figure 4) was analysed to understand how different factors influenced the response. The model parameters showed a good fit (R^2=0.91), indicating strong significance (John et al. 2020).

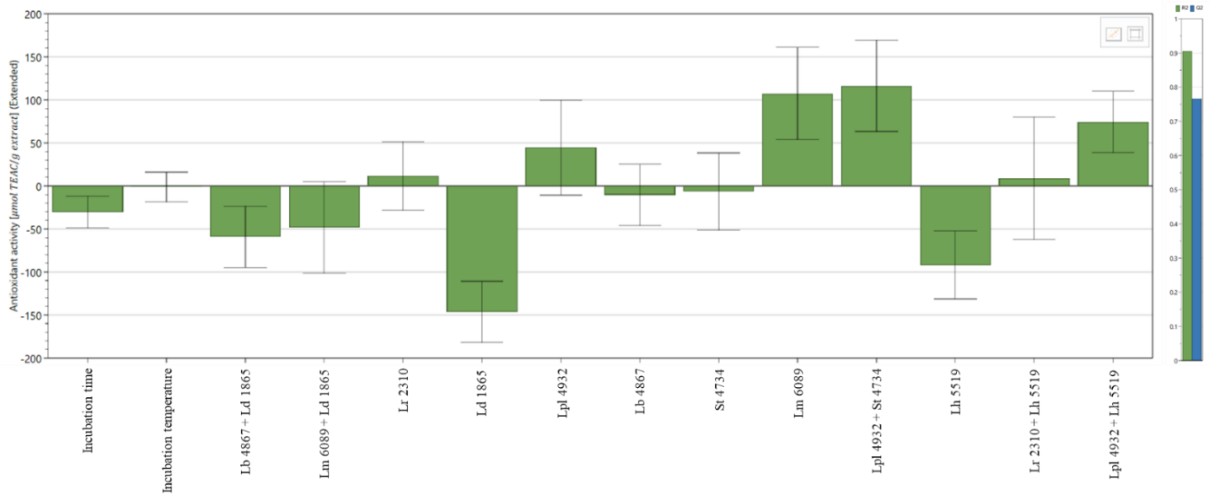


Figure 4. Factors significantly affecting antioxidant activity in fermented cladodes of prickly pear

The prediction accuracy (Q^2) was 0.79, indicating good predictive ability (Consonni et al. 2010). Factors are considered significant if their error bars do not cross zero, corresponding to a p-value of less than 0.05. Differently from incubation temperature, the quantitative factor incubation time influenced the antioxidant activity. Indeed, the shorter fermentation process of cladodes, the greater the antioxidant activity. Considering the strains inoculated in the sample, it was observed that only three inoculum condition affect the response: Lm, Lpl + St and Lpl + Lh. Conversely when Lpl grow in monoculture did not affect the concentration of antioxidant activity. These data are in agreement with the study of Hashemi et al. (2020) who observed a greater increase during the fermentation of bergamot juice when *L. plantarum* was inoculated in coculture.

To evaluate the total polyphenol content of each fermented cladodes the analysis with HPLC-DAD was performed. In Figure 5 are depicted the results respect to the unfermented sample in which was detected a concentration of 15 mg GAE/g extract.

A significant increase in concentration was observed in samples fermented with Lb for 24 h at 25°C, while in several conditions the total phenolic content significantly decrease. Fermentation can lead to a reduction in polyphenol content, primarily due to oxidation and condensation reactions that transform polyphenols into different compounds.

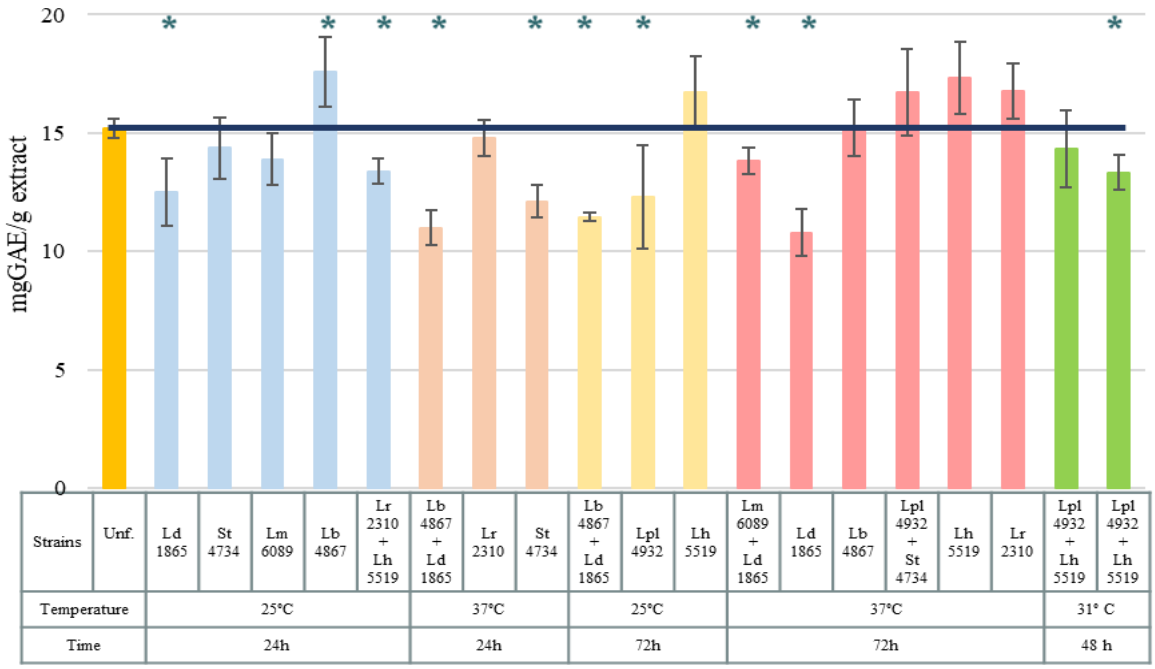


Figure 5. Total phenolic compounds were measured for all conditions obtained after fermentation of prickly pear cladodes. Results were reported as mean ± standard deviation of two biological replicates each extracted and analysed in triplicate. The stars indicate the conditions with a significant difference (P<0.05) compared to the unfermented sample, highlighted with a T-test.

This decrease arises from various processes, including the rearrangement of phenolic structures, the cleavage of phenolic compounds, the binding of phenolics with other molecules, and a decrease in their extractability (Adebo et al. 2018).

As for the antioxidant activity, HPLC-DAD data were analysed also with Modde software to obtain information about factors that influence the total phenol content during fermentation (Figure 6). Also, in this case the model has significant statistical parameters (R^2=0.89 and Q^2=0.77) and it is possible to perform the analysis of coefficient.

Temperature in this case seems to influence the response; when the cladodes were fermented at low temperature a higher concentration of phenolic compounds were reached.

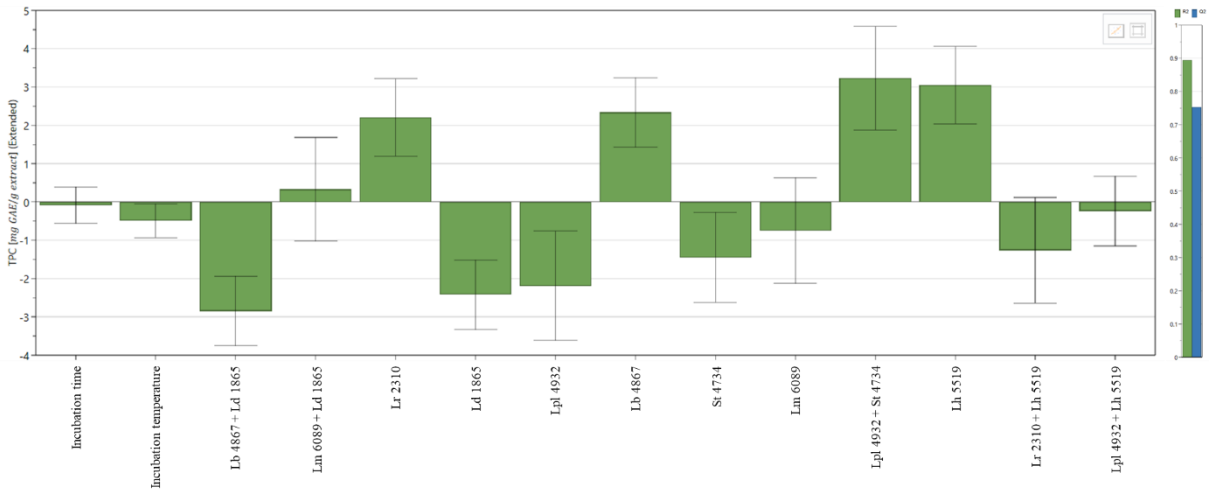


Figure 6. Factors significantly affecting the total phenolic content in fermented cladodes of prickly pear.

The coculture with Lpl and St confirm a positive correlation as for the antioxidant activity. Further, also Lr, Lb and Lh grown in monoculture have a positive effect on the total phenolic content. As reported by Pontonio et al. (2019) fermentation of plant materials leads to an increase in total phenolic compounds mainly due to microbial hydrolysis reactions and biochemical changes in the products.

All data were analysed to find out other conditions, beyond the observed one, that can give a greater response in terms of polyphenol content and antioxidant activity. Using the prediction function of MODDE software, it allows to make predictions based on the factors that were considered more relevant. In our case we have chosen all the factors that have a significant effect depicted in the coefficient plot of antioxidant activity (incubation time, Lpl + St) and total phenol content (incubation temperature, Lpl + St, Lh, Lr and Lb). Table 2 reported the four predicted conditions that can boost both the response with the lower and upper level 95% confidence intervals calculated. Among the conditions indicated by the software as the most efficient, there is the coculture condition, in line with what was previously reported. However, in contrast to previous observations, the software suggests other process conditions that can enhance antioxidant activity and polyphenol content.

Table 2. Data from the MODDE Pro v 13.00.1 software prediction tool and experimental validation of the condition. For the different fermentation conditions, the minimum, average and maximum concentration values were reported

Strains	Prediction		Validation					
	Antioxidant (µmol TEAC/g extract) min - med - max	Total phenol content (mg GAE/g extract) min - med - max	Antioxidant activity (µmol TEAC/g extract)			Total phenol content (mg GAE/g extract)		
Lpl 4932 + St 4374	781 - 834 - 887	16.20 - 17.74 - 19.28	1041.42	±	154.88	16.82	±	0.66
Lh 5519	588 - 627 - 667	16.54 - 17.54 - 18.54	910.02	±	173.96	15.56	±	0.94
Lb 4867	673 - 708 - 744	15.91 - 16.91 - 17.92	979.36	±	167.77	16.29	±	1.39
Lr 2310	694 - 729 - 764	15.56 - 16.80 - 18.04	1031.37	±	169.18	16.90	±	0.57

For example, incubating at 25°C for 48 hours may lead to a higher response. These conditions were tested to validate them and to ensure a good predictive model. As shown in Table 2, all the conditions tested had a positive effect on total polyphenol content and antioxidant activity in line what was previously predict by the software.

Conclusion

This study highlights the potential of lactic acid fermentation to valorize prickly pear cladodes by enhancing their antioxidant activity and polyphenolic content. Through an experimental design approach, key factors such as incubation time, temperature, and microbial strains were identified as critical determinants influencing fermentation outcomes.

The results demonstrate that certain strains and co-cultures, particularly involving *Lactiplantibacillus plantarum* and *Streptococcus thermophilus*, are highly effective in maximizing antioxidant activity and phenolic content. Additionally, predictive modeling using MODDE software validated fermentation conditions that optimize these responses, underscoring the utility of fermentation as a sustainable method for bioactive recovery from agricultural by-products.

These findings suggest that fermentation not only improves the nutritional and functional properties of prickly pear cladodes but also offers a viable pathway for developing innovative food ingredients and reducing agricultural waste.

References

Acharya, Prasenjit, Chandrashekhar Biradar, Mounir Louhaichi, Surajit Ghosh, Sawsan Hassan, Hloniphani Moyo, and Ashutosh Sarker. 2019. “Finding a Suitable Niche for Cultivating Cactus Pear (Opuntia Ficus-Indica) as an Integrated Crop in Resilient Dryland Agroecosystems of India.” Sustainability (Switzerland) 11 (21). https://doi.org/10.3390/su11215897.

Adebo, Oluwafemi Ayodeji, Patrick Berka Njobeh, and Eugenie Kayitesi. 2018. “Fermentation by Lactobacillus Fermentum Strains (Singly and in Combination) Enhances the Properties of Ting from Two Whole Grain Sorghum Types.” Journal of Cereal Science 82 (July):49–56. https://doi.org/10.1016/j.jcs.2018.05.008.

Ayadi, M. A., W. Abdelmaksoud, M. Ennouri, and H. Attia. 2009. “Cladodes from Opuntia Ficus Indica as a Source of Dietary Fiber: Effect on Dough Characteristics and Cake Making.” Industrial Crops and Products 30 (1): 40–47. https://doi.org/10.1016/j.indcrop.2009.01.003.

Carpena, M., L. Cassani, A. Gomez-Zavaglia, P. Garcia-Perez, S. Seyyedi-Mansour, Hui Cao, J. Simal-Gandara, and M. A. Prieto. 2023. “Application of Fermentation for the Valorization of Residues from Cactaceae Family.” Food Chemistry 410 (June). https://doi.org/10.1016/j.foodchem.2022.135369.

Consonni, Viviana, Davide Ballabio, and Roberto Todeschini. 2010. “Evaluation of Model Predictive Ability by External Validation Techniques.” Journal of Chemometrics 24 (3–4): 194–201. https://doi.org/10.1002/cem.1290.

Fernández-López, José A, Luís Almela, José M Obón, and Rosario Castellar. 2010. “Determination of Antioxidant Constituents in Cactus Pear Fruits.” https://doi.org/10.1007/s11130-010-0189-x.

Figueroa-Pérez, Marely G., Iza F. Pérez-Ramírez, Octavio Paredes-López, Candelario Mondragón-Jacobo, and Rosalía Reynoso-Camacho. 2018. “Phytochemical Composition and in Vitro Analysis of Nopal (O. Ficus-Indica) Cladodes at Different Stages of Maturity.” International Journal of Food Properties 21 (1): 1728–42. https://doi.org/10.1080/10942912.2016.1206126/ASSET/C1DB66B0-CFB4-4A70-8875-C8E48F218CB2/ASSETS/IMAGES/LJFP_A_1206126_F0001_B.GIF.

Filannino, Pasquale, Ivana Cavoski, Nadia Thlien, Olimpia Vincentini, Maria De Angelis, Marco Silano, Marco Gobbetti, and Raffaella Dicagno. 2016. “Lactic Acid Fermentation of Cactus Cladodes (Opuntia Ficus-Indica L.) Generates Flavonoid Derivatives with Antioxidant and Anti-

Inflammatory Properties." PLOS ONE 11 (3): e0152575. https://doi.org/10.1371/JOURNAL.PONE.0152575.

Guevara-Figueroa, Teresita, Hugo Jiménez-Islas, María L. Reyes-Escogido, Anne G. Mortensen, Bente B. Laursen, Li Wei Lin, Antonio De León-Rodríguez, Inge S. Fomsgaard, and Ana P. Barba de la Rosa. 2010. "Proximate Composition, Phenolic Acids, and Flavonoids Characterization of Commercial and Wild Nopal (Opuntia Spp.)." Journal of Food Composition and Analysis 23 (6): 525–32. https://doi.org/10.1016/J.JFCA.2009.12.003.

Hadj Saadoun, Jasmine, Luca Calani, Martina Cirlini, Valentina Bernini, Erasmo Neviani, Daniele Del Rio, Gianni Galaverna, and Camilla Lazzi. 2021. "Effect of Fermentation with Single and Co-Culture of Lactic Acid Bacteria on Okara: Evaluation of Bioactive Compounds and Volatile Profiles." Food and Function 12 (7): 3033–43. https://doi.org/10.1039/d0fo02916e.

Hashemi, Seyed Mohammad Bagher, and Dornoush Jafarpour. 2020. "Fermentation of Bergamot Juice with Lactobacillus Plantarum Strains in Pure and Mixed Fermentations: Chemical Composition, Antioxidant Activity and Sensorial Properties." LWT 131 (September). https://doi.org/10.1016/j.lwt.2020.109803.

Hernández-Becerra, Ezequiel, María de los Angeles Aguilera-Barreiro, Margarita Contreras-Padilla, Esther Pérez-Torrero, and Mario E. Rodriguez-Garcia. 2022. "Nopal Cladodes (Opuntia Ficus Indica): Nutritional Properties and Functional Potential." Journal of Functional Foods 95 (August):105183. https://doi.org/10.1016/J.JFF.2022.105183.

Hur, Sun Jin, Seung Yuan Lee, Young Chan Kim, Inwook Choi, and Geun Bae Kim. 2014. "Effect of Fermentation on the Antioxidant Activity in Plant-Based Foods." Food Chemistry. Elsevier Ltd. https://doi.org/10.1016/j.foodchem.2014.03.112.

John, Warren A., Nina L. Böttcher, Britta Behrends, Marcello Corno, R. N. D'souza, Nikolai Kuhnert, and Matthias S. Ullrich. 2020. "Experimentally Modelling Cocoa Bean Fermentation Reveals Key Factors and Their Influences." Food Chemistry 302 (January). https://doi.org/10.1016/j.foodchem.2019.125335.

Jorge, Ana O.S., Anabela S.G. Costa, and M. Beatriz P.P. Oliveira. 2023. "Adapting to Climate Change with Opuntia." Plants. Multidisciplinary Digital Publishing Institute (MDPI). https://doi.org/10.3390/plants12162907.

Kock, G. C. De. 2001. "The Use of Opuntia as a Fodder Source in Arid Areas of Southern Africa." FAO Plant Production and Protection Paper.

Lee, I. Hsin, Yu Hsiang Hung, and Cheng Chun Chou. 2008. “Solid-State Fermentation with Fungi to Enhance the Antioxidative Activity, Total Phenolic and Anthocyanin Contents of Black Bean.” International Journal of Food Microbiology 121 (2): 150–56. https://doi.org/10.1016/j.ijfoodmicro.2007.09.008.

Liu, Rui, Suyun Li, Bo Yang, Lei Chen, Qingfeng Ge, Guoyuan Xiong, Hai Yu, Mangang Wu, and Wangang Zhang. 2021. “Investigation of the Antioxidant Capacity of Cell-Free Extracts from Lactobacillus Plantarum NJAU-01 Obtained by Different Cell Disruption Methods.” LWT 152 (December). https://doi.org/10.1016/j.lwt.2021.112393.

López-Domínguez, C. M., M. O. Ramírez-Sucre, and I. M. Rodríguez-Buenfil. 2019. “Enzymatic Hydrolysis of Opuntia Ficus-Indica Cladode by Acinetobacter Pittii and Alcohol Fermentation by Kluyveromyces Marxianus: PH, Temperature and Microorganism Effect.” Biotechnology Reports 24 (December). https://doi.org/10.1016/j.btre.2019.e00384.

Mazari, Azzedine, and Liberato Portillo. 2021. “Article in Journal of the Professional Association for Cactus Development.” https://doi.org/10.56890/jpacd.v23i.320.

Medina-Torres, L, E Brito-De, La Fuente, B Torrestiana-Sanchez, and R Katthain. 2000. “Rheological Properties of the Mucilage Gum (Opuntia Ficus Indica).” www.elsevier.com/locate/foodhyd.

Mena, Pedro, Michele Tassotti, Lucía Andreu, Nallely Nuncio-Jáuregui, Pilar Legua, Daniele Del Rio, and Francisca Hernández. 2018. “Phytochemical Characterization of Different Prickly Pear (Opuntia Ficus-Indica (L.) Mill.) Cultivars and Botanical Parts: UHPLC-ESI-MSn Metabolomics Profiles and Their Chemometric Analysis.” Food Research International 108 (June):301–8. https://doi.org/10.1016/J.FOODRES.2018.03.062.

Mondragón-Jacobo, Candelario, Santiago De, J Méndez-Gallegos, and Genaro Olmos-Oropeza. 2001. “Opuntia as Forage CULTIVATION OF OPUNTIA FOR FODDER PRODUCTION: FROM RE-VEGETATION TO HYDROPONICS.”

Montijo-Prieto, Soumi De, María del Carmen Razola-Díaz, Federica Barbieri, Giulia Tabanelli, Fausto Gardini, Maria Jiménez-Valera, Alfonso Ruiz-Bravo, Vito Verardo, and Ana Ma Gómez-Caravaca. 2023. “Impact of Lactic Acid Bacteria Fermentation on Phenolic Compounds and Antioxidant Activity of Avocado Leaf Extracts.” Antioxidants 12 (2): 298. https://doi.org/10.3390/ANTIOX12020298/S1.

