# Characterisation and bioconversion of Andean seeds' macromolecules into bioactive food ingredients

Ibieta Jiménez, Gabriela

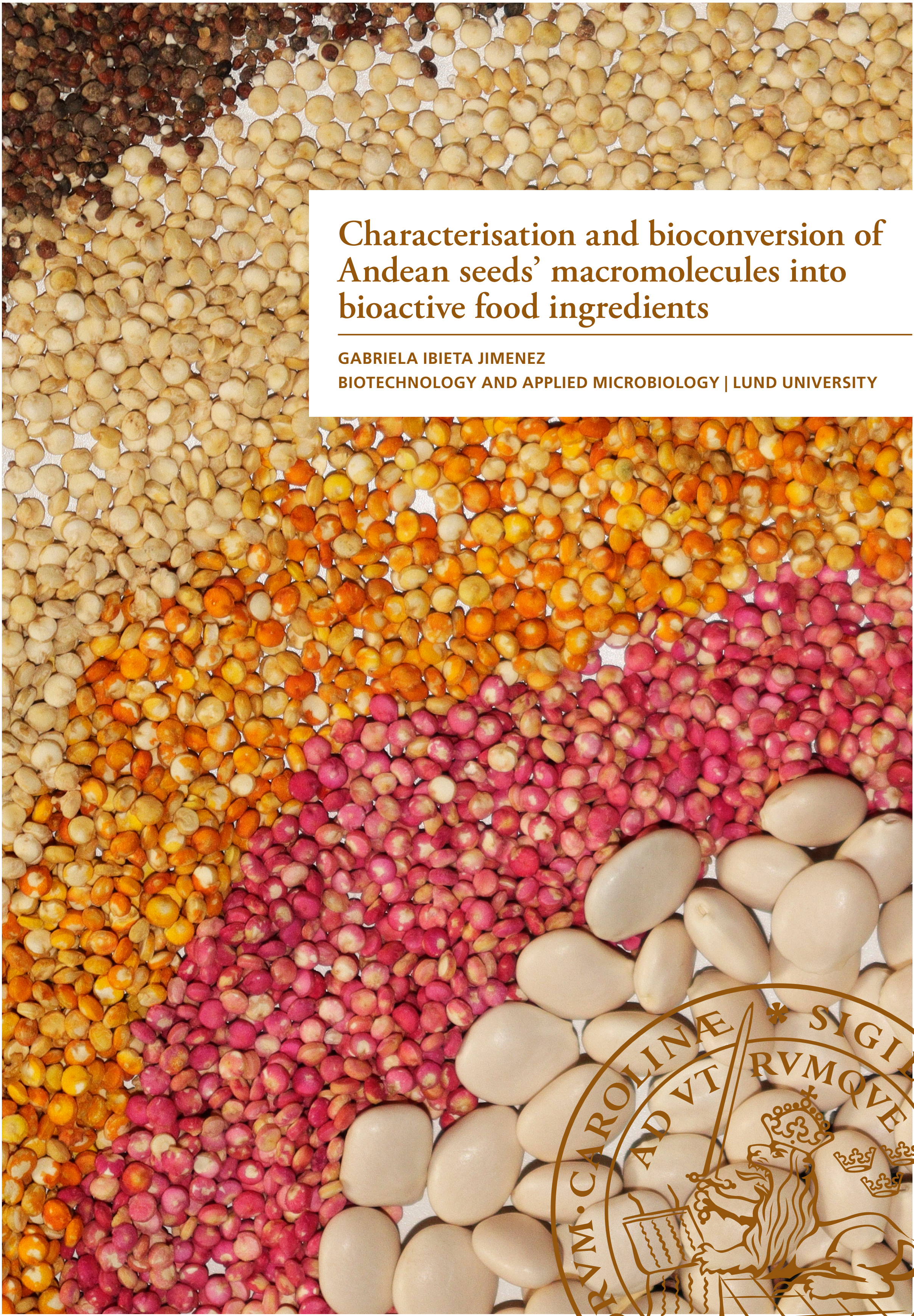
2025

*Citation for published version (APA):*
Ibieta Jiménez, G. (2025). *Characterisation and bioconversion of Andean seeds' macromolecules into bioactive food ingredients*. Biotechnology, Lund University.

*Total number of authors:*
1

LUND UNIVERSITY

PO Box 117
221 00 Lund
+46 46-222 00 00



# Characterisation and bioconversion of Andean seeds' macromolecules into bioactive food ingredients

GABRIELA IBIETA JIMENEZ
BIOTECHNOLOGY AND APPLIED MICROBIOLOGY | LUND UNIVERSITY

Printed by Media-Tryck, Lund 2025 NORDIC SWAN ECOLABEL 3041 0903

Growing up in a city at 4,000 meters above sea level where there is always someone selling boiled tarwi on the streets and my mom cooking quinoa "cake" very often. I was not aware of the benefits of these seeds and I am sure most people wasn't either. Now, many years after and with all the knowledge I have gained, I hope we can continue investigating and making people aware of the potential of our Andean seeds. Maybe in some years we will see new functional food products around the world based on them.

LUND UNIVERSITY

Faculty of Engineering
Department of Process and Life Science Engineering
Division of Biotechnology and Applied Microbiology

ISBN 978-91-8096-118-9

# Characterisation and bioconversion of Andean seeds' macromolecules into bioactive food ingredients

# Characterisation and bioconversion of Andean seeds' macromolecules into bioactive food ingredients

Gabriela Ibieta Jimenez

DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the Faculty of Engineering at Lund University to be publicly defended on Friday, 7th of November 2025, at 09.00 in the lecture Hall B, Kemicentrum, Lund.

*Faculty opponent*
Professor Katleen Raes, Department of Food Technology, Safety and Health, Ghent University – Campus Kortrijk, Belgium.

**Organization:** LUND UNIVERSITY **Document name:** Doctoral thesis

**Date of issue:** 2025-11-07

**Author(s):** Gabriela Ibieta Jimenez

**Sponsoring organization:** Swedish International Development Cooperation Agency (SIDA)

**Title and subtitle:** Characterisation and bioconversion of Andean seeds' macromolecules into bioactive food ingredients

**Abstract:** The global demand for sustainable, plant-based food systems has increased significantly over the past decade and is projected to continue rising. Andean seeds are interesting contributors, not only for their high nutritional value but also for their ability to grow naturally in very harsh conditions. Among these Andean seeds, four have gained interest due to their nutritional value and potential industrial applications: tarwi (*Lupinus mutabilis*), cañihua (*Chenopodium pallidicaule*), quinoa Real (*Chenopodium quinoa*), and tara (*Caesalpinia spinosa*).

Although many studies have been conducted on Andean seeds, more information is still needed. Even if all the benefits are scientifically proven, there may still be a problem in raising awareness about this. In this sense, this thesis work aims first to characterise two of the main types of macromolecules present in the natural composition of Bolivian Andean seeds, polysaccharides and proteins. Then, the thesis aims to convert these macromolecules into smaller bioactive compounds by using enzymes and biotransformation with lactic acid bacteria (LAB), which are biotechnologies with minimal environmental impact. Tara's galactomannan was structurally characterised, including the first determination of its refractive index increment (dn/dc). The non-digestible polysaccharide fractions from cañihua and tarwi (cellulose and hemicellulose) were characterized and then hydrolyzed to produce fermentable oligosaccharides, which demonstrated prebiotic activity by in vitro stimulating the growth of *L. casei*, *L. rhamnosus*, and *P. freudenreichii*.

Among the Andean seeds studied in this thesis, tarwi stands out for its high protein content; proteomic analysis revealed the presence novel and nutritionally valuable proteins. Moreover, the amino acid composition of its proteins exhibits a valuable precursor: L-glutamic acid, which can be irreversibly converted into γ-aminobutyric acid (GABA). This well-established inhibitory neurotransmitter plays a pivotal role in the central nervous system. GABA production was achieved in tarwi, cañihua, and quinoa seeds via recombinant glutamate decarboxylase (GAD) enzymes from *L. brevis,* and through fermentation, which also yielded beneficial metabolites, including lactic and acetic acid, as well as essential amino acids.

This thesis makes significant contributions to the valorisation of Andean seeds by uncovering their potential as sources of bioactive compounds through sustainable biotechnological processes. It provides a detailed characterisation of native polysaccharides and proteins and demonstrates their transformation into bioactive compounds. These findings not only advance the understanding of the functional properties of Andean seed macromolecules but also open new avenues for their application in the formulation of health-promoting food products. By linking molecular characterisation with microbial biotransformation, the thesis lays the groundwork for the development of high-value ingredients and supports the integration of Andean seeds into global innovation and commercialisation efforts.

**Key words:** Andean seeds, characterisation, bioconversion, carbohydrates, proteins, functional foods.

**Language:** English **Number of pages**: 76

**ISBN (printed version):** 978-91-8096-118-9

**ISBN (digital version):** 978-91-8096-120-2

Recipient's notes Price Security classification

Signature Date 2025-09-29

# Characterisation and bioconversion of Andean seeds' macromolecules into bioactive food ingredients

Gabriela Ibieta Jimenez

LUND
UNIVERSITY

Cover image by William Dóra & Gabriela Ibieta Jimenez

Published by:
Division of Biotechnology and Applied Microbiology
Department of Process and Life Science Engineering
Faculty of Engineering
Lund University

ISBN (printed version) 978-91-8096-118-9
ISBN (digital version) 978-91-8096-120-2

Printed in Sweden by Media-Tryck, Lund University,
Lund, 2025

Printed matter
3041 0903

Media-Tryck is a Nordic Swan Ecolabel
certified provider of printed material.
Read more about our environmental
work at www.mediatryck.lu.se

MADE IN SWEDEN

*To the curiosity that inspires science*

# Table of Contents

# Abstract

The global demand for sustainable, plant-based food systems has increased significantly over the past decade and is projected to continue rising. Andean seeds are interesting contributors, not only for their high nutritional value but also for their ability to grow naturally in very harsh conditions. Among these Andean seeds, four have gained interest due to their nutritional value and potential industrial applications: tarwi (*Lupinus mutabilis*), cañihua (*Chenopodium pallidicaule*), quinoa Real (*Chenopodium quinoa*), and tara (*Caesalpinia spinosa*).

Although many studies have been conducted on Andean seeds, more information is still needed. Even if all the benefits are scientifically proven, there may still be a problem in raising awareness about this. In this sense, this thesis work aims first to characterise two of the main types of macromolecules present in the natural composition of Bolivian Andean seeds, polysaccharides and proteins. Then, the thesis aims to convert these macromolecules into smaller bioactive compounds by using enzymes and biotransformation with lactic acid bacteria (LAB), which are biotechnologies with minimal environmental impact. Tara's galactomannan was structurally characterised, including the first determination of its refractive index increment (dn/dc). The non-digestible polysaccharide fractions from cañihua and tarwi (cellulose and hemicellulose) were characterized and then hydrolyzed to produce fermentable oligosaccharides, which demonstrated prebiotic activity by in vitro stimulating the growth of *L. casei*, *L. rhamnosus*, and *P. freudenreichii*.

Among the Andean seeds studied in this thesis, tarwi stands out for its high protein content; proteomic analysis revealed the presence novel and nutritionally valuable proteins. Moreover, the amino acid composition of its proteins exhibits a valuable precursor: L-glutamic acid, which can be irreversibly converted into γ-aminobutyric acid (GABA). This well-established inhibitory neurotransmitter plays a pivotal role in the central nervous system. GABA production was achieved in tarwi, cañihua, and quinoa seeds via recombinant glutamate decarboxylase (GAD) enzymes from *L. brevis*, and through fermentation, which also yielded beneficial metabolites, including lactic and acetic acid, as well as essential amino acids.

This thesis makes significant contributions to the valorisation of Andean seeds by uncovering their potential as sources of bioactive compounds through sustainable biotechnological processes. It provides a detailed characterisation of native polysaccharides and proteins and demonstrates their transformation into bioactive

compounds. These findings not only advance the understanding of the functional properties of Andean seed macromolecules but also open new avenues for their application in the formulation of health-promoting food products. By linking molecular characterisation with microbial biotransformation, the thesis lays the groundwork for the development of high-value ingredients and supports the integration of Andean seeds into global innovation and commercialisation efforts.

# Popular science summary

In recent years, an increasing number of people worldwide have begun seeking healthier and more sustainable food options. One exciting source of nutrition comes from traditional seeds growing in the Andean region of Bolivia. These seeds - tarwi, cañihua, quinoa, and tara - are not only packed with nutrients but also grow naturally in challenging environments, such as salty soil, high altitudes, and drastically changing temperatures.

Although these seeds have been cultivated and consumed for generations, scientists still have much to learn about their composition and health benefits. My research delves deeper into what makes these seeds special, particularly in terms of their proteins and sugars, and how we can unlock even more health-promoting benefits from them. As part of our search for sustainable processes, we focused on utilising enzymes and friendly bacteria. Enzymes are proteins used as biological catalysts, meaning they can accelerate the rate of reactions without being consumed or altered in the process. In this thesis, we focus particularly on two functions of enzymes: firstly, their ability to break down large structures, such as carbohydrates, into smaller pieces (by hydrolysis), and secondly, their ability to irreversibly convert one molecule into another. In addition to enzyme use, probiotics, which are the friendly bacteria that reside in our gut, were utilised to investigate the health-promoting properties of the seeds. These bacteria utilise fibre or sugars that our own body cannot absorb and digest, and produce compounds that offer numerous benefits to our physical and mental health.

In this sense, we initially studied the composition and structure of the carbohydrates in tara, tarwi, and cañihua seeds. Then, we used enzymes to break down the carbohydrates in tarwi and cañihua, converting them into smaller structures called oligosaccharides. These oligosaccharides were tested on a laboratory scale as prebiotics, that is, substances that cannot be broken down or absorbed by the body, but serve as nutrients for the beneficial bacteria in the intestine. In these studies, the oligosaccharides were utilised by three human probiotic bacteria and converted into molecules with potential positive effects on human health.

Among the seeds studied in this thesis, tarwi has become important due to its high protein content, which is even higher than that of soybeans. Upon closer examination of tarwi's proteins, we found that they are not only nutritious but also novel structures, not previously identified in other species. Looking deeper into the small molecules that build proteins, amino acids, we found high quantities of L-glutamic acid. What makes this amino acid so interesting? The so-called GAD enzymes can irreversibly convert L-glutamic acid into a neurotransmitter called GABA, which is involved in the strong connection between the gut and the brain, known as the gut-brain axis. This is a bidirectional connection, meaning that what we eat can influence our emotional state, and our emotional state can also affect how the probiotic bacteria in our gut function. GABA occurs naturally in foods like tomatoes, potatoes, and asparagus. Still, it can also be produced in other foods through natural processes such as fermentation, even in foods that do not contain it originally. Based on this, we studied the production of GABA in tarwi, cañihua, and quinoa Real by a friendly bacterium, *Levilactobacillus brevis*, in two ways: first, by producing its GAD enzymes in the laboratory and applying them to the seeds, and second, by fermenting the seeds with *L. brevis*. Both approaches could successfully produce GABA. While enzyme treatment is more specific, fermentation also yields other compounds, including lactic and acetic acid, as well as some essential amino acids.

By demonstrating how these traditional seeds can be utilised to produce healthier foods, my research aims to support local producers, promote sustainable agriculture, and inspire the development of new food products that benefit not only the people in Bolivia, but also people worldwide.

# Populärvetenskaplig sammanfattning

På senare år har fler och fler människor världen över börjat söka hälsosammare och mer hållbara matalternativ. En intressant källa till näring utgörs av traditionella frön som växer kring Anderna i Bolivia. Dessa frön - tarwi, cañihua, quinoa, och tara - är inte bara fullproppade med näring, utan växer också naturligt i tuffa miljöer som i salthaltig jord, hög altitud, och växlande temperaturer.

Trots att dessa frön har odlats och använts i generationer, har forskare fortfarande mycket att lära om deras uppbyggnad och hälsofördelar. Min forskning fördjupar sig i vad som gör dessa frön speciella, särskilt vad gäller deras proteiner och sockerarter, och hur vi kan få ut ännu mer hälsofördelar från dem. Som en del av vårt sökande efter hållbara processer, fokuserade vi på att använda enzymer och godartade bakterier. Enzymer är proteiner som används som en biologisk katalysator, vilket betyder att de kan accelerera reaktionshastigheter utan att själva bli konsumerade eller förändrade i processen. Denna avhandling fokuserar speciellt på två tillämpningar av enzymer: för det första, deras förmåga att underlätta nedbrytningen av större strukturer, till exempel kolhydrater, till mindre delar (genom hydrolys), och för det andra, deras förmåga att påskynda irreversibel omvandling av en molekyl till en annan. Utöver enzymanvändning, användes även probiotika, det vill säga godartade bakterier som ingår i vår tarmflora, för att studera frönas hälsofrämjande egenskaper. Dessa godartade bakterier använder till exempel fibrer eller socker som inte kan absorberas eller brytas ner av vår kropp, och producerar föreningar som bidrar till vår fysiska och mentala hälsa.

Inledningsvis studerades sammansättning och strukturer av kolhydrater i tara-, tarwi- och cañihuafrön. Sedan användes enzymer för att bryta ner kolhydraterna i tarwi och cañihua, för att omvandla dem till mindre strukturer som kallas oligosackarider. Dessa oligosackarider studerades sedan på laboratorienivå som prebiotika, det vill säga ämnen som inte kan brytas ner eller absorberas av kroppen, men som fungerar som näring åt tarmens goda bakterier. Oligosackariderna kunde utnyttjas av tre mänskliga probiotiska bakterier och omvandlas till molekyler med potentiellt positiva effekter på människors hälsa.

Bland de frön som har studerats i denna avhandling har tarwi visat sig vara viktig på grund av sin höga halt av protein, vilket är högre än i sojabönor. Vid närmare analys av tarwiproteinerna framkommer att de inte bara är näringsrika, utan också har unika strukturer som hittills inte har identifieras i någon annan art. Vid närmre undersökning av de molekyler som bygger proteiner, aminosyror, hittade vi stora kvantiteter av L-glutaminsyra. Så varför är denna aminosyra så intressant? L-glutaminsyra kan irreversibelt omvandlas av så kallade GAD-enzymer till signalsubstansen GABA som är inblandad i den starka förbindelsen mellan tarmen och hjärnan, även kallad tarm-hjärna-axeln. Detta är en dubbelriktad axel, vilket innebär att vad vi äter kan påverka vårt känslomässiga tillstånd och vårt känslomässiga tillstånd kan också påverka hur de probiotiska bakterierna i vår tarm fungerar. GABA förekommer naturligt i livsmedel såsom tomater, potatis och sparris. Det kan också produceras i livsmedel genom naturliga processer, såsom fermentering, även om de inte innehåller ämnet från början. I den här avhandlingen studerades produktionen av GABA i tarwi, canihua och quinoa Real av en godartad bakterie, *Levilactobacillus brevis*, på två sätt: först, genom att producera dess GAD-enzymer i laboratoriet och applicera dem på fröna, sedan genom fermentering av fröna med *L. brevis*. Båda metoderna kunde framgångsrikt producera GABA. Medan enzymbehandling är mer specifik,  leder fermentering till produktion av ytterligare andra ämnen, såsom mjölksyra, ättiksyra, och även några essentiella aminosyror.

Genom att visa hur dessa traditionella frön kan användas för att producera hälsosammare mat, syftar min forskning till att stödja lokala producenter, främja hållbart jordbruk, och inspirera utvecklingen av nya livsmedelsprodukter som inte bara gynnar människorna i Bolivia, utan även människor över hela världen.

# Resumen científico popular

El número de personas que buscan opciones alimentarias más saludables y sostenibles ha aumentado significativamente en los últimos años. Una fuente prometedora de nutrientes se encuentra en semillas tradicionales que crecen en la región andina de Bolivia. Cuatro semillas, el tarwi, la cañihua, la quinua y la tara no solo resaltan por sus nutrientes, sino también porque se desarrollan de forma natural en condiciones ambientales desafiantes, como suelos muy salinos, gran altura sobre el nivel del mar y cambios de temperatura drásticos.

A pesar de que estas semillas han sido cultivadas y consumidas durante muchas generaciones, aún queda mucho por estudiar sobre su composición y potenciales beneficios para la salud. Este trabajo de investigación se enfoca en el estudio de las proteínas y azúcares que componen a las semillas y en cómo podemos transformarlos en compuestos con beneficios en la salud. En la búsqueda de procesos sostenibles, nos enfocamos en el uso de enzimas y bacterias beneficiosas. Las enzimas son proteínas que actúan como catalizadores biológicos, es decir, aceleran reacciones sin consumirse ni transformarse. En esta tesis, nos centramos en dos funciones de las enzimas: primero, en su capacidad para descomponer estructuras grandes como los carbohidratos en fragmentos más pequeños (hidrólisis) y segundo, en su habilidad para convertir una molécula en otra distinta. Adicionalmente al uso de enzimas, los probióticos, que son microorganismos que habitan en el intestino humano, fueron usados para estudiar las propiedades benéficas de las semillas Andinas. Estas bacterias, usan fibras o azúcares que no pueden ser digeridos por el cuerpo humano y producen compuestos con efectos positivos en la salud física y mental.

