UNIVERSIDAD
DE GRANADA

Faculty of Pharmacy

Department of Nutrition and Food Sciences

PhD Program Nutrition and Food Sciences

TANNINS

BIOACTIVITY AND EFFECT ON GUT MICROBIOTA

Doctoral thesis

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2021

Editor: Universidad de Granada. Tesis Doctorales
Autor: Silvia Molino
ISBN: 978-84-1117-222-6
URI: http://hdl.handle.net/10481/72470

Acknowledgements

I would like to thank the numerous contributions to the development of this thesis from professors, researchers and students from the Department of Nutrition and Food Sciences of the Faculty of Pharmacy, Universidad de Granada.

My special thanks go to my three thesis directors José Ángel Rufián Henares, Maria Pilar Francino Puget and Mariano Fernandez Miyakawa who advised me throughout the different steps of this work.

A very big thank to Michele Battaglia and Samuele Giovando who played a crucial role in the success of this industrial doctoral programme. Thank you for particularly supporting my activity within Silvateam and making sure that my academic research fitted with the activity within the company.

I am grateful to all the external laboratories that welcomed me to develop the research for this thesis. Thanks to Alberto Lerma Aguilera, María José Gosalbes and Nuria Jiménez-Hernández of the Area de Genòmica i Salut, Fundació per al Foment de la Investigació Sanitària i Biomèdica de la Comunitat Valenciana (FISABIO). Thanks to Laura Gómez Mascaraque, André Brodkorp and Fahrad Garavand of the Moorepark Food Research Centre, of Teagasc (Ireland).

One more remark: a lot of gratitude to my family, my friends and my colleagues for their patience and support.

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ACSL Long chain acyl-CoA synthetases
AMP Adenosine monophosphate
ANR Anthocyanidin reductase
ANS Anthocyanidin synthase
ASV Amplicon sequence variants
BMI Body mass index
C4H Cinnamic acid 4-hydroxylase
C4L 4-Coumaric acid CoA ligase
CAT Catalase
CCL2 Chemokine ligand2
CCR C-C Chemokine receptor
CDK Cyclin-dependent kinases
CHE Chestnut tannin extract
CHI Chalcone isomerase
CHS Chalcone synthase
COSY Correlated spectroscopy
COX Cyclooxygenase
CRP C**-** reactive protein
CVD Cardiovascular disease
DAD Diode-array detector
DAHPS 3-Deoxy-D-arabinoheptulosonate 7-phosphate synthase
DCF 2′-7′dichlorofluorescein
DCFH-DA 2′-7′dichlorofluorescin diacetate
DFR Dihydroflavonol-4-reductase
DHHDP Dehydrohexahydroxydiphenoyl
DMSO Dimethyl sulfoxide
DNA Deoxyribonucleic acid
DP Degree of polymerisation
EC Epicatechin
ECG Epicatechin gallate
EFSA European Food Safety Authority
EGC Epigallocatechin
EGC Epigallocatechin
EGCG Epigallocatechin gallate
ESI Electrospray ionization
F3H Flavanone-3-hydroxylase
FBS Foetal bovine serum
FDR False discovery rate
FTIR Fourier transform infrared spectroscopy
GAR+ Global antioxidant response
GLUT-4 Glucose transporter type 4
GPx glutathione peroxidase
GSH reduced glutathione
H_2O_2 Hydrogen peroxide
HCV Hepatitis C virus
HECTOR Heteronuclear correlation spectroscopy
HHDP Hexahydroxydiphenoyl
HIV Human immunodeficiency virus
HPLC High pressure liquid chromatography
HSV Herpes simplex virus
IAV Influence A Virus
IC50 Inhibit concentration 50%
ICAM Intercellular adhesion molecule
IG Immunoglobulin
IL Interleukin
iNOS Inducible nitric oxide synthase
PI Iodide propidium
IR Infra-red
JNK c-Jun N-terminal kinases
LAR Leucoanthocyanidin reductase
LC Liquid Chromatography
LDA Linear discriminant analysis
LDL Low density lipoprotein
LEfSe Linear discriminant analysis (LDA) effect size
LPS Lipopolysaccharide
MALDI-TOF Matrix-assisted laser desorption/ionization time of flight
MAPK Mitogen activated protein kinase
MDA Malondialdehyde
ME Microencapsulation efficiency
MIC Minimum inhibitory concentrations
MIP-1a Macrophage inflammatory protein-1a

MMP Matrix metallopeptidase
MRSA Methicillin-resistant Staphylococcus aureus
MS Mass spectrometry
MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)

NF-κB Nuclear factor kappa-light-chain-enhancer of activated B cells
NIR Near-infrared spectroscopy
NLRP3 Family pyrin domain containing 3
NMR Nuclear magnetic resonance
NO Nitric oxide
NOESY Nuclear Overhauser effect spectroscopy
PAL Phenylalanine ammonia lyase
PBS Phosphate-buffered saline
PCA Principal component analysis
PCoA Principal coordinate analysis
PFA Paraformaldehyde
PGG Pentagalloylglucose
PPARγ Peroxisome proliferator-activated receptor gamma
PRP Protein rich in proline
PUFAs Polyunsaturated fatty acids
QUE Quebracho tannin extract
RNA Ribonucleic acid
ROESY Rotating frame Overhauser effect spectroscopy
ROS Reactive Oxygen Species
SARS-CoV-2 Severe acute respiratory syndrome Coronavirus-2
SCFA Short fatty acid
SOD Superoxide Dismutase
sPLS sparse Partial Least Squares
TE Tara tannin extract
TG Triglyceride
TNF Tumour Necrosis Factor
TOCSY Totally correlated spectroscopy
TOF Time of flight
TPC Total phenolic content
TPTZ 2,4,6-Tri(2-pyridyl)-s-triazine
TSS Total-sum scaling
TTC Total tannin content
UM Urolithin metabotype
UPLC Ultra performance liquid chromatography
USDA United States Department of Agriculture
VCAM Vascular cell adhesion protein
VEGF Vascular endothelial growth factor
VLDL Very low-density lipoprotein

Abstract

Changes due to modern life behaviours have led to a progressive modification of dietary patterns. The consequent increasing interest of consumers for new healthy foods has given a big push to the development of innovative functional products.

Tannins are appealing bioactive ingredients for designing dietary supplements as they have been described to help in prevent or delay the evolution of several diseases. However, these polyphenols are not yet really exploited in the food industry due to a lack of information on various aspects. Therefore, this doctoral thesis has been developed to determine the different interactions and effects that tannins can have on the human body depending on their chemical origin and the administered amount. The second step focused on the study of the incorporation of tannins in food. The search was directed for technological strategies to mask possible alterations in the flavour and to avoid possible detrimental changes in the structure of food products, while maintaining unaltered the bioactive effect exerted by tannins. Thanks to the results obtained from the *in vitro* studies it was possible to design a small nutritional intervention with healthy volunteers.

This research highlighted the powerful bioactive effect of tannins after gastrointestinal digestion and fermentation, exerted as antioxidant activity and as a prebiotic effect that induces beneficial shifts in the microbiota, in terms of composition and functionality. Tannins, as promising tools for the promotion of a healthy gut environment could be applied as bioactive ingredients boosting the effects induced by food, or as dietary supplement, with potential long-term benefits for metabolism and immunity.

Introduction

1.1 Tannins

1.1.1 Historical outline and definition

It is not certain what the origin of the term tannins is. Its original sense was “to convert skins to leather” and it is normally attributed to the French word ‘tanin’ (1802). However, it could have more ancient origins, going back to the Celtic source (such as Breton) indicating an oak tree. From that, the term was acquired by the Medieval Latin tannare "tan, dye a tawny colour" (c. 900), from tannum "crushed oak bark," used in tanning leather (Harper, 2020). Whatever the etymology of the term, it is clear that the meaning of the word tannins has always been associated with the use of these substances since ancient times.

Tannins can be found in nature in numerous different families of the higher plants all over the world, such as in chestnut, quebracho and oak wood, myrobalan, trillo, valonia, sumac or plant galls; they can rise to a molar mass of up to 20000 D, depending on their origin, thus their chemistry could differ widely. These molecules are concentrated in most part of the plant, i.e. bark, wood, leaves, fruit, roots, and seeds. It is accepted that the biological role of tannins in the plant is associated to growth regulation, as well as the preservation against external perturbations, such as infection, insects, or animal herbivory. Tannins can exhibit many different appearances, as light yellow or white amorphous powders or as shiny, nearly colourless, loose masses, with a characteristic smell and astringent taste.

It is considered that the oldest application of these vegetal compounds was the stabilization of animal skin protein against putrefaction, thanks to their property of precipitating proteins. Some authors report that it may have been by the end of the Neolithic period and in the eastern Mediterranean region that man began to use, initially, parts of plants (i.e. leaves, barks and roots) to prevent animal skin degradation, transforming them into a more durable and useful material. The empirical vegetable tanning technique, improved during Classical Antiquity, was introduced in the north-western regions of Europe after the Roman conquest, reaching, a great importance in leather production that lasted until the end of the 19th century.

Thanks to the expansion of their use, tannins have also started to be experimented with and applied in different field, from medicinal application to the textile and food industry. In particular Asian (Chinese and Japanese) natural medicine used different plant-based remedies whose active principles were tannin-containing plant extracts, to treat different pathologies and disorders, such as diarrhoea, gastrointestinal tumours or inflammation (Khanbabaee *et al*., 2001).

Thus, in time the nomenclature of tannins underwent multiple interpretations, misunderstandings, and changes, due to the progress in the field. Not all tanning substances can be called tannins, and many tannins do not possess tanning properties but are counted as tannins because of their structural characteristics.

So, what is the definition of tannins?

In 1796 Seguin, adhering only to the use in tanneries, coined the word "tannin" to define "the substances present in vegetable extracts, which are responsible for converting animal skin into leather" (Hatchett, 1805). However, this description is oversimplified because not all substances tested for their ability to leather are of vegetal origin, and, in the counterpart, many tannins do not have the ability to tan, even though they have the same chemical structure as those that do.

Later, in 1962, Bate-Smith and Swain gave the most common definition, still in use nowadays, describing plant tannins as "water soluble phenolic compounds with a molar mass between 300 and 3000", adding also some of their properties: to perform the phenol reactions (e.g. blue colour with iron(iii) chloride) and to precipitate alkaloids, gelatines and other proteins (Swain and Bate-Smith, 1962). However, this definition still excludes molecules with higher molar mass, and recently, molecules with a molar mass of up to 20000 D have been isolated that should also be classified as tannins based on their molecular structures.

Similar limitations were found in the definition by Griffith, who described tannins as "macromolecular phenolic substances" and divided them in two major groups, the 'hydrolysable' and 'condensed' tannins, but ignoring low molecular and monomeric tannins with a molar mass below 1000 D. Afterwards, other authors tried to define and classify tannins based on their structure but with restrictions, leaving out some tannin groups.

To formulate a definition that would take into account all the tannins, their structural characteristics and their properties is *a priori* not an easy mission. Finally, in 2001, Khanbabaee *et al.* formulated an updated definition based on the molecular structures of the currently known tannins, and their origin and role in plant life (Khanbabaee *et al*., 2001).

> "*Tannins are polyphenolic secondary metabolites of higher plants, and are either galloyl esters and their derivatives, in which galloyl moieties or their derivatives are attached to a variety of polyol-, catechin- and triterpenoid cores (gallotannins, ellagitannins and complex tannins), or they are oligomeric and polymeric proanthocyanidins that can possess different interflavanyl coupling and substitution patterns (condensed tannins).*"

1.1.2 Classification

Since it is not so easy to give a comprehensive definition of tannins due to the enormous structural diversity, it is not so obvious to reach a universal consensus on classification. These compounds can be divided by their structural characteristics or chemical properties.

One basic classification of tannins is related to constancy of the chemical structure. Knowing stability in the composition and chemical structure of any target compound is crucial for characterizing its biological and pharmacological properties. Thus, Okuda and Ito (2011) report the grouping of tannins and other polyphenols related to tannins as Type A polyphenols, which include those with constant chemical structure, and Type B, characterized by a variable composition.

Serrano *et al*. (2009) proposed to classify between extractable and non-extractable tannins. The former group includes tannins that can be extracted with aqueous-organic solvents, so they could be analysed by spectrophotometric (i.e. Ferrum chloride, Folin-Ciocalteu, Vanillin-HCl) and chromatographic (i.e. HPLC and MALDI-TOF-MS) techniques. Non-extractable tannins need acid and basic hydrolysis, which allow to quantify monomers.

The most common way to classify tannins is the analytical subdivision based on their structural characteristics. A simplistic grouping adopted by several authors divides tannins into two large categories: hydrolysable tannins and condensed tannins. Treatment of many tannins with hot water or tannases enables hydrolysation into monomeric products, and leads to classifying them as ‘hydrolysable tannins’. The remaining non-hydrolysable oligomeric and polymeric proanthocyanidins have been classified as ‘condensed tannins’. However, this classification doesn’t do justice to the complex structural diversity of the tannins.

The term ‘hydrolysable tannins’ comprises two distinct groups: the gallotannins and the ellagitannins. There are some ellagitannins that for historical reasons were classified as hydrolysable tannins, but others were not, such as vescalagin. Partially hydrolysable tannins, which contained the characteristic structural element of the monomeric ellagitannins (hexahydroxydiphenoyl (HHDP) units) together with C-glycosidic catechin units, were originally classified as ‘non-classified tannins’, in 1985 (Nonaka *et al*., 1985).

In following years, they have been further categorised between ‘complex tannins’ and flavanoellagitannins (Kashiwada *et al*., 1992; Ferreira *et al*., 1995). In particular, the last group, which sometimes has been called ‘condensed flavanoid tanning substances’, includes tannins consisting of catechin units. These polymeric flavanotannins, constituted by coupled flavan-3-ol (catechin) units, are known as proanthocyanidins (oligomeric and polymeric) and belong to the condensed tannins.

Evaluating all the structural characteristics, in 2001 Khanbabaee and van Ree proposed a more detailed classification, dividing the tannins into four major groups: gallotannins, ellagitannins, complex tannins, and condensed tannins (**Figure 1.1.1**).

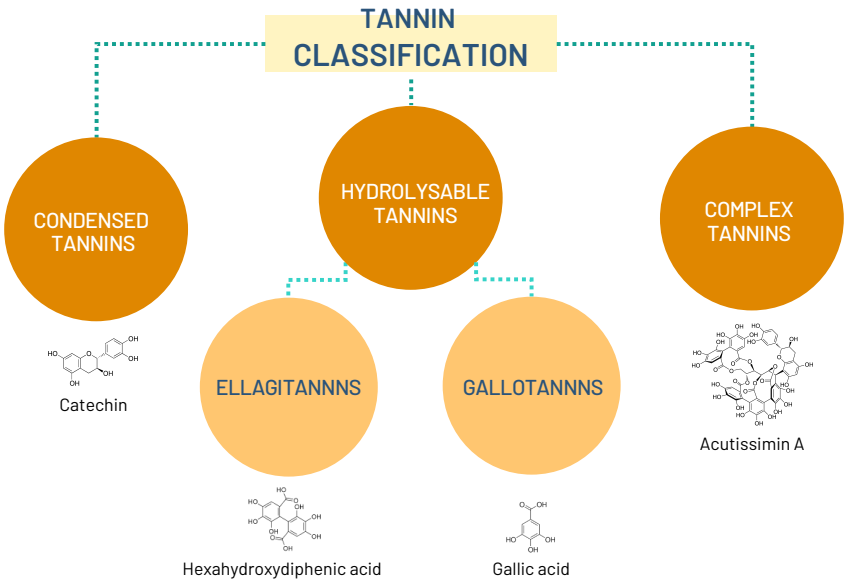


Figure 1.1.1 Tannin classification.

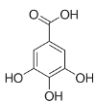
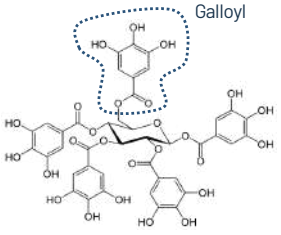
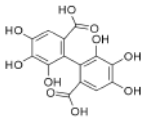
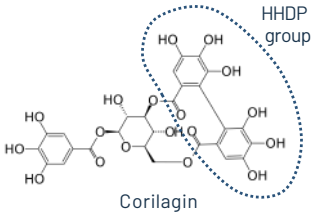
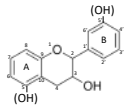
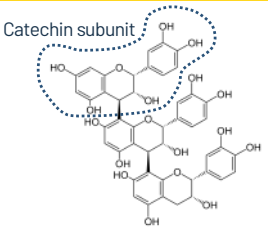
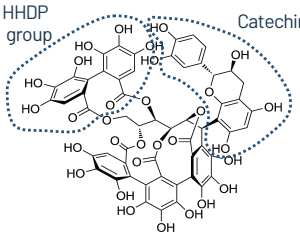
Gallotannins

Gallotannins are scarcely found in nature and are the most basic hydrolysable tannins, containing a polyphenolic and a polyol residue. Gallotannins are all those tannins in which galloyl units or their meta-depsidic derivatives are bound to diverse polyol-, catechin-, or triterpenoid units (**Table 1.1.1**).

Even though there is a wide variety of polyol residues, most of the gallotannins isolated from plants contain a polyol residue derived from a saccharide, in particular d-glucose. The hydroxyl functions (-OH) of the polyol residues may be partly or fully replaced with galloyl units. If this substitution is partial, the remaining –OH can be substituted or not by other residues. The meta-depsides (or 'syn-gallotannins') also belong to the gallotannins group. Their galloyl residues are esterified with the polyol residue and with one or more linked galloyl units in the meta position relative to the galloyl unit carboxyl groups.

The described compounds are found in many plant families and are key intermediates in the biosynthesis of nearly all hydrolysable plant polyphenols. On the other hand, gallotannins in which the polyol residues are coupled to cinnamoyl or coumaroyl groups are relatively scarce (Arbenz and Avérous, 2015).

Table 1.1.1 Structures of tannins.

TYPE OF TANNIN		EXAMPLE OF STRUCTURE
Hydrolysable tannins	**Gallotannins**	 Gallic acid  Galloyl Pentagalloyl glucose
	Ellagitannins	 HDDP  HHDP group Corilagin
Condensed tannins		 Monoflavonoid  Catechin subunit Procyanidin C1
Complex tannins		 HHDP group Catechin subunit Acutissimin A

Ellagitannins

Ellagitannins, widespread in many plant families, are the largest group of known tannins including more than 1000 natural compounds. Ellagitannins are constituted from gallotannins by at least two galloyl units which are C–C linked to each other, leading to the axially chiral monomer HHDP unit, and they do not contain a glycosidically linked catechin unit (**Table 1.1.1**). The chirality is due to the presence of large substituents located in the ortho positions of the biaryl axis, and by the restricted rotation around this axis, causing isomerism. Recurrently, it can be observed that the source of this chirality is caused by the esterification in the ortho of the two carboxyl groups with a polyol (generally D- glucopyranose) (Arbenz and Avérous, 2015).

Condensed tannins

Condensed tannins are the most common group of naturally occurring tannins and represent almost 90% of the worldwide production. These tannins are generally found in nature complexed with proteins, depending on their chemical structure and consequently on their affinity.

To be called condensed tannins, these compounds must be formed by the repetition of 3 to 8 units (or building blocks) and the precursors must be a flavan-3-ol (catechin) or a flavan-3,4-diol (leucoanthocyanidin) (**Table 1.1.1**).

Each flavonoid is composed of two phenolic rings (A and B) having different reactivities and two possible configurations comprising or not a hydroxyl group in positions 5 and 5′. The different configurations set up four different possibilities of basic building blocks to form condensed tannins (**Figure 1.1.2**).

Monomeric catechins and leucoanthocyanidins do not possess tanning properties, but they have then the interesting capacity of being converted into oligomers and polymers that do have tanning capacities, by the action of acids or enzymes. The type of connection between the various flavanoid units depends on the nature of the rings. All oligomeric and polymeric proanthocyanidins are formed by the linkage of the C-4 of one monomer with the C-8 or C-6 of the next building block. C4–C6 is prevalent in tannins mainly composed of profisetidins and prorobinetidins, while C4–C8 is prevalent in tannins mainly composed of procyanidins and prodelphinidins.

The degree of polymerisation (DP) can vary between two and more than fifty units. Oligomers and polymers consisting of two to ten catechin units are also known as flavolans. Beside the size, also the coupling pattern of the catechin units in condensed tannins can vary considerably (Arbenz and Avérous, 2015; Sieniawska and Baj, 2017)

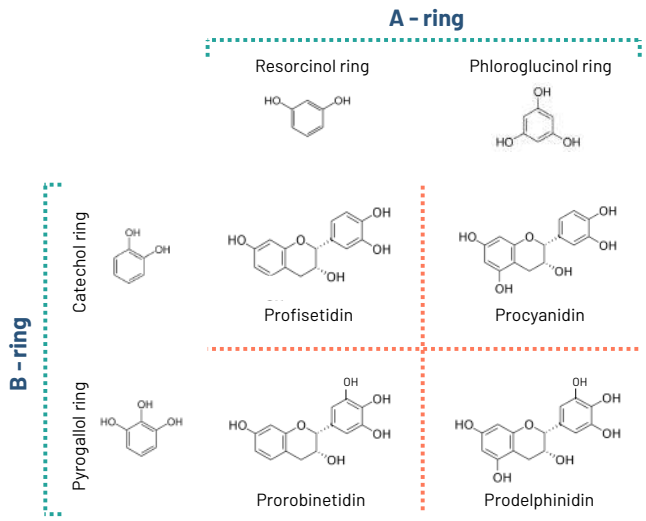


Figure 1.1.2 Structures of the four monoflavonoids.

Complex tannins

Complex tannins are tannins containing both condensed and hydrolysable tannins, in which a catechin unit is bound glycosidically to a gallotannin or an ellagitannin unit (**Table 1.1.1**). The ones most commonly found are flavano-ellagitannins, procyanidin-ellagitannin, and flavono-ellagitannins. An example from this substance class is acutissimin A, containing a flavogallonyl unit (nonahydroxytriphenoyl unit) bound glucosidically to C-1, and linked via three further hydrolysable ester bridges to the d-glucose derived polyol (Khanbabaee *et al*., 2001).

1.1.3 Botany

Tannins are highly hydroxylated secondary compounds, synthesized through plant secondary metabolism. These compounds are present also as smaller molecules, in plant organs with intense physiological activity. Tannins are found either dissolved in vacuolar sap of some parenchymatic cells such as cortical parenchyma, secondary xylem, and pith, or in specialized cells like idioblasts.

Vegetable tannins can be accumulated practically in any part of a plant – seeds, fruit, leaves, wood, bark, root. The function of tannins has been related to plant-environmental interactions. Tannins have been commonly identified as protection agents against external insults such as infections and as preventing plant-herbivore interactions thanks to their acid taste and the property of precipitating proteins. Thus,

a particular pathological condition increases tannin production by the plant. A clarifying example are plant galls produced as a result of insect attack.

The tannin content may vary depending on the plant species, the specific part of the plant and external factors (i.e. time of the year). However, the level of vegetable tannins normally found in most plant tissues, such as fruit and leaves, is in the range 2–5% of the fresh weight (Furlan *et al*., 2011). Other authors reported that tannin content can range from 0.2 to 25% of dry weight. The variation depends on many factors such as plant species, harvest time, habitat of plants, and extraction method (Cuong *et al*., 2020).

1.1.4 Plants containing tannins

In many cases, tannins can be used as chemotaxonomic markers, especially for Angiosperm orders and families. Okuda *et al.* (2000) re-designed the Cronquist system of plant classification, in which orders, families and genera are correlated with the oxidative structural transformation of plant polyphenols (**Figure 1.1.3**).

Biogenetic transformation of hydrolysable tannins is considered to occur along the following route (*vide supra*), starting from gallotannins: galloyl group in gallotannin (I) → HHDP group in ellagitannin (II) → dehydrohexahydroxydiphenoyl (DHHDP) group in dehydroellagitannin (III) → transformed DHHDP groups in transformed dehydroellagitannin (IV). The authors started from the thesis of Gottlieb *et al.* (1898) that stated: ``in plant groups of high hierarchic rank, morphological evolution is accompanied by progressive oxidation of secondary metabolites belonging to a particular bio- synthetic class". Thus, the series of oxidative transformations of hydrolysable tannins may be useful for correlating progressive oxidation with morphological evolution.

Hydrolysable tannins are especially useful for the correlation with plant evolution, more than other types of plant metabolites, because they are strong antioxidants whose potency depends on their oxidation stage. In this classification, tannins are missing in just one subclass, the Asteriidae.

Since in recent years new techniques of DNA sequencing have been applied, most of the subclasses, orders and families proposed by Cronquist have been revisited, and angiosperm systematics have been reorganized in a new proposal based on phylogenetic paradigms - the Angiosperm Phylogeny Group (Chase *et al*., 2016). This new system presents a synthesis of angiosperm phylogeny hypothesis, based mainly on the combination of DNA sequences. However, also this new classification distinguishes the Asterids clade by families with the presence of typical alkaloids and iridoids. As this clade is mainly formed by herbaceous plants, it can be deduced that tannins are not directly associated to this form of life.

Viewing the evolution of plant defence in a phylogenetic perspective, the biosynthetic machinery needed to produce plant defence must be well-conserved and of single origin. Indeed, it is supported by the correlations between qualitative plant defences (alkaloids, iridoids, glucosinolates) and the absence of tannins (a quantitative defence). All these bases explain why tannins can be found ubiquitously in woody plants, but they are relatively untraceable in herbaceous species (Furlan et al., 2011).

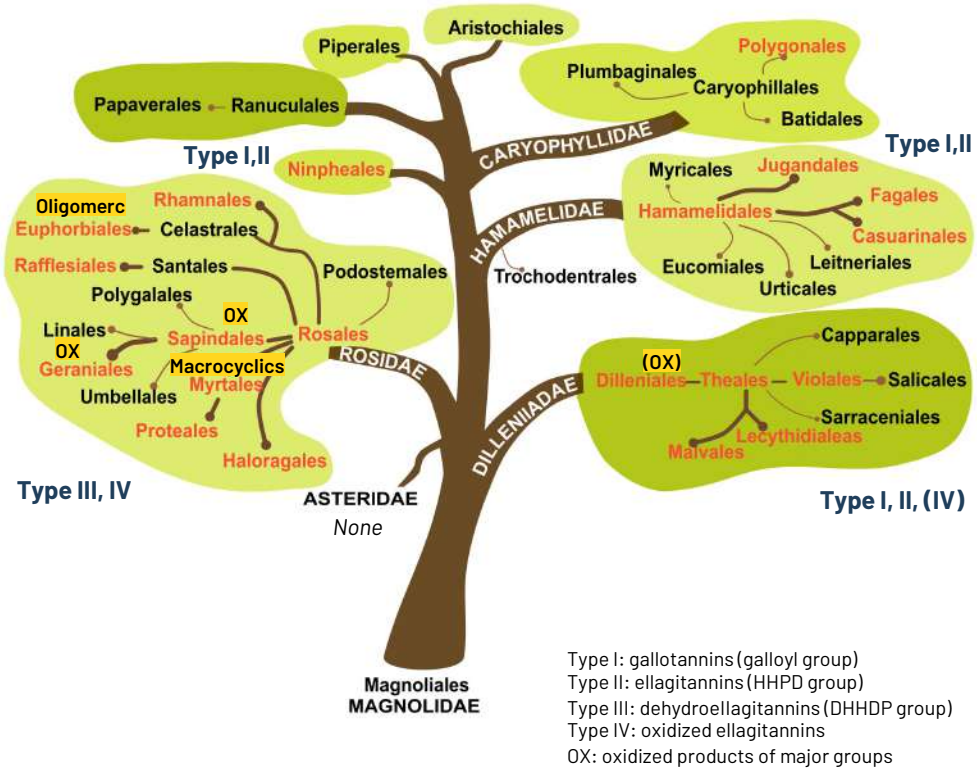


Figure 1.1.3 Polyphenolics evolution - Ellagitannins; Cronquist's evolutionary representation applied to this schema.

There are two aspects that should be pointed out:

- Plants which have a strong capacity to synthesise proanthocyanidins generally do not metabolise substantial quantities of esters of gallic and hexahydroxydiphenic acids, and vice-versa. For the few plants which can biosynthesise galloyl esters of flavan-3-ol substrates, the presence of co-occurring proanthocyanidins is very little or inexistent.

- In the Northern hemisphere, leaves of plants, which synthesise substantial quantities of condensed or hydrolysable tannins during normal growth generally, produce strong anthocyanin pigmentation during autumnal senescence (Haslam, 2007).

Finally, the category of complex tannin monomers is widely present in Fagaceae, Combretaceae and Myrtaceae, reflecting the broad distribution of the C-glucosidic tannin monomers and flavans in these families. Some species of Theaceae and Melastomataceae also produce this type of tannin, such as camelliatannins A and B. Some of these complex tannin monomers are also found as units in oligomers. In the following, the four tannin sources used to realise the studies for the present thesis are illustrated at more depth.

Quebracho (Schinopsis spp.)

The most popular quebracho trees are represented by *Schinopsis lorentzii* and *Schinopsis balansae* species, whose most distinctive character is the red hardwood. There is also another tree species, the white quebracho (*Aspidosperma quebracho-blanco*) belonging to the family *Apocynaceae*. However, when we use the word 'quebracho' generically, we are referring to plants belonging to the *Anacardiaceae* family (*Schinopsis* spp.) (Venter *et al*., 2012b). Quebracho trees are the dominant plants in the western and eastern sectors of the Chaco forest, located in northern Argentina and eastern Paraguay. These species are so ubiquitous across the Chaco region that the first settlers called this formation "Región del Quebracho" (Ferrero and Villalba, 2009). Quebracho trees are used to produce many different products for construction, thanks to the hardness of the wood. These plants are also exploited for their high wood content of tannins, which is around 15–21% in *S. lorentzii* and 20–21% in *S. balansae* (Pizzi, 2019; Caprarulo *et al*., 2021).

