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Studies with galacto-oligosaccharides and lactic acid bacteria for the valorization of food by-products

DOCTORAL THESIS

Gonçalo Nuno Gouveia Martins
DOCTORATE IN CHEMISTRY

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Gonçalo Nuno Gouveia Martins

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"Mais importante que as respostas,
são as perguntas que se fazem"

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Abstract

Food wastes and by-products' generation raises humanitarian, economic and environmental concerns. The UN's 12th Sustainable Development Goal promotes waste reduction and co-products' valorisation along the food production chain. Prebiotic sugars galacto-oligosaccharides (GOS) resist digestion in the upper gastrointestinal tract, being metabolized by beneficial gut bacteria, supporting their proliferation, and promoting consumers' health. GOS show cryoprotective potential towards lactic acid bacteria during freezing, freeze-drying, and storage, by replacing water molecules and forming a glass-like structure around the bacteria, preventing cell damage. α-GOS can be obtained from natural sources and β-GOS by enzymatic synthesis from lactose. Removal of glucose formed during the synthesis increases the mixture's health benefits. For the valorisation of food by-products, α-GOS from chickpeas' and lentils' cooking wastewaters were used for growing and the stabilization of two food-grade *Lactiplantibacillus plantarum* strains, and β-GOS were produced by two β-galactosidases immobilized in halloysite nanotubes and purified by fermentation with surplus yeast from the Madeiran brewing industry.

Chickpeas' yielded the most α-GOS, while lentils' water contained more GOS of higher degree of polymerization and fewer simple sugars. *L. plantarum* CIDCA 83114 grew in cooking water-containing media, similarly to the standard microbiological media that uses glucose as carbon source. After freezing, freeze-drying, and storage for 3 weeks at 37 °C, GOS wastewaters were the most successful cryoprotectants towards *L. plantarum* WCFS1 strain, outperforming reference materials (sucrose and fructo-oligosaccharides). After the enzymatic synthesis and purification by fermentation in a repeated batch operation, both mixtures' final composition consisted in 41 % β-GOS, with unreacted lactose and galactose present, but only 1 % glucose.

Food industry's by-products are valuable sources of bioactive compounds and materials. Legumes' cooking waters support the growth and protection of food-grade bacteria, while surplus yeast can be used in β-GOS' purification. These added-value products can circle back to the food industry, tackling waste management and environmental concerns, while improving consumers' health by the production of prebiotics, and probiotics with increased shelf-life.

Keywords: Fermentation, High-Performance Liquid Chromatography with Refractive Index Detection, *Lactiplantibacillus plantarum*, *Saccharomyces cerevisiae*, yeast

Resumo

O 12.º Objetivo de Desenvolvimento Sustentável da ONU promove a redução do desperdício e a valorização de sub-produtos na cadeia de produção, prevenindo problemas humanitários, ambientais e económicos. Os açúcares prebióticos galacto-oligossacáridos (GOS) resistem à digestão, sendo metabolizados por bactérias intestinais benéficas, promovendo a sua proliferação, contribuindo para a saúde do consumidor. Os GOS podem ser crioprotetores de bactérias lácticas durante o congelamento, liofilização e armazenamento, substituindo moléculas de água e envolvendo as bactérias numa estrutura protetora. Pode obter-se α-GOS de fontes naturais, enquanto β-GOS são produzidos por síntese enzimática a partir da lactose. Remoção da glucose formada aumenta os benefícios para a saúde. Para a valorização de sub-produtos alimentares, águas de cozedura de grão-de-bico e de lentilhas contendo α-GOS foram usadas para crescimento e estabilização de estirpes alimentares de *Lactiplantibacillus plantarum*, e foram produzidos β-GOS por β-galactosidases imobilizadas em nanotubos de haloisita que foram purificados por fermentação com excedente de levedura da indústria cervejeira.

O grão-de-bico libertou mais α-GOS, já as lentilhas possuíam α-GOS de maior grau de polimerização e menos monossacáridos. A *L. plantarum* CIDCA 83114 cresceu com as águas de cozedura de forma semelhante ao meio de referência com glucose como fonte de carbono. Após congelamento, liofilização e armazenamento por 3 semanas a 37 °C, estas misturas foram os melhores crioprotetores da *L. plantarum* WCFS1, destacando-se dos materiais de referência (sacarose e fruto-oligossacáridos). Após a síntese enzimática e purificação num processo repetido, ambas as misturas finais consistiam em 41 % β-GOS, lactose por consumir e galactose, contudo com apenas 1 % de glucose.

Sub-produtos alimentares são valiosas fontes de compostos e materiais bioativos. As águas de cozedura de leguminosas promovem o crescimento e conferem proteção a bactérias da indústria alimentar, enquanto que o excedente de levedura pode ser usado na purificação de β-GOS. Estes produtos de valor acrescentado podem reinserir-se na indústria alimentar, respondendo a questões de gestão de resíduos e ambientais, ao mesmo tempo que é feita uma aposta na saúde do consumidor, pela produção de prebióticos e probióticos com tempo de vida útil acrescido.

Palavras-chave: Cromatografia Líquida de Alta Precisão com Deteção por Índice de Refração, Fermentação, *Lactiplantibacillus plantarum*, Levedura, *Saccharomyces cerevisiae*

Resumen

El 12º Objetivo de Desarrollo Sostenible de la ONU promueve la reducción del desperdicio y la valorización de subproductos en la cadena de producción, previniendo problemas humanitarios, ambientales y económicos. Los azucares prebióticos galacto-oligosacáridos (GOS) resisten a la digestión, siendo metabolizados por bacterias intestinales benéficas, promoviendo su proliferación, contribuyendo a la salud del consumidor. Los GOS pueden ser crioprotectores de bacterias lácticas durante el congelamiento, liofilización y almacenamiento, sustituyendo moléculas de agua y encapsulando las bacterias en una estructura protectora. Pueden obtenerse α-GOS de fuentes naturales, mientras que los β-GOS son producidos por síntesis enzimática a partir de lactosa. La remoción de la glucosa formada en la síntesis mejora los beneficios para la salud. Para la valorización de subproductos alimenticios, aguas de cocción de garbanzos y de lentejas conteniendo α-GOS fueron usadas para el crecimiento y estabilización de cepas alimentarias de *Lactiplantibacillus plantarum*, y β-GOS fueran sintetizados por β-galactosidasas inmovilizadas en nanotubos de haloisita. Más tarde los β-GOS fueran purificados por fermentación con excedente de levadura de la industria cervecera.

El garbanzo liberó más α-GOS, pero las lentejas contenían α-GOS con mayor grado de polimerización y menos monosacáridos. La *L. plantarum* CIDCA 83114 creció en las aguas de cocción de forma semejante que en los medios de referencia con glucosa como fuente de carbono. Después del congelamiento, liofilización y almacenamiento por 3 semanas a 37 °C, estas mezclas fueron los mejores crioprotectores de la *L. plantarum* WCFS1, destacándose de los materiales de referencia (sacarosa y fructo-oligosacáridos). Luego de la síntesis enzimática y purificación en un proceso repetido, ambas mezclas finales contenían 41 % β-GOS, lactosa y galactosa, pero solo 1 % de glucosa.

Los subproductos alimentarios son valiosas fuentes de compuestos y materiales bioactivos. Las aguas de cocción de legumbres promueven el crecimiento y protegen bacterias de la industria alimentaria, en tanto que el excedente de levadura puede ser usado en la purificación de β-GOS. Estos productos de valor aumentado pueden reincorporarse en la industria alimentaria, respondiendo a cuestiones de gestión de residuos y ambientales, mientras la salud del consumidor es mejorada, por la producción de prebióticos y probióticos con tiempo de vida útil aumentado.

Palabras-clave: Cromatografía Líquida de Alta Eficacia con Detección por Índice de Refracción, Fermentación, *Lactiplantibacillus plantarum*, Levadura, *Saccharomyces cerevisiae*

List of publications

1. **Gonçalo N. Martins**, Mariana Vieira, Carina Caires, Javier González Perez, Paula C. Castilho, Andrea Gómez-Zavaglia. Use of Brewer's Surplus Yeast for the Purification of Galacto-oligosaccharides and as a Nitrogen Source for Bacterial Cultivations' Media. *Food and Bioprocess Technology* 19, 135 (2026): https://doi.org/10.1007/s11947-026-04218-z

2. Angela D. Carboni, **Gonçalo N. Martins**, Paula C. Castilho, M. Cecilia Puppo & Cristina Ferrero. Influence of Thermal Treatment and Granulometry on Physicochemical, Techno-Functional and Nutritional Properties of Lentil Flours. *Foods* 13 17(2024): 2744. http://dx.doi.org/10.3390/foods13172744

3. **Gonçalo N. Martins**, María Guerrero S., Angela D. Carboni, Stéphanie Cenard, Fernanda Fonseca, Andrea Gomez-Zavaglia & Paula C. Castilho. Use of Legume Wastewater Extracts on the Storage Stability of Freeze-Dried *Lactiplantibacillus plantarum* WCFS1. *Food and Bioprocess Technology* (2024): http://dx.doi.org/10.1007/s11947-024-03554-2

4. Stéphanie Passot, Stéphanie Cenard, Pascale Lieben, Sarrah Ghorbal, **Gonçalo N. Martins**, Paula C. Castilho & Fernanda Fonseca. Chickpeas' Cooking Wastewater as an Alternative Source of Galacto-Oligosaccharides for Improving the Freeze-Dried Resistance of Lactic Acid Bacteria. *ACS Food Science & Technology* (2024): http://dx.doi.org/10.1021/acsfoodscitech.4c00120

5. **Gonçalo N. Martins**, Angela D. Carboni, Ayelén A. Hugo, Paula C. Castilho & Andrea Gomez-Zavaglia. Chickpeas and Lentils' Soaking and Cooking Wastewaters Repurposed for Growing Lactic Acid Bacteria. *Foods* 12 12 (2023): 2324. https://www.mdpi.com/2304-8158/12/12/2324

6. Angela D. Carboni, **Gonçalo N. Martins**, Andrea Gomez-Zavaglia & Paula C. Castilho. Lactic Acid Bacteria in the Production of Traditional Fermented Foods and Beverages of Latin America. *Fermentation* 9 4 (2023): 315. https://doi.org/10.3390/fermentation9040315

7. **Gonçalo N. Martins**, Onofre Figueira, Paula C. Castilho. Immobilization of β-Galactosidase in Calcium Alginate Beads. In: Gomez-Zavaglia, A. (eds) *Basic Protocols in Encapsulation of Food Ingredients. Methods and Protocols in Food Science* (2021). Humana, New York, NY. https://doi.org/10.1007/978-1-0716-1649-9_15

8. M. Micaela Ureta, **Gonçalo N. Martins**, Onofre Figueira, Pedro F. Pires, Paula C. Castilho & Andrea Gomez-Zavaglia. Recent Advances in ß-galactosidase and Fructosyltransferase Immobilization Technology. *Critical Reviews in Food Science and Nutrition* 61 16 (2021): 2659-2690. https://doi.org/10.1080/10408398.2020.1783639

9. **Gonçalo N. Martins**, Maria M. Ureta, E. Elizabeth Tymczyszyn, Paula C. Castilho & Andrea Gomez-Zavaglia. Technological Aspects of the Production of Fructo and Galacto-Oligosaccharides. Enzymatic Synthesis and Hydrolysis. *Frontiers in Nutrition* 6 (2019): http://dx.doi.org/10.3389/fnut.2019.00078

List of oral communications (presenting author)

1. **Gonçalo N. Martins**, Andrea Gómez-Zavaglia & Paula C. Castilho. Galacto-oligosaccharides and lactic acid bacteria for the valorization of food by-products. 12th CQM Annual Meeting, 25th July 2025, Funchal, Portugal. In person

2. Guilherme Ribeiro, **Gonçalo N. Martins**, Angela D. Carboni & Paula C. Castilho. Enzymatic extraction of ferulic acid from food by-products: a review. 12th CQM Annual Meeting, 25th July 2025, Funchal, Portugal. In person

3. Regina Sousa, **Gonçalo N. Martins** & Paula C. Castilho. Cooperative activity of xylanases from *Aspergillus oryzae* and Bacillus species for xylo-oligosaccharides' extraction from Brewer's Spent Grain. 12th CQM Annual Meeting, 25th July 2025, Funchal, Portugal. In person

4. **Gonçalo N. Martins**. Estabilización de bacterias ácido-lácticas con aguas residuales de cocción de lentejas y garbanzos. Curso de Posgrado Internacional Virtual: Avances en el desarrollo de materiales bio-basados derivados de resíduos agroalimentarios, 4th December 2024, La Plata, Argentina. Online

5. **Gonçalo N. Martins**, Bruno Drumond, Regina Sousa, Mariana Vieira, Javier Gonzalez, Carina Caires, Alexandra Lopes & Paula C. Castilho. Valorisation of Madeiran brewing industry's by-products. XI jornadas de I+D+i & 3rd International Workshop on STEM y VIII Jornadas del Programa de Doctorado en Instalaciones y Sistemas para la Industria (Curso 2024/25), 17th October 2024, Seville, Spain. Online

6. **Gonçalo N. Martins**, Bruno Drumond, Regina Sousa, Mariana Vieira, Javier Gonzalez, Carina Caires, Alexandra Lopes & Paula C. Castilho. Turning Madeiran brewing industry's by-products into "buy-products". 11th CQM Annual Meeting, 4th October 2024, Funchal, Portugal. In person

7. Stéphanie Passot, Stéphanie Cenard, Pascale Lieben, Sarrah Ghorbal, **Gonçalo N. Martins**, Paula C. Castilho & Fernanda Fonseca. Oligosaccharides as Potential Protectants for Preserving Freeze-Dried Lactic Acid Bacteria. CRYO Conference 2024, 24th July 2024, Washington, USA. In person

8. Paula C. Castilho, **Gonçalo N. Martins**, Javier González, Carina Caires & Mariana Vieira. Purification of GOS (and FOS): oxidation vs fermentation methods. XVIII Congreso Argentino de Ciencia y Tecnologia de Alimentos, Buenos Aires, Argentina, 5th October 2023. In person

9. Angela D. Carboni, **Gonçalo N. Martins**, Ayelén A. Hugo, Paula C. Castilho & Andrea Gómez-Zavaglia. Reaprovechamiento de aguas provenientes del procesamiento de legumbres: Extracción de compuestos prebióticos. Economía circular en sistemas de producción agroalimentarios de Iberoamérica da rede CYTED 323RT0142 - Red Iberoamericana de Innovación para la Sostenibilidad de la Cadena de Valor de Alimentos (Ibero-Circular), 3rd October 2023, La Plata, Argentina. In person

10. **Gonçalo N. Martins**, Maria Guerrero S., Angela D. Carboni, Fernanda Fonseca, Sonia Campoy, Andrea Gómez-Zavaglia & Paula C. Castilho. Stabilization of *Lactiplantibacillus plantarum* WCFS1 during freezing, freeze-drying, and storage using legume wastewater extracts containing galacto-oligosaccharides. 10th CQM Annual Meeting, 1st-2nd June 2023, Funchal, Portugal. In person

11. Javier González, **Gonçalo N. Martins**, Andrea Gómez-Zavaglia & Paula C. Castilho. Purification of galacto-oligosaccharides by enzymatic reaction with glucose oxidase. 10th CQM Annual Meeting, 1st-2nd June 2023, Funchal, Portugal. In person

12. **Gonçalo N. Martins**, Andrea Gómez-Zavaglia & Paula C. Castilho. Applications of GOS extracts: Sugar sources and protective compounds for lactic acid bacteria. PREMIUM Final Meeting, 3rd-4th May 2023, Cambridge, United Kingdom. Online

13. **Gonçalo N. Martins**, Angela D. Carboni, Ayelén A. Hugo, Andrea Gómez-Zavaglia & Paula C. Castilho. Discards from legumes as bacteria cryoprotectants. 9th CQM Annual Meeting, 29th September 2022, Funchal, Portugal. In person

14. **Gonçalo N. Martins**, Onofre Figueira & Paula C. Castilho. Immobilization of β-galactosidase in calcium alginate beads. 8th CQM Annual Meeting, 8th October 2021, Funchal, Portugal. In person

List of poster presentations (presenting author)

1. Bruno Drumond, **Gonçalo N. Martins** & Paula C. Castilho. Enzymatic extraction of ferulic acid from brewer's spent grain. XI jornadas de I+D+i & 3rd International Workshop on STEM y VIII Jornadas del Programa de Doctorado en Instalaciones y Sistemas para la Industria (Curso 2024/25), 17th October 2024, Seville, Spain. Online

2. Angela D. Carboni, **Gonçalo N. Martins**, Cristina Ferrero & María C. Puppo. Textural modifications of high-protein bakery products. XI jornadas de I+D+i & 3rd International Workshop on STEM y VIII Jornadas del Programa de Doctorado en Instalaciones y Sistemas para la Industria (Curso 2024/25), 17th October 2024, Seville, Spain. Online

3. **Gonçalo N. Martins**, Maria Guerrero S., Angela D. Carboni, Fernanda Fonseca, Sonia Campoy, Andrea Gomez-Zavaglia & Paula C. Castilho. Stabilization of *Lactiplantibacillus plantarum* WCFS1 during freezing, freeze-drying, and storage using legume wastewater extracts. 3rd Food Chem Conference, 11th October 2023, Dresden, Germany. In person

4. **Gonçalo N. Martins**, Angela D. Carboni, Ayelén A. Hugo, Paula C. Castilho & Andrea Gomez-Zavaglia Extraction, obtention, and yielding of galacto-oligosaccharides from chickpea and lentils. 3rd Food Chem Conference, 11th October 2023, Dresden, Germany. In person

5. **Gonçalo N. Martins**, Angela D. Carboni, Paula C. Castilho, María C. Puppo, Cristina Ferrero & Andrea Gómez-Zavaglia. Impacto económico del descarte de aguas provenientes del procesamiento de legumbres. 1º Congreso Nacional de Innovación Social, La Plata, Argentina, 31st May 2023. In person

6. Angela D. Carboni, **Gonçalo N. Martins**, Ayelén A. Hugo, Andrea Gómez-Zavaglia & Paula C. Castilho. Application of soaking and cooking waters as prebiotics. XVI Encontro de Química dos Alimentos, 25th October 2022, Castelo Branco, Portugal. In person

7. Angela D. Carboni, **Gonçalo N. Martins**, Cristina Ferrero, María C. Puppo & Paula C. Castilho. Techno-functional and physicochemical properties of lentil flour with different particle sizes. XVI Encontro de Química dos Alimentos, 25th October 2022, Castelo Branco, Portugal. In person

Secondments

- May 2023 - Biosearch Life, a Kerry Company – Granada, Spain (1 week)
- September-October 2022 - Biosearch Life, a Kerry Company – Granada, Spain (1 month)
- March-April 2022 - Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CIDCA) – Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) – La Plata, Argentina (1 month)

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Preface and Structure

This section is divided in three parts, **Context**, **Introduction**, and **Structure**, in order to provide the reader with a basic understanding of how this thesis was planned, the scientific importance of studying these subjects, and, finally, how the thesis was structured and organized.

Context

The design of this thesis' workplan was greatly influenced by the participation of the research team comprised by me and my supervisors in the Horizon 2020 international consortium and research project PREMIUM – Preservation of Microorganisms using Oligosaccharides and Eco-friendly Processes (nr. 777567); and to a lesser extent by previous research done during my master's degree.

The PREMIUM project set out to study the role of oligosaccharides, specifically galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS), in the preservation of lactic acid bacteria during the freezing, the freeze-drying, and storage of the bacteria to prevent loss of viability. Both groups at the University of Madeira and CIDCA-CONICET[1] were tasked with producing prebiotic oligosaccharide mixtures (FOS and GOS) by hydrolysis from plant sources, e.g., production wastes, and by enzymatic synthesis, respectively for each group. In 2018, as the PREMIUM project was picking up, I answered a PhD scholarship call by the FCT to pursue research in this subject. Although it was unsuccessful, I continued working on these topics, and in late 2018, I did a three months secondment to CIDCA-CONICET and one review paper was published on the obtention of GOS and FOS by both hydrolysis and synthesis.[1] After that, we started studying enzyme immobilization for oligosaccharide's production, and a review paper was published on the immobilization of fructosyltransferases and β-galactosidases for enhancing FOS and GOS production [2], as well as a book chapter detailing the immobilization of β-galactosidase in calcium alginate beads.[3] In 2019, GOS samples prepared by hydrolysis of chickpeas were delivered to fellow partners in the National Research Institute for Agriculture, Food and the Environment (INRAe) in France, who studied their potential as promising bacterial cryoprotectors.[4]

[1] CIDCA-CONICET = *Centro de Investigación y Desarrollo en Ciencia y Tecnologia de los Alimentos - Consejo Nacional de Investigaciones Científicas y Técnicas - Universidad Nacional de La Plata - Comisión de Investigaciones Científicas de la Provincia de Buenos Aires*

During my master's degree in Applied Biochemistry, I performed one small project for the valorisation of residues of the Madeiran brewing industry, namely Brewers' Spent Grain and Surplus Yeast, and from there came the idea for the oligosaccharides' mixtures' purification method using yeast fermentation, specifically surplus yeast that is produced by the food industry as a waste product.

When I started by PhD in 2021 after receiving a PhD studentship by the FCT in collaboration with the CQM (UI/BD/152066/2021), there were two secondments planned in 2022 in the scope of the PREMIUM project, to CICDA-CONICET and to Biosearch Life (a Kerry Company), in Spain. With this timetable in mind the workplan for my PhD was shaped to align with the project's goals, and my own research interests. And so, in summary, for this thesis, we proposed the study of the obtention of GOS from both hydrolysis of Portuguese and Argentinean legumes', and by enzymatic synthesis using immobilized enzymes. Purification would be performed by fermentation with surplus yeast. The produced GOS mixtures would be studied as sources of cryoprotectant compounds towards lactic acid bacteria, as well as culture media ingredients.

Introduction

Food wastes and by-products' generation raises humanitarian, economic and environmental concerns. The UN's 12th Sustainable Development Goal promotes waste reduction and co-products' valorisation along the food production chain. In fact, interest in the recovery of valuable components from by-products of agricultural and food processing industries has been growing, leading to new market products or replacements, without compromising food accessibility or price, while decreasing waste production [5]. Food wastes often contain oligosaccharides with prebiotic properties such as galacto-oligosaccharides. GOS can be obtained by extraction/hydrolysis or enzymatic synthesis. Briefly, hydrolysis consists in the isolation of compounds naturally occurring in some plants or seeds. Enzymatic synthesis uses lactose as substrate, and through the use of β-galactosidase enzymes GOS are produced. There are two types of GOS: those produced by enzymatic synthesis from lactose have β-(1→4) or β-(1→6) glycosidic linkages (β-GOS) and a terminal glucose unit; and those with α-(1→6) linkages (α-GOS) which contain terminal glucose or sucrose residues. The latter are known as raffinose-family of oligosaccharides (RFO) and can be found in pulses.[1] Soaking and cooking treatments are often used in the preparation of these food to prevent symptoms of flatulence cause

by these sugars after consumption of legume seeds.[6] These wastewaters can be recovered instead of being discarded and new applications for them can be designed, such as in this thesis, for the cultivation and protection of lactic acid bacteria.

GOS are the object of study in this work due to their prebiotic potential, meaning they are substrates selectively utilized by host microorganisms conferring health benefits. [7] When they are incorporated in food products they show several nutritional properties, reducing their caloric value and improving the consumers' health, contributing to body weight control or relieving gastrointestinal issues such as constipation. [1] Mono- and disaccharides, particularly glucose and lactose, are regarded as contaminants remaining in the final products after hydrolysis and, in the case of lactose, after enzymatic synthesis. They reduce the prebiotic potential of the GOS mixture, and by themselves generate health concerns in cases of diabetes or lactose intolerance, for example. For this reason, their removal is paramount. Several physical methods are found in the literature [1] and have been assayed by our group, namely physical methods using packed columns with activated charcoal or precipitation with co-solvents.[8] These procedures however generate wastes and are laborious. Fermentation with yeasts and treatment with glucose oxidase are two effective and clean chemical methods. Glucose oxidase turns the sugar in gluconate, however, this needs to be removed, for instance by precipitation as calcium gluconate by the addition of calcium hydroxide to the medium.[9] On the other hand, glucose can be efficiently metabolized by yeasts such as *Saccharomyces cerevisiae* recovered as surplus yeast from the brewing industry, and turned into ethanol or CO_2. The yeast can be easily removed by centrifugation and the ethanol by evaporation. Additionally, considering the low cost of inputs and the simple facilities, the enzymatic industrial production of GOS are attractive processes. The cost of enzymes and their recovery, however, are the most pressing issues, because certain experimental conditions (Temperature, pH, and others) can destabilize the enzymes, inhibiting their action or lowering their usefulness, but these can be tackled by enzyme immobilization [2, 10, 11]. The main advantage being that after the immobilization, an insoluble form of the enzyme is obtained, allowing for its recovery and reuse. Commercial forms of β-galactosidase are engineered for the removal of lactose from food products, so the hydrolytic activity is favoured. It is possible that immobilization can change the odds towards transferase activity, increasing GOS' formation over hydrolysis. Previous works have combined β-galactosidase and yeasts for lactose degradation or GOS' formation and glucose removal.

[12, 13] In this work, a similar strategy will be assayed for GOS' production by immobilized β-galactosidases and fermentation by the Madeiran brewer's surplus yeast, increasing GOS yield, and tackling by-product valorization.

Oligosaccharides such as GOS have also been studied in preservation processes towards lactic acid bacteria. During drying and storage of starter cultures, bacteria suffer oxidative stress and membrane damage, leading to loss of viability. GOS and other carbohydrates increase the shelf-life of the stabilized product by forming rigid matrixes surrounding cells, preventing movement and degradation reactions or by preventing damage during drying by forming hydrogen bonds with the cells' membrane polar groups and keeping them in a pseudo-hydrated state. [14–19] Lactic acid bacteria consist of a broad group of bacteria used in the preparation of fermented foods, with important associated health-benefits. Some have probiotic potential when consumed in foods or by dietary supplementation, promoting the consumers' health and well-being, by assisting in colonic fermentation of food, promoting the growth of beneficial (synbiotic) gut bacteria, and releasing compounds such as short-chain fatty acids with health benefits. [20] In this work it is hypothesized that lactic acid bacteria spoilage can be prevented by their stabilization with GOS-containing wastewaters from the cooking of legumes.

Structure

The thesis titled "Studies with galacto-oligosaccharides and lactic acid bacteria for the valorization of food by-products" results from the study of different by-products of the food industry and their up-cycling using different approaches. Much of the work was published in specialized journals, and so, this thesis was put together by their combination. The final paragraphs of this section explore the structure of this document.

The first chapter "Literature review" includes three review papers and details the various approaches for the obtention of GOS, both from natural sources and by enzymatic synthesis using free and immobilized enzymes, and the importance of lactic acid bacteria in the food industry for their role in the fermentation of food, specifically in the preparation of traditional fermented foods of Latin America.

The second chapter "Galacto-oligosaccharides' production and application" combines three research papers discussing how GOS were obtained from soaking and cooking water discards from the cooking of lentils and chickpea seeds. These water

extracts were characterized by High-Performance Liquid Chromatography and were later used for culture medium ingredients and as sources of cryoprotectant compounds for *Lactiplantibacillus plantarum* strains. GOS were also obtained by enzymatic synthesis from lactose, using β-galactosidase enzymes and enzyme immobilization technologies. Later, the mixtures were purified from glucose by fermentation with surplus yeast recovered from a Madeiran brewery. Another valorization strategy for the surplus yeast involved the production of yeast extract through different physical and chemical methods, and the produced extracts were used in bacterial cultivation.

The third and final chapter provides general concluding remarks and is titled appropriately so ("Conclusions"). It was also important to discuss possible future strategies for continuing the research started with this thesis.

Minor spelling or formatting incorrections found in the original publications when writing this document were corrected to ensure a clearer reading experience.

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Chapter I

Literature review

i. Technological Aspects of the Production of Fructo and Galacto-Oligosaccharides. Enzymatic Synthesis and Hydrolysis

frontiers in Nutrition

REVIEW
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Technological Aspects of the Production of Fructo and Galacto-Oligosaccharides. Enzymatic Synthesis and Hydrolysis

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Abstract

Fructo- and galacto-oligosaccharides (FOS and GOS) are non-digestible oligosaccharides with prebiotic properties that can be incorporated into a wide number of products. This review details the general outlines for the production of FOS and GOS, both by enzymatic synthesis using disaccharides or other substrates, and by hydrolysis of polysaccharides. Special emphasis is laid on technological aspects, raw materials, properties, and applications.

Keywords

Fructo-oligosaccharides, galacto-oligosaccharides, enzymatic synthesis, hydrolysis, properties and applications, alternative substrates

Introduction

The first reference to prebiotic concept dates from 1954, when Gyorgy reported that a component of human milk (N-acetyl-glucosamine) promoted the growth of a strain from the genus *Bifidobacterium*. A few years later, Petuely [1] recognized lactulose as a *bifidus factor*. Almost 20 years after, Japanese researchers reported that several non-digestible oligosaccharides were *bifidus factors* [2, 3]. The term prebiotic as such, was

defined in 1995 [4], as “non-digestible food components that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, thus improving host health” [5].

Since then, the original definition was subjected to several revisions. According to the most recent one, prebiotics are “substrates that are selectively used by host microorganisms conferring a health benefit” [6]. Research in different domains (glycomics, proteomics, etc.), reveals more complex interactions of putative prebiotics with the host, thus this definition is far from being the last one. From a scientific point of view, it is a subject still under development and the advances in this issue impact not only on the scientific community, but also on regulatory agencies, food industries, consumers and healthcare professionals [7].

Regardless the definition, fructo- and galacto-oligosaccharides (FOS and GOS) are widely known because of their prebiotic properties. Additionally, their nutritional properties are also important, they are low caloric sweeteners, give a feeling of satiety, contribute to body weight control, relieve constipation, have a low glycemic index and are not cariogenic [8]. GOS and FOS are used in the formulation of dairy products, different types of beverages, bakery products, and some sweets, converting them in functional foods [9]. Moreover, they are extensively employed in infant formula to stimulate the development of newborn microbiota [10, 11].

As GOS and FOS can be incorporated in many products, their demand has exponentially increased worldwide over time [12]. Japan has been pioneer in the production and consumption of FOS and GOS. It was the first country to incorporate non-digestible oligosaccharides in foods, being a world leader in the use of prebiotics as functional ingredients.

In 2006 the functional food market was estimated to be $20 billion in the United States, $15 billion in Europe, and $12 billion in Japan, growing at an annual rate of 7.5 % [13]. Particularly the prebiotic market reached $200 million in 2015, with an increase rate of about 15 % per year (www.reuters.com/article/pressRelease). What is more, according to Global Market Insights, INC (Delaware, USA), the global prebiotic market is expected to surpass $8.5 billion by 2024 [14]. It is remarkable that the increase of the prebiotic market is much higher than that of the food market as a whole, whose increase is about 2 % per year.

Considering the economical and nutritional importance of FOS and GOS, this review will be focused on their obtaining.

From a technological point of view, these prebiotics can be produced either from natural sources or by enzymatic synthesis using disaccharides or other substrates as raw materials. Furthermore, the hydrolysis of polysaccharides present in many fruits and vegetables is another way for obtaining FOS and GOS. Different methods for producing FOS and GOS will be presented, with special emphasis on raw materials, suitable for both synthesis and hydrolysis reactions. Additional properties and applications of FOS and GOS will be also discussed.

FOS

Fructo-oligosaccharides (FOS) are composed of a small number of fructose units linked by (2→1)-β-glycosidic bonds and having a single D-glucosyl unit at the non-reducing end. Particularly, short chain FOS are mixtures of the smallest oligosaccharides, namely 1-kestose [degree of polymerization (DP) equal to 3], nystose (DP4) and 1^F-fructofuranosylnystose (DP5) [4]. They can be obtained either by enzymatic synthesis or by hydrolysis of inulin from natural sources mainly from roots of chicory, artichoke, yacon, dahlia or agave. This later method leads to higher molecular weight FOS.

FOS obtained by Enzymatic Synthesis

The production of FOS obtained by enzymatic synthesis involves transfructosylation reactions where fructosyltransferases (β-fructofuranosidase, EC 3.2.1.26 or β-D-fructosyltransferase, EC 2.4.1.9) act as biocatalysts [10, 11, 15–17]. Meiji Seika Kaisha Ltd. pioneered the production of FOS by enzymatic synthesis using the organism of *Aspergillus niger*. Nowadays, this company is one of the leaders of short chain FOS market all over the world, their products are labeled under the brand names Actilight® in Europe and Meioligo in Asia [18]. Additionally, NutraFlora® from Ingredion group companies is another brand of short chain FOS that leaders the market in North and South America and Australia [19].

Transfructosylation reactions involve the cleavage of the β-2,1-glycosidic bond and the transfer of fructosyl moieties from carbohydrates acting as donors onto any acceptor other than water [17]. Most fructosyltransferases have also a hydrolytic activity, so that the production of FOS is a complex process in which different reactions of synthesis and hydrolysis occur simultaneously both in parallel and in series [17], through consecutive sets of disproportionation reactions. Figure 1 gives a simplified general outline of the mechanism of the mentioned reactions. In such reactions, the FOS synthesized in the first steps act as fructosyl donors and acceptors leading simultaneously to the production of FOS with DP immediately higher (DPn+1) and lower (DPn-1) than those of the FOS acting as reagents [20]. As a result, mixtures of short chain FOS (DP ranging from 2 to 6, i.e., DP3, DP4, DP5, and DP6) [4], together with glucose (secondary product), are obtained. To mathematically describe this mechanism, many authors adapted a kinetic model based on Michaelis-Menten mechanism, assuming that the series of transfructosylation reactions with sucrose, 1-kestose (DP3), and nystose (DP4) as substrates occur in chain, and also considering a competitive glucose inhibition. One of the first approaches in this sense, is the one proposed by Jung et al. [20]. They described the reaction mechanism with sucrose as a substrate that can act either as donor or as acceptor, so that 1 mole of glucose and 1 mole of 1-kestose (DP3) are formed simultaneously, indicating a disproportionation reaction mechanism. This pattern was extended to explain the rest of the pathways involved in the course of the synthesis: 1-kestose (DP3) acts as a substrate and sucrose and nystose (DP4) are produced, afterwards nystose (DP4) acts as a substrate and kestose (DP3) and fructofuranosyl nystose (DP5) are formed. Applying mathematical integration of the several reaction patterns proposed, authors were able to calculate the Michaelis-Menten kinetic constant and the maximum rate of appearance of each product. Duan et al. [21], modified this mathematical model by adding the fact that glucose acts as a substrate inhibitor even for sucrose, 1-kestose (DP3) and nystose (DP4). The same kinetic approach was mathematically described by Alvarado-Huallanco and Maugeri Filho [22], using purified and non-purified fructosyltransferase from *Rhodotorula* sp. In this latter model, the authors considered that hydrolysis occurs when nystose (DP4) concentration reaches about 5 % (w/v). In addition, a much lower value for the nystose hydrolytic constant was found when purified enzyme was used. In the same direction, Guio et al. [23] modified the original model [20], considering the effect of immobilized glucose isomerase, incorporated to improve FOS conversion. In addition, Detofol et al. [24] proved the accuracy of this approach both on

batch and on continuous reactors. According to Vega and Zúniga-Hansen [17], this assumption just partially describes the progress of the reaction because it considers that the same substrate is acting as a donor and acceptor for the fructosyl moiety. However, the active site of fructosyltransferases contains a pocket that accommodates a single sucrose molecule in the substrate-bound structure. Therefore, they proposed a mathematical model based on a mechanism in which sucrose and FOS interact with the enzyme species applying multi-response non-linear regression. This concept was also developed by Khandekar et al. [25] who presented a five-step, ten-parameter kinetic model based on the Michaelis-Menten concept but including the step of binding sites of the enzyme, with sucrose as substrate and glucose as an inhibitor, and also the occurrence of FOS hydrolysis. These contributions regarding the mathematical models explaining the mechanisms involved in the synthesis of FOS were the most important ones reported in the last years. It is worth to mention that the more accurate the model, the better its capacity to explain technological aspects, namely the improving of reaction conditions or yield of the products.

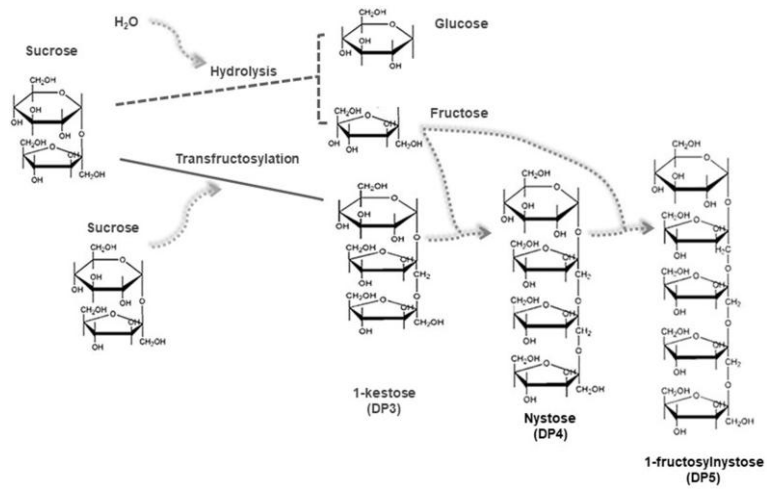

Figure 1 – Simplified scheme for the enzymatic synthesis of FOS through transfructosylation reactions

The composition of the obtained FOS can be modulated by adjusting different parameters, namely substrate concentration, enzyme source, time, temperature and pH, all of them interacting with each other. Therefore, when searching for the optimal value of one of these parameters, the values of the other ones must also be taken into account. Table 1 summarizes several research works on this field, specifying enzyme source, reaction temperature, pH, time, substrate concentration, amount of enzyme, and the resulting FOS yield.

Table 1 – Different enzymes sources, conditions performed, and yields for the synthesis of FOS. [a]$Y_{FOS\ max}$: Maximal yield of FOS; [b]n.i.: not informed; [c]d.b.: dry basis; [d]UT/mL: transfructosylation activity/mL of reaction volume; [d1]UT/g: transfructosylation activity/g of dry support; [e]FU/mL: fructosyltransferase units/mL of reaction volume; [f]U/mL: One unit of enzyme activity; the amount that produce 1 µmol of reduced sugar per minute/mL of reaction volume; [g]U/g: One unit of enzyme activity; the amount that produce 1 µmol of reduced sugar per minute/g of dry support; [h]Informed yield: weight percentages of total sugar; [i]Substrates: sucrose, sugarcane molasses and sugarcane juice; [j]Yield informed as amount of DP4 produced; [k]Substrates: sucrose and sucrose analogs; [l]Substrates: maltose or sucrose; [m]Analysis based on transfructosylated products and acceptor specificity

Enzyme source	T (°C)	pH	Sucrose (g/100 ml)	Enzyme amount	Reaction time (h)	$Y_{FOS\ max}$ (g FOS/100 g sucrose)[a]	Observation	References
COMMERCIAL								
Rohapect CM (AB Enzymes GmbH)	45–60	5.5–6.5	53–72 %	3.4–7.4 UT/mL[d]	3 and 5	41–64 %		[15]
Viscozyme L (Blumos SA)	55	5.5	10–60 %	56 FU/mL[e]	6	65–85 %		[10]
25 enzyme preparations from fungal strains	45–60	4.5–6	40–80 %	4.2–15 UT/mL[d]	6	59–64 %	5 enzymes selected.	[16]
Viscozyme L (Novozyme)	50	5.5	60 %	1,230 UT/g[d1]	2.5 (50 batches)	40–6 %	purified immobilized enzyme.	[26]
Pectinex Ultra SP-L & Rapidase TF (Novozyme)	60	5.6	63 %	0.3 U/mL[f]	144	62 %[h]	immobilized enzyme.	[27]
MICROORGANISM								
A. japonicus TIT-K J1	37	5.4	10, 30, 50 %	0.2, 0.56, 0.96 U/mL[f]	24	65–68 %		[28]
A. japonicus	50	5	45–70 %	5.75 g cell/100 mL	4	51–59 %		[29]
A. aculeatus	50-70	4.8–6.4	20–60 %	20–100 U/mL[f]	4-24	55 % DP3[h]; 43 %DP4[h]		[30]
A. niger	55	6	10, 30, 60 %	0.66 U/mL[f]	88	55-45 %[h]		[31]
S. cerevisiae (invertase)	40–55	5.5	21–85 %	0.5–8.0 U/mL[f]	8	10 % (d.b.)[c]		[25]
Rhodotorula sp.	50	4.5	50–70 %	5 UTF/mL	96	50–58 %		[22]
Rhodotorula sp.	48	6	50 %	0.022 U/mL[f]	48-56-72-96	44–60 %	immobilized and free enzyme.	[24]
Aureobasidium sp. ATCC 20524	30	5.5	40 %	270 U/g[g]	20 mL/h (26 days)	1,512 g DP3	immobilized enzyme; continuous reactor	[32]
Cryptococcus sp.	50	4.5	50 %	1 FTA/mL	48	34 %		[33]
A. niger IMI 303386	39	6.5	50 %	0.4 U/mL[f]	72	62 %[h]		[34]
B. subtilis natto CCT 7712	35-55	7.7	20-40 %[i]	n.i[b]	12–36	388 mg/mL[j]		[35]
Levansucrase SacB of *B. subtilis*	37	6	9 %[k]	1.47 U/mL[f]	24	54 %		[36]
A. niger AS0023	50	5.8	50 %	5×10^6 KU	5	62 %[h]		[37]
A. foetidus	40–70	3–7	30 %[l]	n.i[b]	12	29–48 %		[38]
Arthrohacter sp. K-l	40	6.5	10 %	3.4 U/mL[f]	5–20	n.i[b]	[m]	[39]
A. niger; *A. awamori*, *S. cerevisiae*	40	5	50 %	6 U/g sucrose[f]	8–72	50–37 %[h]		[40]
A. niger, A. pullulans.	50–65	4–8	70 %	1:9 (w/w) cell:sucrose	8	35–38 %		[41]
A. oryzae	55	5–6	60 %	0.14 (v/v) Culture/sucrose	4–24	55 %		[42]
A. oryzae	60	5.5	75 %	275 U/g sucrose[f]	7	57 %		[23]

Regarding substrate concentration, in general terms, higher initial concentration of sucrose (i.e., >40 %), enhances the production of shorter FOS [i.e., 1-kestose (DP3) and nystose (DP4)], with low production of glucose. On the contrary, lower concentrations of sucrose lead to the production of larger FOS [i.e., DP5 and DP6] with a higher production of glucose [10]. Some authors claimed that using very high initial concentration of sucrose (85 % w/v) is a technological strategy for the production of commercial syrups. This way, the final evaporation step is simplified [43]. The modulation of the synthesis regarding initial substrate concentration is important to accurately obtain FOS mixtures with better prebiotic effects, taking into account that the shorter the chain length the greater the prebiotic effect [44]. As stated before, the synthesis of FOS occurs through consecutive sets of disproportionation reactions in which the FOS synthesized in the first steps act as fructosyl donors and acceptors leading simultaneously to the production of FOS with DP immediately higher. Consequently, when the maximum conversion of DP_n is reached, it is followed by a decrease of DP_n, leading to an increase in DP_{n+1}. Taking this into account, it is crucial to know these kinetic parameters (maximum conversion and time at which is reached) to modulate the product composition, not forgetting their dependence on other reaction conditions (pH, temperature, enzyme source, enzyme concentration). In this sense, many authors have studied the effect of substrate concentration on the enzymatic synthesis of FOS, under different conditions [10, 16, 28, 29, 31, 40, 45]. However, only few authors investigated the effect of more than one parameter at the same time. For example, Nemukula et al. [30] proposed a joint analysis of the effect of sucrose concentration, enzyme concentration, reaction time, temperature and pH for obtaining the maximal FOS yield, using response surface methodology. In line with such study, Vega and Zúniga-Hansen [15] studied the interaction of sucrose concentration, temperature and enzyme concentration on FOS, DP3, and volumetric yield. These approaches enabled to determine the most appropriate cost-effective condition to operate (operation temperature 50 °C, pH 5.5, 6.6 TU/mL of enzyme, and 71 % w/v of initial sucrose concentration), which enabled obtaining 63.8 % of FOS yield (short chain FOS grams per 100 g of initial sucrose).

Concerning the enzyme source, all enzymes used for producing FOS (both by synthesis and by hydrolysis) generally belong to the glycoside hydrolases family (GH) and are either included into the GH32 or GH68 families (CAZy classification) [46]. Particularly, enzymes with fructosyltransferase activity can be found in plants, yeasts and

molds (GH32) and in bacteria (GH68) [47]. Most commercial enzyme preparations have both fructosyltransferase and hydrolase activities; this combination gives them advantages over specific enzymes, such as low price, versatility and high stability under reaction industrial processes conditions, but the disadvantage of non-probiotic monosaccharides (i.e., glucose and fructose) being also produced as result of the enzymatic reaction (Table 1). Therefore, preparations with high transfructosylase activity are preferred for the synthesis of short chain FOS. Vega and Zúniga-Hansen [16] studied twenty-five commercial enzyme preparations from the global market (Europe, USA and South America) to obtain short chain FOS from sucrose, weighing up both transfructosylation activity and transferase/hydrolase ratio. As an example, the enzyme *Viscozyme L* from *Aspergillus aculeatus* (Novozyme, Denmark) simultaneously has high transfructosylation activity and high transferase/hydrolase ratio [16, 26, 48] (Table 1). The enzymatic transfructosylation of sucrose with bacterial or fungal fructosyltransferases [23, 33, 42, 49] or fungal β-fructofuranosidases [32, 34] have also shown promissory results. In this regard, using extracellular β-fructofuranosidases from different fungus, together with cultivation with *Picchia pastoris* increases the production of FOS DP3 (26.47 %) and DP4 (57.98 %) [50]. Other reports describe the capacity of *Bacillus subtilis* natto CCT 7712 to produce high amounts of DP5 (nystose) from low-cost substrates, such as sucrose, sugarcane molasses, and sugarcane juice [35]. Each type of enzyme was tested for FOS production under different conditions and the results and main particular observations are presented in (Table 1).

Besides the enzyme characteristics, biocatalysts can be free [10, 11, 15–17, 23, 36] or immobilized [26, 27, 51] in the reaction medium. Immobilization consists on turning the enzyme into a physically confined form in a defined region, blocking its mobility but maintaining its catalytic activity. Many authors reported a higher catalytic efficiency of enzyme membrane reactors employing free enzymes for relatively long periods [52]. Although the enzymatic production of FOS using immobilized enzymes may not work optimally due to limited substrate or product mass transfer to and from the enzymes, it is a relatively new alternative, whose main advantage is offering the possibility of re-using the enzyme. This great advantage denotes the need of further research to overcome the mentioned inconvenients regarding immobilized enzymes.

Optimal pH and temperature strongly depend on the enzyme source. As it is shown in Table 1, the reaction can be performed in a widely pH range of (3–7), and the

temperature can vary from (35–70 °C). Nevertheless, in general terms there can be mentioned that more bounded ranges of optimal pH and temperature can be defined by gathering together more than one type of enzyme. In this respect, a large number of reports have placed the optimum pH and temperature for activity of fructosyltransferase between 4.5–6.5 and 40–60 °C, respectively [30, 53, 54] (Table 1). These two parameters fundamentally affect reaction rates. In this sense, Vega et al. [16] who studied the effect of temperature reaction in a range of 45–60 °C, found that the increase of reaction temperature causes an increase in the reaction rate. A similar behavior has been reported by other authors [15, 41, 55]. It is important to mention that over 60 °C the enzyme could present thermal damage and its activity decreases considerably [26]. Regarding pH, it has strong impact in the ionization state of the constituent amino acids, thus affecting the enzyme's primary and secondary structure and consequently its activity [56]. A pH of around 5.5 was reported to be optimal for fructosyltransferase production in *Penicillium purpurogenum* [57], *Aureobasidium pullulans* [58], and *Syncephalastrum racemosum* Cohn [59].

In general, the synthesis of FOS yields about 60 % FOS, under the form of syrup. Most commercial FOS products are mixtures containing different amounts of FOS, products with 55–99 % of purity. The presence of glucose (and residual sucrose) obtained as secondary product of reaction decreases the prebiotic effect of the mixtures, increasing their caloric and cariogenic value, and thus preventing their incorporation into health, dietetic and diabetic foods [60]. To enhance the purity of FOS, mono and disaccharides can be removed. One option is the continuous removal of glucose and residual sucrose during the synthesis using enzymes and membrane reactors [61, 62]. Another option is the purification process after the synthesis. There are many strategies in the research background of FOS production, but generally both activated charcoal adsorption and enzymatic methods are the most extensively used.

Purification of FOS using activated charcoal consists on the adsorption of sugars onto the activated charcoal, in a reversible process. As activated charcoal is non-polar or hydrophobic, sugars are adsorbed according to their hydrophobic character due to van der Walls forces, which is directly related to their molecular weight (the higher the molecular weight, the more CH groups and the more hydrophobic the sugar is). Hence, FOS are more strongly adsorbed than mono and disaccharides, enabling their separation [60]. In practice, purification involves the filling of columns with activated charcoal (sorbent) and

the re-circulation of the obtained syrups until an equilibrium between the sorbent and the moving phase is reached. After that, the non-adsorbed sugars are removed by circulating milli-Q pure water through the column. Finally, the retained oligosaccharides are recovered by elution with different ethanol gradients [40, 63–65]. The products of elution are also syrups that can be concentrated and even dehydrated to obtain powders [11]. The mechanisms involved in the purification of oligosaccharides using activated charcoal are determined by their molecular interactions. Packer et al. [66] deeply analyzed the efficiency of using graphitized carbon to separate oligosaccharides or their derivatives (hydrazones and alditols) released from glycoproteins from solutions containing salts (of hydroxide, acetate, phosphate), detergents (sodium dodecyl sulfate and Triton X-100), and proteins (enzymes, glycoproteins). Reagents such as hydrazine or sodium borohydride were reported to release oligosaccharides. Fractionation of neutral and acidic oligosaccharides, which are sialylated, sulfated or phosphorylated, is also possible by elution with water-acetonitrile mixtures. Although the use of such desorbents might be useful for FOS purification, the alimentary use of FOS must not be forgotten. Therefore, when FOS are to be purified, only GRAS (Generally recognized as safe) products are allowed.

Enzymatic oxidation of glucose is an alternative to purify the synthesized FOS. This method is as efficient as the former, but much easier to scale-up. The glucose can be oxidized using glucose-oxidase as biocatalyst, producing gluconic acid, which can be precipitated by the addition of $Ca(OH)_2$. This calcium gluconate can be also used as source of calcium. This way, the glucose generated during the enzymatic synthesis of FOS can be transformed into other products of high added value [11]. Additionally, if the synthesized FOS are treated with immobilized cells of *Zymomonas mobilis*, glucose, fructose and sucrose can be simultaneously eliminated [67]. Other methods to remove mono and di saccharides from FOS syrup, are membrane technology, mainly ultra and nanofiltration [68–70], and also microbial treatment through the fermentation of glucose, fructose and sucrose to ethanol and carbon dioxide [60, 71, 72]. This method involves additional process to treat fermentation products, and depending on the microorganism selected and the raw material used, additional nutrients may be necessary [67].