Navarrete-Bolaños, J. L., E. Fato-Aldeco, K. Gutiérrez-Moreno, J. E. Botello-Álvarez, H. Jiménez-Islas, and R. Rico-Martínez. 2013. “A Strategy to Design Efficient Fermentation Processes for

Traditional Beverages Production: Prickly Pear Wine." Journal of Food Science 78 (10). https://doi.org/10.1111/1750-3841.12237.

Nefzaoui, Ali, Paolo Inglese, and Tesfay Belay. 2010. "Improved Utilization of Cactus Pear for Food, Feed, Soil and Water Conservation and Other Products in Africa Editors."

Ozturk, Tarik, María Ángeles Ávila-Gálvez, Sylvie Mercier, Fernando Vallejo, Alexis Bred, Didier Fraisse, Christine Morand, Ebru Pelvan, Laurent Emmanuel Monfoulet, and Antonio González-Sarrías. 2024. "Impact of Lactic Acid Bacteria Fermentation on (Poly)Phenolic Profile and In Vitro Antioxidant and Anti-Inflammatory Properties of Herbal Infusions." Antioxidants 13 (5). https://doi.org/10.3390/antiox13050562.

Pontonio, E., M. Montemurro, D. Pinto, B. Marzani, A. Trani, G. Ferrara, A. Mazzeo, M. Gobbetti, and C. G. Rizzello. 2019. "Lactic Acid Fermentation of Pomegranate Juice as a Tool to Improve Antioxidant Activity." Frontiers in Microbiology 10 (JULY). https://doi.org/10.3389/fmicb.2019.01550.

Procacci, Silvia, Emanuel Bojórquez-Quintal, Giovambattista Platamone, Oliviero Maccioni, Vania Lo Vecchio, Vincenzo Morreale, Chiara Alisi, et al. 2021. "Opuntia Ficus-Indica Pruning Waste Recycling: Recovery and Characterization of Mucilage from Cladodes." Natural Resources 12 (4): 91–107. https://doi.org/10.4236/NR.2021.124008.

Quines-Lagmay, Venus C., Beom Gyun Jeong, William L. Kerr, Sung Gil Choi, and Jiyeon Chun. 2020. "Antioxidative Properties of Eastern Prickly Pear (Opuntia Humifusa) Fermented with Lactic Acid Bacteria and Cell Wall-Hydrolyzing Enzymes." LWT 122 (March). https://doi.org/10.1016/j.lwt.2020.109029.

Quinzio, Claudia, Carolina Ayunta, Beatriz López de Mishima, and Laura Iturriaga. 2018. "Stability and Rheology Properties of Oil-in-Water Emulsions Prepared with Mucilage Extracted from Opuntia Ficus-Indica (L). Miller." Food Hydrocolloids 84 (November):154–65. https://doi.org/10.1016/J.FOODHYD.2018.06.002.

Serra, Ana Teresa, Rui O. Duarte, Maria R. Bronze, and Catarina M.M. Duarte. 2011. "Identification of Bioactive Response in Traditional Cherries from Portugal." Food Chemistry 125 (2): 318–25. https://doi.org/10.1016/J.FOODCHEM.2010.07.088.

Stavi, I. 2022. "Ecosystem Services Related with Opuntia Ficus-Indica (Prickly Pear Cactus): A Review of Challenges and Opportunities." Agroecology and Sustainable Food Systems.

Stintzing, Florian C, and Reinhold Carle. 2005. “Review Cactus Stems (Opuntia Spp.): A Review on Their Chemistry, Technology, and Uses.” Mol. Nutr. Food Res 49:175–94. https://doi.org/10.1002/mnfr.200400071.

Timpanaro, G., A. Urso, D. Spampinato, and V. T. Foti. 2015. “Cactus Pear Market in Italy: Competitiveness and Perspectives.” Acta Horticulturae 1067 (January):407–15. https://doi.org/10.17660/ACTAHORTIC.2015.1067.56.

Yamina, Boudaoud, and Abdessemed Dalila. 2021. “PHYSICOCHEMICAL CHARACTERIZATION OF JAMS, ARTINASAL, AND INDUSTRIAL PRODUCED FROM THE PRICKLY PEAR FRUIT OF OPUNTIA FICUS-INDICA L” 14:2021. https://doi.org/10.22159/ajpcr.2021v14i2.40227.

Opuntia cladodes fermentation: an insight into antimicrobial activity and volatile profile

Marrella Martina, Figuccia Sonia, Ricci Annalisa, Hadj Saadoun Jasmine, Del Vecchio Lorenzo, Cirlini Martina, Bernini Valentina, Lazzi Camilla

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Abstract

Opuntia ficus indica (OFI) cladodes have garnered significant attention due to their rich content of bioactive compounds, which hold promise for diverse applications across various fields. Despite this potential, limited research has examined the effects of cladodes fermentation. Notably, lactic acid fermentation is known to enhance aromatic profiles and modify bioactive compounds, offering a transformative approach to improving sensory and functional properties.

This study delves into the lacto-fermentation of OFI cladodes using various strains and conditions to assess its impact on antimicrobial activity and volatile compound profiles. Both fermented and unfermented OFI extracts demonstrated a relevant antimicrobial activity, particularly against *Bacillus coagulans*, as well as partial inhibition of *Rhodotorula mucilaginosa, Zygosaccharomyces bailii*, and *Leuconostoc mesenteroides*. Intriguingly, fermentation induced significant changes in the volatile composition, with certain compounds such as ethanol, 2-hexen-1-ol, 2-furanmethanol, p-mentha-1,8-dien-7-ol, 2-octenal, acetoin, and acetic acid ethenyl ester detected exclusively in the fermented matrices.

This study underscores the potential of lactic acid fermentation as a strategy to refine the aromatic profile of OFI cladodes while preserving their antimicrobial properties. By generating a unique mixture of volatile compounds, fermentation paves the way for the application of OFI extracts as natural additives in industries such as food, feed, or perfumery. These findings lay the groundwork for utilizing fermented OFI cladodes as multifunctional ingredients of natural origin.

Introduction

Opuntia ficus indica (OFI), a member of the Cactaceae family, originates from Mexico, where it is valued for its culinary and medicinal uses. The plant is also widely distributed in Africa, India, Australia and some countries of the Mediterranean area since it grows preferably in arid or semi-arid environments as most tropical and sub-tropical plants (Pimienta-Barrios 1994; Stintzing and Carle 2005; Maiuolo et al. 2024). Its capability to grow in hostile environments is due to the photosynthetic mechanism of the cladodes, known as Crassulacean acid metabolism (CAM), which is an adjustment of the photosynthesis to a low presence of water or CO2 (Cushman and Bohnert 1999).

The entire plant is composed of flowers, fruits also called prickly pear, cladodes, roots and leaves, visible only on the tender cladodes (Martins et al. 2023). Each part find applications in several fields, as cosmetic, nutraceutic, pharmaceutic, food industry, and also as alternative fuels, fount of nectar for bees, preserve fauna and control of soil erosion (Nharingo and Moyo 2016; Ventura-Aguilar et al. 2017; Alves et al. 2008; Inglese et al. 2017; Stintzing et al. 2005b; Martins et al. 2023). One of the fields of most interest regards human health. To name some of the applications of the single constituents of the plant, flowers have an anti-hemorrhoid effect, fruits are administered as antiulcerative and antidiarrheal agents, while syrup obtained from cladodes is effective in the whooping cough treatment (El-Hawary et al. 2021). Recently, cladodes obtained attention because of the presence of bioactive compounds, which could be easily extracted and find applications in different fields. Cladodes are, in fact, rich in malic acid, pectin, mucilage, vitamins, minerals, and carotenoids. One of the main component that make them interesting is polyphenolic fraction, whose content change based on the plant stage (Valentini et al. 2018; Rocchetti et al. 2018; De Santiago et al. 2018; Maiuolo et al. 2024).

Polyphenolic compounds present in vegetal foods are widely investigated for their capability to

inhibit the growth of microorganisms such as foodborne pathogens; it was demonstrated in several studies that vegetal extracts negatively affect the growth of different pathogenic bacteria, as Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa (Brambilla et al. 2017; Nostro et al. 2016), Lysteria monocytogenes, Bacillus cereus and Pseudomonas spp. (Ricci et al. 2021a).

Specifically, methanolic cladode extracts have demonstrated to cause membrane damage and reduction of ATP levels in Vibrio colerae (Sánchez et al. 2010) and to be toxic for Salmonella typhimurium, Enterobacter aerogenes, and Enterococcus faecalis. On the other hand, aqueous extracts have shown to interfere with the development of Klebsiella pneumoniae and Pseudomonas aeruginosa (Nawas 2018; Barba et al. 2022). Both of methanolic and aqueous extracts can inhibit the growth of Staphylococcus aureus and Escherichia coli (Nawas 2018; Blando et al. 2019; Barba et al. 2022).

Even if the fermentation of *Opuntia* spp. residues have been gaining attention in the last years, only few studies have explored the impact of lactic acid fermentation on cladodes (Carpena et al. 2023; Filannino et al. 2016; Barba et al. 2022). Lactic fermentation has an impact on the volatile components, which can be leveraged to obtain ingredients of interest in the food industry. Indeed, the fermentation can enhance aromatic notes or diminish undesirable off-flavours in different products. The modifications of the aromatic profile are the result of the bacterial metabolism, which can generate various catabolites depending on the available nutritional compounds. These catabolites include both aromatic compounds and aroma precursors, each contributing distinct sensory characteristics (Hadj Saadoun et al. 2021).

Considering the potential of the fermentation process and the lack of studies on *Opuntia*, this work wants to investigate the lacto-fermentation of *Opuntia* cladodes exploiting different LAB species, in mono and co-colture. In particular, the effect of bacterial metabolism on the antimicrobial activity and the volatile profile was evaluated. This work open up the perspective to use cladodes as a

starting substrate to produce fermented biomasses with distinctive blend of volatiles that could be used as ingredients in food formulations, as well as antimicrobial extracts that could serve as preservatives to inhibit the growth of spoilage microorganisms.

Materials and Methods

Sample collection and fermentation

Opuntia ficus indica preparation

OFI cladodes were collected on a farm located in Apulia (Italy) in April 2023.

Before fermentation, they were chopped using a commercial blender (Kenwood, China), sterilized at 121°C for 20 minutes in glass jars (45 g) and stored at -20 °C until their use.

Cladodes fermentation

Five lactic acid bacteria (LAB), *Leuconostoc mesenteroides* 6089, *Levilactobacillus brevis* 4867, *Lactobacillus delbruecki* 1865, *Lacticaseibacillus rhamnosus* 2310 and *Lactobacillus helveticus* 5519, belonging to the University of Parma Culture Collection (UPCC), were used as starters in the OFI cladodes fermentation. Bacterial strains were used as mono and co-cultures to steer the fermentation process. All the strains were maintained at −80 ˚C in Man Rogosa and Sharpe (MRS) broth (Oxoid, UK) and added with 12.5% glycerol (v/v). Before their use, LAB were revitalized three times with 3% (v/v) inoculum in the MRS broth and incubated at their optimal temperature for 24 h, 30°C for *L. brevis* 4867, *L. mesenteroides* 6089, 37°C for *L. rhamnosus* 2310, *L. helveticus* 5519 and 42°C for *L. delbrueckii* 1865.

Before fermentation the strains were cultivated in MRS broth overnight to reach the microbial load of ca. 9 Log CFU/mL. Cell cultures were then centrifuged (10 minutes, 10.000 rpm, 4 °C) (Centrifuge 5810R, Eppendorf SRL), washed in Ringer's solution three times and eventually resuspended in water. The matrix was inoculated with an initial microbial load of 6 Log CFU/g with single cultures (*L. delbruecki, L. mesenteroides* and *L. brevis)* or with co-culture obtained by mixing the strains in equal

concentration (*L. rhamnosus* and *L. helveticus)*. All samples were incubated for 24 h at 25°C as well as the control unfermented samples (sterilised but not inoculated). LAB load was checked after inoculation (T_0) and at the end of fermentation (T_{24}) by microbial plate count on MRS agar. The pH (Beckman, CA, USA) was measured for each sample before and after fermentation. The fermentations were carried out in duplicate and the results reported as the mean values of the data ± standard deviation.

Antimicrobial activity

Samples preparation and extraction

After the fermentation process, all the samples were stored at -20°C and freeze-dried (Lio 5P, Milano, Italy). The samples extraction was carried out using the accelerated solvent extractor (ASE) system (Thermo Fisher, Italy). 0.2 g of sample powder was mixed with diatomaceous earth and placed in 5 mL stainless steel reaction cells containing a filter at the bottom. Ethanol/water 50/50 v/v was used as solvent and the extraction was conducted at 65°C for 5 minutes. Extracts were put in a concentrator (Eppendorf, Italy) until solvent evaporation and re-suspended in water to a concentration of 300 mg/mL.

Microbial strains tested

In order to test the antimicrobial activity of the extracts obtained from fermented cladodes, four strains were chosen for their capability to induce food spoilage: *Rodotorula mucilaginosa* DSM 70403, *Zygosaccharomyces bailii* ATCC 8766, *Bacillus coagulans* LMG 17451 and *Leuconostoc mesenteroides* UPCC 4258. *R. mucilaginosa* DSM 70403, *Z. bailii* ATCC 8766 were kept at -80 °C in

Sabouraud broth (Oxoid, Milano), *B. coagulans* in Tryptic Soy Broth (Oxoid, Milano) and *L. mesenteroides* in Man Rogosa Sharp broth (WVR Chemicals) added with 12.5% glycerol (v/v). Each strain was cultured twice in the above mentioned media at their optimal temperature growth, 25° C for yeast and 37° C for bacteria, before the analyses.

Agar well diffusion assay

The antimicrobial activity was primarily carried out employing the agar well diffusion assay. The strains tested were diluted until they reached the concentration of 8 Log CFU/mL. A sterile cotton swab was dipped into the obtained suspension, and it was spread on Tryptic Soy Agar (TSA), Sabouraud agar and MRS agar (respectively for *B. coagulans, R. mucilaginosa, Z. bailii* and *L. mesenteroides*). Wells with a diameter of 7 mm were created in the agar and filled with 30 µL of extract (300 mg/mL). The antimicrobial activity was evaluated for extracts obtained from fermented and unfermented matrix subjected to sterilization process. Plates were incubated at 25 °C for yeast and 37 °C for bacteria in aerobic conditions and the antimicrobial activity was evaluated by measuring the total inhibition zone diameter (mm) observable after 24 and 48 h of incubation. Analyses were performed in triplicate and average values ± standard deviations were reported.

Evaluation of the antimicrobial effect of extracts on microbial growth

The antimicrobial activity of the extracts was also studied by using the Tecan microplate reader (Infinite® 200Pro, Tecan Group Ltd.). The analysis was carried out in 96-well microplates (VWR). In each well, 100 µL of medium broth (Sabouraud broth for yeast strains, TSB for *B. coagulans* and MRS broth for *L. mesenteroides*), and 5 µL of each fermented extract at the concentration of 300 mg/mL were added to reach the concentration of 15 mg/mL. Each strain was then inoculated (5 µL) to reach

a concentration of 2 Log CFU/mL. The instrument was set to take a spectrophotometric measurement (Abs 600 nm) every 10 minutes for 48 hours.

HS-SPME/GC-MS technique applied for VOCs determination

Volatile organic compounds (VOCs) profile was obtained by the application of head space solid phase micro-extraction (HS-SPME) coupled with GC-MS technique, following the indications given by Ricci et al. (2018a). In particular, 2 g of sample were extracted and analysed applying the same conditions reported by Ricci et al. (2018a), in terms of sampling time and temperature, SPME fiber type, GC separation, and MS detection. All the detected compounds were identified on the basis of their registered mass spectra compared with those listed in the instrument library (NIST14). At the same time, VOCs concentration was calculated on the basis of a reference compound (Toluene) added in proper quantity to each sample prior to the analysis, that was repeated twice.

Statistical analysis

All conditions were performed with two biological replicates. For antimicrobial activity and for VOCs determination each sample was analysed in duplicate. Quantitative data are reported as mean ± standard deviation (SD).

Using the IBM SPSS Statistics 29 software, the data obtained from the antimicrobial activity were subjected to the T-test, and stars were used to indicate the conditions with a significant difference ($p<0.05$) compared to the unfermented sample.

VOCs data obtained were subjected to one-way Analysis of Variance, through R version 4.2.2, with mean separation conducted using Tukey's test ($p \leq 0.05$). Significant differences were evaluated for the same compound among the different samples obtained from the fermented and unfermented

matrices.

The obtained data were statistically elaborated by means of SIMCA 17 (Sartorius, Gottingen, Germany) used to perform principal component analysis (PCA), and MetaboAnalyst 6.0 that was used to build heat map.

Results and discussions

Cladodes fermentation

OFI cladodes were fermented with different strains: *L. mesenteroides* 6089, *L. brevis* 4867, *L. delbruecki* 1865 and, for a co-culture approach, with *L. rhamnosus* 2310 and *L. helveticus* 5519. All the samples were incubated at 25°C for 24 hours. After 24 h of fermentation, all the strains and co-culture grown in the matrix (ca. 2 Δ Log CFU/g) except for *L. delbruecki* as reported in Table 1. To the best of our knowledge, only one study available in literature reported a growth for *L. brevis* and the absence of growth for *L. mesenteroides*, in line with our results (Filannino et al. 2016). On the other hand, no known studies have previously used *L. delbruecki* for fermentations of OFI cladodes.

Table 1. Growth ability of all strains used for the fermentation of OFI cladodes, all the samples were incubated at 25°C for 24 h. For each sample was mensurated the pH at the end of the fermentation process, the results were reported as mean ± standard deviation of two biological replicates

Strains	Sample	T_0 (Log CFU/g)	T_{24} (Log CFU/g)	LAB growth (Δ Log CFU/g)	Initial pH T_0	Final pH T_{24}
L. delbruecki	L.d.	4.30 ± 0.00	4.09 ± 0.06	-0.21 ± 0.06	4.58 ± 0.01	4.63 ± 0.04
L. mesenteroides	L.m.	5.78 ± 0.01	7.78 ± 0.31	2.00 ± 0.33	4.54 ± 0.02	4.45 ± 0.01
L. brevis	L.b.	6.00 ± 0.03	8.27 ± 0.16	2.26 ± 0.12	4.57 ± 0.04	4.49 ± 0.07
L. rhamnosus + L. helveticus	L.r. L.h.	5.98 ± 0.01	8.26 ± 0.20	2.27 ± 0.21	4.57 ± 0.00	4.37 ± 0.03

Nowadays, lactic acid fermentation applied on vegetal matrices is gaining particular relevance and species and strains selection represent an essential aspect to valorize these substrates better (Filannino, et al. 2018). The essential condition for the selection is the microbial growth in an environment characterised by low pH and high polyphenols content, as a vegetal matrix could be. Cladodes pH, in particular, is not excessively low (mean value 4.60 ± 0.03), reason for which all the

strains succeeded in growing, except for *L. delbruecki*, even if pH modifications were not observed (Table 1).

Antimicrobial activity evaluation

Traditional food preservation strategies have long been employed to control microbial spoilage, contamination and spoilage by microorganisms remain still challenges that are not fully resolved. Plants, with their diverse array of constituents, are valuable sources of novel, potentially biologically active molecules with antimicrobial properties (Negi 2012).

In this study, those microorganisms that can cause spoilage in food products were considered. In particular, the attention was focused on those food products where the addition of the cladode-based antimicrobial extract would be feasible. Products such as fruit and vegetable juices can be spoiled by lactic acid bacteria and yeasts, while in canned vegetables, Bacillaceae are among the main spoilage agents. These are the reasons why in this work the antimicrobial activity was evaluated against *L. mesenteroides, Z. bailii*, *R. mucilaginosa* and *B. coagulans*. All the extracts have shown antimicrobial activity against *B. coagulans* (Figure 1) in a preliminary evaluation through agar well diffusion assay. Nevertheless, the antimicrobial activity observed was comparable to the activity detected for unfermented samples. Instead, no activity was observed against *L. mesenteroides*, *R. mucilaginosa* and *Z. bailii*.

Among spoilage microrganisms, lactic acid bacteria, such as *Lactobacillus* and *Leuconostoc*, may be regarded as spoilage agents in various foodstuffs such as juice and vegetable affecting the texture and colour (Aneja et al. 2014). Moreover, the production of diacetyl and acetoin metabolites affects the flavour of fruit juices.