En este contexto, estudiamos la composición y estructura de los carbohidratos presentes en las semillas de tara, tarwi y cañihua. Luego, utilizamos enzimas para hidrolizar los carbohidratos de tarwi y cañihua obteniendo estructuras más pequeñas llamadas oligosacáridos. Estos oligosacáridos fueron evaluados como prebióticos en laboratorio, los prebióticos son sustancias que no pueden ser digeridas en el cuerpo humano, pero son nutrientes para las bacterias beneficiosas en nuestros intestinos. En estos estudios, los oligosacáridos demostraron su capacidad para estimular el crecimiento de tres cepas probióticas humanas y se convirtieron en moléculas con efectos positivos para la salud.

El tarwi, en particular, destaca por su elevado contenido proteico, que es incluso superior al de la soya. Analizando las proteínas de tarwi, descubrimos que no solo tienen un alto valor nutricional, sino que también son estructuras no reportadas previamente. Además, estudiando a las pequeñas estructuras que componen a las proteínas, los aminoácidos, detectamos niveles elevados de ácido L-glutámico. ¿Qué hace a este aminoácido interesante? Con el uso de una enzima llamada GAD, el ácido L-glutámico puede convertirse en ácido γ-aminobutírico (GABA), que es un neurotransmisor clave en el eje intestino-cerebro. Éste eje es una conexión bidireccional, lo que significa que los alimentos que consumimos pueden afectar nuestro estado emocional y nuestro estado emocional también puede afectar el funcionamiento de las bacterias beneficiosas que viven en nuestros intestinos. El GABA se encuentra naturalmente en alimentos como el tomate, la papa y los espárragos. Pero, también es posible producirlo en alimentos que no lo contienen.

Como parte de la tesis, estudiamos la producción de GABA en tarwi, cañahua y quinua Real con la bacteria beneficiosa *Levilactobacillus brevis* de dos maneras: primero, produciendo sus enzimas GAD en laboratorio y aplicándolas a las semillas; y segundo, fermentando las semillas con *L. brevis*. Ambas estrategias funcionaron para producir GABA: las enzimas lo hicieron de forma específica, mientras que la fermentación también produjo otros compuestos como ácido láctico, ácido acético y algunos aminoácidos esenciales.

Este trabajo demuestra el potencial de las semillas andinas como materia prima para el desarrollo de alimentos funcionales, contribuyendo a la valorización de cultivos tradicionales, al fortalecimiento de la agricultura sostenible y a la creación de productos que beneficien tanto a productores locales como a consumidores a nivel global.

# List of papers

I. **Molecular characterisation of a galactomannan extracted from Tara (*Caesalpinia spinosa*) seeds.**
Gabriela Ibieta, Atma-Sol Bustos, Jimena Ortiz-Sempértegui, Javier A. Linares-Pastén, & J. Mauricio Peñarrieta.
Scientific Reports, 13(1), article 21893, 2023
https://doi.org/10.1038/s41598-023-49149-3

II. **Characterisation of non-digestible polysaccharides from tarwi and cañihua seeds.**
Gabriela Ibieta, Jimena Ortiz-Sempértegui, Tam Nguyen, Baozhong Zhang, J. Mauricio Peñarrieta, & Javier A. Linares-Pastén, 2025 (manuscript).

III. **Prebiotic potential of enzymatic hydrolysates derived from non-digestible polysaccharides of tarwi and cañihua seeds.**
Gabriela Ibieta, Jimena Ortiz-Sempértegui, J. Mauricio Peñarrieta, & Javier A. Linares-Pastén, 2025 (submitted to LWT journal).

IV. **Microwave-assisted extraction and proteomic analysis of tarwi (*Lupinus mutabilis*) seeds' proteins.**
Gabriela Ibieta, Katja Bernfur, Jimena Ortiz-Sempértegui, J. Mauricio Peñarrieta, & Javier A. Linares-Pastén, 2025 (manuscript).

V. **Enhancing the functional value of Andean food plants: Enzymatic production of γ-aminobutyric acid from tarwi, cañihua and quinoa real seeds' proteins.**
Gabriela Ibieta, Jimena Ortiz-Sempértegui, J. Mauricio Peñarrieta, & Javier A. Linares-Pastén.
LWT, vol 220, article 117564, 2025.
https://doi.org/10.1016/j.lwt.2025.117564

VI. **γ-aminobutyric acid (GABA) production and soluble free amino acid profile change in Andean seeds by *Levilactobacillus brevis* fermentation**
Gabriela Ibieta, Jimena Ortiz-Sempértegui, Carl Grey, J. Mauricio Peñarrieta, & Javier A. Linares-Pastén, 2025 (submitted to Frontiers in Nutrition journal).

# Author contributions

**I.** I planned and designed the study in collaboration with ASB, JMP, and JALP, and I performed all the experiments. The experiments by AF4 were conducted in collaboration with JOS under the supervision of ASB. I conducted the data analysis and visualisations from AF4, together with ASB. I wrote the original draft, and all co-authors contributed to the review and editing of the manuscript.

**II.** I planned and designed the study under the supervision of JALP. I performed all the experiments except for the X-ray diffraction, which TN conducted in collaboration with BZ. I performed the interpretation and data analysis, as well as the visualisations. I wrote the original draft, and all co-authors contributed to the review and editing of the manuscript.

**III.** I planned and designed the study under the supervision of JALP. I performed all experimental work with the help of JOS. I conducted the interpretation, performed the data analysis, and wrote the original draft. The visualisations were done in collaboration with JALP. All co-authors contributed to the review and editing of the manuscript.

**IV.** I planned and designed the study under the supervision of JALP. I performed all experiments except for the MALDI-TOF analysis, which was performed by KB. I conducted the interpretation, data analysis, visualisations, and wrote the original draft. All co-authors contributed to the review and editing of the manuscript.

**V.** I planned and designed the study under the supervision of JALP. I performed all experiments with the help of JOS. I conducted the interpretation, data analysis, statistical analysis, visualisations, and wrote the original draft. All co-authors contributed to the review and editing of the manuscript.

**VI.** I planned and designed the study under the supervision of JALP. I performed all experiments, and the LC-MS analysis was conducted in collaboration with CG. I conducted the interpretation, data analysis, visualisations, and wrote the original draft. All co-authors contributed to the review and editing of the manuscript.

# Publications not included in the thesis

The following papers are not included in the thesis, but I contributed to them during my PhD, either as first or second author.

i. **Chemical Characterisation of New Oils Extracted from Cañihua and Tarwi Seeds with Different Organic Solvents**
Jimena Ortiz-Sempértegui, Gabriela Ibieta, Cecilia Tullberg, J. Mauricio Peñarrieta, & Javier A. Linares-Pastén,
*Foods*, *13*(13), 1982, 2024.
https://doi.org/10.3390/foods13131982

ii. **Bioactive Peptides as Functional Food Ingredients: Production, Mechanisms of Action, Market Trends, and Future Perspectives with Emphasis on Andean Crops**
Gabriela Ibieta, Jimena Ortiz-Sempértegui, J. Mauricio Peñarrieta, & Javier A. Linares-Pastén, 2025 (submitted to Journal of Functional Foods).

# Abbreviations

| | |
|---|---|
| AF4 | Asymmetrical Flow Field-Flow Fractionation |
| COS | Cellooligosaccharides |
| FTIR | Fourier-Transform Infrared spectroscopy |
| GAD | Glutamate Decarboxylase |
| GABA | γ-aminobutyric acid |
| LAB | Lactic Acid Bacteria |
| MALDI-TOF | Matrix-Assisted Laser Desorption Ionization–Time of Flight |
| MAE | Microwave-assisted extraction |
| MW | Molecular weight |
| PLP | Pyridoxal-5′-phosphate |
| SCFAs | Short-Chain Fatty Acids |
| SEM | Scanning Electron Microscopy |
| TG | Tara Gum |
| XOS | Xylooligosaccharides |
| XRD | X-ray Diffraction |

# Introduction

Bolivian Andean seeds have gained research interest due to their nutritional value. Studies have thus far focused mostly on their characterisation, considering protein content, fatty acid composition, and naturally occurring bioactive compounds, such as antioxidants and bioactive peptides. However, deeper characterisation is needed, as well as investigating the transformation into new bioactive compounds. As there is a global need for new sustainable sources and technologies to obtain food products with added nutritional and functional value (health benefits), Andean seeds appear as interesting contributors, not only for their high nutritional value but also for their ability to naturally grow in very harsh conditions including soil with high salt content, arid, high altitude above sea level and freezing temperatures during night.

Besides growing in harsh conditions, Andean seeds can also benefit the soil where they grow, unlike other seeds, which can lead to deforestation or infertile soils. To address deforestation and climate change, the cultivation of Andean seeds, such as tarwi (*Lupinus mutabilis*), cañihua (*Chenopodium pallidicaule*), and quinoa (*Chenopodium quinoa*), is of great interest due to their ability to grow and the positive impact they have on the soil. Tarwi contributes to soil fertility by fixing higher amounts of Nitrogen compared to other legumes [1]. Recent research also suggests that intercropping quinoa with legumes improved the yields, including enhanced plant and soil nutrients, and increased soil enzyme activity [2].

There is a lack of deep characterisation of two of the main macromolecules in Andean seeds; carbohydrates and proteins. Most of literature found has quantified the amount of these macromolecules in the seeds but their structures remain quite understudied. For example, cañihua and tarwi have been studied in terms of their starch content, however, their cellulose and hemicellulose have not been characterised. Then, tarwi has been used for novel formulations for its high protein content, still, its proteins have not been characterised, and recently, one variety has been fully sequenced, being the first study on this field [3]. Therefore, characterising tarwi's proteins is of high importance, particularly to identify conglutins, which are the main group of proteins found in other lupin species. They are rich in essential amino acids and have functional properties, particularly γ-conglutins, have demonstrated anti-diabetic properties by improving glucose uptake and regulating insulin pathways. β-conglutins exhibit anti-inflammatory and antioxidant activities [4].

A key aspect when investigating the nutritional potential of food matrices emerges in the context of the gut microbiome. It has been widely studied that the microorganisms living in the digestive tract, especially in the colon (probiotics), require non-digestible carbohydrates that can reach these areas without being absorbed by the human body (prebiotics). The gut microbiome is an ecosystem of microbiota, including probiotics. When healthy, it supports various functions in the human body, such as the absorption of micronutrients, protection against pathogens, regulation of the immune system, and strengthening of biochemical barriers. In this sense, it is crucial to have a diverse range of nutrients in the daily diet to reap all the benefits of a healthy microbiome.

The connection between the gut microbiome and the brain has garnered significant interest in research over the last decade. It has been proven that a healthy microbiome not only promotes physical wellness but also contributes to mental health. This connection is known as the gut-brain axis, a bidirectional communication pathway between the central and enteric nervous systems, linking emotional and cognitive centres of the brain with peripheral intestinal functions. Recent research in this field has identified a promising molecule, γ-aminobutyric acid (GABA), which is well known as an inhibitory neurotransmitter that plays a crucial role in the central nervous system. Imbalances of GABA are associated with neurological diseases, such as Alzheimer's and Parkinson's disease, and psychological disorders, including anxiety, depression, and stress [5]. Recent research shows that GABA is also a potent mediator of the gut-brain axis.

The importance of maintaining a healthy gut microbiome is evident for both physical and mental health. To obtain molecules with a positive effect on the gut microbiome, the use of enzymes and fermentation with LAB are two important biotechnologies that can transform macromolecules, such as proteins and carbohydrates, into smaller molecules with biological benefits for humans.

In this sense, is it possible to use Andean seeds as raw material to produce potential functional food ingredients using enzymes and LAB? The following chapters describe how this thesis addressed the question.

## Amis and scope of the thesis

This thesis has two main objectives: (1) first, to characterise two of the main macromolecules present in the natural composition of Bolivian Andean seeds, polysaccharides and proteins. (2) Then, the thesis aims to convert these macromolecules into smaller bioactive compounds by using enzymes and biotransformation with lactic acid bacteria (LAB), which are biotechnologies with minimal environmental impact.

The outcomes of this thesis are presented in six papers: Papers I to III focus on polysaccharides, while Papers IV to VI address proteins. A description of each article is provided below.

**Paper I** studied a well-known galactomannan extracted from the endosperm of tara seeds (*Caesalpinia spinosa*), which is used as a thickener and stabiliser in many food products. It was molecularly characterised by asymmetrical flow field-flow fractionation (AF4). The characterisation includes monosaccharide composition, molar mass, root mean square radius, hydrodynamic radius, conformation, and densities, as well as the determination of the specific refractive index increment (dn/dc) for the first time for this galactomannan.

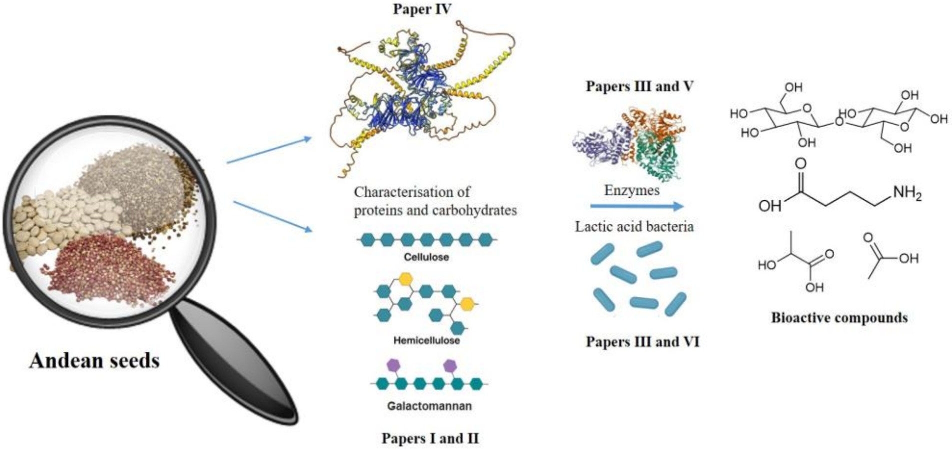
**Paper II** dived into the characterisation of the non-digestible polysaccharides of tarwi and cañihua, studying the cellulose and hemicellulose fractions, which are part of the so-called dietary fibre. The seeds were subjected to an alkali extraction, and the extracted polysaccharides were analysed in terms of monosaccharide composition and d-glucoronic acid content. Additionally, FTIR analysis was performed, as well as the total phenolic compounds and total starch in these fractions. Finally, X-ray diffraction and SEM are used to gain a better understanding of the structure of these polysaccharides.

**Paper III** studied the production of fermentable oligosaccharides from the non-digestible polysaccharides of cañihua and tarwi by using a sequential enzymatic treatment. The hydrolysis process began with a cellulose blend comprising cellobiosidase I and II, β-glucosidase, and endo-1,4-β-D-glucanase. Followed by a treatment with α-amylase and a final treatment with a mix of xylanase and glucuronoxylanase. The products of hydrolysis were used to grow three LAB: *Lacticaseibacillus rhamnosus*, *Lacticaseibacillus casei*, and *Propionibacterium freudenreichii*. Products of fermentation and consumption of oligosaccharides during fermentation were analysed.

**Paper IV** examined the proteins of tarwi seeds, given this legume's high protein content, which accounts for approximately 46% of the seed's total weight. Microwave-assisted alkaline extraction yielded more soluble protein and fewer glycoproteins compared to neutral and acidic extractions. MALDI-TOF analysis revealed novel protein structures, due to no 100% similarity to proteins found in other species. Among the most nutritional and functional proteins, β- and δ-conlgutins were identified.

**Paper V** focused on the production of GABA from naturally occurring L-glutamic acid in tarwi, cañihua, and quinoa Real seeds with two recombinant GAD enzymes, GadA and GadB, produced by *Levilactobacillus brevis*. GAD enzymes can irreversibly convert L-glutamic acid into gamma-aminobutyric acid (GABA) in the presence of the pyridoxal-5′-phosphate (PLP) cofactor. The production of GABA in tarwi seeds was higher, correlating with its higher protein and L-glutamic acid content.

**Paper VI** studied a different approach to producing GABA in Andean seeds. Fermentation by *L. brevis*, which stands out for its ability to produce GABA among LAB. In this paper, the fermentation of Andean seeds by *L. brevis* produced SCFAs and GABA, and the change in soluble free amino acids due to fermentation was also determined.



# Bolivian Andean seeds, sustainable production, and nutritional potential

The Andean region of Bolivia, as the entire country is highly biodiverse, supporting a wide variety of crops, many of which are only known to the locals. Very few receive more attention, as research has shown their nutritional benefits. The best example is quinoa; whose production originates from the Andean region. Specifically, Real ("royal") quinoa is the one produced near the most extensive salt flats in Uyuni, Bolivia [6]. Nowadays, due to its nutritional benefits, quinoa is cultivated in many countries across all continents [7]. Other interesting seeds that grow in the Andean region of Bolivia are cañihua (*Chenopodium pallidicaule*), tarwi (*Lupinus mutabilis*), and tara (*Caesalpinia spinosa*). The first two due to their nutritional value, and the last one is attributed to its industrial applications. The gum found in the seed is used in the food industry as an emulsifying and thickening agent [8].

Despite the great variety of crops grown in this area, the Andean region of Bolivia is also affected by malnutrition, obesity, and diet-related chronic diseases, which highlights the significant inequalities observed worldwide [9]. Most of the crops cultivated in the Andes are underutilised species, and they play an important role in food security systems because they grow in infertile and arid soils, which are often crucial to household nutritional strategies [10]. In this sense, it becomes crucial to support the production of crops that, in addition to having low nutrient requirements during growth, also benefit the soil and provide nutritional benefits to consumers.

## Tarwi (*Lupinus mutabilis*)

It is a legume species native to the Andes, also known as Andean lupin; its production is essential for conserving agroecological systems. The plant can fix $N_2$ through nodules formed in its roots by the bacterium Rhizobium; it has been shown to fix up to 500 kg $ha^{-1}$ of $N_2$ per year [11]. Tarwi also grows in poor soil conditions with very low nutrient content [12]. One of the most important benefits for the producers in Bolivia is that the seeds can be stored for long times, around one or two years, allowing the producers to sell them when there is more demand for them or when the prices can go a bit higher, for example, at fairs or bigger markets [13]. Locals consume tarwi seeds as “mote”, a simple process that involves boiling. Outside the Andean regions, people are not familiar with or do not consume them. This is why it becomes very important to increase research and visibility of tarwi seeds.

The nutritional value of tarwi seeds primarily relies on their protein content, which ranges between 41% and 51%, depending on the cultivation location, variety, and environmental factors [14], exceeding that of any other lupin species. It also has oil content ranging between 14% and 24%. The Bolivian seeds used in this thesis have a protein content of 41.6 % to 46.4% (**Papers IV** and **V**) and an oil content of 18% when extracted with hexane [15].

Tarwi seeds have significant industrial potential, as the commercialisation of plant-based proteins and their derivatives has increased substantially over the last few decades. Tarwi production originated in the Andean region, and many European countries, including the Netherlands, Germany, Portugal, and Spain, are still adapting the seeds to their local environments. On the other hand, tarwi produced in the Andes is still undervalued. It is therefore necessary to increase innovative research on Andean tarwi to valorise its production, properties, and industrial potential through the use of new technologies.

Some studies have focused on the bioactive compounds present in tarwi seeds. Most of the bioactive compounds are found in the lipid fraction of the seeds. Many authors have identified carotenoids, tocopherols, and phenolic compounds that contribute to the antioxidant capacity of tarwi [16]. Tarwi seeds used in this thesis (Figure 1) were characterised in terms of the lipid fraction, and it was shown that the extraction of oil with ethanol yields higher total phenolic compounds, as well as an increased concentration of tocopherols compared to the oil extracted with other solvents. Interestingly, it also shows a more diverse fatty acid profile compared to commercial oils [15].

**Figure 1.** Tarwi seeds used in this thesis, with an average seed size of 1 cm.

## Quinoa real (“royal quinoa”) (*Chenopodium quinoa*)

Quinoa has gained significant interest in research over the past few decades. Initially, its origins correspond to the Andean region of South America; however, this seed exhibits a wide genetic diversity, resulting in a varied composition and numerous varieties worldwide. Quinoa Real is the designation of origin of the quinoa produced near the largest salt flats in the world, “Salar de Uyuni” in Uyuni, Bolivia. The environmental characteristics that support its growth include high soil salinity, intense UV radiation, and high altitude above sea level (higher than 3600 m). The main characteristics of the seeds are their large size and high saponin content compared to other quinoa varieties [6].