No matter purification process, the mixture of purified FOS still contains different concentrations of FOS with different DP. As they are usually employed in the formulation of functional foods or in infant formula, purification of each oligosaccharide is not strictly

necessary. However, for mechanistic or physiological investigations, the availability of pure FOS with a given DP is necessary. The isolation is possible using preparative HPLC although it is not an easy process, especially for the production at a large scale. Indeed, pure FOS are expensive and are only available for analytical purposes.

FOS obtained by Hydrolysis of Inulin

In general, the presence of mono and disaccharides in the final product is one of the drawbacks of synthesis of FOS over the hydrolysis from inulin, making the yield and purity of the latter much higher. In this regard, the production of FOS using endo-inulinases yields 81 %, compared to the 55 % resulting from fructosyltransferases activity [73].

Plant inulin have chains of up to 60 units of fructose, which length, composition and dispersity vary with plant species, life cycle phase, time of harvest and the conditions of extraction and post-extraction. Fresh plant material is always used to extract native inulin, and precautions must be taken to inhibit the plant own inulinase activity and to prevent acid hydrolysis. Even so, the extraction of inulin is always accompanied by the extraction of FOS, sucrose, fructose and glucose in variable amounts. Inulin is soluble in water in moderate extent (about 10 % at 20 $^{\circ}$C), producing a low-viscosity solution. It can form a tridimensional microcrystalline gel network at higher concentrations; this will give a fat-like mouthfeel. Inulin is about 10 times less sweet than sucrose and that sweetness is eliminated when short chain inulin molecules are removed. This process increases the gel-forming capabilities.

Commercially available inulin is currently produced by the industry from two species belonging to Compositae: Jerusalem artichoke (*Helianthus tuberosus*) and chicory *(Cichorium intybus*); however, commercial inulin from dahlia (*Dahlia pinnata*) tubers can also be found [74]. Additionally, it can be extracted from the tubers of *Cynara cardunculus* (artichoke) and *Polymnia sonchifolia* (yacon) [75]. Agave, garlic and shallots are also potential sources [76]. Jerusalem artichoke is one of the most important raw materials for the industrial production of fructose and inulin since it is easy to cultivate, accumulates about 50–70 g/kg of its fresh weight as inulin-type fructans and the crop yield estimate is 5.4 ton/ha [77]. However, both inulin contents and degree of polymerization vary extensively with time of harvest [78]. This may lead to variation in

the composition, something common in natural products, but a possible issue for some applications in which a very precise composition is required.

Inulin may be commercially obtained in different forms: native inulin with an average degree of polymerization (DP) of 10–12, containing short chain inulin fractions (DP 2–10) and high performance inulin (HP) with DP higher than 20. Small inulin oligomers mixture with DP<10 are often designated by oligofructose or short-chain FOS. The long-chain inulin or inulin HP is produced by physical separation techniques.

Mensink et al. [79] revised the origin, physico-chemical properties and DP of commercially available inulins. They highlight that two batches of inulin with the same average DP can have different size distributions and therefore there characteristics can be very different. Inulins with higher DP have lower solubility in water, higher melting temperatures (crystalline inulins) or higher glass transition temperatures (amorphous inulins), higher chemical stability (do not hydrolyse easily), form stronger gels and their aqueous solutions have higher viscosity.

The fructose units of inulin are linked by β-(2→1) D-fructosyl-fructose bonds and the chain thus formed is usually terminated with one glucose unit linked through an α-D-glucopyranosyl or α-(1→2) bond in the same way as in sucrose. Inulins that show this terminal glucose unit are designated by α-D-glucopyranosyl-[β-D-fructofuranosyl]$_{n-1}$-D-fructofuranosides (FOS or GF_n), while those that lack this glucose unit and are therefore constituted of fructose only are called fructopyranosyl-[α-D-fructofuranosyl]$_{n-1}$-D-fructofuranosides (or inulo-oligosaccharides -IOS or FF_n) [80].

The extraction of inulin and FOS from vegetables is carried out by grinding and solubilization in hot water, with further enzymatic treatment with sucrases (to eliminate the sucrose still present), α-amylase and maltase (for degradation of short chain carbohydrates) [81].

Enzymatic hydrolysis of inulin is the most common procedure, however other methods such as acid hydrolysis and auto-hydrolysis can also be employed for this purpose.

Enzymatic Hydrolysis

There are two types of hydrolytic enzymes that break down inulin, endo- and exo-inulinases. As in October 2018, BRENDA (the free comprehensive enzyme system– www.brenda-enzymes.org) has 58 endo- and 70 exo-inulinases described. These enzymes can be obtained from bacteria, fungi, yeast, and plants, although commercially available products come from the fungus *Aspergillus* spp., in particular *A. niger* (www.brenda-enzymes.org).

Endo-inulinases (E.C.3.2.1.7) are enzymes capable of cleaving linkages between fructosyl moieties/residues within the fructan chain. They have been widely used for the production of FOS, especially since the commercial inulinase form is the endolytic type (Figure 2). These enzymes can also be used to determine the overall content of inulin and FOS in plants and foodstuffs by measuring the amount of fructose, glucose, and sucrose before and after the enzymatic hydrolysis [76].

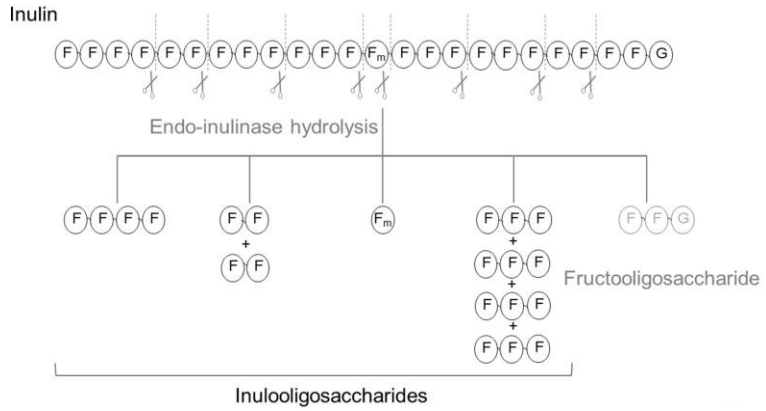

Figure 2 – Scheme of the hydrolysis of inulin using endo-inulinase as biocatalyst. Each initial GFn yields one fructooligosaccharide and several inulo-oligosaccharides

Exo-inulinases hydrolyze terminal, non-reducing 2,1-linked and 2,6-linked β-D-fructofuranose residues in inulin, levan and sucrose releasing β-D-fructose. Most exo-inulinases are capable of hydrolyzing inulin in a very effective way, producing fructose in yields as high as 90–95 %, so they are used mainly for the production of ultra-high-fructose syrup.

The enzyme source can dictate the outcome of the hydrolysis: amount and type of products generated. For instance, the production of FOS using endo-inulinases from *Xanthomonas oryzae* No. 5 [82] results in FOS with DP≥5 as the major compounds, while

the same enzyme from *Pseudomonas* sp. No. 65 [83] produces mainly DP2 (inulinobiose) and DP3.

In an assay with endo-inulase from *Pseudomonas* sp. No. 65, DP2 (inulobiose) and DP3 FOS were the main products (31 and 23 %, respectively) when using pure inulin from chicory and, with raw chicory extract, the hydrolysate consisted of 19 % DP2, 19 % DP3, 14 % DP4, and 19 % >DP5, with fructose, glucose, and sucrose being detected in both cases. Additionally, dual systems with different endo-inulinases can also be used for the production of FOS from inulin, accordingly to the user's needs [84].

The high cost of the enzymes increases the overall cost of this process [85]. This has been partially overcome through genetic engineering and molecular biology techniques, many modified enzymes with enhanced properties compared to their natural counterparts have been obtained [86–88].

Endo-inulinase genes from microbial species have been successfully cloned in another and the expressed enzyme used for the hydrolysis of inulin with noteworthy results [89]. It is possible to design a system suited to the user's needs, such as high expression, intra or extracellular enzymatic production and thermoresistance. Recently, an endo-inulinase encoding gene was cloned and transfected into *Baccillus subtilis* WB800-R and the enzyme produced was used for the hydrolysis of inulin resulting in yield of around 69 g/L of FOS, mainly DP3, 4 and 5, in the crude extract, with a conversion rate of pure inulin into FOS of 75 % [90].

Wang et al. [91] reported a simple and highly efficient one-step bioprocess for production of high-content FOS from inulin by yeast fermentation, using a recombinant yeast strain JZHSTSC, in which a heterologous endo-inulinase gene was expressed and the inherent invertase gene SUC2 was disrupted. This yeast simultaneously hydrolyzed inulin into FOS by secretion of endo-inulinases and removed mono-sugars by assimilation, resulting a product with high purity of FOS (≈90 %).

In a similar process, but in a two-step way, Han et al. [92] achieved similar results by using a recombinant *Yarrowia lipolytica* strain Enop56, in which an optimized endo-inulinase gene from *Aspergillus niger* was overexpressed. The hydrolysis in these conditions lead to the formation of FOSs with DP 3–5 as major products and to <5 % of mono- and disaccharides (non-prebiotic). As before, large amounts of FFn

oligosaccharides were obtained. Since both GFn and FFn oligosaccharides show identical functional and physiological properties, this is not a disadvantage [93].

Several studies of cloning and modification have been performed on fungal inulinases in order to improve efficiency, achieving yields up to 90 % of oligofructose with degrees of polymerization between 3 and 6 [94].

Acid Hydrolysis

Glibowski and Bukowska [95] found out that heating 5 % inulin solution in a strong acidic environment (pH 1–3) caused intensive hydrolysis, even mild temperatures (40 °C) which somehow contradicts the notion that inulin is not digested by the human gastrointestinal tract.

It has been reported that both fructose and fructooligosaccharides can be produced from inulin by chemical hydrolysis (pH 1–2 at 80–100 °C), but fructose degrades easily at low pH resulting in the formation of di-fructose anhydride, a colored byproduct with almost no sweetening capacity, and hydroxymethylfurfural, a known by-product and inhibitor for fermentative organisms.

Acid hydrolysis becomes relevant in the obtaining of FOS from agave, since the amount of fructan accumulated in the mature plants [13–17 % (w/w) fresh weight] is similar to what is found in the current source of inulin, chicory [15.2–20.5 % (w/w) fresh weight]. The main difference resides on the structure of the fructose polymers: while in chicory inulin fructose molecules are joined through β(2–1) linkages in linear chains, fructans present in agave, especially in *Agave tequilana*, have a relevant content of β(2–6) linkages resulting in branching fructose molecules (levan type fructans) in chains with DP 3–29. Due to their complex structures, commercial *endo*-inulinases have little hydrolytic activity over these polymers, while specific endo-levanases are difficult to obtain and fructanases, combining *endo* and exo-inulinase activities, lead fructose as the main hydrolysis product, even at low conversions. Avila-Fernadez et al. [96] used a limited acid hydrolysis by HCl and cation exchange resins for the production of FOS from agave fructans; the reaction need to be controlled to prevent hydrolysis to fructose. β-(2,6)-FOS were prepared from microbial high-molecular-mass levan by acid hydrolysis and refined by cation-exchange chromatography, resulting in oligosaccharides with a DP

within 2 and 20 and the same β-(2,6) linkage type. The long-chain β-(2,6)-FOS were more resistant against acid or enzymatic hydrolysis than the short-chain β-(2,6)-FOS.

Hence, acid hydrolysis is suitable when the aim is the production of fructose syrups as an alternative to exo-inulinase hydrolysis or for bioethanol production from biomass [97].

Autohydrolysis

Long-term storage provides adequate conditions for the chemical breakdown of inulin and FOS. This is also the reason why older plants typically have lower inulin contents than younger ones: plants also contain enzymes that can hydrolyze inulin. The main effects are the shortening of the FOS chains and eventually the production of free sugars, that is, glucose, fructose and sucrose.

Extracted inulins may contain a large amount of sugars (mono-, di- and small oligosaccharides) [84]. Typically, extraction is done by boiling the cleaned and cut or ground up tubers, or other inulin containing plant part, in water. Process conditions (pH, water–root ratio, boiling time, etc.) affect the DP of the produced inulin. Higher oligomers are more hydrolyzed than the lower oligomers, since they have a relatively high content of fructosyl end chains.

The isolation of those small oligosaccharides, which will have a glucosyl end and are thus similar to FOS obtained by synthesis, can be an interesting approach.

Cho et al. [84] found 38 % of FOS (DP3 to >5) in the initial carbohydrate composition of chicory juice, together with 33 % inulin and 27 % mono and disaccharides. Precipitation of inulin and removal of mono and disaccharides would lead FOS as the main product.

Other Species Should be Considered as Direct Sources of Oligosaccharides

Benkeblia et al. [98] extracted FOS (DP3 to DP12) from onions in average amounts of 270 mg/g together with free mono and disaccharides in amounts of 450 mg/g; only the fraction of DP5-DP12 degraded with time at 20 °C. Shiomi et al. [99] revised the metabolism of FOS in onions, concluding that the maximum amounts are found during

dormancy, after the activity of fructosyltransferases during bulbing and before the extensive activity of exo-hydrolases that takes places during sprouting.

Yacon (*Smallanthus sonchifolius* Poepp. and Endl.) is a root crop native to the Andean region, but has also been cultivated in other regions. Yacon tubers are traditionally consumed as fresh fruit due to their crunchy texture and high juice contents, having a moderate sweet taste. Saccharides compose up to 80 % of the total dry matter content of yacon tubers, with a large dependence on cultivar. These saccharides consist of fructose, glucose, sucrose and FOS, which are usually as their dominant group of saccharides [100].

Campos et al. [101] studied 35 different yacon accessions and found that the content of reducing sugars (RS), sucrose (S) and FOS based on dry matter vary wildly depending on accession. The highest FOS contents found was 65.0 g FOS/100 g DM. The content of RS in yacon accessions was inversely correlated to the FOS content.

Sumiyanto et al. [102] analyzed the fructans content in tuberous roots of yacon and found values between 70 and 80 % of the dry weight during the harvest period of October-December and very little variation in the amount of other solids over this period of time.

The fructooligosaccharides in yacon represent mainly oligosaccharides from DP3 to DP10 with terminal sucrose (inulin-type fructooligosaccharides) [103]. Regarding other nutrients of yacon, many studies reported that it contains low protein, lipid and ash content, thus making this tuber a potential source of FOS.

The large variations in mono and disaccharide content may be due to the accession, edaphoclimatic conditions during growth of yacon, and particularly the post-harvest procedures. A common postharvest strategy consisting on exposing the tubers to direct sunlight in order to increase their sweetness will cause the breakdown of FOS to FOS with lower DP and/or free fructose and glucose. Processes such as drying will also modify the profile of carbohydrate content of yacon tubers [104].

A derivative of yacon that is industrially available is yacon syrup, produced by juicing the fresh roots, filtering and concentrating by evaporation of water [105]. Since the water contents is diminished to about 20 %, the syrup can be stored for several months without significant reduction of FOS content or significant depolymerization [106].

The enzymatic hydrolysis of inulin generally produces chains longer than DP5, with a lower prebiotic activity than those produced synthetically (DP3-5). Depending on the application of the generated FOS, these points should be considered [90]. There is the possibility of making use of the action of exo-inulinases (E.C. 3.2.1.80), which remove frutosyl residues from the non-reducing end of the inulin molecule, thus shortening the chain, but these also hydrolyze sucrose and raffinose, thus produces a mixture containing high amounts of free glucose and fructose, since this enzyme is able to hydrolyze the glycosidic bond α-(1,2), which connects glucose to the main inulin chain, so additional steps of purification are also needed.

The major drawback of inulin as a source of FOS is the fact that it is not a single structure. The chemical structure of fructans vary widely depending on the species. For example, as mentioned before, inulin from some plants has a 2,1-linked -D-fructosyl back bone with 2,6-linked -D-fructosyl side chains in variable percentage, as in garlic and *Agave tequilana* [96], while other have only linear chains. Degree of polymerization differences are another issue: inulin from chicory (*Cichorium intybus*) has a much lower DP (about 20) than inulin obtained from globe thistle (*Echinops ritro*) with mean DP 30 or global artichokes (*Cynara scolymus*) with mean DP65.

The species mentioned in this section also contain their own inulinases, which is the major drawback for inulin recovery. Leroy et al. found that throughout the period of artichoke storage, a decrease in inulin content and mean DP occurs, owing to its *in natura* depolymerization [107]. *A. tequilana* was investigated as potential inulin source, the youngest plants exhibited the highest levels of free monosaccharides and low molecular weight fructans with potential application as prebiotics, while the DP reached a maximum of 3–30 in 4-year-old plants and then decreased to 4–24 in the oldest (>6 years) ones [108].

Another important issue is the need to extract and purify inulin from the natural matrix usually requires juices extraction and a succession of freezing, thawing and (ultra)centrifugation in order to remove low DP and other contaminants. Filtrates are deionized by passing through strong anionic and cationic resin exchangers, before a final step of freezing/thawing/centrifugation. As a result, very pure inulin (>98 % purity) can be obtained. Depending on the application, this process, albeit tedious and costly, can be very effective specially to obtain FFn oligosaccharides from long chain inulins, since each resulting molecule contains a terminal glucose (GFn) for several FFn oligomers after high

purity endo-inulinase hydrolysis. The general mode of endo-inulinase action is that the hydrolytic activity for inulin increases with the degree of polymerization of fructosyl residues [73]. For global artichoke, F_3 and F_4 were the main fructose polymers [80].

However, Cho et al. [82] carried out IOS production from chicory root juice, using endoinulinase from *Xanthomonas oryzae* No. 5, and compared with FOS from pure inulin. From their results, hydrolysis with endo-inulinase over the extract does not affect ≤DP4, mainly converting inulin in DP5 and >DP5. So, if the aim is the use of FOS, the initial purification on inulin is not necessary and the removal of mono and disaccharides could be left as final step.

Physical techniques, such as ultrasound, have also been reported as methods for the production of low molecular weight FOS fragments from Jerusalem artichoke inulin extractions [109]. Furthermore, ultrasound extraction of *Flammulina velutipes* polysaccharides has also been reported as a method for production of FOS [110].

GOS

GOS are composed by a variable number of galactose units, within 2 and 10. Similarly to FOS, GOS can be obtained either by synthesis or by extraction and hydrolysis. The type of linkage between units varies according to their origin and obtaining process. Plant based GOS are α-GOS whereas GOS prepared from lactose are β-GOS.

α-GOS are important components of seeds, namely pulses, and show a terminal sucrose unit and the linkage between monosaccharide moieties can be [Gal-α(1→6)-Gal], [Gal-α(1→4)-Gal], [Gal-α(1→3)-Gal] and [Gal-α(1→6)-Glu-β(2→1)-Fru]. This is called the raffinose family (RFO). Another relevant α-GOS is melibiose, a reducing disaccharide with a linkage (Gal-α(1→6)-Glc) (isomer of lactose).

β-GOS, also known as oligogalactosyllactose, oligogalactose, oligolactose, transgalactosylated oligosaccharide, and transgalacto-oligosaccharide, show a terminal glucose unit and the galactose units are linked mostly by β(1→4) and β(1→6) bonds [111–113].

Although tri- to hexa-saccharides, with 2 to 5 galactose units (DP3-6), tend to be the main components of GOS-containing products, disaccharides (DP2) consisting of

galactose and glucose with β-glycoside bonds such as [Gal-β(1→6)-Glc], [Gal-β(1→6)-Gal], [Gal-β(1→4)-Gal] or [Gal-β(1→3)-Gal] which are different from lactose, [Gal-β(1→4)-Glc], are also present and defined as GOS since they have physiological characteristics are similar to longer chains.

The prebiotic effect of α- and β-GOS is mainly associated to tri and tetrasaccharides (DP3 and DP4, respectively) [114–116].

GOS Obtained by Enzymatic Synthesis

GOS can be commercially synthesized from lactose through transgalactosylation reactions, using β-galactosidases (EC 3.2.1.23) as biocatalysts [117]. The main companies leading GOS market are Yakult Honsha Co Ltd. in Japan with Oligomate 55 syrup and Oligomate 55P powder (both with 55 % dry matter of oligosaccharides), and TOS-100, a purified powder containing 99 % oligosaccharides [118]. Also, Friesland Foods Domo in The Netherlands commercializes TOS-syrup (75 % w/v content of GOS) and Vivinal GOS syrup (with 75 % w/v of solids which 59 % are GOS) [4].

α-GOS can also be produced by transgalactosylation reactions of α-galactosidase (α-Gal) or by conversion of raffinose family oligosaccharides by levansucrase. However, there is very little data on transgalactosylation reactions of α-Gal [119, 120], and therefore, all the discussion will be based on the better known β-GOS production.

β-galactosidases from fungi of the genus *Aspergillus* and yeasts of the genus *Kluyveromyces, Rhodotorula, Bullera singularis* and *Sterigmatomyces*, as well as bacteria of the genus *Lactobacillus* or *Bacillus* are generally used as biocatalysts for the industrial synthesis of GOS [121, 122] both for food and pharmaceutical applications [123]. These enzymes are widely known for their glycoside hydrolase activity, leading to the cleavage of β-galactosides into monosaccharides. However, in certain conditions, they can be used as biocatalysts for the synthesis of GOS. Indeed, β-galactosidases identify different types of glucose-glucose bonds [i.e., β(1→2), β(1→3), β(1→4)], as well as β(1→6) and β(1→3) glucose-galactose bonds, and catalyze the transfer of a galactose moiety from a β-galactoside to an acceptor containing a hydroxyl group. The accepted mechanism for the enzymatic catalysis involves two steps (Figure 3):

i. The formation of an enzyme–galactosyl complex, with simultaneous liberation of glucose;

ii. The transfer of the enzyme–galactosyl complex to a nucleophilic acceptor containing a hydroxyl group. If the nucleophilic acceptor is water, galactose is obtained as a product (hydrolysis reaction) (Figure 3) [124]. If the nucleophilic acceptor is another sugar, di, tri or higher DP GOS are produced (Figure 3). The mechanism has been mathematically described by many kinetic models. Boon et al. [125] reported that the best approach for describing GOS synthesis by β-galactosidases is a kinetic model that considers lactose hydrolysis and oligosaccharide synthesis, so there must be taken into account that water or lactose can attack the galactosyl-enzyme complex, and also it must be included glucose inhibition. From a mathematical viewpoint, the problem can be raised by integrated rate equations and fitted by non-linear regression at different concentrations of substrate [126] so each parameter can be estimated separately and independent of the initial lactose concentration [127].

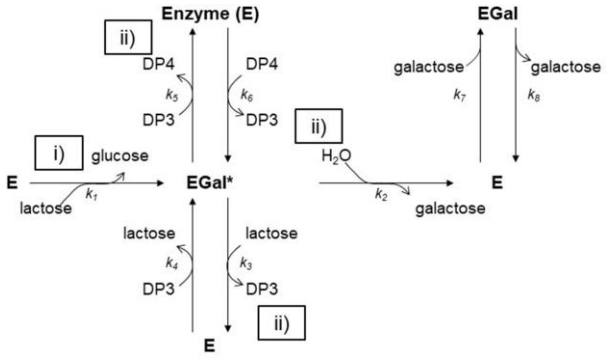

Figure 3 - Reaction mechanism for the hydrolysis and transglycosylation of lactose by β-galactosidase. (i) The lactose molecule on the active site of the enzyme forms an acyl-enzyme complex with liberation of glucose; (ii) The enzyme-galactose complex, can react with carbohydrate molecules

High concentrations of lactose compete with water for the transfer of galactosyl moieties (ii). Therefore, under these conditions β-galactosidases catalyze the formation of GOS [128]. On the contrary, lower concentrations of lactose promote lactose hydrolysis rates to glucose and galactose [112]. To stimulate the synthesis of GOS, two main approaches are used: the equilibrium approach and the kinetic approach. Both approaches tend to favor transgalactosylation over hydrolysis, the former through high substrate concentration (less water available in the medium) and the adequate enzyme/substrate ratio, depending on the enzyme source [122] and the later through

enhancing the kinetic variables that promotes the most favorable rate of product formation [129].

β-galactosidases are the most frequent catalysts used in the synthesis of GOS, although their main application is the hydrolysis of lactose to generate products suitable for lactose allergic people [129]. Different species possess different specificities for building glycosidic linkages and therefore produce different GOS mixtures. For example, the β-galactosidase from *K. lactis* produces predominantly β-(1→6)-linked GOS, the β-galactosidase from *Aspergillus oryzae* produces mainly β-(1→3) and β-(1→6) linkages [130], *Bacillus circulans* β-galactosidase forms mainly β-(1→4)-linked GOS [131], whereas β-galactosidases from *Lactobacillus* spp. preferably form β-(1→6) and β-(1→3) linkages in transgalactosylation mode [132, 133]. Another important factor regarding enzyme source is the maximum GOS yield and the lactose conversion, that is, the percentage of initial lactose that is consumed during the synthesis. This latter is a very important factor because it has very important nutritional and technological consequences (both the hydrolysis -monosaccharides- and the synthesis products -GOS- are much more soluble than lactose, thus it is possible to go from a suspension to a syrup during the enzymatic reaction). The decrease in the lactose concentration is desirable in people with lactose intolerance. Just to mention some examples, β-galactosidase from *Aspergillus oryzae*, yields 28 % of GOS with a lactose conversion of 58 % [112], β-galactosidase from *Bacillus circulans* yields 54 % GOS [134], and β-galactosidase from the thermophilic archaeon *Thermus caldophilus,* 75 % GOS with 50 % of lactose conversion [118].

Besides the type of enzyme, generally, the reaction conditions (i.e., initial substrate concentration, temperature, pH or presence of inhibitors or activators of the enzyme) affect the enzyme activity [135, 136]. For this reason, all these parameters strongly determine the yield and composition of the GOS obtained, as well as the concentration of mono and disaccharides present in the products of reaction. Table 2 presents a detailed list with enzymes from different origins used for GOS synthesis, together with the respective reaction conditions and yields. As each type of enzyme has different optimal conditions (lactose concentration, pH, time, temperatures), they have to be deeply investigated to achieve the best performance (lactose conversion, yield of GOS) to obtain the desired composition of GOS.

Table 2 – Different enzyme sources, conditions performed and yields for the synthesis of GOS. [a]$Y_{GOS\ max}$: Maximal yield of GOS; [b]GAU/g: the amount of enzyme which releases 1 µmol of O-nitrophenol per minute/ g lactose; [c]pNPG/g unit of para-nitrophenol galactoside/ g lactose; [d]IU/g: the amount of enzyme producing 1 µmol of O-nitrophenol per minute/g lactose; [e]IUT /g: the amount of enzyme that catalyzes the transglycosylation of 1 µmol of galactose per minute; [f] U/mL: the amount of enzyme producing 1 µmol of O-nitrophenol per minute/mL substrate solution; [g]U/g: amount of β-galactosidase needed to liberate 1 lmol glucose per min/g lactose; [h]Informed yield: weight percentages of total sugar; [i]Substrate: whey permeate; [j]Glucose and galactose (10 or 50 g/L) were added to evaluate inhibition effect; [k]Substrate: lactose or lactulose

Enzyme source	T (°C)	pH	Lactose (g/100 mL)	Enzyme amount	Reaction time (h)	$Y_{GOS\ max}$ (g GOS/100 g lactose)[a]	Observations	References
COMMERCIAL								
Maxilact 2000 (*K.lactis,* DSM); Lactozym 2000L (*K. fragilis*, Novozyme); Ha-Lactase (*A. oryzae*, Chr. Hansen)	40	6.5	10, 20, 30 %	40 GAU/g[b]	1	14 %[h]		[129]
Biolacta FN5 (*B. circulans*, Vitachem)	4–60	6.6	10 and 5 % (skim milk)	0.1 pNPG/g[c]	0–32	54 %		[134]
Biolactasa-NTL CONC X2 (*B. circulans*, Biocon)	60	6	50 %	40 IU/g[d]	5	39 %	Free and immobilized enzyme	[137]
Lactase (*A. Oryzae*, Enzeco Fungal)	40–55	4.5	40–60 %	5–300 IUT/g[e]	10	17–30 %		[138]
A. oryzae (Merck), Lactozyme 2600 L (*K. lactis*, Novozymes), strain K12 (*E. coli,* Worthington)	35	4.5–7	30 %[i]	50 U/g[b]	12	25 %	3 enzymes compared	[139]
E. coli (Sigma-Aldrich)	10–60	5.5–8	2.5–15 %	4.5, 9,14 U/mL[f]	24	49 %	j	[140]
A. oryzae Genencor International	40	4.5–6	5–50 %	4.5 g of support	37 mL/h (2 days)	25 %[h]	Immobilized enzyme, continuous reactor	[130]
A. oryzae (Enzeco Fungal Lactase)	50	4.5	20 %	388, 250,100 IUT/g[e]	2.5	33–47 %	Immobilized enzyme	[141]
A. oryzae and K. lactis (Sigma), *Bacillus sp.* (Taiwan Fructose Co.)	30–50	5	34 %	4.5–10 U/g[g]	18	n.i[b]		[142]
MICROORGANISM								
Sulfolobus solfataricus	70–90	5–7	30–60 %	1.2–4.8 U/mL[f]	48–60	50–53 %		[143]
S. solfataricus and *Pyrococcus. furious*	70	5.5	4.5–17 %[i]	1–2 U/mL[f]	200	50 %	Continuous and batch reactor	[144]
Bifidobacteria (BbgIV)	45–65	6.5	43 %	10 U/g[g]	24	49–53 %[h]	Free and immobilized enzyme	[145]
Lactobacillus delbrueckii subsp. *bulgaricus*	30-50	6.5	20 %	1.5U/mL[f]	5, 8, 12	48.2–49.5 %[h]		[133]
Rhodotorula minuta IF0879	60	5	25 %	0.24 U/mL[f]	50	39 %[h]		[146]
P. acidipropionici and Lactozym® Pure 6500L (Novozymes)	45	6.5	30 %[k]	1.3 U/mL[f]	24	24 %[h]		[147]
S.thermophilus DSM2 0259	37, 50	6.5	65 %[i]	2.7 U/mL[f]	9	50 %[h]		[148]
A. oryzae and *C. laurentii*	55 45	4.5	20 %	1 IU/g[d]	3-96	33-34 %	Combination of enzymes	[149]
A. oryzae and *K. lactis*		6.4		50 IU/g[d]		25 %		
L. reuteri	25–37	6, 6.5	13.5, 30, 60 %	195 U/g	70	38 %		[132]

Similar to the synthesis of FOS, the initial concentration of lactose determines the chemical composition of the synthesized GOS, the more concentrated the substrate, the larger the synthesized GOS [129, 142]. Some authors [150] claimed that the initial lactose concentration is directly related with the enzyme activity explaining that higher concentrations favor an increase in GOS yield [121, 127, 138]. However, Adamczak et al. [129] investigated the effect of different lactose concentrations and commercial enzymes (Table 2), concluding that the lowest lactose concentration used (10 %) was the one resulting in the greatest GOS yield 13.7 % when Ha-Lactase from *Aspergillus oryzae* was employed as a biocatalyst. Besides, although the solubility of lactose in water is rather low (220 g/L at 25 °C [151]), this is not a limitation for the synthesis of GOS. Even when suspensions of lactose with constant shaking can be used as a substrate, the employ of thermostable enzymes enables the synthesis at higher temperatures, which also increases the solubility of lactose. Also, Gosling et al. [134] used a commercial enzyme preparation and 5 and 10 % w/v lactose as a substrate, achieving a yield of 50 % of GOS regarding initial lactose content. In this sense, Petzelbauer et al. [144] achieved high conversions of lactose into GOS by using a thermostable enzyme that allows to operate at 70 °C, thus allowing a continuous hydrolysis of lactose (Table 2). Moreover, it was reported that when GOS synthesis was carried out with saturated lactose solutions, the specific enzyme productivity increased while maximum yield slightly decreased with temperature [138]. When partially dissolved lactose was employed, an increase in temperature produced an increase in both yield and specific productivity [138]. In addition, the continuous removal of the synthesized GOS drives the reaction over time to consume different concentrations of lactose [152]. At this point, it should be pointed out that in spite of the several attempts to counterbalance the low solubility of lactose, it must not be forgotten that lactose is a very cost-effective substrate and its price is not a limiting factor for the synthesis of GOS. Only when the lactose used as a substrate takes part of a more complex matrix, such as when using milk or whey permeate, the effect of higher temperatures should be especially considered. In such cases, thermostable enzymes are a good strategy to enhance GOS yields, but the increase in reaction temperature during synthesis must be controlled, as Maillard reaction can occur due to the presence of amino side-chains of proteins and sugars [150].

The reaction temperature is directly related with the lactose concentration (lactose solubility, as mentioned before) and the stability of β-galactosidases (stability of

enzymes). During the last decades increasingly interest have raised to find thermostable and thermoactive versions of β-galactosidases [153–157]. One of the main enzymes used for the synthesis of commercial GOS is BgaD, obtained from *Bacillus circulans*, and used for the synthesis of GOS commercialized as Vivinal (Orafti), BiOligo® (Ingredion) Purimune™ and Yakult Oligomate 55®. The enzyme is stable up to 65 °C (optimal temperature ca. 60°C), thus enabling high lactose concentrations (Table 2). Other thermostable β-galactosidases (recombinant) were studied even at temperatures above 80 °C [143, 158]. These enzymes showed an increase in reaction yields given that higher temperatures favor higher rates, high lactose solubility, and favorable equilibrium for transgalactosylation reactions [144, 159].

Regarding pH, several studies claimed that the optimal pH for GOS production is in a range of 6–7 [143, 160–163]. However, a more certain pH value must be adjusted considering the enzyme source [150] (Table 2). In particular, commercial β-galactosidase from *Aspergillus oryzae* is more efficient in acid than in neutral solutions. Nevertheless, Rodrigues Mano et al. [139] confirmed that transgalactosylation activity for this enzyme have a stronger dependence on lactose concentration than on the pH of the solution.

Experimental research outlined that galactose and/or glucose commonly act as inhibitors for many β-galactosidases. Although galactose is recognized to have a greater inhibitory effect than glucose because it directly competes with lactose to form the galactosyl-enzyme complex [150, 153, 164, 165], this issue is quite controversial and strongly depends on the enzymes and reaction conditions. There are reports showing that for some enzymes neither glucose nor galactose are inhibitors [122], some enzymes have only galactose as inhibitor [165], and some others are inhibited by both sugars [158]. As galactose is a competitive inhibitor of most of the β-galactosidases (especially in the hydrolysis of lactose), high concentrations of lactose can counterbalance this inhibitory effect [113]. On the contrary, galactose is used to enlarge the chains of GOS during the transgalactosylation reaction [113]. Glucose was claimed to have a greater inhibitor effect in some cases [125] and to have similar inhibitory effect [166] respect to galactose. In this regard, glucose is an inhibitor of β-galactosidases from *Lactobacillus reuteri* [132], *Sulfobacterium solfataricus* [155], *Thermus* sp. [167], *Kluyveromyces lactis* [168], *Thermus* sp. [169], and *Caldicellulosiruptor saccharollyticus* [158]. As the inhibitory effect of glucose mainly occurs during GOS production [113]. Therefore, the desirable enzymes are those with low inhibition of lactose hydrolysis by glucose. The inhibitory or

activator effects of glucose and galactose are also dependent on the enzyme source and on the concentration of reagents and products [170]. Hence, β-galactosidase from *Kluyveromyces fragilis* was reported to be affected by both combined and individual effects of lactose, glucose and galactose. Glucose is an activator at low concentrations of lactose and galactose and an inhibitor at higher concentrations of these sugars. In turn, galactose becomes an activator of the enzyme at high concentrations of glucose and low concentrations of lactose.

The enzyme is one of the major cost factors for the synthesis of commercial GOS. Therefore, immobilization of β-galactosidases deserved great attention in the last decade, as a way to improve their stability, enable their reutilization and facilitate their removal from the reaction medium. All these advantages enhance the yield of GOS in relation to the enzyme concentration (higher g GOS/ IU of enzyme). Immobilization technique requires a carrier that interacts with the enzyme through physical adsorption, entrapment or covalent binding [171, 172]. Different parameters define the efficiency of the support, namely mechanical resistance, enzymes interaction, particle size, specific surface area, among others. Regarding mechanical properties of the support, they rather depend on the final configuration of the reactor than on the application. For example, for a fixed-bed reactor, rigidity is a desired characteristic for the support to bear high pressures, thus, silica-based materials, carbon materials, porous glass, and other mineral materials are good choices in this case [173]. On the other hand, if the process is carried out in a stirred-tank reactor, flexible materials (i.e., agarose beads, celulose beads, Lentikats-polyvinyl alcohol polymers shaped like a lens) are more adequate [174]. With respect to enzymes interaction, physical adsorption on different scaffolds (i.e., cellulose, starch, charcoal carbon, diatomeaceus earth, Shephadex, cotton cloth, chitosan) has the advantage of being cost-effective with little influence on the enzyme conformation [171]. Although the weakness of the binding forces represents a disadvantage of these methods, a treatment with glutaraldehyde can stabilize the enzyme adsorption. In what concerns entrapment methods, enzymes are enclosed in polymeric matrices (i.e., alginate beads, carrageenins, polyacrylamide) or in membranes (i.e., nylon, cellulose, polyacrylamide). These methods are simple and mechanically resistant but enzyme desorption is more difficult compared to the physical adsorption, and requires cross-linking [172]. Finally, covalent binding scaffolds establish covalent bonds with the functional groups of the enzyme (amino, carboxyl, hydroxyl, and sulfydryl groups), taking care of protecting the active site. They

include eggshell, nylon, zeolite, gelatin, and Sephabeads-epoxy for thermo-stable enzymes. Particle size is another factor to consider depending on the operative characteristics of the synthesis. In general, large particles may be retained more easily than small ones, but they may produce preferential ways in column reactors or present diffusional problems given that long pores may decrease the rate of enzyme adsorption. At last, pore size and specific surface area of a porous particulate support are related parameters: in general, the larger the pores, the smaller the specific area. There must be reached a compromise solution considering loading capacity and size of protein/substrates [175].

For β-galactosidases immobilization several scaffolds were analyzed depending on the enzyme source, both in batch or in continuous operations, and reactions were carried out within 37 and 55 °C and pH within 3 and 6.5. Enzymes from *A. orizae* were immobilized in covalently bound cotton cloth [130], in activated chitosan [83, 141, 176, 177], in the form of self-supported cross-linked aggregates [178], in amino-epoxy sepabeads [141], in glyoxyl agarose [137, 141], in magnetic polysiloxane–polyvinyl alcohol beads [127], in magnetic particles coated with polyaniline [179] in magnetite nanoparticles [176]. In turn, β-galactosidases from *Bacillus circulans* were immobilized in epoxy-EupergitC [180, 181], in microporous polyvinylidene fluoride or polyvinylidene difluoride (PDVF) membrane [182], or in activated agarose [137]. Finally, enzymes from bifidobacteria were immobilized in DEAE-cellulose [145], Q-Sepharose [183], amino-ethyl agarose [184]. Enzymes from *Kluyveromyces lactis* were immobilized in glutaraldehyde activated chitosan [185] or in the form of whole permeabilized cells containing the enzyme [186, 187] and enzymes from lactobacilli, in microcrystalline cellulose [188], in PVC silica sheets, active carbon, porous glass beads [189]. Among all these strategies, the immobilization in activated agarose [137, 141], in activated chitosan [176, 177], in magnetic polysiloxane–polyvinyl alcohol beads [127], in the form of self-supported cross-linked aggregates [178], and in the form of whole permeabilized cells containing β-galactosidase [186] appear as the most promising ones in terms of maximum yield of GOS and highest productivity (g GOS per liter per hour) [113].

Beyond all these reaction parameters and immobilizing strategies that can be modulated to enhance enzyme activity, the yield of GOS resulting from the enzymatic reactions is in general, relatively low. These can be easy deduced by comparing Table 1 with Table 2. The maximum GOS yield regarding the initial lactose concentration rounds

50 % (Table 2) while that of FOS regarding initial sucrose concentration often overcomes 60 % (Table 1). Moreover, their composition, both in type of linkage and molecular size distribution strongly depends on the enzyme used [190]. Glucose, galactose and lactose that did not react are the main secondary products of the enzymatic reactions. Likewise in the synthesis of FOS, they shall be removed.

To this aim, similar chromatographic methods such as size exclusion chromatography [191–193] and charcoal-celite chromatography [150, 194] have been proposed.

Selective fermentation is another strategy to remove monosaccharides [135, 142, 194, 195]. It basically consists on an anaerobic glycolysis by yeasts, in which the monosaccharides are converted into ethanol and CO_2 [72]. This method has the advantage that can be performed directly during the synthesis, and the disadvantage that removal of yeast cells and ethanol are necessary to obtain the purified GOS [135].

Another technology available for GOS purification is ultrafiltration [122], a process where fluid containing enzyme and product flow at a high rate across a membrane surface at a certain fluid pressure. Commonly, membrane pore-size is designed to retain the enzyme while smaller molecules (GOS) are permeated [13]. Given that ultrafiltration usually does not ensure the complete elimination of monosaccharides (low molecular weight), nanofiltration appears to be a potential industrially scalable method for purification and concentration of oligosaccharide mixtures [196–199].

Additionally, *in situ* adsorption or precipitation of the undesired sugars [194, 200] are other alternatives for the removal of glucose and lactose. More recently, using immobilized enzymes enabled the simultaneous synthesis of GOS and elimination of mono and disaccharides [201]. To this aim, β-galactosidase from *Aspergillus oryzae* was immobilized in glyoxyl-agarose of different particle sizes (fine and macro). At higher lactose concentrations, the hydrolytic potential of the enzyme was of 16 and 30 %, and the ratio of transgalactosylation to total reaction, 70–84 %.

Obtaining of GOS by Hydrolysis from Vegetal Matrices

Plant based GOS, with α-galactosidic linkages instead of β- ones, are vastly distributed and ubiquitous in the plant kingdom [202]. Raffinose, a trisaccharide (Gal-

Glc-Fru) is the smallest RFO. Further elongation with Gal residues leads to the DP4 stachyose (Gal-Gal-Glc-Fru), verbascose (DP5), ajugose (DP6), etc. (**Figure 4**) [203]. Relevant amounts of α-GOS occur especially in generative parts of plants, such as seeds and fruits; GOS have diverse functions such as physiological protection, germination inhibition under low water availability conditions, and play a role in cold acclimation of many plants [204].

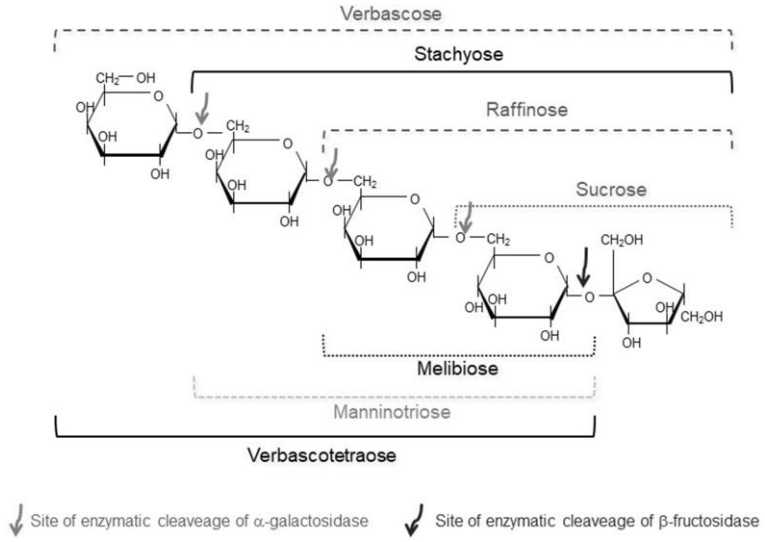

Figure 4 - Structures of α-GOS with (Raffinose family, RFO) and without a terminal fructose

Sugars belonging to the raffinose family have been implicated as protective agents in the cellular dehydration tolerance in plant seeds. Experiments on liposome preservation have demonstrated that the effect of degree of polymerization since RFO were progressively better to stabilize liposomes against leakage of aqueous content and against membrane fusion after rehydration, due to the higher glass transition temperature of the longer chain oligosaccharides [205].

α-GOS can be obtained by extraction from plants, mainly from legume seeds (pulses), such as soybean, lupin, lentil, chickpea, pea and cowpea. α-GOS from soybean are the only legume oligosaccharides in the market and the main producer is Japan. More recently, a French company, Olygose, has developed a type of GOS called Alpha-GOS®. Previously, this compound was a by-product of pea protein production. After research was conducted on the effectiveness of GOS as a prebiotic, Olygose began to produce Alpha-GOS® intentionally, from peas sourced from local farmers in France.

Extractable amounts vary from 1 to 10 %, depending on species and cultivar [206, 207]. Espinosa-Martos found that GOS content of soybean seeds vary with the degree of

maturity. Immature seeds contain less amount of GOS than fully matured ones, but no influence of biological or intensive agricultural practices in GOS content were reported [208].

Unlike FOS, there is no inulin equivalent (no long polymer) from which GOS could be obtained by hydrolysis. DP3 and DP4 are the most abundant GOS but chains of DP7 have been extracted from chickpeas. Usually sucrose is extracted along GOS which some authors claim can be purified by ethanol precipitation [209]. However, others found no evidence that sucrose and soy galacto-oligosaccharides could have a differential behavior, both having a similar distribution between the two eluents: water and 70 % ethanol. Kim et al. [210] optimized the conditions for oligosaccharide extraction and evaluated an ultrafiltration system for the purification of galacto-oligosaccharides from defatted soybean meal. Their main conclusion was that their system was more efficiency in the removal of protein than in the concentration of oligosaccharides, and no different distribution of GOS and sucrose is observed.

Both extraction and purification procedures must be optimized for each matrix, based on its composition. Considering that seeds are usually rich in lipids, a deffatening step must be performed prior to sugar extraction. Seeds are also high protein parts of the plant and soluble proteins and peptides are normal heavy contaminants on a first extraction. Soluble fiber, such as pectins, are also present on the aqueous extracts [211].

The viability of industrial production of GOS by extraction from natural sources depends strongly on the demands of the application, concerning purity. In order to achieve high purity, a complex set of procedures must be implemented, each step leading to loss of yield.

Like β-GOS, α-GOS are not hydrolyzed in the upper part of the human gastrointestinal tract, due to the absence of the enzyme α-galactosidase. In the colon, they are fermented together with soluble dietary fibers by the colon microbiota, generating significant amounts of short-chain fatty acids [212]. These fermentation substrates stimulate the growth of lactobacilli and bifidobacteria and the decrease of enterobacteria in the intestinal microflora. This prebiotic action is beneficial for the host's well-being and health [213, 214]. However, fermentation also produces gases (carbon dioxide, hydrogen and methane) that generate bloating and flatulence. Indeed, GOS of RFO are considered an important factor in the development of flatulence caused by consuming

legumes [215]. On the contrary, melibiose did not promote gas formation, thus suggesting that the fructose moiety present in raffinose was responsible for the gas production (Figure 4) [216].

Recently, research has been devoted to so-called "alternative" RFOs in plants. These are novel plant GOS that did not get much attention in the past. The stachyose derivative manninotriose (Gal-α(1→6)-Gal-α(1→6)-Glc) (Figure 4) was found for the first time as main carbohydrate in a garden weed *Lamium purpureum* known as deadnettle [217].

This non-fructosylated raffinose family of α-GOS includes melibiose, manninotriose and verbascotetraose and has been found naturally in foodstuffs [218–220]. *In natura*, they may be the result of the activity of plant acid invertases (β-fructosidases), which are able to split sucrose into fructose and glucose by hydrolysis of the 2→1 glycosidic bond. This appears to be the base of the recent commercial product of Olygose, mentioned before, that starts with RFO from peas and uses invertases to remove the terminal fructosyl unit from the α-GOS chain.

The European Food Safety Authority (EFSA) analyzed the claim that this group of α-GOS, like RFO and β-GOS, is resistant to hydrolysis and absorption in the small intestine and decided in favor [221]; at the same time, they do not have the same gas production negative effects.

Other Substrates for the Synthesis of FOS and GOS

Nowadays, Life Cycle Assessments (LCA) and the treatment of by-products from the food industry are gaining importance because of the environmental concern. In this context, using sucrose and/or lactose arising from different by-products or underutilized materials has acquired great importance. As sucrose and lactose are highly available in such kind of products, different attempts have been carried out to use them as raw materials for the synthesis of FOS and GOS, thus adding value to these underutilized products.

Some examples of products available for the synthesis of FOS include carob flour, containing *ca.* 50 % sucrose, which has been used as substrate with similar yields than pure sucrose in equivalent concentrations [222]. The use of grape must, mainly composed

of glucose and fructose, for the synthesis of FOS is a recent and very interesting strategy to add value to a by-product highly available in wine producing countries [223]. In addition, sugar syrup and molasses from beet processing containing sucrose were reported to be low-cost and available substrates for the enzymatic synthesis of FOS [54].

In turn, using by-products rich in lactose has been a quite extended strategy for the synthesis of GOS. This is the case of whey permeate. Whey is the by-product remaining from the production of cheese. It is majorly composed of proteins of high biological value (i.e., β-lactoglobulin) and lactose. Whey is generally spray-dried and powders are manufactured as three main products [136]: whey protein concentrate, containing 70– 85 % of the milk whey proteins and 50 % of the milk lactose; Whey protein isolate, containing more protein (90–98 %) than whey protein concentrates; Whey permeate, essentially composed of lactose and some minerals.

Whey proteins are usually incorporated in the formulation of bakery, meat and dairy products, as well as in infant and sportive food products. The remaining whey permeate is currently used for the production of refined lactose. The obtaining of GOS from whey permeate enables the valorization of whey surplus that economically are not feasible to dry [50]. In this regard, milk, sweet and acid whey have also been used as substrate for the synthesis of GOS [149]. In addition, different attempts have been used to obtain GOS from whey permeate. Golowczyc et al. [224] used this by-product first to obtain GOS, and then as culture and dehydration medium for probiotic lactobacilli. In turn, Nestle company uses demineralized sweet whey permeate as a food grade source of lactose for the synthesis of GOS. To this aim, the partially demineralized whey permeate containing lactose is evaporated to achieve 50 % total dry matter, and then incubated with beta-galactosidases from *A. oryzae* to obtain GOS with DP within 2–5. After synthesis, the enzyme is denatured and inactivated by heating, and the products, containing GOS, mono and disaccharides, purified by membrane nanofiltration, and finally dehydrated.

Properties and Applications

As mentioned before, the main characteristic of FOS and GOS is their prebiotic effect: both of them are non-digestible food ingredients that selectively stimulate the growth and/or activity of potentially health-enhancing intestinal bacteria [6]. Short chain FOS and GOS (DP<5) were especially recognized to encourage the growth of beneficial

bacteria in the colon. They act as fermentative substrates, and undergo fermentation in the colon of the host [42, 114]. This capacity discourages the growth of potential pathogens in the colon, enhancing the defense mechanisms of the host and protecting against enteric infections. Additionally, this increases mineral absorption and immunomodulation for the prevention of allergies and gut inflammatory conditions; furthermore, they are being investigated as possible reducers of risk factors for colon cancer [4, 42].