The tannins which characterise quebracho wood extracts are condensed, predominantly profisetinidins. Quebracho tannins are mainly based on combinations of resorcinol, catechol and pyrogallol building blocks, thus the following monoflavonoids and their oligomers are also expected to be present in the extract. In commercial extracts, big structures, up to heptamers, octamers, nonamers and

decamers, have been found. Tannins are known to be not readily soluble in water at this high molecular weight. Quebracho tannin polymers are characterized by an almost completely 'linear' structure, which determine a susceptibility to hydrolysis of the interflavonoid bond between one building block and another (Pash, H.; Pizzi, A.; Rode, 2001).

Chestnut (Castanea sativa Mill.)

Chestnut, *Castanea sativa* (Mill.), Fagaceae, is the predominant sweet chestnut tree in central Europe (Živković *et al*., 2009). The most important application of sweet chestnut is the production of timber, but it also has an important economic value in food and feed markets. The high presence of phytocomplexes, as tannins, gives the chestnut an additional value, with a wide range of possible applications. The tannin concentration in the spray-dried extract is about 75%, of which around 89% is characterised by the presence of hydrolysable tannins (both gallo- and ellagitannins), in particular castalagin (53%), vescalagin (35%), castalin (3%) and vescalin (8%). Investigations have reported an important percentage of other molecules such as acutissimin A, kurigalin or chestanin. Evidences of dimerization and hydrolysis of ellagitannin has also been reported, resulting in the growth of structural blocks (Caprarulo *et al*., 2021).

Tara (Caesalpinia spinosa)

Tara, *Caesalpinia spinosa*, is a tropical tree found in several Andean countries, but mainly in Peru, used traditionally by the rural populations for a wide range of purposes. Tannins are obtained from the pods, which are manually collected twice a year and are also a great source of gum. The tannin extract is characterized by the presence of an oligomeric composition and the presence of different chemical moieties and building blocks. Tara tannin is based on an ellagic unit, which is decorated with different numbers of gallic acid units. The ellagic acid contains an ester group which stems from hydrolysis reactions that take place during the extraction process (Radebe *et al*., 2013).

Gall of Rhus chinensis Mill. and Quercus infectoria Oliv.

Chinese galls (form *Rhus chinensis* Mill.) and Turkish galls (from *Quercus infectoria* Oliv.) are used mainly in the Far East, China and India. Chinese gallnuts are formed by different species of gallnut aphids, particularly the Chinese sumac aphid, *Schlechtendalia chinensis* Bell. The aphid parasitizes the leaves or petioles of the plant and lead to the production of reddish-brown gallnuts, normally harvested in the autumn after removal of the larvae. The Turkey gall tannin is an extract of Turkey galls that are produced by larvae of Gallic bee insects (*Cynips gallae-*

tinctoriae) which parasitize in young branches of the Fagaceae Gallic tree (*Quercus infectoria*) growing in the oriental Mediterranean coasts.

Due to their chemical composition, both galls have found similar applications for industrial productions and in particular they have been exploited for their great pharmacological and medicinal values. The extract is characterized by the presence of 50∼70% of gallotannin (arrangements of gallic acid units around a glucose core) and 2∼4% of gallic acid and ellagic acid. Even though the galls present from a qualitative point of view a similar composition, they have a different content in singular compounds. Thus, ellagic acid, galloyl-HHDP-glucose and pedunculagin can be used as markers to distinguish Chinese gall and Turkish gall extract in HPLC-ESI-MS/MS (Ma *et al*., 2020b).

1.2 Chemistry

1.2.1 Biosynthesis

The fundamental metabolic route underlying the synthesis of the precursors of tannins, such as gallic acid and p-coumaric acid, is the shikimate pathway. This pathway is found mostly in fungi, bacteria, and plant species, and it generate fundamental building blocks (i.e. phenylalanine) of a wide number of compounds, such as flavonoids, alkaloids, lignin, and antibiotics. The extreme importance of this pathway for the organisms producing these metabolites is denoted by a high flux of the photosynthetic fixed carbon (20–50%) and a restricted chemical diversity.

The shikimate pathway is located in plastids and starts with phosphoenolpyruvate (a metabolite from the glycolytic pathway) and erythrose-4-phosphate (a metabolite from the pentose-phosphate pathway) (**Figure 1.2.1**). It is regulated at many points, e.g. transcriptional regulation for the DAHPS (3-Deoxy-D-arabinoheptulosonate 7-phosphate synthase) and inhibition by phenylalanine of arogenate dehydratase activity. Phenylalanine is converted to p-coumaryl-CoA, a precursor of flavonoids, by the action of three different enzymes: PAL, C4H, and C4L.

In plants many isogenes of PAL, are present and their expression is often regulated, as they are used differentially for generation of lignin and condensed tannins. Gallic acid is produced from 3-dehydroshikimate, as has been demonstrated by different biochemical and molecular techniques (i.e. isotope labelling) (Vogt, 2010).

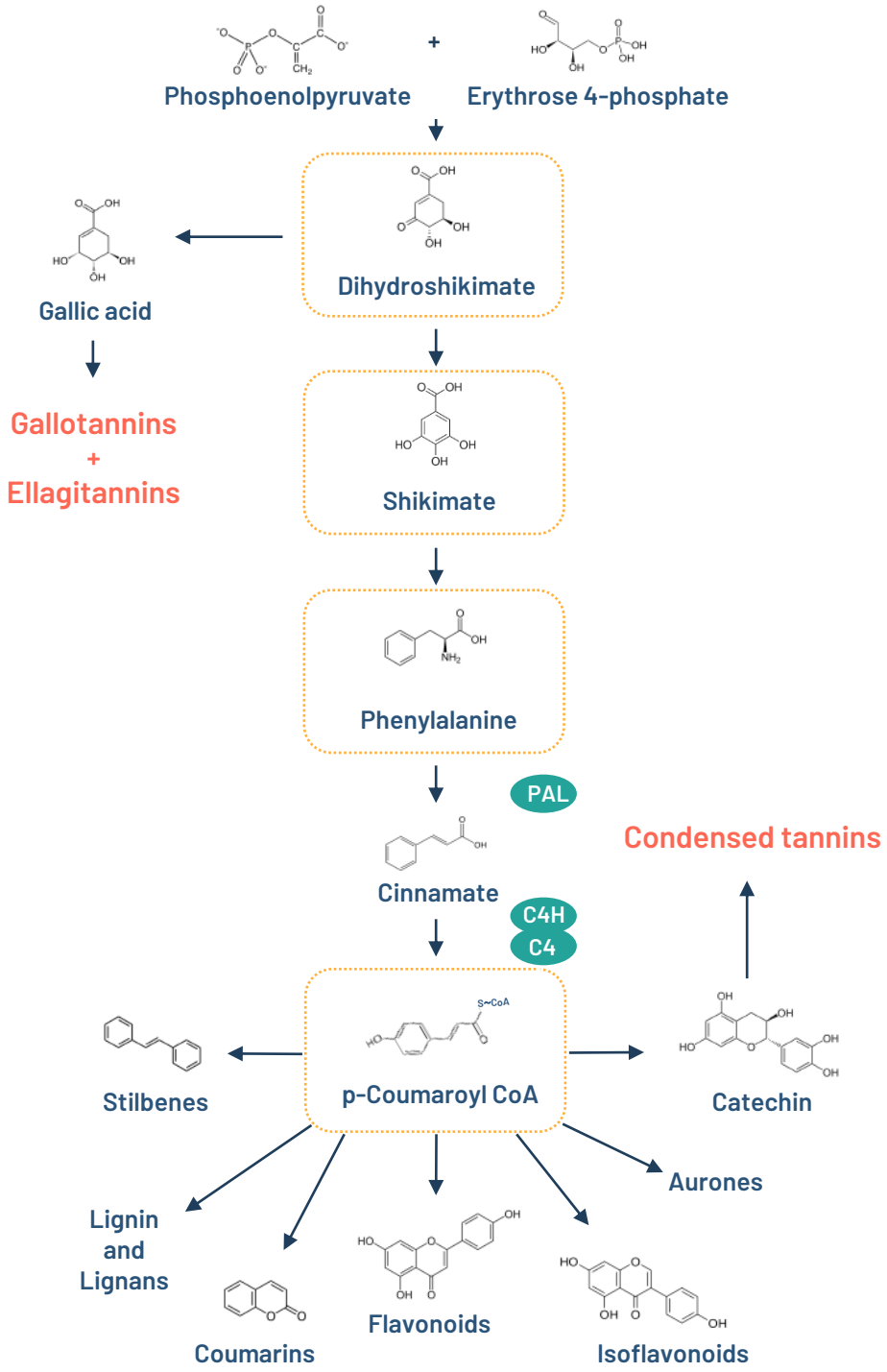


Figure 1.2.1 Diversification of phenylpropanoids based on the general phenylpropanoid pathway. The metabolites of the shikimate pathway and the central metabolite p-Coumaroyl CoA, are circled in yellow.

Biosynthesis of hydrolysable tannins

The esterification of gallic acid with glucose starts the synthesis of hydrolysable tannins and the first intermediate is β-glucogallin (1-O-galloyl-β-D-glucose) (**Figure 1.2.2**). This reaction has been demonstrated with different techniques of molecular biology and by *in vitro* assays, using *Escherichia coli* (Mittasch *et al*., 2014). Subsequently, β-glucogallin, without any additional cofactor, acts as donor and acceptor of galloyl groups to produce further substituted glucose. The substitution of glucose hydroxyls by galloyl groups follows an ordered process: β-glucogallin → 1,6-digalloylglucose → 1,2,6-trigalloylglucose → 1,2,3,6-tetragalloylglucose → 1,2,3,4,6-pentagalloylglucose (PGG). PGG is the basic compound needed to synthetise gallotannins and ellagitannins (Montes-Ávila *et al*., 2017).

Biosynthesis of gallotannins derives from the galloylation of PGG, and forms structures with one or more meta-depsidic digalloyl moieties, two galloyl groups joined by an ester bond. The depsidic bond formation is catalysed by galloyltransferases dependent on β-glucogallin, and a certain degree of specificity has been registered. Ellagitannins are formed by intra- and intermolecular oxidation processes of PGG, mediated by a laccase-type polyphenol oxidase. The oxidation of PGG to tellimagrandiin II and then to its dimer cornusiin E, lead to the formation of the HHDP biaryl unit, which defines the hydrolysable tannins as ellagitannins. HHDP released from the hydrolysis of ellagitannins has a strong tendency to form ellagic acid through spontaneous lactonization (Montes-Ávila *et al*., 2017).

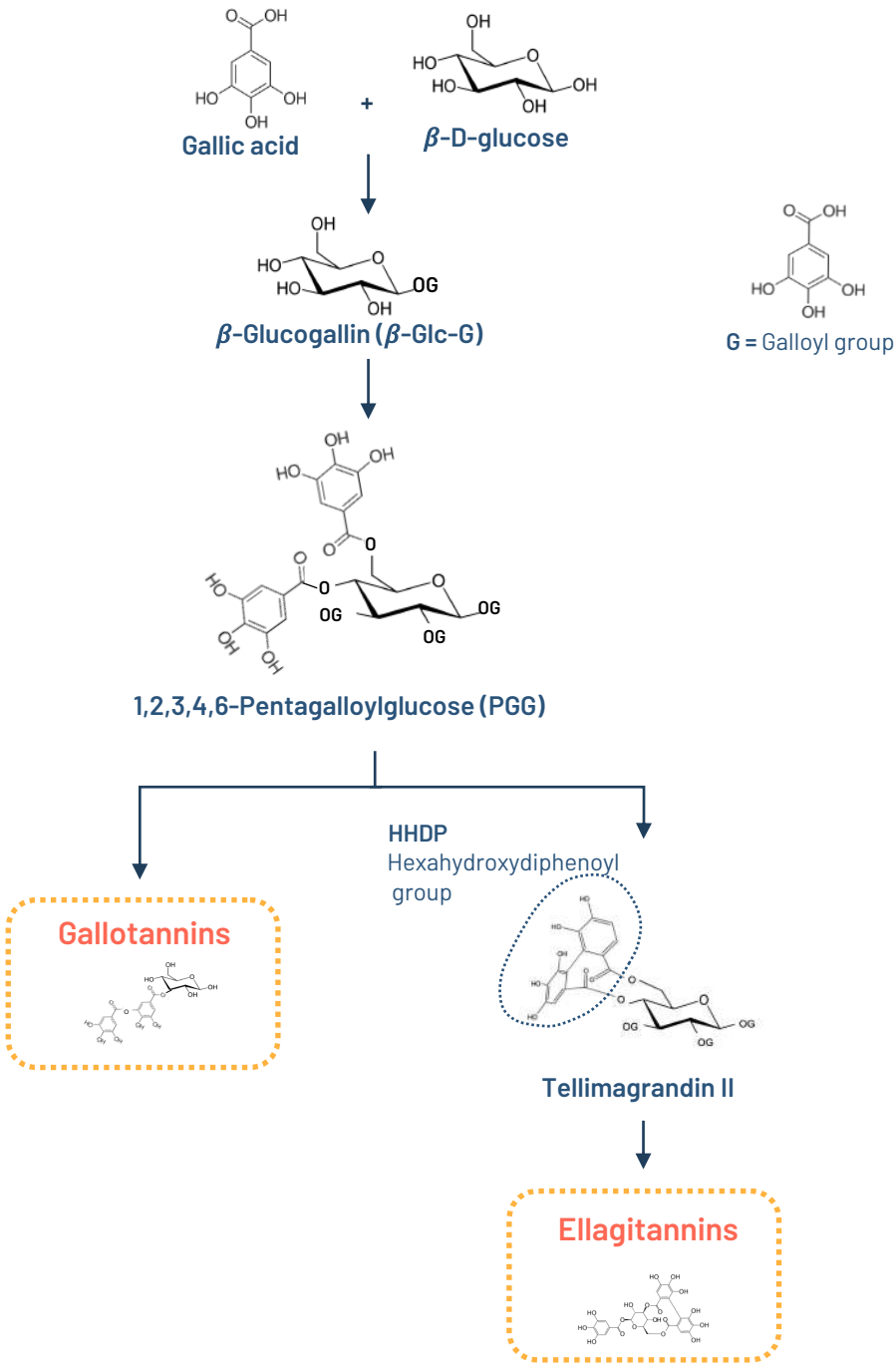


Figure 1.2.2 Biosynthesis of hydrolysable tannins. The first two reactions produce 1,2,4,6-pentagalloylglucose (PGG), a common intermediate of hydrolysable tannins. These are followed by the formation of metadepsidic bonds in gallotannins. Alternatively, the PGG could be oxidised by a laccase polyphenol oxidase forming a HHDP group and give origin to ellagitannins.

Biosynthesis of condensed tannins

In the early stages of formation of proanthocyanidins, they share the same biosynthesis pathways with other flavonoids, which are generated by chalcone synthase (CHS), starting from p-coumaryl-CoA. Then, p-coumaryl- CoA condensed with three molecules of malonyl-CoA forms a chalcone. The enzyme chalcone isomerase (CHI) converts the chalcone to (2S)-naringenin which is a precursor of many types of flavonoids (i.e. flavonoids, isoflavonoids, flavanones). In the pathway forming proanthocyanidins, flavanone-3-hydroxylase (F3H) acts on naringenin to produce dihydrokaempferol; this in turn can be further hydroxylated in the B-ring by flavonoid-3′-hydroxylase or by flavonoid-3′-5′-hydroxylase to the corresponding dihydroflavonols dihydroquercetin and dihydromyricetin, respectively.

The dihydroflavonols are transformed to leucoanthocyanidins by the enzyme dihydroflavonol-4-reductase (DFR), and subsequently, the enzyme anthocyanidin synthase (ANS) transforms leucoanthocyanidins into anthocyanidins (cyanidin, pelargonidin and delphinidin) (Montes-Ávila *et al*., 2017). Specific enzymes for proanthocyanidin biosynthesis are the anthocyanidin reductase (ANR), which transforms the anthocyanidins to epi-flavan-3-ols (epicatechin, epiafzelechin and epigallocatechin); and the leucoanthocyanidin reductase (LAR), which transforms leucoanthocyanidins to flavan-3-ols (catechin, afzelechin and gallocatechin). Epi-flavan-3-ols and flavan-3-ols are direct monomeric precursors of proanthocyanidins (**Figure 1.2.3**).

So far it has not been possible to clarify the route of polymerization of flavan-3-ols. Generally, it is well accepted that the production of monomers takes place in the cytosol and then they are gathered together with glycosylated flavan-3-ols, through specific transporters, to vacuoles where they are polymerized. Other specific transporters are needed to incorporate such monomers into the vacuoles. Another very common transformation of monomers but still not fully understood is the galloylation. Biosynthesis of proanthocyanidins is also regulated by numerous factors such as light, temperature, tissue, and development stage of the plant, among others (Montes-Ávila *et al*., 2017).

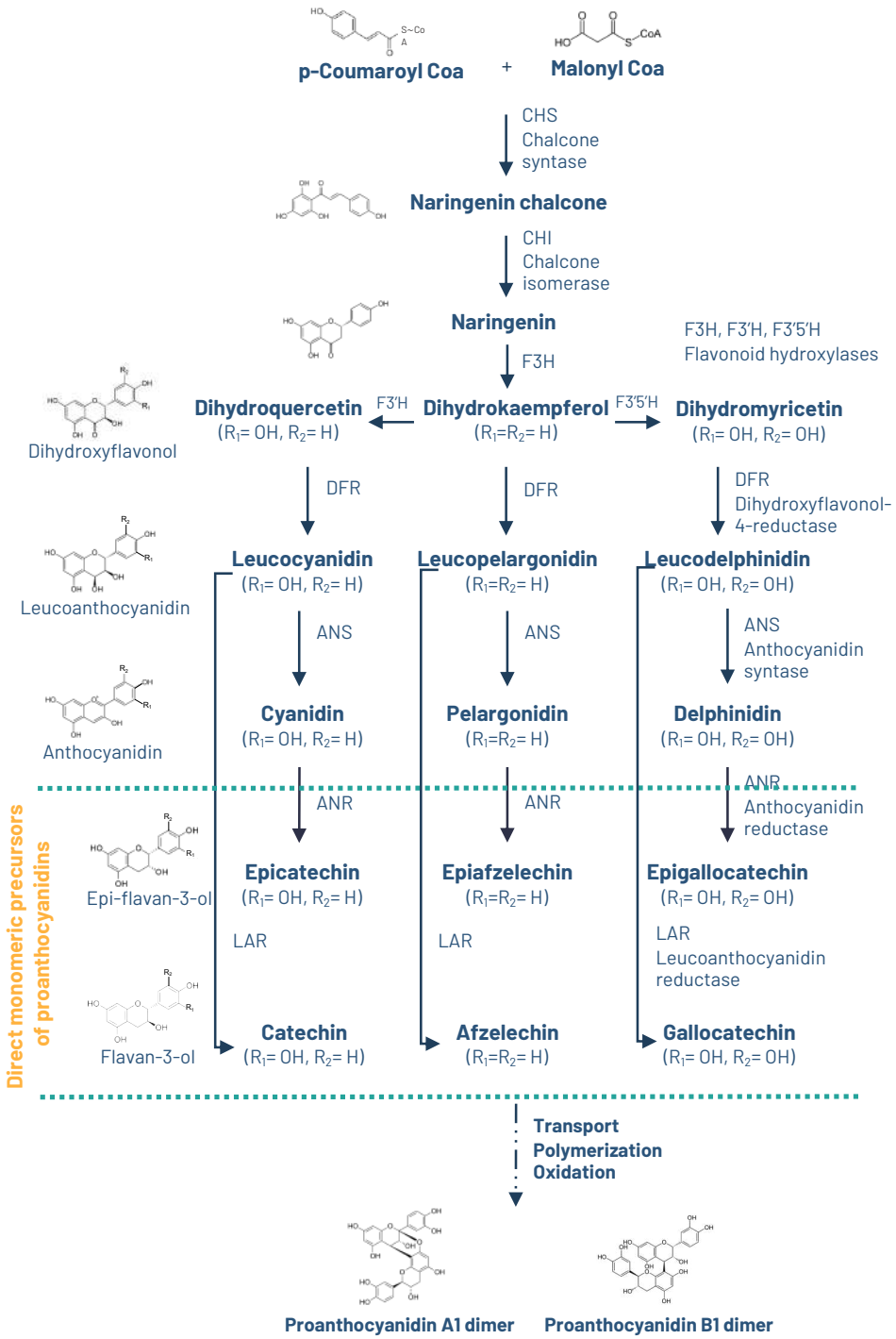


Figure 1.2.3 Condensed tannin biosynthesis.

1.2.2 Interaction with molecules

The ability of tannins to interact with different types of molecules is such an important and characteristic aspect of these polyphenols that it is a key element for their definition. The long tradition of using tannins in leather tanning made their application popular before the molecular mechanisms behind it was elucidated. Later, for many years, tannins have been distinguished from other phenols thanks to their ability to bind and precipitate proteins, alkaloids and metals. Today, through scientific research, this ability of tannins to interplay with different types of molecules is exploited and finds application in many fields, such as the food technology, leather or pharmaceutical industries. In the following section, the interaction of tannins with specific compounds will be discussed in depth.

Tannins–Proteins

Tannins have a peculiar affinity for binding to proteins, by establishing cross-links implying different kinds of bonds. The principal driving forces implicated are hydrophobic interactions and hydrogen bonds (de Freitas and Nuno, 2012). The association between tannins and proteins occurs in a specific and selective way and has been summarized in three specific steps (**Figure 1.2.4**):

- **Step 1**: The earliest interactions are characterized by hydrogen bonds and hydrophobic interactions, resulting in the generation of protein−tannin complexes. Hydrophobic interactions comprise entropy-driven van der Waals forces, while hydrogen bonds are enthalpy-driven electrostatic interactions. Tannins can bind to multiple sites on the protein, leading to a condensation of the protein−tannin complex and resulting in a spherical structure.

- **Step 2**: Cross-links between protein−tannin complexes determine a self-association, with the formation of bigger structures.

- **Step 3**: The association of the large aggregates produces colloidal size particles, which induces the precipitation of protein−tannin complexes (de Freitas and Nuno, 2012; Renard *et al*., 2017).

The tannin-protein interactions depend on many protein factors, such as their size, charge, side chains, and conformation. On the other hand, the tannin molecular weight and degree of galloylation can influence their affinity for proteins, probably because tannin size determines the number of interaction sites. Nevertheless, it has to be taken into account that a large tannin structure can cause steric hindrance and impede access to binding sites, limiting solubility (Baxter *et al*., 1997). Protein−tannin interaction can also be limited by the interference of other molecules,

such as polysaccharides (i.e., arabic gum, pectin, gellan, polygalacturonic acid, and xanthan), which could compete with proteins for binding tannins. This issue is not yet totally understood because it has been shown that the presence of polysaccharides, in particular in mannoproteins, can inhibit the evolution of tannin aggregate particle size but not their generation (Riou *et al*., 2002). The inhibition of protein– tannin interactions can work through two different mechanisms: i) formation of a ternary soluble complex among protein, tannin, and polysaccharide, ii) encapsulation of tannins by polysaccharides, competing with protein aggregation (**Figure 1.2.4**) (Molino *et al*., 2019).

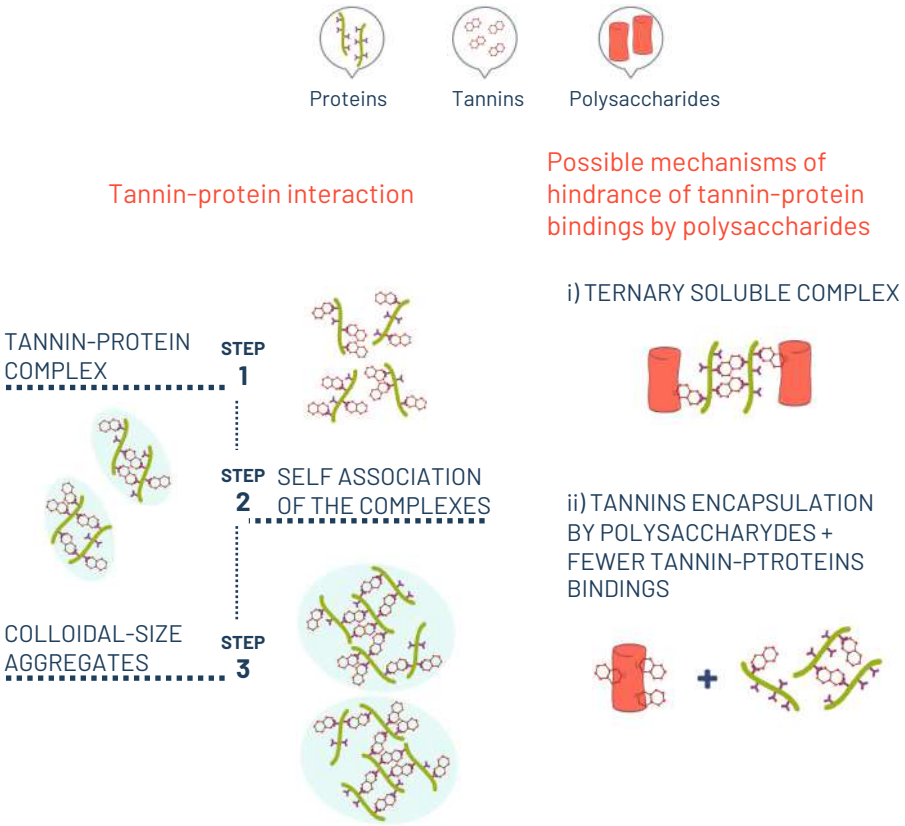


Figure 1.2.4 Scheme of tannin-protein and tannin-polysaccharide interactions.

The binding between proteins and tannins was first described and studied in relation to its fundamental role in leather tanning. However, in recent years there has been increasing interest in the application of tannins to food and their sensory aspects. In view of this, a large part of the studies on the interaction of these macromolecules has been focused on the mechanisms that take place with the first contact of tannins with salivary proteins, in the mouth. These interactions are principally driven by the concentration of the proteins, but other environmental factors, including temperature, pH, and ionic strength, can influence the formation of salivary protein aggregates or their precipitation. In saliva, several tannin-binding proteins can be found, with a predominance of proteins rich in proline (PRPs) and in minor proportion, glycine, and glutamic/glutamine residues.

PRPs are effective in complexing tannins, especially in the presence of a repeated proline region, which increase the binding affinity. Among histatins, another types of salivary proteins, it has been reported that histatins 3 and 5, other salivary proteins, increase precipitation of condensed tannins more than histatin 1 as a result of a different content of histidine and phosphoserine (Naurato *et al*., 1999).

Moreover, a study reported that quebracho tannins and tannic acid are more efficient precipitating salivary histatin 5 than PRP-1, at pH 7.4 (de Freitas and Nuno, 2012). The interaction with salivary proteins does not seem to interfere with the bioavailability of tannins, and some authors suggested that proteins may act as carriers of these bioactive compounds (Molino *et al*., 2019).

Tannins–Polysaccharides

Interactions between tannins, particularly condensed tannins, and polysaccharides have been studied and widely demonstrated in the context of clarification and astringency control of beverages. The models adopted to study the tannin−polysaccharide interactions are represented by grapes, apples, and pears (rich in proanthocyanidins), normally employed to produce wine and ciders (Li *et al*., 2018). Proanthocyanidin−polysaccharide associations are spontaneous and quick and direct binding events and occur during the processing of vegetables and fruits.

The complexation mechanisms are similar to those of the aggregation between tannins and proteins (*vide supra*). The associations with the different macromolecules can be differentiated by different kinetics and colloidal consequences. Hydrogen bonds and hydrophobic interactions are the key players. Ionic strength and a lower temperature can boost the interactions between tannins and polysaccharides, and high DP corresponds to higher affinities (de Freitas and Nuno, 2012).

Tannins–Organic Non- Protein N Compounds

Since the capacity of tannic acid to bond and precipitate choline, an amine and the precursor of the neurotransmitter acetylcholine, was demonstrated, researchers started to investigate the possibility of tannins forming complexes with other organic N-Compounds other than proteins (Kalina and Pease, 1977). Adamczyk *et al.* (2001) showed that tannins can react with an extensive set of organic N compounds, including arginine (from all amino acids), nitrogen bases, polyamines, chitin, and chitosan (Adamczyk *et al*., 2011). Such reactions with numerous N compounds call for a change in our way of thinking about tannins: they can react with non-protein organic N compounds similarly to their reaction with proteins (Adamczyk *et al*., 2011).

Like in tannin–protein interactions, reactions are influenced by the concentration, chemical structure, and pH of the solution; and a greater ability to form multiple hydrogen bonds corresponds to a higher ease of forming complexes with tannins. For proteinaceous amino acids, polyamines, and nitrogen bases, a higher reactivity towards tannins was found with higher molecular masses and more amine groups: of all amino acids, arginine has the highest number of amine groups and almost the highest molecular mass (174 Da); for poly- amines, spermine has the highest molecular mass (202 Da) and amount of amine groups (Croteau *et al*., 2000). For nitrogen bases, the two having no amine groups exerted the weakest reactivity towards tannins.