Little research has been conducted on quinoa Real's actual benefits. A notable advantage is its protein content, which surpasses that of cereals like rice, maize, barley, and wheat. The five quinoa varieties studied in this thesis (Figure 2) have a protein content ranging from 17.56% to 20.96% (**Paper V**), which is higher compared to the average protein content in other quinoa varieties worldwide. Other studies show that quinoa Real has a high fibre content. Black, red, and white quinoa real were studied, and they showed a high total dietary fibre content, with most of it being insoluble dietary fibre [17].

Another interesting characteristic of quinoa is its gluten-free nature. Research on the formulation of gluten-free products based on quinoa has increased in the last decade. For example, gluten-free pasta has been developed, utilising guar gum as an additive to mimic the structural properties of gluten. The resulting pasta exhibited a higher protein content, total phenolic content, and antioxidant capacity, as well as a significant increase in Ca, Fe, K, Mg, P, and Zn compared to a gluten-free pasta made from rice and corn [16]. Similarly, gluten-free shortbreads with 60% quinoa flour increased their dietary fibre content and polyphenol content in 5 fold compared to the controls; additionally, the content of essential amino acids increased [19].

Quinoa Real lacks information on the composition of bioactive compounds; most studies have been conducted on other varieties of quinoa. Quinoa seeds contain bioavailable vitamins, including vitamin C, vitamin B6, vitamin B5, thiamine, and folate. Vitamin C in 100 g of quinoa is 16 mg, which is undetectable in wheat, corn, or rice. Additionally, 100 g of quinoa contains high levels of vitamin B6 and folic acid, meeting approximately an adult's daily requirement. In particular, 100 grams of quinoa contain 80% of the recommended daily intake for children and 40% of the recommended daily intake for adults in terms of riboflavin. Furthermore, quinoa is a good source of vitamin B2, fulfilling approximately 40% of the daily recommended intake for an adult [20].

Quinoa Real “Pandela Rosada”

Quinoa Real “Kellu”

Quinoa Real “Challa Mok’o”

Quinoa Real “Chillpi”

Quinoa adapted to tropical climate

**Figure 2.** Quinoa real varieties and quinoa adapted to tropical climate used in this thesis. Average quinoa Real seed size 2.5 mm, and average quinoa adapted to tropical climate seed size 1.5 mm.

## Cañihua (*Chenopodium pallidicaule*)

Cañihua is considered part of the so-called “forgotten crops”; it is a pseudo cereal native to the Andes. Its production remains at subsistence levels, despite its remarkable agricultural characteristics, such as high resistance to climate change, a hostile environment, and low nutrient demand [21]. However, very little is known about this crop outside its place of cultivation, limiting its consumption.

Its nutritional composition relies mostly on its carbohydrate content, which is around 63% to 67% [22]. Then, proteins are the second principal component, accounting for approximately 15%. Cañihua seeds used in this thesis (Figure 3) contain 15.9% protein (**Paper V**) and 6.73% oil when extracted with hexane [15].

Cañihua belongs to the *Chenopodiaceae* family, but its saponin content is lower than that of other members of the same family, such as quinoa, and it does not give the bitter taste to the product. However, some studies have shown that cañihua varieties from Bolivia and Peru can contain saponins, and likewise quinoa, it can be divided into sweet and bitter varieties [23].

In the same way as quinoa, cañihua is gaining interest for its lack of gluten to formulate gluten-free products. For example, a study determined that the optimal composition of gluten-free bread had 100% cañihua flour (without any other flour additive) and xanthan gum [24].

The bioactive compounds found in cañihua include tocopherols, which appear to be superior to those found in cereals. For example, the oil from cañihua seeds used in this thesis when extracted with ethanol, has the highest tocopherols concentration (α, γ, and δ) as well as higher total phenolic content and antioxidant capacity compared to the same oil extracted with hexane and petroleum ether [15].

**Figure 3.** Cañihua seeds used in this thesis. Average seed size is 1 mm.

## Tara (*Caesalpinia spinosa*)

Tara is native to the Andes in South America; Peru is the largest producer, and production in Bolivia has increased in the last decade. In fact, in 2021, the first tara pod packaging facility was built, and the plant is capable of producing 6,000 tons per year. Tara is produced in orange pods that contain four to seven dark seeds (Figure 4). The pod is utilised in various industries due to its high tannin content [25]. The pods of the seeds used in this thesis were previously characterised in terms of total tannins, which ranged between 70% and 85% of the whole pod [26]. The pods have also garnered interest in the food industry due to their high concentration of phenolic compounds in ethanolic extracts, which have been shown to possess high antioxidant capacity (10.17 TE mmol $L^{-1}$ $g^{-1}$) [27]. An ethanolic extract, designed as P2Et, from tara pods has been widely studied, exhibiting antitumor and immunomodulatory activities in breast cancer, leukaemia, and melanoma. The safety dose in humans is determined to be a maximum of 600 mg/day [28].

On the other hand, tara seeds are also of interest in the food industry due to the galactomannan in the endosperm of the seed, known as tara gum (TG), which is already listed as a food additive in many countries as a stabilizer and a thickener [29]. More recent research includes TG as a delivery system for vitamin $D_3$, in a complex with gelatin A. This encapsulation increased thermal stability and protected vitamin $D_3$ from physiological conditions in the gastrointestinal tract [30].

TG is already well-known, and numerous studies have been conducted to investigate its potential bioactivity, as well as to explore structural modifications that enhance its properties. Four important modifications are: (1) carboxymethylation with monochloroacetic acid (MCA), the resulting modified gum presents characteristics of an anionic polysaccharide and reductions in molecular weight, and the pseudoplastic behaviour was maintained along with a lower viscosity compared to its native form. These results show potential complexing with other biomolecules and for the controlled release of bioactives [31]. (2) grafting with polyacrylic acid, which resulted in the formation of a 3D structure having numerous pores in it, that can absorb huge amounts of water and possibly form a barrier to prevent water molecules from overflowing. The superabsorbent polymer also showed antimicrobial properties, which were evaluated using bacterial strains of *E. coli* and *S. aureus* [32]. (3) cross-linking, where TG was incorporated into acrylic acid by gamma radiation to produce super-absorbent hydrogels. The obtained crosslinked hydrogels exhibited higher mechanical strength and an optimum swelling ratio, making them suitable for use as absorbents for body fluids, such as urine [33]. Finally, (4) sulfation with piperidine-N-sulfonic acid, the resulting sulfated TG had anticoagulant activity [34].

**Figure 4.** Tara seeds used in this thesis. Average seed size is 1 cm.

# Polysaccharides from Andean seeds

## Characterisation

Characterisation of polysaccharides extracted from seeds is quite challenging. First, due to difficulties in extracting or isolating them. Second, because their solubility in water might be low, or it may need to be enhanced by thermal treatment. This last factor can interfere with some analyses that require the high solubility of the polysaccharide. In this section of the thesis, we will examine the factors influencing the isolation and characterisation of polysaccharides in three Andean seeds: tara, tarwi, and cañihua.

## Galactomannan from tara

Galactomannans are water-soluble non-ionic polysaccharides used in the food and pharmaceutical industries. Usually, galactomannans are referred to the gums naturally present in the endosperm of seeds, and their structure is a linear β-(1→4) d-mannopyranosyl backbone (mannan) partially substituted by α-(1→6)-linked d-galactopyranosyl residues [35]. The significant difference between galactomannans from different plant sources lies in the galactose content and its distribution in the mannose backbone [36]. In general, it has been reported that guar gum has a Man/Gal ratio of around 2:1, the TG Man/Gal ratio is around 3:1, and locust bean gum has a ratio of around 4:1 [37]. This relationship has also been associated with the solubility of the galactomannan; the higher the substitution with galactose units in the mannose main chain, the higher the solubility [38]. The Man/Gal ratio is also associated with the viscosity of the polysaccharide; a higher ratio is associated with extended structures and higher apparent viscosity [39].

In tara seeds, the endosperm comprises 22-24% of the total seed weight, with the rest being 40% germ and approximately 26-28% hull or husk [40]. The isolation of the endosperm becomes difficult due to the hull's hardness. Although different methods are used, some of them employ strong acids or destroy either the endosperm or the germ. In this sense, the method used in **Paper I** is based on the process used for commercial TG isolation, which involves thermal-mechanical grinding to separate the endosperm from the germ and hull. Figure 5 shows the three separated parts. When the endosperm is separated, it is ground and suspended in hot water. Finally, ethanol is used to precipitate the pure gum, which then requires drying [40].

**Figure 5.** . The three main components of tara seeds are the germ, endosperm, and hull, as determined after thermal-mechanical separation for characterization in **Paper I.**

Once the gum is extracted, an important factor for AF4 analysis is the solubility of the sample in water. In general, galactomannan-based polysaccharides are soluble in water and form highly viscous and stable aqueous solutions [34]. The Bolivian TG isolated for this thesis was easily dissolved in the carrier liquid (10 mM $NaNO_3$) to proceed with the AF4 analysis (Figure 6). A crucial parameter in the characterisation of polysaccharides is the specific refractive index increment (dn/dc), especially when using light scattering and refractive index detection. Dn/dc represents the change in refractive index of a solution concerning a change in concentration and is essential for the accurate determination of molecular weight and size. According to the determination principle, a slight shift in dn/dc can significantly impact the result of MW obtained from light scattering [41]. The dn/dc value for TG was not determined before; therefore, the first step in characterising the Bolivian TG was the determination of the (dn/dc) for the first time (0.1454). The molecular characterisation of TG reveals a Man/Gal ratio of 3.37, with a molecular weight ranging from $2.460 \times 10^7$ to $3.699 \times 10^7$ Da, distributed in a single population.

Besides the chemical characteristics and MW of the gum, it was also possible to use a tool (Kratky plot) that analyses the flexibility and degree of unfolding of macromolecules. This analysis in **Paper I** indicated that TG is a random coil monodisperse, suggesting that the chains have a low branched density, are highly flexible, and disordered in solution, adopting a tangled, randomly coiled shape rather than a rigid or extended one, and a very narrow molecular weight distribution, meaning that most of the polymer molecules have similar sizes [42].

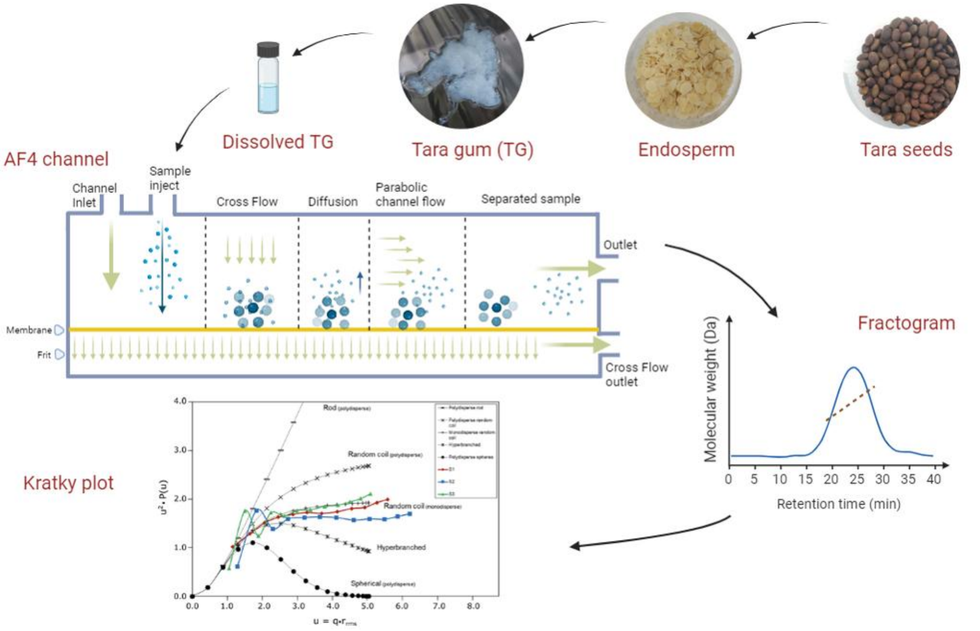


**Figure 6.** Isolation of TG from tara seeds and AF4 channel separation to identify molecular properties of the gum (**Paper I**).

## Cellulose and hemicellulose from cañihua

So far, the most studied carbohydrate fraction of cañihua is starch, which has been reported to range from 44.4 to 52% in Bolivian seeds [43]. Approximately 20% of this starch is amylose [44]. The amylose content falls within the range typically found in many starch-rich foods, indicating that the starch is relatively easily digested in the human digestive tract and serves as a rapid energy and glucose source [45]. Moreover, starch does not compose the full carbohydrate content of cañihua seeds, and there are non-digestible fractions that have not been well characterised.

**Paper II** began with the hypothesis of isolating and characterising these non-digestible fractions, specifically the cellulose and hemicellulose fractions. However, the analysis by AF4 and NMR to understand the structure was not possible due to the low solubility of the hemicellulose fractions in the three solvents tried (available in deuterated form for NMR): $H_2O$, DMSO, and chloroform. The soluble solutions achieved were not sufficiently concentrated for analysis, and it was decided to try other techniques, such as SEM, FTIR, and X-ray diffraction, to gain insights into the structure of these polysaccharides.

Typically, to isolate hemicellulose and cellulose from seeds the ground seeds are subjected to defatting, which involves extracting the fat fraction. Then, either an alkaline or acidic treatment can be applied using a strong base, such as sodium hydroxide, or a strong acid, like sulphuric acid to break down the hemicellulose, which is then precipitated with ethanol. Further purification is then applied. The residual solid material can be bleached using oxidising agents or treated with acids, such as nitric and acetic acids. Finally, the cellulose fraction is precipitated with ethanol and purified [46, 47]. In **Paper II**, the hemicellulose and cellulose fractions of cañihua were extracted by using first a strong alkaline treatment (hemicellulose) and then an acidic treatment (cellulose). Both fractions are shown in Figure 7.

**Figure 7. A)** Freeze-dried hemicellulose fraction of cañihua seeds after alkaline extraction. **B)** Freeze-dried cellulose fraction after hemicellulose extraction of cañihua seeds.

The carbohydrate fractions of cañihua seeds similarly to the seeds, have a dark brown colour. This factor still requires further investigation due to the resistance of the compounds responsible for the colour after all the strong procedures during extraction. Both fractions also have very low solubility in water. The hemicellulose fraction, as determined by monosaccharide composition analysis, is mainly composed of glucose and arabinose with a lower content of galactose and xylose. Hemicelluloses can vary in their composition even when comparing different parts of the same plant [48]. The characterisation of cañihua's hemicellulose also shows that most of the glucose is the soluble part of it, as well as a high antioxidant capacity. Literature suggests that the antioxidant capacity in hemicellulose may be attributed to phenolic compounds, such as ferulic acid and p-coumaric acid, or functional groups like hydroxyls and carboxyl groups [49]. The method used to detect antioxidant capacity was the Ferric Reducing Antioxidant Power (FRAP) assay, which measures the overall reducing potential of the polysaccharide structure [50]. The results in **Paper II** show a high antioxidant capacity, but not as high total phenolic compounds. The method for detecting phenolic compounds is based on the reaction with Folin-Ciocalteu reagent. This assay is designed for free phenolic compounds, and therefore, it would be necessary to break the bonds to detect structures such as ferulic acid [51].

The cellulose fraction of cañihua is composed mainly of glucose, as expected, and XRD shows two of the four typical peaks of cellulose; however, some crystalline impurities are also observed. SEM imaging of the cellulose fraction does not look like the regular fibre structures observed in SEM imaging of other cellulose samples. Figure 8 shows cañihua cellulose SEM imaging.

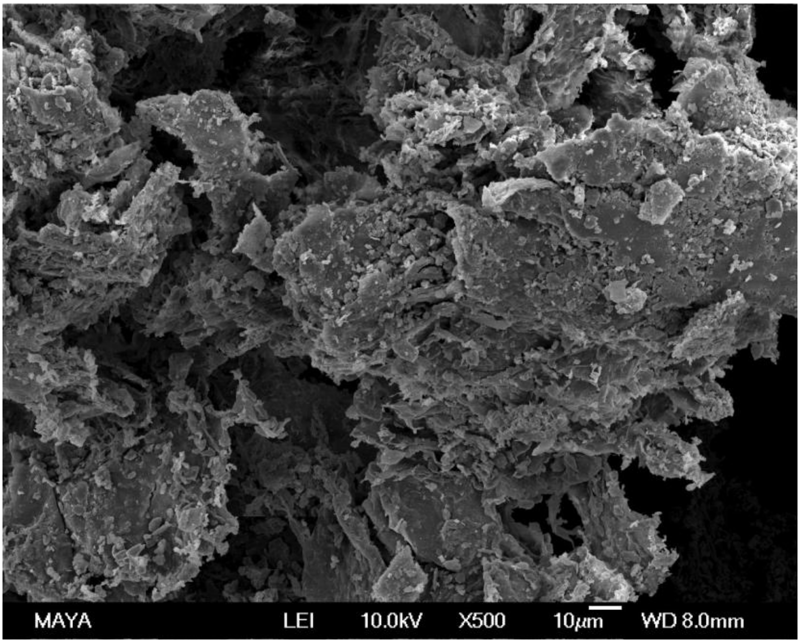


**Figure 8.** Cañihua cellulose SEM imaging, reference 10 µm.

## Cellulose and hemicellulose from tarwi

A similar issue with the solubility was observed for cellulose and hemicellulose fractions of tarwi seeds (Figure 9). In this case, the carbohydrate fraction is not the main component of the seeds, and it is also considered a non-starch leguminous seed. It has been found to contain between 27% and 33% carbohydrates, depending on the cultivar [52]. One study on tarwi from a local market in Ecuador quantified the starch content of the seeds at 5.9%, with 33% of the starch being amylose and 67% being amylopectin [53]. The content of amylose makes it harder to digest compared to other starch compositions, such as those found in cañihua (described before), but still lower than in resistant starch-rich foods. However, starch is not the main component of tarwi carbohydrates, and the composition of tarwi polysaccharides has not been reported.

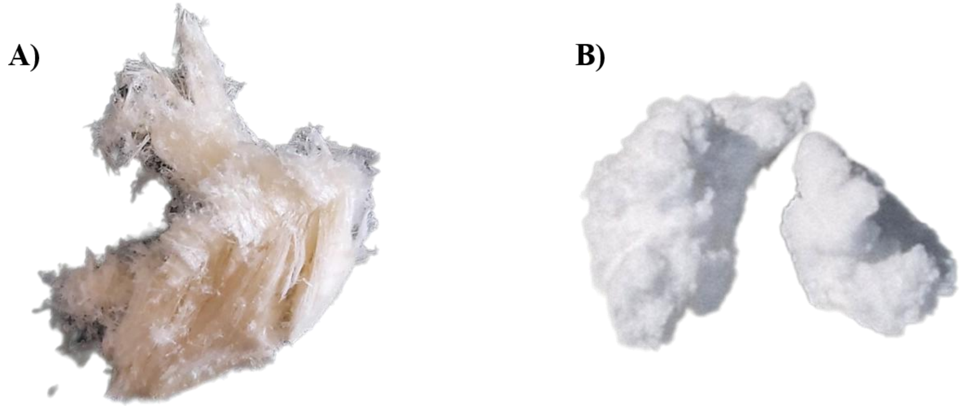


**Figure 9. A)** Freeze-dried hemicellulose fraction of tarwi seeds after alkaline extraction. **B)** Freeze-dried cellulose fraction after hemicellulose extraction of tarwi seeds.

The carbohydrate fractions of tarwi are white, and cellulose is a fine powder. The hemicellulose fraction has mainly arabinose in its monosaccharide composition, followed by galactose and smaller amounts of xylose. The SEM imaging reveals the presence of fibres (Figure 10 B). The analysis of this fraction also shows antioxidant capacity and the presence of phenolic compounds, but in lower amounts compared to the same fraction of cañihua seeds.

The SEM imaging of the cellulose fraction (Figure 10A) reveals mixed a structure combining fibres with laminar structures, which correlates with broader peaks in XDR that define non-crystalline structures.