Strongly related to the non-digestible characteristic, FOS and GOS are identified as dietary fiber. The European regulation on food labeling obliges the manufacturers to identify these ingredients as dietary fiber [42, 114]. In fact, the recent legal definition of fiber is "carbohydrate polymers with 3 or more monomeric units, which are neither digested nor absorbed in the human small intestine obtained from food raw material by physical, enzymatic, or chemical means and which have a beneficial physiological effect demonstrated by generally accepted scientific evidence" [225]. Among their nutritional properties, GOS and FOS are carbohydrates that reduce basal hepatic glucose production without any effect on insulin stimulated glucose metabolism, which makes them suitable for diabetic diets [226, 227]. Furthermore, they affect lipid metabolism control counteracting triglyceride metabolism disorder and reducing free cholesterol level [42, 227].

Beyond their already known nutritional and prebiotic properties, FOS and GOS have technological properties that are strongly determined by their composition. Both inulin and oligofructose are quite stable toward disadvantageous technological conditions, namely low pH, high temperatures and low dry solids conditions. In extreme conditions, FOS and inulin are not hydrolyzed when the pH is above 4.5 and the storage temperature is below 10 $^{\circ}$C. The greater the degree of polymerization, the more stable the oligosaccharide. On this basis, they were used in a wide spectrum of technological applications either as syrups or as powders.

The (2→1) glycosidic bonds of inulin make it indigestible to humans and it can therefore be used as a low-calorie sweetener, fat replacer and dietary fiber [228]. Short chain FOS are those used for sugar reduction. The technical properties of oligofructose, such as solubility, taste and viscosity, make it a suitable ingredient to reduce the sugar content and increase the fiber content of many food products (i.e., jams, candies, gums, marshmallows) without affecting their organoleptic properties [42, 117, 122]. FOS and

inulin have been successfully incorporated as sugar replacers in the formulation of dairy products (mainly in yogurts) following the concept “sugar out, fiber in” and “fat out, fiber in.” Bakery products, including bread, cookies, cakes and muffins, are other group of products that have benefited from the addition of FOS and inulin in replacement of sugar. Cereals (i.e., breakfast cereals, cereal bars) represent another food category that suits the “sugar out, fiber in” concept, and in which oligofructose has been adequately used in replacement of part of the sugars, leading to products resembling the sugar texture very closely. For example, due to its excellent binding properties and good moisture retention, oligofructose is currently used as a binder of granola bars, leading to an improvement of their shelf-life (oligofructose acts as a humectant, inhibiting the hardening during storage).

Inulin is able to form gels, whose rheological properties are directly related with their crystallization behavior. The primary non-spherical inulin crystallites combine to more or less spherical aggregates which interact to form a weak structured gel where a significant amount of water is immobilized. When inulin is incorporated in a food product the formation of these crystalline aggregates results in an enhanced creaminess and mouthfeel even at dosages much lower than those needed for gel formation [42, 70, 229]. These properties make them excellent textures modifiers. Indeed, the addition of inulin to a low fat food product improves his creaminess and texture. The fat replacement and texturizing properties are related to the particle gel behavior. Hence, inulin is an excellent fat-replacer for water containing food systems, where inulin is present as small particles mimicking the mouthfeel and mouthcoating properties of fat. After shearing, inulin particles are formed with a size between 1 and 3 μm which is also the size of fat droplets after homogenization. This property enables the reduction of the caloric content of many products, including dairy products (yogurts, dairy desserts, custards, ice-creams), bakery (cake systems, puff-pastry, croissants, scones). Another category of foods benefiting from the fat-replacement properties of inulin are emulsified meat products, sauces, prepared meals, meal replacers, sausages and pates, which can be obtained with a creamier and juicier mouthfeel and improved stability thanks to the better water immobilization when replacing fat with inulin. Finally, the solubility of inulin and FOS makes them suitable to enrich beverages (dairy beverages, dairy analogs based on soy, rice, almonds or oat, near waters, fruit beverages), converting them in fiber enriched ones.

As a whole, inulin and FOS are natural ingredients highly versatile, whose applications are beyond their functional properties, making them very attractive in the food industry. The combination of the nutritional properties of fiber with the possibility to reduce sugar and fat give fructans a unique position in the ingredient world.

Regarding GOS, Japanese companies were pioneers in introducing them to the market, during the 1990s. At present, most of the applications of GOS are associated to their incorporation into infant products, with the aim of formulating products that more closely approximated human milk. Although their incorporation into food products is clearly regulated in the legislations of USA, European Union, Australia, New Zealand, Argentina and Brazil, their incorporation into other food products is rather limited in comparison to that of FOS. In this regard, in Austria, Finland, Italy, Belgium, the Netherlands and Japan, GOS are used as food ingredients in the formulation of dairy products, fruit juices, bread and bakery products, meal replacers, fermented and flavored milks, and cereal bars. Food for elderly and hospitalized people and poultry, pig and aquaculture products are among other applications of GOS as ingredients [114].

Similar to FOS, the composition of GOS determines their physico-chemical properties as food ingredients. GOS are usually commercialized as mixtures of oligosaccharides (>55 %), lactose (<20 %), glucose (<20 %), and a small amount of galactose, in powder or high concentrated syrups. As GOS have the capacity of remaining stable at high temperature treatments (up to 160 °C) and at low pH (2–3) [117], they are considered more stable than FOS [230]. The shelf-life of GOS exceeds 18 months without microbial spoilage. GOS containing monosaccharides have relatively low Tg (*ca.* 50 °C), thus making them very difficult for spray-drying processes. To counterbalance this disadvantage, the use of whey protein concentrates or maltodextrins has been reported [231]. In spite of that, mono and disaccharides present in the matrices make the products highly hydroscopic, so that, they must be stored under dry conditions. This hygroscopic character (that is, humectant properties) makes them suitable ingredients to prevent the excessive drying of bread and other bakery products, thus providing a better taste and texture.

One of the most important applications of GOS is as ingredients for infant formulas. Basically they are added to mimic human milk oligosaccharides, which are claimed to be responsible for a number of physiological effects that impact on the development of newborns [4, 197, 232–234]. Additionally, in the food industry, GOS are

used as sweeteners, not only in such formulas, but also in fermented products (as milk products and breads), jams, refreshing water and fruit juices [115]. Regarding fermented products, GOS are especially suitable for them because of their stability. For example, during bread making GOS resisted yeast fermentation and baking conditions. What is more, the taste and texture of bread remained preserved [117]. In the case of yogurt, GOS besides of being unchanged during the fermentation lactic acid bacteria, studies with consumers suggested that the yogurt with GOS had better sensory attributed (mouthfeel experience) than yogurt without GOS [234]. In the case of beverages, particularly fruit juices and soft drinks, GOS are preferred to be incorporated as prebiotic ingredient due to their acid stability and their ability to form clear solutions [213].

Because plant based GOS are not produced from dairy, they are completely lactose free. Growing infant formula demand in China and India as well as application growth in cereals, ice creams and dairy replacement products is expected to have a positive impact on plant based GOS research and development in the near future.

As it was mentioned, besides their application in the food industry, GOS are also relevant in the healthcare industry as constituents in clinical nutrition products [234]. These types of products are food and beverages designed for people with a lowered defense system who have specific nutritional needs. These kind of products often contain fiber (both insoluble and soluble) to provide an intestinal function as close as possible to normal food and to prevent constipation or diarrhea. In this sense, from a nutritional point of view, GOS are assumed to be fiber for being non-digestible polysaccharides, so they are suitable for use in different types of medical nutrition concepts, including tube- and sip feed and powdered supplements. Moreover, their stability is extremely important for liquid formulas. In many cases, patients express lactose intolerance. This is why GOS mixtures for this purpose must be lactose-free [213].

GOS prebiotic effect in not limited to human health. They are also interesting ingredients for pet food. They help to maintain animal immune system in right conditions promoting a healthy intestinal environment. Several studies pointed out that GOS consumption favored the generation of lactic acid bacteria such as lactobacilli or bifidobacteria and protected them from pathogens [213]. In this line, during the last years, GOS applications in the poultry, pig and aquaculture industries have been rising up. They promote animal’s health and growth, improved gut microbial ecology, and reduced

diseases, mortality, and fecal odor. Additionally, there was demonstrated that GOS could eliminate methane production by ruminants [114].

Conclusions

FOS and GOS have been the most investigated compounds with demonstrated prebiotic properties. As they can be obtained either by synthesis or by hydrolysis, they are highly variable in terms of structures. The chirality of GOS obtained by synthesis and by extraction/hydrolysis is opposed (β- in synthesis and α- from extraction/hydrolysis) even though the linkage is identical. Both types of GOS have relevant prebiotic effects. Hence, research work focused on understanding the relationship structure-functionality contributes to the development of the functional food market toward specific health needs.

FOS and GOS are complex structures containing mixtures of oligosaccharides with different degrees of polymerization. Their technological properties strongly depend on their composition which in turn, is a result of the obtainment process. For this reason, an accurate engineering of their production is of great importance to achieve the desired properties. Such engineering depends on many factors, not only technological but also economical. In this regard, the synthesis of FOS and β-GOS has a very important advantage, as substrates (sucrose and lactose) are cost-effective and the reactions can be standardized as there is no variability on the substrates. On the contrary, the natural variability of the raw materials normally used to obtain FOS and α-GOS by hydrolysis can eventually lead to difficulties to standardize the production. However, as α-GOS are assuredly lactose free, their commercial production can be important for relevant market sectors. Standardizing FOS and GOS production by enzymatic synthesis requires the control of the combined effect of reaction conditions (temperature, pH, time, and substrate concentration), enzyme source and activity on the process yield and product composition. In this line, as enzymes are the most expensive input for an economically feasible process, the selected ones are not specific and thus, the reaction conditions must be optimized to achieve a maximum productivity and yield of FOS and GOS. The improvements in immobilization technologies have certainly contributed to overcome this problem in the last years.

Taking into account the advantages and disadvantages of both hydrolysis and synthesis processes, and also the technological properties of the obtained products, an

adequate engineering of the processes appears as an important strategy to make the production of FOS and GOS an economically feasible industrial process. This viewpoint is of special interest for small and medium companies, considering the high turnover of FOS and GOS production, which makes the investment in the prebiotic market a very profitable activity.

Author Contributions

MU, ET, and AG-Z wrote the issues related to the synthesis of GS and FS, as well as the applications and conclusions. GM and PC wrote the issues related to the obtaining of FS and GS by hydrolysis.

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Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer SC declared a shared affiliation, with no collaboration, with several of the authors, MU and AG-Z, to the handling editor at time of review.

ii. Recent Advances in β-galactosidase and Fructosyltransferase Immobilization Technology

CRITICAL REVIEWS IN FOOD SCIENCE AND NUTRITION
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REVIEW

Recent advances in β-galactosidase and fructosyltransferase immobilization technology

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Abstract

The highly demanding conditions of industrial processes may lower the stability and affect the activity of enzymes used as biocatalysts. Enzyme immobilization emerged as an approach to promote stabilization and easy removal of enzymes for their reusability. The aim of this review is to go through the principal immobilization strategies addressed to achieve optimal industrial processes with special care on those reported for two types of enzymes: β-galactosidases and fructosyltransferases. The main methods used to immobilize these two enzymes are adsorption, entrapment, covalent coupling and cross-linking or aggregation (no support is used), all of them having pros and cons. Regarding the support, it should be cost-effective, assure the reusability and an easy recovery of the enzyme, increasing its stability and durability. The discussion provided showed that the type of enzyme, its origin, its purity, together with the type of immobilization method and the support will affect the performance during the enzymatic synthesis. Enzymes' immobilization involves interdisciplinary knowledge including enzymology, nanotechnology, molecular dynamics, cellular physiology and process design. The increasing availability of facilities has opened a variety of possibilities to define strategies to optimize the activity and re-usability of β-galactosidases and fructosyltransferases, but there is still great place for innovative developments.

Keywords

β-galactosidase, immobilization methods, fructosyltransferase, supports

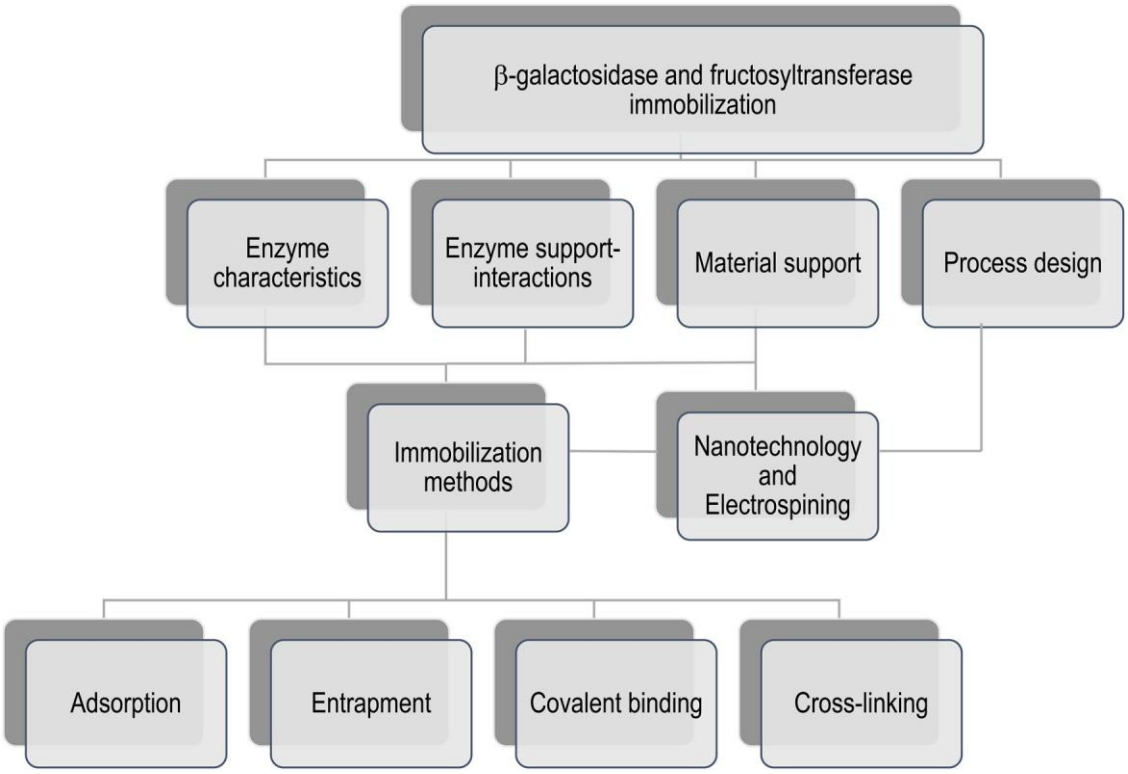
Introduction

Enzymes are biocatalysts responsible for specific chemical reactions, where a set of reactants (substrates) are converted into specific products during complex metabolic processes essential to sustain life [1]. They are proteins that act as catalysts in living organisms, participating in biological processes, and regulating the rate at which chemical reactions proceed, without altering their equilibrium [2, 3]. Furthermore, enzymes are integral parts of industrial processes since some of the reactions in which they participate would not be feasible without their aid. When enzymes are used in such applications, the overall cost of the process is lowered because enzymes are eco-friendly (reusable and biodegradable), obtained from renewable resources (microorganisms), and they increase the process's efficiency, requiring mild operating conditions (temperature, pH, energy), similar to physiological ones, and producing less waste and overall cleaner products [4,5].

Industrial chemical reactions can occur under extreme conditions, in terms of temperature, pH and presence of salts, surfactants, and organic solvents. These conditions may greatly lower the usefulness of enzymes due to their destabilization, so it is important from a technological and economical point of view to ensure enzyme stability [6]. This can be achieved by implementing different methods, such as using innovative natural enzymes (screening the microbiota and metagenomics), the development of new enzymes (mutagenesis, directed evolution and enzyme engineering design), catalyst engineering (chemical modification, immobilization on solid matrices or auto-aggregation), medium engineering (use of non-conventional reaction media or the addition of cryoprotectants and surfactants, to aid during dehydration and storage), catalyst reactivation (reactivation of the enzyme after achieving activity exhaustion) and process engineering (design of scale-up processes that maintain or improve enzymes' stability, activity and specificity) [1, 7-9].

During the last decades, enzyme immobilization had emerged as a suitable methodology having shown successful results [1, 5, 9-11]. This strategy promotes enzyme stabilization and guarantees reusability of the catalyst, simplifying its removal from the reaction medium. This review will focus on immobilization strategies addressed to achieve optimal industrial processes. To this aim, special care was put on the physical and chemical fundamentals explaining the enzyme-support interaction, not forgetting the properties of the carrier materials, a key factor to optimize immobilization. All these

aspects were specifically reported for two types of enzymes: β-galactosidases and fructosyltransferases, which are used to obtain GOS and FOS, respectively. These oligosaccharides are among the most widely employed prebiotic compounds with several applications in the food and pharmaceutical industries, indeed their demand has exponentially increased worldwide over time [12]. Particularly, enzymes' cost is one of the most critical issues in the industrial production of prebiotics. For this reason, design processes need to consider both economic and technological aspects, and enzymes' immobilization emerges as a promising strategy to improve their stability and reusability. Scheme 1 provides a graphical representation of how the manuscript information was organized to guide the reader through the main discussed items.



Scheme 1 - Organization of the relevant issues discussed in this review

Enzymatic Production of GOS and FOS: Immobilization as a Process Design Strategy

Immobilization proved to be a good alternative among the previously mentioned methods used to achieve enzyme stabilization mainly for being cheaper, simpler and providing a better chance of reusing and recovering the enzyme. In particular, when dealing with enzymes that are expensive, rare or difficult to purify, the reusability, stability over storage and transport, and ease of recovery are critical issues [13].

Immobilization involves the attachment or incorporation of enzymes into a support material. An insoluble, reusable, and more resistant form the enzyme is obtained, which can participate in chemical reactions with different process conditions, ideally without significant loss of stability or activity [6, 14]. Immobilized enzymes (IE) are used in several fields, such as in the food and pharmaceutical industries, in medicine, for the production of biofuels, detergents and cleaning products, and in bioremediation and waste waters' treatment. There are several reviews on this topic that delve into the use of several enzymes, the immobilization procedures and their applications [13-18].

Each active site of an enzyme is composed of a few amino acid residues and this part of the molecule is responsible for its catalytic activity. The rest of the enzyme is important for structural stability during catalysis [1] and environmental alterations (temperature, pH) can cause conformational changes and, consequently, loss of activity [6]. This happens because the amino acid residues of the active site are very close in the folded protein, held in position by intermolecular forces, but can be very distant in the primary structure, and denaturation can lead to their separation, thus losing the catalytic activity [19]. This effect, however, can be beneficially used, for example, when designing an immobilization procedure for controlling (stopping) the reaction. Immobilization should not block or hinder access to the active site of the enzyme, although it can be used for the steric exclusion of inhibitors. This can lead to an increase in activity, often mistaken for the obtaining of a more advantageous conformation of the enzyme after immobilization, when, in fact, there is loss of inhibition [20, 21].

Reactions using free enzymes (FEs) are homogenous systems, i.e., the enzyme is in solution among the substrates, co-factors, products and other species relevant to the process. Working with IE implies heterogeneous systems since the enzyme exists in a different phase than the solutes (the enzyme is now insoluble). The kinetics of the two systems are not the same. Limitations in mass-transfer together with the reduction of enzymes' conformational mobility due to enzyme-carrier interactions often compromise enzyme activity and, consequently, the reactions' kinetics [1, 10]. When designing new systems, the gain in stability *per se* may not compensate for slower reaction rates. There are no standardized concepts about the most appropriate immobilization technique for the different biocatalysts in the industry. In fact, this selection must be done with an optimization *via* trial and error comparing the activity, stability, and reusability of the FE with those of the IE.

What is more, industrial reactors can be designed to work in batch or in continuous mode. In the former, limited amounts of reactants are placed in a confined environment during the time needed for the reaction to be completed. Afterwards, the enzyme must be separated, often leading to its inhibition or inactivation. Eventually, substrate and enzyme are restocked, and the process is repeated. In this mode, soluble enzyme is commonly used, although IE can be used (i.e., in the recirculation batch reactor, IE is recovered and reused). In continuous processes, there is constant and simultaneous renewal of reactants and removal of products. The IE can be used until a significant loss of activity is observed so optimization is needed to determine the number of cycles the enzyme can perform. Continuous mode is more advantageous since it requires fewer steps, namely the preparation of the reaction medium, transformation, recovery of the medium post-reaction, purification/removal of enzyme and obtaining of the pure product can be done simultaneously and, consequently, at lower costs [1, 22].

FOS and GOS Enzymatic Synthesis

Galacto- and fructo-oligosaccharides (GOS and FOS) are non-digestible oligosaccharides with prebiotic properties that can be incorporated into a wide number of products. From a nutritional point of view, they are low caloric sweeteners that give a feeling of satiety, contribute to body weight control, relieve constipation, have a low glycemic index and are not cariogenic [23]. According to the latest definition, prebiotics are “substrates that are selectively used by host microorganisms conferring a health benefit” [24]. These compounds are used in the formulation of many food products, beverages, and especially in infant formula, to stimulate the development of newborn microbiota [12, 25]. Both GOS and FOS can be obtained by hydrolysis or by enzymatic synthesis. The former consists in the hydrolysis of compounds naturally occurring in some plants or seeds (i.e., soybean, lupin, lentil, chickpea, pea and cowpea for GOS and roots of chicory, artichoke, yacon, dahlia or agave for FOS). This strategy promotes the obtaining of large molecular weight compounds (degree of polymerization, DP, higher than 8) and a mixture of different other compounds besides prebiotics. For this reason, this kind of processes need specific purification steps, depending on the natural source used to obtain GOS and FOS. Industrial enzymatic synthesis using a disaccharide as substrate (i.e., lactose and sucrose for GOS and FOS, respectively) is a strategy that

allows the obtaining of short chain GOS and FOS (DP ranging from 3 to 7). Besides that, this is a versatile and easy way to control once the reaction mechanistic is well established, which depends on a large extent on understanding the enzyme activity characteristics [12].

Particularly, the enzymes used to obtain FOS and GOS, fructosyltransferases and β-galactosidases, have both transferase and hydrolase activities. These enzymes are capable of catalyzing the transfer of functional groups (glycosylic) and, at the same time, the hydrolysis of organic molecules (sucrose and lactose, respectively). From the one side, this double function considerably decreases the enzymes' costs. From the other side, the non-specificity represents a technological challenge because the products' yield is lower than that obtained using more specific enzymes. For this reason, the industry must implement production processes considering both economic and technological aspects. In line with this, industrial scale production of GOS and FOS by enzymatic synthesis can be performed through batch or continuous processes, either with soluble FE or with IE. The pros and cons of the different processing modes applied for obtaining GOS and FOS for enzyme immobilization studies are presented in this review and schematized in Figure 1.

β-Galactosidase

β-galactosidase, also called lactase, beta-gal or β-gal (EC 3.2.1.23), is the enzyme most commonly used to obtain glucose and galactose from lactose. This enzymatic reaction turns a wide variety of products adequate for lactose intolerant consumers [26]). The reaction is sometimes also employed as a technological strategy to overcome the possibility of lactose deposition and to obtain a sweeter flavor (glucose and galactose are more soluble and sweeter than lactose) [27].

As an advantage, β-galactosidase is widely distributed in nature and can be found in plants (especially in almonds, peaches, apricots, and apples) but often has low lactase activity [28]. On the contrary, β-galactosidase from microorganisms (yeast, bacteria, and fungi) [29] and from mammalian's intestinal tract presents a high lactase activity. β-galactosidases used for the synthesis of GOS are usually from yeast (*Kluyveromyces lactis, Kluyveromyces fragilis, Rhodotorula minuta*), bacteria (*Escherichia coli*, *Bacillus circulans*, *Bacillus* sp., *Lactobacillus reuteri*) and fungi (*Aspergillus oryzae*) [12]. The most studied β-galactosidase is that produced by *E. coli*, but because of its origin

(coliforms), it is not a suitable option for food applications if it is not appropriately purified [26]. Therefore, β-galactosidase is produced from recognized safe microorganism sources [yeasts (*Kluyveromyces*) and fungus (*Aspergillus*)] at industrial scale. Although enzyme molecular size varies depending on the enzyme source, there is an estimation of its molecular size since early 70s. Melchers and Messer (1973) defined that *E. coli* β-galactosidase is tetrameric, being composed of four identical subunits of 135,000 daltons [30]. Also, Yang, Marchio, and Yen (1994) reported that it has an average protein diameter of 12 nm, but when exposed to low concentrations of salts aggregation was observed, reaching particle sizes of around 1000 nm and 2000 nm [31].

In general terms, the dynamics of this enzyme was described by Mahoney (1998) as a simple three steps mechanistic: first, thanks to the enzyme active site, the lactose-enzyme complex is formed; then, galactosyl transfer occurs, resulting in the formation of a galactosyl-enzyme complex, while glucose is released; finally, the enzyme will transfer galactose to a nucleophilic acceptor containing a hydroxyl group [28]. When the acceptor is water, galactose is formed, and when the acceptor is another sugar, the product is an oligosaccharide.

Fructosyltransferases

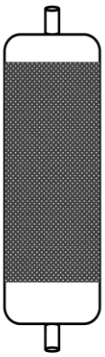
Fructosyltransferases (β-fructofuranosidase, invertase, EC 3.2.1.26 or β-D-fructosyltransferase, EC 2.4.1.9) are the biocatalysts for the transfructosylation reactions leading to the obtaining of FOS. They can be extracted from plants, yeasts and molds and from bacteria (GH32 and GH68 families from CAZy classification, respectively) [32]. Depending on the enzyme source, some characteristics like molecular weight will differ from each other. In this sense, Sangeetha, Ramesh, and Prapulla (2005) provide a detailed study including several fructosyltransferase sources [33].

The activity of this enzyme consist on the cleavage of the β-2,1-glycosidic bond and the transfer of fructosyl moieties from carbohydrates acting as donors onto any acceptor other than water [34]. Synthesis and hydrolysis occur simultaneously both in parallel and in series [12]: FOS (DPn) synthesized in the first steps act as fructosyl donors and acceptors leading simultaneously to the production of FOS with DP immediately higher (DPn + 1) and lower (DPn-1); mixtures of short chain FOS (DP ranging from 3 to 6, i.e., DP3, DP4, DP5 and DP6), together with glucose (secondary product), are obtained [12,35].

a) Continuous Process

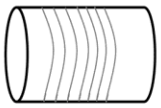
Main yield factors: flow rate, substrate concentration, residence time, effective volume, mass diffusion coefficient.

Packed-Bed Reactor



Adsorption
GOS: Albayrak and Yang 2002
FOS: Hayashi et al. 1994; Yun, Kang, and Song 1995; Yun and Song 1996, 1999.
Entrapment
GOS: Ateş and Mehmetoğlu 1997; Jovanovic-Malinovska et al. 2012.
FOS: Cheng et al. 1996; Fernandez-Arrojo et al. 2013; Zambelli et al. 2016.
Covalent binding
GOS: Klein et al. 2012; 2013; Chen et al. 2009; Song et al. 2013; Warmerdam et al. 2014.
FOS: Hayashi et al. 1991; Lorenzoni et al. 2015.
Cross-linking
GOS: Eskandarloo and Abbaspourrad 2018.

Membrane Reactor

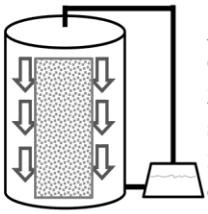


Adsorption
FOS: Nishizawa, Nakajima, and Nabetani 2000 (forced flow).

b) Batch Process

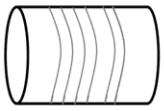
Main yield factors: effective occupied volume, reaction time, substrate concentration, number of cycles, mass diffusion coefficient.

Recycle Reactor



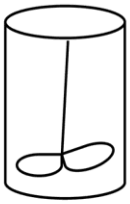
Adsorption and Cross-linking
GOS: Matella, Dolan, and Lee 2006; Albayrak and Yang 2002 (the same system **without recirculation operates as a continuous single-path reactor**).

Membrane Reactor



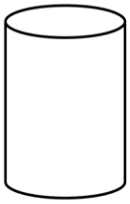
Adsorption and Covalent binding
GOS: Güleç 2013.

Stirred Reactor



Adsorption
GOS: Gaur et al. 2006; Botelho-Cunha et al. 2010; Carević et al. 2018.
Entrapment
GOS: Tanriseven and Doğan 2002; Jovanovic-Malinovska et al. 2012.
Cross-linking
GOS: Zhou and Dong 2001; Guerrero et al. 2015.

Fermentation Flask



Adsorption
FOS: Mussatto et al. 2009; Mussatto, Rodrigues, and Teixeira 2009; Mussatto et al. 2012; Ganaie, Pathak, and Gupta 2011; Castro et al. 2017.
Entrapment
GOS: Yu and O'Sullivan 2018.

Figure 1 – Process and reactor design used to perform GOS and FOS production with IE. (a) Continuous mode and (b) batch process

β-Galactosidase and Fructosyltransferases: their Mechanisms of Enzymatic Action

Understanding the mechanism of the kinetics involved in the catalytic enzyme performance is crucial for process design. In particular, for immobilization, considering all these mechanisms is of great importance to choose both an appropriate immobilization method and an appropriate support. β-galactosidase and fructosyltransferase belong to the glucosyl hydrolases family (GH-A superfamily). This family of enzymes has been widely studied with the objective of unraveling their catalytic mechanism [36, 37]. The enzymatic hydrolysis of glycosidic bonds is carried out with retention or inversion of the anomeric configuration, thus hydrolases are classified as either retaining or inverting [38]. Particularly, β-galactosidase is a retaining hydrolase and fructosyltransferase is an inverting.

β-Galactosidase

Using x-ray diffraction data together with directed site mutations, kinetic experiments and *in silico* studies, the *E. coli* β-galactosidase structure, binding sites and catalytic mechanism are well understood [39, 42]. The enzyme has two binding modes: a shallow mode, with weak interactions and poor specificity that allows weak binding from several different substrates; and a deep mode, at the catalytic site, with strong interactions and high specificity. As it is shown in Figure 2, in Following Davies' nomenclature, the deep mode corresponds to subsites -1 and +1, and the shallow mode, to subsites +2 and higher [43].

For the catalytic process, the lactose substrate is captured in the deep mode at the catalytic pocket with its binding pose controlled mainly by the β-D-galactosyl moiety, which is pinned down at the -1 subsite by a hydrophobic stacking with a tryptophan residue and a complex network of hydrogen bonds between its hydroxyl groups and six polar residues (among them, GLU461, GLU537, Figure 2). The glucosyl moiety is stabilized at subsite +1, by a hydrophobic interaction with a tryptophan residue. The weak interactions at subsite +1 result on higher mobility and less specificity than subsite -1. Both kinetic and quantum mechanics/molecular mechanics *in silico* studies indicate that an Mg^{2+} ion located at the catalytic pocket, complexed with three water molecules and

three residues, including GLU461, increases the specificity toward the β-D-galactosyl moiety [44].

The proposed catalytic mechanism for cleavage of the lactose glycosidic bond involves several steps. The first step is the formation of two hydrogen bonds, first between the carboxylate of the GLU537 residue below the galactosyl ring and the hydroxyl at C2, and second, between the carboxylic acid of GLU461 residue, above the ring, and the glycosidic oxygen (Figure 2). On the second step, the second hydrogen bond results in proton donation after the nucleophilic attack by the GLU537 carboxylate on the anomeric carbon, forcing the glycosidic bond cleavage. The Mg^{2+} ion modulates the acidity of GLU461, facilitating the bond cleavage. After the glycosidic bond has been cleaved, the weak interactions at subsite +1 allow the glucose molecule to leave. Calculations also suggest that a change on the galactosyl ring to a half-chair conformation is also important for the glycosidic bond cleavage [44]. This ring conformation is consistent on all GH-A enzymes, although differences between families have been found [45].

After the glycosidic bond cleavage, two fates await the covalent enzyme-galactosyl complex. The complex can suffer hydrolysis, following a nucleophilic attack by a water molecule, or a new glycosidic bond can be formed with the freed glucose or another substrate resulting on the transglycosylation. On the second case, a lactose or longer GOS can be captured by the shallow binding mode placing the galactosyl end at the subsite +1. For the *E. coli* β-galactosidase, the quantum mechanics/molecular mechanics study allowed the elucidation of the reaction path showing that transglycosylation leads β(1-6) oligosaccharides as the thermodynamically favored products, galactosyl-β(1-6)-glucose (allolactose) being the preferred one [44]. The catalytic mechanisms for both hydrolysis and transglycosylation are also represented by the diagrams on Figure 2.

The true nature of the catalytic mechanism is relevant for the establishment of a proper kinetic model. Experimental data led to the conclusion that glucose acts as a competitive inhibitor. *In silico* studies with human β-galactosidase (also a retaining hydrolase) suggest that a similar catalytic mechanism is the origin for the inhibiting effect [46], since glucose can enter the catalytic site but the absence of an hydroxyl in the adequate position prevents the formation of the covalent bond to the enzyme [39].

β-galactosidase from *Aspergylus oryzae* belongs to GH-35 family, together with human galactosidase, while the enzyme from *E. coli* belongs to the GH-2 family [47]. A comparison of several enzymes has shown that all members of the GH-A superfamily have the same two glutamic acid residues at the catalytic site, one to act as a nucleophile and the other, as a Brönsted proton donor. Therefore, the mechanism should be the same as that for other enzymes from the superfamily [36, 48-52]. Crystallographic and kinetic studies confirm this finding [53]. All enzymes from the GH-A super family are structurally and mechanistically related [45].

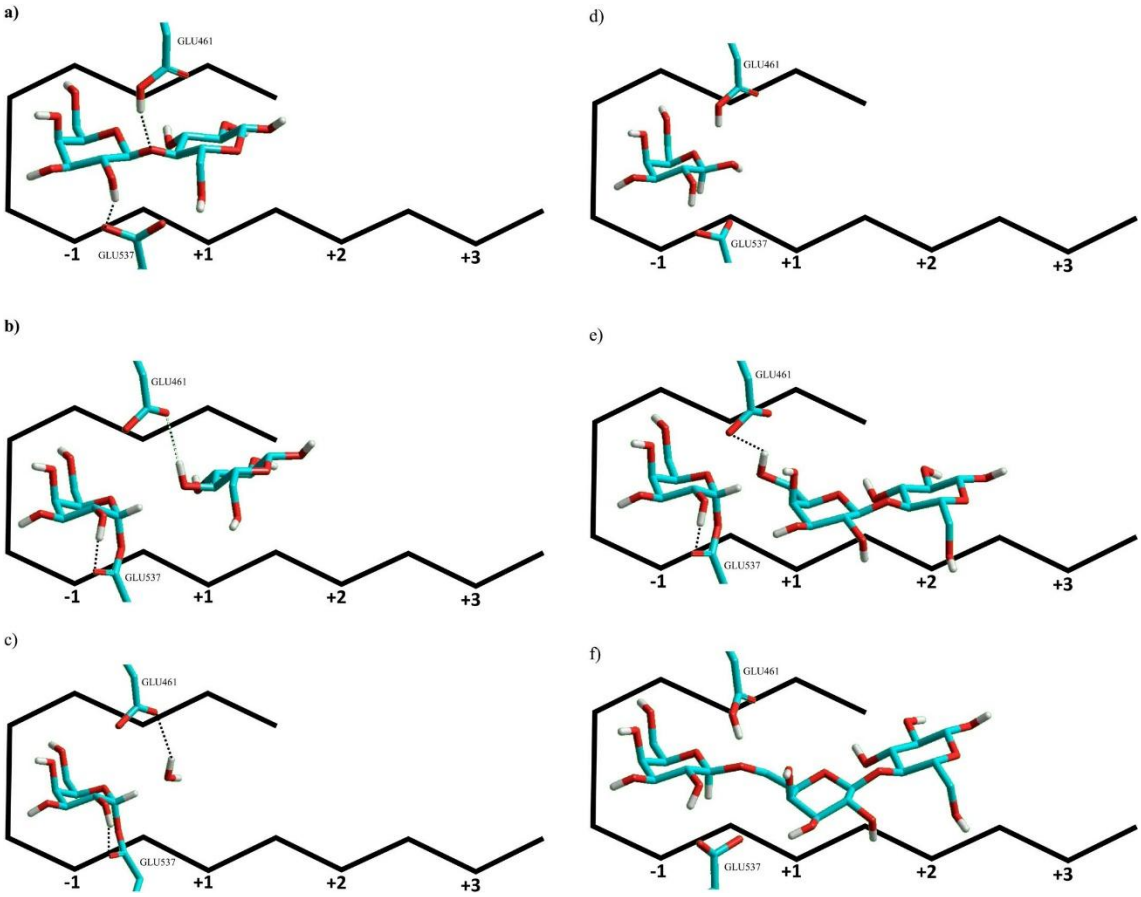


Figure 2 – Diagram representing subsites -1 to +3 and six different situations from β-galactosidase catalytic mechanism: (a) binding of lactose on subsites -1 and +1; (b) glycosidic bond cleavage; (c) enzyme-galactosyl plus water at subsite -1; (d) galactose ready to leave subsite -1; (e) enzyme-galactosyl plus lactose at sub-sites +1 and +2; and (f) GOS ready to leave

Fructosyltransferase

Fructosyl hydrolases and transferases belong to the GH-32 family, including retaining and inverting enzymes. The general mechanism has been elucidated with data from X-ray diffraction, directed site mutations, kinetic experiments and *in silico* studies.

It follows a similar path than that of galactosidases, but with three instead of two key residues: one aspartic acid acts as a nucleophile and an aspartic acid, as acid/base donor; a second aspartic acid modulates the nucleophile with a stabilizing effect on the covalent enzyme-fructosyl intermediate. The presence of a Ca^{2+} ion has been noted and its role might be the same as the Mg^{2+} ion discussed before [54-60]. As with β-galactosidase, quantum mechanics/molecular mechanics calculations for transfructosylase from *Aspergillus japonicus* allowed the calculation of the reaction path, through a retaining mechanism, and the prediction of the most stable products to come from transfructosylation instead of sucrose hydrolysis [61]. The results confirmed those previously obtained, suggesting that transfructosylation and sucrose inversion are performed by different enzymes [62].

The modeling of glucosidases' and fructosyltransferases' kinetics has been widely studied. Invertase, also a hydrolase, was used by Michaelis and Menten on their studies leading to the general enzyme kinetics model [63]. Due to their industrial importance, hydrolases' kinetics have been studied in the free form, immobilized and with several types of reactors [34, 35, 62, 64-73]. Several authors have proposed empirical based kinetic models with support from mechanistic conclusions, adapted to every experimental setup fitting the models' parameters (Michaelis constants or kinetic constants) to existing experimental data. Figure 3 represents the hydrolysis (Figure 3a) and the transglycosylation of a disaccharide (Figure 3b).

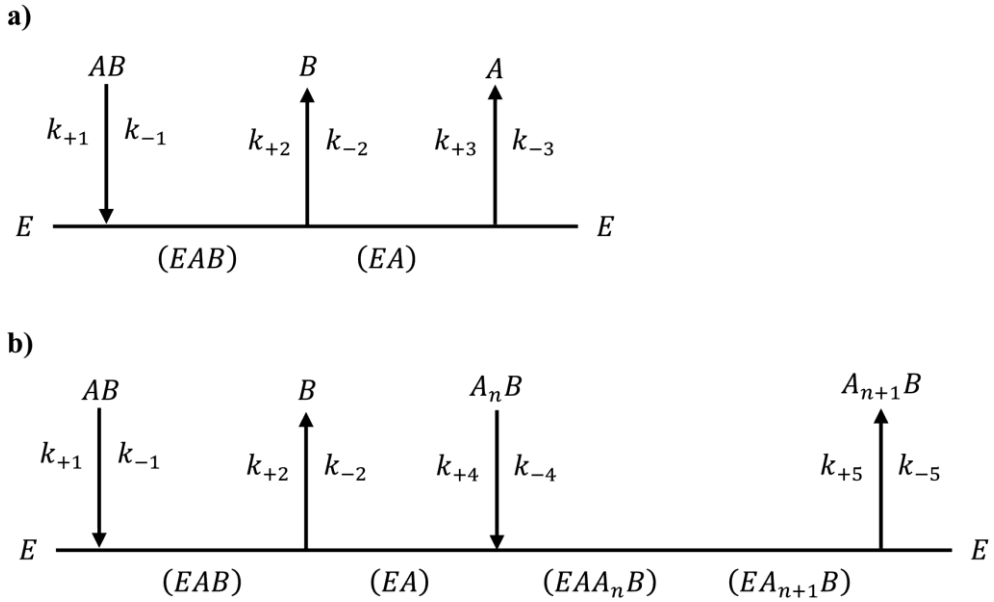


Figure 3 – Proposed mechanism for: (a) hydrolysis and (b) transglycosylation of a disaccharide. *A* is a galactoside or fructoside, *B* is a glucoside, and *E* is the enzyme

Contribution of Immobilization Technology for β-galactosidase and Fructosyltransferase

Different classifications for the various types of immobilization can be found in the literature. The most common one is the distinction between physical or chemical methods [74]. The first group includes the methods involving *physical* interaction between the enzyme and the support or those in which the enzyme is *physically* restrained by the carrier, hindering its release to the medium. The chemical methods depend mostly on the establishment of covalent bonds [13, 75]. Other classifications distinguish the presence or absence of carriers/supports [19] or those that differentiate the reversible or irreversible nature of the interactions [76]. There are also authors that do not classify them, and simply discuss the different methods [2, 9, 14, 18, 77]. This latter perspective is that assembling most with the focus of this review. In this line, although there are many immobilization methods and types of enzymes in the food industry, we will discuss those particularly used for fructosyltransferase and β-galactosidase. In this sense, adsorption, entrapment, covalent coupling, cross-linking and aggregation are the main methods used to immobilize these type of enzymes, all of them having pros and cons. Figure 4 gives a general outline of the mechanisms of each method.

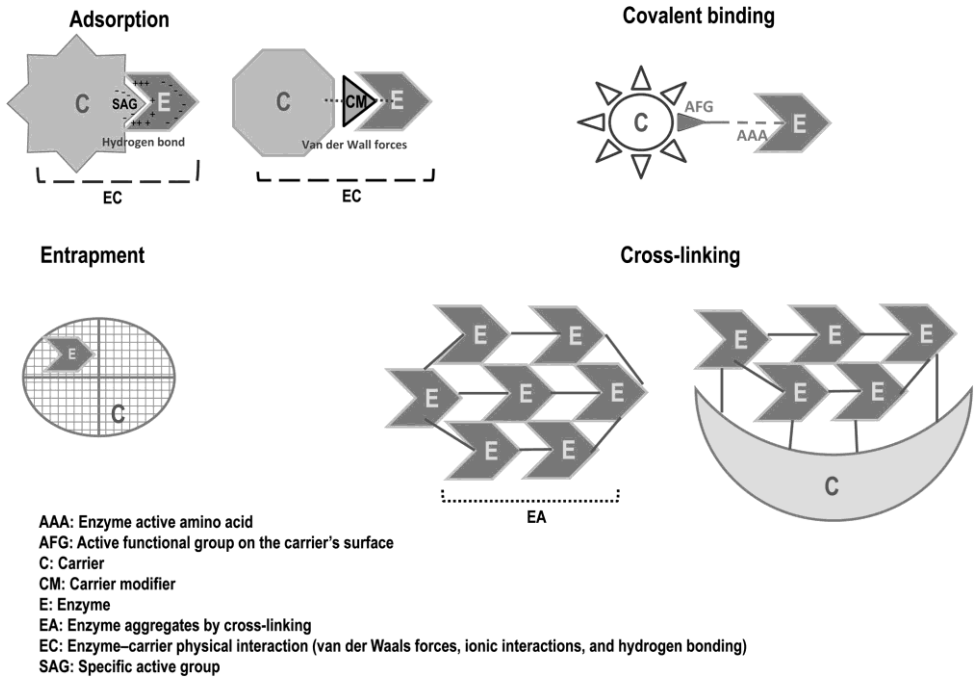


Figure 4 – Simple representation of the principal immobilization methods (adsorption, entrapment, covalent binding and cross-linking) used to immobilize β-galactosidases and fructosyltransferases

Adsorption is often considered a simple method consisting on physical interactions such as van der Waals forces, ionic interactions and hydrogen bonding, between the carrier and the enzyme without modifying the natural structure of this last one [10, 27, 78]. For this type of immobilization, hydrophobicity/hydrophilicity character and charge (isoelectric point) of the enzyme are very important for the selection of the support [9]. The carrier needs to provide specific functional groups (SAG in Figure 4) on the surface for the conformation of the enzyme-carrier binding. If this interaction does not occur spontaneously, intermediates (carrier modifiers) can be applied (CM in Figure 4). Silica, silica gel, alumina, alumina gel, activated charcoal, ion exchange resins, hydroxyapatite (inorganic), chitosan, calcium alginate, agarose gel, cellulose, synthetic polymers, fiber membranes, cotton cloth (organic) are common supports to immobilize enzymes by adsorption [78, 79].

Entrapment involves the occlusion of an enzyme in a synthetic or natural polymeric network (gel, fiber or microcapsule) that act as a permeable membrane to substrates and products, while the enzyme is retained inside [74, 77, 79]. This is a fast, cost-effective and feasible strategy of immobilization but it has some drawbacks, the main one being the mass transfer limitation due to diffusional problems between the substrates, products and the carrier [2, 78]. Additionally, physical retain is generally weak and does not prevent enzyme leakage. Therefore, very often additional treatments with specific reactants are required to effectively retain the catalyst, usually by covalent attachment. Consequently, the difference between entrapment and covalent binding is not clear. In this sense, Sheldon (2007) proposes that the term entrapment involves the synthesis of the polymeric network during the immobilization process [77]. As a drawback, this last strategy makes the supports not renewable.

Enzyme immobilization via covalent binding is one of the first methods implemented, therefore, one of the most widely investigated. In brief, it consists in a chemical interaction between the aminoacids from the active site of the enzyme with the active functional groups from the carrier surface (AAA and AFG in Figure 4) [80]). Carriers need often to be activated, and this process involves two stages: the addition of a reactive compound and the modification of the polymer backbone to activate the matrix [21, 78]. Covalent bonding generates strong and stable linkages and it usually prevents leakage of the enzyme from the carrier's matrix [78, 80]. However, the main drawback is

the fact that both the enzyme and the support remain unavailable if the enzyme is irreversibly deactivated during either the immobilization or the synthesis processes [77].

Cross-linking consists in intermolecular linking of molecules by the use of bifunctional reagents (substances that contain two identical or two different functional groups or groups of different reactivities) [81]. The principal bifunctional cross-linking reagent used for enzyme immobilization is glutaraldehyde (GA), which has the ability to react with different enzyme moieties (primary amino groups, thiols, phenols, imidazoles) and also, the capacity to polymerize [82]. In this sense, there are two main approaches: to use GA as an intermolecular enzyme crosslinker in support-free methods, and to use GA to activate the support and prepare the IE. The first one can overcome the diffusional limitations of mass transfer by using different supports, it does not require purified enzyme, and it is highly specific. Nevertheless, its main disadvantage is that it can modify the enzyme's configuration, leading to a loss of activity. This may condition the selectivity of the enzyme for a certain substrate or the formation of side-products [83].

In the next paragraphs the reader will find a chronological description of the evolution to immobilize β-galactosidases and fructosyltransferases, with special emphasis on those works which gave a useful insight in the field during the last decades. Although this section is divided considering each method of immobilization, it must be mentioned that in many works authors compared different immobilization methods or used a combination of them. Hence, the sequence selected for presentation is just for a matter of organization rather than a strict classification. The examples mentioned in this section are listed in both Table 1 and Table 2 and are discussed with greater detail below. It is worth mentioning that in the case of β-galactosidase immobilization, most of the works are focused on the hydrolysis reaction rather than on its transferase activity. For this reason, the table includes a column indicating the application procured by each author to design the immobilization process.