Similar to bacteria, yeast can also cause food spoilage and *Z. bailli* is a yeast that can spoil acid food

with an high sugar content with a particular resistant to common preservatives (Snyder 2024; Aneja et al. 2014; Bento de Carvalho et al. 2024). As *Z. bailli, R. mucilaginosa* is also capable of surviving in juices leading to alterations in quality and volatile organic compound profiles (Fikri et al. 2024; Fikri et al., 2023).

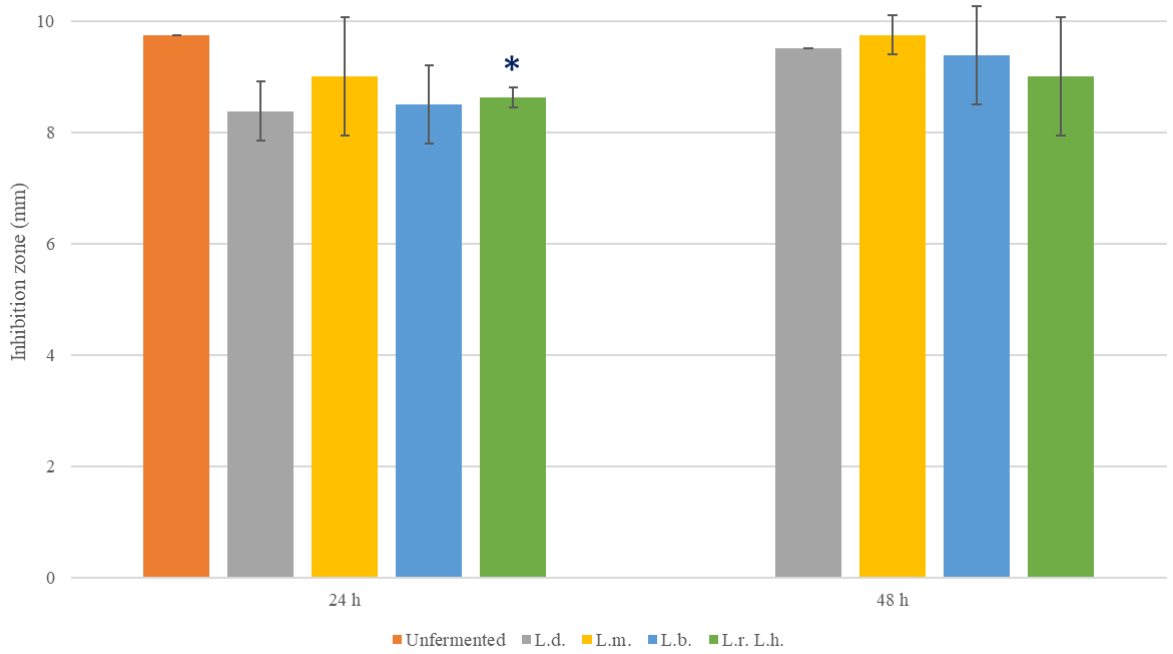

Figure 1. Antimicrobial activity of fermented and not fermented cladodes OFI extracts after 24 and 48 h against B. coagulans. Orange bar: unfermented extract, grey bar: extract obtain after L. delbruecki 1865 fermentation, yellow bar: extract obtain after L. mesenteroides 6089 fermentation, light blue bar: extract obtain after L. brevis 4867 fermentation, green bar: extract obtain after co-culture (L. rhamnosus 2310 and L. helveticus 5519) fermentation. Inhibition zone is reported in mm as mean values ± standard deviation of two biological replicates.

Some Bacillaceae, as *B. coagulans*, are responsible for the spoilage of canned food (André et al. 2017), where fermentation is undesired. *B. coagulans* is capable of growth at low pH (approximately 4) and exhibits resistance to heat treatment through sporulation (Palop et al. 1999; André et al. 2017). These species does not produce gas but causes an increase in lactic acid, which, in addition to acidifying the product, can also result in sensory changes (André et al. 2017).

The antimicrobial activity of OFI cladodes has been the topic of several studies (Blando et al. 2019a; Lamia et al. 2022; Nam et al. 2023; Welegerima et al. 2018) but no one has previously investigated the effect on the spoilage microbial target considered in our study.

In the study conducted by Lamia et al. (2022), the OFI cladode extracts were examined for their antimicrobial activity against a range of bacterial species, including *Escherichia coli*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Bacillus subtilis* and *Pseudomonas aeruginosa*. The findings indicated that the extracts exhibited a broad inhibitory effect against Gram-positive bacteria and a slightly reduced efficacy against Gram-negative bacterial strains (Lamia et al. 2022). It is frequently observed that the antimicrobial activity of plant extracts is carried out by phenolic compounds, which are involved in membrane damage, protein and cell wall binding and enzyme inactivation. Furthermore, these compounds can act as pro-oxidants, leading to damage of DNA, lipids and other biological molecules (Blando et al. 2019a). The OFI cladodes extracts contained a range of phenolic substances, including tannins, glycosides, alkaloids, flavonoids, saponins, steroids, alkaloids and amino acids (Welegerima et al. 2018). In the study conducted by Blando et al. (2019a), the antimicrobial activity of OFI cladodes is linked in particular to isorhamnetin. However, other compounds, such as saponins, seems to inhibit the growth of microorganisms (Nam et al. 2023).

For the strains that have not shown inhibition through the agar well diffusion assay (*L. mesenteroides, R. mucilaginosa* and *Z. bailii*) the extract's activity was also evaluated in a liquid medium. Differently to agar well diffusion assay, in this test an initial lower artificial contamination (2 Log CFU/ml) was taken into account.

As reported in Figure 2, *L. mesenteroides*, *R. mucilaginosa* and *Z. bailii* showed a different growth curve in presence or absence of the extracts.

An extended Lag phase was observed for *L. mesenteroides* in the presence of all the extracts (fermented and unfermented). In the case of the extract obtained after fermenting the matrix with

L. mesenteroides (Figure 2, yellow line), the growth after the Log Phase was basically absent. The same results were observed for the extract obtained after fermenting the matrix with *L. brevis* (Figure 2, light blue line). Taken together, the results suggest that the antimicrobial activity observed may be partially attributed to bioactive compounds present in *Opuntia ficus-indica* cladodes, including phenolic compounds and other secondary metabolites such as flavonoids and saponins. Several studies have shown that lactic fermentation of plant matrices can enhance the concentration and/or bioavailability of such compounds (Hur et al. 2014; Filannino et al. 2018). During fermentation, hydrolytic enzymes produced by LAB may release bound phenolics from the plant cell wall matrix or convert them into more active forms, increasing their antimicrobial potential.

In particular, the strong inhibition of *L. mesenteroides* growth observed in the presence of the extracts fermented with *L. mesenteroides* and *L. brevis* might be related to the production of specific phenolic acids, indeed, different authors have reported that the fermentation process leads to the production of 2-hydroxyisocaproic acid and ferulic acid, both of which possess antimicrobial properties as well as, 3-phenylactic acid, 4-hydroxyphenylactic acid, and 2-hydroxyisocaproic acid (Vougiouklaki et al. 2022; Sakko 2012; Axel et al. 2016).

While some inhibition was also seen with unfermented extracts, the enhanced or distinct activity in specific fermented samples suggests a role of fermentation in modulating the antimicrobial profile. Although a direct chemical correlation between individual phenolic compounds and antimicrobial activity was not established in this study, the concomitant presence of inhibitory effects and the known antimicrobial potential of phenolic-enriched extracts support this hypothesis.

Eventually, *R. mucilaginosa* and *Z. bailii* showed growth inhibition when the extracts were added without appreciable differences between extracts obtained after fermentation or from the unfermented matrix. In detail, the Lag Phase in the presence of the extracts was not extended but the growth rate of Log Phase was reduced.

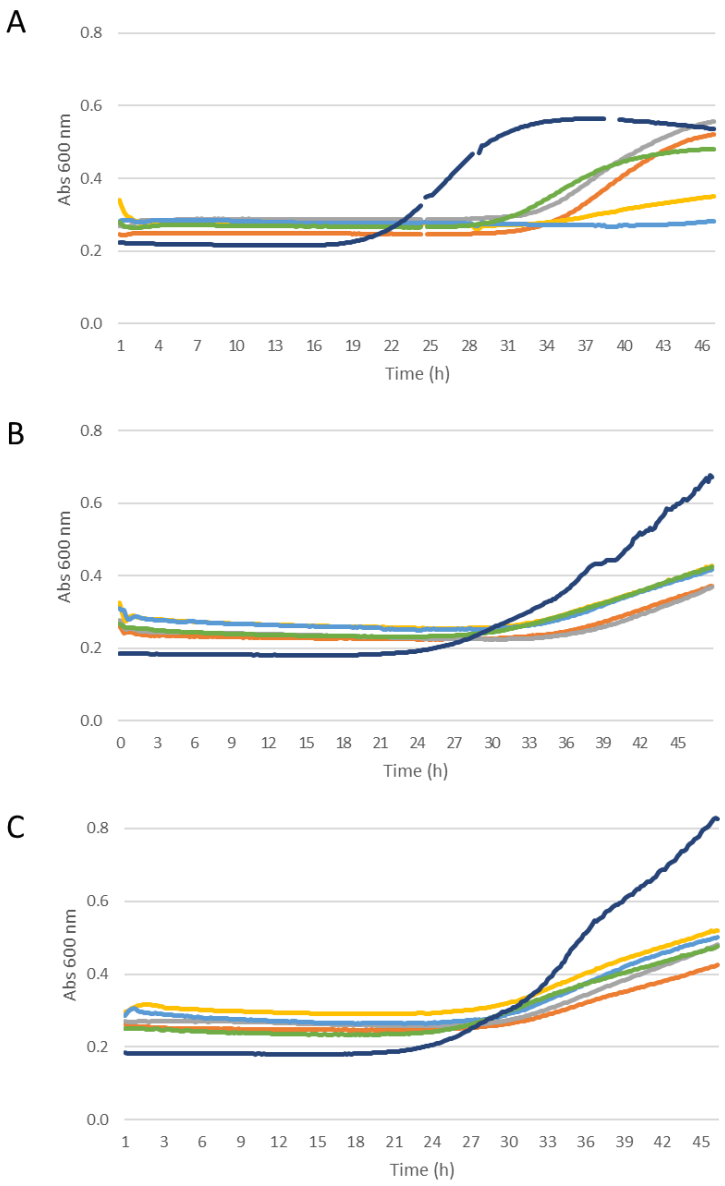

Figure 2. Evaluation of the antimicrobial effect of extracts on microbial growth. Graph A shows the growth of L. mesenteroides, B of R. mucilaginosa and C of Z. bailii in the presence or absence of the extracts. The unfermented extract was reported in the first line (in orange), L. delbruecki 1865 in the grey line, L. mesenteroides 6089 in the yellow line, L. brevis 4867 in the light blue line, in the green line with a co-culture used L. rhamnosus 2310 and L. hehelveticus 5519, and in the blue line the positive control. Results were reported as mean of two biological replicates.

Therefore, the antimicrobial activity against yeasts could be attributable to the composition of the matrix, probably due to the presence of phenolic compounds, rather than to lactic fermentation.

Volatile profile of fermented cladodes

The volatile organic compounds (VOCs) profile was characterised by applying headspace solid phase micro-extraction (HS-SPME) coupled with the GC-MS technique. A total of 38 compounds for each sample, belonging to the classes of aldehydes, alcohols, ketones, acids, ester, terpenic and phenolic compounds, were quantified and reported in Table 2.

Alcohols and aldehydes are the predominant VOCs observed. Within these classes, great differences in concentrations between the fermented and control samples are evident. After fermentation, an increase in alcohols is observed with heterofermentative LAB (for *L. mesenteroides* 72.62 ± 6.16 µg/mL, for *L. brevis* 77.98 ± 12.40 µg/mL compared to unfermented sample 64.23 ± 8.78) while a reduction is evident in the samples fermented with *L. delbruecki* (51.14 ± 14.35 µg/mL) and the co-culture *L. rhamnosus* and *L. helveticus* (45.22 ± 15.94 µg/mL).

The alcohol 2-hexenol, already present in the raw matrix at a concentration of 53.24 ± 6.16 µg/mL, is characterised by fresh green notes. This compound was already reported in previous paper after lactic acid fermentation of plant substrates (Ricci et al., 2018). Heterofementative fermentation with *L. mesenteroides* and *L. brevis*, lead to a concentration of 61.28 ± 5.03 and 62.40 ± 10.79 µg/mL, respectively. Conversely, in samples inoculated with *L. delbruecki* or *L. rhamnosus* and *L. helveticus* co-culture, the concentration of 2-hexenol showed a decrease (41.21 ± 12.41 and 35.51 ± 12.48 µg/mL, respectively.

Table 2. Concentrations (µg/mL) of volatile compounds identified in OFI cladodes (not fermented) and OFI cladodes fermented obtained after L. delbrueckı 1865 fermentation, after L. mesenteroides 6089 fermentation, after L. brevis 4867 fermentation and after co-culture with L. rhamnosus 2310 and L. helveticus 5519 fermentation. For each compound, a specific variable number (VOC), used in statistical and graphical analyses, was assigned. Values are reported as mean ± standard deviation of two biological replicates. n.d., not detected in the adopted conditions.

Molecules	**Flavour note**	**VOC**	***Not fermented***	***L. mesenteroides***	***L. brevis***	***L. delbruecki***	***L. rhamnosus + L. helveticus***	***Reference***
Alcohol								
Ethanol	strong, alcoholic	VOC1	n.d.	n.d.	3.86 ± 0.10	n.d.	n.d.	(Ricci (b) et al. 2018)
1-Pentanol	fermented	VOC7	0.32 ± 0.05 ^{c}	0.80 ± 0.07 ^{a}	0.62 ± 0.13 ^{b}	n.d.	0.57 ± 0.08 ^{b}	(Ricci (a) et al. 2018)
2-Hexen-1-ol	fruity, green, leafy	VOC11	n.d.	0.47 ± 0.07 ^{a}	0.48 ± 0.22 ^{a}	n.d.	n.d.	(Ricci (b) et al. 2018)
1-Hexanol	herbal	VOC13	4.61 ± 1.81 ^{a}	5.84 ± 1.00 ^{a}	6.01 ± 1.27 ^{a}	3.37 ± 1.56 ^{a}	3.81 ± 2.37 ^{a}	(Cirlini et al. 2012)
2-Hexenol	fresh green note	VOC16	53.24 ± 6.16 ^{ab}	61.28 ± 5.03 ^{a}	62.40 ± 10.79 ^{a}	41.21 ± 12.41 ^{b}	35.51 ± 12.48 ^{b}	(Ricci (a) et al. 2018)
1-Octanol	fresh green note	VOC24	1.23 ± 0.04 ^{a}	1.02 ± 0.14 ^{b}	0.84 ± 0.11 ^{b}	0.37 ± 0.07 ^{c}	0.88 ± 0.16 ^{b}	(Ricci (a) et al. 2018)
2-Octen-1-ol	citrus, green, plastic, soap	VOC25	0.78 ± 0.00 ^{a}	0.61 ± 0.02 ^{b}	0.42 ± 0.02 ^{c}	0.70 ± 0.13 ^{ab}	0.81 ± 0.01 ^{a}	(Jung et al. 2023)
2-Furanmethanol	alcoholic, chemical, caramellic, bready	VOC29	n.d.	0.93 ± 0.13 ^{a}	0.81 ± 0.20 ^{ab}	0.61 ± 0.08 ^{b}	0.31 ± 0.03 ^{c}	(Ricci (b) et al. 2018)
Benzyl alcohol	floral	VOC35	0.39 ± 0.02 ^{b}	0.38 ± 0.03 ^{bc}	0.75 ± 0.08 ^{a}	0.31 ± 0.01 ^{cd}	0.24 ± 0.02 ^{d}	(Yao et al. 2021)
3-Hexen-1-ol	green, leafy	VOC14	1.63 ± 1.81 ^{ab}	1.29 ± 0.01 ^{ab}	1.80 ± 0.31 ^{ab}	2.66 ± 0.74 ^{a}	0.85 ± 0.16 ^{b}	(Ricci (b) et al. 2018)
1-Octen-3-ol	earthy	VOC20	2.02 ± 1.10 ^{a}	n.d.	n.d.	1.91 ± 0.23 ^{a}	2.25 ± 0.76 ^{a}	(Ricci (b) et al. 2018)
Total			64.23 ± 8.78 ^{abc}	72.6161 ± 6.16 ^{ab}	77.9788 ± 12.40 ^{a}	51.1399 ± 14.354 ^{bc}	45.22 ± 15.94 ^{c}	

Aldehyde								
Pentanal	-	VOC3	2.54 ± 0.30 ^{b}	1.27 ± 0.05 ^{c}	2.16 ± 0.16 ^{bc}	1.56 ± 0.01 ^{c}	8.49 ± 1.04 ^{a}	-
Hexanal	fresh green note	VOC4	1.38 ± 0.44 ^{a}	0.08 ± 0.01 ^{b}	0.09 ± 0.01 ^{b}	0.12 ± 0.00 ^{b}	0.06 ± 0.01 ^{b}	(Ricci (a) et al. 2018)
2-Hexenal	fresh green note	VOC5	3.91 ± 0.26 ^{a}	1.25 ± 0.01 ^{b}	1.20 ± 0.11 ^{b}	3.61 ± 1.08 ^{a}	2.78 ± 1.17 ^{a}	(Ricci (a) et al. 2018)
Octanal	lemon, citrus	VOC6	0.50 ± 0.07 ^{a}	0.27 ± 0.06 ^{b}	0.21 ± 0.03 ^{b}	0.26 ± 0.01 ^{b}	0.16 ± 0.23 ^{b}	(Ramírez-Rodrigues et al. 2011)
2-Heptenal	fatty, fruity	VOC10	3.36 ± 0.51 ^{a}	2.23 ± 0.23 ^{b}	1.84 ± 0.13 ^{b}	3.22 ± 0.55 ^{a}	3.16 ± 0.71 ^{a}	(X. Feng et al. 2020)
Nonanal	fatty, rose	VOC15	3.06 ± 1.02 ^{a}	0.81 ± 0.37 ^{b}	0.17 ± 0.24 ^{b}	n.d.	0.86 ± 0.02 ^{b}	(Yao et al. 2021)
2-Octenal	green	VOC17	n.d.	1.62 ± 0.08 ^{a}	n.d.	0.82 ± 0.02 ^{b}	n.d.	(Cirlini et al. 2012)
Furfural	bready	VOC21	10.80 ± 3.61 ^{a}	n.d.	n.d.	7.72 ± 2.05 ^{ab}	4.15 ± 0.94 ^{b}	(Cirlini et al. 2012)
Benzaldehyde	bitter almond	VOC23	2.33 ± 0.03 ^{a}	1.56 ± 0.18 ^{b}	1.03 ± 0.29 ^{cd}	1.39 ± 0.05 ^{bc}	0.86 ± 0.31 ^{d}	(Hadj Saadoun et al. 2021)
2-Decenal	waxy	VOC27	1.48 ± 0.20 ^{a}	0.67 ± 0.08 ^{c}	0.50 ± 0.14 ^{c}	0.96 ± 0.15 ^{b}	0.95 ± 0.03 ^{b}	(Cirlini et al. 2012)
2-Undecenal	plasticky	VOC32	0.73 ± 0.17 ^{a}	0.64 ± 0.23 ^{ab}	0.42 ± 0.13 ^{b}	0.61 ± 0.01 ^{ab}	0.44 ± 0.02 ^{b}	(Osawa et al. 2013)
Total			30.09 ± 2.76 ^{a}	10.42 ± 0.71 ^{c}	7.61 ± 0.76 ^{c}	20.29 ± 1.76 ^{b}	21.93 ± 0.06 ^{b}	
Ketone								
1-Octen-3-one	metallic mushroom	VOC8	1.11 ± 0.05 ^{a}	0.80 ± 0.16 ^{c}	0.45 ± 0.02 ^{d}	0.99 ± 0.03 ^{ab}	0.90 ± 0.11 ^{bc}	(Lubran et al. 2005)
Acetoin	sweet, buttery, creamy, dairy	VOC9	n.d.	n.d.	n.d.	n.d.	2.36 ± 1.29	(Rajendran et al. 2023)
6-Methyl-5-heptene-2-one	fruity	VOC12	0.82 ± 0.29 ^{a}	0.60 ± 0.19 ^{ab}	0.36 ± 0.15 ^{b}	0.66 ± 0.11 ^{ab}	0.90 ± 0.07 ^{a}	(Zhou et al. 2022)