Tannins–Metal ions

The dihydroxyphenyl groups of tannins are excellent chelators of Fe(III). They also form complexes with Al(III) and Cu(II). The formation of the complexes determines a precipitation at neutral pH as long as the concentration of the ligand (tannins) is not too high relative to the metal ion concentration (Santos-Buelga and Scalbert, 2000). The ferric-ion chelation is produced by the bond between o-dihydroxyphenyl groups and several ferric ions. Each ferric ion can bind to at least three o-dihydroxyphenyl groups in different tannin molecules. Then, a lattice is generated, leading to the precipitation of iron along with tannins. The iron-chelating efficiency of tannins can be related so several structural factors. For instance, in the case of gallotannins, a higher number of the galloyl groups is associated to a more efficient chelation, while the increase in degrees of galloylation leads to a lower iron binding capacity, due to the steric hindrance (Engels *et al*., 2011).

Complexation of such metal ions by tannins has been largely studied for the possible antinutrient consequences (see Antinutritional properties section). The formation of stable tannin-Fe(III) complexes in the gut inhibits the absorption of non-haem dietary iron through the gut barrier, after the intake of polyphenol-rich beverages such as wine or tea (Cook *et al*., 1995; Hurrell *et al*., 1999). Such

inhibition occurs when the ligand is consumed together with Fe(III) (South *et al*., 1997). However, the reduction of the iron absorption depends on the presence of other nutrients; for example, the ingestion of a complex composite meal reduces the tannin inhibitory effect, in comparison to a simple bread roll meal (Cook *et al*., 1995). Similarly, the absorption of the radiolabelled iron complexed in haemoglobin haem was affected by tea polyphenols when the protein was cooked as in gravy (Disler *et al*., 1975). Other nutrients such as ascorbic acid can remove the inhibition of iron absorption by polyphenols, including tannins, probably by reducing the complexed Fe(III) into the poorly co-ordinated iron(II) (Siegenberg *et al*., 1991).

The large majority of polyphenols regularly consumed with our diet, and not only tannins, have o-dihydroxyphenyl groups in their structures. Even though both low- and high- molecular-weight polyphenols are likely to inhibit non-haem iron bioavailability, there is limited information about the effect of their chemical structure on iron absorption and more *in vivo* experiments are needed. Little is known about the influence of proanthocyanidins and other polyphenols on the gut absorption of metal ions other than Fe(III), but this influence may also be relevant.

There is an apparent link between metal dyshomeostasis and neurodegenerative diseases, in particular, the presence of Al(III) in drinking water has been often associated with an increase in Alzheimer disease risk (Santos-Buelga and Scalbert, 2000; Molino *et al*., 2016). This apparent link sparked the idea that metal chelation could be used therapeutically. Several publications reported that the intake of proanthocyanidins present in wine or tea were associated with a reduction of the risk of senile dementia (Cook *et al*., 1995; Hurrell *et al*., 1999). Furthermore, it has been demonstrated in animal models of neurodegeneration that various iron chelators/antioxidants possess neuroprotective effects (Mandel *et al*., 2011).

On the counterpart, tea consumption caused an increase in copper absorption, in the plasma level of ceruloplasmin and in the copper retention in tissues, particularly in the liver. The influence of tea on Cu(II) absorption has been studied in rats (Greger and Lyle, 1988). Finally, zinc has, in general, a low affinity for polyphenols at acidic and neutral pH. This is consistent with the lack of a significant effect of tea, wine and beer on its bioavailability in humans (Santos-Buelga and Scalbert, 2000).

Considering the available data, based on the relative affinities for different metals, tannins could inhibit Fe(III) and Al(III) and possibly increase Cu(II) metal ion bioavailability. Indeed, once absorbed, tannins are largely metabolised and one of the main consequences is the loss of the o-dihydroxyphenyl functionality to form simple phenols (Santos-Buelga and Scalbert, 2000).

1.2.3 Extraction techniques

There are no universal standard extraction conditions for plant tannins and, in general, different procedures are optimized in accordance with the sample. The choice of the right extraction process is crucial to obtain a reliable sample and to avoid the formation of artefacts. Many factors can alter tannin extracts; for example, the presence of a great number of hydroxyl groups and the consequent ability of tannins to form reversible complexes with other natural macromolecules such as polysaccharides and proteins may occur during normal physiological plant development or during the extraction process. Other artifacts that may occur during tannin extraction could be due to the light-sensitivity of proanthocyanidins or the sensitivity to hydrolysis of hydrolysable tannins. In view of this, specific precautions have to be adopted, taking into account fundamental factors such as solvent polarities, the time and temperature of extraction, and the sample/solvent ratio. The composition and the yield of the extracts are strictly dependent on them (Serrano *et al*., 2009).

Traditional extraction techniques comprise maceration and decoction, used since ancient times for tannin extraction from medicinal plants. Maceration is the simplest technique of extraction and consists of a first step in which plant powder is placed in a closed vessel and soaked with the corresponding amount of solvent for a few to hundreds of seconds. While the first stage is characterized by osmose followed by diffusion, in later stages, osmose occurs simultaneously with diffusion. This technique has always been considered a popular choice thanks to its ease of application and low price, as uncomplicated utensils and equipment are needed. Nevertheless, the time of the extraction is long, high quantities of solvent are required and the yield is not very high.

Decoction is similar to maceration, but a temperature of 100°C degrees is required throughout the process. The decoction method is more effective than the maceration for extracting condensed tannins. For these, aqueous solutions are good solvents for extraction both by decoction and by maceration. With decoction the thermal energy of the solvent helps in pulling out tannins from the material together with the affinity of the solvent. Similarly to maceration, this method is easy and cheap to apply and it is suitable for all production scales (Cuong *et al*., 2020).

Nowadays, especially for industrial production, tannin extraction from plant samples is mainly carried out by solid–liquid extraction using different aqueous and organic solvents, such as acetone, ethanol, methanol, or mixtures of these solvents with water. Then, it is also usual to carry out a further fractionation of the raw extracts by liquid–liquid extraction using non-miscible solvents, in order to simplify the sample processing. In general, low molecular weight compounds (such as simple gallic acid esters and proanthocyanidins with low DP) can be separated with ethyl

acetate, but hydrolysable and condensed tannins with high molecular weight are insoluble, as complexes of tannins and macromolecules.

In recent years, several authors have proposed innovative extraction techniques introducing ultrasound-assisted extraction (Bagheri and Esmaiili, 2017), microwave-assisted extraction (Nayak *et al*., 2015), supercritical fluid extraction (Feliciano *et al*., 2014), and high hydrostatic pressure extraction (Varela-Santos *et al*., 2012). Afterwards, it is common to carry out a purification of the sample to better characterize mixtures. Supports normally used for tannin separation are Sephadex LH-20 and reversed phase materials, with mobile phases such as mixtures of alcohol–water or acetone–water. Other typical stationary phases for analytical tannin separations are Supercosil, Phenomenex Aqua, Chromatorex ODS, Discovery HS, Hypersil ODS-C18 silica gel, Purospher, Hichrom, Synergy Hydro RP, Latex C-18, among others (Montes-Ávila *et al*., 2017).

1.2.4 Analytical methods

Due to the high variety and complexity of tannin plants extracts, there is not a universal analytical method to study all of them, especially the highly polymerised tannins, which are the most difficult to analyse. For this reason, most of the developed techniques are focused on analysis of lower molecular weight tannins.

There are some spectrophotometric techniques to which tannins test positive, such as ferrum chloride and Folin-Ciocalteu. These reactions are quite quick and easy to carry out, but they are not specific for tannins since other phenolic compounds also give positive results. For years, some colour reactions have been developed for group determinations for spectrophotometric quantification of tannins. Hartzfeld *et al.* proposed in 2002 a specific colour reaction to detect esters of gallic acid or ellagic acid (Hartzfeld *et al*., 2002). On the other hand, treatment with mineral acids or with Vanillin-HCl enables the detection of proanthocyanidins, which generate red reaction products (Okuda *et al*., 1989). Proanthocyanidins can be also detected by making them react with dimethylamino-benzaldehyde and obtaining blue reaction products (Treutter, 1989).

HPLC (high pressure liquid chromatography) is currently the preferred method to establish the composition and concentration of mixtures of tannins in a given sample. The compounds can be separated with HPLC columns of normal (e.g. silica gel) and reverse-phase (e.g. C8 or C18) support. Then, they can be detected in many different ways, with HPLC detectors, a diode-array detector (DAD) or a mass-spectrometry detector. Nevertheless, separation of highly polymerised tannins is complicated to resolve by HPLC techniques, as the number of possible molecule isomers increases with DP. Currently, there is no reference in the literature to a suitable method for analysis of tannins with a high DP.

As of recently, the NMR (nuclear magnetic resonance) spectroscopy technique is increasingly being used in food analysis, because many NMR techniques can determine the composition of mixtures of components in natural products. Some of them are: ^{1}H and ^{13}C, two-dimensional homonuclear (2D 1H-1H), correlated NMR spectroscopy (COSY), heteronuclear chemical shift correlation NMR (C-H HECTOR), totally correlated NMR spectroscopy (TOCSY), nuclear Overhauser effect in the laboratory frame (NOESY), and rotating frame of reference (ROESY) (Naczk and Shahidi, 2006). The strengths of this technique are the minimal and non-destructive procedures of sample preparation and the possibility of obtaining an excellent non-target analysis, to perform screenings of samples.

The main drawbacks are related to the high cost of the equipment and the relatively low NMR sensitivity compared to other techniques such as HPLC or gas chromatography. To better understand the linkages between the components, spectroscopic techniques are needed. Even though near-infrared (NIR) spectroscopy does not provide clear information about the features of carbonic structures present in the different types of tannins and their origins, it is a non-destructive powerful tool, fast to use and accurate. With the Fourier transform infrared (FTIR) spectroscopy it is possible to detect fundamental molecular vibrations, providing a more comprehensive study of the structural differences, allowing tannins to be distinguished from other polyphenols.

Proanthocyanidins can be clearly identified by the presence of three strong bands at 1288–1283cm^{-1}, 1160–1155 cm^{-1}, and 1116–1110 cm^{-1} and two other weaker bands at 976 and 844–842 cm^{-1}. Specifically, the 1288–1283 cm^{-1} bands are characteristics for the flavonoid-based tannins, and they are missing in the spectra of hydrolysable tannins. On the counterpart, the IR (infra-red) spectra of hydrolysable tannins (i.e. gallo- and ellagitannins) present two characteristic strong bands at 1731–1704 cm^{-1} and 1325–1317cm^{-1} (Falcão and Araújo, 2013). Reid *et al.* (2013) used the solid-state 13C NMR to identify the proanthocyanidins of black wattle (Acacia) and quebracho (*Schinopsis lorentzii*). The authors highlighted that this particular technique has the advantage of being applicable to source materials in their native state (Reid *et al*., 2013).

Thanks to its analytical power, mass spectrometry (MS) has been relevant for structural studies on tannins, giving information on their components and molecular weights. The MS principle consists in ionizing chemical compounds to generate charged molecule fragments and measuring their mass-to-charge ratios. Among other sources used, direct flow injection electrospray ionization (ESI) mass spectrometry analysis has been used to establish tannin fingerprints of complex extracts (Ignat *et al*., 2011).

If used in tandem (MS/MS) mass spectrometry can provide an even more detailed structural analysis of the different molecules. The matrix-assisted laser

desorption/ionization time of flight (MALDI-TOF) technique can be used to detect tannin oligomers of higher molecular weight, up to heptamers and nonamers (Fulcrand *et al*., 2008). MALDI-TOF yields spectra easier to interpret than ESI-MS, because it produces single charged molecules rather than multiply charged molecules. On the counterpart, a problem related to MALDI-TOF, especially for higher oligomers, is that a given mass peak only provides the elemental composition. Thus, it is not easy to distinguish the different isomers.

Finally, liquid chromatography–mass spectrometry (LC-MS) techniques are one of the best analytical approaches to study polyphenols, including tannins, in vegetal samples, because it allows the characterization of complex structures such as procyanidins and prodelphinidins (Fulcrand *et al*., 2008).

1.3 Tannins in diet

1.3.1 Occurrence in Food – Food sources

As tannins are considered to be ubiquitous in nature, they have been suggested to constitute a significant fraction of the polyphenols ingested in a Western diet. The content of tannins in food sources has been calculated both trough analytical method or indirectly by using databases such as that elaborated by USDA, which allows to calculate the proanthocyanidin content of selected foods (USDA, 2015).

Berries are the best sources of proanthocyanidins in the diet, even though they are ubiquitous in almost all fruits, while in general terms vegetables and cereal products are not a relevant source. Lingonberry, cranberry, black elderberry, black chokeberry, black currant, and blueberry are some of the edible berries with predominance of proanthocyanidin content (Krenn *et al*., 2007). One study evaluating 99 food items from vegetal origin reported that the highest contents per fresh weight were determined in chokeberries and rose hips (Hellström *et al*., 2009). The authors highlighted that there was some variation in proanthocyanidin contents between different sampling times. Indeed, the high content of proanthocyanidins can be perceived in some fruits, due to the astringent taste, when they are not ripe, as in the case of bananas, persimmon, carob bean, and Chinese quince.

Proanthocyanidins are more concentrated in the peel of fruits or the bran of grains. For instance, proanthocyanidin concentration has been calculated to be higher in apples with peel than in apples without peel, as the DP is lower in the flesh than in the peel (Gu *et al*., 2004). Some parts of fruits, particularly rich in proanthocyanidins, are also consumed as dietary supplements, as in the case of grape seeds. Beverages derived from fruits rich in condensed tannins such as wine, beer and some fruit juices are also good sources of proanthocyanidins (Serrano *et al*., 2009). As mentioned before, legumes, nuts and other minority cereals such as sorghum and barley contain proanthocyanidins, but they are not detectable in staple crops such as corn, rice and wheat (Serrano *et al*., 2009).

As regards hydrolysable tannins, they can be found as gallotannins and ellagitannins. Gallotannins are not universally present in higher plants. They can be encountered within specific taxonomic limits in both woody and herbaceous dicotyledons. Even though more than 1000 natural ellagitannins have been identified in nature, they can be found in a reduced number of foods.

Ellagitannins identified in foods are principally punicalagin (particularly in pomegranate), sanguiin H6 and lambertianin C (especially in raspberry), pedunculagin (mainly in walnuts), vescalagin and castalagin (in chestnut), and casuarictin (in strawberries) (Serrano *et al*., 2009; Ortega Villalba *et al*., 2020). The

occurrence of ellagitannins in foods is restricted to a few fruits, such as berries of the genus *Rubus* (i.e. raspberry, blueberry, blackberry, cloudberry and cranberry), the genus *Fragaria* (strawberry), pomegranate, and their derivatives such as juices, jams and jellies. In the berries from the genus *Rubus* and genus *Fragaria*, ellagitannins provide the main part of the phenolic content (50–88%). Ellagitannins can also be found in nuts, such as pecans, walnuts, brazil nuts, peanuts, cashews as well as in white and red grapes and oak-aged wines and beer (as an additive) (Ortega Villalba *et al*., 2020). However, berries have almost three times more equivalent ellagitannin content than nuts and at least 15 times more than other types of fruits (Bakkalbasi *et al*., 2009).

The research on ellagitannin food sources of local importance revealed jabuticaba, guava, and grumixama cherries as particularly rich in this type of tannins (Alezandro *et al*., 2013; Teixeira *et al*., 2017; Rojas-Garbanzo *et al*., 2019). According to a Brazilian research team, jabuticaba berries from a particular variety cultivated in south Brazil present the highest registered ellagitannin content in fruits.

As cereal bran contains significant quantities of phenolic compounds, benzoic and hydroxycinnamic acids, which are present in the plant cell wall mainly ester-linked to polymers, cereals could be considered as partial sources of hydrolysable tannins in the diet. Similarly to condensed tannins, the ellagitannin content in food sources could be affected by a number of factors such as variety and part of the fruit, ripeness, geographic origin, climate, season, among others (Bakkalbasi *et al*., 2009).

1.3.2 Dietary intake

There is no available and detailed information on dietary intake of polyphenols and consequently of tannins, even though they are part of and major antioxidants of our daily diet. It has been estimated that the mean daily intake of polyphenols can be from 2590 to 3016 mg/day (Saura-Calixto *et al*., 2007). More recently, the daily intake of tannins has been calculated to be from several tens to several hundreds of milligrams per day (Prior and Gu, 2005; Landete, 2011). However, the information available in the literature is scanty.

The limited knowledge on tannin content in food is due to several factors, which make it difficult to evaluate their intake with precision. Most of the time, data regarding food composition are insufficient and imprecise. The richest foods in tannins are vegetables and fruit, the content of which can vary greatly depending on the time and geographical area they come from.

Another big limitation is related to the different techniques of extraction and quantitation used, which can increase even more the variability in the evaluation. The huge variety of molecules that constitute the tannin group also needs to be taken into account. Finally, the complexity of making an accurate estimation of tannin

consumption is affected also by the different diet patterns preferred in the various parts of the world.

Dietary intake of hydrolysable tannins

Fruits and in particular berries are the main sources of hydrolysable tannins in western diets with a low general daily intake, estimated around 5 mg per day (Ortega Villalba *et al*., 2020). In France, strawberry is the main dietary source, eaten as fresh product or in the form of processed products (yogurts, pastries, syrups, sweets, preserves). This leads to an annual consumption of 1.7 kg, contributing 0.2–0.3 mg/day of hydrolysable tannins (Clifford and Scalbert, 2000).

Wine is also particularly rich in hydrolysable tannins, but its contribution is difficult to estimate, because the tannin content depends on wine aging in oak barrels. In some cases, this beverage could be only a minor source of hydrolysable tannins, as only a small proportion of the wine consumed is aged in oak barrels, and seldom in ET-rich new barrels. The consumption of Bavarians is estimated to range between 4.9 and 5.4 mg/day, and that in Finland is higher at 12 mg/day (Landete, 2011).

In the Finnish Food Composition Database Fineli (available at http://www.fineli.fi) kept by the National Public Health Institute of Finland it is possible to calculate the daily intake of hydrolysable tannins of the Finnish as 12 mg per day, almost exclusively through the consumption of certain berries of the family *Rosaceae* (Koponen *et al*., 2007). In Scandinavian countries the consumption of berries depends on the seasonality of the production, increasing considerably in summer, with a principal contribution by cloudberry, raspberry, rose hip, strawberry, and sea buckthorn, with content ranging from 1 to 330 mg/100 g (fresh weight basis) (Ortega Villalba *et al*., 2020).

In contrast to these data, other authors indicate that the intake of dietary hydrolysable tannins may be much higher than previously estimated, especially if some foods (such as berries and nuts) rich in hydrolysable tannins are normally consumed in the diet (Tomás-Barberan *et al*., 2009).

Dietary intake of condensed tannins

The consumption of oligomeric and polymeric proanthocyanidins is higher than that of monomeric proanthocyanidins. In general, the consumption of flavan-3-ols has been estimated to range from 10mg/day to 0.5 g/day (Montes-Ávila *et al*., 2017). Several authors have calculated the daily intake of condensed tannins based on food composition and consumption survey data. In the United States the mean intake for the general population is around 53.6 mg per day, counting with a higher consumption in children (2–5 and 6–11 years) and older males (40–59 and >60 years) (Prior and Gu, 2005).

In the Spanish population, proanthocyanidin intake is estimated to range approximately from several tens to several hundred milligrams per day (Santos-Buelga and Scalbert, 2000). It is interesting that there are discrepancies between the proanthocyanidin intake in diet calculated trough the Spanish consumption survey data (450 mg per person per day) and that based on the proanthocyanidin composition data published by Gu *et al.* (2004) (around 240 mg per person per day) (Gu *et al*., 2004; Saura-Calixto *et al*., 2007). The higher values estimated by Saura-Calixto *et al.* (2007) could be explained by the fact that some food items which contribute to the proanthocyanidin intake of the Spanish population were not included in the proanthocyanidin composition data from Gu *et al.* (2004).

Tea is not considered an important source of proanthocyanidins; it is only a major source of monomers. However, in the countries of northern Europe there is a large consumption habit, and it cannot be overlooked. In the Netherlands, the mean intake of proanthocyanidin monomers is 50 mg per day, being tea the major contributor (65.2–87.3%), whereas tea consumption in the US provides 12.7–34.2 mg per day (Prior and Gu, 2005).

1.3.3 Sensorial aspects

Tannins are directly responsible for several sensorial aspects of food, in particular its astringency and bitter taste. Astringency corresponds to the taste experience of dryness and puckering mouthfeel all over the oral surface (Lee and Lawless, 1991). This variegated group of sensations is the result of the interaction between tannins and salivary proteins, which leads to physical changes in the salivary mixture, with a deep decrease in viscosity. The mechanoreceptors present in the mouth perceive the sensation of roughness when the food comes in contact with the tongue, and while the tongue is moving over the palate.

The generation of the sensation is driven by two different mechanisms:

- decrease of viscosity of saliva and increase of friction as a result of the interaction between tannins and salivary protein-rich proteins;

- perception in the oral texture of the protein−tannin precipitates as discrete particles. In this case, the generation of a drying and grainy sensation depends on the concentration and dimension of the colloidal aggregates, in addition to the hardness of the precipitate (Molino *et al*., 2019).

Tannin structure and DP play a relevant role for the sensation of astringency. The interaction between tannins and salivary proteins is favoured when tannins present high molecular weight (see **Section 1.2.2**, Interaction with molecules). In general, it is assumed that proanthocyanidins are the major factors responsible for astringency intensity. Smaller compounds are not considered to be astringent in equal measure, likely because smaller dimensions do not facilitate the generation of cross-linking bonds (Brossaud *et al*., 2001; Silva *et al*., 2017).

The astringent sensation is not always necessarily correlated to the precipitation of tannin−protein complexes. Obreque-Slier *et al.* (2010) showed that soluble aggregates of hydrolysable tannins and gelatine were perceived with a marked astringent mouthfeel in a sensory test, but they failed to determine precipitation *in vitro* (Obreque-Slier *et al*., 2010). From that, it could be inferred that the unbound remaining tannins could interact with the epithelial cells of the oral surface, resulting in an increased perceived astringency, especially at lower pH (Payne *et al*., 2009).

The astringent mouth feel could be influenced also by some external factors, such as acidic pH, that lead to higher puckering sensation (Llaudy *et al*., 2004). The presence of polysaccharides in the food matrix could determine an altered astringency response. The smoothing of the astringency may be driven by the inhibition of tannin-protein interactions. In this sense, polysaccharides are applied in oenology to improve astringent sensation, conversely increasing the roundness and sweetness of wine (Ozawa *et al*., 1987; Carvalho *et al*., 2006).

In the mouth, tannins could also interact with taste receptors and give a bitter flavour to food. Soares *et al.* (2018) showed that bitterness is a result of the combinatorial pattern of TAS2R activation. More specifically, the concentration of natural tannins in food is responsible for a different grade of bitter taste by specifically activating TAS2R5 (condensed tannins) or TAS2R7 (hydrolysable ellagitannins). In general, it is well accepted that larger tannins are less bitter than those with a smaller structure.

The evaluation of bitterness may vary based on the type of assay. Indeed, the absence of salivary proteins in *in vitro* taste receptor activation assays could determine discrepancies with the taste threshold of sensory assays (Soares *et al*., 2018). *In vivo*, saliva, and in particular salivary proteins, interacts with tannins by inducing a decrease of perceived bitterness.

The interaction with salivary proteins could also reduce the activation of TAS2Rs and determine a perception more astringent than bitter of some tannins (Hufnagel and Hofmann, 2008). Finally, the bitter-making potential of tannins could also be reduced by proteins present in food (Bohin *et al*., 2013).

1.3.4 Metabolism and Bioavailability

The whole metabolic fate and bioavailability of tannins are not yet fully understood. In order to exert their biological activities, tannins have to be bioavailable in the target tissue. Thus, their absorption in the gut and their bioavailability play a key role for the realization of the biological properties of tannins. Monomeric phenols have been studied in depth both in animals and humans taking into account the metabolism, the absorption and the bioavailability (Sieniawska and Baj, 2017). However, polymeric tannins represent a difficult subject to investigate due to their complexity and their variability in chemical structure. Approximately 90% of the consumed tannins can reach the colon almost intact but high molecular weight structures cannot be absorbed and need to be further metabolized by the gut microbiota. For this reason, molecules appearing in blood or excreted in urine can be very different from those ingested (Serrano *et al*., 2009).

As the absorption and metabolism of each type of tannin differs greatly, and given that the gut microbiota has a clear impact on the metabolism of plant tannins, the metabolism of hydrolysable tannins and condensed tannins will be discussed individually.

Metabolism and bioavailability of hydrolysable tannins

Due to the low occurrence of gallotannins in food and their low intake, the metabolism of these compounds has been poorly investigated. In contrast, the metabolic fate of ellagitannins has been extensively documented. After intake by healthy volunteers of gallotannin-rich fruits several metabolites were identified in urine, such as methyl gallic acid, gallic acid sulphate, gallic acid glucuronide, galloylquinic acid, catechol sulphate, catechol glucuronide, pyrogallol sulphate, deoxypyrogallol sulphate, methyl pyrogallol sulphate and pyrogallol glucuronide (Barnes *et al*., 2016; Mosele *et al*., 2016a).

The identified metabolites are the result of the degradation of gallotannins through enzymatic activity during digestion and partly through the metabolization by the gut microbiota. As there is no information about metabolic pathways of gallotannins, Mosele *et al.* (2016) tried to investigate in depth the steps of gallotannin degradation trough human *in vitro* digestion and fermentation (Mosele *et al*., 2016b). The authors observed that in low acidic conditions simulating the gastrointestinal tract hydrolysis caused a slight degradation of the original gallotannins depending on the level of gallic acid esterification.

After enzymatic digestion, higher molecular weight tannins from dodeca- to octa-O-galloyl glucoses were transformed to lower weight hepta- to tetra-O-galloyl glucoses with the additional production of free gallic acid digallic acid and galloyl-shikimic acid. Finally, as a result of the activity of the gut microbiota gallic acid,

protocatechuic acid and p-hydroxybenzoic acid were produced. After 24 h of fermentation, gallic acid was further degraded to a simple catechol (Mosele *et al*., 2016b).

Another study reported that penta-galloyl glucose can be transported across intestinal Caco-2 cells monolayers, although a large portion of it is degraded during the process to tri- and tetra-galloyl glucose, probably due to the presence of esterase (Cai *et al*., 2006). As pyrogallol sulphate was present in blood and urine, that absorption and metabolism may take place in the small intestine as well. Although little data is available in the literature, the information available so far suggests that mostly low molecular weight metabolites may be responsible for the biological activity of this class of tannins (Kiss and Piwowarski, 2016).

Ellagitannins are not bioavailable as such, probably due to their large size (approximately from 634 Da up to 3740 Da). The association with proteins in saliva or in food could inhibit their metabolization as well (Mena *et al*., 2015). For this reason, they have never been detected in human plasma after intake of ellagitannin-rich foods (Garcia-Muñoz and Vaillant, 2014; Tao *et al*., 2019). Ellagitannins are degraded in different manners in the upper gastrointestinal tract depending on several factors such as their chemical structure, the food matrix, and their susceptibility to acid/base hydrolysis in the stomach and duodenum (Garcia-Muñoz and Vaillant, 2014).

Most ellagitannins are sensitive to acidic and basic hydrolysis, releasing the lactone ellagic acid, which is poorly bioavailable in the stomach or small intestine. Low bioavailability of ellagic acid is probably related to its scarce water solubility, and to its ability to bind irreversibly to cellular DNA and proteins, or to form poorly soluble complexes with calcium and magnesium ions that affect transcellular absorption (Serrano *et al*., 2009; Nuñez-Sánchez *et al*., 2014).

A subset of ellagitannins resulted to be resistant to enzymatic digestion, reaching almost intact the large intestine where they can potentially exert their biological activity and can be partially converted into ellagic acid by enzymes from the microbiota. Then the ellagic acid can be further metabolised by the gut microbiota into urolithins (Uro, dibenzopyran-6-one metabolites) through reduction of one of the two lactone groups followed by decarboxylation and sequential dehydroxylation involving a step-by-step reduction to tetrahydroxy (urolithin D), trihydroxy (urolithin C), dihydroxy (urolithin A and isourolithin A), and monohydroxy dibenzopyranones (urolithin B) (**Figure 1.3.1**) (Aguilar-Zárate *et al*., 2017; Milala *et al*., 2017; Zhang *et al*., 2018b).

Even though the classification appeared to be consistent across multiple intervention studies, the factors determining the distribution of urolithin metabotypes are not yet clear (García-Conesa *et al*., 2018). In a Western adult population, UM (urolithin metabotype) -A is the most abundant metabotype with 55% followed by

UM-B (34%) and UM-0 (11%). Nonetheless, Cortés-Marín *et al.* (2018) reported that age may play a role in the individual's capacity to metabolize ellagic acid into urolithins A and B. The percentage of individuals with metabotype A was higher in the case of children (80%) and decreased steadily after adolescence while metabotype B increased (Cortés-Martín *et al*., 2018). In the same study, it emerged that increased physical activity determine a prevalence of UM-B, especially in young people (from 5 to 18 years old). Apparently, gender, body mass index, weight, health status, or diet do not have an influence on the metabotype (Cortés-Martín *et al*., 2018).

The ability to produce urolithins could be influenced by long-term exposure to ellagitannin-rich food by enhancing growth of the bacteria involved in urolithin metabolism. After a long-term exposure (up to 6 months) to pomegranate ellagitannin extract, adults presenting a metabotype 0 became urolithin excreters of UM-A or UM-B (González-Sarrías *et al*., 2017).