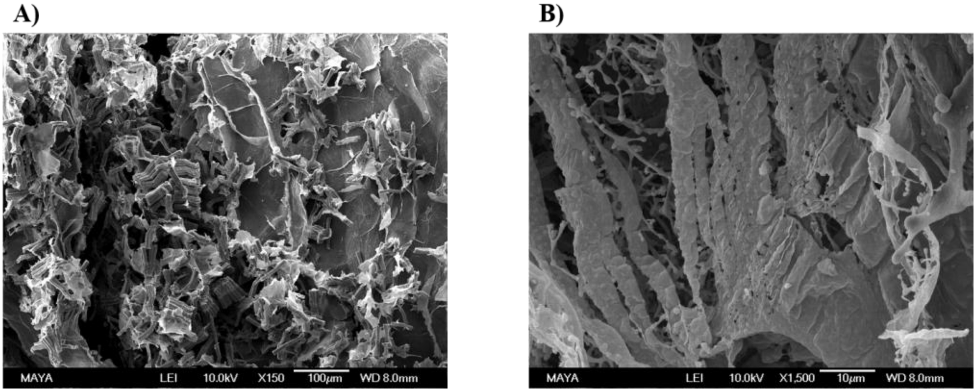


**Figure 10. A)** SEM imaging of tarwi cellulose. **B)** SEM imaging of tarwi hemicellulose.

XRD of cellulose fraction of tarwi (Figure 6, **Paper II**) confirms the mixed structures observed by SEM; the main peaks for cellulose I, which is the native form of cellulose, are observed at 15°, 16°, 22°, and 34°. However, there is also a broad peak at 20°, which is associated with the cellulose II structure formed through mercerization (alkali treatment) or regeneration (dissolving and recrystallizing) of native cellulose. These findings suggest that the tarwi cellulose structure may be partially influenced by the extraction process.

However, there is also a need to transform these non-digestible carbohydrate fractions of food into smaller forms that can reach the gut microbiome, where healthy bacteria can ferment them. With this aim, the next section of the thesis will explore bioconversion into potential fermentable oligosaccharides.

# Bioconversion of polysaccharides into functional food ingredients

The nutritional characteristics of Andean seeds, along with the bioactive compounds identified in them, were described. Most of the bioactive compounds found are classified as antioxidants and vitamins. However, other types of bioactive compounds that can be obtained through enzyme transformation and fermentation, such as prebiotics and SCFAs, have not been previously studied in Andean seeds. In this sense, the two approaches — enzymatic and LAB fermentation — will be addressed in this section to transform carbohydrates from Andean seeds into potential bioactive compounds.

## Enzymatic transformations

Enzymes are an eco-friendly alternative to replace chemicals, and the main advantages are their specificity, lower energy usage compared to chemical processes, non-toxicity, and the absence or very little amount of by-products in their reactions. Microbial sources of enzymes have become of interest because they grow at fast rates and can be genetically modified to produce enzymes that can perform optimally for the desired application [54].

## Enzymatic production of fermentable oligosaccharides

Fermentable oligosaccharides are short-chain carbohydrates that are not absorbed or digested in the small intestine, therefore, they can reach the colon, where they are fermented by gut bacteria, including LAB, producing mainly SCFAs as products of fermentation. The first approaches of fermentable oligosaccharides included chains of fructose, and sorbitol [55]. However, as research interest in the field increased, more oligosaccharides were discovered within these characteristics, including fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS), xylo-oligosaccharides (XOS), and cello-oligosaccharides (COS). The last abbreviation (COS) is also used for chitooligosaccharides in some research articles; to avoid confusion, in this thesis we refer to cellooligosaccharides as COS.

The most common method for obtaining oligosaccharides from natural sources, such as food samples or agro-industrial waste, is enzymatic hydrolysis of polysaccharides. Enzymatic hydrolysis shows outstanding properties compared to chemical synthesis, for example, high selectivity, specificity, activity under mild operation conditions, biodegradability, minimal environmental impact, and high yields compared to the chemical synthesis that requires a high number of steps, low-moderate yields, use of organic solvents and high temperatures, and sometimes the use of special equipment [56]. Enzymes have been considered expensive and labile catalysts, but this concept is no longer valid. Robust enzymes, well-endowed to perform hydrolysis, are now available.

For the production of oligosaccharides from tarwi and cañihua, a sequential enzymatic hydrolysis was studied in **Paper III**, starting with a cellulose blend composed of cellobiosidase I and II, β-glucosidase, and endo-1,4-β-D—glucanase, followed by a treatment with α-amylase, to a final treatment with a mix of xylanase and glucuronoxylanase (Figure 11).

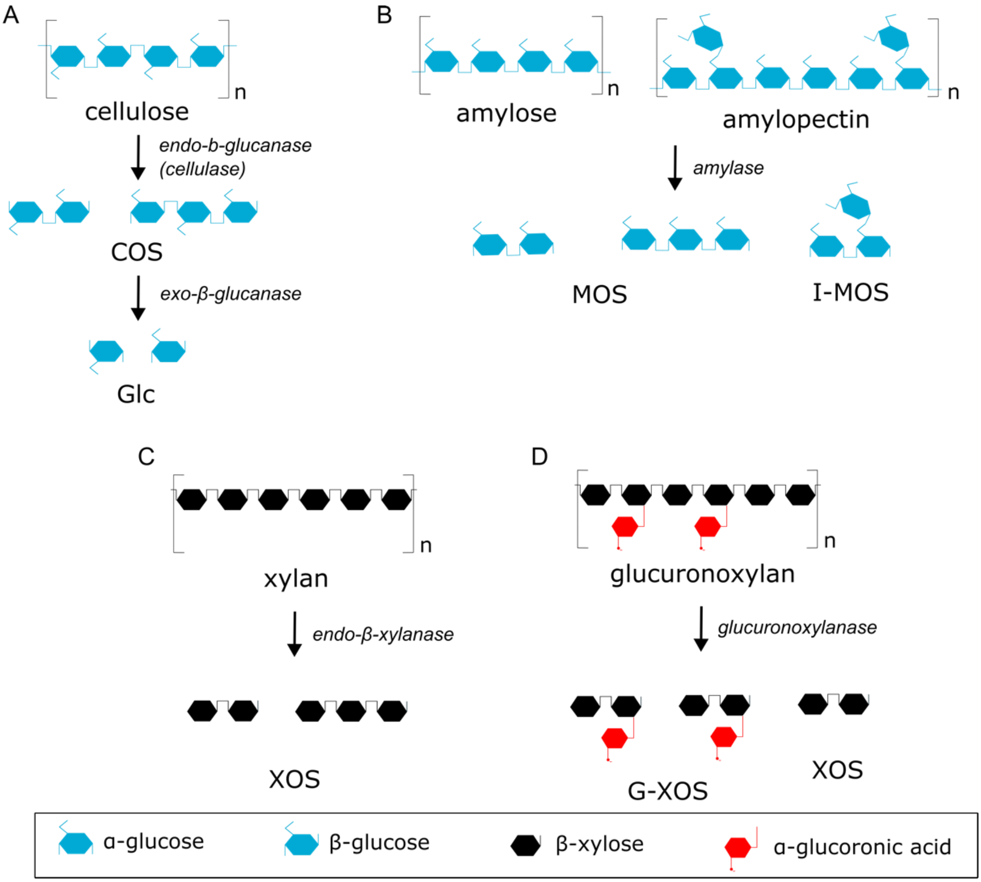


**Figure 11.** Enzymes used for sequential enzymatic hydrolysis of polysaccharides of tarwi and cañihua seeds (**paper III**).

The cellulose blend contains three kinds of cellulase enzymes. Endoglucanases have an affinity for amorphous cellulose regions and promote a random attack on internal β-glycosidic bonds, releasing oligomers of different degrees of polymerisation. Exoglucanases are enzymes that break down cellulose by hydrolysing it from its ends. This group can act on the reducing ends of the molecule (CBH I, exo-1,4-β-cellobiosidase) or on non-reducing ends (CBH II, exo-1,4-β-d-glucanase). Finally, β-glucosidases hydrolyse the oligosaccharides into glucose molecules.

Hemicellulose is a group of branched polysaccharides found in plant cell walls, which is composed of different sugars such as xylose, glucose, mannose, and galactose. One of the most abundant hemicelluloses is xylan, which is formed by units of xylose linked by β-(1,4)-glycosidic bonds and branched by α-(1,2)-glycosidic bonds with substitution taking place often with arabinofuranosyl, 4-O-methylglucuronic acid, acetyl, or phenolic groups at positions $C_2$ or $C_3$ [57]. Xylanase is the enzyme used for the breakdown of xylan into XOS, and it hydrolyses xylan in a random way [58].

The xylanases used in **Paper III** are two variants of a thermostable alkali-tolerant xylanase from *Alkalihalobacillus halodurans* (former *Bacillus halodurans* S7), BhXynH10A and BhXynH10K80R [59]. BhXynH10A has higher activity on tarwi, and BhXynH10K80R on cañihua seeds. The activity of the enzymes was previously analysed using xylan as substrate and xylose as a standard reference. This step of the sequential enzymatic hydrolysis included a glucuronoxylanase, glucuronoarabinoxylan endo-1,4-β-xylanase (CtXyn30A) from *Clostridium thermocellum*, which is also a thermostable enzyme. The activity of CtXyn30A was analysed using quinoa xylan and wheat straw xylan as substrates and solutions of xylose as a standard curve. The activity of BhXynH10A, BhXynH10K80R, and CtXyn30A was determined in units from the concentration (µM) of xylose produced and detected by the DNS (3,5-dinitrosalicylic acid) assay, which measures the ability of xylose to reduce DNS to 3-amino-5-nitrosalicylic acid under alkaline conditions. The results of the enzymatic assays (Figure 12) were used to determine the amount of enzyme required for the sequential enzymatic treatment in **Paper III**.

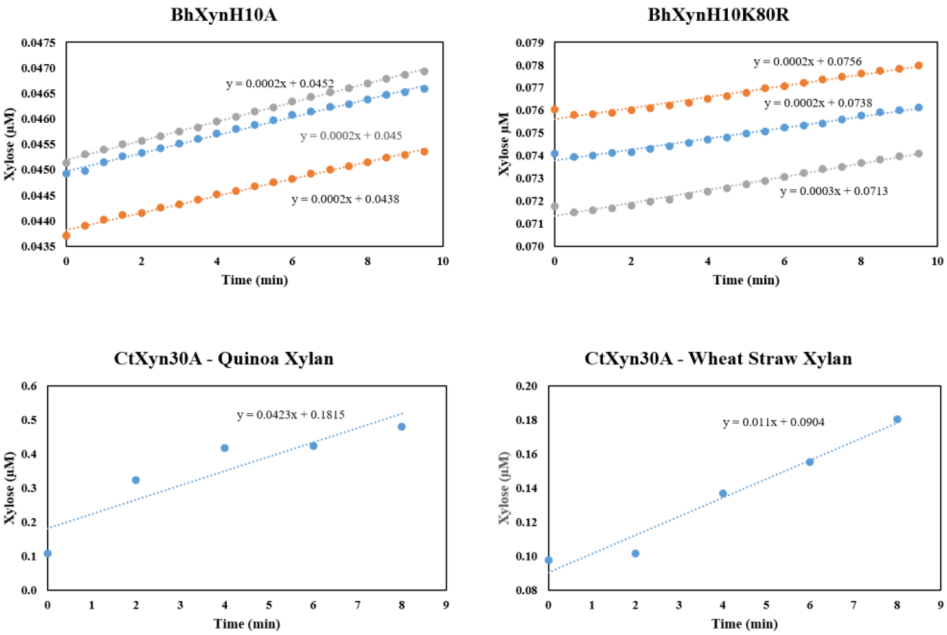


**Figure 12.** Enzymatic activity of recombinant produced enzymes: BhXynH10A, BhXynH10K80R and CtXyn30A.

### *Cellooligosaccharides (COS) and Xylooligosaccharides (XOS)*

Two potential prebiotic oligosaccharides were studied in this thesis: COS and XOS, both considered low-calorie prebiotics due to their minimal contribution to calorie intake. XOS are xylose units bound through β-1,4-glycosidic linkages (Figure 13), and they have been largely studied as prebiotics, obtained from various sources and exhibiting numerous benefits upon consumption. The major XOS are xylobiose (X2), xylotriose (X3) and xylotetraose (X4), and it has been shown that they can differentially improve gut microbiota and metabolite profiles in the chicken gut against Avian pathogenic *Escherichia coli* (APEC). X3 exhibited the greatest inhibition against APEC abundance and virulence [60]. The results of **Paper III** show that X2 is present in almost all hydrolysates.

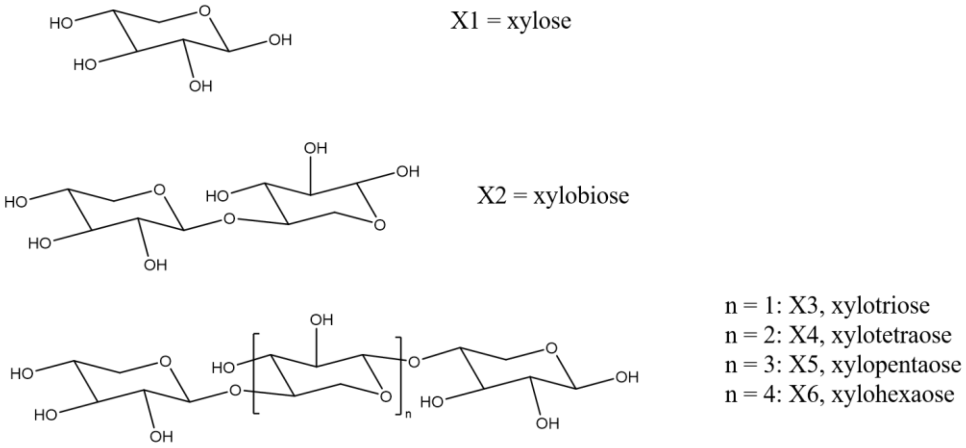


**Figure 13.** XOS structures, from X1 to X6. Molecules drawn using ChemDraw.

*In vitro* studies have proven that XOS can stimulate the growth of probiotic strains; however, the utilisation of XOS was strain-specific, attributed to its homo-fermentative property. Studies show that XOS aids in the selective stimulation of beneficial, non-pathogenic organisms, with probiotic lactic acid bacteria showing maximum growth, including *Lacticaseibacillus. brevis*, *Lacticaseibacillus plantarum*, *Lacticaseibacillus acidophilus*, *Lacticaseibacillus casei* and *Lacticaseibacillus lactis*. The main products of fermentation identified were lactic and acetic acid [61, 62]. Another study showed that the presence of feruloyl substituents in XOS might help in the growth of beneficial bacteria and produce higher butyric acid due to the slow fermentation of branched XOS compared to non-branched XOS [63].

COS, on the other hand, are oligomers of β-1,4-linked d-glucose units (Figure 14). They are an emerging group of oligosaccharides in the field of prebiotics; they are still considered new, and they have been mainly obtained from enzymatic hydrolysis of cellulose. However, large-scale production remains unexplored [64]. Cellulose is usually the most abundant carbohydrate portion in plants and seeds; however, it is also the most challenging as a source of fermentable oligosaccharides due to recalcitrance against enzymatic hydrolysis. However, in comparison to chemical-physical methods, enzymatic hydrolysis is more attractive due to the high specificity, need of mild conditions and very few by-products production. This was observed in **Paper III**, where lower amounts and a variety of COS were obtained compared to XOS through sequential enzymatic degradation.

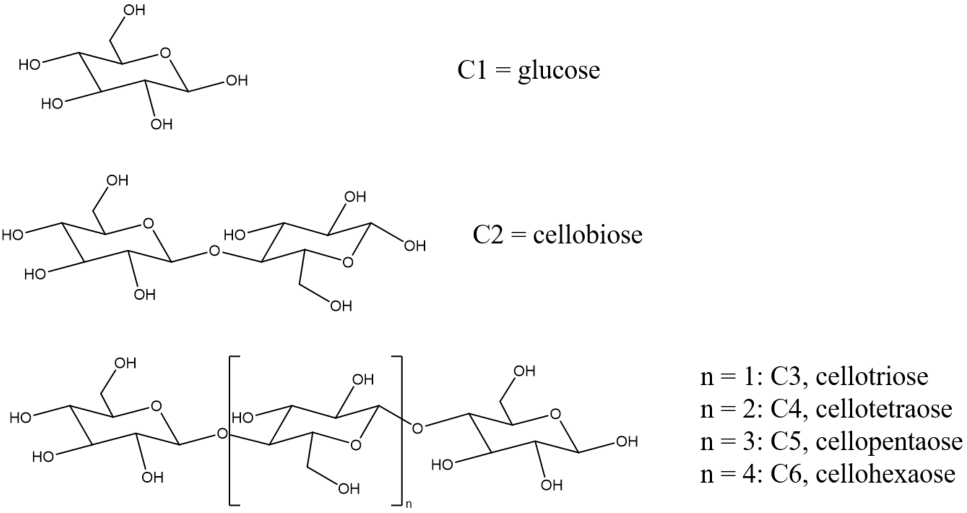


**Figure 14.** COS structures, from C1 to C6. Molecules drawn using ChemDraw.

During the characterisation of the hemicellulose fraction of tarwi in **Paper II**, the results of seaman hydrolysis, followed by identification and quantification of monosaccharides, showed that the main monosaccharides present are arabinose and galactose, with smaller amounts of xylose and mannose. Other studies also indicate that legumes are a rich source of galactooligosaccharides (GOS). In this sense, the findings in **Paper II** require further study to obtain other types of oligosaccharides as fermentable prebiotics from tarwi seeds.

## LAB fermentation of XOS and COS

LAB fermentation with XOS as substrates has been widely studied; the main product of this fermentation is lactic acid and depending on the presence of substitutions in the XOS structure, it can also produce other SCFAs. One study showed that non-substituted XOS and arabino-XOS are fermented by faecal inocula (FI) from four human volunteers more quickly compared to complex structures of acetylated XOS (AcXOS) and XOS containing a 4-O-methylglucuronic acid group (GlcA(me)XOS) [65]. Kabel et al. also demonstrated that, after 40 hours of fermentation of nXOS and AXOS, acetate and lactate were the primary products formed. At the same time, the fermentations of AcXOS and GlcAmeXOS resulted in a lower lactate production, whereas the concentration of propionate and butyrate increased. Another study found that *Lacticaseibacillus acidophilus*, *Bifidobacterium adolescentis*, and *Levilactibacillus brevis* could ferment XOS to varying extents, as indicated by their differences in growth, to produce acetate as the predominant SCFA [66].

The fermentation of COS by LAB has also been studied before. One study utilised enzymatic hydrolysis of cellulose extracted from agro-industrial waste (sugarcane straw and coffee husk); the obtained COS primarily promoted the growth of *Lactobacillus acidophilus* and *Levilactobacillus brevis* after 24 h of fermentation, although it presented low efficiency with *Bifidobacterium* sp. [67]. They observed that the main product of fermentation was acetic acid, and glucose was the most consumed substrate, followed by cellobiose.

The products of fermentation and the extent of XOS and COS consumption depend on many factors. In **Paper III**, the consumption of XOS was clearer than the consumption of COS. For example, *L. casei* consumed C1, X2, and X3 (Figure 15) compared to the content of these oligosaccharides in the hydrolysate before fermentation. All fermentation processes produced acetic acid and lactic acid by L. casei and *L. rhamnosus*, but not in all products of fermentation by *Propionibacterium freudenreichii*, which mainly produced propionic acid. Interestingly, in three of the hydrolysates: — CC, TA, and TM — the main SCFA produced is propionic acid. Understandably, propionic acid is mainly produced by *P. freudenreichii*, but the production of propionic acid from *L. casei* and *L. rhamnosus* needs further investigation.

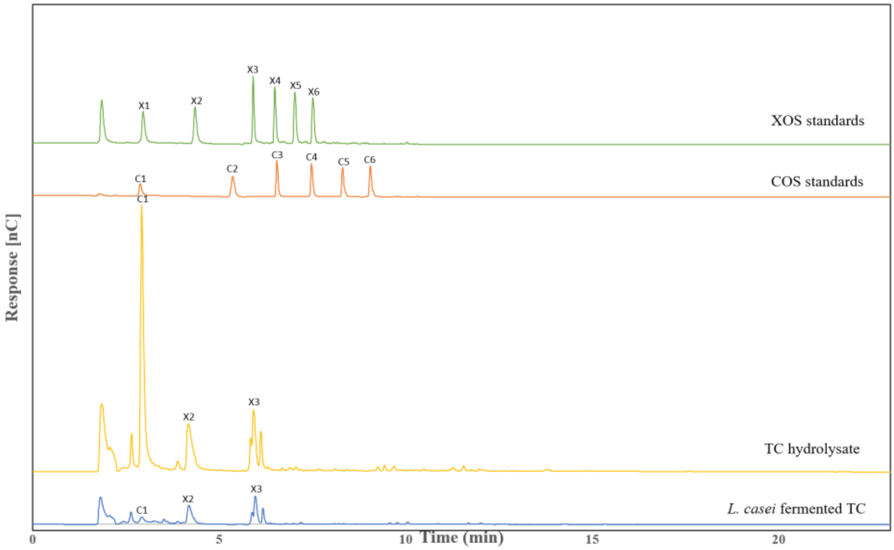


**Figure 15.** XOS and COS consumption by *L. casei* during fermentation of tarwi seeds hydrolysed by cellulose blend (TC), determined by HPAEC-PAD; TC hydrolysate (yellow), TC hydrolysate after fermentation by *L. casei* (blue), XOS standards (green), COS standards (orange).