Table 1 – Characteristics of the principal innovative strategies to immobilize β-galactosidase

Immobilization method	Support	Application	Enzyme source	Results/observations	Reference
Adsorption and cross-linking of enzyme aggregates	Cotton cloth	GOS synthesis	*A. oryzae* (Genencor Int.l); (Sigma and Bio-Cat)	Multilayer immobilization was achieved. The method required highly concentrated and pure enzymes (that is, 100000 U/mg). If not, it could cause enzyme inactivation	[84, 85]
1-physical adsorption 2-covalent binding 3-aggregation by cross-linking	1. Celite 2. Chitosan 3. –	Oligosaccharide synthesis	*A. oryzae*	Covalent coupling to chitosan was more appropriate for oligosaccharide synthesis, as compared to adsorption or aggregation. Enzyme aggregates appear to be more suitable for lactose hydrolysis applications	[86]
Adsorption	Duolite A-568 (resin beads)	GOS synthesis	Commercial *K. lactis* (*Lactozym 3000 L HP-G*, Novo Nordisk A/S)	Higher the concentration of enzyme, higher the loading obtained, but with loss of enzyme activity and leading to substrate hydrolysis over GOS synthesis	[164]
Adsorption and covalent binding	Cellulose acetate membranes	GOS synthesis	*K. lactis*	The IE via adsorption caused higher lactose conversion but lower initial GOS yields than the soluble enzyme system. The most efficient strategy was covalent bonding with plasma polymerization of 2-mercaptoethanol modification, which achieved high immobilization yield and high enzyme activity	[87]
Adsorption by ionic interaction	Resins with different functional groups	GOS synthesis	*L. acidophilus ATCC 4356*	Resin carriers with amino groups and larger pores and longer spaces were selected in order to enhance enzyme activity yield	[90]
Entrapment	Cobalt alginate beads	Lactose hydrolysis	*A. oryzae*	GA enabled to avoid enzyme leakage. The hydrolysis products contained higher amounts of Co^{2+} than the limits allowed in foods so, removal is needed	[98]
Entrapment	Alginate and gelatin fibers	Lactose hydrolysis	Commercial *A. oryzae* (Sigma)	More stability at high pH and temperatures than FE. Activity did not decrease for 35 days	[99]
Entrapment	PVA lenses and sol–gel carriers	GOS synthesis	Commercial *A. oryzae*	PVA IE was better biocatalyst than sol–gel enzyme in terms of lactose conversion and operational stability	[100]
Entrapment	Chitosan beads and alginate beads	GOS synthesis	*L. lactis*	First the recombinant DNA was degraded in the whole cells using UV treatment. Chitosan was more appropriate for immobilization than alginate for GOS production at high temperature (85ºC)	[101]

Entrapment and adsorption by ionic interaction	Sodium alginate or l-carrageenan	Lactose hydrolysis	Commercial *K. lactis* (Lactomax, Prozyn)	Immobilization of the enzyme might negatively affect its activity, however, is able to reduce the denaturation rate of the enzyme exposed to low pH	[88]
Entrapment and adsorption by ionic interaction	Sodium alginate	Lactose hydrolysis	*A. oryzae* (Sigma)	The binding between β-galactosidase and alginate resulted from the equilibrium between enthalpy and entropy contributions. The thermal stability was improved compared to FE	[102]
Covalent binding	Chitosan	Lactose hydrolysis	*B.stearothermophilus*	Support activation with GA was performed. Superior activity, reusability, thermostability, and storage stability was achieved compared to FE	[109]
Covalent binding	1. Polyaniline coated with magnetite. 2. Polysiloxane-PVA polymer	GOS synthesis	*1. A. oryzae* (Sigma) *2. K. lactis*	1. Similar product composition was obtained and similar kinetic behavior was observed compared to the FE. 2. Immobilization yield was 99% with 78.5% of enzyme activity recovery. Authors studied experimentally the reaction mechanism to produce GOS and proposed a mathematical model	[111; 112]
Covalent binding	Chitosan macroparticles	Lactose hydrolysis and GOS synthesis	Commercial *K. lactis* (Maxilact LX 5000)	pH and temperature operational range was larger with IE in PBR. The combination of continuous flow with a high content of lactose can increase enzyme stability	[107, 108]
Covalent binding	Silica gel	Whey lactose hydrolysis	*K. lactis*	Pretreatment with lactose prior to immobilization. The pretreated β-galactosidase activity was 2.6 times higher than non-pretreated. Temperature and pH stability and reusability of the IE was improved by the pretreatment	[113, 114]
Covalent binding	Glyoxyl Sepharose	*o*-NPG hydrolysis	Commercial *K. lactis* (Lactozym 3000)	Multi-point attachment enhanced thermal and pH stability, and made the enzyme less susceptible to inactivation in the presence of solvents (dioxane 30%)	[115]
Covalent binding	Porous acrylic beads with oxirane functionality (Eupergit C)	GOS synthesis	Commercial *B. circulans* (Biolacta N5)	The use of IE in a continuous PBR was compared to FE in a batch reactor. 90 days was the half-life time of the IE. PBR productivity was more than six times higher than FE in a batch reactor	[116]
Cross-linking	Gelatin	Lactose hydrolysis	*E. coli*	IE preserved its activity for 3 months, being more stable to pH variations than FE	[126]
Cross-linking	Graphite electrode surface	Lactose hydrolysis	*K. lactis*	Higher specific activity than FE. Stable and active at 37 °C and 50 °C	[127]

Cross-linking	PVDF membrane	GOS synthesis	Commercial *B. circulans* (Biolacta N5)	The units of enzyme immobilized per gr of membrane increased with enzyme concentration. The formation of GOS product increased with the initial lactose concentration. A recirculation loop allowed improving the process. All this was satisfactorily incorporated in a mathematical model	[128, 129]
Cross-linking and aggregation	–	GOS and f-GOS synthesis	Commercial *A. oryzae* (Enzeco)	Immobilization favored the generation of disaccharides over higher oligosaccharides and allowed enzyme reuse increasing lactulose production per unit mass of biocatalyst and in cumulative productivity	[130, 131]
Cross-linking	Glyoxyl-agarose, amino-glyoxyl, carboxy-glyoxyl and chelate-glyoxyl agarose	Lactulose, GOS and f-GOS synthesis.	Commercial *A. oryzae* (Enzeco)	Glyoxyl-agarose and amino-glyoxyl-agarose derivatives retained the selectivity of the FE for lactulose synthesis while carboxy-glyoxyl-agarose and chelate-glyoxyl-agarose favored the synthesis of transgalactosylated oligosaccharides	[131-133]
Cross-linking	Glass beads	GOS synthesis	Commercial *A. oryzae* (Megazyme)	IE retained higher enzymatic activity than the FE at higher temperatures. It was studied in a PBR	[135]
Cross-linking	Electrospun gelatin nanofiber mats	GOS synthesis	*A. oryzae*	Hexamethylenediamine was used as activation agent in order to increase their stability in water. Achieved GOS yield using this catalyst was 31 %, higher than that obtained with FE	[11]

Table 2 – Characteristics of the principal innovative strategies to immobilize fructosyltransferase

Immobilization method	Support	Enzyme source	Results/observations	Reference
Adsorption via ion exchange	DEAE-cellulose	*β-fructofuranosidase Aureobasidium sp*	pH stability was improved with immobilization and also the enzyme became less susceptible to metal ions. Long-term continuous operation in a column reactor yielded a total production of FOS of 105-127 mg/mL	[91]
Adsorption via ion exchange	Porous styrene-derived resin	*Fructosyltransferase A. pullukzns*	High porous support and a column system produced high volumetric activity, more stability against pH changes, and long-term continuous operation	[92-94]
Adsorption and covalent binding	Ceramic membrane	*β-fructofuranosidases A. niger*	Smaller pore size membranes showed larger immobilized activity, and covalent bonding presented higher immobilization capacity (units of enzyme activity U per m2 of membrane) than adsorption	[95]
Adsorption	Synthetic fiber, polyurethane foam, stainless steel sponge, loofah sponge and cork	*P. expansum*	The best carriers were the synthetic fiber and the polyurethane foam. Repeated batch fermentation with high FOS yields were possible thanks to the enzyme activity that remained constant for 6 cycles	[97]
Entrapment	Calcium alginate gel	*β-fructofuranosidase A. japonicus*	Great mechanical strength of the support and immobilized *A. japonicus* mycelium achieved an increase in enzyme stability (pH and temperature). Sucrose diffusion did not affect the conversion yields of FOS	[103]
Entrapment	Potato dextrose agar	*A. japonicus*	The method enhanced the hydrolyzing activity of this enzyme but decreased the transfructosylating activity	[96]
Entrapment	Sodium alginate	*A. pullulans*	It was able to immobilize *A. Pullulans* culture, and intra and extra cellular enzyme were studied for FOS production. Intra cellular enzyme was allowed obtaining higher FOS yield	[104]
Entrapment	Dried alginate gel beads	Commercial *Fructosyltransferase A. aculeatus (Pectinex Ultra SP-L)*	The IE resulted stable during batch operation and when compared to calcium alginate gel beads, promoted higher volumetric activity and enhanced the space-time-yield of fixed-bed bioreactors for continuous operation.	[105-106]
Covalent binding	Shirasu porous glass	*β-fructofuranosidase Aureobasidium sp.*	Immobilization in a strong support allowed producing continuously FOS in a packed glass column. A selective production of 1-kestose was possible operating at fast flow rate of concentrated sucrose	[117]
Covalent binding	Oxriane-containing polymer	*β-fructofuranosidases A. niger* and *A. japonicus*	Both IE showed an increase in Michaelis Menten constants, but regarding pH and temperature stability and product composition, there were no differences between free and IE	[119]

Covalent binding	Polymethacrylate-based polymer (Sepabeads EC)	Commercial *fructosyltransferase A. aculeatus (Pectinex Ultra SP-L)* and *A. niger (Rapidase TF)*	Efficient immobilization of commercial enzymes can be achieved without adding external salt or buffer. The reaction course of FOS formation under batch operation was not affected by enzyme immobilization	[120]
Covalent binding	Porous acrylic beads with oxirane functionality (Eupergit C)	*Fructosyltransferase A. aculeatus* *Pectinex Ultra SP-L*	High immobilization efficiency and more stability for higher pH and temperatures were achieved. The enzyme retains its activity constant for 20 days	[121[
Covalent binding	Acrylic copolymers	*Fructosyltransferase A. pullulans*	IE behaves quite similar than free enzyme when analyzing the influence of pH and temperature. IE decreases considerably its activity when operating at sucrose concentration higher than 500 g/dm^3	[122]
Covalent binding	GA-activated chitosan	Partially purified commercial *β-fructosyltransferase (Viscozyme L)*	Immobilization enhanced the thermal stability comparing to free enzyme and retained its activity after 50 cycles of batch FOS synthesis. The combination of enzyme partial purification and immobilization allowed obtaining high FOS yield.	[70, 123]
1. Covalent binding 2. Adsorption	1. Polymethacrylate porous beads with epoxy functions 2. Epoxy resin	*A. terreus* expressed in *K. lactis*	Covalent immobilization was promoted because it maximized enzyme activity, stability and enhanced the yield of FOS	[124]
Covalent binding	Fe_3O_4 chitosan-magnetic nanoparticles	Commercial *A. aculeatus* (Pectinex Ultra SP-L)	Hydrolytic and transfructosylating activities and retention was 70 and 86 %, respectively, after 6 cycles of reuse. High thermostability was achieved obtaining a maximum FOS concentration of 101.56 g/L	[125]
Cross-linking	Commercial anion-exchange resins and polymethacrylate carriers	Commercial f*ructosyltransferase A. pullulans*	Six different commercial carriers (with different functional groups) were tested for the immobilization. The addition of the cross-linking agent produced a drop of the activity. The most appropriate based on specific activity and storage stability of the IE were immobilized through direct attachment and consisted on styrene with quaternary amine groups and polymethacrylate with epoxide groups.	[136]
Cross-linking	–	Mutant-type *A. pullulans* NAC8	CLEA reusability was 100% of residual activity after four catalysis cycles, for this reason authors promoted this method for industrial synthesis of FOS. The produced FOS had prebiotic properties comparable to those obtained from commercially obtained FOS.	[137]
Cross-linking	–	Inulosucrase (R483A-Lrlnu) of *L. reuteri* 121	When comparing CLEAs with soluble inulosucrase to produce IFOs, there was obtained a product composition with a lower degree of polymerization when using the immobilized form, obtaining a product with higher prebiotic effect	[138]

Adsorption

Adsorption of β-galactosidase with polyethyleneimine on cotton cloth has been applied by Albayrak and Yang (2002) [84]. The immobilization process consisted in a combination of adsorption (polyethyleneimine solution to cotton cloth and exposing it to the enzyme solution) and cross-linking (polyethyleneimine-enzyme aggregates with GA). The procedure performed in monolayer presented an enhancement of the immobilization yield when increasing the enzyme concentration up to a maximum loading value. In contrast, the maximum enzyme immobilization yield, in multilayer mode, was achieved when the polyethyleneimine to enzyme ratio was near 1/20–1/25. When testing GOS formation from lactose with the multi-layered polyethyleneimine-IE technique in a packed-bed reactor, high productivity was achieved and GOS formation kinetic was not affected compared with soluble enzyme.

However, Matella, Dolan, and Lee et al. (2006), who applied the same approach to immobilize β-galactosidase from *Aspergillus oryzae*, claimed that adsorption was not effective and inactivation occurred, but their enzyme contained significant amounts of dextrin (neutral charge), which could avoid the electrostatic interaction between the enzyme and the polyethyleneimine-cloth [85]. Therefore, when applying this type of configuration, special care must be paid in the presence of substances that can alter the electrochemical nature of the interaction between enzyme and the carrier.

Gaur et al. (2006) immobilized the enzyme through physical adsorption on celite, and compared with other immobilization methods, finding that adsorption was the method with less enzyme recovery (only 2 % of activity yield) [86].

Güleç (2013) immobilized β-galactosidase from *Kluyveromyces lactis*, comparing simple adsorption and covalent attachment onto cellulose acetate membrane surface [87]. For adsorption, authors used plain and oxygen-plasma modified membrane, applying radio frequency and low-pressure, finding that plasma activated surface membrane was able to immobilize higher amounts of the initial enzyme concentration since surface hydrophilicity increased with oxygen activation. The IE via adsorption caused higher lactose conversion than the soluble enzyme system but the initial GOS yields (30–34 %) of the IE was lower than that of the soluble enzyme (39 %), so for GOS synthesis IE demanded higher reaction time to reach the same yield than operating with soluble enzyme.

These references confirm that charge (isoelectric point) of the enzyme and hydrophobicity/hydrophilicity characters are crucial for the efficiency of adsorption as immobilization method. Additionally, according to our exhaustive search, more updated bibliography promoted this method in combination with entrapment [88] or covalent binding and cross-linking [89], both discussed in the next paragraphs. Also, Carević et al. 2018 applied this immobilization technology, but given that this work was focused on the study of different resins as supports, it will be discussed later, in Support materials and approaches for enzymatic production of GOS and FOS [90].

Immobilization of fructosyltransferase via ion exchange was early investigated using different supports. Hayashi et al. (1994) evaluated the immobilization of β-fructofuranosidase with diethylaminoethyl (DEAE) cellulose in a continuous reaction [91]. They found that IE was less susceptible to inhibition by metal ions and the temperature stability was comparable with that of FE. The long-term stability of IE enabled the continuous production of FOS. Yun, Kang, and Song (1995) and Yun and Song (1996, 1999) used a porous styrene-derived ion exchange resin and packed it into a glass column to reproduce an industrial system [92-94]. The combination of a high porous support and the column system allowed a high volumetric activity, more stability against pH changes, and long-term continuous operation, while product composition was very similar with FE and IE. Additionally, authors compared the same system design with IE and immobilized whole cells, claiming that IE reactor operated more efficiently regarding stability and FOS production.

Nishizawa, Nakajima, and Nabetani (2000) tested immobilization of β-fructofuranosidase on ceramic membranes operating in a forced-flow membrane reactor in order to compare physical adsorption and covalent bonding efficiency [95]. They used GA to activate membranes of different pore size, promoting the formation of covalent bonds with aldehyde groups at the surface of porous membrane. In a few seconds, the forced-flow membrane reactor achieved FOS yields similar to those obtained after few hours using batch systems, thanks to the high quantity of IE within the membrane.

Mussatto, Aguilar, et al. (2009) got a deeper insight on the mechanisms of FOS synthesis with IE in vegetal fiber, finding that immobilization allowed for the reduction of the time necessary for repeated batch fermentation from 42 to 14 days (including the microorganism growth and the fermentation process for seven batches) [96].

Nevertheless, the main disadvantage of this method was that the hydrolyzing activity of this enzyme increased along cycles, while the transfructosylating activity decreased. Later, this research group (Mussatto et al. 2012) found that among several carriers, synthetic fiber and the polyurethane foam were the best options to operate batch fermentation to obtain FOS [97].

Although adsorption is a simple method, enabling high enzyme loading, high immobilization yields are usually associated to low enzymatic activities. In general terms, the engineering of this method needs to contemplate a compromise between the enzyme concentrations, the amount of support, and the amount of actually active IE. From literature data, it can be concluded that the immobilization strategy for these two kinds of enzymes involves ionic interaction. In this sense, any additive or change in the nature of the enzyme (i.e., isoelectric point, hydrophilicity) can affect the effectiveness of the immobilization and the enzyme activity yield. According to recent findings about this method, it seems that the innovation focus is more on the type of support than on the immobilization strategy itself.

Entrapment

Ateş and Mehmetoğlu (1997) developed a method for immobilizing β-galactosidase in cobalt alginate beads *via* entrapment, and analyzed the utilization of IE in a plug flow reactor, where there was retention of 83 % of the relative activity and increased stability at high temperatures [98]. These results were much better than the β-galactosidase immobilization through entrapment as enzyme fibers composed of alginate and gelatin and hardened with GA, which preserved 56 % of activity of FE but also conferred more stability at higher pH and temperature. In both cases, the systems were specially designed for lactose hydrolysis [99].

Jovanovic-Malinovska et al. (2012), studied the synthesis of GOS with immobilized β-galactosidase in polyvinyl alcohol (PVA) lenses and in sol-gel carriers and compared it to the synthesis with the FE [100]. Authors found that polyvinyl alcohol immobilization was the most appropriate method. It retained 95 % of its initial activity after seven repeated uses and retained more of the initial activity after 3 months of storage than sol–gel-immobilized β-galactosidase. Also, polyvinyl alcohol-IE achieved higher

lactose conversion rates than sol–gel enzyme. IE was adapted to operate in a PBR to produce GOS from both lactose and whey.

Recently, Yu and O'Sullivan (2018) developed a method to produce GOS with immobilized whole cells of *Lactococcus lactis* containing high levels of a hyper-thermostable β-galactosidase from *Sulfolobus solfataricus* [101]. The approach involved as first step the degradation of the recombinant DNA with UV treatment and then immobilization, comparing two supports: chitosan and alginate beads. Although both supports were able to entrap whole cells, alginate beads swelled during prolonged exposure to high temperatures, so chitosan was the appropriate carrier to perform GOS synthesis.

Another interesting approach is the combination of entrapment and adsorption via ionic interaction. Souza, Garcia-Rojas, and Favaro-Trindade (2018), complexed β-galactosidase with different polysaccharides (sodium alginate or k-carrageenan), mixing them with the enzyme solutions, and varying the pH (from neutral to acid) [88]. Authors evaluated lactose hydrolysis at different pH values, until achieving a change in the three-dimensional conformation as result of the interaction of the amino groups of the enzyme with the sulfate and carboxyl groups of the polysaccharides. Although the complex was affected by pH variation, this alteration was observed in a lesser extent when alginate was used as the polymer (when compared to k-carrageenan). Low pH also reduced the enzyme denaturation rate. More recently, Souza et al. (2019) got an insight on the immobilization through complexation using alginate, stating that when the enzyme-complex was exposed to high temperatures for a long time, thermal stability was improved, compared to FE [102].

The latest innovations in β-galactosidase immobilization through entrapment indicate that using alginate as a carrier seems to be proper when lactose hydrolysis is the final objective but when immobilization is designed for GOS synthesis, carriers less common, like fibers or chitosan, are more adequate to implement the entrapment.

In early studies, the mycelium from *Aspergillus japonicus* was immobilized using calcium alginate gel, to improve enzyme (β-fructofuranosidase) stability in terms of pH and thermal changes [103]. When performing FOS synthesis, the obtained yields were very similar to those obtained with FE, and only 17 % of enzyme activity was lost over one month of continuous operation. Authors claimed that mass transfer was effective

thanks to a high ratio of transfructosylating to hydrolyzing activity. A similar approach was applied by Ganaie, Pathak, and Gupta (2011), by immobilizing whole cells of *Aureobasidium pullulans* with sodium alginate through extruded drops [104]. Authors evaluated separately extracellular and intracellular enzyme performance for FOS production. The last one showed a higher conversion yield (54 % wt/wt FOS) than extracellular mass (46 % wt/wt FOS).

Also using the entrapment method but with a commercial enzyme preparation, Fernandez-Arrojo et al. (2013) immobilized the enzyme in calcium alginate gel beads and then, dried the gel with the entrapped enzyme [105]. This strategy was successful, and IE resulted stable because it did not swell in the concentrated sucrose solution, thus avoiding enzyme leakage. The IE was tested for FOS synthesis using both batch and continuous fixed bed reactors at lab scale. The continuous operation promoted higher volumetric activity and enhanced the space-time-yield of fixed-bed bioreactors. FOS yield was stable for long term operation and the enzyme system could be stored at room temperature without microbial attack.

More recently, Zambelli et al. (2016) implemented this immobilization method for whole cells, as most of the cited works do, of *Cladosporium cladosporioides* and performed FOS' synthesis in a continuous flow reactor [106]. The strategy promoted a significant improvement of reactor productivity (1.7 times, compared to batch processes), being stable during 7 days of continuous FOS production without varying significantly the product composition.

In general terms, immobilization via entrapment is a good strategy to improve pH and temperature stability for both β-galactosidase and fructosyltransferase. In the particular case of fructosyltransferase, this immobilization method is most employed for immobilizing whole cells rather than isolated enzymes. The main disadvantage of this method is that in general, supports cannot be reusable when the enzyme activity runs out.

Covalent binding

In addition to adsorption methods, Gaur et al. (2006) also immobilized β-galactosidase by covalent coupling to chitosan previously activated with GA [86]. This method led to a high activity yield and the enzyme presented higher temperature stability

while GOS yield was comparable with that obtained with FE. A similar immobilization method using an analogous support was implemented by Klein et al. (2012, 2013), claiming that optimal pH was enhanced from 6.5 to a wider range between 6.5 and 8.0 [107, 108]. Although the optimal temperature was the same for both FE and IE, this latter immobilization method led to a higher enzyme activity in a wider range of temperatures. Authors also assayed the enzyme thermal stability under different lactose concentrations (50 g/ L and 400 g/L) indicating that a higher lactose concentration promotes retaining enzyme activity. These results encourage the production of GOS in a continuous PBR using immobilized β-galactosidase in chitosan macroparticles.

Chen et al. (2009) immobilized a thermostable β-galactosidase from Bacillus stearothermophilus using Tris(hydroxymethyl)phosphine (THP) and GA and chitosan as support, but the focus in this work was to enhance lactose hydrolysis in a PBR [109]. In line with this, Lima et al. (2013) investigated the same immobilization method but focusing on the selection of the best strain of *Kluyveromyces* promoting the highest hydrolytic activity [110]. The strategy increased thermal stability (compared to that of FE) and the enzyme could be reused for 10 cycles, retaining more than 70 % of its initial activity.

Again, with GA as a driver for the covalent attachment, Neri et al. (2011) immobilized β-galactosidase using polyaniline coated with magnetite as support [111]. Although highlighted that similar product composition was obtained and similar kinetic behavior was observed, compared to the FE, with the advantage of an easy way to remove the IE from the reaction mixture by a magnetic field, being reusable.

Using the same immobilization technique González-Catanño et al. (2017) employed polysiloxane-polyvinyl alcohol polymer activated with GA, which led to an immobilization yield of 99 %, with 78.5 % of enzyme activity recovery [112]. Authors studied experimentally the reaction mechanism to produce GOS and proposed a mathematical model estimating rate constants, considering a pseudo steady-state hypothesis for two concomitant reactions. The first one involved lactose hydrolysis forming glucose and galactose, the latter reacting with lactose to form trisaccharides, and with each other (glucose and galactose) to form transgalactosylated disaccharides. In the second one, the galactosyl-enzyme complex reacts with the obtained transgalactosylated disaccharides, and although trisaccharides are still being formed, they are simultaneously

hydrolyzed, leading to glucose release. Song et al. (2010) implemented this immobilization method, but using silica gel as the support, inducing a reaction between the protein amine and carboxyl groups and electrophilic moieties previously introduced onto the solid surface treating it with 3-APTES and GA [113]. As a strategy to protect the enzyme active sites, before immobilization β-galactosidase was previously treated with lactose solution. This produced a higher activity yield than non-pretreated enzyme, and a higher thermal stability, as many IE mentioned above. An interesting finding of this work is the fact that not only pH but also buffer molarity affected both FE's and IE's activity, showing that the IE works properly in a more basic medium and tolerates a wider range of buffer molarity. In a more recent work, Song et al. (2013) analyzed the continuous synthesis of lactulose from whey lactose finding that the inhibitory effect of galactose and glucose decreased with the immobilization in a PBR [114].

Bernal et al. (2013) immobilized β-galactosidase in a glyoxyl Sepharose support comparing both one-point and multi-point attachments [115]. Immobilization was performed at pH 10 to promote the inactivation of the enzyme by displacing cation, which allowed immobilizing in 20 min and retaining 82 % of the enzyme activity. Multi-point attachment enhanced thermal and pH stability, increased the rigidity of the three-dimensional structure, and made the enzyme complex less susceptible to inactivation in the presence of solvents (dioxane 30 %). Authors also optimized the degree of multi-point attachment, given that they observed that excessive multi-point linkage (longer incubation time during immobilization) caused a decrease in the enzyme stability which was explained by a modification or distortion of the structure. Güleç (2013) applied covalent binding to immobilize β-galactosidase onto cellulose acetate membranes, modifying membrane's surface with plasma polymerization of ethylene-diamine (EDA) and plasma polymerization of 2-mercaptoethanol in order to introduce –NH2 and –SH groups on the membranes [87]. Additionally, plasma polymerization of EDA-modified membrane was coated with layers of IE using polyethyleneimine. Although high enzyme loading (65–83 %) was achieved, both methods decreased enzyme activity (11–12 %) and GOS yield, probably due to negative effects on active amino groups. The most efficient strategy was to immobilize β-galactosidase onto thiolated membrane surfaces, created by plasma polymerization of 2-mercaptoethanol with high immobilization yield (70 %) and especially high enzyme activity (46 %).

Warmerdam et al. (2014) used well-known commercial porous acrylic beads (Eupergit C) with oxirane functionality to immobilize covalently β-galactosidase through reaction of its thiol and amino groups with the epoxide groups of the carrier [116]. Although after immobilization the enzyme experienced activity loss, it was stable for 90 days and its productivity during one run in the PBR was more than six-fold higher than the productivity of the FE during one run in a batch reactor.

Using chitosan as support, de Albuquerque et al. (2018) and Nguyen, Styevkó, et al. (2019) have recently immobilized covalently β-galactosidase [5, 21]. The former used GA to activate and to improve catalysts stability for lactulose synthesis using cheese whey and fructose as substrate. The methodology allowed the obtaining of 17.3 g/L of lactulose, and 86 % of lactose conversion, suggesting that the immobilization improve not only enzyme stability but also, its conformation and its kinetic properties. On the other hand, Nguyen, Styevkó, et al. (2019) used chitosan-coated magnetic Fe3O4 nanoparticles and assayed the immobilized catalyst to produce GOS from lactulose [21]. Also in this case enzyme stability was improved in terms of half-life and thermal and pH tolerance. A maximum GOS yield of 17 % mol/mol was obtained after 36 h of reaction using 2.34 M of initial lactulose concentration.

Regarding fructosyltransferase, Hayashi et al. (1991) [117] applied this method with an inorganic support (Shirasu porous glass), previously activated with an aqueous silanization process coupling a monolayer of silane onto the support surface activated with GA [118]. Enzyme activity was tested in a PBR using a fast flow rate of concentrated sucrose solution as substrate, leading to the production of short chain FOS.

Also with an inorganic support, Chiang et al. (1997) used oxirane containing methacrylamide-based polymeric beads to covalently immobilize β-fructofuranosidase from *A. niger* and *A. japonicas* [119]. Although both IE and FE presented their maximum activity at 60 °C, at lower temperatures IE retained a higher percentage of its maximum value than FE. Obtaining FOS with both IEs in a batch reaction presented a similar pattern to that of the FE's reaction.

A similar approach took Ghazi et al. (2005) immobilizing two commercial enzymes using two kinds of polymethacrylate-based polymers (Sepabeads EC) [120]. Authors assayed the influence of pH and ionic strength, finding that pH 5.5 favored the binding of the carboxylic heads of the aspartic and glutamic side chains whereas pH 9,

the binding of amino and thiol groups. Additionally, low ionic strength produced more protein bound to the support, which also increased with the porosity of the support. The pattern and the yield of FOS production under batch mode with IE, was similar to the production with FE and other immobilization methods, respectively. After 36 h of reaction they were able to obtain 61.5 % of FOS relative solid composition. In line with this, Tanriseven and Aslan (2005) immobilized the same commercial enzyme using also a commercial support (Eupergit C) [121]. The covalent attachment was possible through the amino, mercapto, or hydroxyl groups. The maximum efficiency (ratio of activity of IE to the activity of FE) was 96 %. In addition, immobilization enhanced the thermal stability of the enzyme comparing with FE. The production of FOS using FE and IE was very similar regarding product composition, and the latter retained its activity for 20 days performing batch reactions of 1 h at 60 °C.

Onderková, Bryjak, and Polakovič (2007) covalently immobilized fructosyltransferase from Aureobasidium pullulans using a commercial support composed of butyl acrylate copolymerized with ethylene glycol dimethacrylate [122]. As a first step, the amount of enzyme to be immobilized was optimized. At optimal conditions, the carrier preserved 98 % of its activity for one month. While immobilization poorly affected thermal stability (comparing with FE), pH stability was favored, shifting the optimum to the alkaline region. FOS production with FE and IE presented similar apparent initial rate, which increased while sucrose concentration increased. Authors pondered the mechanical resistance of the carrier which was able to perform 11 cycles of FOS synthesis in a stirred vessel with only 8 % of activity loss.

Lorenzoni et al. (2014), covalently immobilized partially purified β-fructofuranosidase, from a commercial enzyme preparation (Viscozyme L), on GA-activated chitosan particles [70]. The best biocatalyst activity was obtained with 120 mg/g of enzyme per dry support, achieving an immobilization yield (ratio between immobilization and initial activity) of 90 % and immobilization efficiency (ratio between observed and immobilized activity) of 33 %. Immobilization enhanced considerably the thermal stability of the biocatalyst comparing to FE at 60 °C, and retained its activity after 50 cycles of batch FOS synthesis. Lorenzoni et al. (2015) evaluated inverted sugar and FOS production using this biocatalyst under two PBR and two fluidized bed reactors (FBR), producing 98 and 94 % (grams of invert sugar per grams of initial sucrose), respectively, and 59 and 54 % (grams of FOS per grams of initial sucrose), in the PBR

and FBR, respectively [123]. In both modes of production, varying the flow rate was possible to modulate the product composition in terms of DP3 and DP4 concentrations.

Burghardt et al. 2019 presented a detailed study of neoFOS production (β-(2,6) glycosidic bonds FOS) using immobilized fructosyltransferase [124]. Authors compared covalently immobilized enzyme using polymethacrylate porous beads with epoxy functions and epoxy resin with ionic immobilization (adsorption) using anion exchange membranes. The former option was preferable because it maximized enzyme activity, stability and enhanced the yield of FOS. Ionic immobilization using membranes seemed to suffer enzyme desorption during the washing step after immobilization. de Oliveira et al. (2020) [125] immobilized fructosyltransferase with a similar approach than applied by Nguyen, Styevkó, et al. (2019) with β-galactosidase [21]. Immobilization carried out using Fe3O4-chitosan-magnetic nanoparticles as support and GA as enabler agent. The immobilized biocatalyst, showed both hydrolytic and transfructosylating activities and retained 70 and 86 % of them after 6 cycles of reuse. In addition, high thermostability was achieved obtaining a maximum FOS concentration of 101.56 g/L, with predominant presence of 1-kestose in the reaction mixture.

Covalently immobilization of fructosyltransferase and β-galactosidase is one of the most studied techniques for immobilization. During the last years, scientific results show that there are many commercial supports that can be applied for this purpose; nevertheless, most of the protocols of enzymatic covalent immobilization required the treatment with a reactive compound that acts as activator of the support surface, GA being the most widespread.

Cross-linking

Sungur and Akbulut (1994) used a gelatin carrier system and two cross-linking agents (GA and chromium (III) acetate) to immobilize a β-galactosidase [126]. Authors managed to use minimum amounts of crosslinkers, obtaining a stable and hardened gelatin and avoiding enzyme leakage. Activity yield was 25 % and 22 % for GA and chromium (III) acetate, respectively, and decreased only 9 % after 42 days of use. Furthermore, enzyme activity was less susceptible to pH variations during immobilization, accentuated with the cross-linker chromium (III) acetate. From literature

search, it seems evident that the enhancement of enzyme activity yield has been prioritized given that GA has been the principal cross-linking agent applied in most studies. In this line, Zhou and Dong (2001) immobilized β-galactosidase using graphite slab and GA as cross-linking agent and found that the average specific activity (ratio of the activity of IE to that of the FE) was between 17 and 25 % [127]. Immobilization increased K_m and decreased V_m Michaelis–Menten's constants. The IEs were stable and active in operational conditions for lactose hydrolysis.

The works of Albayrak and Yang (2002), and Matella, Dolan, and Lee et al. (2006) show the use of cross-linking as a final step for coating polyethyleneimine-enzyme aggregates to cotton cloth, with GA as cross-linking agent [84, 85]. Authors stated that this strategy promoted a strong permanently fix bond of enzyme aggregates, avoiding leaching out when exposed to acetate buffer. In addition, low temperatures favored higher enzyme activity. Multilayered polyethyleneimine IE was used in a plug-flow reactor achieving stable and continuous operation with an enhancement in GOS productivity comparing with others previously reported for this type of process configuration.

Gaur et al. (2006) studied a cross-linking method, without any support, to immobilize β-galactosidase along with adsorption and covalent bonding processes [86]. The strategy was to form enzyme aggregates by adding ammonium sulfate and GA under controlled conditions. The activity yield with this method was 13.5 % (lower than covalent bonding but higher than adsorption). Also, β-galactosidase immobilized through cross-linking aggregates (as well as covalent immobilization) was thermally more stable and its half-life was enhanced comparing to FE. The disadvantage of this method was that it promoted lactose hydrolysis over GOS synthesis.

These cited works were able to enhance GOS production with this type of immobilization, testing the synthesis under continuous production or repeated-batch operation. For this reason, it is not clear if the disadvantage of the strategy applied by Gaur et al. (2006) rises in the immobilization procedure or if it is a matter of reactor configuration [86].

In order to design an enzymatic membrane reactor to produce and purify GOS, Palai and Bhattacharya (2013); Palai, Singh, and Bhattacharya (2014) immobilized β-galactosidase in a polyvinylidene fluoride (PVDF) membrane using GA as cross-linking agent [128, 129]. The units of enzyme immobilized per mass of membrane increased with

enzyme concentration up to a maximum loading capacity. The formation of GOS increased with the initial lactose concentration. Authors developed a six-step-eleven-parameter model based on Michaelis–Menten kinetics, which was able to reproduce the experimental results. Furthermore, they improved the process' design by incorporating a recirculation loop that allowed producing GOS selectively and, again, mathematically modeled this process. The storage stability of the IE was studied, the enzyme retained 50 % of its initial activity after 30 days of storage at 20 °C.

Guerrero et al. (2015; 2018) analyzed diverse strategies to immobilize β-galactosidase by aggregation and cross-linking comparing performances in a repeated-batch operation with a single batch operation with FE, using lactulose as substrate [130, 131]. Immobilization was produced by precipitating β-galactosidase from *A. oryzae* with different concentrations of ammonium sulfate and testing the addition of different concentrations and times of exposure of GA. Authors found that increasing the ratio between GA and protein promoted an increase in specific activity and the immobilization yield up to a certain point, from which consumption of more cross-linking agent did not have any benefit. Thermal stability was not improved under these conditions of immobilization. Regarding lactulose synthesis, immobilization favored the generation of disaccharides over higher oligosaccharides. When comparing with single batch FE operation, immobilization allowed enzyme reuse, increasing lactulose production per unit of mass of biocatalyst. Furthermore, Guerrero et al. 2019 tested this catalyst in continuous packed-bed reactor to produce lactulose from fructose and lactose analyzing the effect of flow rate, substrates ratio and biocatalyst ratio [22]. Under optimal conditions, maximum lactulose yield was 0.6 g/g of total sugars, and lactose conversion was 28 %. Authors claimed that operation with recycle had no significant effect on yield.

Guerrero et al. (2017; 2018); Urrutia et al. (2013) also analyzed the use of monofunctional and heterofunctional glyoxyl-agarose supports, as previously reported by Mateo et al. (2010), which consisted on epoxide-agarose with different additional functional groups [131-134]. This approach involved first the adsorption of the enzyme to the support and then multi-point covalent attachment by means of the amino groups in the enzyme lysine residues and the aldehyde groups of the support. Authors determined the reaction kinetics and the product composition, obtaining a higher immobilization yield (39.4 %) with amino-glyoxyl-agarose support. When analyzing enzyme performance during lactulose synthesis, higher yields were obtained with monofunctional glyoxyl-

agarose. Moreover, they determined that glyoxyl-agarose and amino-glyoxyl-agarose derivatives retained the selectivity of the FE for lactulose synthesis while carboxy-glyoxyl-agarose and chelate-glyoxyl-agarose favored the synthesis of transgalactosylated oligosaccharides. The restrictions that immobilization produced on the enzyme activity had low effect on transgalactosylation because of the use of high substrate concentrations, concluding that immobilization had a more critical impact on the hydrolysis of lactose. Additionally, Urrutia et al. 2018, using the same experimental design, tested chitosan partially functionalized with aldehyde groups as support [89]. In this case, authors studied two cross-linking agents: GA and epichlorohydrin. The cumulative GOS yield after 10 batches using immobilized enzyme was 4.7 and 4.0 times higher, compared to that obtained with soluble enzyme.

Also, using GA as cross-linking reagent, Eskandarloo and Abbaspourrad (2018) immobilized β-galactosidase on the surface of glass beads, activated with 3-APTES [135]. Different enzyme concentrations were analyzed, founding what many other works reported previously: the amount of IE per mass of support increased when the concentration increased, up to the maximum capacity of the support. However, they showed that increasing enzyme concentration resulted in a considerable decrease in immobilization efficiency (unit of IE per unit of enzyme taken in the solution). Immobilization increased the optimal operational temperature and the highest enzymatic activity was achieved at higher temperatures than FE. Similar behavior was observed with higher pH, related to diffusional constraints, or to secondary interactions between the enzyme molecules and the supports. When studying GOS production in a PBR, it was observed that the GOS yield increased with repeated cycles of operation and demonstrated the high efficiency and reusability of its process configuration with this type of IE.

Recently, Sass and Jördening (2020) promoted an innovative strategy to immobilize β-galactosidase on electrospun gelatin nanofiber mats [11]. The findings involved the determination of optimal conditions for solvent system during electrospinning process and the subsequent cross-linking of gelatin nanofiber mats using hexamethylenediamine (HMDA) in order to increase their stability in water. GOS yield using this catalyst was 31 % higher than that obtained with FE (27.7 %).

Cross-linking of fructosyltransferase has also been evaluated, although not as extensively as β-galactosidase. Platková et al. (2006) studied six commercial anion-exchange resins and polymethacrylate carriers, both by direct attachment, or by the attachment accompanied by GA cross-linking [136]. Increasing operational pH caused a decrease in enzyme activity, probably due to the presence of hydroxyl groups. For all biocatalysts, the addition of the cross-linking agent produced a drop of the activity. The carriers that promoted higher enzyme activity were styrene with quaternary amine groups and polymethacrylate with epoxide groups. The lower enzyme activity in other supports was attributed to enzyme inactivation during the process and diffusional problems. Ademakinwa et al. (2018) prepared and evaluated cross-linked enzyme aggregates (CLEAs) of fructosyltransferase to produce FOS using GA as cross-linking agent [137]. Authors indicated that the best precipitant for CLEAs production was ammonium sulfate being able to maintain 100 % of residual activity over four rounds of catalysis. The secondary structure of CLEAs was determined from FTIR spectra, showing that cross-linking with GA promoted protein aggregation causing the transformation of helical and beta sheets structures into beta turns. When analyzing FOS synthesized with CLEAs, authors stated that they had prebiotic properties comparable to those obtained from commercial FOS. Although authors promoted this method for industrial FOS biocatalysts, no comparison between CLEAs and FE activities (i.e., product yield, enzyme stability) at different process conditions (i.e., pH, stirring, temperature) has been made. Charoenwongpaiboon et al. (2019) also made use of CLEAs [139]. In order to immobilize inulosucrase, they used a fructosyltransferase with higher transglycosylation activity than β-fructofuranosidase, and capable to synthesize both inulin and inulin-type fructo-oligosaccharides from sucrose. In this study the optimum conditions for CLEAs preparation were determined in terms of recovered activity and again ammonium sulfate was promoted as the best precipitant, together with 0.5 mM GA and pH in a range of 5–7. Under these optimum conditions, CLEAs retained 42 % of original inulosucrase activity. Comparing with FE, the optimum pH of inulosucrase changed from 5 to 4 after immobilization, while the optimum temperature was the same. Nevertheless, immobilization produced higher pH and thermal stability. There was found that the CLEAs promoted the synthesis of inulin-type FOS with the DP ranging from 3 to 8, while the soluble inulosucrase catalyzed the synthesis of inulin-type FOS with the DP up to 13. Authors concluded that CLEAs were useful to produce insulin-type FOS with higher

prebiotic effect than FE and also presented operational stability in the batch synthesis conditions.

In an overall view, cross-linking is a methodology derived from covalent binding, which is among the latest advances in enzyme immobilization. Although it seems to be one of the most delicate methods, given that it can modify the enzyme's configuration leading to inactivation, it presents the advantage of being highly specific. As it consists on the intermolecular linking of different enzyme moieties (primary amino groups, thiols, phenols, imidazoles) with the carrier, immobilization can be modulated to enhance the active sites of the enzyme and to increase the specificity for the substrates avoiding the inhibitors. Although this strategy is still under development, recent advances in nanotechnology will allow going further in the understanding of the intermolecular configurations and interactions, thus optimization of IE selectivity and effective activity yield could be improved.

Support Materials and Approaches for Enzymatic Production of GOS and FOS

The selection of an enzyme carrier also depends not only of the material's properties (e.g., surface area, particle size, pore structure, presence or absence of functional groups on its surface) but also on practical issues (e.g., cost, availability, stability, and the type of reactor). For instance, depending on the application, a specific material can successfully immobilize the catalyst but may not survive the industrial processing conditions. On the other hand, if a given material can resist the reactor's conditions but its affinity with the enzyme is insufficient, this can be overcome with the use of surface modifiers, changing the properties of the support [79]. Besides its simplicity, the main advantage of this method, is its ability to preserve the native structure and the activity of the enzyme. However, the weak interactions between the protein and the carrier result in leaching of enzyme from the support over time.

In terms of the properties, enzyme supports should grant the process some advantages over the use of the soluble enzyme. The most critical issue is the reduction in the overall cost of the industrial process and this can be achieved using a cost-effective support (not always an easy task), by increasing the reusability of the enzyme (enabling

the implementation of continuous processes), by facilitating the recovery of the catalyst and the purification of the final product, and by increasing enzyme stability and durability, while performing the transformation(s) and during storage and transport [83].

The support should also have thermal, mechanical and physical endurance to withstand the (sometimes) harsh conditions of the industrial process. It ought to grant the enzyme with increased specificity toward the substrate, reduce catalyst inhibition and be inert. It should also present easy regenerability, avoid contamination, namely by bacteria, and be eco-friendly (biocompatible and biodegradable). However, the selection of the support and its properties are closely related with the type of immobilization procedure chosen for the enzyme. Hence, physical and chemical properties (e.g., hydrophilicity/hydrophobicity, pore size, presence of surface functional groups, or resistance to certain pH or temperature) must be also considered taking into account the application foreseen [2, 13, 14, 75].

Selection of the Support Material: a Key Factor for Enzyme Immobilization

Taking into consideration the different methods for enzyme immobilization to produce GOS and FOS, a suitable support material should be chosen. The selection of the material depends not only on the immobilization method and the type of the enzyme, but also on the conditions of the catalytic process and the enzyme-support interactions that may occur [83, 139]. These interactions may interfere with the properties of the whole biocatalytic system, so special care must be taken in order to enhance the enzyme specificity [75, 140]. Besides, supports should preferentially be low cost and eco-friendly as well as inert, in order to not interfere and not increase the costs of the overall enzymatic operation. Supports that have high stability, thermal and mechanical resistance, a high rate of regeneration and reusability are preferred [14, 141]. Additionally, the reusability of biomacromolecules such as carbohydrates and proteins-based biopolymers is a promising strategy to obtain biopolymeric nanoparticles that are antibacterial, biocompatible, immunogenicity, and biodegradable [142]. It is important to remark that the support should act as a barrier that preserves the enzyme structure against extreme process conditions (pH, temperature, mechanical damage) avoiding denaturation and inactivation. Furthermore, the chosen material should provide an efficient establishment of the enzyme-support complex and there should be a good affinity between the functional

groups of the enzyme and the support, so an effective binding can occur. Given that an ideal and universal support would not be feasible to obtain, the choice of the most appropriate material will involve analyzing the pros and cons of its properties and usability [14, 75].

There is a variety of materials that can and have been used as support to enzyme immobilization. Based on their chemical composition, these supporting matrices can be divided in two main categories: inorganic and organic. The latter can then be subdivided into natural and synthetic organic supports [143]. Silica and other oxides, such as aluminum, titanium or zirconium oxides, are the most commonly inorganic supports, as well as hydroxyapatite, activated carbon, glass and ceramic, as described in Table 3. Usually, inorganic matrices are preferred for their lower reactivity, thermal and mechanical resistance, high stability, rigidity and porosity. Some of them can ensure a fixed volume and shape attributable to the invariance of their pore diameters [14, 144]. Since most of these materials are not chemically reactive and the functional groups are mainly hydroxyl groups, a previous treatment to modify and activate the matrix is required. The matrix modification generally occurs prior to the activation and it consists on the addition of amino groups, through a treatment with aminoalkyl triethoxysilanes. For the matrix activation, different methods make use of dialdehydes, such as cross-linking agents, being GA the most common one [14, 143].

Several reported works merit the use of organic material, since these can be chemically modified and also surpass the limitations of inorganic materials such as reduced biocompatibility, low affinity to biomolecules and the inorganic supports inadaptability to be reshaped and to be used with different methods of immobilization [79, 141]. The main disadvantage of organic matrices is the low chemical and mechanical resistance, which impair their usage in systems with aggressive thermal and pH conditions leading to the impossibility of regeneration of the matrix. Among the most used organic materials there are reports of the usage of a broad variety of polymers, natural and synthetic [79]. Natural polymers, such as carbohydrate species can form inert and strong aqueous gels (hydrogels) such as alginate, chitosan, starch, cellulose and carrageenan. These mentioned carbohydrates can be easily obtained, with low associated costs, since most of them are by-products of different industries [76, 141]. As for the most common synthetic polymers described, there are reports of the application of polyvinyl alcohol, polyvinyl chloride, polyurethane, polyaniline, diethylaminoethyl cellulose

(DEAE-cellulose), Eupergit and activated nylon [14, 140, 143]. These synthetic polymers are relatively easy to produce and can be used in different methods of immobilization, where they can be modified to satisfy the desired specific requirements of the enzymes and the reactional conditions of a specific enzymatic process, without interfering with other properties such as thermal and chemical resistance [79, 140].

In order to obtain some products through cascade reactions, some innovative methods, like co-immobilization processes, with multienzyme systems, have gained special attention as well as the materials chosen for such reactions [75, 145].

In summary, there is wide range of possibilities regarding the type of support, as shown in Table 3. Nevertheless, particularly for β-galactosidase and fructosyltransferase, the most assayed supports include chitosan, alginate-based and sol-gel carriers, resins with different functional groups, acrylic or glass porous beads and membranes (cellulose, polyvinylidene fluoride and ceramic).

Table 3 – Principal materials and applications implemented as supports for enzyme immobilization

Type of material	Immobilizing matrix	Enzymes	Immobilization Method	Reference
Inorganic	Silica	Penicillin acylase Lipase Trypsin	Covalent cross-linking with GA Cross-linking with GA Adsorption	[173] [174] [175]
	Glass	Pronase (Protease mixture) β-fructofuranosidase Lipase	Covalent binding Covalent binding Adsorption	[176] [117] [177]
	Celite	α-chymotrypsin β-galactosidase Lipase	- Covalent binding Adsorption	[178] [161] [179]
	Activated charcoal	Amyloglucosidase Papain Protease	Adsorption Adsorption Adsorption	[180] [181] [182]
	Alumina	Invertase Trypsin Penicillin G acylase	Adsorption Adsorption Adsorption	[183] [184] [185]
	Hydroxyapatite	Urease Levansucrase Protease	Adsorption Ionic binding Adsorption	[186] [187] [188]
Biopolymers	Alginates	Glucose oxidase β-galactosidase β-glucosidase	Encapsulation Covalent binding Entrapment	[189] [190] [191]
	Chitosan	Laccase Inulinase Lipase	Adsorption cross-linked GA Covalent binding Adsorption	[192] [193] [179]
	Cellulose	Peroxidase	Covalent binding	[194]

		Lipase Glucose oxidase	Covalent binding Entrapment	[195] [196]
	Agarose	α-chymotrypsin α-amylase β-galactosidase	Covalent binding Entrapment -	[197] [198] [199]
	Gelatin	Urease Tyrosinase α-amylase	Cross-linking with GA Entrapment Cross-linking with GA	[200] [201] [202]
Synthetic Polymers	Polyacrylamide	Invertase Alkaline phosphatase Tyrosinase	Entrapment Entrapment Entrapment	[203] [204] [201]
	PVA	Laccase α-amylase Alcohol dehydrogenase	Cross-linking with GA Entrapment Adsorption/ Covalent binding	[205] [206] [207]
	Polyurethane	Lipase Inulinase Papain	Covalent binding - Covalent binding	[208] [209] [210]
	PEG	Cellulase α-chymotrypsin	Covalent binding Cross-linking with cystamine	[211] [212]
	PVDF	β-galactosidase Lipase Tyrosinase	Covalent binding - Covalent binding	[129] [213] [152]
Resins	DEAE-cellulose	Invertase Epoxide hydrolase Nuclease p1	Adsorption Adsorption -	[203] [214] [215]
	Duolite A-568	β-galactosidase Cellobiose 2-epimerase Invertase	Adsorption Adsorption Adsorption	[164] [216] [217]
	Dowex®	Invertase Phospholipase D	Adsorption Adsorption	[218] [219]
	Sephadex G-25	Glutaminase Lipase	Adsorption Adsorption	[220] [179]
Nanomaterials	Chitosan-coated magnetic nanoparticles	β-galactosidase β-fructofuranosidase	Covalent binding -	[221] [170]
	Silica nanoparticles	Glutamate dehydrogenase/ Lactate dehydrogenase Horseradish peroxidase Endo-inulinase	Covalent binding Entrapment Adsorption/covalent binding/cross-linking with GA	[222] [223] [224]
	Hydroxyapatite nanoparticles	β-glucosidase	Adsorption	[225]
	Polyurethane-gold and polyurethane-silver nanoparticles	Maltogenase	Adsorption	[226]
	Electrospun cellulose nanofiber membrane	Lipase	Covalent binding	[227]
	Electrospun PVA fibers	Lipase	Entrapment	[228]
	Electrospun polyethersulfane nanofibers	α-amylase	Covalent binding (through EDC)	[229]

Nanotechnology and Electrospinning as New Approaches to Produce Support Materials for Enzyme Immobilization

Due to an increased popularity over the years for industrial applications and many other areas such as medicine and pharmaceuticals, agriculture and even biodiesel production, the search for new materials and immobilization in micro and nano-scales allowed the continuous development of different enzyme supports and immobilization methods.

Advances in the nanotechnology field allowed for immobilizing enzymes using different nanostructured forms, such as nanofibers, nanotubes, nanoparticles, nanoporous, nanosheets and nanocomposites. These materials provide large surface area to volume ratio which improves enzyme loading, leading to a more efficient immobilization and stabilization of the enzymes facilitating reaction kinetics [146]. They also have the ability to control particle and pore size, tailoring the thickness of nanofibers and nanotubes. Additionally, the need for the use of surfactants and toxic reagents, such as cross-linking agents, is reduced and in some cases specific particles with conductive or magnetic properties can be used in order to control the immobilized system [75, 76, 141, 147]. Nanoscale particles, in general, can be designed and redesigned according to the required necessities of the enzyme system [148]. This way, enzymes have been successfully immobilized into many nanoparticles and nanomaterials, like those described in Table 3, with positive results verified toward the improvement of enzymatic performance. Different nanomaterials have been used as supports, such as polymers, silica, graphene, gold and magnetic particles [148, 149].

Immobilization in nanomaterials can be adapted accordingly to desired conditions, however it still depends on factors, such as the type of enzyme, the support itself and the immobilization conditions, which will condition aspects such as immobilization yield and specific activity.

Electrospinning is one of the simplest techniques used to produce nanofibers characterized by their exceptional length, possibility to have a diversified composition and the uniformity of the fiber's diameters. Electrospun nanofibers have been appointed as immobilization supports with a great potential to overcome the problems presented by the other materials. The obtained fibers generally have high porosity and interconnectivity

that allow the system to benefit from low diffusion resistance leading to an efficient mass transfer process, high reaction rate and conversion.

The surfaces of the fibers can be modified in order to benefit the specific enzyme activity, loading onto the fiber a huge quantity of enzyme [147, 150]. Spun membranes can also be produced and used as filters, allowing the enzyme-membrane system to act simultaneously as a biocatalyst and a separation material having huge interests for the enzymatic membrane-bioreactors field [147].

The chosen polymer to spin should not only be able to form fibers or membranes by electrospinning, but should also be able to interact with the enzyme. The selection of materials for electrospinning comes with a specific range of solvents associated that should not interfere with the activity or conformation of the enzyme [151].

Polyvinylidene fluoride is a common electrospinning material, inert due to the absence of reactive groups, but different procedures have been developed toward modifications of the surface to make it more reactive for biomolecules immobilization (e.g., enzymes) [152].

A good immobilization material, depends not only on the enzyme but also the method of immobilization and the processing conditions. The same material does not behave equally with different enzymes, due to differences in the binding, leakage, matrix effect, and diffusional barriers, among others [153].