Acetophenone	sweet, pungent	VOC28	1.16	±	0.08	b	0.77	±	0.09	c	0.30	±	0.02	e	1.47	±	0.04	a	0.55	±	0.15	d	(Yao et al. 2021)
Benzyl methyl ketone	-	VOC31	0.91	±	0.16	a	0.82	±	0.00	ab	0.72	±	0.07	b	0.79	±	0.06	ab	0.53	±	0.01	c	-
Total			3.99	±	0.26	b	2.99	±	0.43	b	1.82	±	0.21	c	3.91	±	0.23	b	5.24	±	1.12	a	
Acid																							
Acetic acid	sharp, pungent, vinegar	VOC19	1.27	±	1.79	c	113.91	±	0.83	a	34.79	±	2.93	b	1.58	±	0.62	c	1.13	±	0.89	c	(Ricci (b) et al. 2018)
Butanoic acid	rancid, sweat	VOC26	0.31	±	0.24	b	0.58	±	0.11	a	0.18	±	0.01	b	0.15	±	0.04	b	0.26	±	0.08	b	(T. Li et al. 2023)
Hexanoic acid	sour, fatty	VOC34	1.67	±	0.01	b	0.92	±	0.05	c	3.35	±	0.30	a	0.95	±	0.04	c	0.59	±	0.06	d	(Yao et al. 2021)
2-Hexenoic acid	fruity, sweet, warm, herba	VOC36	2.59	±	0.34	a	2.28	±	0.43	a	1.56	±	0.24	b	0.93	±	0.10	c	0.61	±	0.04	c	(Ricci (b) et al. 2018)
Nonanoic acid (Pelargic acid)	waxy, cheese	VOC38	0.58	±	0.11	ab	0.99	±	0.59	a	0.58	±	0.05	ab	0.49	±	0.03	ab	0.34	±	0.01	b	(Yao et al. 2021)
Total			6.42	±	1.99	c	118.68	±	0.25	a	40.47	±	3.53	b	4.10	±	0.49	c	2.93	±	1.06	c	
Ester																							
Acetic acid ethenyl ester (Ethenyl acetate)	sweet, fruity	VOC2	n.d.				n.d.				n.d.				n.d.				5.01	±	0.81	a	(Luttrell 2013)
Methyl salicylate (Betula oil)	wintergreen mint	VOC33	2.90	±	0.77	b	1.23	±	0.24	c	18.21	±	0.54	a	2.97	±	0.25	b	1.44	±	0.26	c	(Ricci (b) et al. 2018)
Total			2.90	±	0.77	c	1.23	±	0.24	d	18.21	±	0.54	a	2.97	±	0.25	c	6.45	±	0.55	b	
Terpene derivative																							
Linalool oxide	earthy, floral	VOC18	2.30	±	0.29	ab	1.33	±	0.44	c	2.40	±	0.46	a	1.65	±	0.10	bc	1.52	±	0.10	bc	(Yao et al. 2021)

L-α-Terpineol	aged lemon and citrus	VOC30	0.75	±	0.21	b	0.63	±	0.02	bc	1.03	±	0.12	a	0.48	±	0.03	c	0.51	±	0.10	c	(Chigo-Hernandez et al. 2022)
p-Mentha-1,8-dien-7-ol	-	VOC37	n.d.				0.37	±	0.04	b	0.65	±	0.11	a	0.42	±	0.05	b	0.25	±	0.05	c	-
Total			3.05	±	0.64	b	2.33	±	0.64	b	4.08	±	0.66	a	2.55	±	0.30	b	2.28	±	0.08	b	
Phenolic																							
2-Methoxy-4-vinylphenol	woody, green	VOC39	2.91	±	1.02	a	1.90	±	0.01	ab	2.60	±	0.44	a	2.28	±	0.69	ab	1.19	±	0.19	b	(Yao et al. 2021)

After the fermentation process, there was a notable reduction in the total aldehyde concentration, from 30.09 ± 2.76 µg/mL in the control sample to a minimum of 7.61 ± 0.76 µg/mL in the fermented *L. brevis* sample. The initial matrix exhibited 10.80 ± 3.61 µg/mL of furfural, indicative of bready-toasted notes (Cirlini et al. 2012), which was reduced in all fermented samples. Especially after the fermentation with *L. mesenteroides* and *L. brevis,* the furfural concentration observed was below the limit of detection.

The total ketones were recorded at 3.99 ± 0.26 µg/mL in the control sample and have been reduced by using *L. mesenteroides* and *L. brevis*, at 2.99 ± 0.43 and 1.82 ± 0.21 µg/mL, respectively. There was no change in the sample treated with *L. delbruecki*, while an increase was observed in the sample fermented with the co-culture, reaching 5.24 ± 1.12 µg/mL total ketones. Among the ketone compounds, only the sample fermented with the co-culture exhibited the presence of acetoin (2.36 ± 1.29 µg/mL). This compound imparts a creamy or buttery note, and its origin can be attributed to the conversion of citrate, through the activity of citrate permease and citrate lyase of LAB, or through the conversion of diacetyl by diacetyl acetoin reductase (Rajendran et al. 2023).

The total acids in the control sample are 6.42 ± 1.99 µg/mL. A notable increase was observed in the sample containing *L. mesenteroides* (118.68 ± 0.25 µg/mL). However, a decrease is evident in the samples containing homofermenting strains. Considering acetic acid, this compound reached the concentration of 113.91 ± 0.83 and 34.79 ± 2.93 µg/mL with *L. mesenteroides* and *L. brevis*, respectively. This acid was produced during fermentation through the consumption of sugars or citric acid (Liu et al. 2023; Ricci et al. 2018a).

The total esters in the control sample are 2.90 ± 0.77 µg/mL, which subsequently decrease to 1.23 ± 0.24 µg/mL following fermentation with *L. mesenteroides*. Indeed, in the other samples, increases were observed, reaching 18.21 ± 0.54 µg/mL, 2.97 ± 0.25 µg/mL, and 6.45 ± 0.55 µg/mL in the samples fermented with *L. brevis*, *L. delbruecki*, and the co-culture *L. rhamnosus* and *L. helveticus*,

respectively.

In particular, an important increase in methyl salicylate was observed in the sample fermented with *L. brevis*, reaching a concentration of 18.21 ± 0.54 µg/mL compared to the control sample (2.90 ± 0.77 µg/mL).

In the last two classes, terpene compounds and phenols, the only few detected were found: linalool oxide, L-α-Terpineol, p-Mentha-1,8-dien-7-ol, and 2-methoxy-4-vinylphenol, respectively. These are compounds that have previously been identified by Ammar et al. (2012) in OFI flowers at different stages of flowering and also in cladodes (Wright and Setzer 2014). During fermentation, they remained unchanged or exhibited slight reductions. A previous study on OFI demonstrated that linalool oxide and 2-methoxy-4-vinylphenol are VOCs that protect the plant from pests and have a role in the communication (Rodríguez-Leyva et al. 2024). Finally, p-Mentha-1,8-dien-7-ol was exclusively present in samples undergoing fermentation, with its highest concentration (0.65 ± 0.11 µg/mL) observed after fermentation with *L. brevis*. This result is consistent with previous studies reporting the appearance or increased levels of this compound only after fermentation with LAB strains in various matrices (Hadj Saadoun et al. 2021; Liu et al. 2023).

In order to elucidate the distinctions between the samples, a principal component analysis (PCA) was conducted using VOCs concentrations as variables (Figure 3). PC1 explains 37.5% of the total variance, while PC2 accounts for a further 22.2%. Collectively, the two components account for approximately 59.7% of the total variance.

It can be observed that the VOCs are divided into three distinct groups, designated as A, B and C groups. In the upper right-hand quadrant (A), the compounds that characterise the control (unfermented) can be observed. In the lower right-hand quadrant (B), the samples obtained from fermentation with the homofermentatative *L. delbruecki* and the co-culture *L. rhamnosus* and *L. helveticus* (the former heterofermentative and the latter homofermentative) are shown. Finally, C

contains the VOCs characterising the samples fermented with heterofermentative strains (*L. mesenteroides* and *L. brevis*). This distinction between the samples highlights that the VOCs indicate the different bacterial metabolisms that define the distinct aroma profiles observed in the post-fermented product.

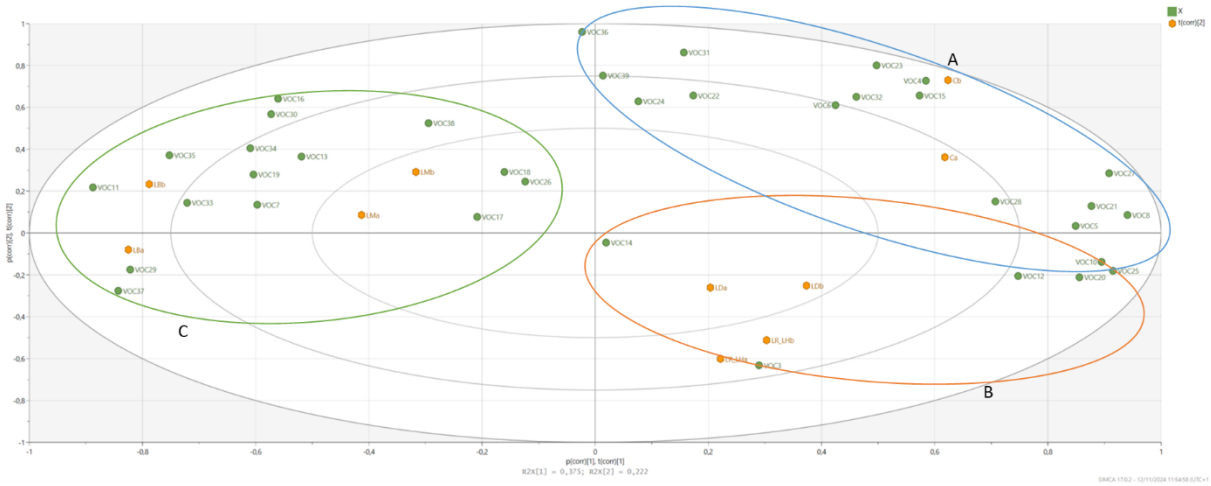

Figure 3. Bi-plot of PC1 vs. PC2, obtained using the concentrations of the volatile compounds found in the fermented and unfermented cladode samples.

The VOCs that characterise the control sample (group A) are predominantly aldehydes (hexanal, 2-hexenal, octanal, 2-heptenal, nonanal, furfural, benzaldehyde, 2-decenal, 2-undecenal) and ketones (1-octen-3-one, acetophenone, benzyl methyl ketone). These compounds tended to decrease following fermentation.

In the group C, containing samples with heterofermentative LAB (*L. mesenteroides* and *L. brevis*), an increase in the concentration of specific alcohols (2-hexen-1-ol, 1-hexanol, 2-hexenol, 2-furanmethanol) and some acids (acetic acid, butanoic acid, hexanoic acid, 2-hexenoic acid, nonanoic acid), was observed.

Samples containing *L. delbruecki* and the co-culture *L. rhamnosus* and *L. helveticus* (group B) exhibited a reduced number of VOCs, with the presence of 3 alcohol (2-octen-1-ol, 3-hexen-1-ol, 1-

octen-3-ol), an aldehyde (pentanal) and a ketone (6-methyl-5-heptene-2-one).

A heat map based on the concentration of VOCs was generated to further elucidate the similarities or the differences between the samples (Figure 4).

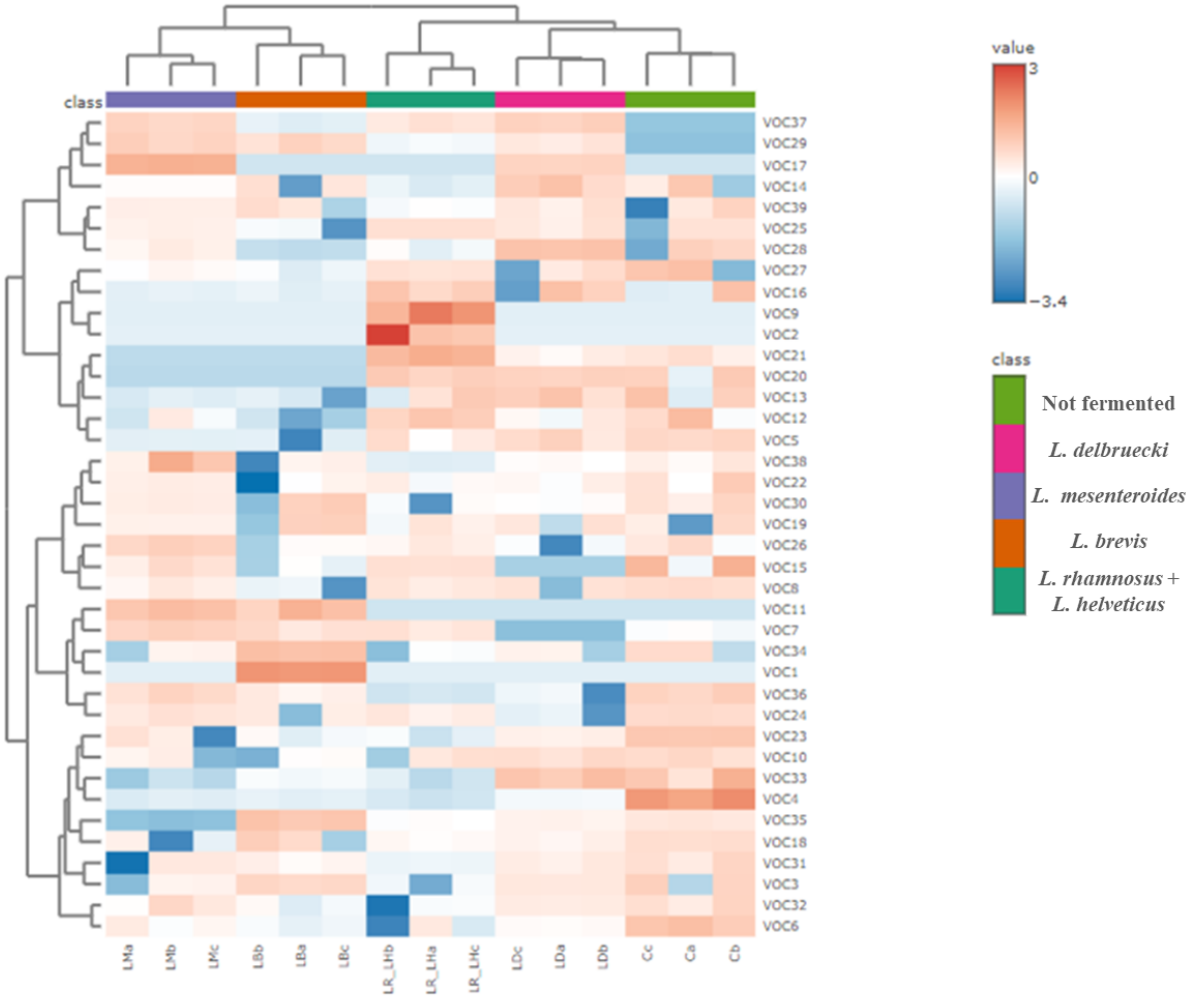

Figure 4. Heat-map visualization of the volatile profiles of the OFI cladodes fermented with L. delbruecki 1865 (fuxia bar), L. mesenteroides 6089 (purple bar), L.brevis 4867 (orange bar), co-culture L. rhamnosus 2310 and L. helveticus 5519 (dark green bar) and the control unfermented samples (green bar). The color scale represents the scaled abundance of each variable with red indicating high abundance and blue indicating low abundance. Samples are reported with the strains abbreviation LD, LM, LB, LR_LH and C for the non-fermented control sample, and the letters a, b or c refer to the biological replicates. The aromatic compounds indicated here with VOC from 1 to 39, correspond to the compounds reported in the Table2.

It can be observed that the control sample exhibits a high degree of similarity to the sample in which *L. delbruecki* was inoculated and that no growth was observed after 24 hours.

The VOCs profile of fermented co-culture constituted by *L. rhamnosus* and *L. helveticus* was similar to the previous group however some differences can be underline. Indeed, the presence of homofermentative strains, which produce almost exclusively lactic acid during fermentation, resulted in an increase in acetoin and acetic acid ethenyl ester.

A distinct cluster is formed by fermentations with *L. mesenteroides* and *L. brevis*, both of which are heterofermentative. In these samples, higher concentrations of p-mentha-1,8-dien-7-ol, 2-furanmethanol, and 2-octenal were recorded, while benzaldehyde and 2-heptenal were reduced. Additionally, an increase in methyl salicylate and hexanal was observed in the fermented sample with *L. brevis*.

Although the use of co-cultures has demonstrated an increase in the production of bioactive compounds for beneficial effects, volatile flavour compounds, and sensory scores in various matrices (Hadj Saadoun et al. 2021; Chen 2024), studies on the development of co-cultures in OFI cladodes remain limited, until this study.

To the best of the authors' knowledge, this study is the first that investigates the effect of lactic acid fermentation on OFI cladodes. Previous research has primarily focused on the aromatic profile of fermented *Opuntia* species, employing yeasts such as *Kluyveromyces marxianus*, *Pichia fermentans*, and *Saccharomyces cerevisiae*, as well as bacteria like *Oenococcus oeni* (Medeiros et al. 2000; Rodríguez-Lerma et al. 2011; Navarrete-Bolaños et al. 2013). For instance, Medeiros et al. (2000) studied the fermentation of OFI with *K. marxianus*, aiming to produce aromatic compounds from this substrate. The fermentation resulted in the production of fruity aroma compounds, including alcohols, esters, and aldehydes, with ethanol and acetaldehyde identified as the dominant volatiles. Other researchers have focused on fermenting OFI fruit juice rather than cladodes, using alcoholic

and lactic acid fermentation methods (Wang et al. 2024; Navarrete-Bolaños et al. 2013; Rodríguez-Lerma et al. 2011). Alcoholic fermentation followed by malolactic fermentation has been shown to produce a variety of aromatic compounds, resulting in fruity notes (Rodríguez-Lerma et al. 2011; Navarrete-Bolaños et al. 2013). In contrast, lactic acid fermentation, carried out by *Lactiplanctibacillus plantarum*, *Lacticaseibacillus paracasei*, and *Limosilactobacillus fermentum*, led to the production of acid compounds such as nonanoic acid and butanoic acid (Wang et al. 2024). The present study also detected these compounds in both control and fermented samples. Moreover, fermentation with *L. plantarum* up-regulated certain esters associated with fruity and floral flavours, enhancing the sensory properties of the substrate by modifying its flavour profile (Wang et al. 2024).

Conclusion

This study shows how lactic fermentation can be a valid strategy for the valorisation of *Opuntia* cladodes. Through this innovative approach, it is possible to obtain biomasses and extracts characterised by a specific blend of volatile compounds that preserve the antimicrobial properties already present in the starting material, which can be used as multifunctional ingredients with prospects for use in various sectors, from food to cosmetics and animal feed.

References

Alves, Marta Assunção, Andréa Carla Mendonça de Souza, Guillermo Gamarra-Rojas, and Nonete Barbosa Guerra. 2008. "Fruto de Palma [Opuntia Fícus-Indica (L) MILLER, Cactaceae]: Morfologia, Composição Quimica, Fisiologia, Índices de Colheita e Fisiologia Pós-Colheita." Revista Iberoamericana de Tecnología Postcosecha 9 (1): 16–25. http://repositorio.ufc.br/handle/riufc/13993.

Ammar, Imène, Monia Ennouri, Bassem Khemakhem, Thabèt Yangui, and Hamadi Attia. 2012. "Variation in Chemical Composition and Biological Activities of Two Species of Opuntia Flowers at Four Stages of Flowering." Industrial Crops and Products 37 (1): 34–40. https://doi.org/10.1016/j.indcrop.2011.11.027.

André, Stéphane, Tatiana Vallaeys, and Stella Planchon. 2017. "Spore-Forming Bacteria Responsible for Food Spoilage." Research in Microbiology 168 (4): 379–87. https://doi.org/10.1016/j.resmic.2016.10.003.

Aneja, Kamal Rai, Romika Dhiman, Neeraj Kumar Aggarwal, and Ashish Aneja. 2014. "Emerging Preservation Techniques for Controlling Spoilage and Pathogenic Microorganisms in Fruit Juices." International Journal of Microbiology. Hindawi Publishing Corporation. https://doi.org/10.1155/2014/758942.

Axel, Claudia, Brid Brosnan, Emanuele Zannini, Lorenzo C. Peyer, Ambrose Furey, Aidan Coffey, and Elke K. Arendt. 2016. "Antifungal Activities of Three Different Lactobacillus Species and Their Production of Antifungal Carboxylic Acids in Wheat Sourdough." Applied Microbiology and Biotechnology 100 (4): 1701–11. https://doi.org/10.1007/s00253-015-7051-x.