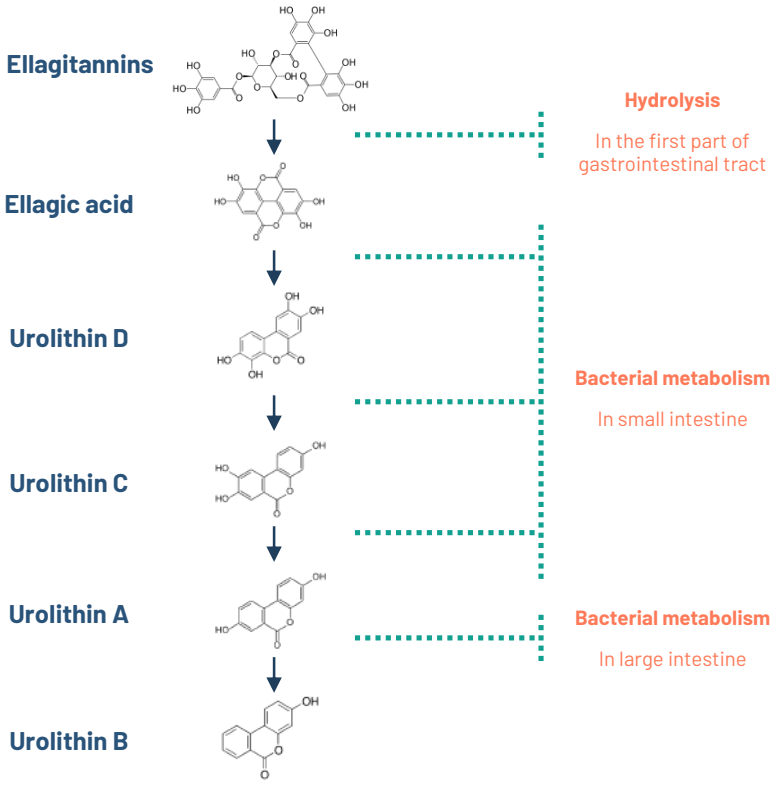


Figure 1.3.1 Metabolism of ellagitannins.

At present, it is still being studied which bacteria are responsible for the production of urolithins. An *in vitro* study with human microbiota indicated that in particular two bacterial strains, *Gordonibacter urolithinfaciens* and *Gordonibacter pamelaeae* from the Eggerthellaceae family, have the ability to transform ellagic acid into urolithin C (Selma *et al*., 2017). A further specific strain, isolated from the human microbiota, *Ellagibacter isourolithinifaciens*, was shown to be able to metabolize ellagic acid up to isourolithin-A (Selma *et al*., 2017; Beltrán *et al*., 2018). But, until now, no specific bacterial strains that could produce urolithins B from ellagic acid has been identified.

Regardless of metabotypes, other factors could impact the rate of urolithin production, such as the food source and the chemical structure of ingested ellagitannins. For instance, more urolithins in prostate have been detected in patients consuming walnuts than in those who were taking pomegranate juice, even though the latter had a higher ellagitannin content (González-Sarrías *et al*., 2010). Finally, it seems that there exists a kind of saturation of the metabolic pathways as the amount of urolithins excreted *in vivo* remains apparently independent of the quantity of ellagitannins ingested and the food source (Cerdá *et al*., 2005b).

Metabolism and bioavailability of condensed tannins

The metabolization of proanthocyanidins can be separated into two parts: the gastric and intestinal part. It is thought that the acidic environment of the gastric fluid may possibly lead to degradation of oligomeric and polymeric proanthocyanidins present in food into oligomers, monomers, and some further metabolites, but a consensus has not yet been reached. However, one study of Serra and co-workers (2010) revealed that the total proanthocyanidin content remained stable during *in vitro* gastric digestion (Serra *et al*., 2010). Conflicting results in the scientific literature may depend on several factors such as experimental conditions and different type and content of proanthocyanidins (Tao *et al*., 2019).

Other external factors (i.e. content of macronutrients in food) may also affect the digestion of condensed tannins. For instance, the high presence of fat and the consequent formation of emulsions could have a protective effect for proanthocyanidins against degradation (Gìltekin-Özgìven *et al*., 2016). On the other hand, a high content of carbohydrate in a food matrix could increase the absorption rate of catechin and epicatechin, and reduce that of epigallocatechin, epigallocatechin gallate and other oligomers, due to a carbohydrate-specific effect on gastrointestinal physiology or an unidentified carbohydrate–flavanol transporter (Serra *et al*., 2010).

In the intestine, condensed tannins are absorbed in different parts based on their structure and molecular weight. Proanthocyanidin monomers can be readily absorbed in the proximal intestinal tract, while oligomers and polymers need to be

further converted into metabolites by the gut microbiota, before being absorbed in the gastrointestinal tract (Wu *et al*., 2017b; Pereira-Caro *et al*., 2018).

The deficiency of specific receptors for condensed tannins on the cell membrane results in a passive transport through the epithelium via the paracellular route, thanks to the hydrophilic characteristic of proanthocyanidins. The absorption through the paracellular route could enable maintaining of the original structures, without any conjugation or methylation processes (Appeldoorn *et al*., 2009). Transcellular uptake of proanthocyanidins could occur also *via* endocytosis, with an interaction between proanthocyanidins and cell membrane (Da Silva *et al*., 2012; Zumdick *et al*., 2012). However, intestinal epithelial cells play a significant role in modification of proanthocyanidins, by inducing methylation and glucuronidation and increasing their bioavailability (Raab *et al*., 2010).

Once absorbed, proanthocyanidins and their metabolites can reach different organs and tissues via the circulatory system, where they are further metabolized, or they can exert their health-promoting effects. It has been reported that proanthocyanidins with a DP lower than 3 could cross the blood–brain barrier, possibly exerting neuroprotective effects (Wu *et al*., 2017a). In the liver, proanthocyanidins can be further modified by phase II enzymes. However, in some cases the presence of galloyl moieties inhibits phase II metabolism, thus ECG (epicatechin gallate) and EGCG (epigallocatechin gallate) can reach human plasma unmodified (Stalmach *et al*., 2009; Clifford *et al*., 2013).

As in the case of ellagitannins, the majority of condensed tannins reach the large intestine, to be fermented by microbiota and transformed into metabolites and further derivatives, such as phenolic acids and valerolactones (Barros *et al*., 2016). The metabolites can be then reabsorbed and detected in blood (Mena *et al*., 2017) and Monagas *et al.* (2010) reported beneficial health effects exerted also by these metabolites (Monagas *et al*., 2010). However, a high amount of condensed tannins is eliminated with feces, due to the poor bioavailability of the big and complex structures of some proanthocyanidins.

Figure 1.3.2 illustrates the possible pathways for dimer B1 metabolization by gut microbiota proposed by Tao *et al.* (2019). The first reaction of proanthocyanidin catabolism requires an interflavan bond cleavage or C-ring cleavage, followed by A-ring oxidation, dehydroxylation, and beta-oxidation (Selma *et al*., 2009; Stalmach *et al*., 2009). Then, the monomers could be divided into two molecules through hydrolysis at C3.

EGCG could be catalysed into EGC (epigallocatechin) and gallic acid, and ECG into EC (epicatechin) and gallic acid. EGC and EC and their metabolites follow the metabolic pathways as depicted in **Figure 1.3.2**, while gallic acid can be transformed into pyrogallol (Roowi *et al*., 2010). Dimer B2 might go through several different pathways. In general, it is well accepted that dimer B2 can be catalysed into two

monomers (catechin or EC) due to interflavan cleavage. Other proposed transformations are the breaking of the interflavan bond after C-ring cleavage at lower units, or alternatively the A-ring oxidation at its lower unit after C-ring cleavage at its upper unit (Kohri *et al*., 2003; Appeldoorn *et al*., 2009; Stoupi *et al*., 2010; Serra *et al*., 2011).

As regards condensed tannins with a DP higher than 3, information is scanty. The depolymerization of oligomeric and polymeric proanthocyanidins into flavanols (monomers) is not proposed to be the major metabolic pathway in the gut. It seems that the gut microbiota plays a key role in the conversion of proanthocyanidins with a DP higher than 2. Indeed, several studies detected that after consumption of condensed tannins the presence of flavanols in the blood stream due to possible acid hydrolysis in the stomach was minimal, while metabolites were measured in the urine, indicating the importance of the catalytic activity of the microbiota (Ottaviani *et al*., 2012; Wiese *et al*., 2015). Further research is needed to clarify the pathways of oligomeric and polymeric proanthocyanidins.

Proanthocyanidin metabolism is also influenced by the different composition of the food matrix in which they are contained. For instance, condensed tannins were easily metabolized by microbiota in presence of small doses of ω-3 polyunsaturated fatty acids (PUFAs), which cooperate in the release and metabolism of proanthocyanidins (Molinar-Toribio *et al*., 2017).

As regards the bacteria involved in condensed tannin degradation, it seems that many species work in combination by acting simultaneously or sequentially (Montes-Ávila *et al*., 2017). Some bacteria are involved in the metabolism of oligomers and polymers. For instance, *Eubacterium oxidoreducens*, belonging to the Firmicutes, obtained from human feces has been demonstrated to be able to insert oxygen in the A- ring to form a new hydroxyl group, which might trigger A-ring cleavage (Stoupi *et al*., 2010).

Some species of Firmicutes also turned out to be involved in proanthocyanidin catabolism. The activity of *Flavonifractor plautii* aK2 and *Flavonifractor plautii* DSM 6740 has been identified in the production of 1-(3',4'-dihydroxyphenyl)-3-(2",4",6"-trihydroxyphenyl)propan-2-ol to δ-(3',4'-dihydroxy-phenyl)-γ-valerolactone and δ-(3',4'-dihydroxyphenyl)-γ-valeric acid (Kutschera *et al*., 2011).

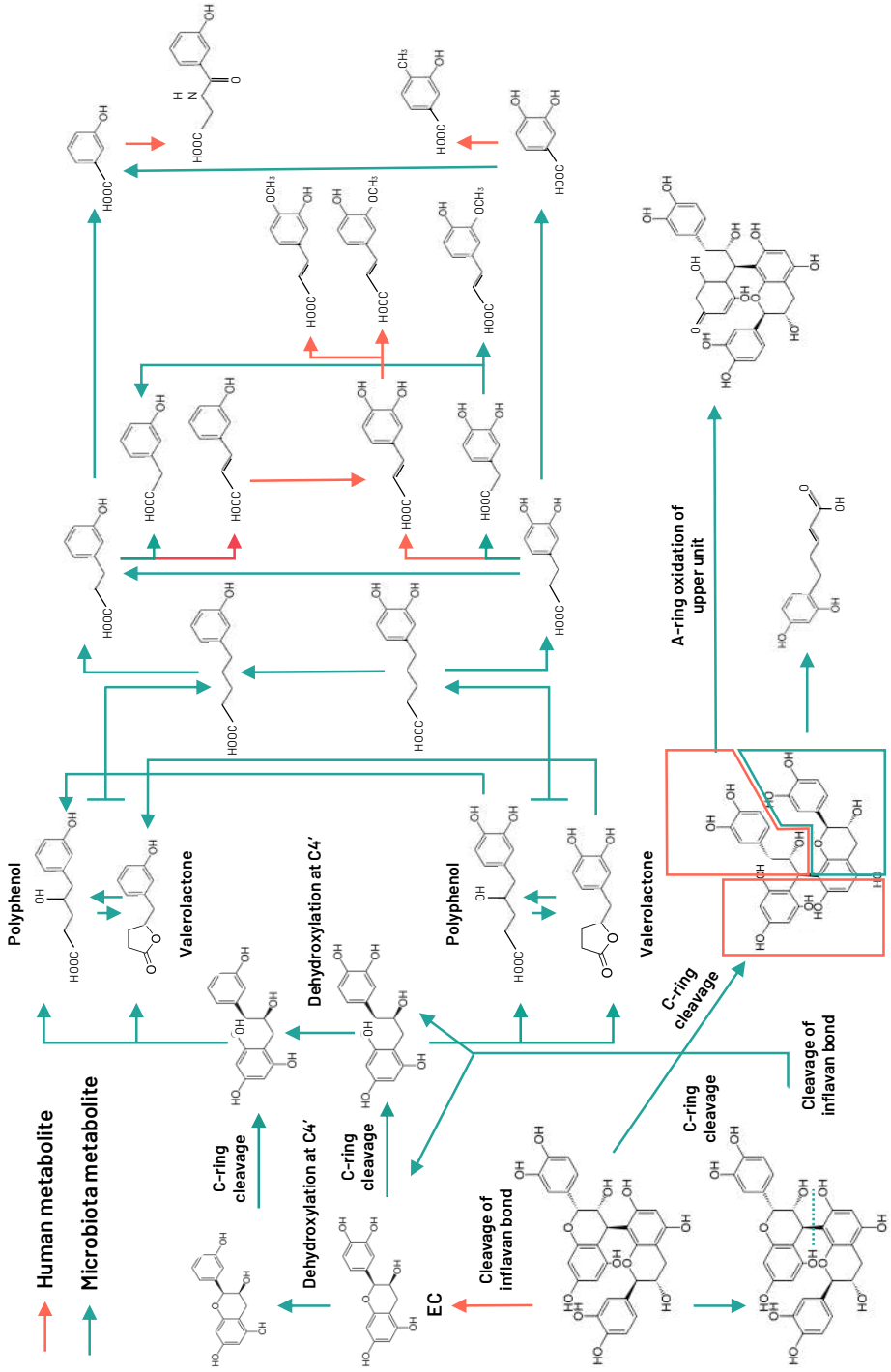


Figure 1.3.2 Possible metabolic pathways of dimer B1 digested by gut microbiota.

*Streptococcus thermophilu*s and *Lactobacillus casei-01* are bacteria known as probiotics and are related to proanthocyanidin metabolism. (Li *et al*., 2013) In particular, *Lactobacillus casei-01* might determine the production of various types of phenolic acids, such as 3,4-hydroxyphenylacetic acid, 4-hydroxy-phenylpropionic acid, m-coumaric acid, and p-coumaric acid, by decomposing proanthocyanidins (Li *et al*., 2013).

Just a few bacteria involved in proanthocyanidin metabolism have been identified and more mechanisms and bacteria species need to be identified and described in order to explain the complex process of proanthocyanidin degradation.

1.4 Bioactivity of tannins

Despite the unpleasant organoleptic properties, tannins have been linked to plenty of properties and applications for human health, which have been extensively reviewed (Sieniawska and Baj, 2017; Rauf *et al*., 2019). Various *in vitro* and *in vivo* (human and animal) studies show that tannin intake may prevent the onset of several chronic diseases, as reported by recent reviews, systematic reviews and meta-analyses (Wang *et al*., 2014; Salvadó *et al*., 2015; Turati *et al*., 2015).

The recognized bioactivity has been ascribed to tannins present in food ingested in the diet or purified from different parts of plants. In addition to their well-documented free radical scavenging and antioxidant activity, other beneficial effects include anti-inflammatory, anticancer, antioxidant, prebiotic, and cardioprotective properties, which have encouraged their application in the food, nutraceutical and pharmaceutical industries (Rauf *et al*., 2019; Ortega Villalba *et al*., 2020). Moreover, there is a long historical use of tannins for healing external skin inflammation and injuries, the efficacy of which has been scientifically demonstrated (Sieniawska, 2015).

It has to be clarified that many *in vitro* studies could give inconsistent or untranslatable results about bioactivity of compounds in humans, because a major part of the ingested tannins could exert their effects only within the gastrointestinal tract, in view of their scarce bioavailability. Indeed, tannins could exert their biological effects in two different ways: as complex and unabsorbable molecules with binding properties which may produce local effects in the gastrointestinal tract, or as small absorbable compounds.

Small metabolites deriving from tannin degradation, produced by the catalytic activity of the gastrointestinal cells and gut microbiota, could easily pass the intestinal barrier, and reach numerous tissues and organs through the blood and lymphatic circulatory systems (see **Section 1.3.4**, Metabolism and Bioavailability).

1.4.1 Antioxidant properties

Reactive Oxygen Species (ROS) deriving from normal cell activity are physiologically important as they contribute to cell signalling. However, an overproduction of ROS can lead to oxidative stress, namely the imbalance between antioxidants and ROS. Tannins, as other polyphenols, present a high antioxidant activity, and when they have been compared to strong antioxidants, tannins showed to exert a similar or even stronger activity. This is the case of some procyanidins such as procyanidin B1 and procyanidin B3, which demonstrated to be stronger antioxidants than ascorbic acid or α-tocopherol, both *in vitro* and *in vivo* (Iglesias *et al*., 2012; Skrovankova *et al*., 2015). Other condensed tannins, delphinidin and

petunidin-3- glucoside, showed an activity of approximately 50% of that of the synthetic antioxidant butylated hydroxytoluene (BHT) (Beninger and Hosfield, 2003).

Tannin antioxidant properties largely depend on their chemical structure; for instance, the increase of the DP corresponds to an increase of the anti-radical effects (Sieniawska, 2015). The presence of hydroxyl groups and phenolic rings result in tannins acting as primary antioxidants (free radical scavengers) or preventive antioxidants (co-antioxidant agents, inhibitors of oxidation catalysts) to maintain the right antioxidant/prooxidant balance. Tannins can exert antioxidant influence through multiple modes of action: scavenging free radicals, up-regulating antioxidant enzymes, regenerating vitamin E, inactivating transition metal ions, and inhibiting free-radical generating enzymes (**Figure 1.4.1**).

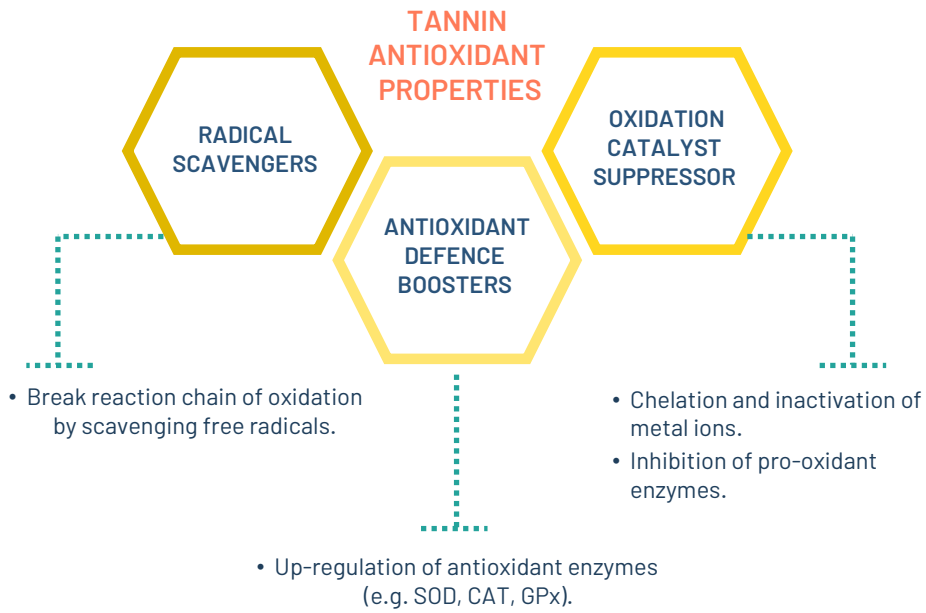


Figure 1.4.1 Tannin modes of action to counteract oxidative stress.

Free radical scavenging power corresponds to the ability to "hunt" and neutralise free radicals thanks to the ability of a molecule to donate electrons or hydrogen atoms. In this sense, tannins can form stabilised and harmless radical structures, breaking the chain reaction of free radical propagation. Moreover, tannins present the ability of inhibiting lipid peroxidation and their content increases under stressful conditions in cellular pro-oxidant states (Corral *et al*., 2021). In this sense, proanthocyanidins help to restore the oxidative balance of the body by scavenging hydroxyl, superoxide and peroxyl radicals and through the inhibition of the lipoxygenases (Georgiev *et al*., 2014).

Fushimi *et al.* (2015) proposed that soluble tannins, found in unripe persimmon, could contribute to lowering rat plasma levels of phosphatidylcholine hydroperoxide, which is a biomarker of membrane lipid peroxidation (Fushimi *et al*., 2015). Hydrolysable tannins of *Castanea sativa* extract could reverse the negative effects caused by induced oxidative stress in a human neuroblastoma cell line (SH-SY5Y), by significantly reducing the production of ROS (Brizi *et al*., 2016).

Another mode of action is thorough the inhibition of oxidation catalysts, or initiators, such as transition metal ions and prooxidant enzymes. Tannins are potent chelators of transition metal ions (i.e., Fe(II)), which prevents the progression of the Fenton reaction that yields hydroxyl radicals. In the study of Hassan and co-workers (2016) it was found that the antioxidant protection given *in vivo* by ethanolic extract of *Nigella sativa* was related to its capacity to counteract Fe(II)-induced lipid peroxidation (Hassan *et al*., 2016).

Tannins exert also a co-antioxidant effect by boosting the antioxidant defence system, inducing the increase of the presence and the activity of endogenous antioxidant enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT), which catalyse the destruction of free radicals. Tannin extract from *Rhodiola rosea* L. boosted the activity of SOD and GPx in mice serum, heart, liver and brain tissues, and consequently reduced malondialdehyde (MDA) content (Zhou *et al*., 2014).

In the meta-analysis of Li *et al.* (2015) the efficacy of proanthocyanidins against oxidative damage was investigated (Li *et al*., 2015a). The authors confirmed that proanthocyanidins might effectively antagonize oxidative damage and enhance antioxidant capacity by enhancing total antioxidative capacity, SOD, GSH (reduced glutathione), GPx and CAT and reducing the MDA levels. However, these beneficial effects can be influenced by several factors such as intervention time, mode of administration and the type of biological sample evaluated (Li *et al*., 2015a). In another study, the oral administration of 200 or 400 mg of persimmon condensed tannins per kg of mouse body weight resulted in the reduction of the activities of different oxidative biomarkers: serum and liver SOD, GPx and liver MAD activities (Li *et al*., 2015a).

As regards the clinical application of hydrolysable and condensed tannins for their antioxidant properties, only little evidence is available. *Quercus robur L*. has been evaluated in three different pilot studies in human volunteers, with the intake of a supplement named Robuvit®. In particular, the study conducted by Horvathova *et al.* (2014) indicated that the daily intake of Robuvit® (containing 300 mg of tannin extract) for one month ameliorated the plasma levels of oxidative stress markers. A decrease of the advanced oxidation protein products and lipid peroxides was registered, while SOD and CAT activity and total antioxidant capacity increased after the supplementation (Horvathova *et al*., 2014).

Finally, the effect of the administration of a procyanidin-rich chocolate was evaluated in a double-blind crossover trial, conducted in 18 volunteers. After two 4-week periods separated by a 4-week washout period, the effects on fecal free radical production and antioxidant activity were examined. Chocolate proanthocyanidins were effective in rebalancing the redox status and free radical production in fecal water with respect to the control group (Record *et al*., 2003).

Although the antioxidant mechanism of tannins has been repeatedly investigated, all the mechanisms of action are not yet fully understood. Moreover, the antioxidant capacity is the basis for triggering further systematic and beneficial effects, such as anti-inflammatory responses.

1.4.2 Anti-inflammatory properties

Inflammation, together with oxidative stress, is a mechanism underlying many diseases, and tannins can have positive collateral effects thanks to their antioxidant and anti-inflammatory properties. Tannins have been proposed in many studies as bioactive ingredients to prevent or treat many disorders and infections. However, a large fraction of the works in the scientific literature are limited to reporting the systemic effects of these natural compounds and avoid describing the specific cellular mechanism or identifying the specific compounds responsible for the effect. Just a minority of the investigations described the specific mechanism of action of the anti-inflammatory effect of several tannins, and the studies have been principally conducted on cell lines or tissues.

A study evaluated the action of different extracts from leaves of *Acacia mearnsii* on RAW 264.7 macrophages, previously exposed to oxidative stress. One of the extracts, particularly rich in tannins (quantified at 12.6 mg/g as procyanidin B2 equivalent) could significantly reduce ROS production and specifically restore the initial levels of nitric oxide (NO) as in non-stimulated cells. When in the same cell culture inflammation was induced through lipopolysaccharide (LPS) stimulation, the *A. mearnsii* extract inhibited the expression of cytokines like interleukin- 1β or -6

(IL-1β, IL-6) and pro-inflammatory enzymes such as cyclooxygenase-2 (COX-2) or inducible nitric oxide synthase (iNOS) (Xiong *et al*., 2016).

When using an extract rich in hydrolysed tannins from *Terminalia chebula*, analogous results were obtained. Indeed, the authors could observe that in LPS-stimulated RAW 264.7 macrophages there was a reduction in NO production and in the protein expression of iNOS and COX-2, thanks to the tannin-reach treatment (Yang *et al*., 2013).

In a simulated microglia model with BV-2 cells, *C. sativa* extract exerted anti-inflammatory effect, by downregulating the expression of IL-1β, tumour necrosis factor-α (TNF-α) and nuclear factor-κB (NF-κB), when the cells were exposed to LPS insult (Chiocchio *et al*., 2020). The use of proanthocyanidins in mice models lowered the LPS-induced iNOS and COX-2 overexpression, through the modulation of NF-κB in different parts of the brain, thus diminishing depressive behaviours in the animals (Jiang *et al*., 2017).

Finally, extracts from *Rhus coriaria* obtained from different types of extraction were tested on the HaCaT cell line. In this case the inflammation was induced by using TNF-α, which stimulates pro-inflammatory signals through the cascade production of interleukins, vascular endothelial growth factor (VEGF), matrix metallopeptidase-9 (MMP-9) and intercellular adhesion molecule-1 (ICAM-1). The *R. coriaria* extracts could contain the inflammatory process in each of the steps before mentioned, except for VEGF, which was just decreased by one of the evaluated extracts (Khalilpour *et al*., 2019). The same extract was then tested *in vivo*, by oral administration in rats, with the aim to prevent or treat necrotizing enterocolitis. As regards the evaluation of the effect on the inflammatory status, the application of the treatment lowered the levels of inflammatory molecules in histological samples, while biochemical results reported lower amounts of IL-6, TNF-α and lipid hyperoxides. It has to be mentioned that overall the use of the tannin-rich extract effectively counteracted the negative effects induced by necrotizing enterocolitis (Isik *et al*., 2019).

In the study of Terra and co-workers (2009) procyanidins obtained from grape seeds were tested in rats to reduce the inflammation induced by a hyper lipidic diet and the oral administration of the extract could induce a down-regulation of C-reactive protein (CRP), TNF-α and IL-6 in liver and white adipose tissue (Terra *et al*., 2009). The same compounds were tested on murine asthma models and they resulted in lowering inflammation in the airways thorough the reduction of inflammatory cells, Th2 cytokines and serum IgE levels (Lee *et al*., 2012).

Some species belonging to the genus *Rubus* resulted in higher contents of tannins and consequently stronger antioxidant and anti-inflammatory properties compared to those of the genus *Vitis* (Park *et al*., 2014). For this reason, many studies were conducted with the genus *Rubus* thanks to its unique potential. The reported anti-

inflammatory effects of the extract of *Rubus fruticosus* were attributed to its content of cyanidin-3-glucoside, which downregulated the activity of NF-κB, COX-1 and -2, NO and/or iNOS (Monforte *et al*., 2018). *Rubus idaeus* extract could inhibit the expression of IL-1β and -6, TNF-α and leptin in hypertrophied adipocytes. On the other hand, the bioactive compounds contained in the extract determined the increase of the expression of antioxidant enzymes, such as SOD and CAT (Kowalska *et al*., 2019). More interesting, from this work it emerged that the application of *R. idaeus* extracts could reduce lipid accumulation and increase lipid mobilization in hypertrophied adipocytes, which indicates a clear correlation between an inflammatory status and metabolism.

The study of Lee *et al.* showed that feeding broilers with 1% of *C. sativa* could modulate metabolism while also acting as an immunoregulator by increasing expression of IL-10 (Lee *et al*., 2021b). In a further work, the authors related the modulation of host immunity via an IL-6 mediated response through the JAK-STAT signalling pathway (Lee *et al*., 2021a).

As tannin metabolites are considered to be responsible of systemic effects, they have been deeply investigated. In particular, the impact of urolithins on inflammatory processes has been well established on various *in vivo* and *in vitro* models (Boakye *et al*., 2018; Lee *et al*., 2019). Urolithin aglycone, an hydrolysable tannin metabolite, seems to be a potent anti-inflammatory compound and some authors proposed that the conjugate may be even stronger. A study investigated the presence in urine of urolithin conjugates (iso-Uro-A-gluc, Uro-A-gluc, and Uro-B-gluc) after ingestion by volunteers of different tannin-rich food (i.e. pomegranate juice, walnuts, hazelnuts, and fresh raspberries) for 5 days. In particular, the activity of the endogenous β-glucuronidases released by human neutrophils was studied, through the cleavage of glucuronides.

β-Glucuronidase is a marker of an inflammatory status generated by inflammatory cells and the lysosomes of necrotic cells, in most solid tumours (Ortega Villalba *et al*., 2020). Urolithin glucuronides and their aglycones have been studied also for their beneficial effects against cardiovascular diseases, particularly characterized by chronic inflammation. In the study of Giménez-Bastida et al. (2012), Uro-A glucuronide exhibited strong vascular protective effects by inhibiting monocyte adhesion and endothelial cell migration, with a downregulation of the levels of chemokine ligand 2 (CCL2) and interleukin-8 (IL-8) (Giménez-Bastida *et al*., 2012).