Main Supports for β-galactosidase and Fructosyltransferase Immobilization

β-galactosidase can be immobilized in different types of materials. Bearing in mind that β-galactosidases have both hydrolytic and transgalactosylase activities, the conditions for these two types of reactions are different and thus, different materials should be considered depend on the exact goal activity: transgalactosylation reactions require higher substrate concentrations, higher temperatures and lower water activity than hydrolysis. Hence, such specific characteristics must be considered when selecting the most appropriate immobilization support [154].

β-galactosidase immobilization has been thoroughly studied with different reports since the early 1970's. Woychik and Wondolowski (1972, 1973) have studied the

immobilization of this enzyme in porous glass beads with and without GA as cross-linker [155, 156]. The 1972 report followed a method previously described by Weetall (1969) (used for trypsin and papain), which consisted in the covalent attachment of the enzyme into a porous glass through a diazotization process (diazo-linkage) [157]. About 75 % of the enzymatic activity was retained using such method and it did not affect any of the enzyme's properties, such as optimum pH and temperature. The method used by Woychik and Wondolowski (1973) also enabled the retaining of 75 % of the enzyme's initial activity and had a better activity at lower pH (80 % of the optimum activity at pH 4.5) [156]. Moreover, the immobilized system allowed for a greater efficiency of lactose hydrolysis in column when compared with the stirred batch reactors (Woychik and Wondolowski 1972, 1973) [155, 156]. Some of the inorganic materials used as supports include silica, glass, activated charcoal, celite and alumina (aluminium oxide) [154, 158].

Finocchiaro, Richardson, and Olson (1980) described a method of β-galactosidase adsorbed into alumina previously activated with tolylene-2,4-diisocyanate [159]. This method led to a minimal enzyme leakage, an increment of 16-fold the catalytic activity when compared to untreated alumina, a broader pH profile, and a slightly decrease of the optimum temperature.

Following this chronological main contributions for β-galactosidase immobilization supports, Verma et al. (2012) promoted the use of silicon dioxide nanoparticles activated with GA [160]. This methodology involved multipoint covalent attachment, which improved the enzyme thermal stability. Additionally, when performing lactose hydrolysis, the enzyme complex retained more than 50 % of the enzyme activity up to the eleventh cycle.

In 2017, Fai and his team obtained GOS through a fixed-bed reactor with enzyme covalently bound to celite [161]. When compared to the FE, the optimum pH slightly decreased and the optimum temperature was 10 °C higher when celite was used as a carrier. Moreover, the immobilized system had higher storage stability, maintaining its functionality for 270 days when kept at 4 °C, and when used repeatedly for 10 times.

Eskandarloo and Abbaspourrad (2018) developed a covalent immobilization into modified glass by cross-linking with 3-aminopropyl triethoxysilane (3-APTES) [135]. The obtained enzymatic system revealed increased pH and temperature stabilities, an increased reusability of the enzyme for packed-bed reactions and allowed for its usage in

cycle reactions with the lactose conversion for GOS formation increasing with multiple cycles.

Natural and synthetic polymers such as chitosan, alginate, gelatin, agarose (some of them in the form of Sepharose), polyvinyl alcohol, polyethyleneimine, polyester, polyacrylamide, and some resins, such as DEAE-cellulose and Duolite have also been studied as supporting material for the immobilization of β-galactosidase [80, 162].

Li et al. (2008) reported the production of GOS with β-galactosidase immobilized in calcium alginate [163]. The resulting beads had a wide pH range (from 3.6 to 8.2) with yields around 23 % and optimum temperature at 55 °C. The enzyme immobilized in these beads could be reused up to seven times without any prominent reduction of GOS production.

A GA-activated chitosan support system increased the enzyme operational stability alongside its pH range and thermal stability [108]. The immobilization system obtained was used in a PBR operating for both lactose hydrolysis and GOS production in a stable operation for 15 days.

Botelho-Cunha et al. (2010) assayed adsorption into a commercial porous anion exchange resin (Duolite A-568) and reported no increase in enzyme activity due to diffusional problems [164].

Carevi´c et al. (2018) adsorbed β-galactosidase from *L. acidophilus* via ionic interaction, comparing different resins with different functional groups, focused on lactose hydrolysis application [90]. Carriers with epoxy groups showed the highest yields, but not the highest activities. Hence, there is a compromise between the amount of enzyme immobilized and the amount of IE actually active, mainly because of the probability of unfavorable conformation of the active site during the immobilization process. Carriers with amino groups leverage the activity yield; carriers with larger pore sites also promote higher enzyme activity since the free space facilitates enzyme mobility and substrate diffusion through the active sites.

Jovanovic-Malinovska et al. (2012) worked with a synthetic polymer, polyvinyl alcohol, and observed that, with the enzyme entrapped into to this polymer, 95 % of the initial activity was retained decreasing to 49 % after 3 months [100]. Polyvinyl alcohol had an immobilization efficiency of 88.5 % and a reusability rate close to 100 % even

after 7 cycles of reuse. Furthermore, the authors verified a higher lactose conversion for GOS production with polyvinyl alcohol in batch system (31 % maximum GOS production) when compared with polyvinyl alcohol in packed bed column (23–30 %).

Palai, Singh, and Bhattacharya (2014) immobilized β-galactosidase onto compacted microporous polyvinylidene fluoride membranes via cross-linking with GA [129]. Increasing the initial substrate concentration led to an increased selectivity for GOS formation, despite a GOS yield reduction. Polyvinylidene fluoride membranes were used in a batch mode feed recirculation system, resulting in a maximum yield of 30 % for GOS formation with initial lactose concentration of 50 g/L. This immobilization system allowed for a larger storage time without a significant loss of the enzymatic activity (approximated 50 % after 30 days), when compared with the loss of the corresponding native enzyme (100 % activity loss after 21 days).

Regarding the group of fructosyltransferases used for the synthesis of FOS (β-fructofuranosidase or invertase), the first report of immobilization strategy was described in 1916, and was attempted by adsorption of invertase onto charcoal and aluminum hydroxide [165].

β-fructofuranosidase was immobilized in Shirasu porous glass via adsorption and used for continuous production of FOS in a packed column [117]. The Shirasu porous glass was modified by silanization and activation with GA. When used in a batch system, the IE catalyzed the production of a wider range of FOS, in contrast with the continuous system, in which the fast flow rates of sucrose as substrate just led to the production of 1-kestose.

Nishizawa, Nakajima, and Nabetani (2000) studied the immobilization activity in ceramic membranes with different pore sizes and two immobilization methods: adsorption and covalent binding [95]. For physical adsorption, the membranes did not suffer any alterations. For covalent binding, the membranes suffered a pretreatment and activation with GA, and the immobilization occurred chemically in the inner-most surface of the membranes. The authors reported the possibility of long-term operations in a sustainable and viable manner, with the system having a half-life of IE of 35 days, due to enzyme denaturation, since no leakage was verified during the process. Immobilization ratios were higher in covalently IEs, with a maximum of 64 %, while for adsorption only

6 % immobilization ratio was obtained. Covalent bonded membranes represent a better option to apply a forced flow of substrate for FOS production.

Mussatto, Rodrigues, et al. (2009) studied different porous carriers (polyurethane foam, stainless steel sponge, vegetal fiber, pumice stones, zeolite molecular sieves and foam glass) to immobilize cells of *Aspergillus japonicus* and found that vegetal fibers were the best materials for this purpose [166]. FOS production with these immobilized systems was similar to the one obtained with free cells. On the contrary, porous glass was not suitable for FOS production, mainly because of its instability during agitation, and pumice stones and zeolites did not immobilize large amounts of cells.

Despite that inorganic materials are used for this class of enzymes, most works dealing with their immobilization fall upon organic supports, such as alginates, chitosan, resins and polymers.

Alginates have been those most largely reported. Cheng et al. (1996) immobilized β-fructofuranosidase in calcium alginate beads, via entrapment, and verified an increment in mechanical strength and enzymatic stability, with a wider resistance to lower pH and greater resistance to higher temperatures [103].

Tanriseven and Doğan (2001) encapsulated the invertase in calcium alginate capsules treated with GA [167]. Despite that no alterations regarding the optimum pH and temperatures were noted, a higher stability at higher pH and temperatures, together with a long-term were reported.

Fernandez-Arrojo et al. (2013) and Zambelli et al. (2016) confirmed this stability in batch operation with the enzyme entrapped in dried alginate beads [105, 106]. Even though different research groups make different approaches in their alginate preparations, either with sodium or calcium, in capsules or beads, alginates confer an increase in mechanical strength and stability to the enzymatic system.

In a comparative study between immobilization with alginate and chitosan, both activated with GA, the optimum pH and temperature were slightly lower for alginate but not significant, with both immobilized systems showing pH stability in a wider range (4–7) than FE and relative activities between 80 % and 100 % [168]. After 50 cycles of use, both immobilized systems maintained more than 80 % its activity. Similar results were obtained by Lorenzoni et al. (2014, 2015) with chitosan particles activated with GA, that

lead to not only higher thermal stability but also a retention of high enzymatic activity after 50 cycles of use in a batch production of FOS [70, 123]. For immobilization in chitosan, Nam et al. (2017) proposed that the occurrence of optimum pH and temperature shifts, when compared with the FE may occur due to alterations of the physical and chemical properties of the enzyme during the immobilization process [169].

Other materials with gelation capacity are agar/agarose, gelatin and some anion-exchange resin, such DEAE-cellulose, referring an improvement in pH stability, a reduction of susceptibility to metal ions for the enzyme alongside to a use of the said system in a long-term continuous operation with a high rate of FOS production [91].

Chen, Sheu, and Duan (2014) studied the immobilization of β-fructofuranosidase on chitosan-coated magnetic Fe3O4 nanoparticles to produce FOS with sucrose as substrate [170]. The enzyme was immobilized on the surface of the nanoparticles without the addition of cross-linking agents. Both immobilized and FE showed maximum activity at the same pH (5.5) and optimum temperature (60 °C), as well as similar FOS yields, both around 50 %. However, the immobilized system showed higher activities at wider range of temperatures and pH than the FE, and retained 55 % of the initial activity after FOS production in 10 batches. The nanoparticles can be easily recovered from the obtained FOS solution through application of a magnetic field.

Ganaie, Pathak, and Gupta (2011), and Mussatto et al. (2012) immobilized whole cells of Penicillium expansum to evaluate the production of FOS and β-fructofuranosidase at lab scale [97, 104]. Cells were immobilized by natural adsorption through their direct contact with the different carriers studied (synthetic fiber, polyurethane foam, stainless steel sponge-inorganic materials, loofah sponge and cork-lignocellulosic materials) at the beginning of fermentation. The best carriers were the synthetic fiber and the polyurethane foam, based on their immobilization yield and the enzyme activity. When analyzing repeated batch operations, FOS yields of 87, 72, and 44 %, in the 3 initial cycles (60 h) were obtained and the enzyme activity remained constant during 6 cycles (96 h). A similar approach was taken by Castro et al. (2017), who tested 16 different carriers including synthetic, agro-industrial and mineral materials for immobilization of Aureobasidium pullulans cells [171]. They suggested that the best carriers to enhance the production of FOS were those with high porosity and water absorption capacity, and low critical humidity point. Reticulated polyurethane foam was

one with the highest immobilization yield (over 75 % w/w of the total cells were immobilized) and achieved a high FOS yield compared to free cells.

Finally, applying electrospining methodology, Gabrielczyk et al. (2018) encapsulated fructosyltransferase by emulsion, suspension, and coaxial electrospinning [172]. Additionally, they compared the electrospun fiber enzyme load performance with a commercial epoxyactivated resin support (covalent immobilization). Analyzing the hydrophilic properties, they found that bioactivity of electrospun support in aqueous medium increased in order of the matrix hydrophilicity. Moreover, enzyme loading and specific enzyme activity was higher in fibers than in the resins. From the three electrospinning methods, coaxial fibers showed the higher specific activity. Operational stability of fiber supports was examined in a plug-flow reactor being the core-shell immobilizates more efficient than one-dimensional fibers both in batch and continuous reaction.

Conclusions

Industrial chemical reactions involving enzymes as biocatalysts often occur under extreme conditions, in terms of temperature, pH and presence of salts, surfactants, and organic solvents, thus affecting enzyme stability. Enzyme immobilization had emerged as a suitable methodology that not only improves enzyme stability, but also guarantees reusability of the catalyst, simplifying its removal from the reaction medium. As evidenced from the discussion provided by this review, enzyme immobilization involves interdisciplinary knowledge including not only enzymology but also nanotechnology, molecular dynamics, cellular physiology and process design. Particularly, for industrial syntheses of GOS and FOS enzyme cost is one of the most critical issues. For this reason, enzyme immobilization deserves special consideration in their design process. This review was focused on different immobilization strategies and support materials to enhance the activity and re-usability of fructosyltransferases and β-galactosidases.

The examples provided, as well as the discussion of their main findings and methods' effectiveness lead to accurate benchmarks. In this line, the type of enzyme, its origin, its purity, together with the type of immobilization method selected and the support will affect the performance during the enzymatic synthesis. There is another

factor that comes into play: process design. The same enzyme, immobilized under the same method with the same support, may not have the same yield when operating at batch or continuous process, under (or not) stirring or forced flow. For this reason, the best method will be the one that better adapts to the process design specifications of each case of study.

Despite this general marks, from the consulted bibliography it was shown that the latest advances in *β*-galactosidase and fructosyltransferase immobilization involve developing efficient material supports taking into account enzyme-support interactions, in this sense, resources from nanotechnology and electrospinning field are the most promising ones to achieve this goal. Nanostructured supports offer the main advantage of increasing surface area, thus the enzyme loading, while electrospinning offers the versatility of a simple method to obtain submicron-sized fibers, thus improving mass transfer limitations. Additionally, the implementation of combined immobilization methods, most of them including cross-linking seems to be the most appropriate to obtain an immobilized catalyst that can be adapted to the a variety of process conditions. The increasing availability of technology facilities has opened a large variety of possibilities to define smart strategies to optimize the activity and re-usability of these enzymes. This indicates that there is still a large gap with great place for innovative developments.

Authors' contributions

M.M.U., G.N.M., P.F.P. and O.F. did the literature search and wrote the first draft P.C.C. and A.G.-Z. proposed the structure and revised the manuscript.

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Disclosure statement

The authors declare that they have no competing interests.

Abbreviations

3-APTES, (3-aminopropyl)triethoxysilane. *CLEA*, cross-linked enzyme aggregate. *DP*, degree of polymerization. *EDC*, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride. *FBR*, fluidized bed reactor; *FE*, free enzyme. *FOS*, fructo-oligosaccharides. *GA*, glutaraldehyde. *GH-A superfamily*, glucosyl hydrolases family (CAZy classification). *GOS*, galacto-oligosaccharides. *IE*, immobilized enzyme. *PBR*, packed-bed reactor

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iii. Lactic Acid Bacteria in the Production of Traditional Fermented Foods and Beverages of Latin America

fermentation

MDPI

Review

Lactic Acid Bacteria in the Production of Traditional Fermented Foods and Beverages of Latin America

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Abstract

Traditional fermented foods are inherent to the human diet and represent an important part of the culture of each country. The fermentation process has been traditionally used as a method of food preservation. It allows modifying the technological, sensory, and nutritional attributes of raw ingredients. Latin America has a vast history with these products, but they are not always known worldwide. One of the most used microorganisms in fermented foods is lactic acid bacteria. This review aims to provide insight into the main attributes, benefits, and nutritional characteristics of traditional fermented foods and beverages from Latin America made with lactic acid bacteria. A bibliography analysis of the general aspects of fermented products from this region was carried out, focusing on the foods and beverages (with and without alcohol), their representation in native communities, nutritional value and effect on health, as well as the risk of their consumption. It is concluded that traditional fermented products of Latin America are usually prepared with specific ingredients of the region (such as cassava and corn), and that the lactic acid bacteria present in these foods are not always identifiable due to the inherent variability of artisanal production. The bacteria observed include *Lacticaseibacillus*, *Lactiplantibacillus*, *Lactobacillus*, *Limosilactobacillus*, *Leuconostoc*, *Streptococcus*, and *Weisella*, among others.

Keywords

Fermentation, bacteria, health, nutritional characteristics, food industry, native foods, lactic acid

Generalities of Fermented Foods and Beverages

Fermentation is a simple, low-cost, and versatile process that can be used to transform many food products, both solid and liquid. Fermented foods can be considered as "foods made through desired microbial growth and enzymatic conversions of food components" [1]. The fermentation activity of those microorganisms, including bacteria, fungi, and yeast, leads to several changes in sensory and technological characteristics of foods. The fermentation process is also useful to obtain diverse health and nutritional benefits. However, this process has been primarily used for preserving food by preventing the proliferation of undesirable microorganisms and by the inhibitory effect of the organic acids produced in the process [2].

The elaboration of fermented foods and beverages is inherent to the human diet, and each region of the world has its specific products. According to some authors, records of food fermentation date back more than 6000 years [3]. Fermented foods and beverages have been consumed for a long time by different civilizations and by various social classes [4]. Some well-known examples are beer, wine, and bread production, of which there are ancient archaeological records. Motivation to consume these products varies within communities; for example, native populations tend to choose fermented foods and beverages due to medical, dietary, or spiritual motives [5], whereas residents of industrialized areas consume them due to nutritional benefits and not for religious reasons. Some people follow specific trends, such as the "slow food" movement and the tendency to consume foods perceived as "more natural" [6,7]. In these cases, fermentation is attractive, since it is a slow process and appears to be a "natural" method in the eyes of consumers [8]. The sensory aspects of fermented foods and beverages can be attractive to other customers.

A broad classification divides the fermentation process according to the participating microorganisms as bacterial or yeast and mold fermentation. The first can be further divided into alkaline or lactic acid fermentation. Lactic acid bacteria (LAB) are responsible for lactic acid fermentation and can be classified into homolactic and heterolactic groups, according to the pathway used to ferment the sugars. Homolactic bacteria operate through glycolysis, using glucose as a carbon source, and produce only lactic acid. The heterolactic group decomposes glucose not only in lactic acid, but also in significant amounts of ethanol, acetate, propionate, and carbon dioxide, using other pathways besides glycolysis [9].

Morphological and physiological characterization of LAB describe them as gram-positive, acid tolerant, meso-aerophillic, non-spore forming, catalase negative, and morphologically either rod-shaped (bacilli) or spherical (cocci) bacteria that produce lactic acid as the major end product of carbohydrate fermentation [10].

LAB are one of the most widely used microorganisms for fermented foods. Because of their capacity to produce lactic acid and other compounds, they contribute to extending the shelf life of products, improving the nutritional profile of ingredients, and modifying certain physicochemical characteristics of the food (including a decrease in pH). They are considered GRAS (generally recognized as safe) substances. The US Food and Drug Administration (FDA) classifies food ingredients according to the GRAS label, which identifies those substances that are "generally recognized, among qualified experts, as having been adequately demonstrated to be safe under the conditions of its intended use" [11]. One of the best-known applications of LAB is the production of fermented dairy foods. However, LAB can grow correctly in different food matrices including fruits, vegetables, and grains [12–14], and a wide variety of strains have been isolated from various indigenous fermented foods and beverages [15].

All regions of the world have traditional fermented foods and beverages that are part of their culture. Asian countries have a vast tradition in this subject, possessing in their culture a large number of different foods and typical drinks, some of them well-known worldwide (e.g., kimchi, kombucha, miso, tempeh, soy sauce) [16–18]. Other parts of the world have their typical fermented foods and beverages, such as beer, wine, cider, yogurt, kefir, or sauerkraut in Europe. Different ingredients used for traditional fermented foods led to developing a diversity of microorganisms, including a variety of LAB [19]. These ingredients and bacteria are typical of each geographical area and contribute to the development of distinctive characteristics, such as typical textures and flavors [19,20].

In some regions, such as Latin America, the knowledge about their traditional fermented products is not so widespread, but they still represent an important nutrient source [20]. Latin America includes the countries of South America, Central America, Mexico, and the Caribbean islands, where a language derived from Latin is spoken. The majority of these countries are considered developing areas with nutrient deficiency representing an important problem. According to [21] traditional fermented products made with LAB can be considered as nutraceuticals with interesting health benefits for

these populations. The objective of this review is to summarize the main characteristics of fermented foods and beverages made with LAB traditionally elaborated by the native population of Latin America. In addition, the benefits and risks of these products, as well as the possibility of developing new fermented foods and beverages from traditional ingredients, are addressed.

Fermented Foods and Beverages of Latin America

Latin American countries have a long history of people who have elaborated fermented foods and beverages over the years from certain typical ingredients of the continent. According to some authors, a large variety of fermented foods and beverages in Latin America come from native inhabitants that traditionally elaborate these kinds of products using empirical information [5]. Among these populations, fermentation was and is still used mainly as a food preservation method. To elaborate fermented foods and beverages, microorganisms can be added on purpose (as starter cultures) or unintentionally (naturally present in the food). In most cases of native populations, fermented foods and beverages are produced through spontaneous fermentation, considering the process involves different unknown microorganisms present not only in the ingredients but also in the atmosphere or in different materials used during their elaboration [2]. Ingredients used to produce traditional fermented foods and beverages in Latin American countries include cereals (like maize), animal products (meats from beef, lamb, llama, or guanaco, and cheese), certain tubers, fruits, and vegetables [5,22]. It is common for different countries to prepare similar fermented products, sometimes under different names with small variations. This includes changes in elaboration steps or the use of region-specific seasonings. Teachings on how to produce fermented foods and beverages were commonly transferred from generation to generation, and subsequently, loss of information often occurred. The production of these foods was adapted due to the geographic and seasonal availability of ingredients. Due to these factors, different recipes and ways of preparation have come about [5,8].

A big part of the solid fermented foods consumed in Latin America are made with typical ingredients of the region and are part of the culture of the native communities. However, other fermented foods produced in this region were introduced by European immigration, such as cheeses and dried sausages, and are commonly consumed. This

section will focus on those products prepared with traditional ingredients of Latin America, leaving aside those fermented foods resulting from immigration.

Traditional Latin American beverages include alcoholic and non-alcoholic products, whose recipes are passed from generation to generation. People often consume alcoholic beverages for religious or spiritual purposes, whereas recreational motives are probably the main reason in industrialized populations. Some authors suggest that most of the fermented beverages elaborated by American natives involve the use of LAB [23,24]. Studies show both bacteria and yeast are commonly used in the elaboration of fermented beverages [24]. In such cases, these two microorganisms have a symbiotic relationship, stimulating each other's growth. In fermented beverages, the development of LAB is probably augmented by the substances produced by yeast, while the latter is stimulated by the production of acid generated by the bacteria [5].

The variety of fermented beverages in Latin America is wide and many of them are prepared using cassava (*Manihot esculenta*) or corn (*Zea mays*) as a substrate [23]. A large part of them are alcoholic beverages and have an important role in these cultures Although the ethanol in fermented beverages is mainly due to the presence of yeasts (and these are not the object of study in the present review), they coexist with LAB, some of these bacteria being heterofermentative with the ability of producing ethanol. Fermentation time and alcohol content of traditional beverages varies significantly among products and also according to the country. This is the case of *chicha*, a general name for a drink consumed in different areas of Latin America. Every region that produces it, has its own variations in substrates, alcohol content, and form of elaboration [25]. Alcohol amount can change according to the fermentation time of the beverage. Inhabitants of Brazilian tribes can wait up to 5 days before consuming *caxiri*, a drink based on cassava, corn, and sweet potato, in order to have a product with a high alcoholic content.

The main bacteria genera described as being part of this kind of product include *Lacticaseibacillus*, *Lactiplantibacillus*, *Lactobacillus*, *Limosilactobacillus*, *Leuconostoc*, *Streptococcus*, and *Weisella* among others. It is common that sweet potato is used as an inoculum. Inhabitants of some native populations can chew this ingredient, and the obtained product serves as an inoculum for some beverages, including *calugi*, *caium*, and *chicha* [22]. Starch from the substrates used for fermentation is transformed into fermentable sugars by the action of this inoculum containing amylase enzyme and microorganisms [5].

Table 1 summarizes some of the main characteristics of traditional Latin American fermented foods and beverages (with and without alcohol) resulting from LAB fermentation.

Table 1 – Traditional Latin American fermented foods and beverages (with and without alcohol) made using lactic acid bacteria. F = Food; B = Beverage; h = hours; d = days; m = months; NI = not informed

Food/Beverage Country	**Raw Material**	**Lactic Acid Bacteria Present**	**Fermentation Time**	**Ethanol (%)**	**Reference**
Aloja (B) Argentina	White carob *(Prosopis alba)*	*Lactiplantibacillus plantarum, Enterococcus faecium*	2–3 d	6.5–7.5	[26]
Atole agrio (B) Mexico	Corn *(Zea mays)*	*Lactiplantibacillus plantarum, Leuconostoc, Enterococcus, Weissella cibaria, Weissella confusa*	6–12 h	0	[27]
Calugi (B) Brazil	Cassava *(Manihot esculenta)*, corn *(Zea mays)*, rice *(Oryza sativa)*	*Lacticaseibacillus casei, Lacticaseibacillus paracasei, Lactiplantibacillus plantarum, Streptococcus parasanguis, Streptococcus salivarius, Weissella cibaria, Weissella confusa*	48 h	0	[23, 28, 29]
Cauim (B) Brazil	Cassava *(Manihot esculenta)*, corn *(Zea mays)*, cotton seed (*Gossypium)*, peanuts *(Arachis hypogae*a), pumpkin *(Cucurbita)*, rice *(Oryza sativa)*, sweet potato *(Ipomoea batatas)*	*Lacticaseibacillus paracasei, Lactiplantibacillus plantarum, Lactobacillus pentosus, Leuconostoc pseudomesenteroides, Levilactobacillus brevis, Limosilactobacillus fermentum*	48 h	0	[23, 30]
Caxiri (B) Brazil	Cassava *(Manihot esculenta)*, corn *(Zea mays)*, sweet potato *(Ipomoea batatas)*	*Lactobacillus helveticus, Limosilactobacillus fermentum*	5 d	10–11	[22, 31, 32]

Chicha (B) Argentina	Corn *(Zea mays)*	*Lactiplantibacillus plantarum, Leuconostoc lactis, Weissella viridescens*	2–8 d	0–10	[22, 33]
Chicha (B) Brazil	Corn *(Zea mays)*, sugar cane *(Saccharum officinarum)*	*Weisella cibaria, Weisella confusa*	36 h	0	[34]
Chicha (B) Colombia	Corn *(Zea mays)*	*Lactobacillus, Leuconostoc*	2–6 d	2-12	[35]
Chicha (B) Ecuador and Peru	Cassava *(Manihot esculenta),* corn *(Zea mays)*, rice *(Oryza sativa)*	*Lactobacillus acidophilus, Lactobacillus crispatus, Lactobacillus delbrueckii, Limosilactobacillus fermentum, Limosilactobacillus reuteri*	48–72 h	2-5	[23, 25]
Colonche (B) Mexico	Red tuna *(Opuntia streptacantha Lem)*	*Lactobacillus, Leuconostoc*	3–4 d	4–5	[36, 37]
Masa agria (F) Colombia	Corn *(Zea mays)*	*Lactiplantibacillus plantarum, Lactobacillus coleohominis, Lactobacillus gallinarum, Lactobacillus panis, Lactobacillus pontis, Lactobacillus spp, Limosilactobacillus fermentum*	3–5 d	0	[35]
Masato de yuca (F) Perú	Cassava *(Manihot esculenta)*	*Lactiplantibacillus plantarum, Limosilactobacillus fermentum, Weissella confusa*	NI	0.2	[38]
Polvilho azedo (or Almidón Agrio) (F) Brazil, Colombia, Paraguay	Cassava *(Manihot esculenta)*	*Lacticaseibacillus casei, Lactiplantibacillus plantarum, Lactobacillus spp*	3–20 d	0	[39]
Pozol (B) Mexico	Corn *(Zea mays)*	*Lactiplantibacillus plantarum, Leuconostoc, Streptococcus infantarius, Weissella confusa*	2–7 d	0	[13, 37, 40]

Puba flour(F) Brazil	Cassava *(Manihot esculenta)*	*Lactiplantibacillus, Lactobacillus delbrueckii,Lactobacillus perolans, Lactobacillus sp., Leuconostoc sp., Levilactobacillus brevis, Limosilactobacillus fermentum*	3–7 d	0	[22, 41, 42]
Pulque (B) Mexico	*Aguamiel (maguey sap)*	*Lactobacillus acidophilus, Lactobacillus hilgardii, Lactobacillus paracollinoides. Lactobacillus sanfranciscenci, Lactococcus sp., Leuconostoc citreum, Leuconostoc gasicomitatum, Leuconostoc kimchi, Leuconostoc mesenteroides*	7–28 d	5–7	[43]
Tarubá (B) Brazil	Cassava *(Manihot esculenta)*	*Lactiplantibacillus plantarum, Leuconostoc lactis, Leuconostoc mesenteroides, Levilactobacillus brevis*	8–15 d	Dependent on the fermentation time	[5, 23, 31, 44]
Tejuino (B) Mexico	Corn *(Zea mays)*	*Lactiplantibacillus plantarum, Limosilactobacillus fermentum*	24 h	0	[39,45]
Tocosh (F) Perú	Potato *(Solanum tuberosum)*	*Lacticaseibacillus casei, Lactobacillus farciminis, Lactobacillus sakei, Leuconostoc mesenteroides, Levilactobacillus. brevis, Limosilactobacillus fermentum*	12 m	0	[22]
Yakupa (B) Brazil	Cassava *(Manihot esculenta)*, sweet potato *(Ipomoea batatas)*	*Lactiplantibacillus plantarum, Limosilactobacillus fermentum, Weissella cibaria, Weissella confusa*	24–48 h	0	[23, 31, 46]

Nutritional Profile and Health Benefits of Traditional Fermented Foods and Beverages of Latin America

As mentioned in other sections, the fermentation process provides several technological, sensory, and nutritional changes to the ingredients used as substrates, and one of the main reasons to consume fermented foods and beverages are the health benefits that come along with them. Functional foods are those that provide a health benefit beyond basic nutrition [47], and foodstuffs obtained by LAB fermentation represent a large part of functional foods [16]. Fermented foods continue to be staple meals for certain native inhabitants of Latin America, so it can be assumed that these products represent an important energy source and provide various essential nutrients in the diet of these people [5]. Some of the main modifications produced by LAB in the nutritional profile of food ingredients during fermentation include improving their digestibility and increasing the macro and micronutrient content.

It is known that several raw foods are indigestible to the human organism or are not sensory acceptable without proper processing (e.g., wheat, barley, corn). Fermentation can help in the production of adequate edible foods and beverages prepared with these ingredients [16,48]. Such are the cases of wheat, a cereal that can be transformed into bread, and malted barley, which is turned into beer. Lactic acid fermentation represents a useful alternative for people having lactose intolerance, since some of them can consume fermented milk products (e.g., yogurts, cheese) without reporting undesirable effects [48]. As can be seen in Section 2, the majority of ingredients used in Latin America to produce fermented products are sources of carbohydrates, including oligosaccharides that are non-digestible to humans [49,50]. When fermentation occurs, these carbohydrates are used by microorganisms as energy source, and this leads to an increase in food digestibility [5].

The production of fermented foods and beverages can increase the protein and amino acid content compared to raw ingredients [5], and LAB can produce peptides with biological activity [46]. Regarding minerals and vitamins, fermentation can be a key tool to increase their bioavailability. Certain anti-nutritional factors (i.e., phytates, oxalates, tannins) present in raw foodstuffs can be reduced by fermentation, increasing the bioavailability of minerals such as Ca, Fe, and Zn that would be otherwise quenched by these anti-nutrients [5]. In addition, LAB fermentation leads to a process of microbial biosynthesis, which in turn enhances the levels of certain vitamins, including vitamins of

the B-group [51], and lactic acid, one of the main compounds produced during fermentation by LAB, has the potential to enhance the bioavailability of certain minerals and vitamins [52].

Nutritional improvements produced by LAB on raw food ingredients are summarized in Figure 1.

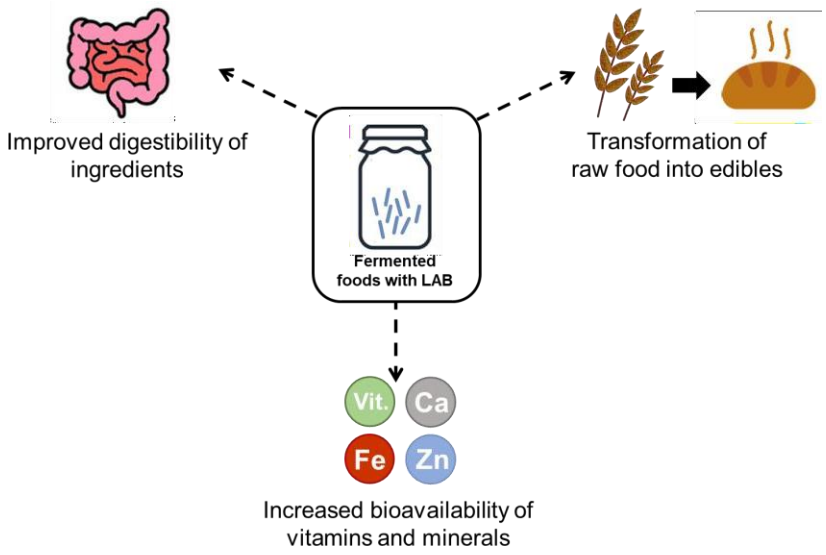

Figure 1 – Nutritional improvements produced by lactic acid bacteria (LAB) on raw food ingredients

The health effects of consuming fermented foods or beverages are diverse and affect various organic systems [53]. According to different authors, improvements in gastrointestinal, hepatic, and cardiovascular disorders are among the benefits obtained by the intake of these products. In addition, they could help in the prevention of pathologies such as obesity, diabetes, hypertension, osteoporosis, and certain allergies or intolerances [13,54,55]. Additionally, fermented products can be labeled as probiotics. It is important to clarify that not all foods and beverages elaborated through fermentation are considered probiotics. This term is limited to those cases when an established health benefit conferred by well-defined and characterized live microorganisms is demonstrated [56]. Non-viable microorganisms that confer a health benefit to the consumer (including parabiotics) are a matter of current research works, as after the application of standardized killing methods (e.g., heat, high pressure, ultraviolet, irradiation, sonication, drying, acid, pH changes), they can exert health benefits (e.g., inhibition of pathogens, immunomodulation, mucosal protection, among others) [57].

The fact that fermented foods provide a wide variety of health benefits may be useful for food industries seeking to attract consumers through health, functional, and nutraceutical labeling [13]. As mentioned, fermentation is used as a food preservation process, making it useful for those looking to conserve their food without the use of synthetic preservatives. However, certain health benefits of native fermented foods may still be unknown due to the fact that many foods contain unidentified but possibly very valuable strains [13]. In this sense, it is necessary to continue the investigations regarding these strains.

Risks of Fermented Foods' Elaboration and Consumption

Regardless of all the named benefits provided by the intake of fermented foods and fermented beverages, it is important to consider that the elaboration of these products has to follow certain steps in order to guarantee food safety and avoid food intoxication and long-term deleterious effects. In populations without access to basic services such as drinking water or electricity, sanitary conditions may be faulty. When fermented foods are prepared in these circumstances, great contamination can occur. Considering this, some investigations have focused on the existence of contamination of fermented foods in low-income populations. Diosma et al. [58] studied the production of kefir in Argentina in communities with low economic resources and observed that despite the existence of situations of vulnerability there was no cross-contamination of said food.

In the case of non-indigenous populations, a big part of the food products is industrially produced, and food quality and safety are ensured. However, with new food tendencies, more residents of industrialized areas are learning how to produce fermented foods domestically in order to obtain health benefits. Fermentation steps are simple and easily applicable domestically with little equipment, but a problem that can occur with this situation is inappropriate bacterial handling which can lead to food contamination. In particular, fermented beverage production is becoming a common procedure, with products such as kombucha and kefir representing one of the most incorporated trends in recent years. In addition, other drinks such as chicha in Argentina have become popular among the inhabitants of industrialized areas, which has led people to elaborate these drinks at home or purchase them online or in non-specialized stores. Although fermentation steps are simple and easily applicable domestically with little equipment, a

problem that can occur with this situation is inappropriate bacterial handling which can lead to food contamination. At the domestic level, it is important to focus on safety issues when preparing or purchasing fermented beverages, as well as when acquiring the microorganisms for their preparation. Providing basic information and knowledge on good manufacturing practices can be a key tool for these cases.

Considering the ingredients of Latin American origin, fermentation is a suitable method of decreasing the risks associated with the consumption of cassava, a food product used traditionally in this region and of rapidly-expanding demand but containing high levels of cyanide, a compounds that may reduce nutrients' bioavailability when cassava is raw or not thoroughly cooked. Additionally, cyanogenic glycosides can cause poisoning and death in humans when cassava is not properly processed [59].

As mentioned in Table 1, there is a wide variety of fermented alcoholic beverages in Latin America. These traditional drinks, along with others consumed worldwide (like beer), can represent a significant portion of the population's diet. Based on this, it is especially relevant to evaluate whether the benefits obtained through the consumption of fermented alcoholic beverages outweigh the possible issues that may arise from alcohol intake itself. It is well known that moderate to excessive alcohol consumption can lead to various health problems, including liver, digestive, and cardiovascular pathologies, as well as overweight, hypertension, and addictions. In this sense, the low alcohol content per serving, as well as its moderate intake could help to achieve an adequate balance and to avoid undesirable effects.

Novel Fermented Products Using Traditional Ingredients

The availability of fermented products in the market has grown in recent years. The health benefits they provide are attractive for consumers and for food/beverage-producing companies that must adapt to new demands and trends. Part of the current trend is related to the production of food and beverages based on plants or suitable for the vegetarian/vegan populations. In addition, the demand for gluten- and lactose-free products has also increased. These interests can align with a sector of consumers in search of foods that provide health benefits. Research on fermented foods and beverages prepared with ingredients such as cassava, corn, quinoa, and potato (traditional from Latin America) represents an interesting way to meet these consumer requirements.

Production at the artisanal or household levels continues to be the most common way of making the majority of traditional fermented foods [8]. However, it is likely that the fermented foods market will increase appreciably due to the augmented consumption of products that provide health benefits, and that the monetary gain may be substantial for those who engage in this market. An increase of $846.73 billion in the size of the fermented food and beverage market is estimated between 2022 and 2027. This market is expected to grow at a CAGR (compound annual growth rate) of 7.16% in this period [60]. It would be important to consider the fact that a large part of the population of Latin America is in an economic situation of poverty. It is essential that when making fermented products on an industrial scale that the ingredients are used in quantities that do not lead to their overexploitation. In addition, it would be useful to investigate the possibility of making fermented products through the use of surplus or discarded foods. In this way, economic and environmental improvements would be achieved [61].

Different investigations at the laboratory level have focused on the elaboration of fermented products from traditional Latin American ingredients with innovative characteristics, including the use of new microorganisms and the production of foods with specific nutritional or sensory attributes. For instance, Menezes et al. (2018) [15] elaborated on a new functional fermented beverage made from an indigenous corn-based drink native to Brazil, combining LAB and yeasts. Carrizo et al. (2019) [62] studied the possibility of bio-enriching quinoa-based pasta by adding LAB and found that it was possible to obtain a product with improved bioavailability of minerals and vitamins.

This niche represents an interesting option for several companies. However, laboratory tests are not always transferable to an industrial scale. Established fermentation conditions, as well as the use of known microorganisms, are essential to the achievement of industrially fermented products that have adequate characteristics and are suitable for sale. These conditions are not always achieved when talking about traditional fermented foods and beverages, where the microorganism composition of these products can constantly change. In this sense, there is still much work to be done in order to achieve a good transfer of technology from laboratories to industry.

Conclusions

Latin American countries have a long tradition of fermented foods, some of them elaborated with LAB. The fermentation process represents a relatively simple and accessible tool for Latin American inhabitants. Different types of consumers can benefit from this versatile technique depending on the objective being pursued. In the case of many Latin American populations, even today, fermentation is probably used mainly as a conservation method or for spiritual purposes. It is undeniable that the consumption of fermented foods and beverages provides the human body with various benefits, so promoting those benefits is a useful strategy to improve the general population's health. However, it is also important to note that fermentation, like other food processing, carries certain risks. It is essential that the people who prepare this type of food carry out proper handling of the ingredients and microorganisms to avoid harmful effects in the short and long term. Additionally, the consumption of fermented alcoholic beverages can lead to the emergence of pathologies, so their intake must be cautioned. The elaboration of novel fermented foods and beverages using traditional ingredients of Latin America represents an interesting approach for (local) food industries, considering that many of these components have characteristics sought by different sectors of consumers, including the fact that some of these ingredients are suitable for populations with specific diseases like gluten-related pathologies and dairy intolerance. However, on an industrial scale, there are still several challenges to overcome, like the lack of knowledge and control of the microorganisms that participate in the fermentation of traditional products. In this sense, the large-scale production of these foods and beverages has the challenge of finding and using the appropriate strains that provide the greatest technological, nutritional, and sensory benefits while being safe for consumers.

Author Contributions

Conceptualization, A.D.C., G.N.M., A.G.-Z. and P.C.C.; writing—original draft preparation, A.D.C. and G.N.M.; writing—review and editing, A.G.-Z. and P.C.C.; supervision, A.G.-Z. and P.C.C. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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Chapter II

Galacto-oligosaccharides' production and application

i. Chickpeas' and Lentils' Soaking and Cooking Wastewaters Repurposed for Growing Lactic Acid Bacteria

foods

MDPI

Article

Chickpeas' and Lentils' Soaking and Cooking Wastewaters Repurposed for Growing Lactic Acid Bacteria

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Abstract

Legumes processing involves large amounts of water to remove anti-nutrients, reduce uncomfortable effects, and improve organoleptic characteristics. This procedure generates waste and high levels of environmental pollution. This work aims to evaluate the galacto-oligosaccharide (GOS) and general carbohydrate composition of legume wastewaters and assess their potential for growing lactic acid bacteria. Legume wastewater extracts were produced by soaking and/or cooking the dry seeds of chickpeas and lentils in distilled water and analysed using high-performance liquid chromatography with refractive index detection. GOS were present in all extracts, which was also confirmed by Fourier transform infrared spectroscopy (FTIR). C-BW extract, produced by cooking chickpeas without soaking, provided the highest extraction yield of 3% (g/100 g dry seeds). Lentil extracts were the richest source of GOS with degree of polymerization ≥ 5 (0.4%). *Lactiplantibacillus plantarum* CIDCA 83114 was able to grow in de Man, Rogosa, and Sharpe (MRS) broth prepared by replacing the glucose naturally present in the medium with chickpeas' and lentils' extracts. Bacteria were able to consume the mono and disaccharides present in the media with extracts, as demonstrated by HPLC and FTIR. These results provide support for the revalorisation of chickpeas' and lentils' wastewater, being also a sustainable way to purify GOS by removing mono and disaccharides from the mixtures.

Keywords

Galacto-oligosaccharides, pulses, *Lactiplantibacillus plantarum*, circular economy, waste management, culture medium

Introduction

Pulses are an important component of the human diet and contain proteins, oligosaccharides, dietary fibre, minerals, and antioxidant polyphenols [1]. Although their consumption has important health benefits (e.g., the reduction of cholesterol and the prevention of the development of diabetes and cancer [1,2]), certain innocuous but uncomfortable associated effects (e.g., flatulence) can make their ingestion undesirable. Therefore, before consumption, soaking and cooking treatments are used to reduce these effects, as well as to enhance the bioavailability of important compounds and improve organoleptic characteristics, such as texture and flavour [3].

According to the latest definition, prebiotics are substrates selectively utilised by the host microorganisms conferring a health benefit [4]. Pulses contain various types of oligosaccharides with prebiotic effects, including galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS), that are not absorbed or hydrolysed in the upper part of the gastrointestinal tract. GOS are composed of a varying number of galactose (Gal) units and a terminal glucose (Glu) or sucrose residue, resulting in different degrees of polymerization (DP). The type of GOS present in pulses, specifically α-GOS, belong to the raffinose-family of oligosaccharides (RFO) and are responsible for causing flatulence. Raw pulses are considered high in GOS, with values ranging from 1 to 10% [5]. Soaking and cooking treatments help to reduce the content of α-GOS, as they are partially leached and degraded by enzymatic action [6].

The processing of legumes, such as chickpeas and lentils, requires the use of large amounts of water, resulting in the generation of waste and high levels of environmental pollution [7]. Reusing industrial by-products or discards is becoming increasingly important to improve the sustainability of food production and reduce waste. This approach is aligned with the principles of the circular economy, an economic system based on business models that prioritize reducing, reusing, recycling, and recovering materials in the production, distribution and consumption processes, instead of the traditional ‘end-of-life’ concept [8]. Although the utilization of food industry waste is increasingly applied to the food itself, the water from the processing of these foods continues to be majorly discarded. Aquafaba (commonly employed as an egg substitute) is one of the most well-known examples of reusing water from the treatment of chickpeas. Some of the studied applications of legumes’ wastewaters include the production of baked

goods, ice-cream, and pastry products [9,10]. Colucci Cante et al. [11] evaluated the fermentation of bean blanching wastewaters as a way to valorise them.

Lactic acid bacteria have played a crucial role in the production of fermented products for centuries. They are capable of fermenting various substrates, producing lactic acid and other metabolites with health-promoting and technological properties – such as reducing spoilage microorganisms, acidification – and enhancing sensory attributes [12]. These microorganisms have been successfully used in the production of lactose-free dairy foods and beverages through lactose hydrolysis, such as bread and other cereal-based foods, and alcoholic beverages, such as wine [13]. Recently, a review of the use of lactic acid bacteria in the production of traditional food products from Latin America through the fermentation of raw materials, such as tubers, cereals, and fish, was published [12]. Lactic acid fermentation was also studied as a way to improve the nutritional and sensorial aspects of legume and fruit beverages, with the finding that fermentation not only was useful to enhance these characteristics, but also to extend the shelf life of products [14,15]. Lactic acid bacteria have a GRAS status ("generally recognized as safe") from the FDA (USA), and as such, they are increasingly being used both in fermentation processes and as functional ingredients [16].

The objective of this work was to evaluate the carbohydrate composition, particularly the GOS, of wastewaters produced during the soaking and cooking of chickpeas and lentils, and their potential as carbohydrate sources for the growth of *Lactiplantibacillus plantarum* CIDCA 83114, a lactic acid bacteria strain isolated from kefir grains. This strain was selected because of to its resistance to preservation processes (including drying treatments), its high stability during storage, its potential probiotic activity, and its ease of growing on simple media [17,18]. The wastewaters were recovered from food producing industries and this work aims to valorise these resources in a circular economy mind-set.

Materials and Methods

Materials

Chickpea (*Cicer arietinum* L.) (Continente, Matosinhos, Portugal) and lentil (Lens culinaris Medikus var. variabilis) (Don Elio, Santa Fe, Argentina) seeds were acquired at local supermarkets. Vivinal® GOS syrup was kindly provided by Friesland Campina Ingredients (Veghel, The Netherlands). The microbiology medium de Man, Rogosa, and Sharpe (MRS) broth was purchased at Sigma-Aldrich® (Burlington, MA, USA). Other reagents used were acquired from common vendors.

Preparation of Legumes' Treatment Wastewater Samples

GOS-containing extracts (wastewaters) from chickpea and lentils were obtained following a similar method to that described by Han and Baik [3]. Three treatments were carried out: soaking, soaking and cooking, and cooking without soaking of the seeds (Figure 1). A 1:5 (*w*/*v*, seeds: distilled water) ratio was used for the three treatments in both legumes.

Raw dried seeds were soaked for 8 h at 20 °C. After that, the seeds were strained, obtaining the waters from the soaking chickpeas (C-SW) and lentils (L-SW). The soaked chickpeas were then cooked in a pressure cooker (C-CW) and the soaked lentils in a pot (L-CW), both for 30 min. Thirdly, extracts were obtained by boiling dry (*i.e.*, without soaking) chickpeas (C-BW) and lentils (L-BW) for 30 min in a pressure cooker and a pot, respectively. After the thermal treatments, the water extracts were cooled to 20-25 °C and centrifuged (15 min, 4000 × *g*) (Heraeus Instruments, Hanau, Germany). The supernatants were filtered using filter funnels with a porosity of 4 (10-16 µm pores). Afterwards, the samples were freeze-dried in a Martin Christ Gefriertrocknungsanlagen GmbH freeze-dryer (Alpha 1-2 LD Plus, Osterode, Germany).

HPLC-RI Analysis

The extracts obtained in Section 2.2 were filtered with 0.45 µm of cellulose acetate filters (Frilabo, Maia, Portugal), and the carbohydrates were determined using high-

performance liquid chromatography with refractive index (HPLC-RI) detection (UltiMate 3000, Dionex, Sunnyvale, CA, USA). A ReproGel-Na column (Dr. Maisch, Ammerbuch, Germany) of 250 × 8 mm and a particle size of 9 μm was used with a CARBOSep CHO 411 pre-column (Concise Separations, San Jose, CA, USA) at 80 °C. The RI detector (Shodex RI-101) was maintained at 50 °C. Degasified filtered ultrapure water was used as mobile phase with a flow rate of 500 μL/min. The obtained chromatograms were analysed with Chromeleon 6.80 software (Dionex Corporation, Sunnyvale, CA, USA).

Because of the absence of GOS standards in the market, different concentrations of Vivinal® GOS syrup were used as standards, to determine the retention times of the sugars of interest and to calibrate the analytical method used. The syrup was composed of Gal, Glu, lactose (Lac), and short chain β-GOS (up to DP = 7) as shown in Table 1 (Friesland Campina DOMO, Amersfoort, The Netherlands, 2017) [19], alongside the retention times determined for each carbohydrate species. A calibration curve for fructose (Fru), whose retention time is 11.4 min, was obtained from a previous work [20]. The HPLC-RI calibration is detailed in Supplementary Material S1. From the determined calibration curves, the sugar composition of the samples was expressed as g/100 g of fresh extract.

Table 1 – Content (%) and retention times of saccharides present in Vivinal® GOS syrup [19]. [a] GOS species with DP = 7, 6, and 5 were treated as one due to poor resolution of the obtained peaks. [b] Lac present in Vivinal® GOS syrup was used as a reference for other similar DP = 2 carbohydrates (*i.e.*, disaccharides) with the same retention time. [c] Total GOS indicates the sum of GOS DP = 3, DP = 4, DP = 5, DP = 6, and DP = 7′s contents

	DP7-5[a]	**DP4**	**DP3**	**Lac/DP2[b]**	**Glu**	**Gal**	**Total**	**Total GOS[c]**
Content (%)	7.4	10.8	22.0	37.4	21.1	1.3	100.0	40.2
Retention time (min)	5.5-5.9	6.4	7.0	8.1	10.4	11.2		

Extraction Yield

The extraction yields were calculated based on the HPLC-RI analysis and the concentration (g/100 g of fresh extract) determined for each carbohydrate detected. The sugar content on a dry basis (g/100 g of dry extracts) and their extraction yield (g/100 g of dry seeds) were calculated by Equations (1) and (2), respectively, after performing °Brix measurements (RX-100, Atago digital refractometer) and determining the volume of fresh extract obtained. The yields for GOS-DP ≥ 5, GOS-DP = 4, GOS-DP = 3, Lac, Glu, Gal, and Fru's extraction were calculated separately from each calibration curve.

$$Sugar\ content\ in\ g/100\ g\ dry\ extract = \frac{Sugar_{\ (g/100\ g\ fresh\ extract)}}{{}^{\circ}Brix_{(g/100\ g\ fresh\ extract)}} \times 100 \quad (1)$$

$$Yield\ in\ g/100\ g\ dry\ seeds = \frac{Sugar_{\ (g/L)} \times V_{extract\ (L)}}{m_{dry\ seeds\ (g)}} \times 100 \quad (2)$$

FTIR Analysis

The freeze-dried samples obtained in Section 2.2 were analysed by a Fourier transform infrared spectrometer (Spectrum Two, Perkin Elmer, Waltham, MA, USA) with attenuated total reflectance (ATR) equipment with a diamond crystal (UATR Two, Perkin Elmer, Waltham, MA, USA). The spectra were registered in the 4000–400 cm^{-1} range by co-adding 32 scans with 4 cm^{-1} spectral resolution at 20 °C. The spectra were analysed using spectrum software (Perkin Elmer, Waltham, MA, USA). The Vivinal® GOS syrup was also analysed as a reference material for a complex carbohydrate mixture.