# Proteins from Andean seeds

## Characterisation of tarwi protein

Among the seeds studied in this thesis, tarwi stands out for its protein content. In this sense, the characterisation of its proteins is the main focus of this chapter.

Tarwi belongs to the well-known group of protein-rich lupin seeds. The three lupin species most cultivated, studied, and commercialised are native to Europe: white lupin (*Lupinus albus*), yellow lupin (*Lupinus luteus*), and narrow-leaved lupin (*Lupinus angustifolius*), while tarwi is not widely cultivated and commercialised, research and industrial interest have grown in the last decade due to tarwi's higher protein content compared to the three most well-known species. Many studies have documented that the nutritional qualities of the three European lupinus and tarwi are comparable to those of soybean; however, lupin seeds are well known for not being genetically modified plants and do not contain isoflavones, the impact on the human body is very controversial [68]. Currently, tarwi is only cultivated and commercialised in South America, and interest in cultivating it in Europe has grown recently. For example, the European project LIBBIO, "*Lupinus mutabilis* for Increased Biomass from marginal lands and value for BIOrefineries" registers the first Andean lupin variety in Europe, developed by Vandinter Semo BV. This variety, named 'Cotopaxi', is characterised by a relatively short growing season, which enables the seed to ripen and be harvested in the Netherlands. The LIBBIO project continues to research the potential of tarwi; genotypes are evaluated in field experiments in seven European countries, under Mediterranean conditions (Greece, Portugal, Spain) and in North-Central European (Austria, Iceland, the Netherlands, Romania) conditions, including *L. mutabilis* genotypes, genetic material from Instituto Superior de Agronomia (ISA, Lisbon, Portugal) and Vandinter Semo (VDS, Scheemda, The Netherlands) [69].

But why is there such a significant interest in tarwi? The answer lies in the nutritional composition of its seeds. Comparing the composition of tarwi with that of the three main lupin seeds and soybean (Table 1) reveals the protein content. It is worth noting that the reference values reported by Bähr et al. in Table 1 are based on dehulled seeds, which may result in a higher protein content compared to whole seeds in other studies.

**Table 1.** Comparison of protein content of soybean to the four lupin seeds: white lupin (L. albus), yellow lupin (*L. luteus*), narrow-leaved lupin (*L. angustifolius*), and tarwi (*L. mutabilis*).

| Seed | Protein (% of dw) | References |
|---|---|---|
| ***L. albus*** | 33-47 | [70] |
| | 32.2 | [71] |
| | 45.3-52.9 | [72] |
| | 34-49 | [73] |
| | 38.2 | [74] |
| ***L. angustifolius*** | 31-37 | [70] |
| | 38.8-44.2 | [72] |
| | 33.9 | [74] |
| | 33-41.5 | [73] |
| ***L. luteus*** | 37-38 | [70] |
| | 55.3 | [72] |
| | 42.2 | [74] |
| | 37.4-55.3 | [73] |
| ***L. mutabilis*** | 32-52 | [70] |
| | 43.3 | [74] |
| | 41.6, 46.4 | **Present work (papers IV and V)** |
| ***Glycine max*** | 47.5-52.1 | [72] |
| | 42.9 | [74] |
| | 27-44 | [73] |

Amino acid composition of tarwi seeds was also analysed for the Bolivian tarwi (**Paper IV**), and Table 2 shows a comparison with literature of the other three lupin seeds and soybean.

**Table 2.** Amino acid composition of Andean lupin (*L. mutabilis*), white lupin (*L. albus*), yellow lupin (*L. luteus*), narrow-leaved lupin (*L. angustifolius*), and soybean (*Glycine max*) expressed in g/100g of protein. N.d. = not determined.

| **Amino acid** | ***L. mutabilis* 1 (paper IV)** | ***L. mutabilis* 2 (paper IV)** | ***L. mutabilis* [75]** | ***L. mutabilis* [76]** | ***L. albus* [75]** | ***L. angustifolius* [70]** | ***L. luteus* [70]** | ***Glycine max* [77]** |
|---|---|---|---|---|---|---|---|---|
| **Alanine** | 2.84 | 3.05 | 3.73 | 3.1 | 3.45 | 3.03 | n.d. | 4 |
| **Arginine** | 8.02 | 8.4 | 10.67 | 14.1 | 10.86 | 12.17 | 9.1 | 7.7 |
| **Aspartic acid** | 8.49 | 9.12 | 10.36 | 12.3 | 10.35 | n.d. | n.d. | 6.9 |
| **Cysteine + cystine** | 1.18 | 1.44 | 1.46 | 2.1 | 1.74 | 1.38 | 2.4 | 2.5 |
| **Glutamic acid** | 19.48 | 21 | 22.45 | 29.7 | 21.66 | 22.74 | n.d. | 19 |
| **Glycine** | 3.33 | 3.58 | 4.25 | 3.8 | 4.07 | 3.98 | n.d. | 3.7 |
| **Histidine** | 2.39 | 2.52 | 2.95 | 3.3 | 2.38 | 3.75 | 3.1 | 3.4 |
| **Isoleucine** | 3.55 | 3.91 | 4.82 | 4.7 | 4.71 | 3.54 | 3.6 | 5.2 |
| **Leucine** | 5.63 | 6.02 | 6.87 | 8 | 7.74 | 5.9 | 7.8 | 8.2 |
| **Lysine** | 4.86 | 5.16 | 5.93 | 5.8 | 5.02 | 5.26 | 4.5 | 6.8 |
| **Methionine** | 0.51 | 0.58 | 0.73 | 0.6 | 0.76 | 0.53 | 0.6 | 1.1 |
| **Phenylalanine** | 2.97 | 3.22 | 3.97 | 4.5 | 4.07 | 3.48 | 3.7 | 5.6 |
| **Proline** | 3.22 | 3.31 | 4.23 | 4.8 | 4.38 | 5.58 | n.d. | 5.3 |
| **Serine** | 4.43 | 4.61 | 4.04 | 6.5 | 4.63 | 4.39 | n.d. | 5.4 |
| **Threonine** | 3.05 | 3.24 | 3.61 | 3.7 | 3.65 | 3.27 | 3 | 4.2 |
| **Tryptophan** | 0.79 | 0.91 | 0.97 | n.d. | 0.76 | 0.6 | 0.9 | 1.3 |
| **Tyrosine** | 3.27 | 3.46 | 4.2 | 4.9 | 4.32 | 3.47 | 2.9 | 4.2 |
| **Valine** | 3.07 | 3.41 | 1.99 | 1.67 | 4.35 | 3.57 | 3.4 | 5.4 |

From the amino acid composition of lupin species and soybean (Table 2), the main amino acid is glutamic acid. There is evidence from human and animal studies showing that glutamate is a major oxidative fuel for the gut and that dietary glutamate is extensively metabolised in the first pass by the intestine [78]. Glutamate is also a crucial precursor for bioactive molecules, such as GABA. In the human body, the glutamate/GABA cycle is a complex process in which glutamate, glutamine, and GABA are metabolised. GABA is synthesized from glutamate in the presynaptic neuron by the enzyme glutamate decarboxylase (GAD, systematic name: l-glutamate 1-carboxy-lyase (4-aminobutanoate-forming), EC 4.1.1.15), which requires pyridoxal phosphate (vitamin B6) as a cofactor. When the reaction does not take place in the human body, the cofactor is pyridoxal-5'-phosphate (PLP) (Figure 16).

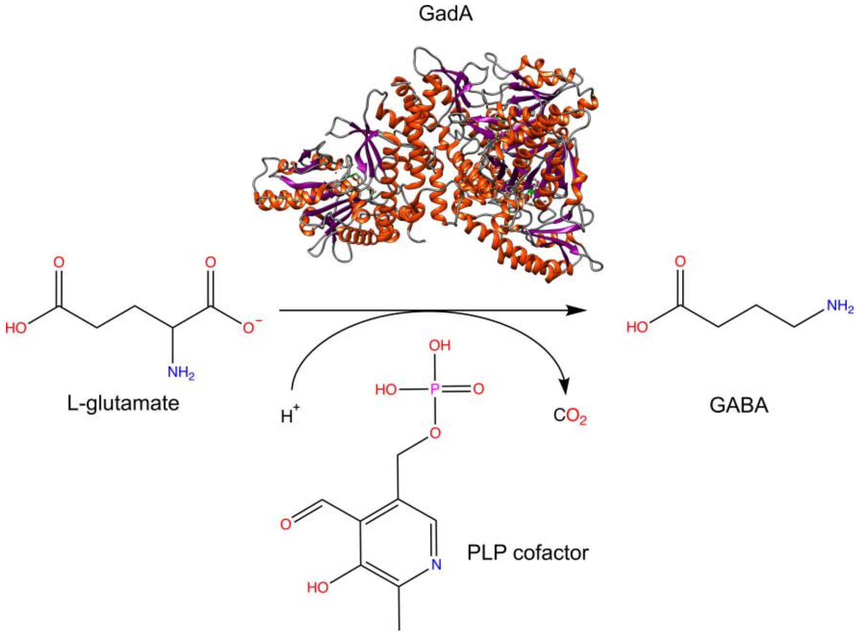


**Figure 16.** Irreversible conversion of GABA from L-glutamate in the presence of the PLP cofactor. Molecules were drawn using ChemDraw (RRID: SCR_016768) and the Gad structure from the Protein Data Bank (PDB code 5GP4).

## Soluble protein extraction and characterisation

Studies on the total protein content and amino acid composition of various tarwi varieties have been conducted. However, deeper characterisation of proteins is still needed. For example, lupin seeds have a general nutritional protein composition consisting of globulins and albumins in a ratio of 9:1. Globulins are high-molecular-weight storage proteins that are divided into four groups: α-conglutin, β-conglutin, γ-conglutin, and δ-conglutin. Albumins, on the other hand, are a diverse group of functional proteins, primarily serving as metabolic enzymes. Thus, compared to the three main European species, tarwi proteins remain unexplored. Size-exclusion chromatography identified conglutins in tarwi proteins, showing that α-conlgutins are the major portion (41.4%), followed by β-conglutins (31.7%), and finally, γ-conglutin (6.94%).

In **Paper IV**, microwave-assisted soluble protein extracts of tarwi were characterised in terms of pH of extractions, their degree of glycosylation, and proteomic analysis was carried out. Early studies on tarwi seeds demonstrated that protein solubility is pH-dependent, with the minimum solubility occurring at pH 4. It showed a rapid increase over pH 5 [79], which correlates with the results found in **Paper IV**, where the lowest soluble protein content was observed at pH 4 and the highest at pH 10. As there is still a lack of research on tarwi, most studies have been conducted in the other three lupin species. Moreover, they appear to exhibit similar behaviour; for example, a study revealed that protein isolates of *L. angustifolius* have the lowest solubility at pH levels of 4-5 and the highest solubility at pH 2 and 10 [80]. **Paper IV** also demonstrated that the glycoprotein content varies with pH; the highest glycoprotein content was observed at pH 4, while the lowest was observed at pH 10. However, glycoproteins have not been studied in tarwi before.

Microwave-assisted extraction (MAE) of protein has been investigated in many food systems, including coffee green beans, peanut flour, coffee silverskin, and soymilk, and most studies show improved yields of extraction and an interesting tendency to increase β-sheets and decrease α-helices, thereby altering the functional properties of the protein [81]. MAE of proteins from *L. angustifolius* revealed that MW treatment preserved the primary structure, while Fourier transform infrared spectrometer (FTIR) analyses showed that high-power MW (1000 W) induced significant protein denaturation changes in the secondary structure, transferring protein structures from highly ordered (β-sheet and α-helix) to disordered forms (random coil and β-turn) [82]. MAE is also considered a beneficial extraction method due to reduced extraction times and less solvent consumption. Interestingly, in **Paper IV**, the optimal conditions for MAE at acidic (pH 4), neutral (pH 7), and alkaline (pH 10) pH were found to be 60 W for 5 min.

Proteomic analysis (**Paper IV**) revealed that all the highest-scoring proteins at the three pH levels (4, 7, and 10) are novel structures, as they exhibit no 100% similarity to previously identified proteins in either lupin species or other species. These findings contribute to the first proteomic characterization of Bolivian tarwi, as well as tarwi in general, which has only been fully sequenced once [3]. Among the proteins identified in tarwi in **Paper IV**, conglutins are of great interest due to their nutritional and functional value; three important conglutins were identified: β-conglutin 6 and 7, and δ-conglutin 1. With the last one being the less studied of all conglutins, the three δ-conglutin 1 present in Bolivian tarwi were detected at all pH fractions when deglycosylated/denatured (ANN01012, ANN01006, and

ANN01010), and interestingly, their structures are very similar (Figure 17). The less confident structure prediction relies on the flexible loops of proteins, and this correlates with the most significant difference between them.

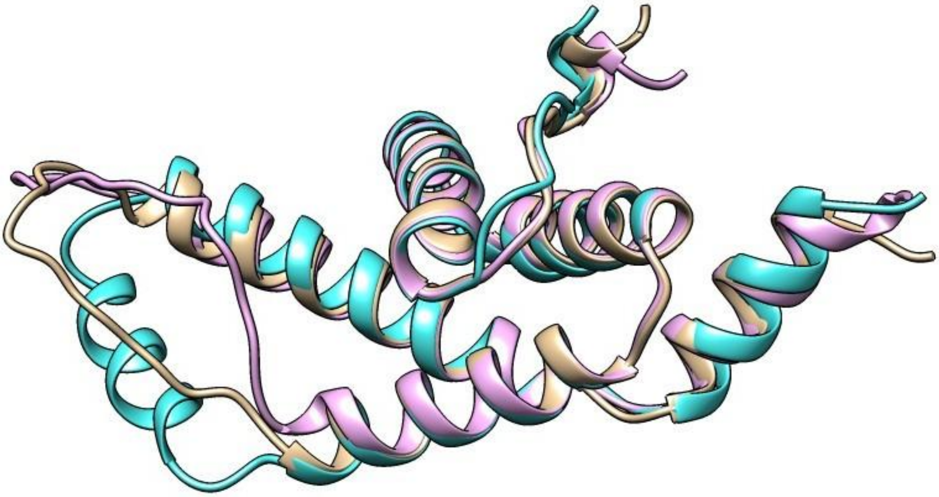


**Figure 17.** Collated δ-Conglutin1 protein structures from tarwi predicted by AlfaFold and visualized by ChimeraX after removal of signal peptide. Light blue structure corresponds to ANN01012, pink structure corresponds to ANN01006, and cream structure corresponds to ANN01010.

## Bioconversion of proteins into functional food ingredients

LAB represents a significant, widespread, and ubiquitous group of Gram-positive bacteria, characterised by unique physiological activities that have been extensively exploited in the food industry. Additionally, many LAB exhibit health-promoting properties that make them potential probiotics. Among LAB, most of the GABA-producing strains belong to the *Lactobacillus* genus [83]. The glutamic acid decarboxylase system enables LAB to withstand acid environments, such as the gastrointestinal tract, while maintaining viability and cellular activity. This mechanism protects against acid conditions, as the decarboxylation of glutamate to GABA by the GAD enzyme consumes intracellular protons, thereby maintaining cytosolic pH homeostasis. The glutamic acid decarboxylase system consists of the glutamate/GABA antiporter, encoded by the *gadC* gene, and the GAD enzyme, encoded by the *gadA* or *gadB* genes. Specifically, the synthesis of GABA follows these steps: glutamate is transported into the cell through an antiporter, where it undergoes decarboxylation; subsequently, the resulting GABA is exported outside the cell through the same antiporter. In LAB, the gad operon is located on the chromosome, and its organisation varies among different species and strains [84]. Among LAB, *L. brevis* is well-known for its ability to produce GABA, and various strains have been studied for this purpose.

## Enzymatic conversion of L-glutamic acid into GABA in Andean seeds

Three Andean seeds were tested in this thesis to convert their naturally occurring L-glutamic acid into GABA: tarwi, cañihua, and quinoa real (**paper V**). Two isoforms of GAD enzyme from *L. brevis* were recombinantly produced: Glutamate decarboxylase-producing strains: recombinant *E. coli* BL21 (DE3) pET-21b::*gadA* (unpublished), and *E. coli* Origami 2 pET-21b::*gadB* [85] (GenBank accession code: KX417371) (Figure 18). The *gadB* gene is in the GAD operon together with transcriptional regulator (*gadR*), glutamate/gamma-aminobutyrate antiporter (*gadC*), and glutamyl-tRNA synthase (*gltX*). In contrast, the *gadA* gene is stand-alone [86].

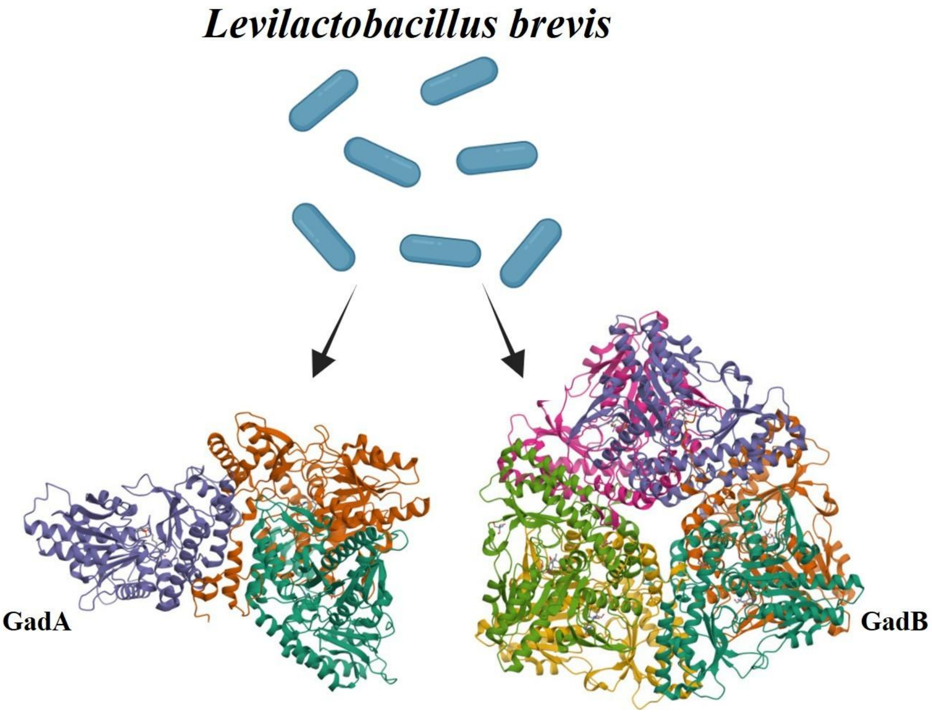


**Figure 18.** *L. brevis* GAD enzymes from the protein data bank, GadA (PDB code 5GP4) and GadB (PDB code 1PMO).

The molecular docking of glutamate into the active site of the holo-form of *L. brevis* CGMCC 1306 GadA was proposed by Huang et al. in 2018, showing several noncovalent interactions, including hydrogen bonds between the O2, O3, and O4 atoms of the substrate L-Glu and various parts of the GAD polypeptide chain. Furthermore, electrostatic interactions between the negatively charged oxygen atom of the α-carboxyl and the γ-carboxyl group of L-Glu and the positively charged nitrogen atom of residue R422, as well as H278 and K279 [87].

GadA and GadB were tested in **Paper V** in three different ways: first, as the sole treatment; second, with a pancreatin pretreatment to obtain more free glutamic acid; and finally, they were also tested together in the same reaction mixture to determine if there is a synergistic effect (Figure 19).