Barba, Francisco J., Cyrielle Garcia, Amandine Fessard, Paulo E.S. Munekata, Jose M. Lorenzo, Aouatif Aboudia, Adbelouahab Ouadia, and Fabienne Remize. 2022. "Opuntia Ficus Indica Edible Parts: A Food and Nutritional Security Perspective." Food Reviews International 38 (5): 930–52. https://doi.org/10.1080/87559129.2020.1756844.

Blando, Federica, Rossella Russo, Carmine Negro, Luigi De Bellis, and Stefania Frassinetti. 2019. "Antimicrobial and Antibiofilm Activity against Staphylococcus Aureus of Opuntia Ficus-Indica (L.) Mill. Cladode Polyphenolic Extracts." Antioxidants 8 (5). https://doi.org/10.3390/antiox8050117.

Brambilla, Lara Z S, Eliana H Endo, Diógenes A G Cortez, and Benedito P Dias Filho. 2017. "Anti-Biofilm Activity against Staphylococcus Aureus MRSA and MSSA of Neolignans and Extract of Piper

Regnellii." Revista Brasileira de Farmacognosia 27:112–17. https://doi.org/10.1016/j.bjp.2016.08.008.

Carpena, M., L. Cassani, A. Gomez-Zavaglia, P. Garcia-Perez, S. Seyyedi-Mansour, Hui Cao, J. Simal-Gandara, and M. A. Prieto. 2023. "Application of Fermentation for the Valorization of Residues from Cactaceae Family." Food Chemistry 410 (June). https://doi.org/10.1016/j.foodchem.2022.135369.

Chen, C., Li, H., Zhu, Y., Zhou, Y., & Luo, Q. 2024. "Effect of Fermentation with Single and Co-Culture of Lactic Acid Bacteria on Chinese Elaeagnus Angustifolia Juice: Evaluation of Bioactive Compounds and Volatile Profiles." Food Bioscience.

Chigo-Hernandez, Mildred Melina, Aubrey Dubois, and Elizabeth Tomasino. 2022. "Aroma Perception of Rose Oxide, Linalool and α-Terpineol Combinations in Gewürztraminer Wine." Fermentation 8 (1). https://doi.org/10.3390/fermentation8010030.

Cirlini, M., C. Dall'Asta, A. Silvanini, D. Begh, A. Fabbri, G. Galaverna, and T. Ganino. 2012. "Volatile Fingerprinting of Chestnut Flours from Traditional Emilia Romagna (Italy) Cultivars." Food Chemistry 134 (2): 662–68. https://doi.org/10.1016/j.foodchem.2012.02.151.

Comi, Giuseppe, Debbie Andyanto, Marisa Manzano, and Lucilla Iacumin. 2016. "Lactococcus Lactis and Lactobacillus Sakei as Bio-Protective Culture to Eliminate Leuconostoc Mesenteroides Spoilage and Improve the Shelf Life and Sensorial Characteristics of Commercial Cooked Bacon." Food Microbiology 58 (September):16–22. https://doi.org/10.1016/j.fm.2016.03.001.

Cushman, John C., and Hans J. Bohnert. 1999. "Crassulacean Acid Metabolism: Molecular Genetics." Annual Review of Plant Biology 50 (Volume 50, 1999): 305–32. https://doi.org/10.1146/ANNUREV.ARPLANT.50.1.305/CITE/REFWORKS.

El-Hawary, S S, M E El-Tantawy, M A Rabeh, and W K Badr. 2021. "Chemical Composition and Antimicrobial Activity of Volatile Constituents of Cladodes, Fruits Peel and Fruits Pulp from Opuntia Ficus Indica (L.) Mill. (Prickly Pear) Growing in Egypt." Egypt. J. Chem 64 (1): 437–44. https://doi.org/10.21608/EJCHEM.2020.21137.2260.

Feng, Xiaoxiao, Yufei Hua, Xingfei Li, Caimeng Zhang, Xiangzhen Kong, and Yeming Chen. 2020. "(E)-2-Heptenal in Soymilk: A Nonenzymatic Formation Route and the Impact on the Flavor Profile." Journal of Agricultural and Food Chemistry 68 (50): 14961–69. https://doi.org/10.1021/acs.jafc.0c06192.

Feugang, Jean M, and Florian Stintzing. 2006. "Nutritional and Medicinal Use of Cactus Pear (Opuntia Spp.) Cladodes and Fruits." https://doi.org/10.2741/1992.

Fikri, Sherazade, Marie Hélène Lessard, Véronique Perreault, Alain Doyen, and Steve Labrie. 2023. "Candida Krusei Is the Major Contaminant of Ultrafiltration and Reverse Osmosis Membranes Used for Cranberry Juice Production." Food Microbiology 109 (February). https://doi.org/10.1016/j.fm.2022.104146.

Filannino, Pasquale, Raffaella Di Cagno, and Marco Gobbetti. 2018. "Metabolic and Functional Paths of Lactic Acid Bacteria in Plant Foods: Get out of the Labyrinth." Current Opinion in Biotechnology. Elsevier Ltd. https://doi.org/10.1016/j.copbio.2017.07.016.

Filannino, Pasquale, Ivana Cavoski, Nadia Thlien, Olimpia Vincentini, Maria De Angelis, Marco Silano, Marco Gobbetti, and Raffaella Dicagno. 2016. "Lactic Acid Fermentation of Cactus Cladodes (Opuntia Ficus-Indica L.) Generates Flavonoid Derivatives with Antioxidant and Anti-Inflammatory Properties." PLOS ONE 11 (3): e0152575. https://doi.org/10.1371/JOURNAL.PONE.0152575.

Hadj Saadoun, Jasmine, Gaia Bertani, Alessia Levante, Fabio Vezzosi, Annalisa Ricci, Valentina Bernini, Camilla Lazzi, Silvia Grassi, and Maria Paciulli. 2021. "Fermentation of Agri-Food Waste: A Promising Route for the Production of Aroma Compounds." Foods. https://doi.org/10.3390/foods.

Hadj Saadoun, Jasmine, Luca Calani, Martina Cirlini, Valentina Bernini, Erasmo Neviani, Daniele Del Rio, Gianni Galaverna, and Camilla Lazzi. 2021. "Effect of Fermentation with Single and Co-Culture of Lactic Acid Bacteria on Okara: Evaluation of Bioactive Compounds and Volatile Profiles." Food and Function 12 (7): 3033–43. https://doi.org/10.1039/d0fo02916e.

Hadj Saadoun, Jasmine, Annalisa Ricci, Martina Cirlini, Elena Bancalari, Valentina Bernini, Gianni Galaverna, Erasmo Neviani, and Camilla Lazzi. 2021. "Production and Recovery of Volatile Compounds from Fermented Fruit By-Products with Lacticaseibacillus Rhamnosus." Food and Bioproducts Processing 128 (July):215–26. https://doi.org/10.1016/j.fbp.2021.06.002.

Hur, Sun Jin, Seung Yuan Lee, Young Chan Kim, Inwook Choi, and Geun Bae Kim. 2014. "Effect of Fermentation on the Antioxidant Activity in Plant-Based Foods." Food Chemistry. Elsevier Ltd. https://doi.org/10.1016/j.foodchem.2014.03.112.

Inglese P., Mondragon C., Nefzaoui A., and Saenz C. 2017. "Crop Ecology, Cultivation and Uses of Cactus Pear." 2017. https://www.cabidigitallibrary.org/doi/full/10.5555/20183168277.

Jung, Yousung, Hye Jin Kim, Dongwook Kim, Bumjin Joo, Jin Woo Jhoo, and Aera Jang. 2023. "Physicochemical Properties and Volatile Organic Compounds of Dairy Beef Round Subjected to

Various Cooking Methods." Food Science of Animal Resources 43 (5): 767–91. https://doi.org/10.5851/KOSFA.2023.E35.

Lamia, Semiria, Abdelkader Ammam, Hachem Kadda, and Belmamoun Reda. 2022. "Polyphenol Content, Antioxidant and Antibacterial Activity of The Aqueous Extract of Opuntia Ficus-Indica Cladodes." Egyptian Academic Journal of Biological Sciences. C, Physiology and Molecular Biology 14 (1): 465–74. https://doi.org/10.21608/eajbsc.2022.245607.

Li, Ting, Juan Wang, Bingzheng Xu, Huibin Sun, Hongqin Liu, Danqing Wang, Yi Shen, et al. 2023. "Comparative Analysis of the Differences among Langya Flavor Baijiu and Strong and Soy Sauce Flavor Baijiu by Targeted Flavor Analysis." Journal of Food Composition and Analysis 122 (September). https://doi.org/10.1016/j.jfca.2023.105479.

Liu, Shuai, Yuhao Li, Xiaoxiao Song, Xiaoyi Hu, Yuxin He, Junyi Yin, Shaoping Nie, and Mingyong Xie. 2023. "Changes in Volatile and Nutrient Components of Mango Juice by Different Lactic Acid Bacteria Fermentation." Food Bioscience 56 (December). https://doi.org/10.1016/j.fbio.2023.103141.

Lubran, Meryl B., Harry T. Lawless, Edward Lavin, and Terry E. Acree. 2005. "Identification of Metallic-Smelling 1-Octen-3-One and 1-Nonen-3-One from Solutions of Ferrous Sulfate." Journal of Agricultural and Food Chemistry 53 (21): 8325–27. https://doi.org/10.1021/jf0511594.

Luttrell, William E. 2013. "Vinyl Acetate." Journal of Chemical Health and Safety. https://doi.org/10.1016/j.jchas.2013.10.007.

Maiuolo, Jessica, Saverio Nucera, Maria Serra, Rosamaria Caminiti, Francesca Oppedisano, Roberta Macrì, Federica Scarano, et al. 2024. "Cladodes of Opuntia Ficus-Indica (L.) Mill. Possess Important Beneficial Properties Dependent on Their Different Stages of Maturity." https://doi.org/10.3390/plants13101365.

Martins, Mariana, Maria H. Ribeiro, and Cristina M.M. Almeida. 2023. "Physicochemical, Nutritional, and Medicinal Properties of Opuntia Ficus-Indica (L.) Mill. and Its Main Agro-Industrial Use: A Review." Plants 2023, Vol. 12, Page 1512 12 (7): 1512. https://doi.org/10.3390/PLANTS12071512.

Medeiros, Adriane B P, Ashok Pandey, Renato J S Freitas, Pierre Christen, and Carlos R Soccol. 2000. "Optimization of the Production of Aroma Compounds by Kluyveromyces Marxianus in Solid-State Fermentation Using Factorial Design and Response Surface Methodology." Biochemical Engineering Journal. Vol. 6.

Nam, Dong Geon, Hee Sun Yang, Ui Jin Bae, Eunmi Park, Ae Jin Choi, and Jeong Sook Choe. 2023. "The Cactus (Opuntia Ficus-Indica) Cladodes and Callus Extracts: A Study Combined with LC-MS Metabolic Profiling, In-Silico, and In-Vitro Analyses." Antioxidants 12 (7). https://doi.org/10.3390/antiox12071329.

Navarrete-Bolaños, J. L., E. Fato-Aldeco, K. Gutiérrez-Moreno, J. E. Botello-Álvarez, H. Jiménez-Islas, and R. Rico-Martínez. 2013. "A Strategy to Design Efficient Fermentation Processes for Traditional Beverages Production: Prickly Pear Wine." Journal of Food Science 78 (10). https://doi.org/10.1111/1750-3841.12237.

Nawas, Tarek. 2018. "Antibacterial Activity of Curcuma Longa, Opuntia Ficus-Indica and Linum Usitatissimum." MOJ Toxicology 4 (3). https://doi.org/10.15406/mojt.2018.04.00102.

Negi, Pradeep Singh. 2012. "Plant Extracts for the Control of Bacterial Growth: Efficacy, Stability and Safety Issues for Food Application." International Journal of Food Microbiology. https://doi.org/10.1016/j.ijfoodmicro.2012.03.006.

Nharingo, Tichaona, and Mambo Moyo. 2016. "Application of Opuntia Ficus-Indica in Bioremediation of Wastewaters. A Critical Review." Journal of Environmental Management 166 (January):55–72. https://doi.org/10.1016/J.JENVMAN.2015.10.005.

Nostro, Antonia, Alessandra Guerrini, Andreana Marino, Massimo Tacchini, Mara Di Giulio, Alessandro Grandini, Methap Akin, Luigina Cellini, Giuseppe Bisignano, and Hatice T. Saraçoğlu. 2016. "In Vitro Activity of Plant Extracts against Biofilm-Producing Food-Related Bacteria." International Journal of Food Microbiology 238 (December):33–39. https://doi.org/10.1016/J.IJFOODMICRO.2016.08.024.

Osawa, C. C., L. A.G. Gonçalves, and M. A.A.P. Da Silva. 2013. "Odor Significance of the Volatiles Formed during Deep-Frying with Palm Olein." JAOCS, Journal of the American Oil Chemists' Society 90 (2): 183–89. https://doi.org/10.1007/s11746-012-2150-7.

Palop, Alfredo, Javier Raso, Rafael Pagan, Santiago Condon, and Francisco J Salá Salá. 1999. "Influence of PH on Heat Resistance of Spores of Bacillus Coagulans in Buffer and Homogenized Foods." International Journal of Food Microbiology. Vol. 46.

Pimienta-Barrios, E. 1994. "Prickly Pear (Opuntia Spp.): A Valuable Fruit Crop for the Semi-Arid Lands of Mexico." Journal of Arid Environments 28 (1): 1–11. https://doi.org/10.1016/S0140-1963(05)80016-3.

Rajendran, Sarathadevi, Patrick Silcock, and Phil Bremer. 2023. “Flavour Volatiles of Fermented Vegetable and Fruit Substrates: A Review.” Molecules. MDPI. https://doi.org/10.3390/molecules28073236.

Ramírez-Rodrigues, M. M., M. O. Balaban, M. R. Marshall, and R. L. Rouseff. 2011. “Hot and Cold Water Infusion Aroma Profilesof Hibiscus Sabdariffa: Fresh Compared with Dried.” Journal of Food Science 76 (2). https://doi.org/10.1111/j.1750-3841.2010.01989.x.

Ricci (a), Annalisa, Martina Cirlini, Alessia Levante, Chiara Dall’Asta, Gianni Galaverna, and Camilla Lazzi. 2018. “Volatile Profile of Elderberry Juice: Effect of Lactic Acid Fermentation Using L. Plantarum, L. Rhamnosus and L. Casei Strains.” Food Research International 105 (March):412–22. https://doi.org/10.1016/j.foodres.2017.11.042.

Ricci, Annalisa, Gaia Bertani, Antonietta Maoloni, Valentina Bernini, Alessia Levante, Erasmo Neviani, and Camilla Lazzi. 2021. “Antimicrobial Activity of Fermented Vegetable Byproduct Extracts for Food Applications.” Foods 10 (5): 1092. https://doi.org/10.3390/FOODS10051092/S1.

Ricci (b), Annalisa, Alessia Levante, Martina Cirlini, Luca Calani, Valentina Bernini, Daniele Del Rio, Gianni Galaverna, Erasmo Neviani, and Camilla Lazzi. 2018. “The Influence of Viable Cells and Cell-Free Extracts of Lactobacillus Casei on Volatile Compounds and Polyphenolic Profile of Elderberry Juice.” Frontiers in Microbiology 9 (NOV). https://doi.org/10.3389/fmicb.2018.02784.

Rocchetti, Gabriele, Marco Pellizzoni, Domenico Montesano, and Luigi Lucini. 2018. “Italian Opuntia Ficus-Indica Cladodes as Rich Source of Bioactive Compounds with Health-Promoting Properties.” Foods 2018, Vol. 7, Page 24 7 (2): 24. https://doi.org/10.3390/FOODS7020024.

Rodríguez-Lerma, G. K., K. Gutiérrez-Moreno, M. Cárdenas-Manríquez, E. Botello-Álvarez, H. Jiménez-Islas, R. Rico-Martínez, and J. L. Navarrete-Bolaños. 2011. “Microbial Ecology Studies of Spontaneous Fermentation: Starter Culture Selection for Prickly Pear Wine Production.” Journal of Food Science 76 (6). https://doi.org/10.1111/j.1750-3841.2011.02208.x.

Rodríguez-Leyva, Esteban, Esperanza García-Pascual, Marco M. González-Chávez, Santiago de J. Méndez-Gallegos, Juan A. Morales-Rueda, Juan C. Posadas-Hurtado, Ángel Bravo-Vinaja, and Avelina Franco-Vega. 2024. “Interactions of Opuntia Ficus-Indica with Dactylopius Coccus and D. Opuntiae (Hemiptera: Dactylopiidae) through the Study of Their Volatile Compounds.” Plants 13 (7). https://doi.org/10.3390/plants13070963.

Sakko, M., Tjäderhane, L., Sorsa, T., Hietala, P., Järvinen, A., Bowyer, P., & Rautemaa, R. 2012. “2-Hydroxyisocaproic Acid (HICA): A New Potential Topical Antibacterial Agent.” International Journal of Antimicrobial Agents.

Sánchez, Eduardo, Santos García, and Norma Heredia. 2010. “Extracts of Edible and Medicinal Plants Damage Membranes of Vibrio Cholerae.” Applied and Environmental Microbiology 76 (20): 6888–94. https://doi.org/10.1128/AEM.03052-09/ASSET/A7D8B97E-E5A6-4DCE-845E-3DF3519C7053/ASSETS/GRAPHIC/ZAM9991014280003.JPEG.

Santiago, Elsy De, Maite Domínguez-Fernández, Concepción Cid, and María Paz De Peña. 2018. “Impact of Cooking Process on Nutritional Composition and Antioxidants of Cactus Cladodes (Opuntia Ficus-Indica).” Food Chemistry 240 (February):1055–62. https://doi.org/10.1016/J.FOODCHEM.2017.08.039.

Snyder, Abigail B., Nicole Martin, and Martin Wiedmann. 2024. “Microbial Food Spoilage: Impact, Causative Agents and Control Strategies.” Nature Reviews Microbiology.

Stintzing, Florian C, and Reinhold Carle. 2005. “Review Cactus Stems (Opuntia Spp.): A Review on Their Chemistry, Technology, and Uses.” Mol. Nutr. Food Res 49:175–94. https://doi.org/10.1002/mnfr.200400071.

Stintzing, Florian C., Kirsten M. Herbach, Markus R. Mosshammer, Reinhold Carle, Weiguang Yi, Subramani Sellappan, Casimir C. Akoh, Ron Bunch, and Peter Felker. 2005. “Color, Betalain Pattern, and Antioxidant Properties of Cactus Pear (Opuntia Spp.) Clones.” Journal of Agricultural and Food Chemistry 53 (2): 442–51. https://doi.org/10.1021/JF048751Y/ASSET/IMAGES/LARGE/JF048751YF00002.JPEG.

Valentini, Valeria, Alessio Allegra, Francesco Adduci, Cristiana Labella, Rosanna Paolino, and Carlo Cosentino. 2018. “Effect of Cactus Pear (Opuntia Ficus-Indica (L.) Miller) on the Antioxidant Capacity of Donkey Milk.” International Journal of Dairy Technology 71 (3): 579–84. https://doi.org/10.1111/1471-0307.12477.

Ventura-Aguilar, Rosa Isela, Elsa Bosquez-Molina, Silvia Bautista-Baños, and Fernando Rivera-Cabrera. 2017. “Cactus Stem (Opuntia Ficus-Indica Mill): Anatomy, Physiology and Chemical Composition with Emphasis on Its Biofunctional Properties.” Journal of the Science of Food and Agriculture 97 (15): 5065–73. https://doi.org/10.1002/JSFA.8493.

Vougiouklaki, Despina, Theofania Tsironi, Joseph Papaparaskevas, Panagiotis Halvatsiotis, and Dimitra Houhoula. 2022. “Characterization of Lacticaseibacillus Rhamnosus, Levilactobacillus Brevis and Lactiplantibacillus Plantarum Metabolites and Evaluation of Their Antimicrobial

Activity against Food Pathogens." Applied Sciences (Switzerland) 12 (2). https://doi.org/10.3390/app12020660.

Wang, Huixian, Xingqiao He, Juanni Li, Jintao Wu, Shuaiming Jiang, Hui Xue, Jiachao Zhang, Rajesh Jha, and Ruimin Wang. 2024. "Lactic Acid Bacteria Fermentation Improves Physicochemical Properties, Bioactivity, and Metabolic Profiles of Opuntia Ficus-Indica Fruit Juice." Food Chemistry 453 (September). https://doi.org/10.1016/j.foodchem.2024.139646.

Welegerima, Gebrekidan, Aragaw Zemene, and Yemane Tilahun. 2018. "Phytochemical Composition and Antibacterial Activity of Opuntia Ficus Indica Cladodes Extracts." Journal of Medicinal Plants Studies 6 (2): 243–46.