Microbiological Assays with Oligosaccharide Mixtures

Lactiplantibacillus plantarum CIDCA 83114 was isolated from kefir grains [21] and maintained frozen at -80 °C in 120 g/L of non-fat milk solids. Cultures were grown in an MRS broth [22] at 37 °C overnight in aerobic conditions to obtain approximately 10^{10}-10^{11} of CFU/mL (stationary phase). Then, they were harvested by centrifugation at 10,000 × *g* for 10 min at 4 °C, and the pellets were washed twice with a phosphate buffered saline (PBS) solution (K_2HPO_4 0.144 g/L; NaCl 9.00 g/L; Na_2HPO_4 0.795 g/L, pH 7) and used to evaluate the microbial growth potential of the water extracts derived from chickpeas and lentils (Section 2.2).

To that aim, the microorganisms were inoculated (1% *v*/*v*) in MRS (5 mL) broth formulated without glucose (composition in Supplementary Material S2) and supplemented at 0.3% *w*/*v*, either with the extracts obtained in Section 2.2 or with Gal, Glu, Lac, or Vivinal® GOS syrup. All the solutions were sterilized by filtration (0.45 µm pore filter diameter, Frilabo, Maia, Portugal). Blank controls were carried out by inoculating the strain in MRS without the addition of extracts or sugars. The samples were incubated at 37 °C for 24 h and then serially diluted in PBS, plated on MRS agar, and

incubated at 37 °C for 48 h in aerobic conditions. The results were expressed as colony-forming units (CFU) per millilitre (CFU/mL). To calculate the growth potential of the samples, the CFU/mL values for the samples were referred to those of the blank control (MRS without sugars) (Equation (3)).

$$log\ CFU/mL = log\left(CFU/mL_{Sugar} - CFU/mL_{Blank}\right) \quad (3)$$

A final microbiological experiment was performed by growing *L. plantarum* CIDCA 83114 at 37 °C for 24 h in a C-BW extract prepared in distilled water at 51 g/L (MRS concentration). After growing, the culture was centrifuged and the supernatant was filtered and analysed in the HPLC-RI for comparison with the initial sample. The FTIR spectra were also recorded for the supernatant after lyophilization and compared with the C-BW extract's spectrum obtained in Section 2.5.

Figure 1 shows a summary of the experimental procedures.

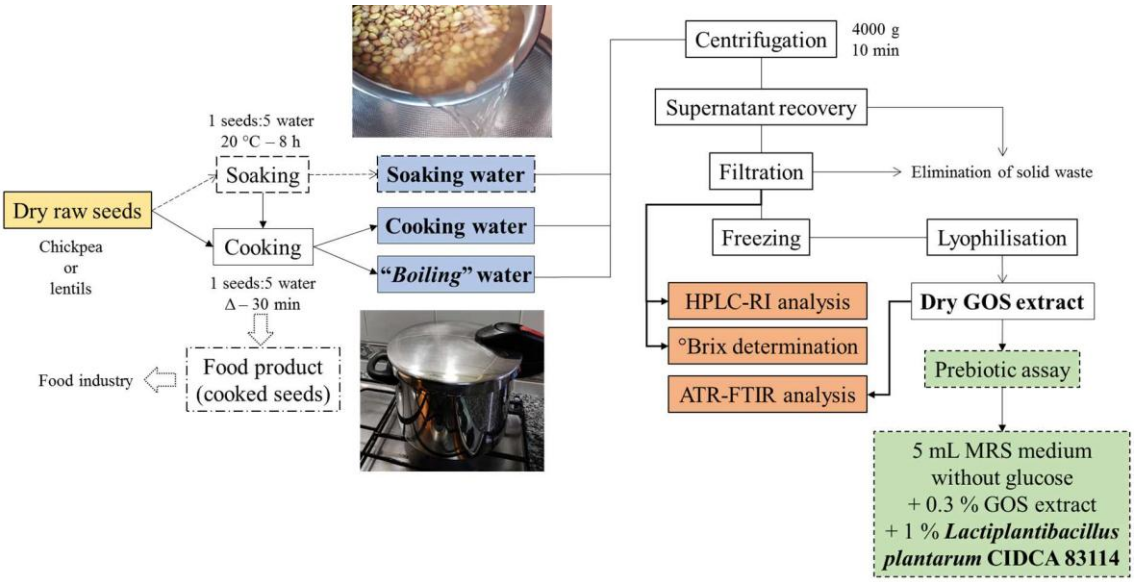

Figure 1 – Experimental procedures for the production and characterisation of chickpea- and lentil-derived extracts and the bacterial growth-potential assessment assay using *Lactiplantibacillus plantarum* CIDCA 83114

Statistical Analysis

The obtained results were evaluated by one-way analysis of variance (ANOVA), using InfoStat software. When this analysis expressed statistical differences ($p < 0.05$), intragroup comparisons were tested using the Tukey test.

Results and Discussion

HPLC-RI Results and Extraction Yield

The chromatograms obtained from the fresh extracts of chickpeas and lentils exhibited similar features, especially in the boiling water extracts (Figure 2) and resembled those of Vivinal® GOS syrup (S1). Considering that the same seeds-to-water weight proportion was used in all procedures, the results indicate that chickpeas are a richer source of saccharides than lentils, because the chromatograms showcase a higher number of peaks, and those in common have higher intensities. GOS were found in higher quantities in the chickpea extracts, and lentils provided much fewer mono- and disaccharides. Fru was only detected in the lentil extracts; however, the juxtaposition of its peak (11.4 min) with that of Gal (11.2 min) may cause its eclipsing in chickpea waters. In all the chromatograms peaks found between 7 and 8 min and at around 9 min have not yet been identified. The prominent peak present in all samples at around 5 min can be attributed to a Glu-composed polysaccharide, most likely starch. This inference is supported by the observation of starch in the presence of iodine during an assay conducted on the legumes' wastewaters from the present study. The chromatograms' resemblance to that of Vivinal (S1) highlights the relevance of its use for HPLC-RI calibration, in view of the absence of GOS standards in the market.

Tables 2–4 show the GOS, DP2 sugars, Glu, Gal, and Fru contents in g/100 g of fresh and dry extract and the extraction yield in g/100 g of dry seeds used for the soaking and cooking, respectively. Fresh extract represents the extract after the centrifugation and filtration steps, which is useful to analyse the sample for direct utilization. Dry extract is expressed on a dry basis and facilitates the manipulation and storage steps.

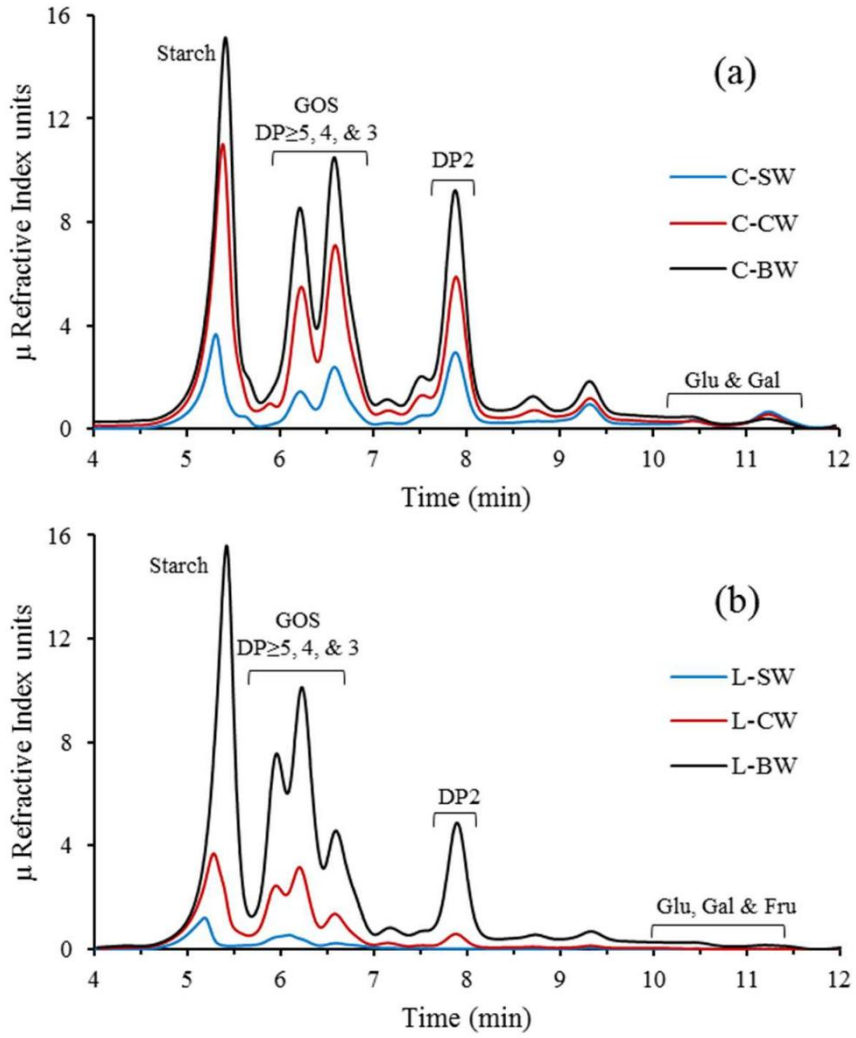

Figure 2 – HPLC-RI chromatograms of soaking (SW), cooking (CW), and cooking without soaking "boiling" (BW) waters from (a) chickpeas and (b) lentils

When evaluating the soaking and cooking treatments, soaking provided the lowest extraction yields, but the obtained mixtures were purer, since the measured ºBrix values (total dissolved solids) of the fresh extracts were closer to the calculated total quantifiable sugar content. C-BW was the fresh extract with the highest total GOS, total mono- and disaccharides, and total saccharides, when compared with the rest of treatments and with lentil extracts. The soaking methods are effortless, and all the extracted material is highly hydrophilic and water soluble. Contrarily, the cooking (C-CW) and boiling (C-BW and L-BW) processes enable the extraction of compounds with lower solubility [9], resulting in a significantly lower concentration of total GOS in the dry extracts (Table 3). It is worth noting that the combined contents of GOS-DP ≥ 5 and DP = 4 are the same for all chickpea extracts (16 g/100 g of dry extract); however, C-SW possesses almost 10% more GOS-DP = 3, twice the amount of DP2 sugars, and thrice the amount of Glu and Gal than C-

CW and C-BW. This trend was not observed for the lentil-derived extracts, with the total saccharide content being approximately 36-40% in all the dry extracts. L-CW was the lentil extract with the lowest total mono- and disaccharides; however, Fru was detected but was not quantified because its area was below the limit of quantification. According to the USDA [23], raw lentil possesses up to 0.27 g of Fru/100 g, and L-BW extraction produced 0.04 g of Fru/100 g of lentils; for chickpea, no Fru values are reported by the USDA [23], and no extraction of this sugar was observed. When comparing the dried extracts of both legumes, it can be noted that the total GOS, total mono- and disaccharides, and total saccharides content were significantly lower in lentils than in chickpeas.

In terms of the total GOS production, as seen in Table 4, the C-BW was the most interesting extract, with a 3% extraction yield (*i.e.*, 3 g of the total GOS (of which half are DP = 3 sugars) were produced out of 100 g of dry chickpea seeds used), and without the need for soaking the legume. Similarly, the L-BW extract stands out as the most efficient source for GOS-DP ≥ 5's extraction, specifically, producing 0.4 g out of 100 g of dry legume seeds. This results in less utilization of resources and generates fewer waste products. In terms of total mono- and disaccharides, chickpeas' extract obtained significantly higher values than lentils'. This is also true for the total GOS content, except for L-BW.

Serventi [24] evaluated the cooking water of legumes (after soaking), finding that this water possesses high amounts of oligosaccharides. In the present study, it was observed that treatments that involve the use of heat (cooking and boiling) led to a higher extraction of GOS than soaking, which can be correlated with the results of Liu and Serventi [25], who showed that the process of cooking after soaking can lead to a loss of 60–85% of oligosaccharides in legumes, compared to soaking (50-75%).

Table 2 – Concentration of different sugars as g/100 g of fresh extract obtained from chickpeas and lentils*. DP = degree of polymerization; SW = soaking water; CW = cooking water; BW = boiling water; N.D. = not detected; <L.O.Q. = below the limit of quantification. † Total saccharides = total quantifiable saccharides. *Results are expressed as average ± SD. Different letters in the same row indicate significant differences among samples ($p < 0.05$); n = 3

Compound (%)	Chickpea Extracts			Lentil Extracts		
	C-SW	C-CW	C-BW	L-SW	L-CW	L-BW
GOS-DP ≥ 5	0.01 ± 0.00	0.03 ± 0.00	0.04 ± 0.00	0.03 ± 0.00	0.08 ± 0.00	0.22 ± 0.00
GOS-DP = 4	0.07 ± 0.00	0.26 ± 0.00	0.39 ± 0.01	0.02 ± 0.00	0.15 ± 0.00	0.49 ± 0.01
GOS-DP = 3	0.15 ± 0.00	0.41 ± 0.00	0.59 ± 0.01	0.03 ± 0.00	0.10 ± 0.00	0.29 ± 0.01
Lac/DP = 2	0.17 ± 0.00	0.33 ± 0.01	0.50 ± 0.01	0.01 ± 0.00	0.04 ± 0.00	0.28 ± 0.01
Glu	0.05 ± 0.00	0.04 ± 0.00	0.07 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.05 ± 0.00
Gal	0.05 ± 0.00	0.05 ± 0.00	0.04 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.02 ± 0.00
Fru	N.D.	N.D.	N.D.	N.D.	<L.O.Q.	0.02 ± 0.01
Total GOS	0.23 ± 0.00^{b}	0.69 ± 0.01^{d}	1.02 ± 0.01^{f}	0.09 ± 0.00^{a}	0.33 ± 0.01^{c}	0.99 ± 0.01^{e}
Total mono- + disaccharides	0.26 ± 0.01^{c}	0.42 ± 0.01^{e}	0.61 ± 0.02^{f}	0.04 ± 0.00^{a}	0.06 ± 0.00^{b}	0.37 ± 0.02^{d}
Total saccharides †	0.49 ± 0.01^{c}	1.11 ± 0.01^{d}	1.64 ± 0.05^{f}	0.12 ± 0.00^{a}	0.39 ± 0.01^{b}	1.36 ± 0.04^{e}
° Brix (total solids)	0.5	1.8	2.8	0.3	1.0	3.7

Table 3 – Concentration of different sugars as g/100 g of dried extracts obtained from chickpeas and lentils*. DP = degree of polymerization; SW = soaking water; CW = cooking water; BW = boiling water; N.D. = not detected; <L.O.Q. = below the limit of quantification. †Total saccharides = total quantifiable saccharides. *Results are expressed as average ± SD. Different letters in the same row indicate significant differences among samples ($p < 0.05$); n = 3

Compound (%)	Chickpea Extracts			Lentil Extracts		
	C-SW	C-CW	C-BW	L-SW	L-CW	L-BW
GOS-DP ≥ 5	1.62 ± 0.13	1.53 ± 0.02	1.59 ± 0.13	10.56 ± 0.30	7.92 ± 0.40	5.95 ± 0.10
GOS-DP = 4	14.73 ± 0.07	14.24 ± 0.22	14.00 ± 0.37	7.15 ± 0.26	15.29 ± 0.35	13.13 ± 0.34
GOS-DP = 3	29.68 ± 0.06	22.70 ± 0.07	21.10 ± 0.47	10.99 ± 0.47	9.86 ± 0.04	7.73 ± 0.18
Lac/DP = 2	33.39 ± 0.35	18.32 ± 0.29	17.97± 0.52	3.88 ± 0.12	4.25 ± 0.24	7.49 ± 0.23
Glu	9.66 ± 0.55	2.34 ± 0.14	2.35 ± 0.10	7.35 ± 0.19	2.12 ± 0.09	1.47 ± 0.11
Gal	9.54 ± 0.27	2.58 ± 0.07	1.60 ± 0.05	0.44 ± 0.07	0.31 ± 0.01	0.42 ± 0.05
Fru	N.D.	N.D.	N.D.	N.D.	<L.O.Q.	0.66 ± 0.20
Total GOS	46.02 ± 0.26^{f}	38.47 ± 0.31^{e}	36.68 ± 0.98^{d}	28.69 ± 1.03^{b}	33.07 ± 0.79^{c}	26.80 ± 0.61^{a}
Total mono- + disaccharides	53.25 ± 0.22^{f}	23.24 ± 0.50^{e}	21.91 ± 0.67^{d}	11.67 ± 0.38^{c}	6.68 ± 0.34^{a}	10.04 ± 0.59^{b}
Total saccharides †	98.61 ± 1.43^{e}	61.71 ± 0.81^{d}	58.60 ± 1.65^{c}	40.36 ± 1.41^{b}	39.75 ± 1.14^{b}	36.84 ± 1.20^{a}

Table 4 – Chickpeas' and lentils' extraction yields expressed as g of sugar/100 g of dried seeds*. DP = degree of polymerization; SW = soaking water; CW = cooking water; BW = boiling water; N.D. = not detected; <L.O.Q. = below the limit of quantification. †Total saccharides = total quantifiable saccharides. *Results are expressed as average ± SD. Different letters in the same row indicate significant differences among samples ($p < 0.05$); n = 3.

Compound (%)	Chickpea Extracts			Lentil Extracts		
	C-SW	C-CW	C-BW	L-SW	L-CW	L-BW
GOS-DP ≥ 5	0.03 ± 0.00	0.10 ± 0.00	0.14 ± 0.01	0.12 ± 0.00	0.25 ± 0.01	0.35 ± 0.01
GOS-DP = 4	0.29 ± 0.00	0.92 ± 0.01	1.21 ± 0.03	0.08 ± 0.00	0.48 ± 0.01	0.78 ± 0.02
GOS-DP = 3	0.58 ± 0.00	1.47 ± 0.00	1.83 ± 0.04	0.13 ± 0.01	0.31 ± 0.00	0.46 ± 0.01
Lac/DP = 2	0.65 ± 0.01	1.19 ± 0.02	1.56 ± 0.04	0.05 ± 0.00	0.13 ± 0.01	0.44 ± 0.01
Glu	0.19 ± 0.01	0.15 ± 0.01	0.20 ± 0.01	0.09 ± 0.00	0.07 ± 0.00	0.09 ± 0.01
Gal	0.19 ± 0.01	0.17 ± 0.00	0.14 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.00
Fru	N.D.	N.D.	N.D.	N.D.	<L.O.Q.	0.04 ± 0.01
Total GOS	0.90 ± 0.00^{b}	2.49 ± 0.02^{e}	3.15 ± 0.01^{f}	0.34 ± 0.01^{a}	1.05 ± 0.03^{c}	1.59 ± 0.04^{d}
Total mono- + disaccharides	1.04 ± 0.00^{d}	1.49 ± 0.00^{e}	1.87 ± 0.00^{f}	0.14 ± 0.00^{a}	0.21 ± 0.01^{b}	0.59 ± 0.03^{c}
Total saccharides †	1.92 ± 0.03^{c}	4.00 ± 0.05^{e}	5.03 ± 0.01^{f}	0.47 ± 0.02^{a}	1.26 ± 0.04^{b}	2.18 ± 0.07^{d}

FTIR

Figure 3 shows the FTIR spectra obtained for the dried chickpeas' and lentils' extracts, and for the reference material, Vivinal® GOS syrup. The spectra of all the extracts were very similar and superimposable. This indicates that the soaking process has no noteworthy qualitative effect on the biomolecules detected by a spectral analysis, meaning that both soaking and cooking processes enable the extraction of similar compounds. The spectrum obtained for the Vivinal® GOS syrup, which corresponds to a mixture of β-GOS, Lac, Glu, and Gal, was also similar to those of the extracts. The bands shared between the samples and the Vivinal® GOS syrup included the broad one at 3500-3000 cm^{-1} (OH stretching) and those in the fingerprint region (1200-800 cm^{-1}), arising from the C-O-C glycosidic linkage, the COH bending, and the C-C stretching vibrational modes, that collectively provide a characteristic pattern for each carbohydrate [26,27]. The differences observed between the Vivinal® GOS syrup and the extracts in this region may be related to the fact that the former does not contain polysaccharides. As stated before, we hypothesize that the peak at 5 min observed in the HPLC chromatograms of the extracts corresponds to starch. This conclusion is further supported by the FTIR analysis. Romano et al. [28] also observed starch-related bands in the 1250-800 cm^{-1} region when evaluating the FTIR spectra of quinoa flour. The main differences between the samples' and the Vivinal® GOS syrup's spectra were related to the relative intensities of the bands observed in the still-undiscussed double-bond stretching and local symmetry regions, around 1800-1500 cm^{-1} and 1500-1200 cm^{-1}, respectively. The bands detected in the former can be attributed to the presence of unsaturated bonds (e.g., in the C=O groups found in carbohydrates) and unspecific CH_2 bending vibrations, whereas those found in the latter can be ascribed to vibrations arising from C-O groups, also observed in carbohydrates [29].

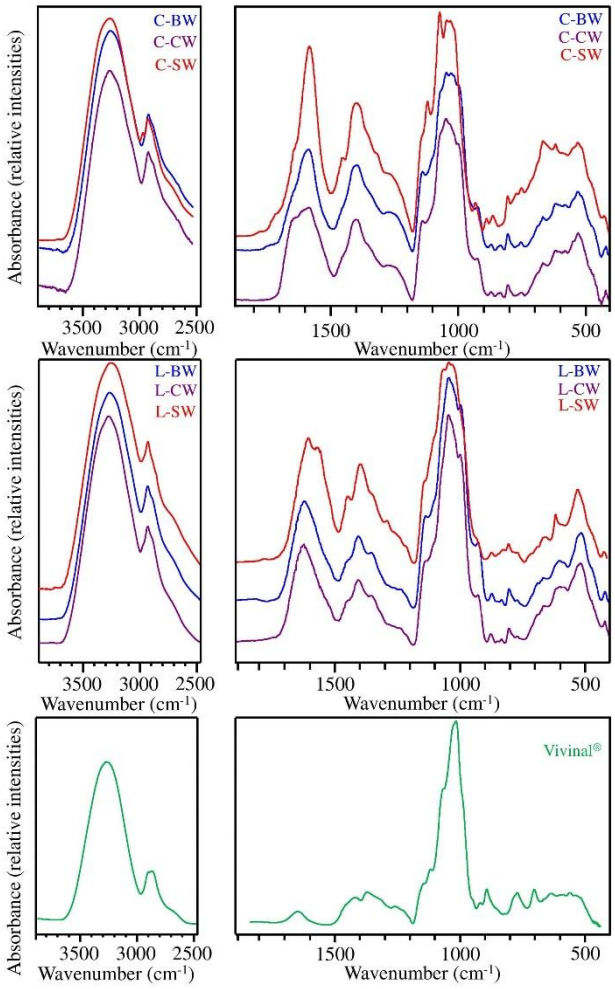

Figure 3 – FTIR spectra of the chickpeas' soaking (C−SW) and cooking (C−CW and "boiling" C−BW) waters (a); of the lentils' equivalent extracts (L−SW, L−CW, and L−BW) (b); and of the Vivinal® GOS syrup (c). νOH and νCH_2 denote the stretching vibrational modes of the OH and CH_2 groups

Microbiological Assay with Oligosaccharide Mixtures

Figure 4 shows the results of the 24 h bacterial growth assays obtained for all the extracts, the standards, and the Vivinal® GOS syrup used as a reference material. All the sample extracts were capable of promoting the growth of *L. plantarum* CIDCA 83114, showcasing a growth potential comparable to that of the standard sugars assayed. The chickpea extracts were the most successful in this regard, leading to bacterial counts close to those obtained for Glu (the sugar present in the standard MRS medium composition) and for Vivinal® GOS syrup, which is composed of β-GOS, unlike α-GOS present in the extracts. L-BW was the best extract from the lentils' counterpart.

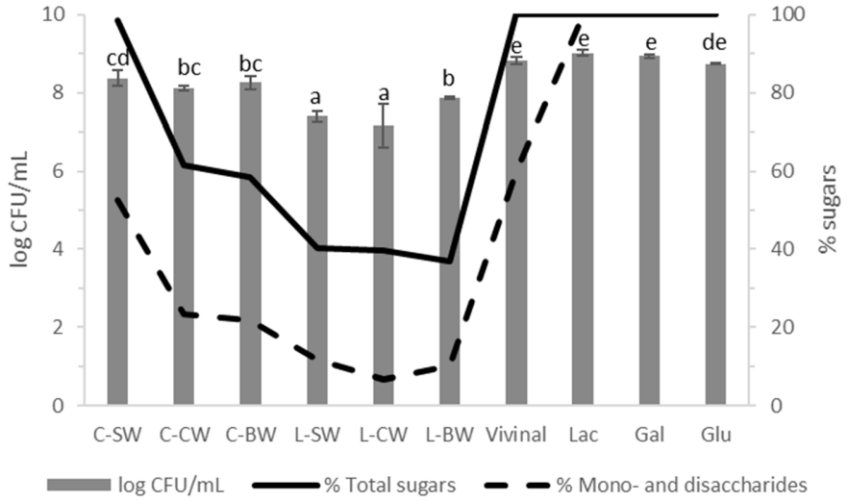

Figure 4 – Results of the growth-potential assessment assay in log CFU/mL (left axis) and the samples' initial sugar composition (right axis) for the chickpea and lentil extracts, and for Vivinal, lactose (Lac), galactose (Gal), and glucose (Glu). Results are expressed as average ± SD. Different letters indicate significant differences among samples ($p < 0.05$); n = 4.

The joint content of mono- and disaccharides does not explain the observed results, where extracts such as C-BW – which only presents 20% of these sugars – showcase bacterial counts close to those obtained for Glu (100%), at 8.2 and 8.7 log CFU/mL, respectively. This indicates that other sugars present, namely GOS, are being utilized by the microorganisms. However, when comparing the total sugar content (total quantifiable sugars) of C-BW (60%) with that of the Vivinal® GOS syrup (100%) this correlation falls short. There are two possible explanations. The first is that *L. plantarum* CIDCA 83114 is capable of more efficiently utilising α-GOS (such as those present in the extracts), than β-GOS (such as those found in the Vivinal® GOS syrup) for its growth, which has been previously reported for some probiotic cultures. Oh et al. [30] studied the growth effect of different α- and β-GOS on several non-probiotic and probiotic bacterial strains and determined that the oligosaccharide structure influences their growth, even amongst different strains of the same species. The second, and most likely reason, is that the unknown polysaccharide found in the extracts (Figure 2) is also being metabolized by *L. plantarum* CIDCA 83114.

To answer this question, *L. plantarum* CIDCA 83114 was grown at 37 °C for 24 h in C-BW (51 g/L) and the final medium was analysed in the HPLC-RI (Figure 5). It was observed that Glu (10.5 min) and Gal (11.4 min) peaks entirely disappeared, while that of DP = 2 sugars (8 min) greatly decreased its intensity after fermentation. The

intensity of GOS peaks at around 6–7 min was not altered. The increase in the GOS-DP = 4 peak (6.3 min) can be explained considering the formation of L-lactic acid, whose retention time is similar to that of GOS-DP = 4. After fermentation, the C-BW extract's chromatogram had 97% of the total area of the initial C-BW sample before fermentation, and a 51 g/L C-BW sample had 18.7 g/L total GOS. As GOS were unaffected by the bacterial metabolic activity, they are expected to still be present at this concentration after fermentation. Interestingly, the polysaccharide peak at around 5 min decreased its intensity. Therefore, it can be concluded that *L. plantarum* CIDCA 83114 preferably utilises sugars where Glu is present. As previously mentioned, the polysaccharide is very likely starch, a molecule composed of Glu units, whereas GOS – in particular RFO – at most contain only one Glu residue. This explains the values of CFU/mL for extracts such as C-CW, C-BW, and L-BW (Figure 4), that showed low concentrations of mono- and disaccharides (as a whole) but whose chromatograms (Figure 2) exhibited a very prominent polysaccharide peak. Future investigations will delve further into this matter.

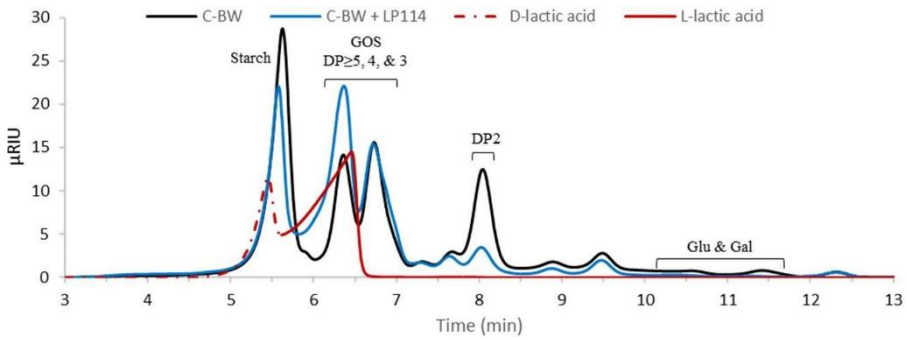

Figure 5 – HPLC-RI chromatograms of C-BW before and after the growth of *L. plantarum* CIDCA 83114 (LP114) in water for 24 h at 37 °C. A chromatogram obtained for a racemic mixture of D- and L-lactic acid is also shown.

After fermentation, the C-BW sample (sans the bacteria) was freeze-dried and analysed using FTIR (Figure 6, top). Although the spectrum is pretty much similar to that of the initial C-BW extract (Figure 3), it exhibited some new bands, which are denoted in Figure 6. The most noticeable change was the shoulder occurring at 1723 cm^{-1}, probably arising from a C=O stretching vibrational mode. Such a functional group is possibly due to the formation of L-lactic acid, as previously identified in the HPLC-RI analysis (Figure 5). The presence of L-lactic acid was further confirmed by spiking the initial C-BW extract powder with a small amount of L-lactic acid and analysing the mixture using FTIR (Figure 6, middle spectrum). As expected, an increase in some bands (corresponding to

L-lactic acid) was observed and such bands corresponded to those new bands previously observed for C-BW fermented with *L. plantarum* CIDCA 83114. These bands were definitely confirmed as belonging to L-lactic acid by analysing the spectrum of the pure compound (Figure 6, bottom), which resembled that found in the National Institute of Standard and Technology database [31].

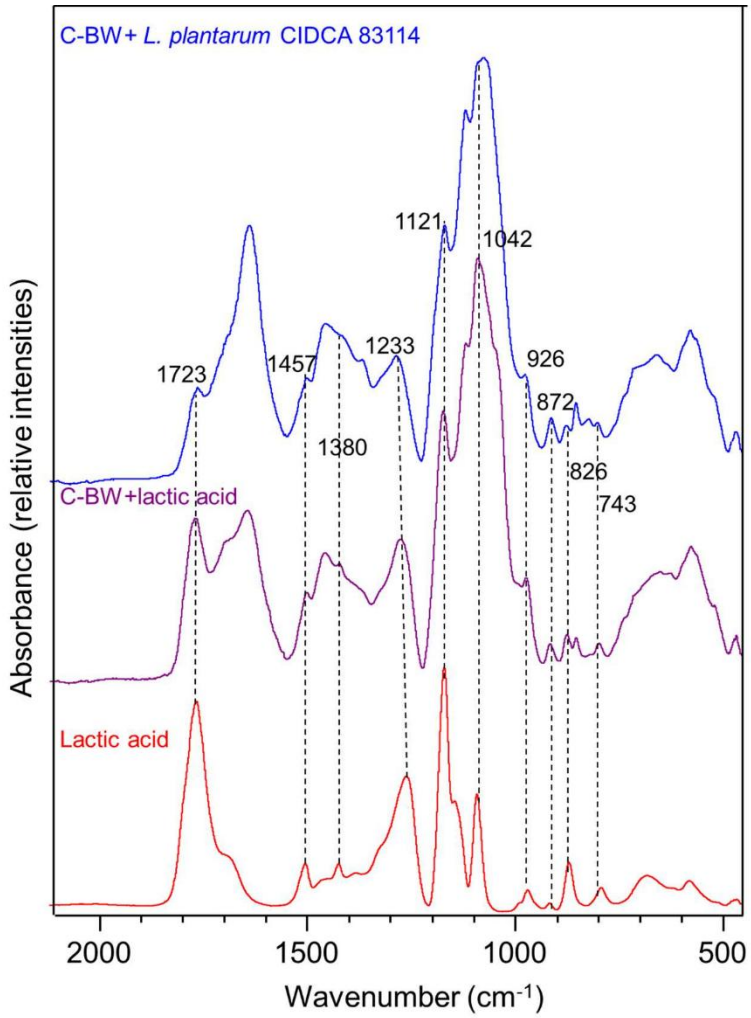

Figure 6 – FTIR spectra of C−BW after fermentation with *L. plantarum* CIDCA 83114 (top), of C−BW spiked with lactic acid (middle), and of lactic acid (bottom)

These results show that legume wastewaters, such as C-BW alone, have the nutritional requirements for growing *L. plantarum* CIDCA 83114, and GOS content does not change according to the fermentation process for this strain and GOS are still available at the end of the process. The fermentation of legume extracts with bacterial strains can therefore be an efficient purification method to remove mono-and disaccharides from α-GOS, before their employment in other industries and applications. If legume extracts

were to be incorporated in food products, the functionality of legume extracts could be improved by the addition of probiotic bacteria with beneficial effects for human health.

Conclusions

Legumes are a rich source of prebiotic compounds, such as GOS. Utilising the wastewaters produced during the soaking and cooking of chickpeas and lentils proved to be a cost-effective and efficient method for their extraction and recovery. Cooking chickpeas provided the highest GOS extraction yields, while lentils' cooking waters were the richest source of GOS-DP ≥ 5 compounds, with low concentrations of mono- and disaccharides. Microbial experiments carried out with *L. plantarum* CIDCA 83114 showed that legume wastewaters can be valorised and effectively repurposed as microbiological growth media for lactic acid bacteria, leading to less waste disposal. Considering that this strain consumed the monosaccharides and greatly diminished the DP = 2 carbohydrates present in the C-BW extract, its use could be further explored as a potential purification method for GOS mixtures.

The obtained results allow for the elucidation of the initial aspects of the use of wastewaters from the processing of legumes, in order to avoid environmental contamination and obtain compounds with prebiotic activity, mainly at the laboratory level. The simplicity of this approach and its ease of scalability make it highly suitable for future implementation by industrial stakeholders.

Author Contributions

Conceptualization: G.N.M., A.D.C., A.A.H., P.C.C. and A.G.-Z.; Data curation: G.N.M. and A.D.C.; Funding acquisition: P.C.C. and A.G.-Z.; Investigation: G.N.M., A.D.C. and A.A.H.; Methodology: G.N.M., A.D.C., A.A.H., P.C.C. and A.G.-Z.; Project administration: P.C.C. and A.G.-Z.; Writing: G.N.M. and A.D.C. Supervising: P.C.C. and A.G.-Z. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement

The data presented in this study are available on request from the corresponding author.

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Conflicts of Interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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Supplementary Materials

Supplementary Material 1 – S1

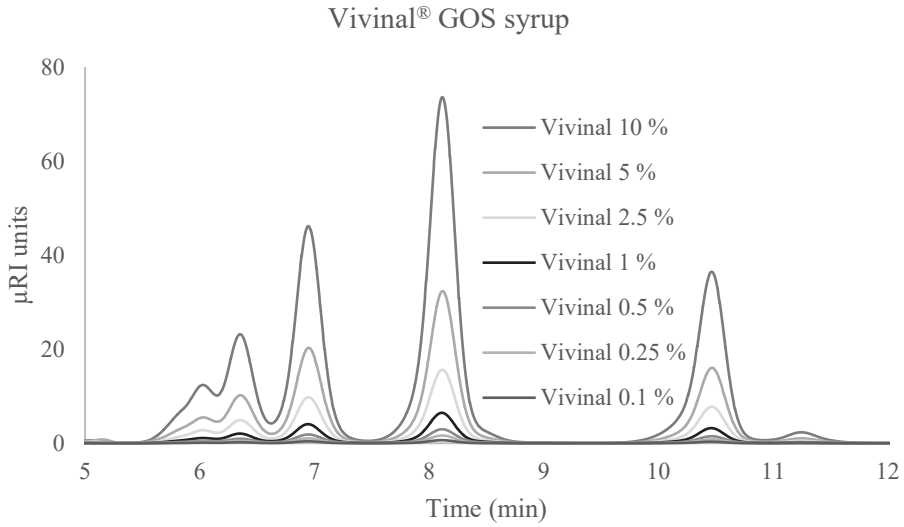
Composition of Vivinal® GOS syrup and HPLC-RI chromatogram, experimental conditions and the retention times, linearity, limit of detection (LOD), limit of quantification (LOQ), and intra- and inter-day precision parameters determined for the HPLC-RI analysis of different carbohydrate species.

A. Composition of Vivinal® GOS syrup as described by Friesland Campina DOMO (2017)[2]

	DP7[a]	DP6[a]	DP5[a]	DP4	DP3	Lac/DP2[b]	Glu	Gal	Total	Total GOS[c]
%	7.4			10.8	22.0	37.4	21.1	1.3	100.0	40.2

[a] GOS species with DP = 7, 6, and 5 were treated as one GOS species. [b] Lac present in Vivinal® GOS syrup was used in this work as a reference for other similar DP = 2 carbohydrates (*i.e.*, disaccharides) with the same retention time. [c] Total GOS indicate the sum of DP = 3 + DP = 4 + DP = 5 + DP = 6 + DP = 7 GOS.

B. Calibration of HPLC-RI method for the quantification of carbohydrates using solutions of Vivinal® GOS syrup at different concentrations

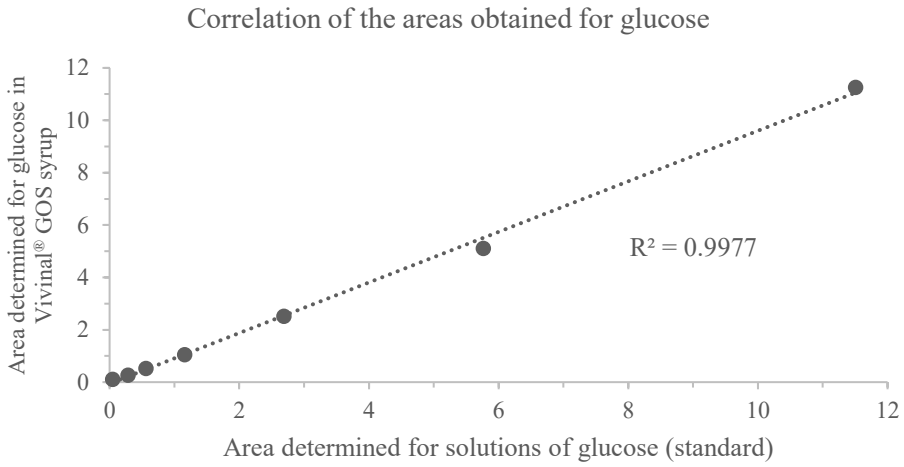


[2] Vivinal® GOS PT. A submission to the UK Food Standards Agency requesting consideration of Substantial Equivalence in accordance with Regulation (EC) No 258/97 concerning novel foods and novel food ingredients. (Non-Confidential Version). Available at: https://acnfp.food.gov.uk/sites/default/files/gos.pdf. Accessed: 13th May 2022.

Carbohydrate species			GOS-DP ≥ 5[a]		GOS-DP = 4		GOS-DP = 3		Lactose/DP = 2[b]		Glucose		Galactose	
Chromatographic run	Solvent		100 % degasified filtered ultrapure water											
	Time (min)		20											
	Temperature (°C)		80											
Injection volume (µL)			5											
RI detector's temperature (°C)			50											
Retention time (min)			5.9-6.2		6.2-6.5		6.9-7.1		7.9-8.1		10.4		11.2	
Linearity[c]	m	b	8.3	0.0	5.9	-0.1	5.8	-0.1	5.6	-0.1	5.3	-0.0	6.8	0.0
	R^2		0.9987		0.9976		0.9976		0.9488		0.9977		0.9995	
	Δ [ ], % (w/w)[c1]		0.007-0.736		0.011-1.076		0.022-2.202		0.037-3.742		0.021-2.112		0.001-0.131	
LOD[d]	3.3*SD/m (% [w/w])		0.0005											
LOQ[e]	10*SD/m (% [w/w])		0.0015											
Precision[f] (% RSD[f1])	Intra-day[f2]		8.8		8.8		8.8		8.8		8.8		8.8	
	Inter-day[f3]		14.4		14.6		14.6		14.3		14.4		15.0	

[a] Galacto-oligosaccharides (GOS) with degree of polymerization (DP) ≥ 5 were treated as one compound due to poor chromatographic resolution between these species' peaks. [b] Calibration for lactose present in Vivinal® GOS syrup was used as a reference for other similar DP = 2 carbohydrates (disaccharides) with the same retention time. [c] Calibration curves determined for each carbohydrate species present in Vivinal® GOS syrup. [c1] Δ [ ] = concentration range in % (w/w) of each calibration curve. [d] LOD (Limit of Detection) and [e] LOQ (Limit of Quantification) were calculated from the calibration curve of galactose by the "standard deviation of the response and the slope" method, in which *m* is the slope of the calibration curve and *SD* is the standard deviation of the area of the lowest point in the curve. The value calculated for galactose was chosen as representative for all carbohydrates since it is the sugar with the lowest concentration assayed. [f] Precision parameters were determined by the analysis of solutions of 1 and 10 % of Vivinal® GOS syrup (n = 6) and calculating the % of the [f1] RSD (Relative Standard Deviation) of the areas obtained for each analysis in the same day ([f2] intra-day) and in three non-consecutive days ([f3] inter-day) of analysis.

C. Correlation of the areas determined for glucose present in the Vivinal® GOS syrup solutions analysed and in solutions prepared using standard glucose (Riedel-de Haën) at the same concentrations found in the Vivinal® GOS syrup solutions



Concentrations of								
Vivinal® GOS syrup solutions	% (w/w)	0.1	0.25	0.5	1	2.5	5	10
Glucose in Vivinal® GOS syrup solutions	% (w/w)	0.02	0.05	0.11	0.21	0.53	1.06	2.11
	g/L	0.2	0.5	1.1	2.1	5.3	10.6	21.1
Standard glucose solutions	g/L	0.2	0.5	1.1	2.1	5.3	10.6	21.1

D. A calibration curve for fructose (Merck) was determined as y = 1.4× + 0.0 (R^2 = 0.9990). Its retention time was attributed to be 11.4 min (Figueira, 2020)[3]

[3] Figueira, O. A. da S. (2020). Profile analysis of oligosaccharides in yacon (*Smallanthus sonchifolius*) roots - extraction optimization and inulin hydrolysis [Universidade da Madeira]. http://hdl.handle.net/10400.13/3143

Supplementary Material 2 – S2

Composition of the de Man, Rogosa, and Sharpe medium prepared without glucose.

Component	Concentration	Brand
Peptone	20 g/L	Biokar (France)
Yeast extract	5 g/L	Oxoid (United Kingdom)
Dipotassium hydrogen phosphate	2 g/L	J. T. Baker (USA)
Sodium acetate trihydrate	5 g/L	Anedra (Argentina)
Triammonium citrate	2 g/L	Mallinckrodt (USA)
Magnesium sulphate heptahydrate	0.2 g/L	ICN Biomedicals (USA)
Manganese sulphate tetrahydrate	0.05 g/L	Sigma (USA)
Tween 80	1 mL for 1 L	Biopack (Argentina)

ii. Use of Legume Wastewater Extracts on the Storage Stability of Freeze-Dried *Lactiplantibacillus plantarum* WCFS1

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RESEARCH

Use of Legume Wastewater Extracts on the Storage Stability of Freeze-Dried *Lactiplantibacillus plantarum* WCFS1

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Abstract

Chickpeas and lentils contain prebiotic carbohydrates, including galacto-oligosaccharides (GOS), that confer health benefits but can also lead to undesirable effects like bloating and flatulence. Legume processing reduces these disadvantages and usually consists of the soaking and cooking of the legume seeds. In their industrial production, the processing water is considered waste. As different carbohydrates have recognized stabilization properties on lactic acid bacteria during dehydration processes, this study aimed to investigate the protective effect of GOS-containing wastewater extracts from cooking chickpeas and lentils, as well as fructo-oligosaccharides, and sucrose (used for comparative purposes), on the stability of freeze-dried *Lactiplantibacillus plantarum* WCFS1, by measuring biological (culturability, storage stability, acidifying activity), chemical (pH), and physical (water content) properties. The best storage stability was observed for mixtures with GOS-containing wastewater legume extracts. Protection of lactic acid bacteria strains with GOS-containing wastewater legume extracts would limit their spoilage in food production, positively impacting the environment.

Keywords

Freeze-drying, galacto-oligosaccharides, lactic acid bacteria, pulses, viability, wastewaters

Highlights

• Legume wastewaters were used for protecting *Lactiplantibacillus plantarum* WCFS1

• Wastewaters containing GOS were better than FOS and sucrose

• *Lactiplantibacillus plantarum* WCFS1 survived in storage at 37 °C for 3 weeks

• Boiling legumes without soaking provided the best protective compounds

Introduction

Chickpeas (*Cicer arietinum* L.) and lentils (*Lens culinaris Medikus* var. *variabilis*) are two of the most important legume crops worldwide [1, 2]. Traditionally, they have been consumed in developing countries, where protein-energy malnutrition is prevalent because they are a staple food and a low-cost source of protein. However, in recent years, the inclusion of these legumes in daily diet has grown significantly in North America, Australia, and many European countries, mainly due as a result of consumers increasingly healthy and balanced diet choices [2-5].

Chickpeas and lentils have a high nutritional quality and are a good source of carbohydrates and proteins, together constituting about 80% of the total dry seed mass [5]. Legumes' carbohydrates contain a wide range of prebiotic substances including galacto-oligosaccharides (GOS), particularly from the raffinose family of oligosaccharides, and fructo-oligosaccharides (FOS) [6, 7]. These prebiotic carbohydrates are selectively utilized by the host microorganisms and fermented into active metabolites (short-chain fatty acids, branched-chain fatty acids, vitamins, bile acid derivatives) conferring significant human health profits [6, 8]. Benefits of legumes include treating and preventing constipation, controlling cholesterol levels, and reducing the risk of obesity, diabetes, and certain types of cancer [4, 7, 9]. Despite these known benefits, chickpeas and lentils contain several compounds that have "anti-nutritional" activity (phytic acid, tannic acid), inhibit mineral absorption, have a bitter taste, increase cooking time, or are non-digestible (α-GOS) and can cause bloating and flatulence [2, 10, 11]. In order to reduce the undesirable attributes of chickpeas and lentils, as well as to increase the nutrients' availability and improve their organoleptic characteristics, legume processing methods are applied [11, 12].

Legume processing frequently consists of two main steps: soaking and cooking. Soaking increases hydration, softening the seed coat, and making it easier for water to penetrate during cooking, allowing for faster cooking. The soaked seeds can then be cooked in different ways, but boiling is the easiest and most common method. Cooking improves palatability causing textural changes and chemical reactions that produce volatile compounds important for flavor development [13, 14]. In the industrial production of legumes, the water resulting from their processing is considered waste and known as *aquafaba* or *liluva*. While aquafaba refers to chickpea cooking water, liluva consists of water from different legumes and different processing methods [15]. The global legume market (chickpeas, lentils, and other grains) represented a CAGR 11.5 Billion US dollars in 2022 [16]. Also in 2022, chickpeas and lentil production was around 18.1 [17] and 6.7 [18] million metric tons, respectively. Given a joint chickpea and lentil production of 24.7 million metric tons, assuming 1/10 of these legumes are sold pre-cooked or canned (2.47 million metric tons), and a standard 1:4 grain to water ratio is used in their cooking, a rough estimate of the amount of cooking wastewater produced would be 9.88 million metric tons, or around 10,000 million liters. Recycling legume wastewater would limit their wastage, positively impacting the environment [14, 15]. Several studies have shown the potential of legume wastewater in food applications as texture improvers (foaming agents, gel lingers, thickeners) and as a source of prebiotics. In addition, these waters have a high content of proteins, soluble and insoluble carbohydrates, saponins, and minerals [19-22].

Lactiplantibacillus plantarum is a lactic acid bacterium with a large and flexible genome that helps it adapt to diverse habitats including dairy products, fruits, vegetables, sausages, the digestive tract/feces of animals and humans, and soil. For decades, *L. plantarum* strains have been used as starter cultures in the production of fermented products because of their organoleptic properties and as probiotic cultures because of their beneficial properties such as antimicrobial activity, immunological effects, and modulation of the intestinal microbiota [23, 26]. Freeze-drying is a standard method for preserving probiotic strains facilitating their transportation and storage. However, it exposes the strains to environmental changes that can lead to structural cell damages, or even their death [27, 28]. A recent review by Penha Rodrigues Pereira et al. (2024) describes the main challenges faced in increasing probiotic survival and how to tackle them [29]. To limit the encountered stresses' potential negative effects during the freeze-

drying process and to improve the survival of bacteria after storage, the addition of protective molecules to the cell concentrates is an effective strategy [30, 31]. Sugars such as sucrose and prebiotics such as FOS and GOS have been demonstrated to be efficient protective compounds of lactic acid bacteria after freeze-drying and storage stages [32-34]. They are commonly used to preserve probiotic strains because they can replace water molecules removed during drying, thus maintaining membrane integrity. Additionally, they have the ability to form glassy states with high viscosity and low molecular mobility, which limits diffusive damaging reactions [32-36].

It was recently reported that *L. plantarum* CIDCA 83114 was able to grow in a culture medium prepared with GOS-containing wastewater legume extracts [37]. In addition, the wastewater from cooking dry chickpeas was capable of protecting *Lactobacillus delbrueckii* subsp. *bulgaricus* CFL1 during freeze-drying and storage by forming a protective glassy matrix surrounding the bacterium [38]. In this work, we intend to continue studying the applicability of these waste products, namely the cooking wastewaters obtained from lentil seeds. Four GOS-containing wastewater extracts were obtained by applying different procedures to chickpeas and lentils and were repurposed for the protection of lactic acid bacteria during freeze-drying and storage. The GOS mixtures' protective capability over *L. plantarum* WCFS1 during the freeze-drying and storage was studied by determining the biological activity (culturability, storage stability, acidifying activity) and chemical (pH) and physical (water content) properties of the cell concentrates.

Materials and Methods

The experimental approach used in this study and the activities measured are shown in Figure 1.