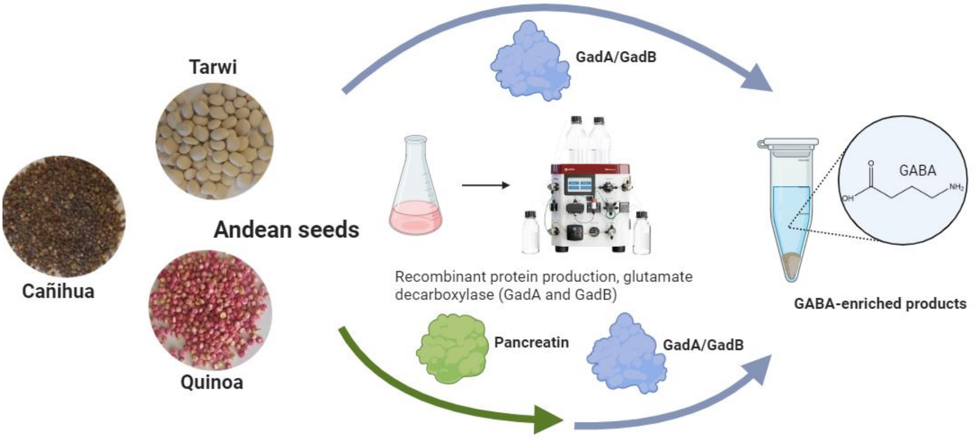


**Figure 19.** Representation of experimental methodology applied in **paper V** for the enzymatic production of GABA in three Andean seeds: tarwi, cañihua and quinoa real varieties. Laboratory material images and enzyme images from Biorender.

The pretreatment with pancreatin had a positive effect only in cañihua and quinoa seeds, which had lower GABA production compared to tarwi. This finding was highly correlated with the protein and glutamic acid content of the seeds. Moreover, another notable result in this work was the incomplete production of GABA, indicating that glutamic acid remained after the enzymatic treatment. This created the hypothesis of a side-catalytic effect of GAD enzymes, the proteolytic activity. There is no literature reporting that GAD enzymes have a proteolytic effect; however, Paper V included an assay in which GAD enzymes showed the ability to partially degrade β-casein. Moreover, the literature indicates that GAD enzymes can be "truncated", meaning that in the presence of a protease that cleaves the full-size enzyme, GAD activity is altered. Specifically, µ-calpain, a calcium-activated protease, has been studied [88]. Sha et al. describe how µ-calpain and other proteases can convert the full-length GadA into truncated GadA (tGadA). This process occurs in vitro, but it also occurs in the brain. Another study describes how truncated GadB exhibits enhanced activity compared to the full-length enzyme. In contrast, the opposite effect is observed in GadA, where the truncated form is less active than the full-length one [89].

The enzymatic conversion of L-glutamic acid into GABA in Andean seeds, as described in this thesis, was performed using whole ground seeds; the presence of proteases in the seed composition is possible. This could lead to the formation of truncated forms of GADs. The pretreatment with pancreatin could have a similar effect, and the field seems to be understudied, which opens up many possibilities for investigating the effect of proteases on GADs' activity, as well as the formation of these so-called truncated forms of GADs that could differ in their activity and exhibit a side-proteolytic effect.

## Production of GABA by fermentation with *L. brevis*

GAD enzymes can be recombinantly produced and purified to produce GABA as described before. However, it is also possible to use the microorganism itself to ferment substrates and obtain GABA as a product of fermentation.

**Paper VI** examines the production of GABA, along with other metabolites such as lactic and acetic acid, and the changes in soluble free amino acids during *L. brevis* fermentation of three Andean seeds: tarwi, cañihua, and quinoa (Figure 20).

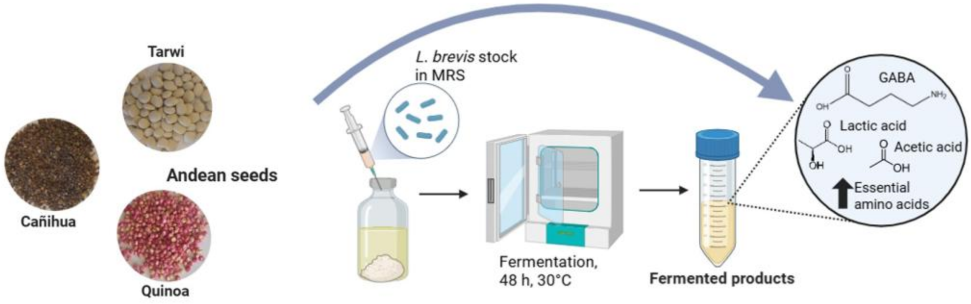


**Figure 20.** Representation of experimental methodology applied in **Paper VI** for the production of GABA by *L. brevis* fermentation of three Andean seeds: tarwi, cañihua and quinoa real varieties. Laboratory material images from Biorender.

Early investigations into GABA production by LAB fermentation utilised monosodium L-glutamate (MSG) as the substrate. However, each decarboxylation requires the consumption of one proton ($H^+$). Therefore, MSG-based GABA fermentation suffers from the continuous feed of an exogenous acid agent. In this sense, more recent research has begun to perform an L-glutamic acid-based GABA fermentation process [90]. Li et al. state in their studies that L-glutamic acid, with its low isoelectric point (3.22) and low solubility (~6.0 g/L), can exert a slight inhibitory effect even when the required L-glutamic acid is added to the fermentation system all at once. L-glutamic acid powder can be slowly dissolved into the fermentation broth as decarboxylation progresses. This L-glutamic-acid-based process can significantly improve fermentation efficiency [91]. In **Paper VI**, the substrate is naturally occurring L-glutamic acid from Andean seeds, which, together with the formation of SCFAs during fermentation, can maintain an acidic environment.

Xylose is more efficient carbon source compared to glucose to improve GABA production by *L. brevis*, one study found between 1.67 and 1.78-fold higher GABA production with xylose compared to glucose, the analysis of the carbon source metabolic pathway indicated that xylose activated the expression of the *xyl* operon, and xylose metabolism produced more ATP and organic acids than glucose, which significantly promoted the growth and GABA production of *L. brevis* CE701 [92]. Cha et al. in their study also determined that the scale of fermentation influences GABA production; a 5 L fermenter produced 336% more GABA than a shake flask. These findings can be further investigated to optimise the production of GABA in Andean seeds by fermentation.

*L. brevis* is considered by EFSA (European Food Safety Authority) to be suitable for the qualified presumption of safety (QPS) approach to safety assessment. This approach requires the identity of the strain to be conclusively established and evidence that the strain does not show acquired resistance to antibiotics of human and veterinary importance [93].

# Potential of obtained products as functional food ingredients

The components that make a food functional can be added to, removed from, naturally enhanced or modified in a food, influencing the health and well-being of the consumer, in addition to its nutritional properties [94]. In the European Union, according to the European Commission Concerted Action on Functional Food Science in Europe (FUFOSE), a functional food has to be satisfactorily demonstrated to affect beneficially one or more target functions in the body beyond adequate nutritional effects, in a way that is relevant to either an improved state of health and well-being or a reduction of risk of disease. Functional foods must remain foods, and they must demonstrate their effects in amounts that can normally be expected to be consumed in the diet. Hence, they are not pills or capsules, but part of a typical food pattern.

In this sense, the potential of the biotransformation products in Andean seeds developed in this thesis as functional foods is described below.

## Fermentable oligosaccharides

Prebiotics are functional food ingredients due to their well-established role as health-promoting agents [94]. One important characteristic of prebiotics is that they must be resistant to digestion; they do not need to be completely indigestible, but significant amounts should be available in the intestine (especially the large intestine) to provide a fermentation substrate [96]. These fermentable functional oligosaccharides are defined as low-molecular-weight, short-chain (degree of polymerisation from 2 to 10) carbohydrate polymers linked by glycosidic bonds that can escape digestion and reach the colon, where probiotic bacteria ferment them. The only carbohydrates that can be hydrolysed by human digestive enzymes are starch-structures.

## Xylooligosaccharides (XOS)

XOS are stable to both heat and acidity during food processing, paving the way for their application in low-pH food products compared to other prebiotics, such as inulin [96]. In addition to their prebiotic effect, XOS possess additional biological activities towards the immune system, acting as anti-inflammatory agents, anti-cancerous agents, and antioxidants [98, 99]. Supplements of XOS have also been shown to reduce cholesterol levels and enhance the biological availability of calcium [97]. XOS may be used as an additive to preserve the protein characteristics of different food products, aiming to enhance their storage stability and shelf life. Therefore, XOS are widely used in nutraceuticals, feed, food additives and pharmaceuticals.

Many studies have shown the efficacy of XOS as a prebiotic in animals. For example, a study in rats compared a high-fat diet to a high-fat diet including XOS at 2g/kg for fifteen weeks: concluding that XOS can alleviate colonic inflammation by regulating gut microbial composition and enhancing SCFAs content in the gut [100]. Less research has been conducted in humans; however, interesting findings include that XOS at a dose of 8–12 g/day is recommended for human consumption. With various clinical trials using XOS, the bifidogenic effect and health benefits were generally observed at a dose of less than 4 grams per day. In contrast, FOS, inulin, and resistant starch required approximately 10–20 g/day, 15 g/day, and 30 g/day, respectively, to show a bifidogenic effect [101]. The recommended daily dose (8–12 g/day) of XOS has not shown any negative effects in humans. The main findings in human studies include increased *bifidobacteria* and certain *lactobacillus* species, as well as higher levels of SCFA [102, 103].

More applications of XOS in food systems include its use as a sugar replacer in cookies and bread. In bread preparation, different proportions of XOS were tested, and a 30% showed dough with excellent stability and better loaf volume [97]. XOS were also tested as fat replacer, sodium reduction, flavour enhancer in processed cheese and at 3.3% showed increased melting rate, decreased viscosity and particle size, decrease in the consistency, increase in elasticity (G′) and firmness (G*), at sensorial level it showed ow bitter taste, improvised salt and acid taste, and high homogeneity [104].

## Cellooligosaccharides (COS)

COS are considered new prebiotic ingredients, and they are emerging for industrial applications. Although COS share many similarities with other well-established prebiotic oligosaccharides, the mechanisms of action and specific health benefits remain under investigation. One study determined the prebiotic effect of cellobiose with six different probiotic strains, and *L. plantarum* ATCC 8014 showed the highest $OD_{600}$ (>5) with lactic acid as the main product of fermentation. Recent research also indicates that the length of the chain of COS is directly related to their prebiotic effect; short-chain COS ($C_2$, $C_3$, and $C_4$) have more significant prebiotic properties. In contrast, those with longer chains ($C_5$ and $C_6$) have more significant techno functional properties and can be used as structuring agents and/or stabilizers in multiphase systems [105].

The main emerging applications of COS that have been studied include: as prebiotic, resistant to digestive enzymes promoting the growth of *Lactobacillus spp*. and *Bifidobacteria spp*. and the secretion of short-chain fatty acids, antimicrobial: inhibits the significant growth of *Helicobacter pylori* and *Clostridium spp*. and high oil retention, formation of powders with high fluidity, resistance to compression and malleability, to heat and acidic pH [105].

Compared to other prebiotic oligosaccharides, to date, there have been only in vitro studies on the prebiotic effect of COS, with no clinical trials published. The application of COS in food products still faces significant challenges, mainly due to their stability under conventional and unconventional processing technologies. Studies on the resistance of COS to thermal conditions, pH variations, and industrial processes, such as pasteurisation, ultrasonication, and high pressure, are limited. The prebiotic effect of COS has been demonstrated in vitro; however, its clinical efficacy still requires robust trials to evaluate its fermentation in the colon, impact on the intestinal microbiota, and potential health benefits [105]. To date, data are insufficient to establish a dosage to obtain a proven prebiotic effect, and all these aspects represent a gap in current research, reinforcing the need for future investigations to enable COS incorporation into foods effectively and safely.

## Short-chain fatty acids (SCFAs)

SCFAs are produced by gut bacteria during the fermentation of prebiotics, highlighting the importance of consuming fibre-rich and fermentable oligosaccharide-rich foods. However, SCFAs can be produced through fermentation by LAB bacteria, along with other metabolites, as observed in **Paper VI**. SCFAs are saturated aliphatic organic acids that consist of one to six carbons, of which acetate (C2), propionate (C3), and butyrate (C4) are the most abundant ($\geq 95\%$). Acetate, propionate, and butyrate are present in an approximate molar ratio of 60:20:20 in the colon and stool [106].

Many studies have focused on the beneficial effects of SCFAs produced in the gut. However, little is known about the effect of SCFAs intake in foods such as fermented foods. A study shows that SCFAs intake protects against high-fat diet-induced obesity in mice, parallel to an increase in plasma SCFAs, without altering cecal SCFAs or gut microbial composition. Dietary SCFA also suppressed hepatic weight and lipid synthesis. Thus, the authors conclude that SCFA supplementation improves hepatic metabolic functions without affecting the intestinal environment [107], thereby promoting the potential of SCFA as functional foods.

Propionic acid has been associated with reductions in lipogenesis and serum cholesterol levels, exerting beneficial effects on weight control and eating behaviour [108]. Formulations of propionic acid for supplementation have been the subject of limited clinical trial studies. A recent study investigated the supplementation of propionic acid, administered twice daily via 500 mg capsules over a 14-day treatment period in patients with multiple sclerosis (MS), which demonstrated a significant 30% increase in Treg cells compared to baseline, with a beneficial effect on immunological, neurodegenerative, and clinical parameters in MS patients, including relapse rate and disability progression. The authors conclude that purified propionic acid supplementation is a safe add-on to existing immune-modulating drugs [109].

Acetic acid holds notable interest due to its low toxicity to epithelial cells, its ability to stimulate bacteria that produce butyrate through cross-feeding, and its anti-inflammatory and protective properties [110]. Supplementation with acetic acid is well-tolerated, has no adverse side effects, and has clinical potential to reduce plasma TAG and FBG concentrations in individuals with type 2 diabetes, as well as to reduce TAG levels in overweight or obese individuals [111].

## GABA-enriched functional foods

Interest in GABA for human consumption has increased over the last decade. Although the mechanism of action of orally consumed GABA is far from clear, interest in it has been long-standing. Early studies in the 1970s claimed that GABA produced in the central nervous system remains there, while GABA produced in the gut does not reach the central nervous system [112]. Later in the 1980s and 1990s, researchers began to believe that small amounts of GABA could cross the blood-brain barrier (BBB) with the discovery of GABA-transporter systems in the brain [113]. Then, in the early 2000s, researchers claimed that substantial amounts of GABA can cross the BBB [114].

Additionally, as GABA is also present in the enteric nervous system, it has been considered that GABA may act on the peripheral nervous system through the gut-brain axis [115]. Although later evidence has shown that biosynthetic GABA can reach the human brain, as evidenced by various EEG responses [116], to date, no data have demonstrated GABA's BBB permeability in humans. Even if blood GABA levels were elevated 30 min after oral GABA intake [117], it's not known if oral GABA intake would increase brain GABA concentrations.

Recently, numerous studies have demonstrated the beneficial health effects of orally consuming GABA, either as a supplement or in food. For example: improved calmness and worry scores in the GABA (vs. control) group at the 4th week of treatment, a trend for improved feelings of awakening in the GABA (vs. control) group at the 4th and 10th weeks of treatment, and lower cortisol were observed in recent clinical studies focusing on the effect of GABA in stress and sleep [118].

Currently, the market offers GABA supplements in powder form, and the formulation of new GABA-enriched food products is an open field for research, particularly in terms of enzymatic and fermentation approaches. Some fermented products that contain GABA include kimchi, miso, and tempeh.

# Conclusions

To transform a regular food into a functional food product, one or more components with health benefits to humans need to be added, naturally enhanced or modified in the original food composition. The results presented in the thesis demonstrate the potential of Andean seeds for the formulation of enhanced plant-based functional products using two environmentally friendly techniques: enzymes and LAB fermentation, as tools to transform naturally occurring macromolecules in the seeds into smaller bioactive compounds, thereby obtaining potential functional food products.

Sequential enzymatic treatment is effective in breaking down hemicellulose and cellulose, as well as releasing proteins trapped within the carbohydrate structure of tarwi and cañihua seeds. The main oligosaccharides obtained from sequential enzymatic treatment were XOS and COS, with XOS exhibiting a higher prebiotic effect than COS for three human probiotic strains. This suggest that consuming these prebiotic oligosaccharides could enable them to reach the gut microbiome and be fermented by the probiotic bacteria. The products of *in vitro* fermentation have numerous health benefits.

GAD enzymes, GadA and GadB, from *Levilactobacillus brevis* were produced in *E. coli* and used to transform soluble L-glutamic acid into GABA in tarwi, cañihua and five varieties of quinoa Real. The production of GABA by fermentation with *L. brevis* was also studied. Using both approaches, the high correlation between total protein content and GABA production is clear, and the highest concentrations of GABA were produced in tarwi seeds. Scaling up from 1 mL reactions to 100 mL reactions with GAD enzymes initially yields a higher GABA content in tarwi, which is promising for industrial applications. On the other hand, fermentation also leads to the production of lactic and acetic acid, alongside an increase in the production of essential amino acids, including threonine, histidine, methionine, isoleucine, leucine, valine, and lysine.

Among the seeds studied in this thesis, tarwi stands out for its high protein content. Soluble proteins were characterised at different pH levels. Alkali extraction (pH 10) shows a higher content of soluble protein compared to neutral and acidic (pH 4). Another interesting finding is that the portion of soluble proteins bonded to carbohydrates (glycoproteins) is higher at acidic pH, indicating that alkali extraction yields higher protein content with fewer glycoproteins. Proteomic analysis revealed the identification of novel protein structures, and their composition also varies depending on the pH and whether the proteins are glycosylated or deglycosylated. Across all fractions studied, nutritional and functional proteins were found, including β- and δ-conglutins.

To conclude, the relevance of this work lies in the lack of knowledge regarding Andean seeds as a source of potential functional food ingredients, which has been addressed through the thesis. It is of high importance to continue investigating and communicating the findings to open up opportunities in the global market for plant-based functional food ingredients.

# Future perspectives

Laboratory-scale enzymatic reactions and fermentation were effective in producing bioactive compounds. Scaling up these processes is necessary to investigate large-scale production; it is equally important to study the formulation of new food products, considering both their organoleptic properties and bioactivity.

Although tara gum has been well characterised (**Paper I**) and it is already used in industry, there is a potential to obtain prebiotic oligosaccharides by enzymatic hydrolysis of this galactomannan, galactomannanoligosaccharides (GMOS). Moreover, the characterisation of non-digestible polysaccharides in **Paper II** revealed that hemicellulose fraction in cañihua has a high glucose content, which warrants further investigation. Tarwi and cañihua hemicelluloses also show arabinose in their composition, and the production of other potential prebiotic oligosaccharide structures can be developed.

During the investigation of GABA production by GAD enzymes (**Paper V**), a side proteolytic effect was detected in both GadA and GadB. They were able to release glutamic acid and partially degrade β-casein. It is a very interesting finding that needs to be addressed to properly understand the mechanism as well as the activity of proteins with different structures.

Tarwi proteins are of great interest; they have been investigated (**Paper IV**), and numerous potential proteins have been identified, providing insights into the extraction conditions that depend on the desired profile. Interestingly, none of the main proteins identified in tarwi are known structures; they all exhibit significant similarities to proteins in other species (mainly lupins), but they are not identical to any known protein. The enzymatic production of bioactive peptides from tarwi proteins is still an ongoing research project.

It has been proven that research can lead to the commercialisation of products. In this sense, it is essential to showcase the potential of Andean seeds to open opportunities for larger markets and support producers in this way. Not only regionally but also internationally, as was the case with quinoa, which is widely known nowadays.

# Acknowledgements

These past years have passed very fast in my mind, it feels like I have just started but at the same time, I go back and realise I have learnt a lot from many people and I would like to thank them for that.

The first people to thank are those who believed in me before the project started; thank you *Javier* for accepting me as your student, for always having your door open for any kind of question, sometimes a question ended up in a long meeting which required me to read a lot of papers afterwards. I have truly learnt a lot thanks to you, and I hope you enjoyed this project as much as I did. Thank you *Mauricio* for accepting me as part of the project and for all your support during my PhD.

*Jimena*, we started this adventure together as friends and as colleagues, thanks for all the scientific, and non-scientific discussions, for the help and for all the fun moments even when we were very stressed on the inside.

To all professors who I learnt from, specially *Olle* and *Lene*, thanks to you I am able to communicate my research in a better way.

To my office mates that made the working environment such a nice place; *Leila K.*, *Supansa*, and *Leonardo*.

To former and current colleagues and friends at the division; *Annika*, *Carl*, *Carlos*, *Fabian*, *Cecilia*, *Christina*, *David*, *Eimantas*, *Elena*, *Emil*, *Emma M.*, *Eva*, *Frans-Peder*, *Israt*, *Lars*, *Leila A.*, *Madeleine*, *María Ester*, *Martin*, *Milad*, *Mahmoud*, *Nélida*, *Nikolina*, *Oliver*, *Pavithra*, *Rajni*, *Roya*, *Sang-Hyun*, *Sara*, *Savvina*, *Siri*, *Sonia*, *Stefano*, and *Zehui*. Thank you all for your help and for the fun times we shared. It has been very nice to be part to the division.