Wright, Cynthia R., and William N. Setzer. 2014. "Chemical Composition of Volatiles from Opuntia Littoralis Opuntia Ficus-Indica, and Opuntia Prolifera Growing on Catalina Island, California." Natural Product Research 28 (3): 208–11. https://doi.org/10.1080/14786419.2013.867345.

Yao, Lingyun, Yifan Mo, Da Chen, Tao Feng, Shiqing Song, Huatian Wang, and Min Sun. 2021. "Characterization of Key Aroma Compounds in Xinjiang Dried Figs (Ficus Carica L.) by GC–MS, GC–Olfactometry, Odor Activity Values, and Sensory Analyses." LWT 150 (October). https://doi.org/10.1016/j.lwt.2021.111982.

Zhou, Zhen, Xiuqing Wang, Xia Cui, and Haijing Wang. 2022. "Rapid Determination of 6-Methyl-5-Hepten-2-One in Fruit with LLE-GC-MS." Journal of Chromatographic Science 60 (3): 280–86. https://doi.org/10.1093/chromsci/bmab071.

Chapter 3

An innovative and sustainable antimicrobial to reduce canned tomato contamination

Martina Marrella, Sonia Figuccia, Jasmine Hadj Saadoun, Alessia Levante, Annalisa Ricci, Valentina Bernini, Camilla Lazzi

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Abstract

The food industry's growing need for sustainable sanitization solutions has increased interest in nature-based antimicrobials. Thanks to innovative approaches for the valorisation of agri-food waste and by-products, we can now rely on antimicrobial molecules obtained via such sustainable processes. This study explores the potential of natural antimicrobials (NA) sourced from tomato production waste for use in cooling water during the canning process. Additionally, it compares the activity to a common antimicrobial molecule, sodium hypochlorite (NaClO), and investigates the interaction between the latter and the NA, focusing on their combined ability to target key microbial contaminants in tomato processing environments.

To evaluate their antimicrobial effects, isolates representing Enterobacteriaceae, Bacillaceae, and Lactobacillaceae were isolated from a tomato processing plant and exposed to NA and NaClO individually and in combination. The impact of these treatments on microbial cells was measured using qPCR coupled with PMA dye to assess membrane damage, while plates counts were used to confirm bactericidal activity. Experiments included spiked water samples simulating real-world conditions with a mixed population of 9 environmental isolates.

The findings demonstrate that NA and NaClO, when applied together, deliver a broad-spectrum bactericidal effect even at lower concentrations and reduced exposure times. This approach not only enhances microbial inactivation but also reduces reliance on traditional chemical sanitizers. By addressing the interactions between microbial communities and antimicrobial agents in realistic scenarios, this study highlights the promise of combining natural and conventional antimicrobials for safer and more sustainable food processing practices.

Introduction

Canned fruits and vegetables are made from different raw fruit and vegetables that are processed and packed into airtight cans, then subjected to thermal treatment to ensure long-term preservation. This process typically involves two main stages: a heating cycle followed by a cooling cycle. During the heating phase, condensing steam is commonly used. In the cooling phase, the process is completed either by spraying water, immersing the cans in water, or using a combination of both methods. By this way, canned foods reach the sterility level required to assure food safety maintaining the quality (Mohamed 2007). Canned tomatoes are the most popular canned vegetable all over the world being widely used as food ingredients. In 2022, their global market amounted to 2.86 million tonnes of finished products and generated sales of EUR 3.57 billion ("Tomato News"). Although these products, due to acidity, do not pose a safety problem, they can still be substrates for the growth of spoilage microorganisms. Their microbiological stability is determined by three different factors, i.e.: the effectiveness of the heat treatment applied, the hermeticity of the container, and the storage temperature (Squitieri et al. 2023). During thermal treatment, conducted at temperatures below 100°C, heat-resistant microorganisms, like spore-forming species *Clostridium butyricum, Clostridium pasteurianum, Bacillus coagulans* or *Alyciclobacillus acidocaldarius* may survive and grow in the product. On the other hand, lactic acid bacteria and enterobacteria, the main spoilage agents in the asporogenic group, along with yeasts and molds, are inactivated by heat. However, it is possible to find these microorganisms in the finished product, and when this occurs, it indicates post-processing contamination that took place during the cooling phase. In fact, defects in the container manufacturing can lead to contamination of the product due to contact with the cooling water, which often harbors

these microorganisms (Squitieri et al. 2023). Maintaining the quality of the process water is therefore very important as it could be a source of cross-contamination. For this reason, process water, including cooling water, undergoes sanitisation treatments to reduce the microbial load and keep the water free of contamination (Gil et al. 2009). Chlorine is one of the most widely used sanitizers used in food industry, especially added to water in the form of sodium or calcium hypochlorite.

Its activity against bacteria, molds and yeast is related to the release of hypochlorous acid and hypochlorite ions which exert antimicrobial activity by destroying cell proteins (Chinchkar et al. 2022). The effectiveness of chlorine relies on its concentration and is affected by different factors, such as contact time, pH and temperature. Although chlorine has many advantages, including broad antimicrobial activity, ease of use and low cost, it is toxic and corrosive in high concentrations. For this reason, alternative nature-based substances are being explored to maintain hygiene in food processing plants, according to the Sustainable Development Goal 6 of UN 2030 Agenda, “Clean water and sanitation” (Chakraborty and Dutta 2022).

To the best of our knowledge, no studies are considering the use of antimicrobials derived from agrifood waste as sanitizers in water. Therefore, this study aims to assess the efficacy of natural antimicrobials derived from tomato production waste for potential use in cooling water during the canning process. Additionally, the study evaluates the synergistic effects of the NA and chlorinated products on the inactivation efficacy of microbial targets relevant to the tomato industry.

Material and methods

Antimicrobials

Two antimicrobials, sodium hypochlorite (Carini Chem Srl, Italy) and fermented tomato extract (NA), were used against microbial targets isolated from the tomato industry. The NA was obtained after the lactic acid fermentation of tomato by-products (mainly peels and seeds) obtained after the industrial process of tomato canning as reported by Ricci et al. (2019; 2021).

Isolation and identification of strains

During two tomato harvest campaigns in 2022 and 2023, extensive sampling was carried out in a tomato processing company at various stages of the process to monitor the presence of microbial spoilage targets of interest.

In details, microorganisms were isolated from tomato washing water, non-compliant sauce tomato cans and industrial plant where cans were cooled, at 30° C, using different agar mediums by culturing on the following media:

Tryptic Soy Agar (TSA) (Merck, Germany), Nutrient agar (Scharlau, Spain), Reinforced Clostridial Medium Agar (RCM), containing the following: Yeast extract 3g, Beef extract 10g, Peptone 10g, Glucose 5g, Soluble starch 1g, Sodium chloride 5g, Sodium acetate 3g, Cysteine hydrochloride 0.5g, Agar 15g, pH 6.8 adjusted with 10 N NaOH, 0001 Agar (containing the following: 'Lab-Lemco' beef extract 1g, Yeast extract 2g, Peptone 5g, Sodium chloride 5g, Agar 15g, pH 7.4).

Colonies grown on the agar plates, were isolated through four purification steps on the same media and purity was assessed by BX51 optical microscope (Olympus, Waltham, MA)

observation. Purified strains were grown in Man Rogosa and Sharpe (MRS) broth (Oxoid, UK) for Lactobacillaceae, Tryptic Soy Broth (TSB, Merck, Germany) for Enterobacteriaceae strains and Nutrient broth for Bacillaceae strains and the broth cultures were stored in cryovials with 20% (v/v) glycerol solution at -80°C.

Bacterial genomic DNA was extracted following the PureFood Pathogen Kit protocol, using the automated extractor Maxwell® Rapid Sample Concentrator (Promega Corporation, Madison, WI, USA). The DNA extraction was checked on agarose gel with Tris-acetate-EDTA 1× running buffer (1%, w/v).

Subsequently, 16S rRNA gene was amplified and sequenced. The primers used were 46Fw 5'-GCYTAACACATGCAAGTCGA-3' and 536Rv 5'-GTATTACCGCGGCTGCTGG-3' (Marchesi et al. 1998). Each PCR mixture contained 1 µL of each primer at concentration [10 µM], 10 µL of 2X GoTaq Master Mix (Promega, Madison, WI, USA), 7 µL of sterile Milli-Q water, and 1 µL of DNA (final volume, 20 µL). The PCR reaction conditions are as follows: initial denaturation at 98°C for 2 min, followed by 35 cycles of annealing beginning at 65°C and ending at 55°C for 15 s (−1°C/cycle), and a final extension at 68°C for 30 s. PCR products were checked on 1.5% w/v agarose gel in 1 × TAE buffer at 90 V/cm for 20 min and visualized on a GelDoc Go gel imaging system (BioRad, Milan, Italy). PCR products were sequenced by BMR genomics (Padova, Italy), and the sequences were compared with reference sequences using the Basic Local Alignment Search Tool (BLAST®, https://blast.ncbi.nlm.nih.gov/Blast.cgi) against the 16S ribosomal RNA sequences database.

In vitro evaluation of antimicrobial activity

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The 29 isolated microorganisms, belonging to Enterobacteriaceae, Bacillaceae and Lactobacillaceae genera kept at -80°C were cultured twice in their growth media at optimal temperature. The antimicrobial activity of sodium hypochlorite and NA obtained from fermented tomato byproduct was evaluated against the 29 isolates as previously reported by Ricci et al. (2019). The Minimum bactericidal concentration was determined as follows. Briefly the 96-well microplates were filled with IsoSensitest medium (Liofilchem, Italy), for Lactobacillaceae strains, TSB for Enterobacteriaceae strains and Nutrient broth for Bacillaceae strains. Different concentrations of sodium hypochlorite (starting from 25 mg/mL to 0.02 mg/mL) or NA (starting from 200 mg/mL to 0.20 mg/mL) were added. Finally, all the microbial cultures were diluted until 4 Log CFU/mL and inoculated in the microplates. Plates were then incubated at the optimal growth temperature of each bacterial strain for 24 h. After the incubation the Minimum Inhibitory Concentration (MIC) was not detected due to the turbidity of high concentration of extracts. Instead to determine the Minimum Bactericidal Concentration (MBC)10 µL from each well were spotted on agar plates and incubated at the optimal temperature for 24 h.

From the results obtained, 9 isolates (3 Lactobacillaceae, 3 Enterobacteriaceae and 3 Bacillaceae) showing the highest MBC values were selected and the MIC and MBC values were determined by inoculating 2 Log CFU/mL of the microbial cultures, as previously described.

Evaluation of microbial growth inhibition in the presence of sub-inhibitory concentration of sodium hypochlorite and NA

The growth capacity of the 9 microbial strains previously selected was evaluated in 96-wells microplates, using the Tecan microplate reader (Infinite® 200Pro, Tecan Group Ltd.) in the presence of the sub-inhibitory concentrations of sodium hypochlorite and NA. In each well, broth medium was added and each microbial culture was inoculated to reach a concentration of 2 Log CFU/mL. The effect of ½ MIC concentrations of sodium hypochlorite/NA was evaluated by measuring the optical density at the wavelength of 600 nm every 10 minutes for 24 hours. The data obtained were normalised by subtracting the uninoculated medium's optical density from the inoculated samples measured optical density. All the experiments were carried out at least in triplicate and the data were reported as mean values.

Evaluation of antimicrobial activity in water

Design of experiment and mock microbial community setup

An experimental design was created to study how the antimicrobials affect microbial viability. Ten experiments, with biological replicates, were performed considering different factors: antimicrobial agents, different concentrations and the contact time (Table 1).

To experimentally create a simplified microbial community, 9 strains which resulted to be more resistant to both antimicrobials, according to the experiment reported in paragraph 2.3.1, were combined 3 at a time for each family.

The following strains were selected: *Enterobacter* sp (7), *Enterobacter* sp (9), *Enterobacter cloacae* (14), *Bacillus* sp (20), *Bacillus* sp (21), *Bacillus licheniformis* (22), *Levilactobacillus*

brevis (23), *Pediococcus pentosaceus* (25), *Pediococcus pentosaceus* (28).

Table 1. Summary of the experimental design created with different factors: two antimicrobial agents (NaClO and NA) with three different concentrations (mg/mL) and contact time (minutes)

Exp Name	NA (mg /mL)	NaClO (mg /mL)	Time (min)
N1	0	0	10
N2	100	0	10
N3	0	0.002	10
N4	100	0.002	10
N5	0	0	60
N6	100	0	60
N7	0	0.002	60
N8	100	0.002	60
N9 – N10	50	0.001	35

Each of the cultured species was mixed and used to inoculate bidistilled sterile water reaching a final concentration of about 4 Log CFU/mL in the water samples, followed by exposure at the conditions selected for experimental design. To evaluate the responses for all conditions, microbial growth was evaluated by plate counting in different agar media (IsoSensitest medium for Lactobacillaceae strains, TSB for Enterobacteriaceae strains and Nutrient broth for Bacillaceae strains), and the results were expressed as ΔLog CFU/mL.

Microbial groups absolute quantification by Real Time qPCR

The absolute quantification of isolates belonging to the families Bacillaceae, Enterobacteriaceae and Lactobacillaceae under the tested conditions was performed by Real Time quantitative PCR (qPCR), using specific primers reported in Table 2. Master mix for rplP primer pair contained: 10 µL of 2 × PowerUp SYBR Green Master Mix (ThermoFisher Scientific,

Milan, Italy), 0.75 µL of premixed forward and reverse primer at a concentration of 18 uM each, 2 µL of DNA and 4.75 µL of water to a final volume of 15 µL. The reaction was run using a QuantStudio® 3 instrument (Thermo Fisher Scientific, Waltham, Massachusetts, USA), and thermal cycle run as follows: a first hold stage of 2 min at 50 °C followed by 10 min at 95 °C, 40 cycles of 15 s at 95 °C and 1 min at 60 °C, during which fluorescence acquisition took place, and a final melting curve stage from 60° to 95 °C with a temperature gradient of 0.1 °C/s.

Table 2. Primers used for the Real Time quantitative PCR (qPCR)

Primer (Probe) name	Sequence (5' --> 3')	Ref.
La-Fw	TGGAAACAGATGCTAATACCG	
La-Rv	CGTCCATTGTGGTAGATTCCCT	(Bizhang et al. 2011)
La-T	FAM-CTGAGACACGGCCCAWACTCCTACGG-BHQ1	
Baci_FW	CACGTGTAGCGGTGAAATGC	
Baci_RV	GTTTACGGCGTGGACTACCA	This work
Baci_pro	CGCTGAGGCGCGAAAGCGTGGGGAGC	
rplP 1F	ATG TTA CAA CCA AAG CGT ACA	(Takahashi et al. 2017)
rplP 185R	TTA CCY TGA CGC TTA ACT GC	

For TaqMan assays, PCR reactions were prepared as follows: 10 µL of 2 x TaqMan™ Fast Advanced Master Mix (ThermoFisher Scientific, Milan, Italy), 0.75 µL of premixed forward and reverse primer at a concentration of 18 uM each and 5 uM of probe, 2 µL of DNA and 4.75 µL of water to a final volume of 15 µL. In this case, the following PCR thermal cycle was set up on the same instrument: a first hold stage at 95° C for 20 s, followed by 40 cycles of 1 s at 95°C and 20 s at 60° C, during which fluorescence acquisition took place.

All the reactions were performed in duplicate, and no template controls (NTC) were included in each experiment.

For absolute quantification, standard curves were constructed using purified genomic DNA of *Lactiplantibacillus plantarum* UPCC 4188, *Enterobacter cloacae* UPCC 6247, *Bacillus coagulans* LMG 17456, calculating the corresponding copy number. The standard curves were constructed from serially 10-fold diluted reference strains DNA at known copy number, covering a dilution range of 6 orders of magnitude, then plotting the resulting threshold cycles (C_q), against the logarithm of the target copy number. The copy number of target genes of each primer pair was calculated in the 20 samples by comparing the C_q of the sample with that of the respective standard curve.

PMA treatment for viable cell assessment trough qPCR

Following challenge tests, water samples were aliquoted, and one of the two aliquots was treated with propidium monoazide (PMAxx) dye (Biotium Inc., Hayward, California) prior to DNA extraction, with slight modifications to the manufacturer's protocol. Briefly, 400 uL of the treated water sample were transferred to a clean Eppendorf tube, and 5 uL of 10 uM PMAxx dye were added onto the solution. The solution was incubated for 10 minutes at room temperature in the dark, followed by exposure to blue light for 15 minutes. Afterwards, the cells were pelleted by centrifugation at room temperature for 10 minutes at 5000 g, and DNA extraction was performed on PMAxx-treated samples as reported in paragraph "Isolation and identification of strains". Each experimental condition was assayed in duplicate.

Results and discussion

Identification of microorganisms isolated

Twenty-nine microbial isolates deriving from washing water, non-compliant sauce tomato cans and cooling industrial plant, recovered on different agar media, were identified by 16S rRNA gene sequencing and resulted were reported in Table 3.

Table 3. Microbial isolates deriving from washing water, non-compliant sauce tomato cans and cooling industrial plant, were identified by 16S rRNA gene sequencing.

ID	Isolation matrix	Family	Genus	Species
1	non-compliant pulps		*Enterobacter*	sp
2	non-compliant pulps		*Enterobacter*	sp
3	non-compliant pulps		*Enterobacter*	sp
4	non-compliant pulps		*Enterobacter*	sp
5	non-compliant pulps		*Enterobacter*	sp
6	non-compliant pulp		*Enterobacter*	sp
7	non-compliant pulps		*Enterobacter*	sp
8	cooling industrial plant		*Klebsiella*	*pneumoniae*
9	cooling industrial plant		*Enterobacter*	sp
10	cooling industrial plant	**Enterobacteriaceae**	*Enterobacter*	sp
11	non-compliant pulps		*Klebsiella*	sp
12	non-compliant pulps		*Enterobacter*	sp
13	non-compliant pulps		*Enterobacter*	*cloacae*
14	non-compliant pulps		*Enterobacter*	*cloacae*
15	non-compliant pulps		*Enterobacter*	*cloacae*
16	non-compliant pulps		*Enterobacter*	*cloacae*
17	non-compliant pulps		*Phytobacter*	*ursingii*
18	non-compliant pulps		*Enterobacter*	sp
19	non-compliant pulps		*Enterobacter*	*cloacae*
20	non-compliant pulps	**Bacillaceae**	*Bacillus*	sp
21	non-compliant pulps		*Bacillus*	sp

22	cooling industrial plant		*Bacillus*	*licheniformis*
23	washing water		*Levilactobacillus*	*brevis*
24	non-compliant pulps		*Limosilactobacillus*	*fermentum*
25	non-compliant pulps		*Pediococcus*	*pentosaceus*
26	non-compliant pulps	**Lactobacillaceae**	*Limosilactobacillus*	*fermentum*
27	non-compliant pulps		*Limosilactobacillus*	*fermentum*
28	non-compliant pulps		*Pediococcus*	*pentosaceus*
29	non-compliant pulps		*Lacticaseibacillus*	*paracasei*

Isolates belonged to different species of the family of Enterobacteriaceae (19 strains, numbered from 1 to 19), Lactobacillaceae (7 strains, numbered from 20 to 26) and Bacillaceae (3 strains, numbered from 27 to 29).

Specifically, strains 8, 9, 10 and 22 were isolated from cooling industrial plant; strain 23 from washing water; while all the others from non-compliant pulps. These microorganisms are known as spoilage target in tomato industries and monitoring their presence is required at various stages of the process because if they are not deactivated by heat treatment or in the case of post-contamination, they can grow in the product, leading to non-compliance.

Evaluation of antimicrobial activity of sodium hypochlorite and NA

To explore sustainable and innovative treatments to inactivate spoilage target microorganisms, the antimicrobial activity of NA (Ricci et al. 2019) was evaluated, as well as the one exploited by sodium hypochlorite (Squitieri et al. 2023; Vannini and Siroli 2025; Pahalagedara et al. 2024). Different concentrations of both antimicrobials were tested against 29 bacterial isolates to determine the MBC values. Sodium hypochlorite is the most common compound employed for water sanification in different fields, including food industry, to avoid the development of unwanted microorganisms that can be carried by water (Somani et al.

2011). NA is already known for its antimicrobial effect against foodborne pathogens (Ricci et al. 2019; 2021b).

Table 4. Minimum bactericidal concentration (MBC) values using 4 Log (CFU/mL) as initial inoculum.