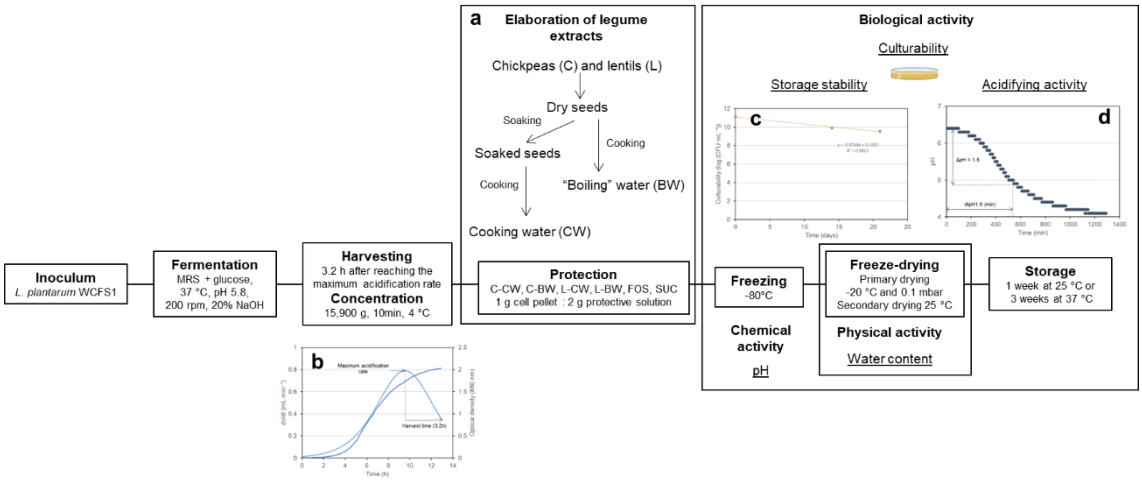

Figure 1 – Diagram of the experimental approach used in this study and showing the activities measured. (a) Experimental procedures for the production of GOS-containing wastewater extracts. (b) Cell growth and acidifying activity of *L. plantarum* WCFS1 showing when the cells were harvested. (c) Method used for determining the storage stability of *L. plantarum* WCFS1 using the slope value of the linear regression. (d) Method used for determining the acidifying activity of *L. plantarum* WCFS1 using the time necessary for a pH drop of 1.5 units. C-CW, chickpea cooking water; C-BW, chickpea boiling water; L-CW, lentil cooking water; L-BW, lentil boiling water; FOS, fructo-oligosaccharide; SUC, sucrose

Chickpeas and Lentil Seeds' Wastewaters

Production

Chickpea and lentil seeds were provided by Continente (Matosinhos, Portugal) and Don Elio (INTA Arroyo Seco, Argentina), respectively. The seeds were kept at 25 °C in dry conditions throughout the making of this work. GOS-containing wastewater extracts were prepared as described previously by Martins et al. (2023) [37]. Briefly, cooking waters were produced from raw and soaked chickpeas and lentil seeds in a 1:5 seeds-to-water mass ratio and cooked for 30 min (Figure 1a). The supernatants were separated and freeze-dried. In total, four extracts were produced: chickpea cooking water (C-CW), chickpea "boiling" water (C-BW), lentil cooking water (L-CW), and lentil "boiling" water (L-BW).

High-Performance Liquid Chromatography

The carbohydrate content of the produced legume extracts was determined by high-performance liquid chromatography with refractive index detection as described previously by Martins et al. (2023) and may be found in Table 1 [37].

Glass Transition Temperature

The glass transition temperature of the freeze-dried GOS mixtures (*Tg*, in °C) was determined by differential scanning calorimetry (DSC) using a Q100 calorimeter calibrated with indium, lead, and zinc (TA Instruments-Waters LLC, New Castle, DE, USA). Samples were equilibrated at 10% relative humidity (RH) with a saturated solution of NaOH. Equilibration lasted *ca.* 2 weeks. Five milligrams of each sample was weighed in gold capsules and hermetically sealed. An empty pan was used as a reference. The thermal history of each sample was removed, and then the samples were heated from -60 to 75 °C with a heating ramp of 10 °C/min. Measures were taken in duplicate.

Table 1 – Carbohydrate composition and *Tg* values of the GOS-containing legume extracts, the commercial FOS sample and Suc used in this work as determined by [a] Martins et al. (2023) [33], [b] Romano et al. (2016) [37], and [c] in this work. C-CW: chickpea cooking water; C-BW: chickpea boiling water; L-CW: lentil cooking water; L-BW: lentil boiling water; FOS: fructo-oligosaccharide; DP: degree of polymerization

		GOS				FOS	Suc
		C-CW	C-BW	L-CW	L-BW	(BeneoOrafti)	
% (g/100 g of dry matter)	Monosaccharides	5[a]	4[a]	2[a]	3[a]	3[b]	0[b]
	Disaccharides	18[a]	18[a]	4[a]	4[a]	5[b]	100[b]
	DP=3	23[a]	21[a]	11[a]	10[a]	25[b]	0[b]
	DP=4	14[a]	14[a]	15[a]	13[a]	29[b]	0[b]
	DP≥5	2[a]	2[a]	8[a]	6[a]	38[b]	0[b]
	Total oligosaccharides	39[a]	37[a]	34[a]	29[a]	92[b]	0[b]
Tg (°C)		23.33[c]	41.00[c]	47.83[c]	40.22[c]	46.92[b]	40.28[b]

Production and Storage of *L. plantarum* WCFS1 Concentrates

Bacterial Strain and Culture Conditions

The strain used in this study was *L. plantarum* WCFS1 (NIZO Food Research B.V., The Netherlands). Twenty-five microliters of stock culture stored at -80 °C was thawed at 40 °C for 5 min before inoculating in 10 mL of MRS broth (Scharlau Microbiology; Barcelona, Spain). This pre-culture was incubated at 37 °C for 18 h, and then 80 µL was used to inoculate a second pre-culture containing 10 mL of MRS broth.

The second pre-culture was incubated at 37 °C for 10 h, after which 10 mL was used to inoculate a 5-L bioreactor (Biostat® B Type 8,840,326, B. Braun Biotech International GmbH; Melsungen, Germany) containing 4.99 L of culture medium.

The culture medium, sterilized at 121 °C for 20 min, was composed of MRS broth supplemented with 40 $g \cdot L^{-1}$ glucose (ADM; Chicago, IL, USA) to avoid starvation stress caused by the depletion of the carbon source after reaching the stationary growth phase. Fermentation was carried out at 37 °C and pH 5.8, which was controlled by the automatic addition of a 20% (w/v) NaOH solution. The stirring was set at 200 rpm to ensure the culture homogenization.

Cell growth and acidifying activity were monitored throughout fermentation. Cell growth was monitored by an infra-red probe (Excell210; CellD, Roquemaure, France), measuring absorbance at 880 nm. Acidifying activity was determined according to the volume of NaOH solution injected into the bioreactor during fermentation to maintain a controlled pH. The maximum acidifying rate (dv/dt, in $mL \cdot min^{-1}$) was determined as the peak of the first derivative of the NaOH consumption curve. Cells were harvested 3.2 h after reaching the maximum acidifying rate (Figure 1b), corresponding to the early stationary growth phase. Three fermentations were carried out in order to consider biological replicates.

Concentration and Protection Conditions

Cell suspensions were concentrated by centrifugation (Avanti® JXN-30 centrifuge, Beckman Coulter; CA, USA) at 15,900 g for 10 min at 4 °C. Cell pellets were resuspended in protective solutions at 23% (w/w) dry matter, using a 1:2 cell pellets-to-protective solution weight ratio. The protective solutions were prepared at 20% (w/w) in 8.9 $g \cdot L^{-1}$ NaCl and sterilized by autoclaving at 121 °C for 20 min. Six protective agents were evaluated: the four GOS-containing wastewater extracts produced as described previously by Martins et al. (2023) [37], a commercial FOS sample (Orafti®P95; Beneo Orafti; Tienen, Belgium), and sucrose (Azucarera; Madrid, Spain) (SUC), whose composition and *Tg* values is in Table 1**.** Freeze-dried cells in the presence of sucrose were included for comparative purposes as they represent the typical formulation for long-term storage of lactic acid bacteria.

Stabilization and Storage Conditions

Protected cell concentrates were frozen at -80 °C for 24 h in cryotubes. The cryotubes were then transferred to pre-cooled shelves at -45 °C in a pilot-scale freeze-dryer (VirTis Genesis 35 L SQ EL-85, SP Scientific; Warminster, PA, USA). The primary drying was carried out at a temperature of -20 °C and at a chamber pressure of 0.1 mbar and the secondary drying at a temperature of 25 °C. The freeze-dried samples were stored for 1 week at 25 °C or for 3 weeks at 37 °C. Aliquots of the protected cell concentrates were also maintained in cryotubes at -80 °C for analyzing the frozen samples.

The process from production to storage of *L. plantarum* WCFS1 concentrates was repeated three times (three biological replicates). In addition, each measurement (culturability, storage stability, acidifying activity, pH, and water content) was measured in triplicate (three technical replicates).

Biological Activity of *L. plantarum* WCFS1 Concentrates

Biological activity measurements were carried out before and after freezing, after freeze-drying, after 1 week of storage at 25 °C, and after 2 and 3 weeks of storage at 37 °C. Before analysis, frozen samples were thawed at room temperature for 5 min, and freeze-dried samples were rehydrated using the same volume of 8.9 $g \cdot L^{-1}$ NaCl present before freeze-drying and incubated for 30 min at 37 °C.

Culturability Measurements

Cell culturability was determined using the agar plate count method. Fresh, thawed, and rehydrated cell suspensions were serially diluted in 8.9 $g \cdot L^{-1}$ NaCl, and 100 µL of the appropriate dilutions was spread into MRS agar plates (VWR International Eurolab; Barcelona, Spain) in triplicate (three technical replicates). Colonies were enumerated (Scan® 500, Interscience; Saint Nom la Bretèche, France) after incubating the plates for 48 h at 37 °C in anaerobic conditions. Plates containing between 30 and 300 colonies were kept for cell concentration evaluation. Culturability measurements were expressed in log ($CFU \cdot mL^{-1}$).

Accelerated Storage Stability Test

Storage stability was evaluated from the culturability loss rate during 3 weeks of storage at 37 °C (Figure 1c), as previously described by Guerrero Sanchez et al. (2022) [31]. For each protective formulation and biological replicate, the logarithmic value of the cell count (log (CFU·mL^{-1})) was plotted as a function of the storage time (*t*, in days). The experimental values were fitted using the following equation:

$$\text{Cell count (log } (CFU \cdot \text{mL}^{-1})) = -K \times t \text{ (days)} + b$$

where the inactivation rate constant *K* (in log (CFU·mL^{-1})·days^{-1}) refers to the absolute value of the slope of the linear regression. The lower the absolute value of the slope *K*, the higher the storage stability was.

Acidifying Activity

The Cinac® system (AMS Alliance; Frépillon, France) was used to measure the acidifying activity of the bacterial suspensions according to the procedure described by Spinnler and Corrieu (1989) [39]. The measurements were performed in a 37 °C water bath. The growth medium composed of 10 g·L^{-1} glucose and 10 g·L^{-1} yeast extract (Condalab; Madrid, Spain) was sterilized at 121 °C for 20 min before being distributed into 250 mL PYREX® round bottle. Each bottle contained 150 mL of medium and was inoculated with 100 µL of fresh, thawed, or rehydrated cell suspensions diluted 10 times in 8.9 g·L^{-1} NaCl. For each sample, the acidifying activity was characterized as the time necessary for a pH drop of 1.5 units (dtpH 1.5, in min). The lower the value of the dtpH 1.5 was, the higher the acidifying activity was (Figure 1d).

Chemical and Physical Properties of *L. plantarum* WCFS1 Concentrates

pH

The pH of *L. plantarum* WCFS1 concentrates was measured at room temperature using a portable pH meter (LAQUA PH1100, Horiba Scientific®; Tokyo, Japan), on freeze, thawed and rehydrated freeze-dried samples prepared according to the procedure

described above in the section of biological activity ("Biological Activity of *L. plantarum* WCFS1 Concentrates" section).

Water Content

The Karl Fisher titration method was used to measure the moisture content of the samples, using a Metrohom KF 756 apparatus (Metrohm AG; Herisau, Switzerland). At least 20 mg of powder was mixed with 3 mL of dry methanol and titrated with hydranal coulomat AG (Honeywell Fluka; Seelze, Germany) until the end point was reached. Measurements were carried out after freeze-drying.

Statistical Analyses

The nonparametric Kruskal–Wallis test and the post hoc Conover Iman test with Bonferroni correction were performed using XLSTAT 19.6 (Addinsoft; Paris, France) to compare data concerning biological and physical activities. A significance level of 95% (p-value < 0.05) was considered.

Results and Discussion

Effect of Protective Formulation on the Survival of *L. plantarum* WCFS1 After Stabilization Processes

Compared to its state before freezing, the biological activity of *L. plantarum* WCFS1 concentrates slightly decreased after the freezing and freeze-drying stages of the production process. This decrease was observed regardless of the protective formulation used, as determined by their culturability and acidifying activity (Figure 2 $\Delta < 6\%$ and Figure 3 $\Delta \leq 15\%$, respectively). No statistical differences in culturability and acidifying activity were observed after these two stages among the protective formulations used (Online Resource 1 and 2). During these processes, microorganisms are exposed to several environmental stresses (thermal, osmotic, mechanical, oxidative) that can cause cell membrane damage, and protein and DNA denaturation, resulting in cell viability and activity loss. However, depending on the bacterial strain, the protective formulation used,

and the stress magnitude, the resistance to freezing and freeze-drying processes can strongly differ [28, 40, 41]. Protective agents play an important role in the conservation of biological activity. It had previously been demonstrated that sucrose, FOS, and GOS are effective protective compounds for lactic acid bacteria during production processes [33, 34]. In addition, no clear differences in culturability were observed after freeze-drying of *Lactobacillus delbrueckii* ssp. *bulgaricus* CIDCA 333 cells protected with two different GOS preparations [34]. The protective capability of sugars and prebiotics on the stabilization of lactic acid bacteria during their freeze-drying and subsequent storage has been mainly associated with two accepted hypotheses. One of them proposes that sugars can replace the water between lipid headgroups when it is removed during drying, maintaining the membrane integrity. The other one is based on sugars capacity to form glassy matrices in which the high viscosity and low molecular mobility constraints molecular interactions [33-36, 42].

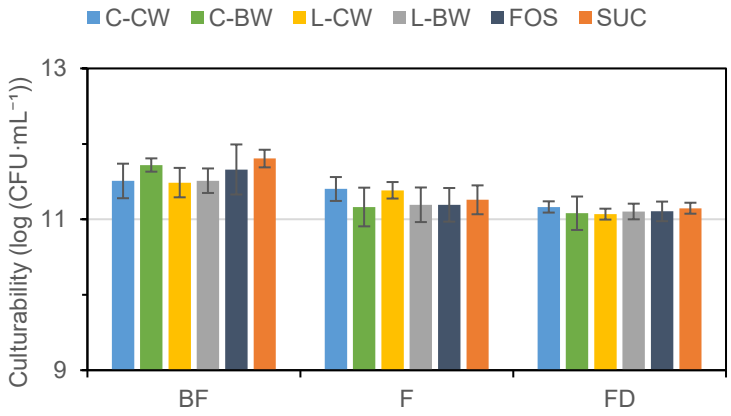

Figure 2 – Biological activity as determined by the culturability (log (CFU·mL^{-1})) of *L. plantarum* WCFS1 concentrates protected with six different protective formulations at different steps of the production process: before freezing (BF), after freezing (F), and after freeze-drying (FD). C-CW: chickpea cooking water; C-BW: chickpea boiling water; L-CW: lentil cooking water; L-BW: lentil boiling water; FOS: fructo-oligosaccharide; SUC: sucrose

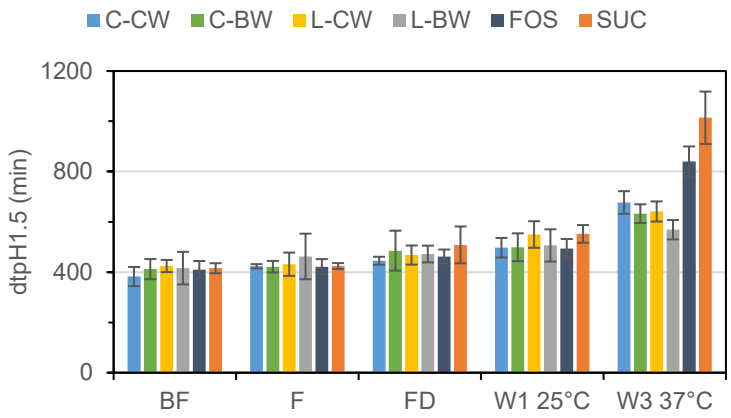

Figure 3 – Acidifying activity, characterized as the time necessary for a pH drop of 1.5 units (dtpH 1.5, in min), of *L. plantarum* WCFS1 concentrates protected with six different protective formulations at different steps of the production process: before freezing (BF), and after freezing (F), freeze-drying (FD), one week of storage at 25 °C (W1 25°C), and three weeks of storage at 37 °C (W3 37°C). C-CW: chickpea cooking water; C-BW: chickpea boiling water; L-CW: lentil cooking water; L-BW: lentil boiling water; FOS: fructo-oligosaccharide; SUC: sucrose

The pH of the *L. plantarum* WCFS1 concentrates was determined after freezing and freeze-drying (Table 2). During these first two stages of the production process, the pH value was maintained. The protective formulations involving FOS and SUC presented the lowest pH (3.8 pH units of average) compared to those involving GOS-containing legume extracts (4.6 pH units of average). During freezing, the hydrogen ions are concentrated as the available liquid water decreases causing an acid shock to the cells. To minimize this shock, the cell concentrates can be neutralized before freezing. However, neutralization of *Bifidobacterium animalis* ssp. *lactis* biomass did not have a positive effect on freeze-drying survival [43]. This was also noted in this work, as the differences in pH of the *L. plantarum* WCFS1 concentrates protected with GOS extracts (less acidic) or with FOS/SUC (more acidic) did not result in a significant change in the cells' biological activity as determined by their culturability and acidifying activity after freezing and freeze-drying.

Table 2 – pH value of *L. plantarum* WCFS1 concentrates protected with six different protective formulations after freezing (F), freeze-drying (FD), one week of storage at 25 °C (W1 25°C), two weeks of storage at 37 °C (W2 37°C), and three weeks of storage at 37 °C (W3 37°C). Data represented are average and standard deviation at least two biological replicates. C-CW: chickpea cooking water; C-BW: chickpea boiling water; L-CW: lentil cooking water; L-BW: lentil boiling water; FOS: fructo-oligosaccharide; SUC: sucrose

Protective formulation		C-CW	C-BW	L-CW	L-BW	FOS	SUC
pH	F	4.43 ± 0.11	4.60 ± 0.16	4.49 ± 0.13	4.43 ± 0.16	3.72 ± 0.04	4.05 ± 0.06
	FD	4.60 ± 0.16	4.72 ± 0.10	4.57 ± 0.03	4.59 ± 0.06	3.65 ± 0.04	3.98 ± 0.29
	W1 25 °C	4.58 ± 0.24	4.71 ± 0.10	4.58 ± 0.15	4.62 ± 0.12	3.73 ± 0.07	3.99 ± 0.23
	W2 37 °C	4.55 ± 0.16	4.64 ± 0.09	4.59 ± 0.09	4.55 ± 0.15	3.90 ± 0.03	4.07 ± 0.14
	W3 37 °C	4.57 ± 0.21	4.55 ± 0.08	4.53 ± 0.15	4.47 ± 0.13	3.93 ± 0.07	4.08 ± 0.14

The survival of dried bacteria is affected by moisture content. Removing the whole water from the cells (0% moisture content) during freeze-drying is harmful to survival as it results in damage to cell proteins. Therefore, a certain amount of water (hydration water) must remain in a dehydrated state for a satisfactory survival rate [44]. However, a high moisture content leads to a decrease in *Tg* resulting in a higher *T-Tg* value (*T* being the storage temperature), which in turn causes a decrease in bacterial viability. When the *Tg* is below the storage temperature (*T-Tg* > 0), the samples change from a glassy stable state into a viscous unstable state (rubbery state), where the mobility of molecules increases. A higher molecular mobility leads to loss of bacterial viability and a decrease of the shelf life of the stored powder [27, 31, 45]. Moisture contents of *L. plantarum* WCFS1 concentrates after freeze-drying with the different protective formulations were in the range of 1.82–3.87% (Table 3). Our results are comparable to those reported for *L. plantarum* TISTR 2075 (1.38–3.83% [46]), *Ligilactobacillus salivarius* subsp. *salivarius* (UCC500) freeze-dried with various protective agents (2.8 to 5.6% [44]), *L. plantarum* NCIMB 8826 freeze-dried in alginate microcapsules coated with chitosan (< 10% [47]), and *L. delbrueckii* ssp. *bulgaricus* CFL1 freeze-dried in a sucrose matrix (2.5 to 3.7% [45]). Therefore, water content in the range of 1.3–5% appears to lead to better bacterial survival after the freeze-drying process. The optimal water content value of a dried bacterial sample is determined by the nature of the protective formulation used and the storage temperature.

Table 3 – Moisture content (%) of freeze-dried *L. plantarum* WCFS1 concentrates protected with six different protective formulations. Data represented are average and standard deviation of one-three biological replicates and at least two technical replicates. No statistical differences (at the 95 % confidence level) were observed between all samples (six formulations) after freeze-drying. C-CW: chickpea cooking water; C-BW: chickpea boiling water; L-CW: lentil cooking water; L-BW: lentil boiling water; FOS: fructo-oligosaccharide; SUC: sucrose

Protective formulation	C-CW	C-BW	L-CW	L-BW	FOS	SUC
Moisture content (%)	3.87 ± 0.19	2.92 ± 1.04	1.82 ± 1.20	2.74 ± 1.03	3.30 ± 1.25	3.85 ± 1.43

Effect of Protective Formulation on the Survival of *L. plantarum* WCFS1 After Storage

After freeze-drying, protected *L. plantarum* WCFS1 cells were stored at two different temperatures (25 °C and 37 °C) for 7 and 28 days, respectively.

In the case of storage at 25 °C, biological activity slightly decreased for all protective formulations after 1 week of storage compared to the levels observed after the freeze-drying stage ($\Delta < 2.5\%$ in culturability and $\Delta < 13.5\%$ in acidifying activity). In addition, no statistical differences were observed among the different protective formulations (Online Resource 1 and 2). After 3 weeks of storage at 37 °C, biological activity abruptly decreased for all protective formulations as compared to that observed after the freeze-drying stage ($\Delta < 16.5\%$ in culturability and $\Delta < 52.5\%$ in acidifying activity), and GOS extracts' formulations were significantly better cell protectants than SUC's and FOS' (Online Resource 1 and 2). During storage, the culturability of freeze-dried bacteria tended to decrease. Water content, storage temperature, and exposure to oxygen are some of the factors that can affect the viability of dried microorganisms. Low storage temperatures lead to the highest bacterial survival because in these conditions, chemical reactions responsible for cell damage are slowed down [48, 49]. The biological activity of *L. plantarum* WCFS1 cells decreased with the increasing storage temperature (from 25 to 37 °C). The decrease in viability during storage of freeze-dried lactic acid bacteria has been related to membrane damage caused by lipid oxidation [47, 50, 51]. However, the loss of membrane integrity and lipid oxidation are not the only detrimental reactions which can occur during storage; the drastic loss of culturability during storage at 37 °C of *L. salivarius* CECT5713 was associated with damage to proteins, nucleic acids, and peptidoglycans of the cell wall [52].

With regard to the culturability results, the absolute slope ($|K|$) values were determined, based on the CFU·mL^{-1} calculated during the 3 weeks of storage at 37 °C, as depicted in Figure 4. The lowest $|K|$ values were obtained for the protective formulations involving GOS-containing legume extracts, showcasing a higher protection of *L. plantarum* cells. The four GOS extract formulations provided the highest protection with similar results, with no statistical differences between legumes (chickpea and lentil) or treatments used to obtain the GOS extracts (cooking and boiling). In addition, no statistical differences between FOS and SUC samples were observed (Online Resource1).

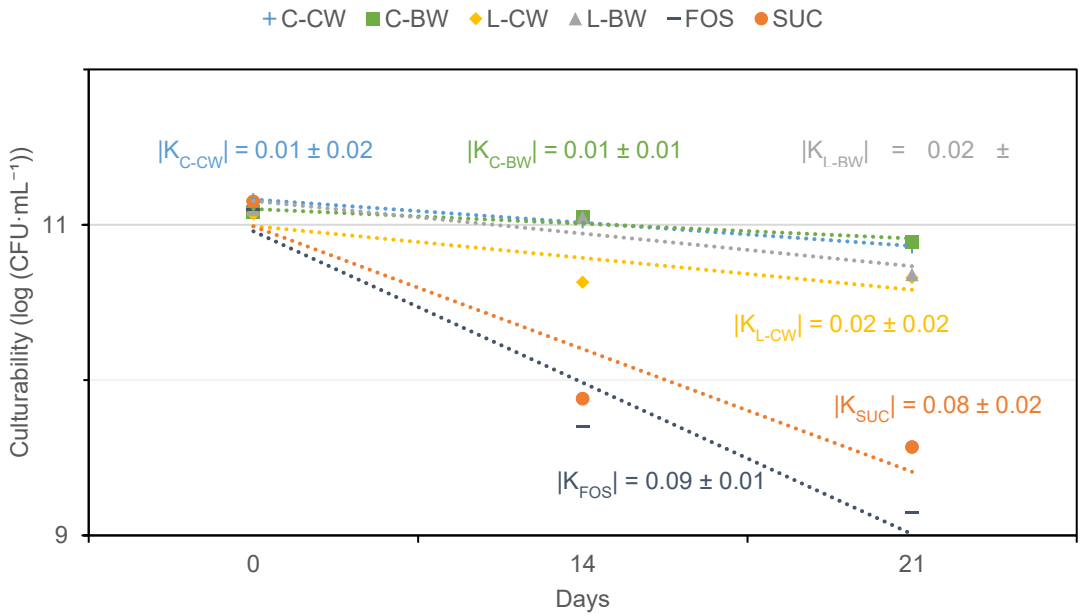

Figure 4 – Culturability (log (CFU·mL^{-1})) of *L. plantarum* WCFS1 concentrates protected with six different protective formulations during 3 weeks of storage at 37 °C showing the absolute value of the slope of the linear regression (|K|). C-CW: chickpea cooking water; C-BW: chickpea boiling water; L-CW: lentil cooking water; L-BW: lentil boiling water; FOS: fructo-oligosaccharide; SUC: sucrose

The acidifying activity results (Figure 3) were in line with the culturability assays, in which the protective formulations involving FOS and SUC were confirmed to have conferred the lowest protection towards *L. plantarum* cells, by showing very pronounced losses of acidifying activity (with no statistical differences between the two samples). GOS extracts yielded the lowest dtpH 1.5 values, with the most successful protective formulation being L-BW, followed by C-BW, L-CW, and C-CW (Online Resource 2). L-BW and C-BW being the sources of the most successful protective formulations suggest that the boiling treatment used to obtain these GOS extracts is the preferred extraction

procedure, providing equal or better results to the cooking counterparts but with less environmental impact, since the soaking of the seeds can be dismissed. The observed results may be explained by a combination of factors. In the present study, average values of *T-Tg* were as follows: 13.7 °C (C-CW), -4.0 °C (C-BW), -10.8 °C (L-CW), and -3.2 °C (L-BW), as calculated from *Tg* values found in Table 1, with T = 37 °C, i.e., storage temperature. Examples of *Tg* curves obtained for each wastewater extract are shown in Online Resource 3. Tymczyszyn et al. (2012) obtained similar results of *Tg* for commercial GOS at 11% RH [53]. All wastewaters obtained *Tg* above 37 °C (except for C-CW), which can be useful for these formulations to help the preservation of the bacterial structure [54, 55]. *Tg* of commercial FOS is 46.92 °C, while *Tg* of sucrose is 40.28 °C (Table 1), results that are similar to those obtained for the wastewaters [33, 37]. The *Tg* results of the present study contribute to the understanding of the correct preservation of the bacteria achieved by the use of legume wastewaters; however, they do not explain the differences observed in terms of culturability, with respect to the other protective samples. Some authors suggest that the presence of mono- and polysaccharides could be useful in protective formulations [33, 54, 56]. In previous work, starch was found in great amounts in the produced GOS extracts (as determined by HPLC-RI and colorimetric tests with iodine [37], whereas it is not present in neither the FOS nor SUC formulations. Li et al. (2016) used gelatinized starch for the entrapment of lactic acid bacteria, finding that this material protected against different conditions. The starch is not the most studied material for its protective and encapsulant properties. However, it would be interesting to evaluate the usefulness of this component in the wastewaters of legumes, since they constitute a big part of these foods. Polysaccharides have been shown to increase *L. plantarum* WCFS1's resistance to freeze-drying by improving cell membrane integrity synergistically with low molecular weight cryoprotectants [57]. Jawan et al. (2022) also observed that combinations of cryoprotectants, including galactose and trehalose, increased the survivability of *Lactobacillus delbrueckii* ssp. *lactis* Gh1 during freeze-drying and storage at different temperatures [58]. However, in the same study, the complex mixture of skim milk did not fare so well in the bacterial preservation. In contrast, Zayed and Roos (2004) reported that the stabilization of *L. salivarius* subsp. *salivarius* (UCC 500) was enhanced by the combination of various cryoprotective compounds, such as the skim milk mixture [44]. The protective effect in that case was attributed to skim milk containing buffering agents such as proteins and minerals, besides cryoprotective compounds. These compounds could possibly also be found in GOS

extracts from the lixiviation of the legumes during soaking and cooking [14], which may explain the higher pH values observed during the whole production process, including storage (Table 2). Principal component analysis (PCA) was performed with Python software using the culturability, acidifying activity, and pH data of the samples after 3 weeks in storage at 37 °C. The results show the separation of two groups along the PC1 axis, one with the GOS wastewaters and another with FOS and SUC (Figure 5). This separation is influenced by the pH of the protective formulations and explains the wastewater effectiveness in protecting the microorganisms in the accelerated storage test.

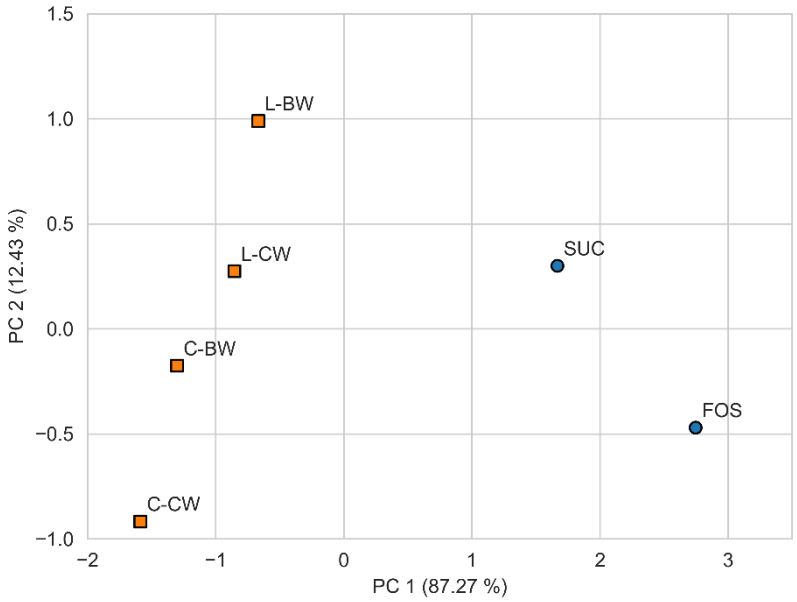

Figure 5 – PCA performed using the culturability, acidifying activity, and pH data of the samples after 3 weeks in storage at 37 °C. C-CW: chickpea cooking water; C-BW: chickpea boiling water; L-CW: lentil cooking water; L-BW: lentil boiling water; FOS: fructo-oligosaccharide; SUC: sucrose

GOS are composed of a variable number of galactose units, within 2 and 10. The type of linkage between units varies according to their origin and obtaining process. Plant-based GOS are α-GOS and are important components of seeds. The prebiotic effect of α-GOS is mainly associated with tri- and tetrasaccharides [7]. In addition to the prebiotic properties, the ability of GOS to act as protectants has been reported. In this work, the four GOS-containing wastewater legume extracts appeared as better protective formulations than fructo-oligosaccharides and sucrose for stabilizing *L. plantarum* WCFS1 during storage at 37 °C (Figs. 3 and 4). *L. plantarum* CIDCA 83114 spray-dried with protective formulations containing GOS and maltodextrin allowed the recovery of 93% microorganisms; in contrast, only 64% microorganisms were recovered when no

GOS were included in the formulation [36]. *Lactobacillus delbrueckii* subsp. *bulgaricus* CFL1 protected with a formulation including C-BW wastewater exhibited a good resistance to freeze-drying and storage stages [38]. Protective formulations with different GOS compositions were also demonstrated to be highly efficient in the preservation of *L. delbrueckii* ssp. *bulgaricus* CIDCA 333 both after freeze-drying and storage at 5 and 25 °C [34]. The efficiency of GOS as protectants could be related to their ability to immobilize cells in a glassy matrix, which reduces molecular mobility and consequently slows down degradation reactions, and to their enough disaccharides content, useful for stabilizing and thus preserving bacterial membranes [38, 53, 59]. In addition to their proven efficacy, the use of α-GOS from legumes' wastewaters to stabilize probiotic cultures is attractive because they may be safely incorporated into food products without raising health concerns regarding lactose intolerance in consumers, as is the case of β-GOS that are obtained from lactose, or other milk-derived products [58]. According to the Global Aquafaba Market Outlook (2023 to 2033) report by Persistence Market Research, in 2022, worldwide sales of aquafaba accounted for 38.8 million US$, with an expected growth in CAGR between 2023 and 2033 of 13.1% [60]. The main cause behind economic growth is the increasing interest in vegan products, namely egg substitutes. The report explains how aquafaba is set to become a viable alternative to egg products when preparing vegan versions of meringues, waffles, and pancakes. The environmental concerns, along with dietary tendencies and increased interest in functional foods, brew favorable conditions for the incorporation of GOS wastewaters and stabilized lactic acid bacteria in novel food products with health benefits.

Conclusions

In the present study, the stability of *Lactiplantibacillus plantarum* WCFS1 with the addition of different protective molecules (GOS extracts from chickpeas and lentils, fructo-oligosaccharides, and sucrose) was evaluated. The biological activity of the bacteria decreased after freezing and freeze-drying, with no influence of the protective molecule used. However, after storage at 37 °C for 3 weeks, wastewaters from legumes' preparation containing GOS were the most effective protective compounds towards *L. plantarum* WCFS1, proving the initial hypothesis correct. The culturability studies showed no significant differences between the legumes, or cooking procedures; however,

the sample with the boiling wastewater obtained from cooking without soaking of lentils showed the best acidifying activity. Our results suggest the boiling method is economically and environmentally advantageous, conferring the best protection for the lactic acid bacterium, with decreased costs and time, with the dismissal of the soaking procedure of the seeds. The protective capacity of the GOS wastewaters appears to be explained by a combination of physical–chemical factors intrinsic to these mixtures, namely their buffering capability, the increased *Tg* values, and the presence of the carbohydrates of various sizes, including the polysaccharide starch, in contrast with the reference materials used for comparison. The increased shelf-life of probiotic cultures is a serious concern, with economic and environmental impact. Prebiotic and probiotic mixtures' incorporation in food products could represent an interesting application for these substances, thus improving the consumers' health through their diet, besides nutraceutical supplementation.

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Author Contribution

G.N.M, M.G.S, A.D.C, and S.C are responsible for the experimental work and methodology. G.N.M, M.G.S, and F.F did the conceptualization. G.N.M. and M.G.S. did the data curation and writing of the original draft. F.F, A.G.-Z, and P.C.C did the supervision, the review of the manuscript, and the funding acquisition.

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Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Competing Interest

The authors declare no competing interests.

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Abbreviations

BF, Before freezing. C-BW, Chickpea "boiling" water. C-CW Chickpea cooking water. CFU, Colony-forming unit(s). DSC, Differential scanning calorimetry. DP, Degree of polymerization. F, Freezing. FD, Freeze-drying/dried. FOS, Fructo-oligosaccharide(s). GOS, Galacto-oligosaccharide(s). L-BW, Lentils' "boiling" water. L-CW, Lentil cooking water. MRS, De Man, Rogosa, and Sharpe. PCA, Principal component analysis. RH, Relative humidity. SUC, Sucrose. Tg, Glass transition temperature

Publisher's Note

Supplementary Information

Online Resource 1

Culturability of *L. plantarum* WCFS1 concentrates protected with six different protective formulations at different steps of the production process and culturability loss rate during storage at 37 °C, characterized as the absolute value of the slope of the linear regression (|K|). Data represented are average and standard deviation of at least two biological replicates and three technical replicates. Superscript letters (a, b) represent statistical differences (at the 95 % confidence level) between the samples at different steps of the process and between the |K| value. BF = before freezing; C-BW = chickpea boiling water; C-CW = chickpea cooking water; F = after freezing; FD = after freeze-drying; FOS = fructo-oligosaccharide; L-BW = lentil boiling water; L-CW = lentil cooking water. SUC = sucrose; W1 25°C = after 1 week of storage at 25 °C; W2 37°C and W3 37°C = after 2 and 3 weeks of storage at 37 °C

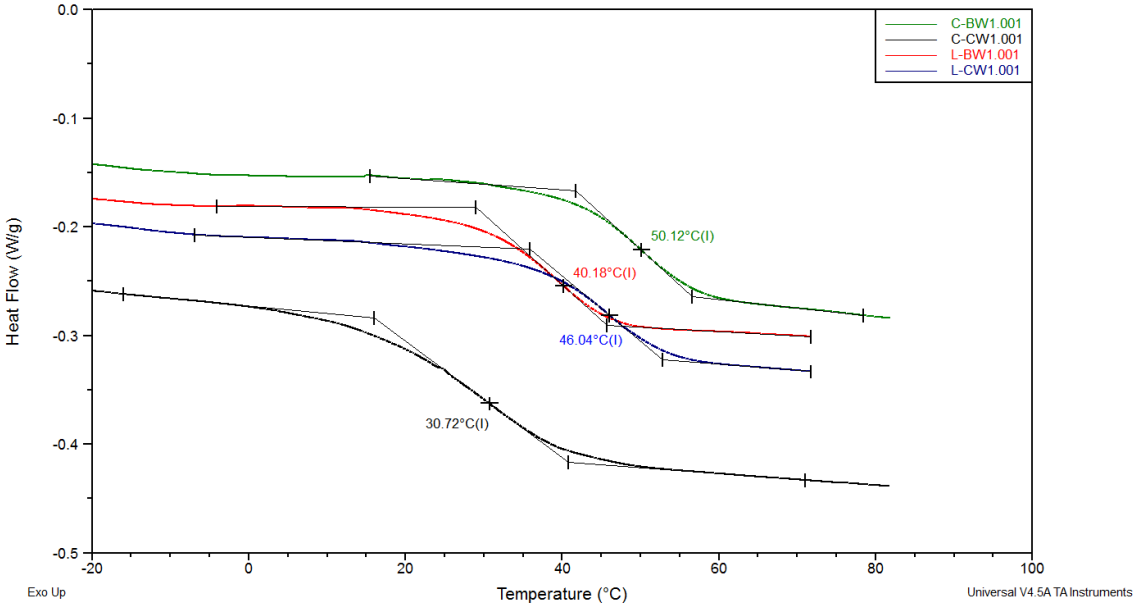
Protective formulation	Culturability (log (CFU·mL^{-1}))						Culturability loss rate (log(CFU·mL^{-1})·day^{-1})
	BF	F	FD	W1 25 °C	W2 37 °C	W3 37 °C	|K|
C-CW	11.51^{a} ± 0.23	11.40^{a} ± 0.16	11.16^{a} ± 0.07	11.01^{a} ± 0.11	11.02^{a} ± 0.25	10.86^{a} ± 0.60	0.01^{a} ± 0.02
C-BW	11.72^{a} ± 0.09	11.16^{a} ± 0.26	11.08^{a} ± 0.22	11.02^{a} ± 0.08	11.05^{a} ± 0.26	10.89^{a} ± 0.36	0.01^{a} ± 0.01
L-CW	11.49^{a} ± 0.19	11.38^{a} ± 0.11	11.07^{a} ± 0.07	10.81^{a} ± 0.14	10.63^{a} ± 0.30	10.66^{a} ± 0.53	0.02^{a} ± 0.02
L-BW	11.51^{a} ± 0.16	11.19^{a} ± 0.23	11.10^{a} ± 0.10	11.01^{a} ± 0.11	11.05^{a} ± 0.33	10.68^{a} ± 0.17	0.02^{a} ± 0.01
FOS	11.66^{a} ± 0.33	11.19^{a} ± 0.22	11.10^{a} ± 0.13	10.95^{a} ± 0.10	9.70^{b} ± 0.49	9.15^{b} ± 0.34	0.09^{b} ± 0.01
SUC	11.81^{a} ± 0.12	11.26^{a} ± 0.19	11.15^{a} ± 0.07	10.99^{a} ± 0.13	9.88^{b} ± 0.28	9.57^{b} ± 0.47	0.08^{b} ± 0.02

Online Resource 2

Acidifying activity of *L. plantarum* WCFS1 concentrates protected with six different protective formulations at different steps of the production process, characterized as the time necessary for a pH drop of 1.5 units (dtpH 1.5, in min). Data represented are average and standard deviation of at least two biological replicates and two technical replicates. Superscript letters (a, b, c) represent statistical differences (at the 95 % confidence level) between the samples at different steps of the process. BF = before freezing; C-BW = chickpea boiling water; C-CW = chickpea cooking water; F = after freezing; FD = after freeze-drying; FOS = fructo-oligosaccharide; L-BW = lentil boiling water; L-CW = lentil cooking water. SUC = sucrose; W1 25°C = after 1 week of storage at 25 °C; and W3 37°C = after 3 weeks of storage at 37 °C

Protective formulation	Acidifying activity (dtpH 1.5 (min))				
	BF	F	FD	W1 25°C	W3 37°C
C-CW	382.3^{a} ± 38.0	423.3^{a} ± 8.5	445.3^{a} ± 15.8	497.0^{a} ± 39.0	677.0^{b} ± 45.0
C-BW	412.0^{a} ± 40.1	421.7^{a} ± 23.0	485.3^{a} ± 79.5	499.0^{a} ± 55.3	632.7^{b} ± 37.4
L-CW	424.3^{a} ± 24.12	431.3^{a} ± 46.4	468.0^{a} ± 38.1	549.7^{a} ± 52.9	641.3^{b} ± 40.1
L-BW	416.0^{a} ± 64.8	462.3^{a} ± 90.7	472.0^{a} ± 33.3	506.3^{a} ± 64.1	568.7^{a} ± 38.6
FOS	409.3^{a} ± 34.9	421.7^{a} ± 30.2	461.7^{a} ± 28.3	494.3^{a} ± 37.6	840.5^{c} ± 59.5
SUC	415.7^{a} ± 19.8	424.3^{a} ± 11.7	508.3^{a} ± 73.3	552.0^{a} ± 35.2	1014.1^{c} ± 104.3

Online Resource 3



DSC thermograms for protective formulations. CW: chickpea cooking water; C-BW: chickpea boiling water; L-CW: lentil cooking water; L-BW: lentil boiling water

iii. Use of Brewer's Surplus Yeast for the Purification of Galacto-oligosaccharides and as a Nitrogen Source for Bacterial Cultivations' Media

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RESEARCH

Use of Brewer's Surplus Yeast for the Purification of Galacto-oligosaccharides and as a Nitrogen Source for Bacterial Cultivations' Media

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Abstract

Brewer's surplus yeast is the second-largest by-product of the brewing industry. Despite its rich nutrient profile, including protein, amino acids, B vitamins, oligosaccharides, and minerals, most of it is discarded. This study aims to valorize the surplus yeast of *Saccharomyces cerevisiae* DSM 2155 generated at a local Madeiran enterprise. The surplus yeast was used to remove monosaccharides remaining as secondary products from the synthesis of galacto-oligosaccharides via fermentation, and for producing yeast extract serving as a nitrogen source for the growth of Gram-negative (*Escherichia coli*) and Gram-positive (*Staphylococcus aureus*, lactobacilli) microorganisms. Two commercial β-galactosidases, Enzeco Fungal Lactase Concentrate (Enzyme Development Corporation, USA) and Biolactase F Conc (Biocon, Spain) were immobilized in glutaraldehyde-activated halloysite nanotubes and used in a repeated batch operation for the synthesis of galacto-oligosaccharides from lactose. After two rounds of synthesis, both synthesis products roughly consisted of 36 % galacto-oligosaccharides, 44-49 % lactose, 15-20 % monosaccharides (glucose and galactose). Fermentation with surplus yeast led to a composition of 41 % galacto-oligosaccharides, 47-51 % lactose, and 8-12 % monosaccharides, due to an almost complete removal of glucose, decreasing its content to only 1 % in the final preparations. In a parallel assay, yeast extract was produced by autoclaving, autolysis, and enzymolysis using Viscozyme L (Novozymes, ND), and used as culture medium for *E. coli, S. aureus* and *Lactiplantibacillus plantarum*. This study demonstrates a dual strategy for valorizing brewer's surplus yeast, yielding nutritionally valuable resources for potential biotechnological applications.

Keywords

Saccharomyces cerevisiae, enzyme immobilization, β-galactosidase, halloysite nanotubes, galacto-oligosaccharides, fermentation

Introduction

According to recent data, approximately 1.05 billion tons of food and beverages were wasted in 2022 globally, representing about 19 % of food available to consumers. Food waste is generated across the supply chain, from agricultural production to household consumption [1]. In fact, 13 % of food and beverages is lost during the supply chain before it reaches retail [2,3]. Adding to the broader issue of this market inefficiency, if improperly managed, residues rich in organic matter from industrial processes can harm ecosystems, thus creating not only waste management but also environment problems [4]. Preventing waste is a major objective in the food and beverage industry; however, achieving full reuse of by-products continues to pose significant challenges. European Union policies actively encourage the valorization of the food industry's by-products [5]. This valorization involves extracting and repurposing high-value components such as proteins, polysaccharides, fibers, and flavor compounds for functional and pharmacological applications, contributing to sustainability and mitigation of environmental impacts [6-8]. These efforts to repurpose food by-products underline the need for sustainable practices in addressing the environmental footprint of food production.

Beer is the most popular alcoholic beverage worldwide, and the third-most popular drink overall after water and tea. In 2023, the global beer production amounted to about 1.88 billion hectoliters, up from 1.3 billion hectoliters in 1998 [9]. Since the brewing industry is a major global sector, brewers prioritize employing techniques that ensure both high product quality and cost efficiency. However, the large-scale production model of modern breweries generates large quantities of co-products that are discarded daily, leading to significant economic and environmental consequences, despite still containing valuable nutrients. These discards include spent grain, hot trub and residual/surplus yeast, which have great potential for valorization in sustainable processes [10]. Addressing the challenge of safely repurposing these co-products for human

consumption or economic use is a critical step toward achieving more sustainable food systems.

Yeast plays a significant role in food-producing industries like brewing, winemaking, and baking [11]. Brewing yeasts are categorized into two main groups: bottom-fermenting yeasts and top-fermenting yeasts [12]. *Saccharomyces cerevisiae* is the most widely used yeast in industrial applications, particularly for fermenting carbohydrates from sources like barley, malt, and wheat to produce alcoholic beverages [13]. During fermentation, the yeast consumes 90 % of fermentable sugars to produce alcohol, allocating only 10 % to biomass production [14,15]. Despite being a small percentage, this 10 % represents substantial industrial waste, due to the large scale of beer production.

Brewer's surplus yeast, primarily *Saccharomyces cerevisiae*, is the second-largest co-product of the brewing industry, following spent grains, with approximately 15 to 18 tons produced per 10,000 hectoliters of beer [16]. Despite its rich nutrient profile, including high protein content, amino acids, B vitamins, and minerals [13,15], most of it is discarded. A portion is used in animal feed, primarily in swine farming, and aquaculture, though it is often sold at prices too low to cover drying costs. It is also used in food production for human consumption, in products such as marmite [17]. However, surplus yeast holds untapped potential for biotechnological applications in pharmaceuticals, functional foods, and biofuels, among others [4,11,12]. Increasing interest in its bioactive compounds, such as β-glucans and oligosaccharides, has led to its exploration as a valuable ingredient in functional foods [16,18]. In the Madeiran Autonomous Region of Portugal, surplus yeast production poses a waste management concern exponentiated by the region's small scale and distance from mainland Portugal which hinders the large-scale waste management of this by-product.

Galacto-oligosaccharides (GOS) (α- and β-type GOS) have been shown to have prebiotic activity, that is, substrates selectively utilized by host microorganisms conferring health benefits [19,20]. α-GOS can be obtained from legume wastewaters, which provide suitable culture media for lactic acid bacteria [21], as well as promising matrices for their dehydration [22,23]. Contrarily to the α-GOS, the β-type GOS are obtained from the enzymatic synthesis from lactose (Lac), using β-galactosidase (β-Gal) enzymes. β-Gal can catalyze both the hydrolysis of Lac into glucose (Glu) and galactose

(Gal) and the transfer of a Gal unit to another molecule, a process called transgalactosylation. This transferase activity is crucial for GOS synthesis, where a Gal molecule is transferred to another Lac molecule, increasing the degree of polymerization (DP) of GOS. The left-over Glu produced in this complex process acts as an inhibitor for the synthesis, decreases the prebiotic potential of the GOS mixture, and increases the caloric value of the preparation when included in food products or used as supplements [24]. The synthesis process can be optimized by both immobilizing the enzyme and removing Glu from the final products. The main advantage with enzyme immobilization is allowing for the enzyme's reutilization. Considering that the enzyme is the main factor increasing process costs, immobilization is a suitable strategy to address this issue, while also contributing to sustainability as result of its reusability, however immobilization may result in loss of catalytic activity due to blockage of active sites, so the correct choice of support and immobilization strategy is paramount [25]. Halloysite nanotubes (HNT) are a type of tubular nanoclay with generally recognized as safe (GRAS) status, and a positively charged $Al(OH)_3$ inner surface and a negatively charged SiO_2 outer surface [26,27], with demonstrated immobilization capacity. Despite the efficiency of this strategy in extending enzyme usability, the GOS yield remains relatively low (ca. 50 %), and the Glu content in the products is still high, given the dual transgalactosylase and hydrolytic activity of the enzymes employed. Therefore, optimizing the synthesis of β-GOS requires the combination of enzyme immobilization with an efficient Glu removal [24]. Fermenting surplus yeast may contribute to this aim, simultaneously tackling economic and environmental concerns, and increasing the content of GOS in the reaction mixture.

Typically, yeast extract is derived from the autolysis of brewers' S. cerevisiae, as it is more economical than baker's yeast. This cost difference is largely attributed to the use of surplus yeast from breweries for brewer's yeast extracts, as opposed to the more resource-intensive production of baker's yeast specifically for this purpose [6,28]. Yeast extract is composed of water-soluble components, including amino acids, peptides, nucleotides, vitamins, minerals, and β-glucans and manno-oligosaccharides from the cell wall [11,13,29]. It finds extensive use in cosmetics, and in the food industry as a flavor enhancer, in animal feed, and is valued for its low production costs and nutritional richness [30-33]. Given its rich composition, yeast extract could also serve as a valuable

nitrogen source in microbial culture media, also exploring different treatments to enhance nutrient availability during microbial growth.