To my friends and colleagues that shared the stressful times in their own projects while in Bolivia and Sweden, and a lot of coffee when we shared office; *Daniela M.*, *Erick*, *Leslie*, and *Valeria P.* And to my friends that are always supporting me in any situation and from any part of the world; *Abigail*, *Claudia*, *Lucia*, *Ronald*, and *Valeria S.*

A mi familia; mamá, gracias a ti pude llegar hasta aquí, gracias por apoyarme en todo lo que hago, a mis hermanos: *Miguel* y *Pablo*, ustedes son parte de mi día a día en la distancia y les agradezco por su ayuda en los pequeños y grandes problemas.

Till min livspartner, *William*, tack för allt ditt stöd, din hjälp och din kärlek. Tack vare dig vet jag att livet ska njutas. Och till *Marie* och *K-A.*, tack för er kärlek och för att för att ni tog mig som en del av er familj.

Finally, I would like to thank SIDA, the Swedish International Development Cooperation Agency, for being the financial support of this project.

# References

1. Barda, M. S., Chatzigeorgiou, T., Papadopoulos, G. K., and Bebeli, P. J., "Agro-Morphological Evaluation of Lupinus mutabilis in Two Locations in Greece and Association with Insect Pollinators," *Agriculture*, vol. 11, no. 3, p. 236, 2021. [Online]. Available: https://www.mdpi.com/2077-0472/11/3/236.
2. Xue, N., Liu, J., and Anwar, S., "Enhancing Soil Physicochemical Properties, Quinoa Yield, and Nutrients through Intercropping of Quinoa with Legumes," *Polish Journal of Environmental Studies*, journal article 2024, doi: 10.15244/pjoes/191963.
3. Pancaldi, F., Gulisano, A., Severing, E. I., van Kaauwen, M., Finkers, R., Kodde, L., and Trindade, L. M., "The genome of Lupinus mutabilis: Evolution and genetics of an emerging bio-based crop," The Plant Journal, vol. 120, no. 3, pp. 881-900, 2024, doi: https://doi.org/10.1111/tpj.17021.
4. Jimenez-Lopez, J. C., "Narrow-leafed lupin (Lupinus angustifolius L.) β-conglutin: A multifunctional family of proteins with roles in plant defence, human health benefits, and potential uses as functional food," Legume Science, vol. 2, no. 2, p. e33, 2020, doi: https://doi.org/10.1002/leg3.33.
5. Braga, J. D., Thongngam, M., and Kumrungsee, T., "Gamma-aminobutyric acid as a potential postbiotic mediator in the gut–brain axis," npj *Science of Food*, vol. 8, no. 1, p. 16, 2024/04/02 2024, doi: 10.1038/s41538-024-00253-2.
6. Bonifacio, A., Aroni, G., and Villca, M., "Catálogo etnobotánico de la quinua real," Fundación PROINPA, Poligraf, Cochabamba, Bolivia, 2012.
7. Taaime, N., Rafik, S., El Mejahed, K., Oukarroum, A., Choukr-Allah, R., Bouabid, R., and El Gharous, M., "Worldwide development of agronomic management practices for quinoa cultivation: a systematic review," (in English), *Frontiers in Agronomy*, Systematic Review vol. 5 - 2023, doi: 10.3389/fagro.2023.1215441.
8. Mukherjee, K., Dutta, P., Badwaik, H. R., Saha, A., Das, A., and Giri, T. K., "Food industry applications of Tara gum and its modified forms" *Food Hydrocolloids for Health*, vol. 3, p. 100107, 2023/12/01/ 2023, doi: https://doi.org/10.1016/j.fhfh.2022.100107.
9. Miranda, M., Bento, A., and Aguilar, A. M., "Malnutrition in all its forms and socioeconomic status in Bolivia" *Public Health Nutrition*, vol. 23, no. S1, pp. s21-s28, 2020, doi: 10.1017/S1368980019003896.
10. Giuliani, A., Hintermann, F., Rojas, W., and Padulosi, S., "Biodiversity of Andean grains: balancing market potential and sustainable livelihoods" 2012.
11. Murillo, A. C. and Canahua, P. R., "Tarwi: Leguminosa andina de gran potencial" *LEISA revista de agroecología*, vol. 32, no. 2, pp. 20-21, 2016.

12. Lucas, M. M., Stoddard, F. L., Annicchiarico, P., Frías, J., Martínez-Villaluenga, C., Sussmann, D., Duranti, M., Seger, A., Zander, P. M., and Pueyo, J. J., "The future of lupin as a protein crop in Europe" *Frontiers in Plant Science*, vol. 6, p. 160197, 2015.
13. Vicente Rojas, J. J., "El cultivo de Tarwi (*Lupinus mutabilis* Sweet) en el Estado Plurinacional de Bolivia" *Revista Científica de Investigación INFO-INIAF*, vol. 1, pp. 88-100, 2016. [Online]. Available: http://revistasbolivianas.umsa.bo/scielo.php?script=sci_arttext&pid=S2308-250X2016000100014&nrm=iso.
14. Molina-Poveda, C., Lucas, M., and Jover, M., "Evaluation of the potential of Andean lupin meal (*Lupinus mutabilis* Sweet) as an alternative to fish meal in juvenile *Litopenaeus vannamei* diets" *Aquaculture*, vol. 410, pp. 148-156, 2013.
15. Ortiz-Sempértegui, J., Ibieta, G., Tullberg, C., Peñarrieta, J. M., and Linares-Pastén, J. A., "Chemical Characterisation of New Oils Extracted from Cañihua and Tarwi Seeds with Different Organic Solvents" *Foods*, vol. 13, no. 13, p. 1982, 2024. [Online]. Available: https://www.mdpi.com/2304-8158/13/13/1982.
16. Estivi, L., Brandolini, A., Gasparini, A., and Hidalgo, A., "Lupin as a Source of Bioactive Antioxidant Compounds for Food Products" *Molecules*, vol. 28, no. 22, p. 7529, 2023. [Online]. Available: https://www.mdpi.com/1420-3049/28/22/7529.
17. Alonso-Álvarez, A. and Haros, C. M., "Characterization of Quinoa Fibre-Rich Fractions Isolated via Wet-Milling and Their Application in Food" *Biology and Life Sciences Forum*, vol. 25, no. 1, p. 12, 2023. [Online]. Available: https://www.mdpi.com/2673-9976/25/1/12.
18. Demir, B. and Bilgiçli, N., "Utilization of quinoa flour (*Chenopodium quinoa* Willd.) in gluten-free pasta formulation: Effects on nutritional and sensory properties" *Food Science and Technology International*, vol. 27, no. 3, pp. 242-250, 2021, doi: 10.1177/1082013220940092.
19. Bravi, E., Sileoni, V., and Marconi, O., "Quinoa (*Chenopodium Quinoa* Willd.) as Functional Ingredient for the Formulation of Gluten-Free Shortbreads," *Foods*, vol. 13, no. 3, p. 377, 2024. [Online]. Available: https://www.mdpi.com/2304-8158/13/3/377.
20. Xi, X., Fan, G., Xue, H., Peng, S., Huang, W., and Zhan, J., "Harnessing the Potential of Quinoa: Nutritional Profiling, Bioactive Components, and Implications for Health Promotion" *Antioxidants*, vol. 13, no. 7, p. 829, 2024. [Online]. Available: https://www.mdpi.com/2076-3921/13/7/829.
21. Quiroga Ledezma, C. C., "Chapter 1 - Native food crops for present and future generations: Their role in nutrition and health" in Sustainability of the Food System, N. Betoret and E. Betoret Eds.: *Academic Press*, 2020, pp. 3-23.
22. Repo-Carrasco-Valencia, R., Acevedo de La Cruz, A., Icochea Alvarez, J. C., and Kallio, H., "Chemical and Functional Characterization of Kañiwa (*Chenopodium pallidicaule*) Grain, Extrudate and Bran" *Plant Foods for Human Nutrition*, vol. 64, no. 2, pp. 94-101, 2009/06/01 2009, doi: 10.1007/s11130-009-0109-0.

23. Gomez Cahuata, J. F., Rosas-Quina, Y. E., and Pachari Vera, E., "Cañihua (*Chenopodium pallidicaule* Aellen) a promising superfood in food industry: a review" *Nutrition & Food Science*, vol. 52, no. 6, pp. 917-928, 2022, doi: 10.1108/NFS-09-2021-0277.
24. Repo-Carrasco-Valencia, R., Vidaurre-Ruiz, J., and Luna-Mercado, G. I., "Development of Gluten-Free Breads Using Andean Native Grains Quinoa, Kañiwa, Kiwicha and Tarwi" *Proceedings*, vol. 53, no. 1, p. 15, 2020. [Online]. Available: https://www.mdpi.com/2504-3900/53/1/15.
25. De la Cruz Lapa, P., "Aprovechamiento integral y racional de la tara *Caesalpinia spinosa-Caesalpinia tinctoria*," *Revista del Instituto de investigación FIGMMG*, vol. 7, no. 14, pp. 64-73, 2004.
26. Ibieta, G. and Peñarrieta, J. M., "Chemical characterization and quantification of tannins in the powder of *Caesalpinia spinosa*: tara boliviana" *Revista Boliviana de Química*, vol. 38, no. 1, pp. 26-35, 2021.
27. Skowyra, M., Falguera, V., Gallego, G., Peiró, S., and Almajano, M. P., "Antioxidant properties of aqueous and ethanolic extracts of tara (*Caesalpinia spinosa*) pods in vitro and in model food emulsions" *Journal of the Science of Food and Agriculture*, vol. 94, no. 5, pp. 911-918, 2014, doi: https://doi.org/10.1002/jsfa.6335.
28. Duran, M. I., Ballesteros-Ramírez, R., Tellez, A., Torregrosa, L., Olejua, P. A., Galvis, S., Urueña, C., and Fiorentino, S., "Safety Evaluation in Healthy Colombian Volunteers of P2Et Extract Obtained from *Caesalpinia spinosa*: Design 3+3 Phase I Clinical Trial" (in eng), *Evidence-Based Complementary and Alternative Medicine*, vol. 2022, p. 7943001, 2022, doi: 10.1155/2022/7943001.
29. Wu, Y., Ding, W., Jia, L., and He, Q., "The rheological properties of tara gum (*Caesalpinia spinosa*)" *Food Chemistry*, vol. 168, pp. 366-371, 2015/02/01/ 2015, doi: https://doi.org/10.1016/j.foodchem.2014.07.083.
30. Santos, M. B., de Carvalho, C. W. P., and Garcia-Rojas, E. E., "Microencapsulation of vitamin D3 by complex coacervation using carboxymethyl tara gum (*Caesalpinia spinosa*) and gelatin A" *Food Chemistry*, vol. 343, p. 128529, 2021/05/01/ 2021, doi: https://doi.org/10.1016/j.foodchem.2020.128529.
31. Santos, M. B., Dos Santos, C. H. C., de Carvalho, M. G., de Carvalho, C. W. P., and Garcia-Rojas, E. E., "Physicochemical, thermal and rheological properties of synthesized carboxymethyl tara gum (*Caesalpinia spinosa*)" (in eng), *International Journal of Biological Macromolecules*, vol. 134, pp. 595-603, Aug 1 2019, doi: 10.1016/j.ijbiomac.2019.05.025.
32. Li, B., Shen, J., and Wang, L., "Development of an antibacterial superabsorbent hydrogel based on tara gum grafted with polyacrylic acid" *International Research Journal of Public and Environmental Health*, 2017.
33. Abd Alla, S. G., Sen, M., and El-Naggar, A. W. M., "Swelling and mechanical properties of superabsorbent hydrogels based on Tara gum/acrylic acid synthesized by gamma radiation" *Carbohydrate Polymers*, vol. 89, no. 2, pp. 478-485, 2012/06/20/ 2012, doi: https://doi.org/10.1016/j.carbpol.2012.03.031.

34. Muschin, T., Budragchaa, D., Kanamoto, T., Nakashima, H., Ichiyama, K., Yamamoto, N., Shuqin, H., and Yoshida, T., "Chemically sulfated natural galactomannans with specific antiviral and anticoagulant activities" *International Journal of Biological Macromolecules*, vol. 89, pp. 415-420, 2016.

35. Vilaró, P., Bennadji, Z., Budelli, E., Moyna, G., Panizzolo, L., and Ferreira, F., "Isolation and characterization of galactomannans from *Prosopis affinis* as potential gum substitutes" *Food Hydrocolloids*, vol. 77, pp. 711-719, 2018/04/01/ 2018, doi: https://doi.org/10.1016/j.foodhyd.2017.10.038.

36. Garros-Rosa, I., Reicher, F., Petkowicz, C. L., Sierakowski, M. R., and Moreira, R. A., "Characterization of the galactomannans from *Parkinsonia aculeata* seeds and their application on affinity chromatography" *Polímeros*, vol. 16, pp. 99-103, 2006.

37. Liu, Y., Lei, F., He, L., Xu, W., and Jiang, J., "Comparative study on the monosaccharides of three typical galactomannans hydrolyzed by different methods" *Industrial Crops and Products*, vol. 157, p. 112895, 2020/12/01/ 2020, doi: https://doi.org/10.1016/j.indcrop.2020.112895.

38. Prajapati, V. D., Jani, G. K., Moradiya, N. G., Randeria, N. P., and Nagar, B. J., "Locust bean gum: A versatile biopolymer" *Carbohydrate Polymers*, vol. 94, no. 2, pp. 814-821, 2013.

39. Xie, F., Yuan, C., Zhang, H., Wu, Y., and Ai, L., "Structure-function relationship between galactomannans and their effects on freeze-thaw stability, retrogradation, and texture of corn starch gels during cold storage" *Food Chemistry*, vol. 398, p. 133915, 2023/01/01/ 2023, doi: https://doi.org/10.1016/j.foodchem.2022.133915.

40. Desai, S., Prajapati, V., and Chandarana, C., "Chemistry, biological activities, and uses of tara gum" in Gums, resins and latexes of plant origin: chemistry, biological activities and uses: Springer, 2022, pp. 1-25.

41. Han, Y., Li, D., Li, D., Chen, W., Mu, S. e., Chen, Y., and Chai, J., "Impact of refractive index increment on the determination of molecular weight of hyaluronic acid by muti-angle laser light-scattering technique" *Scientific Reports*, vol. 10, no. 1, p. 1858, 2020/02/05 2020, doi: 10.1038/s41598-020-58992-7.

42. Pollard, M. A., Eder, B., Fischer, P., and Windhab, E. J., "Characterization of galactomannans isolated from legume endosperms of *Caesalpinioideae* and *Faboideae* subfamilies by multidetection aqueous SEC" *Carbohydrate Polymers*, vol. 79, no. 1, pp. 70-84, 2010/01/05/ 2010, doi: https://doi.org/10.1016/j.carbpol.2009.07.028.

43. Mérida-López, J., Rojas, C. C., Bergenståhl, B., and Purhagen, J., "Functional properties of starch cultivars of two Andean grains grown in Bolivia: Amaranth (*Amaranthus caudatus*) and canihua (*Chenopodium pallidicaule*)," *Heliyon*, vol. 10, no. 15, p. e35140, 2024/08/15/ 2024, doi: https://doi.org/10.1016/j.heliyon.2024.e35140.

44. Fuentes, C., Perez-Rea, D., Bergenståhl, B., Carballo, S., Sjöö, M., and Nilsson, L., "Physicochemical and structural properties of starch from five Andean crops grown in Bolivia" *International Journal of Biological Macromolecules*, vol. 125, pp. 829-838, 2019/03/15/ 2019, doi: https://doi.org/10.1016/j.ijbiomac.2018.12.120.

45. Zhou, X., Chen, Y., Feng, P., Shen, J., Fan, X., Chen, Y., and Yu, W., "Fine structure of starch biomacromolecules and digestibility: The regulative role of amylose and amylopectin in the digestive hydrolysis of starch in rice" *Carbohydrate Polymers*, vol. 350, p. 123040, 2025/02/15/ 2025, doi: https://doi.org/10.1016/j.carbpol.2024.123040.
46. Gautam, D., Rana, V., Sharma, S., Kumar Walia, Y., Kumar, K., Umar, A., Ibrahim, A. A., and Baskoutas, S., "Hemicelluloses: A Review on Extraction and Modification for Various Applications," *Chemistry Select*, vol. 10, no. 24, p. e06050, 2025, doi: https://doi.org/10.1002/slct.202406050.
47. Ali, N. B., Abdedayem, A., Nouri, H., and Ben-Ali, S., "Cellulose extraction by biomass valorization of prickly pear seed waste," (in en), *Comptes Rendus. Chimie*, vol. 27, no. S3, pp. 45-53, 2024, doi: 10.5802/crchim.369.
48. Li, L., Rowbotham, J. S., Christopher Greenwell, H., and Dyer, P. W., "Chapter 8 - An Introduction to Pyrolysis and Catalytic Pyrolysis: Versatile Techniques for Biomass Conversion" in New and Future Developments in Catalysis, S. L. Suib Ed. Amsterdam: Elsevier, 2013, pp. 173-208.
49. Stoklosa, R. J., Latona, R. J., Powell, M. J., and Yadav, M. P., "Influence of phenolic acid content on the antioxidant capacity of hemicellulose from sorghum plant fractions" *BioResources*, vol. 15, no. 4, p. 7933, 2020.
50. Xu, W., Zhang, F., Luo, Y., Ma, L., Kou, X., and Huang, K., "Antioxidant activity of a water-soluble polysaccharide purified from *Pteridium aquilinum*" *Carbohydrate Research*, vol. 344, no. 2, pp. 217-222, 2009/01/26/ 2009, doi: https://doi.org/10.1016/j.carres.2008.10.021.
51. El-Maksoud, A. A. A., Cheng, W., Petersen, S. V., Pandiselvam, R., and Guo, Z., "Covalent phenolic acid-grafted β-lactoglobulin conjugates: Synthesis, characterization, and evaluation of their multifunctional properties" *LWT*, vol. 172, p. 114215, 2022/12/30/ 2022, doi: https://doi.org/10.1016/j.lwt.2022.114215.
52. Mujica, A., Romero-Ucharico, M., Arcos, J., Moscoso, G., Ucharico, A., and Luna, J. C., "Grain Yield, Protein Content, Oil Content, and Vegetative Period of National Tarwi Cultivars (*Lupinus Mutabilis Sweet*) in Puno, Peru" *Revista de Gestão Social e Ambiental*, vol. 18, no. 8, p. e07570, 06/13 2024, doi: 10.24857/rgsa.v18n8-150.
53. Salazar, D., Arancibia, M., Ocaña, I., Rodríguez-Maecker, R., Bedón, M., Caballero, M. E. L., and García, P. M., "Characterization and technological potential of underutilized ancestral Andean crop flours from Ecuador" ed: Multidisciplinary Digital Publishing Institute, 2021.
54. Okpara, M. O., "Microbial enzymes and their applications in food industry: a mini-review" *Advances in Enzyme Research*, vol. 10, no. 1, pp. 23-47, 2022.
55. Barrett, J. S. and Gibson, P. R., "Fermentable oligosaccharides, disaccharides, monosaccharides and polyols (FODMAPs) and nonallergic food intolerance: FODMAPs or food chemicals?" *Therapeutic Advances in Gastroenterology*, vol. 5, no. 4, pp. 261-268, 2012, doi: 10.1177/1756283x11436241.
56. Losada-Garcia, N., Simović, M., Ćorović, M., Milivojević, A., Nikačević, N., Mateo, C., Bezbradica, D., and Palomo, J. M., "Developing and improving enzyme-driven technologies to synthesise emerging prebiotics," *Green Chemistry*, doi: 10.1039/D5GC01723H.