ID	Isolation matrix	Family	Genus	Species	NaClO (mg/mL)	NA (mg/mL)
1	non-compliant pulps		*Enterobacter*	sp	1.56	12.50
2	non-compliant pulps		*Enterobacter*	sp	1.56	25.00
3	non-compliant pulps		*Enterobacter*	sp	0.78	25.00
4	non-compliant pulps		*Enterobacter*	sp	0.39	25.00
5	non-compliant pulps		*Enterobacter*	sp	1.56	12.50
6	non-compliant pulp		*Enterobacter*	sp	1.56	12.50
7	non-compliant pulps		*Enterobacter*	sp	1.56	50.00
8	cooling industrial plant		*Klebsiella*	*pneumoniae*	0.78	12.50
9	cooling industrial plant		*Enterobacter*	sp	0.78	25.00
10	cooling industrial plant	**Enterobacteriaceae**	*Enterobacter*	sp	1.56	12.50
11	non-compliant pulps		*Klebsiella*	sp	1.56	12.50
12	non-compliant pulps		*Enterobacter*	sp	1.56	25.00
13	non-compliant pulps		*Enterobacter*	*cloacae*	1.56	12.50
14	non-compliant pulps		*Enterobacter*	*cloacae*	3.13	12.50
15	non-compliant pulps		*Enterobacter*	*cloacae*	0.78	25.00
16	non-compliant pulps		*Enterobacter*	*cloacae*	0.78	25.00
17	non-compliant pulps		*Phytobacter*	*ursingii*	0.78	25.00
18	non-compliant pulps		*Enterobacter*	sp	1.56	12.50
19	non-compliant pulps		*Enterobacter*	*cloacae*	1.56	25.00
20	non-compliant pulps		*Bacillus*	sp	1.56	12.50
21	non-compliant pulps	**Bacillaceae**	*Bacillus*	sp	0.78	12.50
22	cooling industrial plant		*Bacillus*	*licheniformis*	0.78	25.00
23	washing water		*Levilactobacillus*	*brevis*	1.56	100.00
24	non-compliant pulps		*Limosilactobacillus*	*fermentum*	1.56	50.00
25	non-compliant pulps	**Lactobacillaceae**	*Pediococcus*	*pentosaceus*	1.56	100.00
26	non-compliant pulps		*Limosilactobacillus*	*fermentum*	1.56	25.00
27	non-compliant pulps		*Limosilactobacillus*	*fermentum*	1.56	25.00

28	non-compliant pulps	*Pediococcus*	*pentosaceus*	3.13	100.00
29	non-compliant pulps	*Lacticaseibacillus*	*paracasei*	0.78	50.00

The major antimicrobial effect evaluated against the target microorganisms at 4 Log CFU/mL was obtained with sodium hypochlorite, whose efficacy was detected between 0.39 mg/mL and 3.12 mg/mL for Enterobacteriaceae, between 0.78 mg/mL and 1.56 mg/mL for Bacillaceae and between 0.78 mg/mL and 3.12 mg/mL for Lactobacillaceae (Table 4). Also, NA showed antimicrobial activity, even if at higher concentrations, ranged between 12.5 mg/mL and 50 mg/mL for Enterobacteriaceae, between 12.5 mg/mL and 25 mg/mL for Bacillaceae and between 25 mg/mL and 100 mg/mL for Lactobacillaceae (Table 4).

To study the effect of both antimicrobials at lower levels of microbial contamination, three isolated strains, from each family, were selected for their higher resistance to one or the other compound. MIC and MBC test were thus repeated reducing the initial microbial inoculum to 2 Log CFU/mL, a microbial load more likely to be found in process water. In particular, strains 7, 9 and 14, belonging to Enterobacteriaceae, 23, 25 and 28, belonging to Lactobacillaceae and all the three strains belonging to Bacillaceae were selected (Table 5).

Generally, lower MBC values were observed at lower concentration, except for the strains belonging to Enterobacteriaceae family. Similarly, the strain 21, belonging to Bacillaceae family, showed the same MBC values as the ones detected with the higher microbial inoculum when treated with sodium hypochlorite (Table 5). Regarding MIC analysis, MIC values lower than the correspondent MBC were observed for all the 9 strains when treated with sodium hypochlorite, ranging from 0.39 to 0.05 mg/mL. MIC values detected after the addition of NA resulted alike the correspondent MBC for all the strains belonging to Bacillaceae family (strains 20, 21 and 22) and for the strains 9 and 14 belonging to Enterobacteriaceae family; while MIC

values under the MBC were observed for strain 7 belonging to *Enterobacter* genus and all the strains belonging to Lactobacillaceae family (12.5 mg/mL).

Table 5. Minimum inhibitory concentration and minimum bactericidal concentration (MBC) values using 2 Log (CFU/mL) as initial inoculum.

					MIC		MBC	
ID	**Isolation matrix**	**Family**	**Genus**	**Species**	**NaClO (mg/mL)**	**NA (mg/mL)**	**NaClO (mg/mL)**	**NA (mg/mL)**
7	non-compliant pulps		*Enterobacter*	sp	0.39	6.25	1.56	12.50
9	cooling industrial plant	**Enterobacteriaceae**	*Enterobacter*	sp	0.39	6.25	0.78	6.25
14	non-compliant pulps		*Enterobacter*	*cloacae*	0.39	6.25	3.13	6.25
20	non-compliant pulps		*Bacillus*	sp	0.05	3.13	0.78	3.13
21	non-compliant pulps	**Bacillaceae**	*Bacillus*	sp	0.1	3.13	0.78	3.13
22	cooling industrial plant		*Bacillus*	*licheniformis*	0.05	3.13	0.78	3.13
23	washing water		*Levilactobacillus*	*brevis*	0.39	12.5	0.78	25.00
25	non-compliant pulps	**Lactobacillaceae**	*Pediococcus*	*pentosaceus*	0.2	12.5	0.78	25.00
28	non-compliant pulps		*Pediococcus*	*pentosaceus*	0.39	12.5	0.78	25.00

There is substantial evidence supporting the antimicrobial use of sodium hypochlorite, as demonstrated by several studies (Gomes et al. 2001; Radcliffe et al. 2004; DeQueiroz and Day 2007; Sassone et al. 2008; Vianna et al. 2004; Sena et al. 2006; Arias-Moliz et al. 2014). These studies examined the contact time between microorganisms and sodium hypochlorite, as well as the concentration of the compound required to exert antimicrobial activity. Various microorganisms were tested for their response, in term of growth or biofilm production, to sodium hypochlorite, including *Enterococcus faecalis*, *Staphylococcus aureus*, *Candida albicans*, *Escherichia coli*, *Porphyromonas gingivalis*, *Porphyromonas endodontalis*, *Prevotella intermedia*, *Actinomyces israelii*, *Actinomyces naeslundii*, *Pseudomonas aeruginosa*, and *Fusobacterium nucleatum*.

Fermented NA was obtained from lactic acid fermentation of tomato peels and seeds (Ricci et al. 2019) and also demonstrated to possess antimicrobial properties both *in vitro* and *in situ* in food products against foodborne pathogens as *Salmonella* spp., *Listeria monocytogenes, Escherichia coli*, *Staphylococcus aureus,* and *Bacillus cereus* and against spoilage microorganisms such as *Pseudomonas* spp., *Alicyclobacillus acidoterrestris* and *Clostridium pasteurianum* (Hadj Saadoun et al. 2022; Ricci et al. 2021b). Nevertheless, their effectiveness against members of Lactobacillaceae was never tested before.

Evaluation of microbial growth inhibition in presence of sub-inhibitory concentration of sodium hypochlorite and fermented NA

The inhibition of microbial growth in presence of sub-inhibitory concentration was evaluated against Enterobacteriaceae (7, 9 and 14), Bacillaceae (20, 21 and 22), and Lactobacillaceae (23, 25, and 28) by measuring optical density at 600 nm every 10 minutes over 24 hours.

Sub-inhibitory concentrations of sodium hypochlorite partially inhibited the growth of Enterobacteriaceae (*Enterobacter* spp. and *Enterobacter cloacae*), extending the lag phase and reducing overall the maximum cell density after 24 hours of incubation (Figure 1A). Conversely, sub-inhibitory concentrations of fermented NA only slightly affected the lag phase extension (Figure 1B).

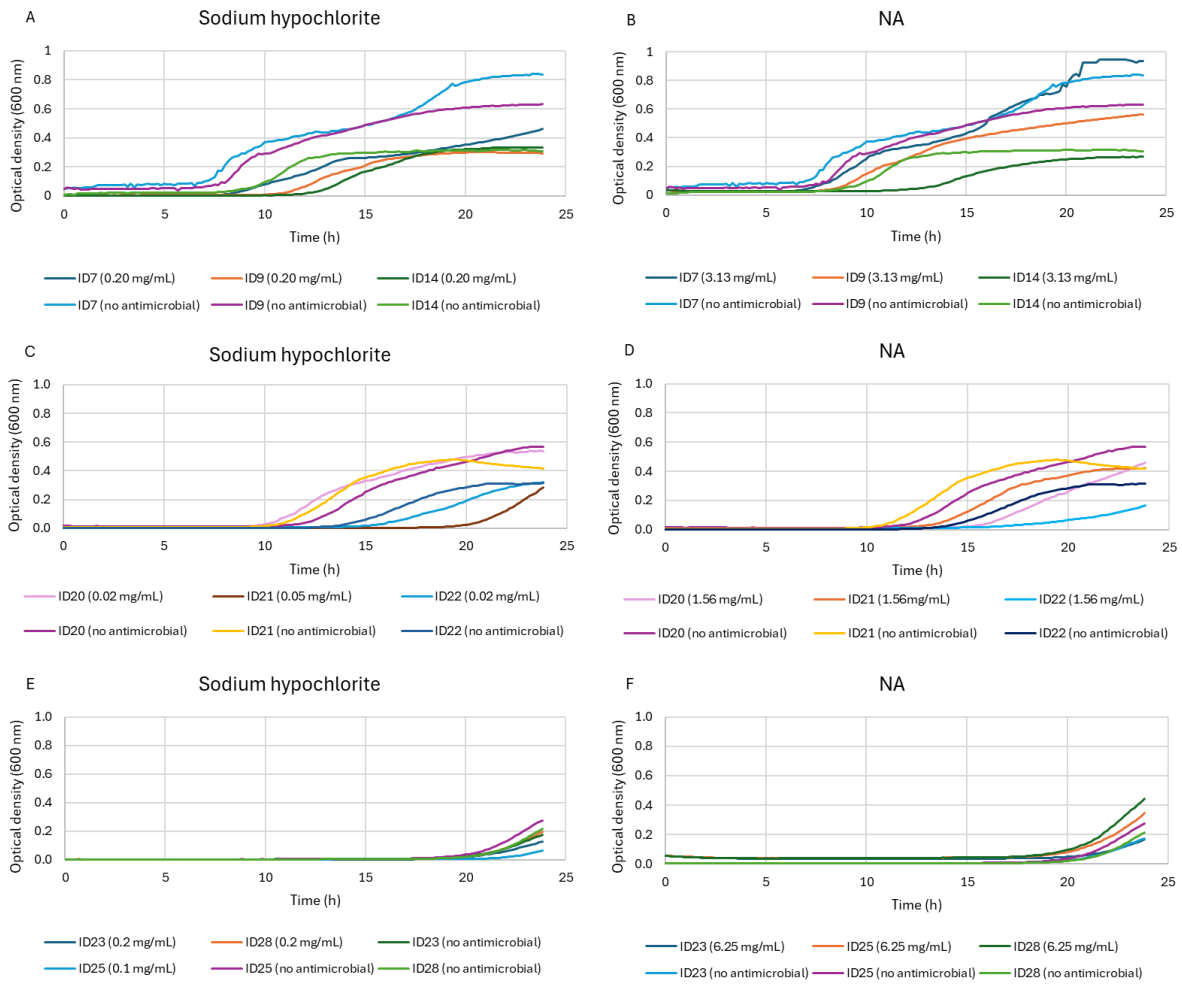


Figure 1. Effect of sub-inhibitory concentrations of sodium hypochlorite and NA (extract) against Enterobacteriaceae (A and B), Bacillaceae (C and D), Lactobacillaceae (E and F).

For Bacillaceae (*Bacillus* spp. and *Bacillus licheniformis*), both sodium hypochlorite and fermented NA visibly influenced the lag phase, prolonging the adaptation period (Figure 1C and D). Finally, in Lactobacillaceae (*Levilactobacillus brevis* and *Pediococcus pentosaceus*), a prolonged lag phase was observed under all tested conditions, including both the presence and absence of antimicrobials (Figure 1E and F).

The effects of sub-inhibitory concentrations have previously been evaluated by other researchers using various naturally derived compounds. Sub-inhibitory concentrations of plant extracts generally can extend the lag phase and/or reduce the growth rate (Nassar et al.

2012). Indeed, Pedreira et al. (2024) reported the sub-inhibitory effect of carvacrol against *Escherichia coli* and *Bacillus cereus* showing a reduction in maximum cell density and a decline in the population growth following the compound concentration increases. Similarly, the slowdown effect on *Staphylococcus mutans* growth was observed with the addition of honey (Nassar et al. 2012).

The same trend was observed by Navarro-Pérez et al. (2021) while studying the effect of sub-inhibitory concentrations of propolis extract against *S. mutans* and *Staphylococcus sanguinis*. At 1/2 MIC, both strains displayed an extended lag phase with a delayed onset of growth. However, the growth rate declined from the beginning for *S. mutans*, while it remained similar to the control for *S. sanguinis*.

Evaluation of antimicrobial activity and cell damage in water

To evaluate the effectiveness of antimicrobial treatment on complex microbial communities, a design of experiment was set up, considering the following variables: NA concentration, NaClO concentration, and time of exposure (minutes) (Table 1). The conditions used were applied to a complex microbial bulk, combining the 3 strains belonging to the same family at a time. In this way, it is possible to create a "mock" microbial community, albeit simple in terms of species composition, that might mimic the complexity observed in environmental conditions (Li et al. 2017). Aliquots of the microbial bulk were subjected to the conditions reported in Table 1, and at the selected timepoints microbial cell damage was evaluated by qPCR after PMAxx treatment. In previous studies, the efficacy of qPCR after PMA treatment has been evaluated on a number of different samples, including environmental matrices, biofilms and probiotic strains (Guo et al. 2024a; Diarra et al. 2023; Truchado et al. 2016). To

evaluate the bactericidal effect in water, plate counts were performed as well.

As reported in figure 2, the isolates belonging to the family Enterobacteriaceae showed the highest sensitivity to all the selected conditions, including control exposure to sterile bidistilled water for 10 minutes.

For this microbial family, it appears that treatment with low concentrations of NaClO or antimicrobials alone is more effective, leading to cellular damage in about 94% of the population (Figure 2, conditions N2, N6, N12, N16) in the case of antimicrobial, or to 100% cellular damage in the case of NaClO treatment alone (Figure 2, conditions N3, N7, N13, N17). If the treated microbial cultures are cultivated on agar plates, we observe a recovery only in the control samples, that grow at the expected concentration of 4 Log CFU/mL. This indicates that the damage observed by qPCR is sublethal, and the cells can recover growth, while in the case of exposure to any of the antimicrobials, alone or in combination, no growth is recovered on the agar plates.

In the case of Bacillaceae, 83% of the cells remained viable when exposed to NaClO under the specified conditions (Figure 2, N3, N7, N13 and N17), regardless of contact time. In the control conditions (Figure 2, N1, N5, N11 and N15) and those in which NA was used, either alone (Figure 2, N2, N6, N12 and N16) or in combination with NaClO (Figure 2, N4, N8, N9, N10, N14, N18, N19 and N20) resulted in a higher percentage of damaged cells, with observations ranging from 89% to 98%.

When placed on an agarised medium, growth was observed only in the control conditions (Figure 2, N1, N5, N11, and N15). In this instance, the exposure time impacted the damaged cells concentration detected following a ten-minute and one-hour period.

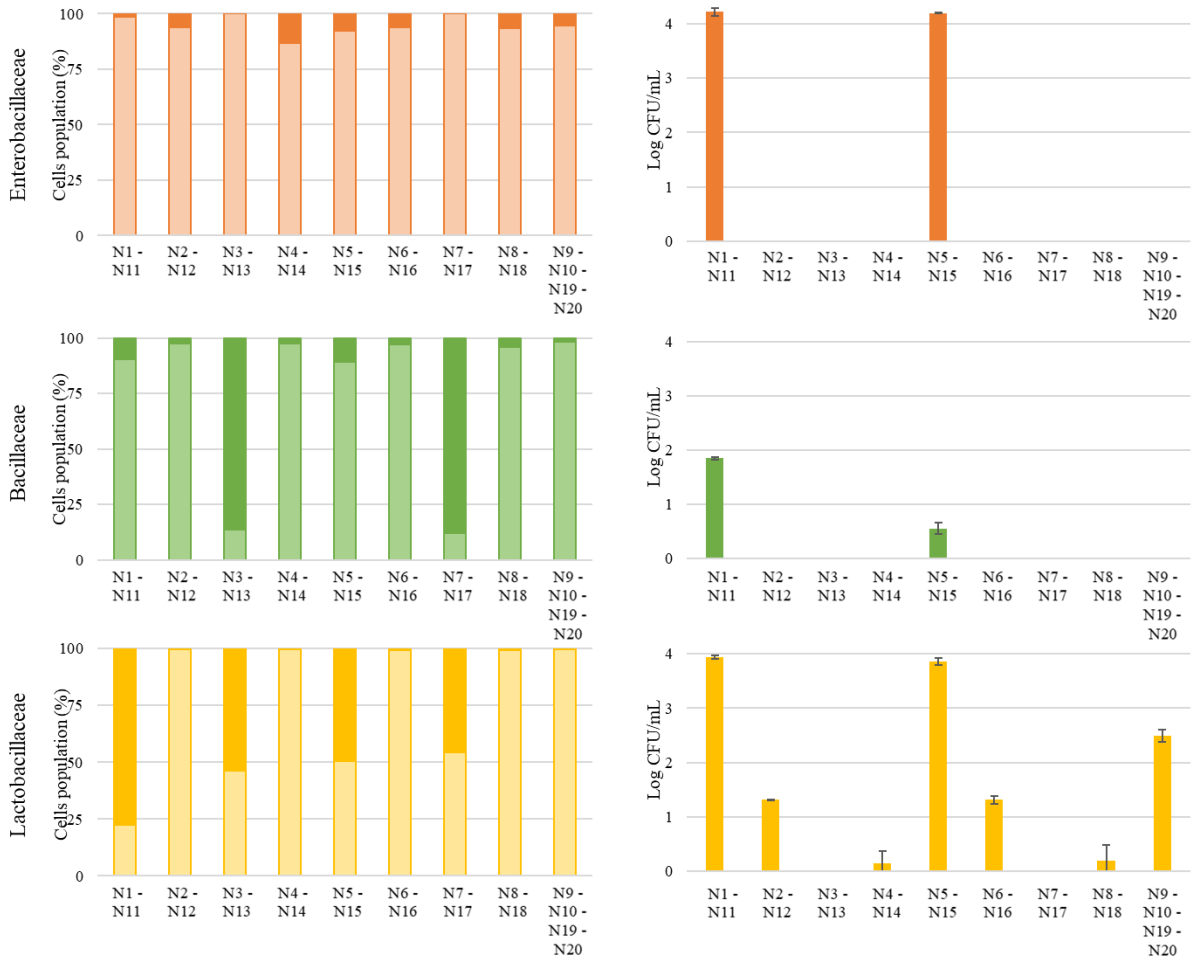


Figure 2 Evaluation of antimicrobial activity in water. On the left are the values measured with Real Time qPCR (total concentration) and after PMAxx treatment with the lighter bars (damaged cell concentration), on the right are the results of the plate counts.

It may be postulated that the incubation period in water caused the cells to sporulate, reducing the antimicrobial effect and, as a consequence, PMA entrance in the cells.

The discrepancy between the qPCR and count data may also be attributed to the presence of "ghost" bacteria, which are metabolically inactive but possess an intact cell wall/membrane. This prevents PMA binding, resulting in a discrepancy between the two analytical approaches (Truchado et al. 2016).

In the case of Lactobacillaceae, more than 99% cell damage was observed in the presence of NA (Figure 2, N2, N4, N6, N8, N9, N10, N12, N14, N16, N18, N19 and N20), independently of

contact time. In controls (N1, N5, N11 and N15) and samples treated only with NaClO (N3, N7, N13 and N17), on the other hand, less cell damage was recorded, which tended to increase with time. In fact, in the controls, the number of damaged cells rose from 22% to 50% after ten minutes and one hour, and in the NaClO samples from 46% to 54% after ten minutes and one hour.