This study aims to valorize by-products from the Madeiran brewing industry, specifically brewer's surplus yeast, which is estimated to be generated by the 100,000 tones each year at Empresa de Cervejas da Madeira, Lda. brewery. To achieve this, surplus yeast was used for two different purposes: a) to purify oligosaccharide mixtures of α- and β-GOS by removing monosaccharides through fermentation, and b) to produce yeast extract for the cultivation of Gram-negative (*Escherichia coli*) and Gram-positive (*Staphylococcus aureus*, and *Lactiplantibacillus plantarum*) bacteria. This dual approach not only adds value to an abundant industrial by-product, but also contributes to more sustainable bioprocesses through waste reduction and resource circularity.

Materials and Methods

Surplus yeast of *Saccharomyces cerevisiae* DSM 2155 strain, generated from *Coral* beer production at Empresa de Cervejas da Madeira, Lda was collected in sterile containers and kept at 4 °C until use. The mixture was centrifuged at 4000 rpm for 10 min, and the cells were washed with distilled water. This process was repeated until the supernatant was clear. The yeast was later recovered by filtration and stored at 4 °C until being used for the purification of oligosaccharide mixtures and for yeast extract production for preparation of culture medium for bacteria, as detailed in Fig 1.

Purification of Oligosaccharides Mixtures Containing Glucose

Commercial Mixtures of GOS and FOS

A set-up of the process was carried out using commercial mixtures of α-GOS (AlphaGOS P, Olygose, France), β-GOS (Vivinal, Friesland Campina, The Netherlands) and fructo-oligosaccharides (FOS) (*Xarope Biológico de Yacon*, Yacon Portugal, Portugal), all of them containing different amounts of Glu in order to assess the yeast's selectivity towards different oligosaccharide species.

α-GOS and FOS were prepared at 5 °Brix and fermented with 10 mg/mL of the collected surplus yeast at 30 °C, with orbital agitation at 150 rpm (Heidolph Unimax 1010 & Heidolph Inkubator 1000, Heidolph) for 6 hours, in aerobic conditions. Previous studies (data not shown) indicated that these experimental conditions of temperature and time were suitable for optimal yeast growth.

For β-GOS, the maximum sugar concentration and working temperature were assessed using Vivinal mixtures at 5, 10, 20, 30 and 40 °Brix, within a temperature range of 30 to 50 °C. This study was performed to understand if fermentation could occur in the same experimental conditions used in β-GOS synthesis (40 °Brix Lac solutions at 50 °C).

The composition of the fermented and non-fermented samples was determined by HPLC-RI analysis. Samples were filtered with 0.45 µm cellulose acetate filters (Frilabo, Portugal) and injected into a ReproGel-Na (Dr. Maisch, Germany) column (250 × 4 mm, 9 µm particle size) and guard-column (20 x 4.6 mm, 9 µm) at 80 °C, in the UltiMate 3000 (Dionex, USA) HPLC. The RI detector (Shodex RI-101) was kept at 50 °C. Degasified filtered ultrapure water was used as eluent at a flow rate of 125 µL/min. The obtained chromatograms were analyzed with Chromeleon 6.80 software (Dionex Corporation, USA). Determinations were carried out in triplicate.

Purification of β-GOS Synthesized with Immobilized β-Gal

Immobilization of the Enzymes

Three commercially available β-Gal were immobilized in HNT (Sigma-Aldrich). The immobilization procedure performed was adapted from that described by Singh & Singh (2022) [27]. 150 mg of HNT was dispersed in a mixture containing 90 mL of 96 % ethanol and 10 mL of hydrogen peroxide (130 v) and was sonicated for 20 min in an Ultrasons H-D (J. P. Selecta, Spain) ultrasound bath. The clay was recovered after centrifugation at 4000 rpm for 5 min (Megafuge 1.0, Heraeus Instruments, Germany), and was later washed thrice with distilled water. HNT was then functionalized with 1.5 % glutaraldehyde (ChemCruz, USA) for 1 h using head-over-heels rotation (Deax 2, Heindolph), after which it was washed with water. For the enzyme immobilization, solutions containing 150 IU of Biolactase F Conc (BFC) (Biocon, Spain), obtained from *Aspergillus oryzae*, Enzeco® Fungal Lactase Concentrate (EFLC), and Enzeco® Lactase

KL (ELKL) (Enzyme Development Corporation, USA), obtained from *Aspergillus oryzae and Kluyveromyces* sp., respectively, were prepared in distilled water and mixed with the functionalized HNT. The clay and enzyme mixtures were left under head-over-heels rotation for 2.5 h. After that, the immobilized catalyst (HNT-β-Gal) was recovered by centrifugation and washed for removal of leftover enzyme. Four independent immobilization procedures were performed for each enzyme.

Synthesis and Purification of GOS Mixtures

HNT-β-Gal was used for the synthesis of GOS. The immobilized enzyme preparations were suspended in 40 % (w/w) Lac solutions prepared in distilled water and incubated for 6 h at 50 °C and 250 rpm (orbital shaking). These experimental parameters were chosen based on the enzymes' manufacturer's recommendations (temperature) and the literature (Lac concentration and time) [34]. After the synthesis, the enzyme/carbohydrate mixtures were centrifuged and the catalysts recovered, washed three times with distilled water, and stored at 4 °C. Then, the carbohydrate mixtures were diluted to a concentration of 20 % (w/w) with distilled water and fermented with the surplus yeast (6 h, 30 °C, 150 rpm). The surplus yeast was removed by centrifugation. The fermented samples were concentrated to obtain 40 % (w/w) solutions, as confirmed by °Brix measurements, and resubmitted to a second round of synthesis and fermentation, as described previously.

After each step of the production process, aliquots were recovered and diluted to a 2 % final concentration with ultrapure water. These samples were centrifuged for 5 min at 5000 rpm, and the supernatants were filtered with 0.45 μm cellulose-acetate filters and analyzed in the HPLC-RI, as explained in section 2.1.1. The synthesis and fermentation were performed in quadruplicate and HPLC measures were made in triplicate.

Yeast Extract Production and Inclusion in Culture Medium for Bacterial Growth

Yeast Extract Production

Three different treatments were used to obtain yeast extracts from *Coral* beer's surplus yeast: physical, autolysis and enzymolysis.

Physical Process

Surplus yeast suspensions (25 %) were autoclaved for 10 min at 125 °C. The mixtures were removed and placed in an ice bath for 10 min. Afterwards, the mixtures were centrifuged for 10 min at 5000 rpm and the supernatants recovered. The produced yeast extract was frozen and lyophilized [35].

Autolysis

10 % yeast suspensions in distilled water were incubated at 50 °C for 24 h with agitation at 250 rpm, and then, centrifuged twice at 5000 rpm for complete removal of cell debris. The supernatants were frozen and lyophilized.

Enzymolysis

This treatment is a variation of autolysis in which the digestion of the yeast cells is aided by the action of external hydrolytic enzymes. The enzymatic cocktail (Viscozyme® L, Novozyme, Netherlands) was added at 1 % (v/v) to the yeast suspensions (10 %) and the mixture was incubated for 24 h at 50 °C and agitation at 250 rpm [36]. The mixture was then centrifuged twice at 5000 rpm for complete removal of cell debris. The supernatants were frozen and lyophilized.

Bacterial Cell Growth Assessment

L. plantarum WCFS1 and CIDCA 83114 were used to evaluate the effectiveness of the yeast extract as culture media for bacterial strains relevant to the food industry. In addition, two reference strains were also used, representing Gram-negative (*E. coli* ER2738, New England Biolabs®) and Gram-positive bacteria (*S. aureus* WDCM 00034). The strains were maintained frozen at -80 °C in 10 % glycerol in LB medium (HiMedia Laboratories, India) (*E. coli*), 10 % glycerol in tryptic soy medium (*S. aureus*) and 120 g/L non-fat milk solids (*L. plantarum*). *E. coli* was reactivated in LB agar for 24 h, *S. aureus,* in tryptic soy agar (VWR Chemicals, Belgium) for 24 h, and both *L. plantarum* strains, in MRS broth (Sigma-Aldrich, USA) for 16 h (stationary phase). In all cases, reactivation was at 37 °C in aerobic conditions.

After reactivation, pre-cultures of all strains were prepared by inoculating an isolated colony of *E. coli* and *S. aureus* in LB broth and tryptic soy broth, respectively. For both *L. plantarum* strains they were inoculated at 1 % (v/v) in MRS broth. The bacterial suspensions were then incubated for 16 h at 37 °C and orbital shaking at 180 rpm, in aerobic conditions.

In a first assay, 50 µL of the pre-cultured bacteria were inoculated on the surface of solid media, prepared by adding 1.5 % bacteriological agar (VWR Chemicals, Belgium) to the produced yeast extracts (8 g/L) and commercial standard liquid media (LB, tryptic soy, MRS), as well as a commercial yeast extract (Biokar Diagnostics, France). Negative controls (media without the addition of yeast extracts) were also included. Plates were incubated at 37 ºC in aerobiosis, and then visually analyzed for the presence of bacteria. Photographs of each plate were recorded in the Azure 400 (Azure Biosystems) apparatus.

In parallel, the pre-cultures of all the four strains were inoculated (1 % v/v) into the three yeast extracts obtained in 2.2.1 (physical, autolysis and enzymolysis) at 20 g/L. Standard culture media (LB, tryptic soy, and MRS) were used as references (positive controls). Microorganisms were grown at 37 ºC under shaking (180 rpm) and growth kinetics were followed by hourly registering the optical densities (OD) up to 16 hours in a spectrophotometer (Genesys, ThermoScientific) at 600 nm. Each assay was performed twice from independent cultures.

A summary of the experimental procedures is shown in Figure 1.

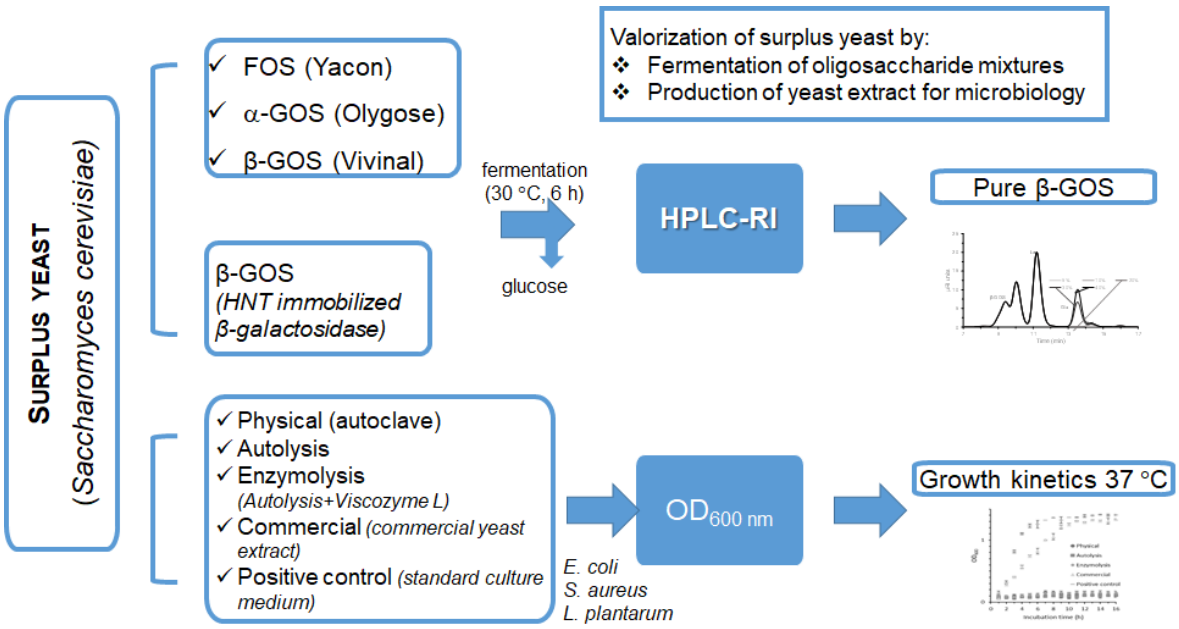


Figure 1 – Graphical abstract

Results and Discussion

Fermentation Substrate's Selectivity Assessment

To establish a purification process for the removal of Glu without compromising the integrity of prebiotic oligosaccharides, commercial FOS, as well as α- and β-GOS, were fermented using surplus yeast (Figure 2). After fermentation of the FOS syrup, the Suc, Glu, and Fru peaks were substantially diminished, lowering the overall di- and monosaccharides' content from 75 to 45 % (Table 1). This resulted in the increase of FOS to almost 50 % of the carbohydrates present in the final mixture; however, the HPLC-RI chromatogram suggests these compounds are also being hydrolyzed. This may explain why there is so much leftover Fru after fermentation. A control experiment was performed with the fermentation of Suc (in the form of white sugar, Sidul, Portugal) at the same concentration as the FOS syrup, and the chromatogram (Online Resource 1) showed the almost complete disappearance of the Suc peak, with the prominent feature of Glu and Fru peaks. There was no FOS formation, further confirming that the increase in FOS content from 25 to 48 % in the yacon syrup's fermentation mainly results from the purification of mono- and disaccharides from the mixture. Additionally, there seems to be an overlap of the inulin and DP5 FOS peaks with those of fermentation by-products,

causing a slight overestimation of the inulin+FOS content. These results show the removal of Suc, Glu, and Fru by the surplus yeast is very effective; however, the possibility of FOS hydrolysis advises caution when purifying these prebiotics. For this reason, subsequent FOS synthesis and purification was not performed. The presence of invertases and the *Suc2* gene correlate positively with the results shown here for the FOS sample fermentation, however this is an unexpected behavior for *S. cerevisiae* strains. This microorganism is regarded as not being able to ferment FOS and has been used for purification of FOS mixtures [37-41], however there are reports on the use of inulin from natural sources by *S. cerevisiae* strains for ethanol production [42-44], whose activity was attributed to the *Suc2* encoded invertase possessing exo-inulinase activity [45], acting on FOS and low DP inulin.

Regarding the α-GOS' fermentation, they were extensively consumed by the yeast, causing the release of monosaccharides, whose content increased from 0 to 27 % (Table 1). Contrastingly, the yeast was not capable of consuming neither Vivinal's β-GOS or Lac, whose contents increased because of the monosaccharides' selective removal from the mixture. The disaccharide melibiose (Mel) was also consumed during the fermentation, indicating this *S. cerevisiae* strain possesses a α-Gal enzyme capable of cleaving the α-type linkages between Glu and Gal residues found in α-GOS, but does not have a β-Gal, proving that using this surplus yeast for purification of β-GOS mixtures is the only practical approach in this context. Similar results regarding β-GOS fermentation with Vivinal were observed by Goulas et al. (2007) [46], in which β-GOS and Lac were not fermented by *S. cerevisiae* but Glu and Gal were, although the latter in a lower extend, as observed here. The reason being that Gal metabolism is inducible, while Glu's is not, meaning the yeast will preferably consume Glu first through aerobic glycolysis.

Table 1 – Composition of the FOS, α-GOS, and β-GOS before (B.F.) and after fermentation (A.F.) as determined by peak area percentage in the HPLC-RI chromatograms. Inulin was quantified altogether with FOS. *Indicates the presence of fermentation products

Composition (%)		FOS/GOS	Disaccharides	Monosaccharides	Fermentation Products
FOS	B.F.	25.3 ± 2.1	14.3 ± 1.1	60.4 ± 1.1	
	A.F.	47.7* ± 2.7	4.5 ± 0.6	41.0 ± 3.8	6.8 ± 0.4
α-GOS	B.F.	91.4 ± 0.3	8.6 ± 0.3	0.0 ± 0.0	
	A.F.	65.0 ± 0.5	7.4 ± 0.2	27.0 ± 0.4	0.6 ± 0.0
β-GOS	B.F.	41.1 ± 0.8	38.3 ± 0.2	20.6 ± 0.6	
	A.F.	50.3 ± 0.5	47.1 ± 0.7	1.4 ± 0.6	1.3 ± 0.2

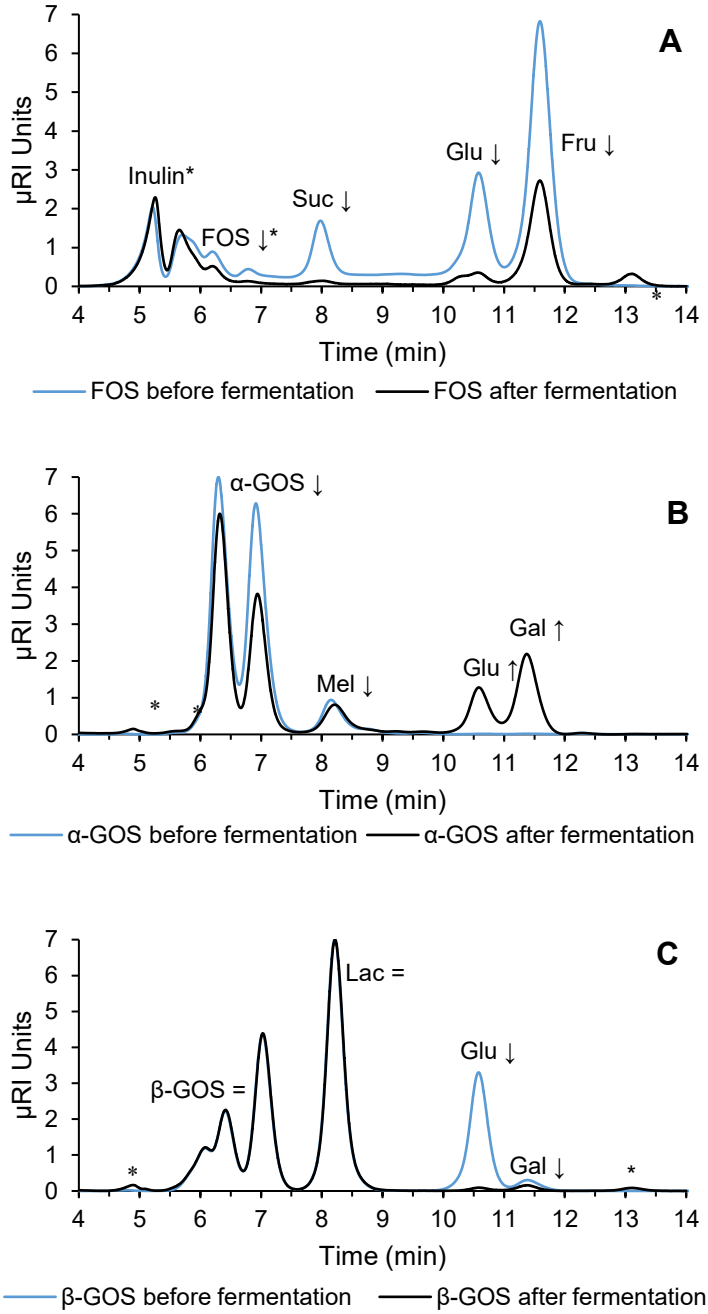


Figure 2 – HPLC-RI chromatograms before and after the fermentation of FOS yacon syrup (**A**), α-GOS powder (**B**), and β-GOS syrup (**C**) by surplus yeast. FOS = fructo-oligosaccharides, Suc = sucrose, Glu = glucose, Fru = fructose, GOS = galacto-oligosaccharides, Mel = melibiose, Gal = galactose, Lac = lactose. Symbols next to each carbohydrate indicate changes in its content after the fermentation. *Indicates the presence of fermentation products

In the study by Yoon et al. (2003) [47], similar results were observed in which D-Glu, D-Fru, D-Gal, and Suc were easily fermented by *S. cerevisiae*. It was also reported that the yeast's invertases converted the α-GOS trisaccharide raffinose into Mel by removal of the terminal fructosyl residue. The disaccharide, in turn, was found to be fully resistant to fermentation. This is supported by the strain's described genome, having both

the *Suc2* and *Mel* genes [48], meaning it has an active Suc metabolism, but can't hydrolyze Mel. However, in our work Mel and α-GOS were in fact hydrolyzed into Glu and Gal, indicating α-Gal activity and the strain's ability to degrade Mel. The α-GOS model used consisted on a commercial sample of derivatives of the raffinose-family of oligosaccharides (RFOs) whose fructosyl moieties were removed [49]. So, the α-GOS with DP3 and 4 present were manninotriose (Glu-Gal-Gal) and verbascotetraose (Glu-Gal-Gal-Gal), respectively, instead of raffinose (Fru-Glu-Gal) and stachyose (Fru-Glu-Gal-Gal). If Mel was completely resistant to fermentation, its content would have increased after fermentation, as per the degradation of the α-GOS. The present results concerning FOS and α-GOS fermentation showcase metabolic differences between *Saccharomyces cerevisiae* strains, and between the DSM 2155 strains' own genotypic and phenotypic features. It is possible that certain activities have yet to be attributed to specific genes in the yeast, or perhaps these differences result from changes occurring during the brewing process, either causing genetic modifications or by production of unspecific hydrolytic enzymes by autolysis caused during the yeast's conditioning and preparation for the fermentation procedures.

β-GOS are produced by enzymatic reaction of β-Gal with Lac solutions typically at 40 % (w/w) and 50 °C, so a proof-of-concept study was performed to assess if a simultaneous synthesis and purification of GOS mixtures by fermentation with surplus yeast was feasible. As discussed, the yeast used does not have the β-Gal needed to consume these sugars, so this proposed approach was deemed feasible. Figure 3 shows the chromatograms produced after the fermentation of Vivinal solutions with concentrations ranging from 5 to 40 % (w/w) with surplus yeast at 30 °C. The yeast was capable of effectively removing all Glu in solutions up-to 20 % (w/w). At 40 % (w/w) Vivinal there was no fermentation, due to osmotic imbalance at this higher sugar concentration. In the work by Goulas et al. (2007) [46] they were able to use *S. cerevisiae* to ferment Vivinal solutions up-to 425 mg/mL (42.5 w/w), however the provenance of the yeast was not mentioned, and it is assumed that a stock culture or commercial product was used, instead of a surplus yeast, and they performed the experiments using buffer solutions for pH control, unlike in this work.

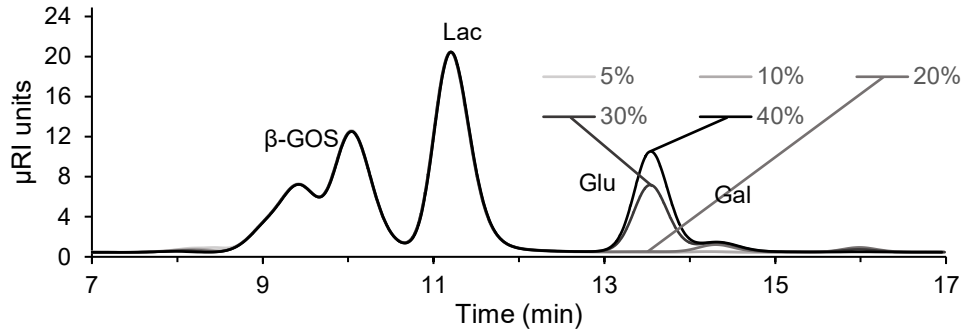


Figure 3 – HPLC-RI chromatogram of Vivinal solutions at 5, 10, 20, 30, and 40 % (w/w) after fermentation by surplus yeast at 30 °C for 6 h. β-GOS = β-galacto-oligosaccharides, Lac = lactose, Glu = glucose, Gal = galactose

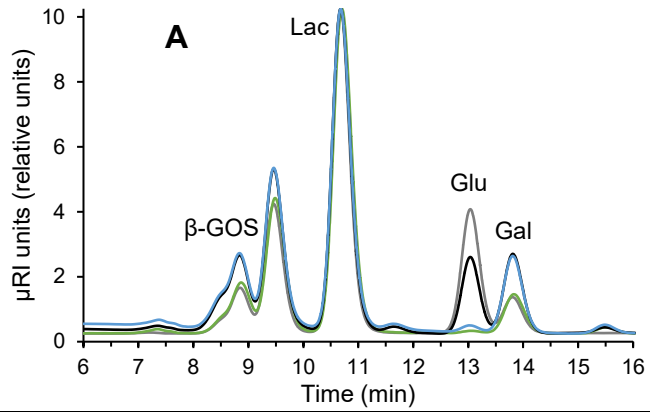
When the temperature increased just to 40 °C there was virtually no fermentation observed (Online Resource 2). Thus, it is concluded that the simultaneous synthesis and fermentation of β-GOS is not possible in the experimental conditions used for the enzymatic GOS synthesis using this surplus yeast. Aburto et al. (2016) [50] designed a one-step batch operation for the simultaneous synthesis and fermentation of GOS from Lac, however they used a baker's yeast and buffers, possibly explaining why they could operate at higher temperatures and sugar concentrations.

Fermentation studies using this surplus yeast and three different oligosaccharide mixtures allowed the establishment of operational conditions for effective Glu removal (20 %, w/w, solutions, and 30 °C). The yeast shows hydrolytic activity towards FOS and α-GOS, suggesting that β-GOS are the most interesting substrate to work with for Glu fermentation from prebiotic mixtures. For this, an experimental design for β-GOS synthesis and purification was established.

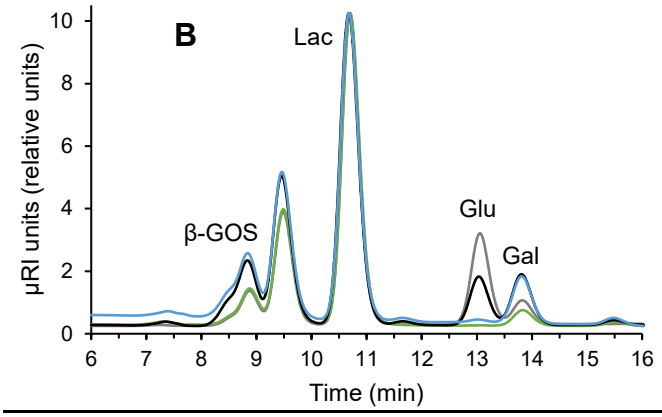
β-GOS Synthesis with Immobilized Enzymes and Purification by Fermentation

Two immobilized β-Gal (Enzeco® Fungal Lactase Concentrate and Biolactase F Conc) were used to synthesize β-GOS using Lac 40 % (w/w) as reagent. After the reaction was finished, the products were fermented with surplus yeast, in a similar procedure as that optimized in the previous section. Figure 4 shows the chromatograms of the carbohydrates' mixtures after each of the two rounds of repeated synthesis and

fermentation, as well as the area percentage of GOS, Lac, and monosaccharides at each stage, for both enzymes.



	Area %*		
EFLC	GOS	Lac	Mono
1S	27.0 ± 0.6	47.2 ± 1.0	25.8 ± 1.4
1F	33.7 ± 0.8	57.8 ± 0.5	8.5 ± 0.9
2S	36.1 ± 0.9	43.8 ± 0.7	20.1 ± 1.4
2F	41.0 ± 1.3	46.8 ± 1.0	12.1 ± 0.7



	Area %*		
BFC	GOS	Lac	Mono
1S	26.8 ± 0.7	52.1 ± 1.1	21.2 ± 0.9
1F	32.8 ± 0.9	63.2 ± 1.6	4.0 ± 0.8
2S	36.1 ± 0.6	48.6 ± 1.3	15.3 ± 1.2
2F	40.8 ± 0.6	50.9 ± 1.1	8.3 ± 0.7

Figure 4 – GOS, Lac, and monosaccharide contents as area percentage obtained from the HPLC-RI chromatogram of GOS solutions produced by synthesis with immobilized enzymes, EFLC (Enzeco® Fungal Lactase Concentrate, **A**) and BFC (Biolactase F Conc, **B**), from Lac solutions at 40 % (w/w) and 50 °C followed by purification by fermentation by surplus yeast at 20 % sugars and 30 °C. 1S = 1st synthesis, 1F = 1st fermentation, 2S = 2nd synthesis, and 2F = 2nd fermentation. *The results express the average and standard deviation of four independent

Both immobilized enzymes produced very similar results, as seen by the resemblance of the chromatograms. After the first synthesis there is the production of DP3, DP4, and DP5 β-GOS, but also of great amounts of Glu, that are readily removed in the first fermentation. At this point, as detailed in Figure 4, the mixtures consisted of 33 % GOS and 60 % Lac. However, given the "low" Lac concentration; in the second synthesis, the enzymes act as hydrolases, as suggested by formation of Glu and Gal in equal amounts, as can be observed in the chromatograms. Once finished the second purification by fermentation, the mixtures' GOS contents are the same as that of Vivinal (41 %, Table 1). There is a higher content of unreacted Lac than in Vivinal, however lower amounts of monosaccharides, particularly Glu that was practically removed, increasing the prebiotic potential of the mixture.

This is not the first time β-Gal has been immobilized in HNT. In the work by Tizchang et al. (2021), the activity of HNT-β-Gal was measured towards Lac degradation and Glu formation, not GOS production [51]. EFLC has also been used after immobilization in different types of carriers, allowing for the synthesis of GOS and lactulose [52-54]. BFC was immobilized in a polyacrylic film, achieving 99 % Lac hydrolysis [55]. However, it's important to note that these reports from the literature pertain to highly optimized procedures, whereas in this work the results for the synthesis and purification of β-GOS mixtures regard proof-of-concept, unoptimized procedures, in terms of solvents (distilled water only), pH, time, temperature, and sugar/enzyme ratio. The fact this straightforward process was able to produce samples with GOS content comparable to those of commercial samples suggests that improving the experimental conditions used should increase GOS purity in the final mixtures. At the beginning of the experiment the 40 % (w/w) were completely attributed to Lac, but during the production process and its degradation by the enzymes, when the GOS mixtures are concentrated back to 40 % (w/w) for the second synthesis, Lac only accounts for 60 % of the overall sugar content of the mixtures. Perhaps for this reason, during the second procedure, the enzymes exhibited more pronounced hydrolytic activity than transgalactosylase activity. When performing the second synthesis, the mixtures could be concentrated further than 40 % (w/w), ensuring the appropriate Lac concentration for GOS formation. Additionally, the proposed approach also allows for the reuse of the catalyst in subsequent GOS syntheses in new batches.

Unshown results regarding the GOS synthesis using immobilized ELKL proved this enzyme cannot perform in the experimental conditions used because of the pH difference, hindering the action of this neutral enzyme.

Online Resource 3 shows an expanded version of Figure 4 with the determined content for each carbohydrate.

Yeast Extract Production and Inclusion in Culture Media for Bacterial Growth

Figure 5 shows the capacity of *E. coli*, *S. aureus*, and *L. plantarum* WCFS1 and CIDCA 83114 to grow in solid media prepared from the yeast extracts obtained by physical treatments (autoclave), autolysis, and enzymolysis, comparing their performance with that of a commercial yeast extract and that of standard media (LB, tryptic soy and MRS, respectively), denoted as "positive controls". This comparative analysis aimed to determine whether our yeast extracts could offer a viable alternative with advantages in both bacterial growth support and sustainability, towards both Gram-negative and Gram-positive bacteria. *E. coli* and *S. aureus* were able to grow in both the produced and commercial yeast extracts. On the contrary, both strains of *L. plantarum* were only able to grow in the commercial yeast extract and in the standard MRS. This is most likely due to the fastidious nutritional requirements of lactobacilli, however brewer's surplus yeast can contain hop acids attached to their cell walls, which were referred to inhibit the grow of other species of *L. plantarum* [56]. Aside from the enhancement of organoleptic properties of beer, hop acids are important in beer production for prevention of microbial contamination. It is estimated that 60-70 % of beer spoilage is caused by lactic acid bacteria, and only tolerant strains can thus survive in media containing such compounds [57]. For this reason, growth kinetics in Figure 6 were carried out just on *E. coli* and *S. aureus*. *E. coli* is a Gram-negative, facultative anaerobe and a predominant commensal in the human gut, with the majority of strains being non-pathogenic, and widely used as a model organism due to its fast growth, metabolic versatility, and extensive genetic characterization [58]. *S. aureus*, in contrast, is a Gram-positive opportunistic pathogen associated with a wide range of human infections and represents a nutritionally more fastidious microorganism [59]. Testing these two bacteria, which differ in cell wall structure, physiology, and nutrient requirements, provides a robust framework to evaluate the nutritional adequacy and versatility of yeast extract as a sole nitrogen source. Moreover, this dual-species model aligns with the broader aim of developing cost-effective and sustainable fermentation media from industrial by-products.

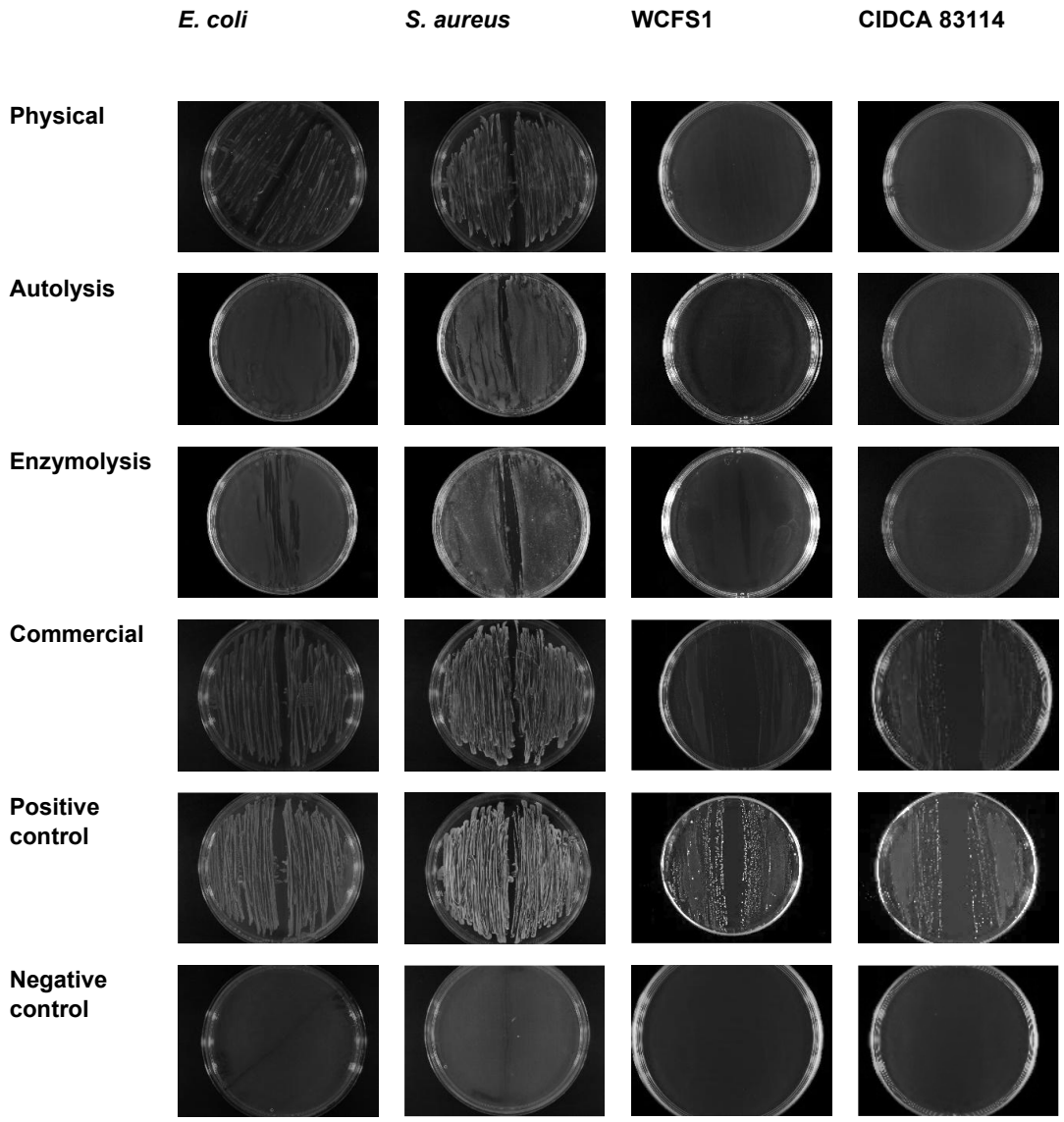


Figure 5 – Photographs of *E. coli*, *S. aureus*, *and L. plantarum* WCFS1 and CIDCA 83114 after inoculation in different solid media: *Physical*, medium with yeast extract produced by autoclaving; *Autolysis*, medium with yeast extract produced by autolysis; *Enzymolysis*, medium with yeast extract produced by autolysis with the addition of Viscozyme L; *Commercial*, medium with commercial yeast extract; *Positive control*, LB (*E. coli*), Tryptic Soy (*S. aureus*), and MRS (*L. plantarum*) media, standard growth media for these bacterial species; and *Negative control*, medium with only agar

E. coli was able to grow in all three yeast extracts obtained in this study. The extract obtained by enzymolysis was the most efficient, and the growth pattern was the closest to that observed in the positive control, standard LB medium. The autolysis and physical treatments led to similar kinetics, with the stationary phase achieved later, similarly to the commercial yeast extract. Physical disruption methods for yeast extract production result in mixtures with high carbohydrate content, by the breakage of cell wall components, whereas the autolysis favors peptide and amino acids' extraction and

production via endogenous peptidase and protease degradation of protein [30]. Viscozyme L shows β-glucanase activity, capable of hydrolyzing β-glucans found in the yeast. Producing yeast extract through enzymolysis with Viscozyme L may result in a final product rich in both shorter oligosaccharides and available peptides. This could explain why this mixture resulted in a growth kinetic for *E. coli* comparable to that of the standard culture medium. *S. aureus* was able to reach the stationary growth phase within the first 5 hours of incubation; however, the produced yeast extracts' media did not provide comparable results with the positive control, as in the case of *E. coli*. This could be explained considering that our extracts are poorer in nutrients than the standard tryptic soy broth.

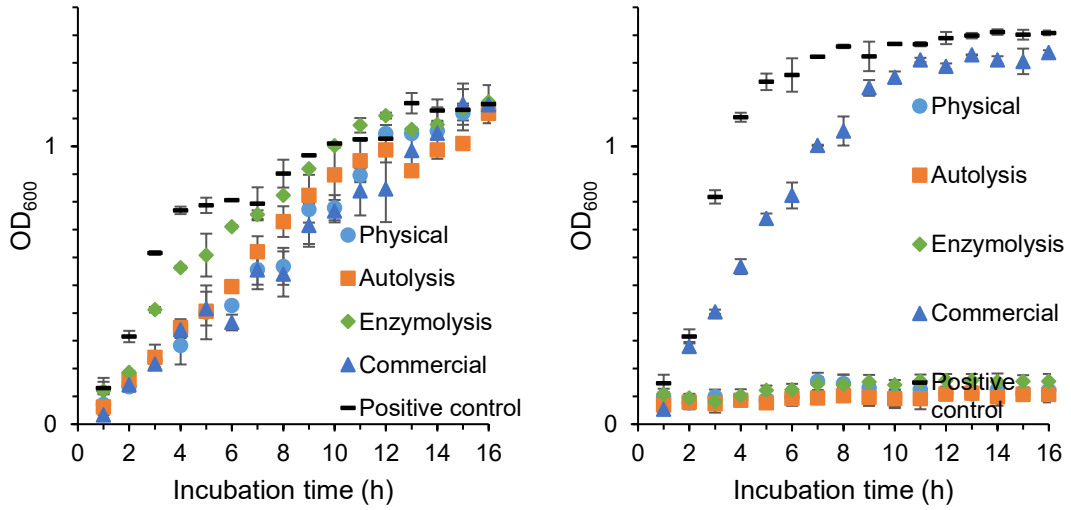


Figure 6 – Plot of the optical density (600 nm) of *E. coli* (left) and *S. aureus* (right) over time in in different liquid media: *Physical*, medium with yeast extract produced by autoclaving; *Autolysis*, medium with yeast extract produced by autolysis; *Enzymolysis*, medium with yeast extract produced by autolysis with the addition of Viscozyme L; and *Positive control*, LB (*E. coli*) and Tryptic Soy (*S. aureus*), standard growth media for these bacterial species

The results from these experiments show that the produced yeast extracts can be used as culture medium ingredients for supporting bacterial growth. With this strategy, it may be possible to culture *S. aureus* and *L. plantarum*, thus allowing for the inclusion of the latter in food products, taking advantage of their associated health benefits.

Conclusion

The surplus yeast used in this study received a second utility after being discarded by the brewing industry. The yeast was used for removal of Glu in carbohydrate mixtures by fermentation, and for production of yeast extract. Future studies could be performed on the optimization of the enzymatic synthesis' production yield, by studying a number of parameters, such as pH, time, temperature, sugar/enzyme ratio, and number of cycles performed. The yeast's selectivity during fermentation opens opportunities to design future strategies for β-GOS or even FOS purification by manipulating the reaction time for a balance between FOS hydrolysis and Glu removal, which is the main goal when purifying prebiotic oligosaccharide mixtures. Another research opportunity could dwell on the possibility that after the fermentation, the yeast could undergo the autolysis or enzymolysis procedures, further valorizing this by-product by producing yeast extract with trace amounts of GOS and other sugars that could be useful for growing food-grade lactic acid bacteria, further enriching the produced culture medium ingredient. Surplus yeast is produced by thousands of tones in Madeira Island each year with no direct application currently, meaning that there is room for the establishment of new industries that can take advantage of this large-scale bioactive raw material, possibly repurposing it back into the food industry itself.

Abbreviations

α-/β-Gal, α-/β-galactosidase. DP, degree of polymerization. FOS, fructo-oligosaccharides. Gal, galactose, Glu, glucose. GOS, galacto-oligosaccharides. GRAS, generally recognized as safe. HNT, Halloysite Nanotubes. HNT-β-Gal, β-galactosidase immobilized in halloysite nanotubes. HPLC-RI, High-Performance Liquid Chromatography with Refractive Index detection. LB, Luria Bertani. Lac, lactose. Mel, melibiose. MRS, de Man, Rogosa, and Sharpe. RFOS, raffinose-family of oligosaccharides. Suc, sucrose.

Conflicts of interest

The authors declare no conflict of interest.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors' contribution

G.N.M, M.V, C.C, and J.G.P are responsible for the experimental work and methodology. G.N.M, M.V., P.C.C, and A.G-Z did the conceptualization. G.N.M. and M.V did the data curation and writing of the original draft. A.G-Z and P.C.C did the supervision, the review of the manuscript, and the funding acquisition.

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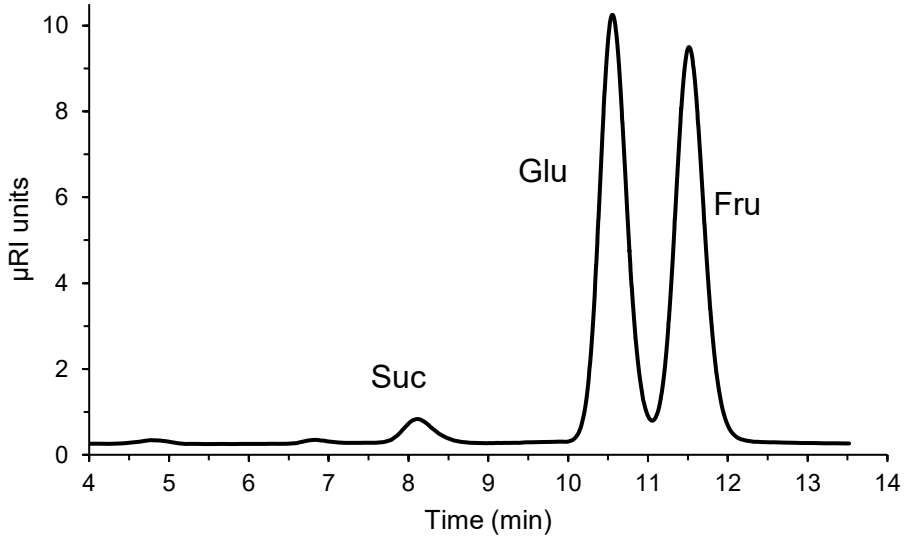
Supplementary Information

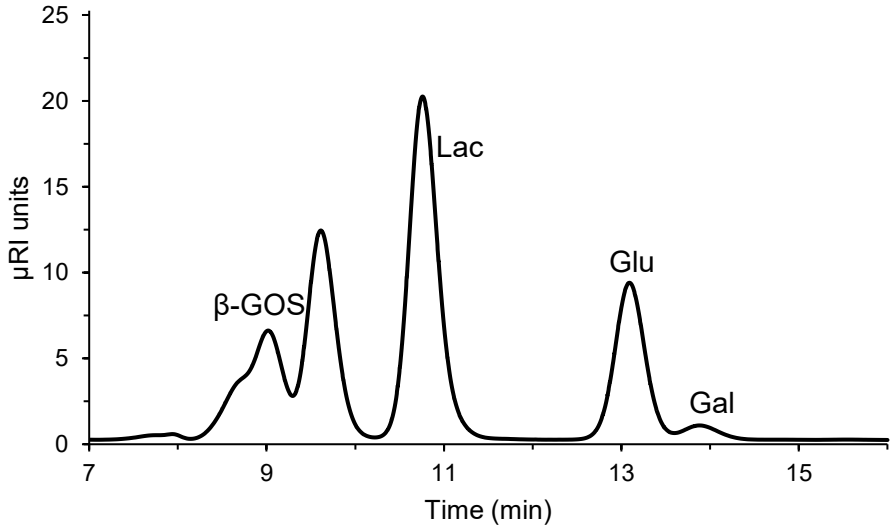
Online Resource 1

HPLC-RI chromatogram of fermentation of sucrose in the form of white sugar. Suc = sucrose. Glu = glucose. Fru = fructose



Online Resource 2

HPLC-RI chromatogram of a Vivinal solution at 20 % (w/w) after fermentation by surplus yeast at 40 °C for 6 h. β-GOS = β-galacto-oligosaccharides. Lac = lactose. Glu = glucose. Gal = galactose



Online Resource 3

DP5, DP4, and DP3 GOS, Lac, Glu and Gal contents as area percentage of the GOS mixtures produced by immobilized EFLC and BFC after each stage of the production process (synthesis and purification by fermentation). The results express the averages and standard deviations of four independent experiments. 1S = 1st synthesis, 1F = 1st fermentation, 2S = 2nd synthesis, and 2F = 2nd fermentation. [a] = total GOS content, [b] = total Glu and Gal contents, and [c] = total Lac, Glu, and Gal contents

	HNT-EFLC				HNT-BFC			
% Area	**1S**	**1F**	**2S**	**2F**	**1S**	**1F**	**2S**	**2F**
GOS DP5	1.7±0.2	2.1±0.2	3.8±0.3	4.4±0.4	1.5±0.2	1.7±0.4	2.9±0.5	4.3±0.4
GOS DP4	6.2±0.2	7.8±0.4	9.7±0.2	11.6±0.5	5.6±0.4	7.1±0.4	9.5±0.0	10.8±0.4
GOS DP3	19.0±0.5	23.7±0.4	22.5±0.6	25.0±0.5	19.7±0.2	24.0±0.2	23.6±0.3	25.7±0.4
Lac	47.2±1.0	57.8±0.5	43.8±0.7	46.8±1.0	52.1±1.1	63.2±1.6	48.6±1.3	50.9±1.1
Glu	19.5±0.7	0.8±0.4	9.1±0.5	1.1±0.2	16.5±0.6	0.2±0.0	7.7±0.4	1.0±0.3
Gal	6.3±0.7	7.8±0.7	11.0±1.1	11.0±0.7	4.7±0.3	3.8±0.8	7.6±1.0	7.4±0.6
GOS[a]	27.0±0.6	33.7±0.8	36.1±0.9	41.0±1.3	26.8±0.7	32.8±0.9	36.1±0.6	40.8±0.6
Mono[b]	25.8±1.4	8.5±0.9	20.1±1.4	12.1±0.7	21.2±0.9	4.0±0.8	15.3±1.2	8.3±0.7
Di+mono[c]	73.0±0.6	66.3±0.8	63.9±0.9	59.0±1.3	73.3±0.7	67.2±0.9	63.9±0.6	59.2±0.6

Chapter III

Conclusions

Food by-products are generated in great amounts each year, many without a clear application or purpose, except for occasional use as animal feed. In this thesis, different waste products were studied in order to understand if they could be inserted in the food industry, for the production of prebiotic GOS and lactic acid bacteria.

Food by-products in the form of α-GOS-containing cooking wastewaters of chickpeas and lentils promoted the growth of lactic acid bacteria when incorporated in culture media. Cooking chickpeas provided the highest GOS extraction yields, while lentils' cooking waters were the richest source of GOS-DP ≥ 5 compounds, with low concentrations of mono- and disaccharides. The rich carbohydrate composition of the samples allowed bacterium cultivation, even when the chickpeas "boiling" water was used as the sole nutrient source, by the hydrolysis of starch for glucose uptake. These legume cooking wastewaters also served as sources of cryoprotectant compounds towards lactic acid bacteria during freezing, freeze-drying, and storage for 3 weeks at 37 °C. The use of a complex matrix was decisive to maintain a higher bacterial survival, by hindering the acidification of the protective medium, as compared to the pure reference materials used. Additionally, there were no significant difference regarding the provenance (chickpeas or lentils) and procedure (cooking vs soaking and cooking). These results suggest the "boiling" method (cooking without soaking) is economically and environmentally advantageous, with the obtained mixture being capable of serving as culture medium and conferring the best protection for the lactic acid bacterium, with decreased costs and time, and with the dismissal of the soaking procedure of the seeds, and the necessary water.

When considering the improvement of β-GOS production, there are two major approaches: improving enzyme activity, or purifying the final GOS mixture. In this work an in-between strategy was designed, taking advantage of the very active and abundant surplus yeast, allowing the production of pure β-GOS mixtures, with only residual glucose. This was accomplished by the use of enzyme immobilization technologies, ensuring the catalysts could be recovered and reused, improving GOS production yield. This procedure can still be further optimized, by studying a number of parameters, such as pH, time, temperature, sugar/enzyme ratio, and number of cycles performed.

Future works could revolve around experimenting the culture of the studied bacteria in media composed of the α-GOS wastewaters and the yeast extract produced from the surplus yeast. There is sufficient evidence that combining these two materials a very rich ingredient for bacterial culture would be obtained. Another point of interest would be completing the studies with both bacterial strains, since *L. plantarum* CIDCA 83114 was used for the cultivation with the wastewaters and *L. plantarum* WCFS1 was used for the stabilization studies.

Prebiotic α- and β-GOS, lactic acid bacteria, and yeast extracts are all food products with associated health benefits and applications. The final conclusion of this work is that it is possible to produce health-promoting added-value food products by the valorization of food industry's by-products. This way tackling waste management and environmental concerns while reintroducing these materials back into the food industry.

The work developed during my PhD gracefully aligned with the expectations of the research group in the scope of the PREMIUM project. In this sense, we were able to contribute to the scientific advances in understanding the protective mechanisms of oligosaccharides towards lactic acid bacteria, particularly with the α-GOS wastewaters from legumes seeds. The study of these materials is still undeveloped, possibly because of the difficulty in their obtention, contrary to the β-GOS, which are readily obtained from lactose.

This thesis is composed of two review papers published before the start of this PhD, and one review and three research papers published during this time. However, other works were developed in related topics, and several communications were performed in regional, national, and international conferences and scientific meetings greatly contributing to the dissemination of my scientific production, and that of the PREMIUM project.