Obesity is characterized by recruitment of adipose tissue macrophages and the consequent release of inflammatory cytokines and chemokines. Quercetin, produced through microbial fermentation of condensed tannins, has been shown to modulate obesity-induced inflammation by reducing MIP-1a (macrophage inflammatory protein-1a) released from adipocytes and macrophages. The downregulation is mediated trough the inhibition of several factors such as C-C Chemokine receptor

CCR1/CCR5, the inhibition of activation of JNK (c-Jun N-terminal kinases), p38 MAPK (mitogen activated protein kinase), and IKK (IκB kinase) as well as IκBα degradation (Noh *et al*., 2014).

1.4.3 Antimicrobial properties

Tannins exert a potent antimicrobial activity, which could be applied in different fields (e.g. as preserver from spoilage in food industry or as additives in livestock) thanks to their negligible toxicity. The inhibition of bacterial growth resulted to be more effective against Gram-positive, rather than Gram-negative bacteria due to the structural differences of the bacterial cell envelope. Tannins act better as bacteriostatic rather than bactericidal agents showing minimum inhibitory concentrations (MIC) ranging from 61.5 to 3200 μg/mL (Boakye, 2016).

The bacterial growth inhibition effects of tannin-rich extracts is strongly related to the structural properties of tannins, in particular to their phenolic hydroxyl groups. Thus, the chemical nature of the tannins plays a key role. For instance, while condensed tannins in mimosa extract exerted a grater bacteriostatic activity, chestnut extract hydrolysable tannins showed bactericidal activity. Furthermore, in general condensed tannins present lower MIC values compared to hydrolysable tannins, probably due to the absence of galloyl groups, which have an important role in the antibacterial activity (Funatogawa *et al*., 2004; Ekambaram *et al*., 2016).

Among hydrolysable tannins, gallotannins seem to be more effective than ellagitannins, but independently of the nature of the compound, the galloyl group is responsible for antibacterial activities. However, other bioactive groups have been identified as responsible for antibacterial activity. For example, the gallagyl moiety present in punicalagin or the valoneoyl group contained in isorugosin were demonstrated to be effective against MRSA (methicillin-resistant *Staphylococcus aureus*) (Shimozu *et al*., 2017).

As regards condensed tannins, the bioactivity is influenced by the number of monomeric subunits and the location of B-ring hydroxylation of the flavan-3-ol monomer. Indeed, in one study a grape seed extract rich in oligomeric units of catechin and epicatechin showed higher antibacterial activity compared to a fraction containing monomers (Mayer *et al*., 2008). Several studies demonstrated that the antibacterial strength increases with the number of flavanol units present in the condensed tannin molecules (Wang *et al*., 2015; Tamura *et al*., 2016). However other factors play a critical role such as the DP. In particular, Sivakumaran *et al*. (2004) studied the relationship between the DP and the antibacterial activity of proanthocyanidin fractions, resulting in a higher influence of the DP rather than the nature of the polymeric composition (Sivakumaran *et al*., 2004).

There are several mechanisms involved in tannin antimicrobial activity, including chelation of metals required for microbial growth (mainly iron), interactions with proteins and the cell wall/membrane (leading to structural destabilization), and enzyme inhibition (**Figure 1.4.2**).

Iron is essential for optimal bacterial growth; the chelation and the consequent subtraction of ferric ion can lead to the inhibition of bacterial growth due to iron deprivation. The bacteriostatic effect of gallotannins isolated from *Mangifera indica L*. on *Clostridium botulinum* is also attributed to their iron-chelating property (Engels *et al*., 2011). The same mechanism has been described also for the antibacterial activity of 2,6-Tri-O-galloyl-β-D-glucopyranose, a gallotannin from the fruits of *T. chebula*, against a multidrug-resistant uropathogenic bacteria (Bag *et al*., 2013).

Interestingly, tannins have inhibitory effects against a variety of pathogenic bacteria, but they are not effective with most probiotic bacteria. Most probiotics are lactic acid bacteria and do not require iron for their growth. Hence, different studies suggested that the genera *Lactobacillus* and *Bifidobacterium* are poorly susceptible to different tannin-rich extracts (Chan *et al*., 2016, 2018a, 2018b).

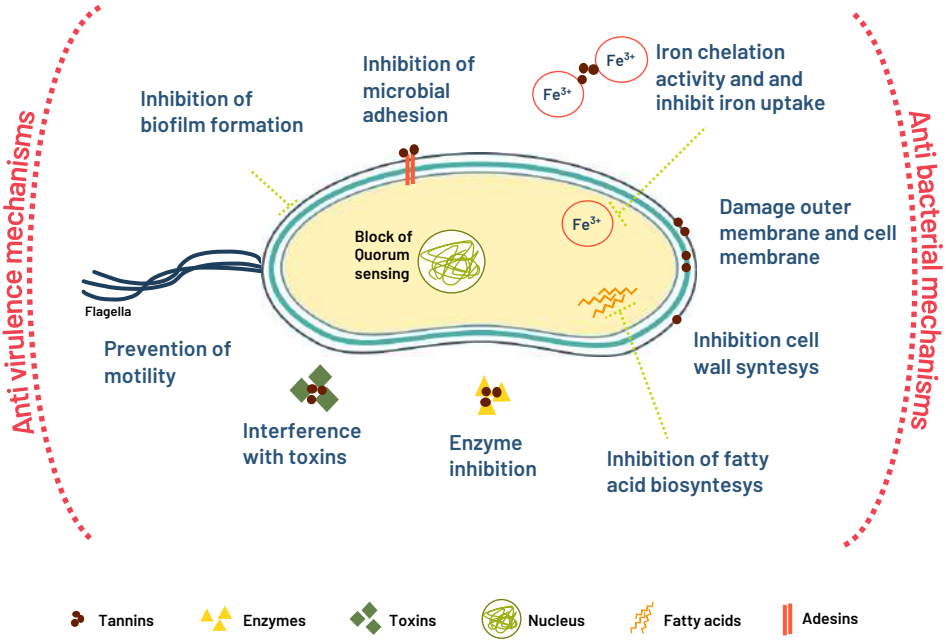


Figure 1.4.2 Schematic representation of the mechanisms of antibacterial action of tannins.

The inhibition of cell wall synthesis by tannins can result from the inactivation of the enzymes or from the direct binding to the cell wall (Trentin *et al*., 2013). Furthermore, tannic acid could destroy the integrity of the bacterial cell wall, directly binding the peptidoglycan layer (Dong *et al*., 2018). More generally, the hindrance of the cell wall synthesis by tannins makes bacteria more susceptible to osmotic lysis.

Tannins can also interact with bacterial cell membranes by affecting the membrane potential or increasing membrane permeability (Funatogawa *et al*., 2004; Trentin *et al*., 2013). This occurred when hexa- and hepta-galloylglucopyranoses were tested against *Staphylococcus typhimurium* and *Bacillus cereus*. The interaction of the bioactive compounds with the bacterial membrane through hydrogen bonds led to alterations in the permeability of cell membranes and the consequent bacterial death (Tian *et al*., 2009).

Some types of tannins, in particular proanthocyanidins, primarily act on Gram-negative bacteria, due to their outer membrane characterized by the presence of LPS (Delehanty *et al*., 2007). Condensed tannins from purple prairie clover destabilized *E. coli* O157:H7 outer membrane by altering the composition of fatty acids and decreasing the fluidity of the membrane (Wang *et al*., 2013). Analogously, proanthocyanidins from *Vaccinium macrocarpon* showed the ability to act on the outer membrane of an *E. coli* responsible for diarrhoea (Alshaibani *et al*., 2017). Finally, tannic acid and PGG could inhibit β-ketoacyl-ACP reductase (FabG), an enzyme fundamental in fatty acid biosynthesis (Wu *et al*., 2010).

Additionally, tannins can act against several bacterial virulence factors, which determine the degree of bacterial pathogenicity and consequently the severity of the disease in the host. Indeed, virulence factors help bacteria to invade and colonize bypassing the host defence system and successfully determine an infection. Tannins can act as quorum sensing inhibitors and attenuate the gene expression of several virulence factors such as toxins and proteins related to biofilm formation, adhesion, and motility. Inhibition of virulence factors represents an attractive therapeutic approach to delay the manifestation and prevention of bacterial infections (Farha *et al*., 2020).

1.4.4 Microbiota modulation

Tannins have been proposed as prebiotics since they meet the definition updated by Gibson *et al*. (2017): ‘a substrate that is selectively utilized by host microorganisms conferring a health benefit’. Thus tannins have been studied in the context of modification of the human gut microbiota and shown to be able to determine positive effects on the diversity and composition of the microbiota, and produce notable shifts in metabolic markers (Gibson *et al*., 2017).

The human gastrointestinal tract hosts up to 1000 bacterial species, and the ability to maintain a microecological balance can reduce the onset of dysbiosis, which in turn is associated with many diseases. The study of beneficial effects of prebiotics is constantly evolving and at the moment involves benefits to the gastrointestinal tract (i.e. inhibition of pathogens and immune stimulation), cardiometabolism, mental health and bone (Ostaff *et al*., 2013; Mosca *et al*., 2016).

Analogously to dietary fibres, most tannins can reach almost intact the large intestine. Herein, these compounds are partially metabolized by microorganisms but, on the other hand, they can also act by modulating the gut microbiota composition and function via the selective inhibition of pathogens and the growth enhancement of beneficial bacteria (Ozdal *et al*., 2016). Some authors reported the ability of quebracho and chestnut tannin extracts to modulate the gut microbiota, with promoting effects for probiotic bacteria (Díaz Carrasco *et al*., 2017, 2018).

Verrucomicrobia and, in particular, the species *Akkermansia muciniphila*, has been recently investigated as a marker of a healthy gut thanks to its anti-inflammatory properties, but also because it could increase insulin sensitivity and boost the gut barrier function (Masumoto *et al*., 2016). Several studies reported that condensed tannins contained in different food matrices could induce in animal models a striking increase of the population of *A. muciniphila* (Anhê *et al*., 2015; Masumoto *et al*., 2016). More recently, Zhang *et al.* (2018) highlighted that in mice fed with a high-fat/high-sugar diet the supplementation with grape proanthocyanidins could attenuate the metabolic syndrome, related to the increase of *A. muciniphila*. However, the authors proposed that the improvement may be due indirectly to the elimination of the microbes that limit the growth of *A. muciniphila* or cross-feeding activities (Zhang *et al*., 2018a).

Tannins have been reported to boost the abundance of other probiotics. In the study on animals of Diaz Carrasco *et al.* (2018), a blend of tannins derived from quebracho and chestnut wood produced an increase of the relative abundance of *Bifidobacterium* and *Lactobacillus* besides other beneficial species like *Faecalibacterium*, among other Clostridiales (Díaz Carrasco *et al*., 2018). The increase of Lactobacillaceae was also reported by other authors in a study in which pigs were fed with a commercial grape seed extract (Choy *et al*., 2014; Williams *et al*., 2017).

The enhancement of probiotic strains by tannins has often been described as being accompanied by a decrease in pathogens. Indeed, in the study of Lee *et al.* (2006) it was reported that the relationship between tea polyphenols and regulation of the intestinal environment was accomplished by inhibiting the growth of pathogens and enhancing that of commensal bacteria, including probiotics (Lee *et al*., 2006). When a blend of condensed and hydrolysable tannins was tested on bovines, it resulted in a good preservation of the overall bacterial complexity of the rumen, besides a

significant reduction of *Prevotella,* sometimes associated with high-fat and pro-inflammatory diets (Díaz Carrasco *et al*., 2017; Kalantar-Zadeh *et al*., 2020). The study of a supplementation of healthy volunteers with cocoa flavan-3-ols showed a boosting effect on the growth of *Lactobacillus spp.* and *Bifidobacterium spp.*, while that of the *Clostridium histolyticum* group was limited (Tzounis *et al*., 2011).

Ellagitannins from extracts of berries were tested *in vitro* against specific bacterial strains and showed a strong inhibitory activity against *Staphylococcus*, but they did not alter probiotic strains, i.e. *Lactobacillus rhamnosus* (Oksman-Caldentey *et al*., 2005). More recently, cranberry extract and grape seed extract rich in condensed tannins were tested in an *in vitro* fermentation and resulted particularly effective in reducing the abundances of *Bacteroides*, *Prevotella*, *Blautia* and *Coccoides–Eubacterium rectale* (Sánchez-Patán *et al*., 2015).

Finally, some researchers reported that tannins can also promote the adhesion and colonization of probiotic bacteria, resulting in an ulterior beneficial effect. Thus, one study found that procyanidins and epigallocatechin activate the adhesion of lactic acid bacteria in an *in vitro* intestinal epithelial tissue model (Kawabata *et al*., 2019).

Tannins play a key role in modulating the intestinal microbiota not only for their growth-promoting effects for beneficial bacteria, but also for the activation of their metabolic functions. This results in the stimulation of short-chain fatty acids (SCFAs) production, among other compounds (Gibson *et al*., 2017; Kawabata *et al*., 2019). SCFAs are crucial for human health as they can modulate cell metabolism and fine-tune the immune response, in addition to contributing as an energy source (Koh *et al*., 2016a).

The *in vitro* digestion and fermentation model of Aura *et al.* (2013) revealed that condensed tannins of Syrah grape can induce a high release of SCFAs (Aura *et al*., 2013). A similar release of SCFAs by pomegranate ellagitannins was described by Bialonska *et al.* (Bialonska *et al*., 2010). The interaction of tannins with the food matrix could result in an additive effect, as in the case of polysaccharides, particularly pectin (Aura *et al*., 2013). This is explained by the fact that especially proanthocyanidins associated with polysaccharides are poorly bioavailable in the upper intestine and reach the colon intact, where they are converted by the gut microbiota into active metabolites.

The bond with polysaccharides acts as a booster likely because polysaccharides act as a nutrient for the microbiota, which in turn can act more efficiently. Apparently, the chain length of proanthocyanidin is determinant in the competition mechanism between the inhibition of microbial enzymes by these molecules and the capacity of colonic microorganisms to metabolize such tannins (Bazzocco *et al*., 2008). Ellagitannins have been investigated in combination with fructooligosaccharides, but it is not yet clear whether their role could be beneficial or counterproductive in the production of SCFAs in animals (Kawabata *et al*., 2019).

1.4.5 Antiviral properties

Today, the use of nature-derived remedies as effective therapies for viral infections is getting attention as antiviral drugs are limited in number, and in many cases, their use is accompanied by the presence of side effects or the formation of viral resistance, which makes the therapy partially ineffective.

Among the plant extracts, tannins have been shown to act effectively against a broad spectrum of viruses comprising both coated viruses (influenza viruses A/H3N2 and A/H5N3, herpes simplex virus type 1, vesicular stomatitis virus, Sendai virus and Newcastle disease virus) (Uozaki *et al*., 2007; Müller Kratz *et al*., 2008) and non-enveloped viruses (poliovirus, coxsackievirus, adenovirus, rotavirus, feline calicivirus and mouse norovirus) (Ueda *et al*., 2013; Vilhelmova-Ilieva *et al*., 2020).

The ability of tannins to bind proteins can be harnessed to establish links to capsid proteins, specific viral enzymes required for viral replication or to newly synthesised viral proteins involved in the composition of new particles. Tannins can affect different stages of viral replication, including the extracellular virions them-selves, their binding to the cell, the penetration and replication process in the host, as well as the assembly of new viral particles and transport proteins, polysaccharides, and viral enzymes. In particular, the permanent bond with proteins of the capsid or supercapside, either to specific viral enzymes required for viral replication or to newly synthesized viral proteins involved in the composition of new viral particles, is an important mechanism to neutralise viruses (Debprasad, 2006, 2007; Vilhelmova-Ilieva *et al*., 2020).

Ellagitannins are considered interesting HSV (Herpex simplex virus) anti-viral agents, as their targets are virus-specific proteins, thus retaining activity against acyclovir-resistant strains of HSV types 1 and 2 (Vilhelmova-Ilieva *et al*., 2020).

Many plant extracts exhibiting tannins as the main component resulted to be a good remedy against the replication of many viruses. Tannin bark extract of *Hamamelis virginiana* L. has been tested at different concentrations for its effect against influenza A virus. The study showed that the life cycle of influenza A is inhibited in the first and, to a lesser extent, the subsequent steps. Among the investigated tannins, those with a high molecular weight could inhibit both IAV (Influence A Virus) receptor binding and neuraminidase activity. Low molecular weight tannin inhibited neuraminidase but not hemagglutination (Theisen *et al*., 2014). The *in vitro* study of Gyuris and co-workers (2009) found that *Euphorbia hirta* extracts exhibit antiretroviral activity thanks to their high content in tannins, by inhibiting the reverse transcriptase activity in a dose-dependent manner (Gyuris *et al*., 2009).

Similarly to natural extracts, diverse isolated tannins also displayed antiviral activity. An example is represented by chebulagic acid and punicalagin, both

hydrolysable tannins, which inhibited the penetration of HCV (Hepatitis C virus) (Lin *et al*., 2013). HIV (Human immunodeficiency virus) has been widely investigated to find targets to prevent its replication. Ellagitannins were found to be able to suppress the HIV replicative cycle through the inhibition of the reverse transcriptase, while in another study HIV replication was prevented by the suppression of the HIV-1 protease and HIV-1 integrase enzymes (Matthøe *et al*., 1999; Notka *et al*., 2003, 2004).

In the last year, an *in-silico* study on 19 different hydrolysable tannins investigated their potential ability to inhibit the activity of SARS-CoV-2 (Severe Acute Respiratory Syndrome Coronavirus-2) through the binding with 3-chymotrypsin-like cysteine protease enzyme (described to be involved in virus transcription). Pedunculagin, tercatain and castalin resulted effective while tellimagradin I, punicalin, chebulagic acid or β- pedunculagin showed potential secondary roles in the inhibition of the activity of this catalytic target (Khalifa *et al*., 2020).

Tannins can also act indirectly by reducing the effects of viral infection, thanks to their antioxidant and anti-inflammatory activity. Indeed, viral infections are characterized by cell and/or tissue injury, due to an hyperproduction of free radicals (e.g., reactive oxygen species ′ROS′ and reactive nitrogen species ′RNS′) and then the onset of a pro-inflammatory state. Acute respiratory viral infections give a clear example of the connection between lung tissue injury and epithelial barrier dysfunction, which has been linked with an increased susceptibility to secondary infections, triggered by a heightened inflammatory response (De Marco, 2013).

In this sense, tannins could represent a valuable strategy to prevent and control viral diseases by diminishing oxidative stress and stress-mediated complications of viral infections. That was demonstrated in mice infected by influenza virus infection and supplemented with tannin-rich apple extract. Their survival rates were improved and researchers could observe a modulation of the immune response, thanks to the exerted antioxidant activity, which acted directly or indirectly on immunocytes (He *et al*., 2011). Similarly, tannins present in *Chaenomeles speciosa* extract and pomegranate juice could reduce the inflammatory and oxidative stress status respectively in avian influenza and influenza viral infection (Haidari *et al*., 2009; Zhang *et al*., 2010).

Notwithstanding the reported promising antiviral effects, it should be kept in mind that tannins are not specifically designed antivirals, thus their beneficial effects could be exploited but in combination with appropriate antiviral therapies. In this regard, tannins could even exert a synergistic effect with antiviral therapies, as it was evidenced by Haidari *et al.* (2009), who studied the effectiveness of punicalagin used together with Oseltamivir against human influenza A (H3N2) virus and reported a synergistic effect (Haidari *et al*., 2009).

1.4.6 Antinutritional properties

Tannins have often been described as nutritionally undesirable. Their capacity to interact with other molecules (i.e. proteins, starch, and digestive enzymes) has been believed to cause a reduction in the nutritional values of foods. However, many issues need to be further explored and clarified. First of all, the vast majority of the studies reporting these disadvantageous properties date back many years, when the knowledge about tannins was still limited, as were probably the methods used for studying them. Indeed, most of the *in vitro* studies reporting the inhibition of enzymes by tannins could not be reproduced *in vivo*. Furthermore, in the past, authors focused their attention on studying the poisoning effects of tannins by using large amounts of these compounds, which are not reliable for a balanced diet or a correct supplementation.

As reported by Chung *et al.* (1998), in animals, toxic effects have been imputed to a disproportionate consumption of high tannin-containing feed. Similarly, the fatalities occurred in humans were due to a use of exaggerated concentrations of tannic acid in specific pathological conditions, such as enemas or burns. In another study in which gastric disorders were reported, subjects were supplemented with 1 g of tannins per day, corresponding to the amount of tannins contained in more than 10 cups of green tea per day (Chung *et al*., 1998; Sieniawska and Baj, 2017).

More specifically, tannins have been claimed to inhibit digestive enzymes, and to interfere with protein, carbohydrate, and metal uptake. Since most enzymes are proteins, it has long been supposed that tannins decrease enzymatic activity. The consequence of their complexation would be then a reduced capacity of the body to digest nutrients (Chung *et al*., 1998).

The current understanding on the inhibiting role of tannins is still limited. The response of enzymes to tannins may vary depending on the enzyme, due to different structure and composition. Recent findings reported only a minor decrease in enzymatic activity in presence of tannins, while other studies found that catalytic activity could be boosted after the reaction with tannins present in low concentrations. As expected, high concentrations of tannins lead to opposite results. In view of that, Adamczyk *et al.* rather propose tannins as modifiers of enzyme activity, by playing with different concentrations and affinity with enzymes (Adamczyk *et al*., 2017).

In *in vivo* studies, just a small part of the ingested tannins gets to interact with digestive enzymes, because they bind the salivary proteins in the mouth and later the mucosa and epithelium of the gut. As described in **Section 1.2.2** (Tannin-protein interaction), salivary proteins constitute the first barrier against tannins, binding and precipitating them, thus generating the characteristic astringent sensation, perceived in mouth as dryness and roughness. Thus, it is thought that the major dietary effect

of tannins within the digestive tract is the formation of less-digestible complexes with dietary proteins, rather than the direct inhibition of the digestive enzymes. On the other hand, the interaction between tannins and proteins does not impair the bioavailability of tannins, and some authors suggested proteins as carriers of these bioactive compounds (van het Hof *et al*., 1998; Livney, 2010). In the same way, tannins could interfere with the digestion and/or absorption of carbohydrates and fibres. As mentioned before, there is a lack of information about this issue as much of the reported information is hypothetical or has been demonstrated only *in vitro*. In order to verify the validity of these assumptions, it would be necessary to carry out large-scale studies, under controlled conditions.

The same is also applicable to iron, whose deficiency has been attributed to tannins, among other contributors. Delimont *et al.* published in 2017 a narrative review based on an extensive examination of the interventional and epidemiological studies in the literature regarding the relationship between tannin consumption and iron bioavailability or iron status. They found that most of the present studies were obscured by several factors, including lack of control of concurrent antinutritional or iron-enhancing factors in diets, tannin-iron interaction in the food matrix as well as assessment of iron deficiency, rather than adequate iron stores. The authors concluded that long-term tannin consumption may impact iron status in a different manner than that predicted by single-meal studies or iron bioavailability models. Indeed, single-meal studies suggest that tannic acid and tea consumption more consistently impair iron bioavailability than does the consumption of condensed tannins. However, the connection between these findings and individual iron status is not established. On the other hand, the authors found that epidemiologic studies and long-term trials show that tannin consumption does not affect the iron status of individuals.

Considering all the results together, the authors conclude by formulating the hypothesis of the development of mechanisms of adaptation to tannins, which reduce the antinutritional effects over time (Delimont *et al*., 2017). This interesting theory could be applied also to proteins and carbohydrates, but large-scale, long-time studies about antinutritional effects and the demonstration of the mechanisms underlying the adaptation to tannins would be needed.

In summary, tannins have been considered nutritionally detrimental, but more recent studies are clarifying many lacunae on the topic. In general, it is advisable not to over-consume tannins, because, as any other phytobiotic, exceeding specific doses may lead to adverse effects.

1.4.7 Control of glycaemic status in diabetes

Several tannin-rich natural extracts have been recognized as potential antidiabetic agents, among them those obtained from *C. sativa, Q. robur, Schinopsis lorentzii or T. chebula*. The antidiabetic potential of tannins is the result of different mechanisms of action.

- The positive modulation of insulin and pro-insulin levels in blood.

By binding polysaccharides from the diet, tannins form complexes reducing the availability of glucose in the in the gastrointestinal tract, and therefore producing a delay in polysaccharide digestion. Furthermore, as discussed in the Antinutritional properties section, tannins could also play a role by inhibiting the enzyme activity involved in polysaccharide digestion (i.e. α-amylase and α-glucosidase) (Serrano *et al*., 2009).

- The insulin-like effect on insulin-sensitive tissues.

The anti-hyperglycaemic effect could be mediated by an insulin-like action of tannins on the liver and peripheral tissues. Furthermore, tannins may control insulin secretion by regulating pancreatic β-cell functionality and the incretin system. The best investigated anti-hyperglycaemic mechanism of proanthocyanidins is the stimulation of glucose uptake into insulin-sensitive tissues, by acting on specific intracellular insulin-signalling pathways (Pinent *et al*., 2004; Corral *et al*., 2021).

- Antioxidant regulation in pancreatic β-cells.

The great antioxidant capacity of tannins may help to counteract the onset of diabetes mellitus. It has been found that there is a low expression of genes involved in the production of antioxidant enzymes in diabetic pancreatic cells. Thus, if oxidative stress is not controlled, it contributes to the development of the disease, by determining the apoptosis of β cells (Serrano *et al*., 2009; Sieniawska and Baj, 2017).

α-glucosidase is one of the enzymes involved in the absorption of carbohydrates from the gut. In one study, hydrolysable tannins inhibited this enzyme and consequently they could be adopted for treatment of patients with impaired glucose tolerance or type 2 diabetes mellitus. Moreover, the authors highlight the doubly beneficial effect of chestnut tannins as inhibitors of α-glucosidase and antioxidants. Among all the evaluated fractions of chestnut extract, trigalloylglucose resulted to be the best one, by exhibiting both the strongest α-glucosidase inhibition and the highest antioxidant capacity (Cardullo *et al*., 2018). When different fractions of *Q. robur* tannin extract were analysed, monogalloylglucose (MGG), an HHDP-glucose isomer, castalin, gallic acid, vescalagin and grandinin showed the strongest antioxidant role, while the strongest α-glucosidase inhibitory activity was exerted by

the sub-fraction containing castalagin (Muccilli *et al*., 2017). Fractions from *S. lorentzii* tannin extract presented both hydrolysable tannins (quinic acid esters with different gallic acid units) and condensed tannins (dimers, trimers, tetramers or pentamers of catechin or catechin-3-O-gallate and fisetinidol, or catechin-3-O-gallate) exhibiting α-glucosidase and α-amylase activity (Cardullo *et al*., 2020).

The antidiabetic potential of *Pterocarpus marsupium* wood extract was investigated in rats. The identified antidiabetic component was epicatechin, which improved the oral glucose tolerance post-sucrose load and exerted regenerative activity on pancreatic β cells (Mishra *et al*., 2013).

Proanthocyanidins were found to induce *in vivo* up-regulation of the expression of GLUT-4 (Glucose transporter type 4) in adipose tissue and muscle and to modulate GLUT-4 translocation to the plasma membrane (Yamashita *et al*., 2012; Kurimoto *et al*., 2013). From these studies it emerges that proanthocyanidins stimulate glucose uptake through molecular mechanisms that involve AMP (adenosine monophosphate)-activated protein kinase and protein kinase B (Salvadó *et al*., 2015). Condensed tannins play a critical role in the liver, which is crucial in maintaining correct glycaemia levels. Many studies have reported that proanthocyanidins can restrain gluconeogenesis and glucose production in liver by targeting hepatic glycolytic and gluconeogenic enzymes (Montagut *et al*., 2010; Gandhi *et al*., 2011; Huang *et al*., 2013; Sundaram *et al*., 2013).

Tannins could also participate in the regulation of the glycaemic state through indirect mechanisms. For instance, some fractions of *T. chebula* were found to suppress the activity of maltase, which is highly involved in diabetic processes (Senthilkumar and Subramanian, 2008). Moreover, the same extract could enhance the peroxisome proliferator-activated receptor-α and/or -γ signalling, which plays an important role in controlling the expression of genes that affect directly insulin sensitivity (Matsumoto and Yokoyama, 2012). Finally, cinnamtannin, a condensed tannin from bay wood, counteracted indirectly type 2 diabetes by reducing some complications, such as platelet hyperactivity and hyperaggregability (Bouaziz *et al*., 2007).

Some studies performed in humans highlighted that the intake of flavan-3-ols may prevent the development of type 2 diabetes (Jacques *et al*., 2013; Zamora-Ros *et al*., 2014). Moreover, a regular consumption of different tannin-rich products (e.g. Pycnogenol®, muscadine grape products, chocolate) helps in controlling glycaemic levels in type 2 diabetes subjects (Liu *et al*., 2004; Banini *et al*., 2006; Curtis *et al*., 2012). On the counterpart, some studies performed by supplementing type 2 diabetes subjects with a flavanol-rich cacao drink or a polyphenol-rich chocolate could not find significant changes in glucose and/or insulin levels (Balzer *et al*., 2008; Mellor *et al*., 2010). However, it has to be taken into account that the discrepancies could

be highly related to the differences in proanthocyanidin amounts administered in each study.