When cells exposed to these conditions were placed on the agar plates, we observed a recovery in the control samples of the initial concentration (4 Log CFU/mL). On the other hand, a growth of approximately 1 Log CFU/mL is observed in the samples treated with NA alone and no growth is recorded in those with NaClO. In the central points, where the two antimicrobials are present at half the concentration of, there is more growth than in the samples treated with NA or NaClO alone. This would indicate an inhibition of the antimicrobial activity carried out by the two antimicrobials individually.

It is therefore necessary to consider not only the sublethal damage to some cells, which are able to recover growth when placed in an optimal condition, but also the contribution of those in a viable but not culturable state (VBNC) (Guo et al. 2024b; Fiore, et al. 2020), as observed in conditions N3, N7, N13 and N17.

Conclusion

In light of the increasing need for effective and sustainable sanitization solutions in the food industry, this study highlights the potential of NA derived from tomato production waste. By focusing on their application in cooling water during the canning process and exploring their synergistic effects with chlorine-based products, the findings offer valuable insights into improving microbial control while aligning with environmental goals. While the antimicrobial effect of NaClO is already well-known, its utilization poses some challenges. In this context, the promising results in microbial control achieved through the application of NA are noteworthy. Viability assessments indicate that exposure to NaClO at a concentration of 2 ppm can cause lethal damage to mixed populations of Enterobacteriaceae, Bacillaceae, and Lactobacillaceae families. Similarly, high concentrations of NA prove especially effective against Enterobacteriaceae and Bacillaceae, though they are less effective against Lactobacillaceae. This reduced effectiveness is unsurprising, as NA is produced by fermentation by members of this same family, which exhibit increased tolerance to the selected conditions. However, the combination of NA and NaClO at lower concentrations achieves a bactericidal effect across all three microbial families, even with reduced exposure time. These results underscore the importance of evaluating the antimicrobial properties of new compounds in complex scenarios, considering the diversity of natural microbial communities. Synergistic effects not only provide broad-spectrum decontamination of microorganisms but also reduce the quantity of antimicrobial compounds required.

References

Arias-Moliz, Maria Teresa, Ronald Ordinola-Zapata, Pilar Baca, Matilde Ruiz-Linares, and Carmen María Ferrer-Luque. 2014. "Antimicrobial Activity of a Sodium Hypochlorite/Etidronic Acid Irrigant Solution." Journal of Endodontics 40 (12): 1999–2002. https://doi.org/10.1016/j.joen.2014.07.031.

Bizhang, Mozhgan, B. I. Ellerbrock, D. Preza, W. H.M. Raab, P. Singh, T. Beikler, B. Henrich, and S. Zimmer. 2011. "Detection of Nine Microorganisms from the Initial Carious Root Lesions Using a TaqMan-Based Real-Time PCR." Oral Diseases 17 (7): 642–52. https://doi.org/10.1111/j.1601-0825.2011.01815.x.

Chakraborty, Sayantan, and Himjyoti Dutta. 2022. "Use of Nature-Derived Antimicrobial Substances as Safe Disinfectants and Preservatives in Food Processing Industries: A Review." Journal of Food Processing and Preservation. John Wiley and Sons Inc. https://doi.org/10.1111/jfpp.15999.

Chinchkar, Ajay V., Anurag Singh, Sukh Veer Singh, Asmita Mukundrao Acharya, and Meenatai G. Kamble. 2022. "Potential Sanitizers and Disinfectants for Fresh Fruits and Vegetables: A Comprehensive Review." Journal of Food Processing and Preservation. John Wiley and Sons Inc. https://doi.org/10.1111/jfpp.16495.

DeQueiroz, G. A., and D. F. Day. 2007. "Antimicrobial Activity and Effectiveness of a Combination of Sodium Hypochlorite and Hydrogen Peroxide in Killing and Removing Pseudomonas Aeruginosa Biofilms from Surfaces." Journal of Applied Microbiology 103 (4): 794–802. https://doi.org/10.1111/j.1365-2672.2007.03299.x.

Diarra, Carine, Coralie Goetz, Mérilie Gagnon, Denis Roy, and Julie Jean. 2023. "Biofilm Formation by Heat-Resistant Dairy Bacteria: Multispecies Biofilm Model under Static and Dynamic Conditions." Applied and Environmental Microbiology 89 (10). https://doi.org/10.1128/aem.00713-23.

Fiore, Walter, Stefania Arioli, and Simone Guglielmetti. 2020. "The Neglected Microbial Components of Commercial Probiotic Formulations." Microorganisms 8 (8): 1–8. https://doi.org/10.3390/microorganisms8081177.

Gil, Maria I., Maria V. Selma, Francisco López-Gálvez, and Ana Allende. 2009. "Fresh-Cut Product Sanitation and Wash Water Disinfection: Problems and Solutions." International Journal of Food Microbiology. https://doi.org/10.1016/j.ijfoodmicro.2009.05.021.

Gomes, B P F A, C C R Ferraz, M E Vianna, V B Berber, F B Teixeira, and F J Souza-Filho. 2001. “In Vitro Antimicrobial Activity of Several Concentrations of Sodium Hypochlorite and Chlorhexidine Gluconate in the Elimination of Enterococcus Faecalis.” International Endodontic Journal. Vol. 34.

Guo, Lizheng, Xiaolei Ze, Yingxin Jiao, Chengyu Song, Xi Zhao, Zhiquan Song, Shuaicheng Mu, et al. 2024a. “Development and Validation of a PMA-QPCR Method for Accurate Quantification of Viable Lacticaseibacillus Paracasei in Probiotics.” Frontiers in Microbiology 15. https://doi.org/10.3389/fmicb.2024.1456274.

Hadj Saadoun, Jasmine, Alessia Levante, Martina Marrella, Valentina Bernini, Erasmo Neviani, and Camilla Lazzi. 2022. “Influence of Processing Parameters and Natural Antimicrobial on Alicyclobacillus Acidoterrestris and Clostridium Pasteurianum Using Response Surface Methodology.” Foods 11 (7). https://doi.org/10.3390/foods11071063.

Li, Ru, Hein Min Tun, Musarrat Jahan, Zhengxiao Zhang, Ayush Kumar, Dilantha Fernando, Annemieke Farenhorst, and Ehsan Khafipour. 2017. “Comparison of DNA-, PMA-, and RNA-Based 16S RRNA Illumina Sequencing for Detection of Live Bacteria in Water.” Scientific Reports 7 (1). https://doi.org/10.1038/s41598-017-02516-3.

Marchesi, Julian R, Takuichi Sato, Andrew J Weightman, Tracey A Martin, John C Fry, Sarah J Hiom, and William G Wade. 1998. “Design and Evaluation of Useful Bacterium-Specific PCR Primers That Amplify Genes Coding for Bacterial 16S RRNA.” APPLIED AND ENVIRONMENTAL MICROBIOLOGY. Vol. 64. https://journals.asm.org/journal/aem.

Mohamed, Ibrahim O. 2007. “DETERMINATION OF AN EFFECTIVE HEAT TRANSFER COEFFICIENT FOR IMMERSION WATER COOLING OF CANNED FOODS.” Journal of Food Process Engineering.

Nassar, Hani M., Mingyun Li, and Richard L. Gregory. 2012. “Effect of Honey on Streptococcus Mutans Growth and Biofilm Formation.” Applied and Environmental Microbiology 78 (2): 536–40. https://doi.org/10.1128/AEM.05538-11.

Navarro-Pérez, M. Luisa, Virginia Vadillo-Rodríguez, Irene Fernández-Babiano, Ciro Pérez-Giraldo, and M. Coronada Fernández-Calderón. 2021. “Antimicrobial Activity of a Novel Spanish Propolis against Planktonic and Sessile Oral Streptococcus Spp.” Scientific Reports 11 (1). https://doi.org/10.1038/s41598-021-03202-1.

Pahalagedara, Amila S.N.W., Elissavet Gkogka, and Marianne Hammershøj. 2024. “A Review on Spore-Forming Bacteria and Moulds Implicated in the Quality and Safety of Thermally

Processed Acid Foods: Focusing on Their Heat Resistance." Food Control. Elsevier Ltd. https://doi.org/10.1016/j.foodcont.2024.110716.

Pedreira, Adrián, Nerea Martínez-López, José Antonio Vázquez, and Míriam R García. 2024. "Modelling the Antimicrobial Effect of Food Preservatives in Bacteria: Application to Escherichia Coli and Bacillus Cereus Inhibition with Carvacrol." Journal of Food Engineering 361:111734. https://doi.org/10.5281/zenod.

Radcliffe, C E, L Potouridou, R Qureshi, N Habahbeh, A Qualtrough, H Worthington, and D B Drucker. 2004. "Antimicrobial Activity of Varying Concentrations of Sodium Hypochlorite on the Endodontic Microorganisms Actinomyces Israelii, A. Naeslundii, Candida Albicans and Enterococcus Faecalis." Endodontic Journal. Vol. 37.

Ricci, Annalisa, Valentina Bernini, Antonietta Maoloni, Martina Cirlini, Gianni Galaverna, Erasmo Neviani, and Camilla Lazzi. 2019. "Vegetable By-Product Lacto-Fermentation as a New Source of Antimicrobial Compounds." Microorganisms 7 (12). https://doi.org/10.3390/microorganisms7120607.

Ricci, Annalisa, Gaia Bertani, Antonietta Maoloni, Valentina Bernini, Alessia Levante, Erasmo Neviani, and Camilla Lazzi. 2021. "Antimicrobial Activity of Fermented Vegetable Byproduct Extracts for Food Applications." Foods 10 (5). https://doi.org/10.3390/foods10051092.

Sassone, Luciana M., Rivail Antonio Sergio Fidel, Cristiana Francescutti Murad, Sandra Rivera Fidel, and Rafael Hirata. 2008. "Antimicrobial Activity of Sodium Hypochlorite and Chlorhexidine by Two Different Tests." Australian Endodontic Journal 34 (1): 19–24. https://doi.org/10.1111/j.1747-4477.2007.00071.x.

Sena, N. T., B. P.F.A. Gomes, M. E. Vianna, V. B. Berber, A. A. Zaia, C. C.R. Ferraz, and F. J. Souza-Filho. 2006. "In Vitro Antimicrobial Activity of Sodium Hypochlorite and Chlorhexidine against Selected Single-Species Biofilms." International Endodontic Journal 39 (11): 878–85. https://doi.org/10.1111/j.1365-2591.2006.01161.x.

Somani, S B, N W Ingole, and N S Kulkarni. 2011. "DISINFECTION OF WATER BY USING SODIUM CHLORIDE (NaCl) AND SODIUM HYPOCHLORITE (NaOCl)." Journal of Engineering Research and Studies.

Squitieri, G., G. Pirone, L. La Pietra, M. Cannavacciuolo, A. Pezzani, G. Ferrari, and D. ... & Cautela. 2023. "Criteria and Methodologies for Determining the Causes of Swelling of

Canned Tomatoes in Tinplate Containers." Comprehensive Reviews in Food Science and Food Safety.

Takahashi, Hajime, Rumi Saito, Satoko Miya, Yuichiro Tanaka, Natsumi Miyamura, Takashi Kuda, and Bon Kimura. 2017. "Development of Quantitative Real-Time PCR for Detection and Enumeration of Enterobacteriaceae." International Journal of Food Microbiology 246 (April):92–97. https://doi.org/10.1016/j.ijfoodmicro.2016.12.015.

"Tomato News." n.d.

Truchado, Pilar, Maria I. Gil, Tanja Kostic, and Ana Allende. 2016. "Optimization and Validation of a PMA QPCR Method for Escherichia Coli Quantification in Primary Production." Food Control 62 (April):150–56. https://doi.org/10.1016/j.foodcont.2015.10.014.

Vannini, Lucia, and Lorenzo Siroli. 2025. "Canned and Heat Preserved Foods." The Microbiological Quality of Food. Elsevier Science Ltd.

Vianna, Morgana Eli, Brenda P.F.A. Gomes, Vanessa Bellocchio Berber, Alexandre Augusto Zaia, Caio Cezar Randi Ferraz, and Francisco José De Souza-Filho. 2004. "In Vitro Evaluation of the Antimicrobial Activity of Chlorhexidine and Sodium Hypochlorite." Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontics 97 (1): 79–84. https://doi.org/10.1016/S1079-2104(03)00360-3.

Conclusion

This PhD study demonstrated the potential of lactic acid bacteria (LAB)-mediated fermentation as a sustainable method for valorizing vegetable by-products and waste, transforming them into value-added products and exploring novel applications. Given the sustainable and environmental benefits of fermentation, this work aimed to increase knowledge of the microbial conversion of two substrates of economic and climate change relevance, the soybean by-product okara and the cladodes of *Opuntia ficus-indica*. Chapter 1 and 2 focused on the production of bioactive compounds, highlighted the contribution of LAB metabolism on the polyphenol profile, antioxidant and antimicrobial activities, as well as the modifications modulating the aromatic profile of in fermented matrices. The biomasses and extract produced by fermentation can be considered as multifunctional ingredients with wide prospects for use in various sectors, from food to cosmetics and animal feed.

In chapter 1, the use of Design of Experiments (DoE) was instrumental in optimizing fermentation conditions and studying the effects of different parameters on bioactive compound production. For okara, optimal conditions (3 Log CFU/g inoculum, 25°C, and 96 hours) were identified, resulting in the release of bioavailable phenolic compounds such as daidzein, glycitein, and genistein. Notably, LAB strains *P. pentosaceus* and *L. paracasei* exhibited robust glucosidase activity, facilitating the conversion of isoflavone glycosides into bioavailable aglycones. Furthermore, the formation of novel phenolic acids, such as phenyllactic and p-hydroxyphenyllactic acids, was observed, particularly with *L. paracasei* and *L. rhamnosus*. These findings demonstrate the ability of LAB fermentation to enhance the functional properties of okara products, aligning with consumer demand for health-oriented, bioactive-rich functional foods.

Similarly, fermentation of cladodes revealed significant increases in total phenolic content and antioxidant activity under specific conditions, such as fermentation with *L. brevis* at 25°C for 24 hours. In chapter 2, DoE analysis enabled the identification of conditions to further enhance these properties. Additionally, the antimicrobial activity of cladode extracts was investigated, revealing varying degrees of efficacy against microbial targets. While *L. mesenteroides* exhibited extended lag phases under all extract conditions, the extract obtained from the fermented with *L. brevis* and *L. mesenteroides* resulted in complete growth inhibition during the Log phase, highlighting the potential of these extracts for microbial control.

Volatile compound analysis of fermented cladodes revealed significant changes in their aromatic profile. While unfermented samples were dominated by aldehydes and ketones responsible for green and herbaceous notes, heterofermentative LAB strains, such as *L. mesenteroides* and *L. brevis*, produced alcohols and acids that contributed fruity and bready notes. These modifications underscore the ability of LAB fermentation to enhance sensory properties, broadening its applicability in food product development.

Chapter 3 instead focused on the potential application as a water disinfectant of an antimicrobial agent of natural origin recently patented by the University of Parma. The extract (NA) studied is derived from a fermentation and extraction process of peels and seeds from the tomato processing industry.

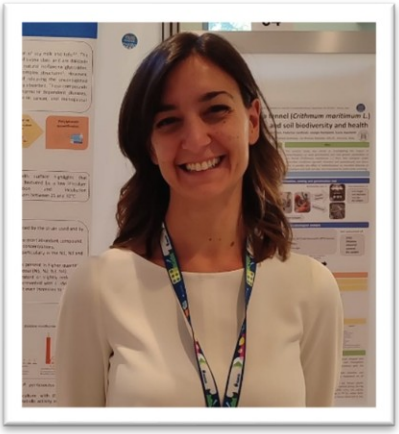
The increasing need for effective and sustainable sanitisation solutions in the food industry and the growing interest in natural antimicrobials has led to the study of its application in cooling water during the canning process in the tomato industry, with promising results.

Preliminary monitoring study identified potential spoilage micro-organisms that were selected as targets for testing the efficacy of the antimicrobial. Viability assessments indicate that exposure at

high concentrations of NA, prove especially effective against Enterobacteriaceae and Bacillaceae, similarly to a traditional sanitizer (sodium hypochlorite), though NA are less effective against Lactobacillaceae. This reduced effectiveness is unsurprising, as NA is produced by fermentation by members of this same family, which exhibit increased tolerance to the selected conditions. When combined with sodium hypochlorite, the NA exhibited synergistic effects, achieving broad-spectrum microbial control while reducing the required concentrations of each agent. This innovative approach aligns with environmental sustainability goals by minimizing chemical usage and leveraging naturally derived compounds for industrial applications.

Overall, the results of this PhD thesis emphasize the versatility and utility of LAB fermentation in addressing global challenges related to sustainability, waste management, and functional ingredient production.

About the author



Martina Marrella was born on 20th March 1989 in Manduria, Italy. She got bachelor's degree in Gastronomic Sciences at the University of Parma discussing the thesis "Study on the preferences and acceptability of new ice cream flavors". She persecuted her study with the Master's degree in Food Science and Technology at the University of Parma discussing the thesis "Algae-based foods: microbiological characterization and product challenge test".

After her master's degree, she worked as a researcher in the food microbiology laboratory on the following projects under the supervision of Professors Camilla Lazzi and Valentina Bernini:

- Strategies for the development of new tomato-based fermented juices
- Development of new lacto-fermented fruit and vegetable products
- Evaluation of the efficacy of different antimicrobials for application in tomato processing
- Behaviour evaluation of Pseudomonas spp. and Escherichia coli during the shelf life of mozzarella cheese by designing and setting up microbiological challenge tests
- The microbial collection of Pecorino Toscano cheese: extension and new selection criteria of autochthonous microorganisms in order to define the 'core' microbial collection
- Evaluation of microflora responsible for the production of off-flavours in tomato pulp

In January 2022, Martina passed the section for the Doctoral School in Food Science at the University of Parma, under the supervision of Prof. Camilla Lazzi. Her study assessed the valorization of vegetable by-products and waste through fermentation by LAB. The research focused on the production of bioactive compounds following lactic fermentation. The research focused on the

production of bioactive compounds, and highlighted the contribution of LAB metabolism on the polyphenol profile, antioxidant and antimicrobial activities, as well as the modifications modulating the aromatic profile of fermented matrices. During the second year of her PhD, she spent time abroad at iBET (Instituto de Biologia Experimental e Tecnológica, Portugal), where chemical characterisation analyses of some fermented products were carried out. The results achieved in the three years of the PhD are described in this thesis.

Scientific activity

Papers published prior to PhD

- Levante, A., Bertani, G., Marrella, M., Mucchetti, G., Bernini, V., Lazzi, C., & Neviani E. (2023). The microbiota of Mozzarella di Bufala Campana PDO cheese: a study across the manufacturing process. Frontiers in Microbiology

- Hadj Saadoun, J., Levante, A., Marrella, M., Bernini, V., Neviani, E., & Lazzi, C. (2022). Influence of processing parameters and natural antimicrobial on Alicyclobacillus acidoterrestris and Clostridium pasteurianum using response surface methodology. Foods

- Marrella, M., Bertani, G., Ricci, A., Volpe, R., Roustel, S., Ferriani, F., Nipoti, E., Neviani, E., Lazzi, C., & Bernini, V. (2022). Pseudomonas fluorescens and Escherichia coli in Fresh Mozzarella Cheese: Effect of Cellobiose Oxidase on Microbiological Stability during Refrigerated Shelf Life. Foods

- Martelli, F., Marrella, M., Lazzi, C., Neviani, E., & Bernini, V. (2021). Microbiological contamination of ready-to-eat algae and evaluation of Bacillus cereus behavior by microbiological challenge test. Journal of Food Protection

- Ricci, A., Marrella, M., Hadj Saadoun, J., Bernini, V., Godani, F., Dameno, F., Neviani, E., & Lazzi. C. (2020). Development of lactic acid-fermented tomato products. Microorganisms

Poster Presentation

- Marrella Martina, Bresciani Letizia, Ricci Annalisa, Agullò Garcia Vincente, Bernini Valentina, Hadj Saadoun Jasmine, Fontechiari Luca, Neviani Erasmo, Lazzi Camilla. Fermented Okara: a source of health-promoting polyphenols. MD2023 - 7th International Conference on Microbial Diversity "Agrifood microbiota as a tool for a sustainable future" (University of Parma). September 26 - 29, 2023.

- Martina Marrella, Naiara Fernández Hernández, Annalisa Ricci, Carolina Ventura, Valentina Bernini, Maria Rosário Bronze, Camilla Lazzi. Changes in the chemical profile of *Opuntia ficus-indica* L. cladodes after lactic acid fermentation. 28th International ICFMH Conference FOOD MICRO 2024 (University of Burgos). July 8 - 11, 2024.

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e Azione IV.5 “Dottorati su tematiche Green”