57. Nabarlatz, D., Ebringerová, A., and Montané, D., "Autohydrolysis of agricultural by-products for the production of xylo-oligosaccharides" *Carbohydrate Polymers*, vol. 69, no. 1, pp. 20-28, 2007.
58. Sharma, D., Dhiman, A., Thakur, A., Kumar, S., and Saini, R., "Functional oligosaccharides as a promising food ingredient: a gleam into health apprehensions and techno-functional advantages" *Journal of Food Measurement and Characterization*, vol. 19, no. 1, pp. 505-536, 2025/01/01 2025, doi: 10.1007/s11694-024-02986-w.
59. Mobarec, H., Villagomez, R., Karlsson, E. N., and Linares-Pastén, J. A., "Microwave-assisted xylanase reaction: impact in the production of prebiotic xylooligosaccharides" *RSC Advances*, vol. 11, no. 20, pp. 11882-11888, 2021.
60. Ren, L., Cao, Q., Ye, H., Dong, Z., Zhang, C., Yan, F., Zhou, Y., Zhou, H., Zuo, J., and Wang, W., "The single degree of polymerization influences the efficacy of xylooligosaccharides in shaping microbial and metabolite profiles in chicken gut to combat avian pathogenic *Escherichia coli*" (in eng), *BMC Microbiology*, vol. 25, no. 1, p. 227, Apr 22 2025, doi: 10.1186/s12866-025-03948-y.
61. Palaniappan, A., Balasubramaniam, V. G., and Antony, U., "Prebiotic potential of xylooligosaccharides derived from finger millet seed coat" *Food Biotechnology*, vol. 31, no. 4, pp. 264-280, 2017.
62. Geetha, K. and Gunasekaran, P., "Purification of endoxylanase from Bacillus pumilus B20 for production of prebiotic xylooligosaccharide syrup; an in vitro study" *Iranian Journal of Biotechnology*, vol. 15, no. 4, p. 232, 2017.
63. Patel, A. and Prajapati, J., "Biological properties of xylooligosaccharides as an emerging prebiotic and future perspective" *Current Trends in Biotechnology and Pharmacy*, vol. 9, no. 1, pp. 28-36, 2015.
64. Ávila, P. F., Silva, M. F., Martins, M., and Goldbeck, R., "Cello-oligosaccharides production from lignocellulosic biomass and their emerging prebiotic applications" *World Journal of Microbiology and Biotechnology*, vol. 37, no. 5, p. 73, 2021/03/29 2021, doi: 10.1007/s11274-021-03041-2.
65. Kabel, M. A., Kortenoeven, L., Schols, H. A., and Voragen, A. G. J., "*In Vitro* Fermentability of Differently Substituted Xylo-oligosaccharides" *Journal of Agricultural and Food Chemistry*, vol. 50, no. 21, pp. 6205-6210, 2002/10/01 2002, doi: 10.1021/jf020220r.
66. Singh, R. D., Muir, J., and Arora, A., "Concentration of xylooligosaccharides with a low degree of polymerization using membranes and their effect on bacterial fermentation" *Biofuels, Bioproducts and Biorefining*, vol. 15, no. 1, pp. 61-73, 2021, doi: https://doi.org/10.1002/bbb.2145.
67. Ávila, P. F., de Mélo, A. H. F., and Goldbeck, R., "Cello-oligosaccharides production from multi-stage enzymatic hydrolysis by lignocellulosic biomass and evaluation of prebiotic potential" *Innovative Food Science & Emerging Technologies*, vol. 85, p. 103335,2023, doi: https://doi.org/10.1016/j.ifset.2023.103335.
68. Czubinski, J., Grygier, A., and Siger, A., "*Lupinus mutabilis* seed composition and its comparison with other lupin species" *Journal of Food Composition and Analysis*, vol. 99, p. 103875, 2021/06/01/ 2021, doi: https://doi.org/10.1016/j.jfca.2021.103875.

69. Bebeli, P. J., Lazaridi, E., Chatzigeorgiou, T., Suso, M.-J., Hein, W., Alexopoulos, A. A., Canha, G., van Haren, R. J. F., Jóhannsson, M. H., Mateos, C., Neves-Martins, J., Prins, U., Setas, F., Simioniuc, D. P., Talhinhas, P., and van den Berg, M., "State and Progress of Andean Lupin Cultivation in Europe: A Review" *Agronomy*, vol. 10, no. 7, p. 1038, 2020. [Online]. Available: https://www.mdpi.com/2073-4395/10/7/1038.
70. Lemus-Conejo, A., Rivero-Pino, F., Montserrat-de la Paz, S., and Millan-Linares, M. C., "Nutritional composition and biological activity of narrow-leafed lupins (*Lupinus angustifolius* L.) hydrolysates and seeds" *Food Chemistry*, vol. 420, p. 136104, 2023, doi: https://doi.org/10.1016/j.foodchem.2023.136104.
71. Erbaş, M., Certel, M., and Uslu, M. K., "Some chemical properties of white lupin seeds (*Lupinus albus* L.)" *Food Chemistry*, vol. 89, no. 3, pp. 341-345, 2005, doi: https://doi.org/10.1016/j.foodchem.2004.02.040.
72. Bähr, M., Fechner, A., Hasenkopf, K., Mittermaier, S., and Jahreis, G., "Chemical composition of dehulled seeds of selected lupin cultivars in comparison to pea and soya bean" *LWT - Food Science and Technology*, vol. 59, no. 1, pp. 587-590, 2014, doi: https://doi.org/10.1016/j.lwt.2014.05.026.
73. Shrestha, S., Hag, L. v. t., Haritos, V. S., and Dhital, S., "Lupin proteins: Structure, isolation and application," *Trends in Food Science & Technology*, vol. 116, pp. 928-939, 2021, doi: https://doi.org/10.1016/j.tifs.2021.08.035.
74. Gulisano, A., Alves, S., Martins, J. N., and Trindade, L. M., "Genetics and Breeding of *Lupinus mutabilis*: An Emerging Protein Crop" (in English), *Frontiers in Plant Science*, Review vol. 10, 2019, doi: 10.3389/fpls.2019.01385.
75. Aguilar-Acosta, L. A., Serna-Saldivar, S. O., Rodríguez-Rodríguez, J., Escalante-Aburto, A., and Chuck-Hernández, C., "Effect of Ultrasound Application on Protein Yield and Fate of Alkaloids during Lupin Alkaline Extraction Process" *Biomolecules*, vol. 10, no. 2, p. 292, 2020. [Online]. Available: https://www.mdpi.com/2218-273X/10/2/292.
76. Intiquilla, A., Jiménez-Aliaga, K., Zavaleta, A. I., and Hernández-Ledesma, B., "Production of Antioxidant Hydrolyzates from a *Lupinus mutabilis* (Tarwi) Protein Concentrate with Alcalase: Optimization by Response Surface Methodology," *Natural Product Communications*, vol. 13, no. 6, p. 1934578X1801300626, 2018/06/01 2018, doi: 10.1177/1934578X1801300626.
77. Nill, K., "Soy Beans: Properties and Analysis" in Encyclopedia of Food and Health, B. Caballero, P. M. Finglas, and F. Toldrá Eds. Oxford: Academic Press, 2016, pp. 54-55.
78. Burrin, D. G. and Stoll, B., "Metabolic fate and function of dietary glutamate in the gut" *The American Journal of Clinical Nutrition*, vol. 90, no. 3, pp. 850S-856S, 2009, doi: https://doi.org/10.3945/ajcn.2009.27462Y.
79. Sathe, S. K., Deshpande, S. S., and Salunkhe, D. K., "Functional Properties of Lupin Seed (*Lupinus mutabilis*) Proteins and Protein Concentrates" *Journal of Food Science*, vol. 47, no. 2, pp. 491-497, 1982, doi: https://doi.org/10.1111/j.1365-2621.1982.tb10110.x.

80. Lara-Rivera, A. H., García-Alamilla, P., Lagunes-Gálvez, L. M., Rodríguez Macias, R., García López, P. M., and Zamora Natera, J. F., "Functional Properties of Lupinus angustifolius Seed Protein Isolates" *Journal of Food Quality*, vol. 2017, no. 1, p. 8675814, 2017, doi: https://doi.org/10.1155/2017/8675814.

81. Ochoa-Rivas, A., Nava-Valdez, Y., Serna-Saldívar, S. O., and Chuck-Hernández, C., "Microwave and ultrasound to enhance protein extraction from peanut flour under alkaline conditions: Effects in yield and functional properties of protein isolates" *Food and Bioprocess Technology*, vol. 10, no. 3, pp. 543-555, 2017.

82. Tang, J., Cases, L., Alves, S., Sun, D.-W., and Tiwari, B. K., "Protein extraction from lupin (*Lupinus angustifolius* L.) using combined ultrasound and microwave techniques: Impact on protein recovery, structure, and functional properties" *Ultrasonics Sonochemistry*, vol. 115, p. 107232, 2025, doi: https://doi.org/10.1016/j.ultsonch.2025.107232.

83. Kanklai, J., Somwong, T. C., Rungsirivanich, P., and Thongwai, N., "Screening of GABA-producing lactic acid bacteria from Thai fermented foods and probiotic potential of *Levilactobacillus brevis* F064A for GABA-fermented mulberry juice production" *Microorganisms*, vol. 9, no. 1, p. 33, 2020.

84. Pizzi, A., Parolin, C., Gottardi, D., Ricci, A., Parpinello, G. P., Lanciotti, R., Patrignani, F., and Vitali, B., "A Novel GABA-Producing *Levilactobacillus brevis* Strain Isolated from Organic Tomato as a Promising Probiotic" *Biomolecules*, vol. 15, no. 7, p. 979, 2025. [Online]. Available: https://www.mdpi.com/2218-273X/15/7/979.

85. Choi, J., Lee, S., Linares-Pastén, J. A., and Nilsson, L., "Study on oligomerization of glutamate decarboxylase from *Lactobacillus brevis* using asymmetrical flow field-flow fractionation (AF4) with light scattering techniques," *Analytical and Bioanalytical Chemistry*, vol. 410, no. 2, pp. 451-458, 2018, doi: 10.1007/s00216-017-0735-6.

86. Banerjee, S., Poore, M., Gerdes, S., Nedveck, D., Lauridsen, L., Kristensen, H. T., Jensen, H. M., Byrd, P. M., Ouwehand, A. C., Patterson, E., and Morovic, W., "Transcriptomics reveal different metabolic strategies for acid resistance and gamma-aminobutyric acid (GABA) production in select Levilactobacillus brevis strains" (in eng), *Microbial Cell Factories*, vol. 20, no. 1, p. 173, Sep 6 2021, doi: 10.1186/s12934-021-01658-4.

87. Huang, J., Fang, H., Gai, Z.-C., Mei, J.-Q., Li, J.-N., Hu, S., Lv, C.-J., Zhao, W.-R., and Mei, L.-H., "*Lactobacillus brevis* CGMCC 1306 glutamate decarboxylase: Crystal structure and functional analysis," *Biochemical and Biophysical Research Communications*, vol. 503, no. 3, pp. 1703-1709, 2018.

88. Sha, D., Jin, Y., Wu, H., Wei, J., Lin, C.-H., Lee, Y.-H., Buddhala, C., Kuchay, S., Chishti, A. H., and Wu, J.-Y., "Role of μ-calpain in proteolytic cleavage of brain l-glutamic acid decarboxylase" *Brain Research*, vol. 1207, pp. 9-18, 2008/05/01/ 2008, doi: https://doi.org/10.1016/j.brainres.2008.02.033.

89. Jigar Pravinchandra, M., Howard, P., and Jang-Yen, W., "Regulation of GABA Neurotransmission by Glutamic Acid Decarboxylase (GAD)" *Current Pharmaceutical Design*, vol. 21, no. 34, pp. 4939-4942, 2015, doi: http://dx.doi.org/10.2174/1381612821666150917094343.

90. Wang, Q., Liu, X., Fu, J., Wang, S., Chen, Y., Chang, K., and Li, H., "Substrate sustained release-based high efficacy biosynthesis of GABA by *Lactobacillus brevis* NCL912," *Microbial cell factories*, vol. 17, pp. 1-8, 2018.
91. Li, H., Sun, T., Jia, M., Wang, L., Wei, C., Pei, J., Lin, Z., and Wang, S., "Production of Gamma-Aminobutyric Acid by *Levilactobacillus brevis* CD0817 by Coupling Fermentation with Self-Buffered Whole-Cell Catalysis" *Fermentation*, vol. 8, no. 7, p. 321, 2022. [Online]. Available: https://www.mdpi.com/2311-5637/8/7/321.
92. Cha, X., Ding, J., Ba, W., You, S., Qi, W., and Su, R., "High Production of γ-Aminobutyric Acid by Activating the *xyl* Operon of Lactobacillus brevis" *ACS Omega*, vol. 8, no. 8, pp. 8101-8109, 2023, doi: 10.1021/acsomega.2c08272.
93. Rychen G., Aquilina G., Azimonti G., Bampidis V., De Lourdes Bastos M., Bories G., Chesson A., Cocconcelli P.S., Flachowsky G., Gropp J., Kolar B., Kouba M., Lopez-Puente S., Lopez-Alonso M., Mantovani A., Mayo B., Ramos F., Villa R.E., Wallace R.J., Wester P., Brozzi R. and Saarela M. "Safety and efficacy of *Lactobacillus brevis* NCIMB 42149 as a silage additive for all animal species" EFSA Journal, vol. 14, no. 11, p. e04616, 2016, doi: https://doi.org/10.2903/j.efsa.2016.4616.
94. Tur, J. A. and Bibiloni, M. M., "Functional Foods" in Encyclopedia of Food and Health, B. Caballero, P. M. Finglas, and F. Toldrá Eds. Oxford: Academic Press, 2016, pp. 157-161.
95. Vera, C., Illanes, A., and Guerrero, C., "Enzymatic production of prebiotic oligosaccharides" *Current Opinion in Food Science*, vol. 37, pp. 160-170, 2021/02/01/ 2021, doi: https://doi.org/10.1016/j.cofs.2020.10.013.
96. Karnaouri, A., Matsakas, L., Krikigianni, E., Rova, U., and Christakopoulos, P., "Valorization of waste forest biomass toward the production of cello-oligosaccharides with potential prebiotic activity by utilizing customized enzyme cocktails," *Biotechnology for biofuels*, vol. 12, no. 1, pp. 1-19, 2019.
97. Palaniappan, A., Antony, U., and Emmambux, M. N., "Current status of xylooligosaccharides: Production, characterization, health benefits and food application" *Trends in Food Science & Technology*, vol. 111, pp. 506-519, 2021/05/01/ 2021, doi: https://doi.org/10.1016/j.tifs.2021.02.047.
98. Singh, A. K., Mishra, B., Bedford, M. R., and Jha, R., "Effects of supplemental xylanase and xylooligosaccharides on production performance and gut health variables of broiler chickens," *Journal of Animal Science and Biotechnology*, vol. 12, no. 1, p. 98, 2021, doi: 10.1186/s40104-021-00617-8.
99. Liu, J., Li, X., Song, F., Cui, S., Lu, W., Zhao, J., Zhang, H., Gu, Z., and Chen, W., "Dietary supplementation with low-dose xylooligosaccharide promotes the anti-Salmonella activity of probiotic *Lactiplantibacillus plantarum* ZS2058 in a murine model" *Food Research International*, vol. 151, p. 110858, 2022, doi: https://doi.org/10.1016/j.foodres.2021.110858.
100. Fei, Y., Wang, Y., Pang, Y., Wang, W., Zhu, D., Xie, M., Lan, S., and Wang, Z., "Xylooligosaccharide modulates gut microbiota and alleviates colonic inflammation caused by high fat diet induced obesity" *Frontiers in Physiology*, vol. 10, p. 1601, 2020.

101. Whelan, K., "Mechanisms and effectiveness of prebiotics in modifying the gastrointestinal microbiota for the management of digestive disorders" *Proceedings of the Nutrition Society*, vol. 72, no. 3, pp. 288-298, 2013.

102. Lin, S.-H., Chou, L.-M., Chien, Y.-W., Chang, J.-S., and Lin, C.-I., "Prebiotic effects of xylooligosaccharides on the improvement of microbiota balance in human subjects" *Gastroenterology Research and Practice*, vol. 2016, no. 1, p. 5789232, 2016.

103. Yang, J., Summanen, P. H., Henning, S. M., Hsu, M., Lam, H., Huang, J., Tseng, C.-H., Dowd, S. E., Finegold, S. M., and Heber, D., "Xylooligosaccharide supplementation alters gut bacteria in both healthy and prediabetic adults: a pilot study" *Frontiers in Physiology*, vol. 6, p. 216, 2015.

104. Ferrão, L. L., Ferreira, M. V. S., Cavalcanti, R. N., Carvalho, A. F. A., Pimentel, T. C., Silva, H. L., Silva, R., Esmerino, E. A., Neto, R. P., and Tavares, M. I. B., "The xylooligosaccharide addition and sodium reduction in requeijão cremoso processed cheese" *Food Research International*, vol. 107, pp. 137-147, 2018.

105. da Silva, M. F., Bueno, D., and Goldbeck, R., "Challenges and trends in the industrial production and application of cellooligosaccharides" *Current Opinion in Food Science*, vol. 63, p. 101308, 2025, doi: https://doi.org/10.1016/j.cofs.2025.101308.

106. den Besten, G., van Eunen, K., Groen, A. K., Venema, K., Reijngoud, D.-J., and Bakker, B. M., "The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism" *Journal of Lipid Research*, vol. 54, no. 9, pp. 2325-2340, 2013, doi: https://doi.org/10.1194/jlr.R036012.

107. Shimizu, H., Masujima, Y., Ushiroda, C., Mizushima, R., Taira, S., Ohue-Kitano, R., and Kimura, I., "Dietary short-chain fatty acid intake improves the hepatic metabolic condition via FFAR3" *Scientific Reports*, vol. 9, no. 1, p. 16574, 2019, doi: 10.1038/s41598-019-53242-x.

108. Facchin, S., Bertin, L., Bonazzi, E., Lorenzon, G., De Barba, C., Barberio, B., Zingone, F., Maniero, D., Scarpa, M., Ruffolo, C., Angriman, I., and Savarino, E. V., "Short-Chain Fatty Acids and Human Health: From Metabolic Pathways to Current Therapeutic Implications" *Life*, vol. 14, no. 5, p. 559, 2024. [Online]. Available: https://www.mdpi.com/2075-1729/14/5/559.

109. Duscha, A., Gisevius, B., Hirschberg, S., Yissachar, N., Stangl, G. I., Dawin, E., Bader, V., Haase, S., Kaisler, J., David, C., Schneider, R., Troisi, R., Zent, D., Hegelmaier, T., Dokalis, N., Gerstein, S., Del Mare-Roumani, S., Amidror, S., Staszewski, O., Poschmann, G., Stühler, K., Hirche, F., Balogh, A., Kempa, S., Träger, P., Zaiss, M. M., Holm, J. B., Massa, M. G., Nielsen, H. B., Faissner, A., Lukas, C., Gatermann, S. G., Scholz, M., Przuntek, H., Prinz, M., Forslund, S. K., Winklhofer, K. F., Müller, D. N., Linker, R. A., Gold, R., and Haghikia, A., "Propionic Acid Shapes the Multiple Sclerosis Disease Course by an Immunomodulatory Mechanism" *Cell*, vol. 180, no. 6, pp. 1067-1080.e16, 2020, doi: 10.1016/j.cell.2020.02.035.

110. Deleu, S., Machiels, K., Raes, J., Verbeke, K., and Vermeire, S., "Short chain fatty acids and its producing organisms: An overlooked therapy for IBD?" *EBioMedicine*, vol. 66, 2021.

111. Valdes, D. S., So, D., Gill, P. A., and Kellow, N. J., "Effect of Dietary Acetic Acid Supplementation on Plasma Glucose, Lipid Profiles, and Body Mass Index in Human Adults: A Systematic Review and Meta-analysis" (in eng), *Journal of the Academy of Nutrition and Dietetics*, vol. 121, no. 5, pp. 895-914, May 2021, doi: 10.1016/j.jand.2020.12.002.

112. Roberts, E., "γ-aminobutyric acid and nervous system function—A perspective" *Biochemical Pharmacology*, vol. 23, no. 19, pp. 2637-2649, 1974.

113. Bassett, M. L., Mullen, K. D., Scholz, B., Fenstermacher, J. D., and Jones, E. A., "Increased brain uptake of γ-aminobutyric acid in a rabbit model of hepatic encephalopathy" *Gastroenterology*, vol. 98, no. 3, pp. 747-757, 1990.

114. Al-Sarraf, H., "Transport of 14C-γ-aminobutyric acid into brain, cerebrospinal fluid and choroid plexus in neonatal and adult rats" *Developmental Brain Research*, vol. 139, no. 2, pp. 121-129, 2002.

115. Cryan, J. F. and Dinan, T. G., "Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour" *Nature reviews neuroscience*, vol. 13, no. 10, pp. 701-712, 2012.

116. Yoto, A., Murao, S., Motoki, M., Yokoyama, Y., Horie, N., Takeshima, K., Masuda, K., Kim, M., and Yokogoshi, H., "Oral intake of γ-aminobutyric acid affects mood and activities of central nervous system during stressed condition induced by mental tasks" *Amino Acids*, vol. 43, no. 3, pp. 1331-1337, 2012.

117. Yamatsu, A., Yamashita, Y., Pandharipande, T., Maru, I., and Kim, M., "Effect of oral γ-aminobutyric acid (GABA) administration on sleep and its absorption in humans" *Food Science and Biotechnology*, vol. 25, no. 2, pp. 547-551, 2016.

118. Hepsomali, P., Groeger, J. A., Nishihira, J., and Scholey, A., "Effects of Oral Gamma-Aminobutyric Acid (GABA) Administration on Stress and Sleep in Humans: A Systematic Review" (in English), *Frontiers in Neuroscience*, Systematic Review vol. 14, 2020-September-17 2020, doi: 10.3389/fnins.2020.00923.