1.4.8 Cardiovascular disease prevention and effect on lipid metabolism

Cardiovascular disease (CVD) is a general term used to indicate conditions affecting the heart or blood vessels. It is related with progressive deposition of fat inside the arteries (atherosclerosis) that leads to the formation of blood clots, inducing damages to arteries at different levels (e.g. the brain, heart, kidneys) (Balakumar *et al*., 2016). These effects underlie damaging molecular processes that progressively lead to oxidative stress, inflammation and platelet aggregation, among other effects. Thanks to their activity against these mechanisms, tannins have been investigated for their cardioprotective potential (Wang *et al*., 2014; Sieniawska, 2015; Bladé *et al*., 2016). A wide number of studies tried to illustrate the mechanisms involved in CVD prevention by tannins. For example, proanthocyanidins have been proposed to be able to inhibit the atherogenic process and to balance blood pressure and lipid homeostasis (Hort *et al*., 2012; Pons *et al*., 2014; Quifer-Rada *et al*., 2016).

Many works have investigated and shown the antiatherogenic and cardioprotective potential of walnut (*Juglans regia*) consumption. Indeed, *J. regia* resulted to be rich in ellagitannins, especially HHDP derivatives, from which the potent antioxidant ellagic acid could be released (Regueiro *et al*., 2014). The peeled fruit of *J. regia* could inhibit in cultured human aorta endothelial cells the expression of the inflammatory biomarkers of vascular cell adhesion protein (VCAM-1) and ICAM-1, induced by the exposure to TNF-α (Papoutsi *et al*., 2008). In the *in vivo* study of Sun and co-workers, *J. regia* extracts were effective in counteracting the detrimental action of the synthetic catecholamine isoproterenol, responsible for producing myocardium pathologies. As a result, myocardial infarction was inhibited by the investigated extract in a dose-dependent manner. Moreover, histopathological analyses, oxidative markers and myocardial tissue lipids highlighted the cardioprotective activity of *J. regia* extract against the damaging effects of isoproterenol (Sun *et al*., 2019).

C. sativa bark extracts have been used to treat primary cultures of neonatal rat cardiomyocytes subjected to oxidative stress, induced by H_2O_2 (hydrogen peroxide). The hydrolysable tannin extract resulted in a reduction in the production of intracellular ROS, improving cell viability in a dose-dependent manner. In cardiac tissues isolated from guinea pigs, *C. sativa* bark extracts diminished the aortic noradrenaline-induced contraction, the heart rate and produced a positive inotropic effect in the left atrium/papillary and a negative chronotropic effect (Chiarini *et al*., 2013).

Other protective mechanisms of action of tannins involve the inhibition of monocyte to macrophage differentiation in atherosclerosis. Indeed, oligomeric proanthocyanidins isolated from *Crataegus oxyacantha L.* decreased vascular cell adhesion protein 1 and chemokine CCL2 levels, thus determining the downregulation of the inflammatory pathway and macrophage markers, such as MMP-2 and -9 and PPARγ (peroxisome proliferator-activated receptor gamma) (Mohana *et al*., 2015). Furthermore, procyanidin B2 was able to downregulate NLRP3 (family pyrin domain containing 3) inflammasome activation in endothelial cells (HUVECs). In particular the bioactive compound inhibited caspase-1 activation and IL-1β secretion in addition to ROS (Yang *et al*., 2014).

Tannins have been evaluated also in human studies, giving positive results. Indeed, tannin extract from grape wine was tested in a double-blind, placebo-controlled crossover study, in mildly hypertensive subjects. After 24-hours from the consumption, systolic and diastolic blood pressure were decreased as was the plasma concentration of the vasoconstrictor endothelin-1. Catechins and procyanidin-rich extracts helped to maintain physiological blood pressure and contributed to preventing the onset of heart disease (Draijer *et al*., 2015).

The onset of CVD is also closely related to lipid metabolism. Dyslipidaemia and impaired regulation of cholesterol levels facilitate the formation of atherosclerotic plaques and thus may progressively lead to CVD. Some phytochemicals, such as tannins, have been shown to be effective in the initial stages of atherosclerosis development and in overt disease. For instance, the administration of tannin extracts from seeds of *Acacia senega* in rabbits subjected to a hypercholesterolemic diet resulted in reducing the levels of total cholesterol, low- and very low-density lipoprotein (LDL and VLDL) cholesterol and triglycerides (TGs) in blood. The anti-atherosclerotic and cardioprotective power resulted also in a lower atherogenic index, besides a reversion in lipid oxidation markers and histological damage (Ram *et al*., 2014).

Until now, the epidemiological studies on the effects of tannins on dyslipidaemia in humans are partially inconsistent, probably due to methodological problems in carrying out the investigations, such as low sample size, differences in the evaluated tannin extracts, dosages, time of treatment as well as the clinical condition of the population studied. On the other hand, numerous studies in animal models proved a hypolipidemic effect of tannins, by reducing plasmatic levels of TGs, apolipoprotein B and LDL, as well as hepatic steatosis (Del Bas *et al*., 2005; Quesada *et al*., 2012; Baselga-Escudero *et al*., 2013; Pons *et al*., 2014).

The hypotriglyceridaemic effect of tannins has been widely investigated. In several studies tannins were found to act through the repression of lipoprotein secretion without affecting their catabolism. More in depth, the primary mechanism of tannins to reduce the release of chylomicrons is to impair the lipid availability in

enterocytes. The promotion of reduced lipid absorption by proanthocyanidins has been observed in rats, with an increased faecal excretion of cholesterol (Tebib *et al*., 1994). Furthermore, *in vitro* studies have shown the ability of condensed tannins in inhibiting the activity of pancreatic lipase as well as the expression of long chain acyl-CoA synthetases (ACSL)-5 and -3, which supply fatty acids for TG synthesis in enterocytes (Moreno *et al*., 2003; Del Bas *et al*., 2009).

Tannins act also by inducing the oxidation of fatty acids, instead of TG synthesis. Similarly to the observations in the intestine, an impaired lipid availability has been observed also in hepatocytes, reducing the production of hepatic VLDL (Del Bas *et al*., 2008, 2009). In this context, a tannin-rich grape seed extract inhibited in the hepatic cell line HepG2 the *de novo* synthesis of TGs and cholesterol, as well as their secretion (Del Bas *et al*., 2008). A proteomic study conducted on rats fed with a high-fat diet corroborated the results, indicating that the chronic consumption of the same extract leads to the repression of the synthesis of fatty acids and TGs (Baiges *et al*., 2010).

Finally, tannins improve lipid homeostasis by increasing the reverse transport of cholesterol to the liver and its elimination via bile acids. Several studies have shown that proanthocyanidins are able to regulate the activation of specific microRNAs, miR-33 and miR-122, which are key controllers of lipid metabolism, involved in the hypolipidemic effect above described (Baselga-Escudero *et al*., 2012, 2013).

1.4.9 Anticancer activity

Cancer is uncontrolled cell growth that can be directly induced by oxidative stress. In this sense, polyphenols, and tannins among them, have been widely investigated thanks to their powerful antioxidant capacity for their eventual application in preventing or controlling cancer development (Kashiwada *et al*., 1992; Serrano *et al*., 2009). Several studies aimed at describing the mechanisms of action of tannins to counteract cancer development and xenograft tumour growth. Thus, many potentially useful molecular targets have been identified, such as the induction of apoptosis and the alteration of genes involved in cell cycle and DNA replication (Sieniawska, 2015).

Proanthocyanidins, especially those extracted from grape seed, were shown to be able to inhibit colon tumour growth by inhibiting the expression of both VEGF and Angiopoietin-1 through the scavenging of ROS (Wen *et al*., 2008; Huang *et al*., 2012a). Similar results were found by Huang and co-workers (2012), who also highlighted the further inhibition of MMP-2 and 9, which together with the before mentioned factors could block angiogenesis (Huang *et al*., 2012b). Apparently, the molecular mechanism involved in the downregulation of MMP-2 and -9 is the phosphorylation of proteins belonging to the MAPK family and activation of NF-κB

(Gollucke *et al*., 2013). The inhibition of these MMPs, involved in tumour development and metastasis, was also observed in prostatic and pancreatic carcinoma cells (Chung *et al*., 2012; Gollucke *et al*., 2013). On the other hand, the suppression of VEGF production by procyanidin-rich extract from sorghum was proven to reduce tumour growth and metastasis (Wu *et al*., 2011).

Condensed tannins could control the apoptosis mediated by p53, a transcription factor that regulates the cell cycle and functions as tumour suppressor. Indeed, on JB6 C141 mouse skin epidermal cells, the activation of p53 resulted in the triggering of Bax/Bcl-2 proteins and caspase-3 (Gollucke *et al*., 2013).

The interference of tannins with the normal regulation of cell-cycle progression can induce the arrest of the Go/G1 phase, through the up-regulation of Cip1/p21 and Kip1/p27 protein levels together with a down-regulation of cyclins (D1, D2 and E) and cyclin-dependent kinases (CDK2, CDK4 and CDK6) (Prasad and Katiyar, 2014). Grapes and pine bark procyanidin-rich fractions could also inhibit cell proliferation by affecting the cell cycle in G2 phase, with a consequent apoptosis observed in HT29 human colorectal cancer cells (Ouédraogo *et al*., 2011). Finally, Kingsley *et al.* (2010) described that proliferation in oral squamous carcinoma cells could be inhibited by the increased expression of apoptosis-specific molecules, such as caspase-2 and caspase-8, after a 24-h proanthocyanidin treatment at 50–70 μg/mL (Kingsley *et al*., 2010).

The *in vitro* anti-cancer properties of proanthocyanidins have been confirmed by several animal studies (Gollucke *et al*., 2013; Sieniawska, 2015). Analogously, hydrolysable tannins exhibited anti-cancer properties both *in vitro* and *in vivo* (Heber, 2008; Seeram, 2008). In particular, ellagitannin in pomegranate extracts selectively inhibits the growth of colon, prostate, breast and lung cancer cells (Kim *et al*., 2002; Malik *et al*., 2005; Seeram *et al*., 2005; Larrosa *et al*., 2006b). The same product could inhibit tumour growth in skin, lung, prostate and colon in animals, after oral consumption (Kohno *et al*., 2004; Pantuck *et al*., 2006; Khan *et al*., 2007; Stoner, 2009).

It is noteworthy to mention that orally taken tannins undergo a metabolization through the combined action of human enzymes and gut microbiota. In the case of ellagitannins, digestion produces mostly urolithin derivatives (Cerdá *et al*., 2005a; Espín *et al*., 2007). Unlike the big structures of ellagitannins, urolithins can be found at micromolar concentrations in the bloodstream and they could also accumulate in some tissues, such as in prostate and intestine (Cerdá *et al*., 2005a; Seeram *et al*., 2007). For this reason, several authors started to investigate also the anticancerogenic potential of tannin metabolites. For instance, Kasimsetty *et al*. (2010) illustrated that the ellagitannins and urolithins deriving from pomegranate juice could possibly prevent colon cancer development, via cell proliferation inhibition and apoptosis

induction (Kasimsetty *et al*., 2010). Urolithins have been demonstrated to counteract cancer *in vitro* through three different mechanisms:

- cell cycle arrest
- reduction of cell proliferation
- modulation of key cellular processes related with cancer development (e.g. MAPK signalling) (Larrosa *et al*., 2006b; González-Sarrías *et al*., 2010).

Besides these effects, urolithins can also act indirectly through the regulation of the immune response by activating NF-κB, which has been shown to play a critical role in cancer development (Landete, 2011). These data suggest that urolithins may be relevant bioactives, which may contribute to the prevention of different types of cancer such as colon, prostate and breast cancer (Larrosa *et al*., 2006a; Seeram *et al*., 2007; Kasimsetty *et al*., 2010).

Despite the large number of published studies, tannins have been investigated just in a few clinical trials for their anti-cancer properties. As tannin-rich pomegranate juice demonstrated to inhibit angiogenesis in prostate cancer, it has been tested in an initial phase II clinical trial. After the treatment, patients with prostate cancer reported significant prolongation of prostate specific antigen doubling time (Pantuck *et al*., 2006).

Tannins have been investigated also for their potential to attenuate the adverse effects of cancer radiotherapy. In a double-blind, placebo-controlled, randomized phase II trial, grape seed proanthocyanidins were orally administered for 6 moths to patients affected by radiation-induced breast induration. After 12 moths, researchers could observe ≥50% reduction in surface area (cm^2) of breast induration in 29.5% of tannin treated patients (Sieniawska, 2015). In another randomized double blind, placebo-controlled, pilot study standardized cranberry capsules (with a content of 72 mg of proanthocyanidin) were used for the prevention and treatment of radiation cystitis in prostate cancer patients. The supplementation determined a statistically significant decrease in cystitis incidence in treated patients (65%) compared with the control group (90%). Furthermore, severe cystitis was reported in 30% of treated patients *vs*. 45% in the control group. The results showed that patients who receive radiation therapy for prostate cancer may benefit from cranberry supplementation (Hamilton *et al*., 2015; Smeriglio *et al*., 2017).

1.5 Nutraceutical applications

Nowadays, there is plenty of evidence for the ethnopharmacological use of tannin-rich extracts and this is clearly due to their numerous biological and chemical properties. Tannins have an excellent potential for innovative nutraceutical applications as food supplements as has been reported by many *in vivo* studies.

Dias *et al.* investigated the use of tannins in patients affected by celiac disease, thus proposing their use for the formulation of nutraceuticals for special categories (Dias *et al*., 2015, 2016, 2018). In this respect, the special category of type 2 diabetes patients could also be a focus fpr developing new products. Indeed, condensed tannins of sorghum encapsulated in kafirin microparticles resulted in an inhibition of amylase activity and prevention of hyperglycaemia symptoms (Dias *et al*., 2018).

Tannins are already commercialized as nutraceuticals, not incorporated in food (**Table 1.5.1**). One example is given by Robuvit, a patented product based on oak tannins, which provide roburins, vescalagin and castalagin, grandinin and vescalin among many other ellagitannins. The company refers to several clinical studies published in the scientific literature and sells the product on Amazon among other vendors. Its formulation is focused on reducing fatigue, boosting energy, and improving both physical performance and mood.

Multiple clinical studies conducted on different classes of populations (e.g. elderly individuals, athletes, and patients affected by chronic fatigue syndrome) reported that Robuvit use resulted in an improvement of mental concentration, sleep, and recovery from fatigue and physical performances (Belcaro *et al*., 2014; Országhová *et al*., 2015; Vinciguerra *et al*., 2015).

Similarly, Atrantíl has been designed in order to provide daily digestive support and to counteract bloating, abdominal discomfort alone or accompanied by constipation or diarrhoea. The producers created a blend with tannins from quebracho and horse chestnut plants, able to inhibit the action of methane producing bacteria. Two studies investigated the efficacy of the product on patients suffering from bloating, constipation and abdominal discomfort. Atrantíl proved to be more than 80 percent effective in relieving the above-mentioned symptoms (Brown *et al*., 2016).

The Italian brand Kastania relies on the benefits of chestnut hydrolysable tannins. The company offers different formulations, always with tannins as a basis. In particular the main foci are intestinal wellbeing, the immune system and the antioxidant and anti-aging action (Kastania).

Table 1.5.1 Commercial products containing tannins.

PRODUCT	BIOACTIVE SUBSTANCE	EFFECTS	REFERENCE
Robuvit	Oak tannins (roburins, vescalagin and castalagin, grandinin and vescalin and other ellagitannins)	• fatigue reduction • boosting energy • physical performance improvement • mood improvement	(Belcaro et al., 2014; Országhová et al., 2015; Vinciguerra et al., 2015)
Atrantíl	Quebracho and Horse-chestnut tannins	• bloating • abdominal discomfort alone or accompanied by constipation or diarrhoea	(Brown et al., 2016)
Kastania	Chestnut hydrolysable tannins	• intestinal wellbeing preservation • immune system modulation • antioxidant effect • anti-aging action	(Kastania)
Pycnogenol	Procyanidins, bioflavonoids and organic acids from French maritime pine bark extract	• skin care • Respiratory and cardiovascular benefits • cognitive function preservation • joint support	(Horphag, 2021)

Finally, Pycnogenol® is a water-soluble flavonoid and represents a natural combination of constant proportions of procyanidins, bioflavonoids and organic acids. The French maritime pine bark extract is obtained from the bark of mono-species pine trees grown exclusively in Les Landes de Gascogne forest in southwest France. The forest is unspoiled and natural, with no pesticides, no herbicides. The seller focuses on four basic properties of the product– it's a powerful antioxidant, acts as a natural anti-inflammatory, selectively binds to collagen and elastin, and finally, it aids in the production of endothelial nitric oxide, which helps to vasodilate blood vessels. Starting form that, Pycnogenol® found a wide variety of applications, e.g. skin care, respiratory and cardiovascular health, cognitive function and joint support among others. The manufacturer, Horphag Research, has invested in extensive scientific research activity assuring the safety and efficacy of Pycnogenol®, with more than 450 scientific publications (counting clinical trials an reviews) (Horphag, 2021).

Sometimes there can exist mistrust on the part of consumers towards substances extracted from parts of plants that are not normally taken in the diet, such as the wood or the bark. However, as regards the EU, it has to be mentioned that EFSA (European Food Safety Authority) has the role of providing a science-based approach to assessing the safety of herbal substances and preparations. It sets out criteria to be taken into account when establishing conditions for the safe use of herbal substances and preparations.

The regulation (EU) 2015/2283 of the European parliament and of the council of 25 November 2015 states that "a food used before 15 May 1997 exclusively as, or in, a food supplement, as defined in Directive 2002/46/EC, should be permitted to be placed on the market within the Union after that date for the same use, otherwise it will be considered to be a novel food for the purposes of this Regulation. However, that use as, or in, a food supplement should not be taken into account for the assessment of whether the food was used for human consumption to a significant degree within the Union before 15 May 1997. Therefore, uses of the food concerned other than as, or in, a food supplement should be subject to this Regulation". Moreover, "the placing on the market within the Union of traditional foods from third countries should be facilitated where the history of safe food use in a third country has been demonstrated. Those foods should have been consumed in at least one third country for at least 25 years as a part of the customary diet of a significant number of people. The history of safe food use should not include non-food uses or uses not related to normal diets".

Where there is no information on human consumption before 15 May 1997 or the information available is insufficient, a simple and transparent procedure, involving the Commission, the Member States and food business operators, should be established for collecting such information. Thus, novel foods should be authorised

and used only if they fulfil the criteria laid down in Regulation (EU) 2015/2283. These products should not be placed on the market or used in food for human consumption unless they are included in a Union list of authorised novel foods.

In addition, the EU published the Compendium, which is a database created in order to help with the safety assessment of botanicals and botanical preparations intended for use in food, including supplements, by facilitating hazard identification. The Compendium does not list all of the bioactive substances present in a given botanical and their potential health effects. However, where adverse effects on animal health were found in the literature, they are reported.

The information reported includes:

- The botanical species and the family to which they belong. It is taken as indicated in the taxonomy database "the Plant List". If not found or if the scientific name of the botanical is not officially accepted ARSGRIN is used or the International Plant Name Index.

- The plant part containing the compounds of concern.

- The preparation, linked to the presence of a substance of possible concern or an adverse effect.

- The substances or chemical groups of concern. As the compendium is intended for hazard identification, information about the concentration in the product on the market, as well as the level of exposure of the consumer to this substance, are not reported, as this belongs to risk assessment.

- The effect description found in the scientific literature.

- The reference retrieved from literature searches for the data given.

Objectives

General objective

The aim of this thesis is to study the bioactivity of tannins extracted from different natural sources, analysing their antioxidant capacity and prebiotic activity, with the objective of designing new functional ingredients or dietary supplements. Thus, different strategies for administering these bioactive components are investigated in order to find the one that has the best impact on the gut microbiota composition and functionality.

Specific objectives

Each chapter was designed for a specific objective, as presented below.

- Chapter **1**

To unravel the possible use of tannin extracts derived from quebracho and chestnut wood and bark as potential functional food ingredients trough the study of the global antioxidant response, the production of short chain fatty acids (SCFAs) and the evolution of the polyphenolic profile after *in vitro* digestion and fermentation.

- Chapter **2**

To study the influence of source, chemical composition and extraction method on the bioactivity of different tannin extracts through *in vitro* gastrointestinal digestion.

- Chapter **3**

To identify the ideal amount of tannins for use as a dietary supplement to obtain the maximum benefit, after *in vitro* digestion and fermentation, in terms of antioxidant capacity exerted and stimulation of SCFA production.

- Chapter **4**

To investigate if the direct association between different tannin extracts and a particular food source could have modulatory effects on gut microbiota composition and quantification of SCFA release.

- Chapter **5**

To compare the performance of two different polysaccharides, alginate and an amidated pectin, as encapsulation matrices for tannins with the extrusion-gelation method, through the evaluation of microstructure, microencapsulation efficiency and release properties.

- Chapter **6**

To improve the pectin-tannin microbead system by adding a one-step gelatine-coating.

- Chapter **7**

To evaluate the performance of the microbeads over *in vitro* gastrointestinal digestion and fermentation, by analysing the antioxidant capacity exerted, the modulation of the intestinal microbiota composition and the production of SCFAs.

To investigate the effect of bypassing the step of enzymatic digestion of tannins on their bioactivity exerted in the intestine.

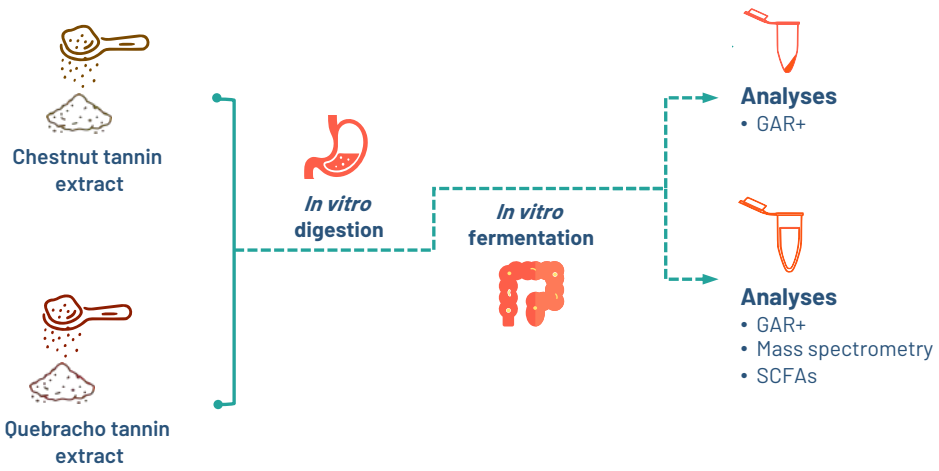
- Chapter **8**

To address the capacity of a tannin blend for promoting a healthy gut environment *in vivo*, through a 4-weeks nutritional intervention in healthy volunteers with tannin capsules, investigating the effects on gut microbiota composition and the production of SCFAs.

Materials and methods

3.1 Chapter 1

Study of antioxidant capacity and metabolization of quebracho and chestnut tannins through *in vitro* gastrointestinal digestion-fermentation



3.1.1 Tannin source plant, chemicals and reagents

Two tannin extracts were provided by Silvateam Spa (San Michele di Mondoví, Italia), as powder. The products were obtained by hot water extraction from quebracho wood (QUE), rich in condensed tannins, and chestnut wood and bark (CHE), characterized by the presence of hydrolysable tannins. Reagents and inulin were from Sigma-Aldrich (Germany) and Alpha-Aesar (United Kingdom), respectively. All chemical reagents used for all the assays, digestion, fermentation, as well as HPLC and UPLC-ESI-MS analysis were of analytical grade.

3.1.2 *In vitro* digestion and fermentation

In vitro digestion

Reagent set up

Table 3.1.1 summarizes the steps followed to set up the preparation of the simulated fluids (oral, gastric, and intestinal) necessary to perform the *in vitro* digestion. As regards enzymes, alpha-amylase from *Bacillus*, pepsin from porcine gastric mucosa, pancreatin from porcine pancreas and bile salts are needed for the procedure.

Table 3.1.1 Reagents needed for the preparation of the simulated fluids of *in vitro* digestion.

Initial salt solution		Simulated salivary fluid (pH 7)	Simulated gastric fluid (pH 3)	Simulated intestinal fluid (pH 7)
	Stock concentrations (mol/l)	Concentration in fluid (mmol/l)	Concentration in fluid (mmol/l)	Concentration in fluid (mmol/l)
KCl	0.5	15.09	6.9	6.8
KH_2PO_4	0.5	1.35	0.9	0.8
$NaHCO_3$	1	13.68	25	85
NaCl	2	-	47.2	38.4
$MgCl_2(H_2O)_6$	0.15	0.15	0.12	0.33
$NH_4(CO_3)_2$	0.5	0.06	0.5	-
$CaCl_2(H_2O)_2$	0.3	1.5	0.15	0.6
HCl	6	1.1	15.6	8.4

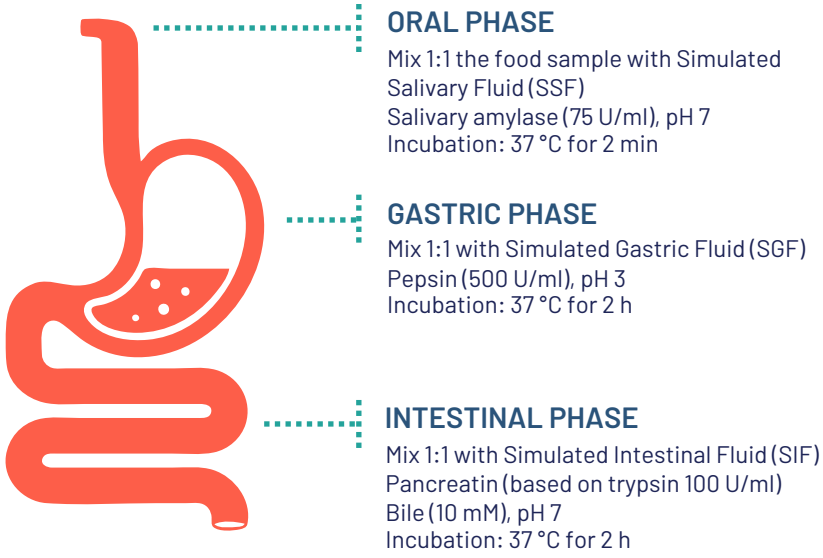


Figure 3.1.1 *In vitro* digestion process.

Procedure

In vitro digestion is composed of three steps that mimic human gastrointestinal conditions (**Figure 3.1.1**). The first step is the 'oral phase', in which 5 mL of simulated salivary fluid with α-amylase (150 U/ml) and 25 µl of $CaCl_2$ are added to 5 g of sample to be digested, following an incubation at 37 °C for 2 min. Then, for the 'gastric phase' 10 mL of simulated gastric fluid with pepsin (4000 U/ml) and 5 µl of $CaCl_2$ are added to the sample and the pH is lowered to 3.0 by adding 1 N HCl. The mixture is then incubated at 37 °C for 2 h. Finally, 'the intestinal phase' mimics the enzymatic digestion that occurs in the small intestine. For this, 20 mL of simulated intestinal fluid with bile salts (20mM), pancreatin (26.74 mg/ml) and 40 µl of $CaCl_2$ are added in the tube and the pH is raised to 7.0 with 1 N NaOH. The mixture is then incubated at 37 °C for 2 h. After that, the tubes are immersed in iced water to stop the enzymatic reactions. A centrifugation of the mixture at 5000 rpm for 10 min at 4 °C enables the separation of the solid fractions from the supernatants (potentially absorbable solution). Then, 10% of the supernatant is added to the solid residue to mimic the fraction that is not readily absorbed after digestion, while the remaining part is stored at -80 °C until further analysis. The digested wet-solid residues derived from the digestion process undergo the fermentation process.

In vitro fermentation

Reagent set up

- Phosphate buffer solution: prepare the phosphate buffer at 0.1 M concentration and adjust the pH to 7.0 with 1M HCl. Dissolve the reagent in less volume of milli-Q water than the final volume, adjust the pH and then, make up to the final volume with water. Phosphate buffer can be stored for up to a month at room temperature.

- Peptone solution: Prepare the peptone solution by dissolving 15 g of peptone in almost a litre of milli-Q water, adjust the pH to 7.0 and then make up to 1 L with water. Make freshly before use. Autoclave before use. Volume lost during autoclaving will have to be compensated with sterile milli-Q water.

- Reductive solution: Prepare the reductive solution by dissolving 312 mg of cysteine and 312 mg of sodium sulphide in 2 mL of 1M NaOH and make up the volume to 50 mL with milli-Q water. Make freshly before use. Cysteine is sensitive to thermal treatment so it cannot be autoclaved. Instead, reductive solution has to be prepared in sterile conditions and under anaerobic environment (80% N_2, 10% CO_2 and 10% H_2).

- Resazurin solution: Prepare resazurin solution at 0.1% (w/v). To prepare this solution, weigh 1 mg of resazurin and dissolve it in 1 mL of milli-Q water. Only 1.25 mL of resazurin solution is needed for each litre of fermentation medium. Make freshly before use. Autoclave before use. Volume lost during autoclaving will have to be compensated with sterile milli-Q water.

- Final fermentation medium: mix 1 litre of peptone solution with 50 mL of reductive solution and 1.25 mL of resazurin for each litre of fermentation medium.

Procedure

The fermentation process has been recently published (Pérez-Burillo *et al*., 2021). It can be divided into seven stages (**Figure 3.1.2**): fecal material collection, preparation of the equipment and reagents, setup of the *in vitro* digestion samples to be fermented, preparation of the fecal slurry, fermentation, sampling and sample processing. Each time fermentation was carried out for the experiments presented in this thesis, fecal material was collected from at least four volunteers and pooled together, in order to minimize the effect of inter-individual variation (David *et al*., 2014).

Figure 3.1.2 Scheme of the fermentation process.

As the aim was to investigate a healthy microbiota, feces were obtained from healthy individuals not taking antibiotics or any dietary supplement, with a normal BMI (between 18.5 and 24.99).

Collection was realized in sterile containers and wearing gloves to avoid any contamination. As far as possible, fecal material was collected the same morning when the experiment was going to be carried out. When it was not possible, the fecal material was stored at 4 °C for a maximum of 24 h. Preparation of the materials and reagents involves firstly the preparation of the fermentation medium with peptone and resazurin, oxygen removal by bubbling nitrogen through it and autoclaving it.

Cysteine and sodium sulfide (reductive solution) are added afterwards to avoid losing cysteine during the thermal treatment. Phosphate buffer for fecal slurry preparation is also made and autoclaved.

Different materials to be used during the experiment are also autoclaved: milli-Q water, pipette tips, and lab spoons. The fecal slurry is prepared at 32% feces (w/v) in phosphate buffer. Each fermentation tube carries 7.5 mL of medium, 2 mL of fecal slurry and 0.5 g of digested wet-solid residues from *in vitro* digestion. Tubes are kept at 37 °C with oscillating shaking at 20 rpm for 20 hours. Right after, microbial activity is stopped by placing the tubes on ice.

Sample deriving from the processes of *in vitro* digestion and fermentation could be processed for a multitude of analyses. **Table 3.1.2** summarises the type of the sample and the appropriate sampling and storage method required for the analyses performed in the present thesis.

Table 3.1.2 Sampling conditions for different analyses.

SAMPLE	SAMPLING AND STORAGE	ANALYSIS	FINALITY
Supernatant from digestion and Supernatant from fermentation	After stopping in ice the digestion/fermentation process, centrifuge the tubes (5000 rpm, 10 min at 4 °C). Pipette 1 ml of the supernatant for each analysis foreseen and store it at -80 °C	**Antioxidant capacity** **Metabolomics**	Measure the effect of the digestion and fermentation on the antioxidant capacity of a food sample Investigate the presence of metabolites and gut microbial functionality
Supernatant from fermentation	After stopping in ice the digestion/fermentation process, centrifuge the tubes (5000 rpm, 10 min at 4 °C). Pipette 1 ml of the supernatant for each analysis foreseen and store it at -80 °C	**SCFAs**	Investigate the prebiotic effect of the fermented sample
Raw sample from fermentation	Take 1 ml and right after centrifuge the tubes (16000xg, 2 min at 4 °C) to remove and discard the supernatant and keep the bacterial pellet. Store the tubes at -80 °C as soon as possible	**16S RNA sequencing**	Reveal gut microbial community structure

3.1.3 Antioxidant capacity

The antioxidant capacity was assessed on three fractions (the supernatants derived from digestion and fermentation and the solid residue remaining after fermentation) with two different methods, presented below: FRAP (ferric reducing ability of plasma) and ABTS (2,2-azinobis 3-ethylbenzothiazoline-6-sulfonic acid). In the case of the supernatants from digestion and fermentation, the liquid sample or its dilution was used directly. The solid residues from fermentation were analysed following the QUENCHER procedure described by Gökmen, Vural Serpena and Fogliano (2009). All the results were corrected considering their respective blanks (enzymes, chemicals and inoculum). For both assays the following material was used: 96-well transparent polystyrene microplate (Biogen Científica, Spain), FLUOStar Omega microplate reader (BMG Labtech, Germany).

$TEAC_{FRAP}$ assay

The $TEAC_{FRAP}$ assay is applied to determine the capacity of antioxidants to reduce the ferric 2,4,6-tripyridyl-s-triazine complex $[Fe^{3}\text{-}(TPTZ)_2]^{3+}$ to the intensely blue coloured ferrous complex $[Fe^{2}\text{-}TPTZ)_2]^{2+}$, in acidic conditions (pH 3.6). FRAP values are calculated by measuring spectrophotometrically the absorbance increase at 593 nm and relating it to a ferrous ions standard solution or to an antioxidant standard solution such as Trolox or ascorbic acid.

Reagent set up

- HCl solution: dissolve 330 µl of HCl 37% in 100 ml of distilled water, to obtain a solution 40 mM
- TPTZ solution: weight 0.0312 g and dissolve them in 10 ml of 40 mM HCl, to obtain a solution 10 mM
- Ferric chloride solution: weight 0.1352 g and dissolve them in 25 ml of distilled water
- Acetate buffer: weight 6.1 mg and dissolve them in 200 ml of distilled water. Adjust the pH to 3.6 and make up the volume to 250ml
- Daily FRAP solution: mix in the order 10 parts of the buffer, one part of ferric chloride solution and one part of TPTZ solution.

Procedure

The ferric reducing ability of each sample solution is estimated according to the procedure described by Benzie & Strain and adapted to a microplate reader (Benzie and Strain, 1996). Briefly, 280 µl of daily FRAP solution, prepared freshly and warmed at 37°C, are mixed in each well of a transparent 96-well polystyrene microplate with 20 µl of sample or water to provide appropriate blank reagent. For the solid samples remaining from fermentation, after a lyophilisation process, 30 mg were added to 6 ml of daily FRAP solution. Readings of maximum absorbance are taken at 595 nm every 60 s using a microplate reader. Temperature of 37°C has to be maintained for all reaction monitoring lasting 30 min. A Trolox stock solution is used to perform the calibration curves and results are expressed as mmol Trolox equivalents per g of fresh sample.

$TEAC_{ABTS}$ assay

$TEAC_{ABTS}$ assay use intensely coloured cation radicals of ABTS to test the ability of antioxidants to quench radicals. The procedure illustrated by Re *et al.* (1999) consists of the generation of the greenish blue $ABTS^{\bullet+}$ with an oxidation with potassium persulfate. The antioxidant activity is determined with the initial absorbance at 734 nm and the drop in absorbance. The more antioxidant a molecule is, the more it induces a decolorization, expressed as percentage of inhibition of ABTS+• (**Equation 3.1.1**) and determined from the ratio of test compound reaction to that of Trolox reaction, the reference standard (Pérez-Burillo *et al*., 2015).

Equation 3.1.1 Percentage of inhibition of ABTS•+

$$ABTS\ value = \frac{Inhibition\ by\ test\ compound}{Inhibition\ by\ trolox} \times 100$$

Reagent set up

- ABTS solution: weight 38.4 mg and dissolve them in 10 ml of distilled water, to obtain a 7 mM solution

- Potassium persulphate: weight 66.2 mg and dissolve them in 100 mm of distilled water, to obtain 2.45 mM solution

- ABTS$^{\bullet+}$ solution: mix solution ABTS and potassium persulphate at 50:50 and leave it in darkness for 16 hours before use it. The solution can be used during a week.

- Daily ABTS$^{\bullet+}$ solution: dilute ABTS$^{\bullet+}$ solution with ethanol solution 50% (v/v), until obtaining an absorbance of 0.70 ± 0.02, at 730 nm.

Procedure

After pipetting 20 µl of sample or Trolox standard solution, 280µL of daily ABTS$^{\bullet+}$ solution is added on a transparent 96-well polystyrene microplate. For the solid samples remaining from fermentation, after a lyophilisation process, 30 mg were added to 6 ml of daily ABTS solution. Absorbance readings are taken every 60 s for 20 min on a microplate reader, at a controlled temperature of 37°C. Trolox stock solutions are used to perform the calibration curves and results are expressed as mmol Trolox equivalents per g of fresh sample.

3.1.4 Global antioxidant response+ (GAR+)

GAR + reflects the overall antioxidant capacity of a product and is calculated as the resultant from the sum of the antioxidant capacity of the supernatant obtained after digestion (potentially bioaccessible fraction), the supernatant obtained after fermentation (potentially bioaccessible fraction) and the solid residue remaining after fermentation (non bioaccessible fraction). All the factors to be considered in estimating the correct antioxidant capacity through the digestion and fermentation processes are indicated in the supplemental information of Pérez-Burillo *et al.* (2008) (Pérez-Burillo *et al*., 2018).

3.1.5 Mass spectrometry (UPLC-DAD-ESI-QTOF-MS) assay

The metabolic profiling studies have been performed using UPLC coupled to MS, on tannin samples (Q1 and C1) submitted to *in vitro* digestion and fermentation. More specifically, the supernatants derived from digestion and the supernatants obtained after the fermentation process were analysed following the protocol of Dunn *et al.* (2011). Before the analysis, the samples needed a previous extraction process, which was achieved following the discontinuous extraction method, adapted to micro-extraction, described by Esteban Muñoz *et al.* (2018).

Sample preparation

One ml of each sample is mixed with diethyl ether (50:50, v/v) and left at 4 °C for 18 h under darkness. The supernatants are collected, and the pellets are mixed with diethyl ether (50:50, v/v). After 5 min the supernatants are collected, and the step is repeated once more. The obtained supernatants have to be combined, desiccated with anhydrous sodium sulphate and evaporated to dryness. The resulting residue is re-collected from the flask with 1 ml of a 50:50 water/methanol solution and the mixture well shacked. Finally, the extracts are filtered with 20 μm Waters Millipore membrane and stored at 4 °C until further use.

Procedure of the chromatographic analysis

Chromatographic separation was performed with an Acquity Ultra performance liquid chromatograph (UPLC) system (Waters, Milford), UPLC column—ACQUITY UPLC BEH C18 2.1 mm×100 mm, 1.7 μm (Waters) with precolumn in-line filter (Waters, Milford). The mobile phase consisted of solvents A (water containing 0.5% acetic acid) and B (100% acetonitrile). The column temperature was set at 40 °C, and the mobile phase flow rate at 0.4 mL/min. Analysis started with 95% A and 5% B; then the concentration of A was decreased to 5% (15 min). Finally, initial gradients were recovered over 0.10 min to re-equilibrate the column for 2.9 min.

Mass data, obtained on a Sinapt G2 TOF in negative ion electrospray ionization (ESI), were recorded in the range of 50–1200 m/z. Source operating parameters are defined hereafter: capillary and cone voltages are set at 2.5 kV and 35 V respectively, the desolvation temperature at 500 °C and the source temperature at 100 °C. UPLC system and Mass spectrometer were controlled by MassLynx® v4.1 software (Waters, Milford, USA). In particular, the analysis of MS spectra was performed with MarkerLynx, a suite included within the MassLynx software.

Identification of the peaks and quantification was carried out with standard solutions (for gallic acid, syringic acid, vanillic acid, p-coumaric acid, ellagic acid and quercetin). The determination of the compounds without standard was performed with the mass spectral data obtained on Q-TOF. The molecules separated by UPLC were analysed by MassLynx in their $[M-H]^-$ deprotonated molecular formulas together with the interpretation of MS fragmentations. The selected compounds were then searched against the Kyoto Encyclopaedia of Genes and Genomes (KEGG) and Chemspider. The peak area of the identified compounds was used to provide semi-quantitative information for comparison purposes.

3.1.6 SCFA analysis

The production of SCFAs as a measure of the gut microbiota functionality was assessed on supernatant deriving from fermentation of the different samples (Q1, C1 and inulin).

Sample preparation

Samples do not require any pre-treatment before injection. Supernatant from the fermentation process (1 ml) is centrifuged and filtered through a 0.22 μm nylon filter.

Procedure

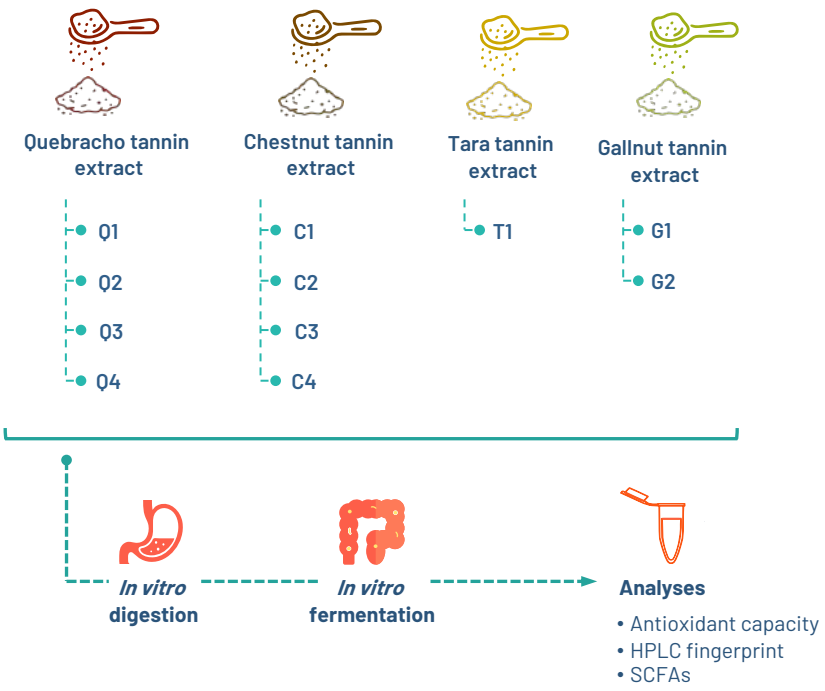
Three different SCFAs were determined: acetic, propionic, and butyric acids. Standard solutions were quantified with concentrations ranging from 10,000 to 5 ppm. Inulin, a commercial prebiotic fibre, was also analysed for comparison. The analysis of SCFAs was carried out on Accela 600 HPLC (Thermo Scientific). The mobile phase was 0.1 M phosphate buffer pH 2.8/acetonitrile 99:1 v/v delivered at a 1.25 mL/min flow rate; the column used was an Aquasil C18 reverse phase (Thermo Scientific) (150 × 4.6 mm, 5 μm), with a total run-time of 30 min. Detection was made at 210 nm with a UV–VIS PDA detector. The results were expressed as mmol of SCFA per g of dry food.

3.1.7 Statistical analysis

All measurements were performed at least in triplicate and expressed as means ± standard deviations. Statistical analysis of the data was carried out with Statgraphics Centurion XVI by using analysis of the unpaired Student's t-test in order to determine the statistical significance ($p < 0.05$) between different groups.

3.2 Chapter 2

Profiling of eleven tannin extracts deriving from different sources through *in vitro* digestion and fermentation



3.2.1 Tannin source plant

11 different tannin extracts produced by Silvateam spa. Four products were condensed tannin extract from quebracho wood (Q1, Q2, Q3 and Q4), four were ellagitannin extract from chestnut wood and bark (C1, C2, C3, C4), and three were gallotannin extracts obtained from tara pods (T1) or gallnuts (G1 ad G2). The samples differed from each other in chemical composition, extraction method or both, as indicated in **Table 3.2.1**.

Tannin extracts are obtained through extraction with hot water or with solvents, such as ethanol, methanol, acetone, methylethylketone, toluene. In the case of wood of chestnut or quebracho, the wood, after being separated from the bark, is chipped into small pieces of 1 - 4 cm and is charged in an extractor battery where it is extracted with water from 100 to 115°C, depending on the plant.

In some cases, a bisulphite solution is added to the hot water for quebracho wood extract, in order to increase the yield of the extraction. The addition of bisulphite solution to the water increases the extraction rate of tannins. The first obtained raw extract, containing around 5% of dry material, is then concentrated to 10% of dry material with a multiple effect concentrator. At this point, the extract is purified through flocculation and further elimination of the hemicelluloses and gums (Radebe *et al*., 2013; Caprarulo *et al*., 2021).

The tara tannin extract is obtained from the pods, collected, air dried, separated from the seeds, milled, and stored. Multiple extractions with organic solvents like acetone/water or ethanol/water mixtures are then performed and with the use of a concentrator, the solvents are stripped with vacuum and with several additions of water. The obtained water-dissolved extract is filtered and purified from sugars, salts and polysaccharides. The purified tannins solution is concentrated to 50% of dry material and spray-dried to powder.

Similarly to tara pods, the galls, after manual collection, air drying and storing, are milled. The extraction is performed several times with organic solvents like acetone, ethanol, ethyl acetate and their mixtures with water to regulate the yield and the purity. Then, the solvents are stripped with vacuum and with several additions of water, in a concentrator. The water-dissolved extract is collected, and filtration and purification are applied to eliminate sugars, hemicelluloses, gums, natural resins, and chlorophylls. In all of the cases, after obtaining a purified tannins solution concentrated to 50% of dry material, it is spray-dried to powder.

Table 3.2.1 List of the samples and the types of tannin extracts.

Product	Source	Method of extraction	Characteristic
Q1	Quebracho wood	Hot water extraction	High amount of insolubles in water
Q2		Hot water extraction + slight sulphation	Low amount of insolubles in water
Q3		Hot water extraction + sulphation	High salt content and high solubility in water
Q4		Hot water extraction, refined	Low in ash content, high solubility in water and in ethanol
C1	Chestnut wood and bark	Hot water extraction	High amount of insolubles in water
C2		Hot water extraction, clarified	Low amount of insolubles in water
C3		Hot water extraction, double clarified	w/o any insoluble compound
C4		Hot water extraction, sedimented	rich in hemicellulose and ellagic acid
T1	Tara pods	Organic solvent extraction	Low in ash content, high solubility in water and in ethanol
G1	Chinese gallnuts	Organic solvent extraction	Low in ash content, high solubility in water and in ethanol
G2	Turkish gallnuts	Organic solvent extraction	Low in ash content, high solubility in water and in ethanol

3.2.2 *In vitro* digestion and fermentation

All of the 11 extracts were submitted to *in vitro* digestion and fermentation according to the protocols previously described in Chapter 1. Briefly, for each sample, 1.5 g of tannin extract was added to falcon tubes along with oral, gastric and intestinal phases, mimicking their enzymatic, time and temperature conditions, to a final volume of 12 ml.

In vitro fermentation was performed at 37 °C for 20 h using faecal samples from five healthy donors (mean body mass index = 21.2; no antibiotics taken for four weeks prior to the assay). The faecal samples were pooled together to reduce the

effect of interindividual variability. The samples were then centrifuged, and the supernatants containing the fermenting microbiota were taken for the procedure.

After *in vitro* gastrointestinal digestion and fermentation, two fractions were picked for the analyses: the digested fraction (supernatant derived from digestion) and a fermented fraction (supernatant derived from fermentation).

3.2.3 Antioxidant capacity assays

The antioxidant capacity was measured on the original tannin powder as on the supernatant from *in vitro* digestion and fermentation with three different methods: Folin-Ciocalteu assay, FRAP assay and ABTS assay. For all the assays the following material was used: 96-well transparent polystyrene microplate (Biogen Científica, Spain), FLUOStar Omega microplate reader (BMG Labtech, Germany)

$TEAC_{FRAP}$ assay

The analyses were performed as previously described in Chapter 1. Briefly, 20 µL of sample were added to a 96-well plate, in triplicate, and mixed with 280 µL of FRAP reagent. The antioxidant reaction was monitored at 37 °C for 30 min. A calibration curve was prepared with Trolox in the range of 0.08–0.001 mg/ml. Results were expressed as mmol Trolox equivalent/g of original extract.

ABTS assay

The analyses were performed as previously described in Chapter 1. Briefly, 20 µL of sample were added to a 96-well plate, in triplicate, and mixed with 280 µL of ABTS daily reagent. The antioxidant reaction was monitored at 37 °C for 19 min. Calibration curve was prepared with Trolox in the range of 0.1–0.001 mg/ml. Results were expressed as mmol Trolox equivalent/g of original extract.

Folin-Ciocalteu assay

The test is generally applied as a method to measure total phenolic content (TPC) in a wide variety of food or vegetable extracts. The Folin-Ciocalteu method is based on the number of phenolic groups or other potential oxidizable groups present in compounds in the sample. The assay relies on the transfer of one electron from an antioxidant to molybdenum (Mo^{4+}), which is reduced, generating blue coloured molybdenum ions, Mo^{5+}, spectrophotometrically detectable at 750 – 765 nm. The results are generally reported relative to gallic acid.

Reagent set up

- Sodium carbonate solution at 10%: dissolve 10g of sodium carbonate in 100 ml of distilled water)

Procedure

As described by Moreno-Montoro *et al.* (2015), 195 µl of distilled water, 15 µl of Folin-Ciocalteu reagent and 60 µl of sodium carbonate are mixed in each well of a transparent 96-well polystyrene microplate with 30 µl of sample or water to provide an appropriate blank reagent. Readings are taken at the maximum absorbance of 745 nm using a microplate reader, during 60 min at 37°C. Gallic acid stock solutions (10 - 100 ppm) were used to perform the calibration curves; results were expressed as mol of gallic acid per g of original extract.

3.2.4 High Performance Liquid Chromatography (HPLC)

A Thermo scientific High Performance Liquid Chromatography was used with a C18 column to separate the pure, digested and fermented samples of all eleven tannins.

Sample preparation

Prior to treatment for the separation in the HPLC, 1.5 mg of pure sample was weighted and dissolved in 1 ml of distilled water. The supernatants deriving from the *in vitro* digestion and fermentation required an extraction process. 1 ml of sample + 1ml of diethyl ether were mixed and left at 4 °C for 18 h under darkness. The resulting supernatants were collected, while the pellet was mixed with diethyl ether (50:50, v/v). After 5 min the supernatants were collected, and the step was repeated once more. All the obtained supernatants were combined, desiccated with anhydrous sodium sulphate and evaporated to dryness. The resulting residue was re-collected from the flask with 1 ml of a 50:50 water/methanol solution and the mixture was well shacked. Finally, the extracts were filtered with 20 µm Waters Millipore membrane and stored at 4 °C until further use.

Procedure

A C18 Higher Chromatography column was used for the separation of all samples. A mobile phase composed of water-formic acid (2%) (solvent A) and acetonitrile (solvent B) was used. The gradient elution applied was: from 0 to 5min, 5% B, from 5 to 11 minutes, 5- 15% B, from 11 to 21 minutes, 15-30% B, from 21 to 28 minutes, 30-35% B, from 28 to 48 minutes, 35-80% B, from 48 to 58 minutes 5% B. This was followed by a recalibration of the column for 5 minutes in the initial conditions. The injection volume was 20 µl per sample. The wavelength used was 280nm. The wavelength 340nm was used for pure sample T4.

3.2.5 SCFA analysis

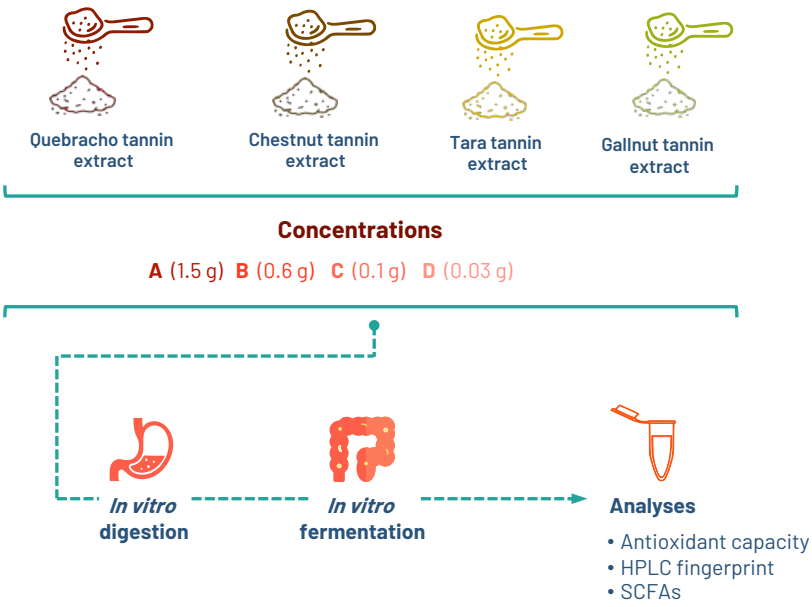
Acetic, propionic, and butyric acids were determined by HPLC. Samples did not require any pre-treatment before injection. Briefly, the SCFA standards were prepared in the mobile phase at concentrations ranging from 5 to 10,000 ppm. 1 mL of supernatant from the fermentation process was centrifuged to remove solid particles, filtered with a 0.22 μm nylon filter, and transferred to a vial for HPLC analysis. The results were expressed as mmol of SCFA per g of original extract.

3.2.6 Statistical analyses

All the statistical analyses were carried out using the SPSS software (version 23, SPSS, Chicago, IL, United States). Pearson's correlation coefficient was calculated to evaluate the linear relation between antioxidant capacities at $p < 0.01$. One-way ANOVA with Bonferroni *post-hoc* test was used to assess the statistical significance of the differences among samples, using as factors the type of extract (quebracho, chestnut, gallnut, tara pod), and type of sample (pure, digested and fermented).

3.3 Chapter 3

Effect of different concentrations of tannin extracts on their bioactivity and microbiota modulation



3.3.1 Tannin source plant

The four extracts that were found to be most representative and best performing of those analysed in the previous chapter were chosen for the present study. Thus, Q2 (quebracho wood extract), C2 (chestnut extract from wood and bark), T1 (tara pod extracts), G2 (gallnut extract) were investigated to evaluate different concentration to be submitted to *in vitro* digestion and fermentation.

3.3.2 *In vitro* digestion and fermentation

Four different amounts of the selected extracts were weighted. So, 1.5 g (A), 0.6 g (B), 0.1 g (C) and 0.03 g (D) of tannin extracts were submitted to *in vitro* digestion, in 12 ml final digestion fluids. The pure extracts followed all the steps of the *in vitro* digestion and fermentation previously described in **Chapter 1**. After digestion and fermentation, the supernatants were frozen at -80°C until further analysis.

3.3.3 Antioxidant capacity assays

Antioxidant capacity was assessed with three different techniques: Folin-Ciocalteu, $TEAC_{FRAP}$ and $TEAC_{ABTS}$ methods. All the assays were performed on the liquid fraction derived from both *in vitro* digestion and fermentation. For all the assays the following material was used: 96-well transparent polystyrene microplate (Biogen Científica, Spain), FLUOStar Omega microplate reader (BMG Labtech, Germany)

$TEAC_{FRAP}$ assay

The analyses were performed as previously described in Chapter 1. Briefly, 20 µL of either digestion or fermentation supernatants was added to a 96-well plate, in duplicate, and mixed with 280 µL of FRAP reagent (freshly prepared each day). The antioxidant reaction was monitored at 37 °C for 30 min. The calibration curve was prepared with Trolox in the range of 0.01–0.4 mg/mL. Results were expressed as mmol Trolox equivalent/kg of food.

$TEAC_{ABTS}$ assay

The method followed the protocol of Re *et al*. (1999) to measure the scavenging capacity in each sample. Briefly, 280 µL of ABTS reagent and 20 µL of digestion-fermentation supernatants were added to a 96-well plate. The antioxidant reaction was followed in triplicate for 30 min at 37 °C. A calibration curve was prepared with Trolox (0.1–0.001 mg/ml), and the results were expressed as mmol Trolox equivalent/g of original extract.

Folin-Ciocalteu assay

The analyses were performed as previously described in Chapter 2. In brief, as described by Moreno-Montoro and colleagues (2015), 30 µl of either digestion or fermentation supernatants was added in duplicate to a 96-well plate and mixed with 15 µl of Folin–Ciocalteu reactive, 190 µl of distilled water and 60 µl of 10% sodium carbonate solution. The antioxidant reaction was monitored at 37 °C for 60 min. The calibration curve was prepared with gallic acid in the range of 10 – 100 ppm. Results were expressed as mg gallic acid equivalent/kg of food.

3.3.4 SCFA analysis

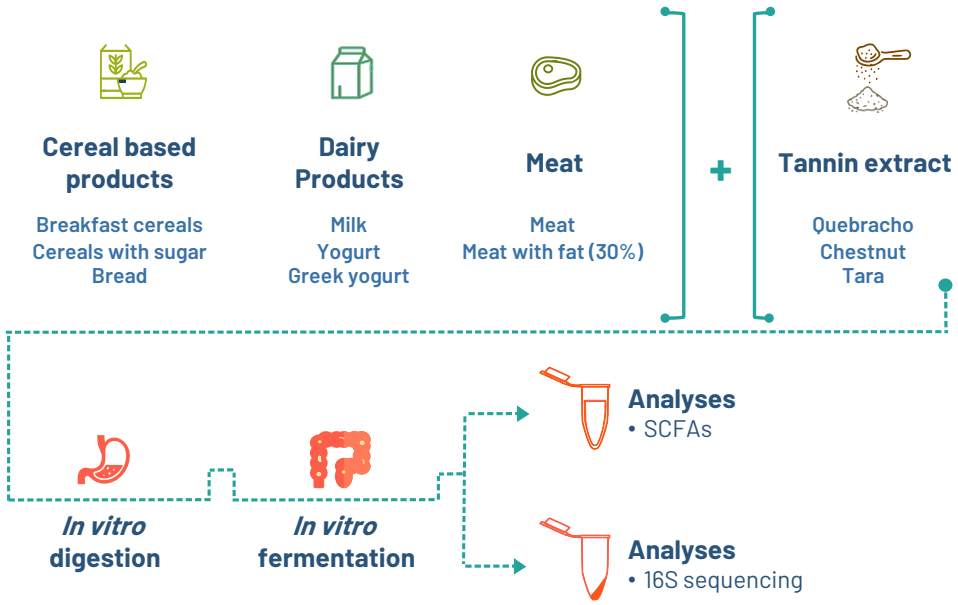
Short chain fatty acids production was assessed by analysing acetic, propionic, and butyric acids, according to the procedure described by Molino *et al*. (2018) (See **Chapter 1**). Results were expressed as mmol of SCFA per g of original extract.

3.3.5 Statistical Analysis

Pearson's correlation coefficient was calculated to show the linear relation between antioxidant capacity at $p < 0.01$. The statistical significance of the data was tested by one-way analysis of variance (ANOVA), followed by the Bonferroni *post hoc* test to compare the means that showed a significant variation ($p < 0.05$). The type of extract (quebracho, chestnut, gallnut, tara pod), and tannin concentration (A, B, C, D) were used as factors for ANOVA. All statistical analyses were performed with the SPSS software (version 23, SPSS, Chicago, IL, United States).

3.4 Chapter 4

Enrichment of food with tannin extracts promotes healthy changes in the human gut microbiota



3.4.1 Plant Material

Three natural tannin extracts were chosen, which are representatives for the three main categories of tannins: quebracho wood extract (QUE; rich in a profisetinidin condensed tannin), chestnut wood extract (CHE; principally characterized by the presence of hydrolysable ellagitannins), and tara pods extract (TE; mainly represented by hydrolysable gallotannins). The three selected extracts correspond to Q2, C2 and T1, previously described in **Chapter 2**.

The tannin content in the extracts, determined by the International Organization of Vine and Wine (OIV) method (Aurand, 2017), was 80% for QUE, 72% for CHE, and 92% for TE. All the extracts were purchased from Silvateam Spa (San Michele di Mondoví, Italia), as powder. The extraction methods were all food grade, characterized by a natural hot water extraction.

3.4.2 Sample preparation

Eight different food items, representing three types of products with different compositions in macronutrients were tested as potential tannin carriers: cereal-based foods [breakfast cereals (C), breakfast cereals with sugar (CS), and bread (B)], meat [meat (M) and meat with 30% fat (MF)], and dairy products [milk (L), low fat yogurt (Y), and full fat Greek yogurt (YG)]. All commercial products were bought in local supermarkets (Granada, Spain). The food samples were ground using an Ultraturrax (model T25, IKA, Spain) at 13,000 rpm. Then, the products were supplemented or not with 0.6% (w/w) of different tannin extracts (QUE, CHE, and TE). This amount of tannin extract added to food was decided based on our previous studies. Since tannins can exert antimicrobial activity, it was critical to find a concentration effective for our purposes but not high enough as to affect negatively the gut microbes. On the other hand, we should use an amount of extracts that does not alter the flavour or the structure of the food matrix. The samples were aliquoted and stored at −80°C until the *in vitro* digestion and fermentation processes.

3.4.3 16S rRNA amplicon sequencing

Sample preparation

DNA Extraction Genomic DNA was extracted from the solid residues deriving from the fermentation process using the MagNA Pure LC JE379 platform (Roche) and DNA Isolation Kit III (Bacteria, Fungi; REF 03264785001), following the manufacturer's instructions, with a previous lysis with lysozyme at a final concentration of 0.1 mg/ml.

After extraction, DNA integrity has to be determined by agarose gel electrophoresis (0.8% w/v agarose in Tris-acetate- EDTA buffer) and DNA samples are quantified using a Qubit 3·0 Fluorometer (Invitrogen). All DNA samples were stored at −20°C until further processing.

Procedure

High-Throughput Amplicon Sequencing Total DNA (12 ng) was used as template for the amplification of the V3-V4 hypervariable region of the 16S rRNA gene. PCR primers were used as described by Klindworth *et al.* (2013), using the forward primer (5'-TCGT CGGC AGCG TCAG ATGT GTAT AAGA GACA GCCT ACGG GNGG CWGCA-G3') and reverse primer (5'-GTCT CGTG GGCT CGGA GATG TGTA TAAG AGAC AGGA CTAC HVGG GTAT CTAA TCC3').

We followed for the library construction the Illumina protocol for the small subunit ribosomal RNA gene (16S rRNA) Metagenomic Sequencing Library Preparation (Cod 15044223 RevA). Primers were fitted with adapter sequences added to the gene-specific sequences to make them compatible with the Illumina Nextera XT Index kit. Then, the amplicons were sequenced in an Illumina MiSeq sequencer according to the manufacturer's instructions in a 2 × 300 cycles paired-end run (MiSeq Reagent kit v3). The data for the present study were deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJEB41013.1

3.4.4 Bioinformatic Analyses

The sequence processing, assembly, amplicon sequence variants (ASVs) generation and annotation were performed in the DADA2 (v1.8.0) package from R (v3.6.0; Callahan *et al*., 2016). The filter and trimming parameters used were the following: maxN = 0, maxEE = c(2,5), truncQ = 0, trimLeft = c(17,21), truncLen = c(270,220), and rm.phix = TRUE. The merging process of the forward and reverse reads required a minimum overlap of 15 nucleotides and a maximum mismatch of 1.

The reads were aligned using Bowtie2 against the human genome (GRCh38.p11) and matches were subsequently discarded (Langmead and Salzberg, 2012). The ASVs were generated by clustering sequences with 100% identity. Taxonomic annotation was assigned by comparison to the SILVA 132 reference database using DADA2 v. 1.12 (Quast *et al*., 2013). Annotation was assigned at species level for 100% similarity matches or at the deepest possible taxonomic level in other cases.

3.4.5 SCFA analysis

Short chain fatty acids production was assessed by analysing acetic, propionic, and butyric acids, according to the procedure described by Molino *et al*. (2018) (See **Chapter 1**).

After the fermentation process, 1 ml of supernatant from the fermentation was centrifuged to remove solid particles, filtered through a 0.22 μm nylon filter, and finally transferred to a vial for UV-HPLC analysis. The sample did not require any pre-treatment before injection. The results were expressed as mmol of SCFAs per ml of fermented soluble fraction.

3.4.6 Statistical Analysis

Amplicon sequence variants with less than 10 counts in total were discarded. The ASV count table was normalized by total-sum scaling (TSS). Alpha and beta diversity measures were computed using various packages in the R platform. The Shannon diversity index, Chao1, and ACE richness estimators and Bray-Curtis dissimilarity index were obtained with the Vegan library (v2.5-2; Oksanen *et al*., 2012). Phylogeny-based measures such as Faith's phylogenetic diversity (PD) and the weighted UniFrac distance were computed using the picante (v1.8.2; Kembel *et al*., 2010) and GuniFrac packages (v1.1; Chen *et al*., 2012), respectively, after sequence alignment with msa (v1.4.3; Bodenhofer *et al*., 2015) and UPGMA tree-building with phangorn (v2.5.5; Schliep, 2011). In addition, principal component analysis (PCA), principal coordinate analysis (PCoA), and heatmaps were generated with R scripts.

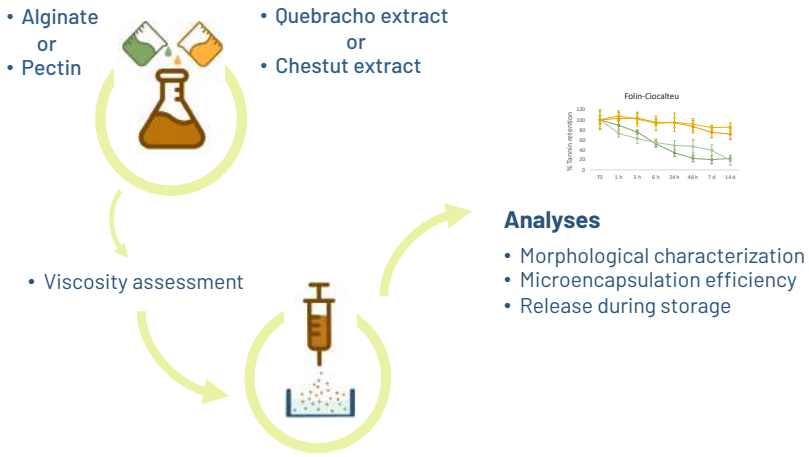
Wilcoxon signed-rank tests with false discovery rate (FDR) adjustment for multiple comparisons were employed to evaluate differences in richness, diversity, and relative abundance of taxa among samples.

The linear discriminant analysis (LDA) effect size (LEfSe) algorithm was applied to identify taxonomical biomarkers from different tannins (Segata *et al*., 2011). It combines Kruskal-Wallis and pairwise Wilcoxon rank-sum tests for statistical significance assessment and feature selection. Default parameters were used for significance ($p < 0.05$) and linear discriminant analysis threshold (<2.0).

Results of SCFA production were expressed as mean values of triplicates ($n = 3$) ± SD. One-way ANOVA with Bonferroni post-test correction was performed with the SPSS software (version 23, SPSS, Chicago, IL, United States) to determine significant differences among mean values on all the measured parameters.

3.5 Chapter 5

Tannin-rich extracts improve the performance of amidated pectin as an alternative microencapsulation matrix to alginate



3.5.1 Materials

Pure (>99%) food grade sodium alginate grade Manugel GHB (60–65% G, 50–100 mPa s) was kindly donated by DuPont Nutrition & Health (Norway) and its detailed characterisation was previously reported by Gómez-Mascaraque *et al.* (2019). Pectin AGLUPECTIN LA-20P (degree of amidation 22 - 25, degree of methoxylation 20 - 25, protein content 0.5% (w/w)) was supplied by JRS Silvateam Food Ingredients S.r.l.

Tannin extracts from quebracho (QUE) and chestnut (CHE) were provided by Silvateam Spa as powder and correspond to Q2 and C2 previously described in **Chapter 2**. Both QUE and CHE were obtained by hot water extraction from wood and bark, respectively. The detailed composition of QUE was previously reported by Pasch, Pizzi, & Rode (2001) and it is a phytocomplex constituted by profisetinidin condensed tannins with a degree of polymerization (DP) up to 6.25 (Pasch *et al*., 2001). The composition of CHE, consisting of a mix of hydrolysable ellagitannins, with 30% of isomers castalagin and vescalagin as representative substances, was previously studied by Pasch & Pizzi (2002). Reagents calcium chloride dihydrate, sodium citrate and potassium bromide were obtained from Sigma-Aldrich (Ireland).

3.5.2 Preparation of the feed formulations

Alginate 1.5% (w/v) and pectin 2% (w/v) aqueous solutions were prepared by magnetic stirring at room temperature. These concentrations were selected according to preliminary trials, achieving the maximum concentration which could be pumped through a 100 μm nozzle as described in **Section 3.5.4**. Lower concentrations were not sufficient to form uniform spheres. Increasing concentrations of QUE and CHE with respect to the mass of the different hydrocolloids (0, 10 and 20% w/w) were subsequently added to the solutions and mixed until a homogeneous dispersion was obtained. For alginate, the ratio of 20 % w/w of tannins did not allow to obtain microbeads due to obstruction of the nozzle. **Table 3.5.1** summarises the coding used to indicate the different systems developed.

Table 3.5.1 Coding used to indicate the different systems developed. Aqueous solution of alginate was prepared at 1.5% (w/v), while that of pectin was prepared at 2% (w/v). The concentrations of tannin extracts are referred with respect to the mass of the different hydrocolloids used (w/w). QUE, quebracho tannin extract; CHE, chestnut tannin extract.

Initial feed formulations		**Freeze-dried microbeads**	
AI	Alginate	A	Alginate
AC10I	Alginate + CHE 10% w/w	AC10	Alginate + CHE 10% w/w
AQ10I	Alginate + QUE 10% w/w	AQ10	Alginate + QUE 10% w/w
AC20I	Alginate + CHE 20% w/w		
AQ20I	Alginate + QUE 20% w/w		
PI	Pectin	P	Pectin
PC10I	Pectin + CHE 10% w/w	PC10	Pectin + CHE 10% w/w
PQ10I	Pectin + QUE 10% w/w	PQ10	Pectin + QUE 10% w/w
PC20I	Pectin + CHE 20% w/w	PC20	Pectin + CHE 20% w/w
PQ20I	Pectin + QUE 20% w/w	PQ20	Pectin + QUE 20% w/w

3.5.3 Rheological properties of the formulations

The viscosity of the prepared aqueous solutions was analysed with an AR-2000 rheometer (TA Instruments, USA) with a parallel plate geometry (60 mm diameter and 500 μm gap) following a procedure adapted from Gómez-Mascaraque *et al.* (2015). Continuous shear rate ramps were performed from 0.1 to 200 s^{-1} over 12 min at a controlled temperature of 20 °C, following pre-shear treatment at 1 s^{-1} for 10 s and equilibration for 1 min.

3.5.4 Preparation of hydrogel capsules

All solutions were filtered through 0.8 μm pore syringe filters for aqueous media (Sartorius, Germany). Microbeads were produced according to Gómez-Mascaraque *et al.* (2019) with slight modifications. We used an Inotech Encapsulator IER-50 (Inotech Biosystems Intl. Inc., Switzerland), to generate alginate or pectin microbeads by extrusion of the solutions through a 100 μm nozzle at a flow rate of 2.5 mL/min into the gelling bath (140 mm diameter) containing 250 mL of 0.1 M $CaCl_2$ solution. The gelling bath was located at a distance of 16 cm from the nozzle and maintained under constant agitation. Alginate or pectin droplet formation and break up was aided by a nozzle vibration frequency of 1240 Hz and an applied voltage of 1.3 kV, as optimized in preliminary trials. The collection time was set at 4 min for each batch, and the microbeads were cured within the gelling solution for

90 min before being filtered and thoroughly washed with deionized water. The produced microbeads were stored at -80 ºC and freeze-dried with a FreeZone benchtop freeze drier (Labconco, USA), until further analysis.

3.5.5 Water content

To estimate the water content, the capsules (ca. 1 g) were filtered and accurately weighted before and after freeze-drying. The quantity of water was calculated according to **Equation 3.5.2**, where m_h is the mass of the hydrated capsules and m_d is the mass of the dried capsules. Measurements were performed in three independent batches.

Equation 3.5.1 Water content

$$Water\ (\%) = \frac{m_h - m_d}{m_h} \times 100$$

3.5.6 Morphological characterization of the hydrogel capsules

Capsule morphology was studied by optical microscopy, taking images at 4x magnification using a digital microscopy system Olympus BX51 (Olympus Corporation, Japan). A digital camera head ProgRes CT3 (Jenoptik, Jena, Germany) and ProgRes CapturePro software (v 2.10.0.0) were used for image capturing. Size distributions were obtained from a minimum of 200 measurements, using the ImageJ (v. 1.52q) software.

3.5.7 Tannin content and antioxidant activity

The content of tannins in the microbeads was estimated using two different methods, summarised below: UV 280 nm assay and Folin-Ciocalteu (quantification of phenolic content) assay. Their antioxidant activity was also assessed through the ABTS assay. Both the feed solutions and the microbeads were analysed. A weight of 30 mg of dried capsules was dissolved in 5 ml of sodium citrate (2% w/v) solution under magnetic stirring for 24h, in order to dissolve the polysaccharides and release their contents.

UV 280 nm assay

The tannin content was directly determined by measuring the optical density at 280 nm using a 10-mm quartz cuvette (Piccardo and González-Neves, 2013), with a Cary 100 Bio UV-Vis spectrophotometer (Agilent, Ireland).

Calibration was performed using aqueous solutions of the original tannin extracts (5 – 100 ppm) as reference standards.

Folin-Ciocalteu assay

The protocol of Moreno-Montoro *et al.* (2015) was slightly modified. Briefly, 60 µL of sodium carbonate (10%) were mixed with 15 µL of Folin-Ciocalteu reagent, 30 µL of sample and 195 µL of distilled water. The samples were incubated at 37 °C for 60 min, before measuring the absorbance at a wavelength of 760 nm. The amount of phenolic compounds was determined according to a calibration curve obtained with aqueous solutions of the original tannin extracts, CHE or QUE, (1 – 100 ppm) as reference standards.

ABTS assay

The antioxidant capacity was estimated in terms of radical scavenging activity, which was evaluated following the procedure by Re *et al.* (1999). Calibration was performed with aqueous solutions of the original tannin extracts, CHE or QUE, used as reference standard (1 – 50 ppm).

For ABTS and Folin-Ciocalteu assays, a Synergy HT microplate reader (Bio-Tek Instruments, USA) with temperature control (37°C) was used to measure the absorbance at the afore mentioned wavelengths on transparent 96-well polystyrene microplates (Biogen Científica, Spain).

All the measurements were performed in triplicate on independent duplicates and results were expressed as % mg tannins/mg microbeads. These three different techniques were used as direct or indirect methods to estimate the presence of tannins in the measured samples. All of them were used to calculate the microencapsulation efficiency (ME%) for both fresh and dried microbeads, and to study the effect of storage on the retention of tannins.

3.5.8 Microencapsulation efficiency (ME %)

The ME % was calculated according to the following equation.

Equation 3.5.2 Microencapsulation efficiency

$$ME(\%) = \frac{TTC_m}{TTC_i} \times 100$$

where TTC_m was the total tannin content in the microbeads, and TTC_i was the total tannin content measured in the initial feed solutions used for microencapsulation, estimated as described in **Section 3.5.7**.

3.5.9 Tannin retention during storage

To study the effect of storage of fresh capsules on the retention of tannins, the content of tannins within the samples was analysed at different times during storage. The first sampling was carried out after 90 min of curation in the gelling bath (T0). After that, 7 batches of 1.5 g of fresh capsules were washed with distilled water and stored in 10 ml of distilled water, under refrigeration (at 4°C). After selected time intervals (1h, 3h, 6h, 24h, 48h, 7d, 14d) one batch of samples was filtered, stored at -80 ºC and freeze-dried until further analysis. Tannin retention is expressed as percentage of the tannins initially encapsulated remaining in the capsules after each selected time period (**Equation 3.5.4**).

Equation 3.5.3 Tannin retention

$$Tannin\ retention\ \% = \frac{TTC_{Tx}}{TTC_{T0}} \times 100$$

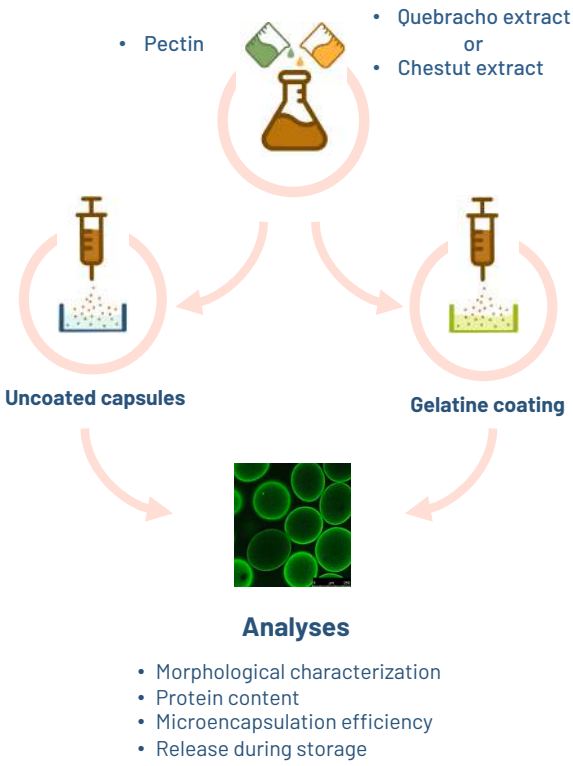
where TTC_{Tx} was the total tannin content in the microbeads measured at each of the selected time intervals and TTC_{T0} was total tannin content in the microbeads measured at T0.

3.5.10 Statistical analysis

One-way ANOVA with Bonferroni post-hoc test was used to assess the statistical significance of the differences among samples. To compare two independent samples *t*-Student's test was performed. All the statistical analyses were performed using the SPSS software (version 23, SPSS, Chicago, IL, United States).

3.6 Chapter 6

Impact of gelatine coating on the performance of tannin-loaded pectin microbeads obtained through external gelation



3.6.1 Materials

Pectin AGLUPECTIN LA-20P (degree of amidation 22 - 25, degree of methoxylation 20 - 25, protein content 0.5% (w/w)) was supplied by JRS Silvateam Food Ingredients S.r.l. (Italy). Gelatine from porcine skin, with reported gel strength of 175 g Bloom, was supplied by Sigma-Aldrich (Ireland). Tannin extracts from quebracho (QUE) and chestnut (CHE) were provided by Silvateam Spa (Italy), as powder, and correspond to Q2 and C2, previously described in **Chapter 2**.

Both QUE and CHE were obtained by hot water extraction from wood and bark. QUE was previously characterized by Pasch *et al*. (2001) and it is a phytocomplex constituted by profisetinidin condensed tannins with a polymerization degree up to 6.25 (Pasch *et al*., 2001). The detailed composition of CHE, consisting of a mix of hydrolysable ellagitannins, with 30% of isomers castalagin and vescalagin as representative substances, was previously described by Pasch & Pizzi (2002). Fast green FCF, calcium chloride dihydrate, sodium citrate and potassium bromide were obtained from Sigma-Aldrich (Ireland).

3.6.2 Preparation of the feed formulations

Porcine gelatine was dissolved in distilled water at 40 °C, under magnetic stirring. Then it was incorporated to $CaCl_2$ solution, to obtain a final $CaCl_2$ concentration of 0.1 M and gelatine concentration of 1% (w/v), avoiding gelation at room temperature. Pectin 2% (w/v) aqueous solution was prepared by magnetic stirring at room temperature. This concentration was selected according to our preliminary trials, achieving the maximum concentration which could be pumped through a 100 μm nozzle, as described in **Section 3.6.3.** Lower concentrations were not sufficient to form spheres. Increasing concentrations of QUE and CHE with respect to the mass pectin (0, 10 and 20% w/v) were subsequently added to the solutions and mixed until a homogeneous dispersion was obtained.

3.6.3 Preparation of the hydrogel capsules

Pectin solutions were filtered through 0.8 μm pore syringe filters for aqueous media (Sartorius, Germany). Microbeads were produced according to Gómez-Mascaraque *et al.* (2019) with slight modifications. We used an Inotech Encapsulator IER-50 (Inotech Biosystems Intl. Inc., Switzerland) to generate microbeads by extrusion of the pectin solutions through a 100 μm nozzle at a flow rate of 2.5 mL/min into the gelling bath (140 mm diameter) containing 250 mL of $CaCl_2$ solution or $CaCl_2$ solution added with gelatine. The gelling bath was located at a distance of 16 cm from the nozzle and maintained under constant agitation. Pectin

droplet formation and break up was aided by a nozzle vibration frequency of 1240 Hz and an applied voltage of 1.3 kV, as optimized in preliminary trials. The collection time was set at 4 min for each batch, and the microbeads were cured within the gelling solution for 90 min before being filtered and thoroughly washed with deionized water. The produced microbeads were directly analysed. **Table 3.6.1** summarises the coding used to indicate the different systems developed.

Table 3.6.1 Coding used to indicate the different systems developed. Aqueous pectin solution was prepared at 2% (w/v). The concentrations of tannin extracts are referred with respect to the mass of the pectin (w/w). Porcine gelatine was incorporated to $CaCl_2$ solution, for a final concentration of 1% (w/v). QUE, quebracho tannin extract; CHE, chestnut tannin extract.

	Type of tannin	Tannin concentration (w/w)	Gelatine
P	-	-	✗
PC10	CHE	10	✗
PQ10	QUE	10	✗
PC20	CHE	20	✗
PQ20	QUE	20	✗
PG	-	-	✓
PC10G	CHE	10	✓
PQ10G	QUE	10	✓
PC20G	CHE	20	✓
PQ20G	QUE	20	✓

3.6.4 Water content

To estimate the water content, the capsules (ca. 1 g) were filtered and accurately weighted before and after freeze-drying. The quantity of water was calculated in accordance with the **Equation 3.5.2**, illustrated in **Chapter 5**. Measurements were performed in three independent batches.

3.6.5 Morphological characterization of the hydrogel capsules

The morphology of fresh capsules was studied by optical microscopy, taking images at 4x and 10x magnification using a digital microscopy system Olympus BX51 (Olympus Corporation, Japan). A digital camera head ProgRes CT3 (Jenoptik,

Jena, Germany) and ProgRes CapturePro software (v 2.10.0.0) were used for image capturing. Size distributions were obtained from a minimum of 200 measurements, by using the ImageJ software (v. 1.52q).

The microstructure of the freeze-dried microbeads was also studied by scanning electron microscopy (SEM). Samples were stuck to the sample holders using carbon tape and sputter-coated with chromium under vacuum, using an Emitech K575X sputter coater (Quorum Technologies, UK). The samples were then imaged using a Gemini field emission scanning electron microscope (ZEISS, Germany) equipped with a secondary electron detector, at an accelerating voltage of 2 kV and a working distance of 4-6 mm.

Fresh capsules were also observed using a Leica TCS SP5 confocal laser scanning microscope (CSLM) (Leica Microsystems CMS GmbH, Wetzlar, Germany) after staining the protein with Fast green FCF. 100 µL of 0.1% Fast green FCF (aq.) were added to 1.5 mL of microbead suspensions. Aliquots of the stained samples were then transferred to cavity slides and covered with 0.13 mm coverslips. Fast green FCF was excited at 633 nm using a He/Ne laser, and the corresponding emission filter was set at 660-710 nm. Leica LAS AV software (v 2.7.3.9723) was used to acquire digital images of 1024 x 1024 pixels in size.

3.6.6 Determination of gelatine content in the capsules

The protein content of the gelatine-coated capsules was estimated by measuring the nitrogen content of the samples through the Dumas method (L. G. Gómez-Mascaraque, Martínez-Sanz, Fabra, *et al*., 2019), using a LECO FP-528 N Analyzer (LECO Instruments UK Ltd., Cheshire, UK).

As the pectin used for the present study was amidated and thus contained nitrogen, the nitrogen content of the capsules prepared in the absence of gelatine was also measured and subtracted from the total nitrogen content obtained for the gelatine-coated capsules. The obtained difference was then multiplied by a nitrogen conversion factor of 6.17, which was the experimental percentage of nitrogen in the commercial gelatine obtained through the same Dumas method (**Equation 3.6.1**). The determinations were conducted in triplicate.

Equation 3.6.1 Gelatine content %.

$$\%\ Gelatine\ content = (\%N_{CPG} - \%N_{CP}) * 6.17$$

where $\%N_{CPG}$ is the nitrogen content in gelatine-coated pectin capsules and $\%N_{CP}$ is the nitrogen content in their non-coated correspondent.

3.6.7 Tannin content and antioxidant activity

Two different methods have been used to estimate the tannin content in the microbeads: UV 280 nm assay and Folin-Ciocalteu (quantification of phenolic content) assay. The antioxidant activity was evaluated with the ABTS assay. The analyses were conducted on both the feed solutions and the microbeads. A weight of 30 mg of prepared dried capsules was dissolved in 5 ml of sodium citrate (2% w/v) solution, during 24h, in order to release their contents.

The methodology used to carry out the analysis with the three different techniques has been previously illustrated in **Chapter 5**.

3.6.8 Microencapsulation efficiency (ME%)

The ME% was calculated following the equation described by **Equation 3.5.2**, in **Chapter 5**.

3.6.9 Tannin retention during storage

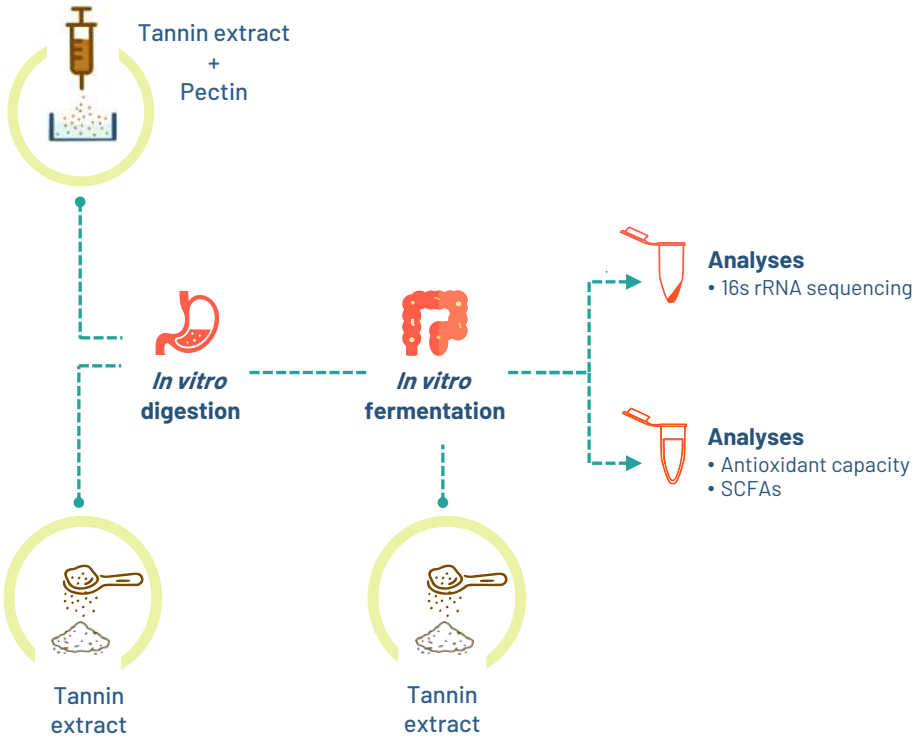
The microbeads were stored and analysed at different times, for a 14-day period, to study the effect of storage of fresh capsules on the retention of tannins. The first sampling was carried out after 90min of curation in the gelling bath (T0). After that, 7 batches of 1.5 g of fresh capsules were washed with distilled water and stored in 10 ml of distilled water, under refrigeration (at 4°C). After selected time intervals (1h, 3h, 6h, 24h, 48h, 7d, 14d) two batches of samples was filtered, stored at -80 ºC and freeze-dried until further analysis. Tannin retention was expressed as percentage of tannins remaining in the microbeads, after each selected time period, in relation to the amount initially encapsulated (**Equation 3.5.3**, Chapter 6).

3.6.10 Statistical analyses

t-Student's test was performed to compare two independent samples. The statistical significance of the differences among samples was calculated by one-way ANOVA with Bonferroni *post-hoc*. All the statistical analyses were performed using the SPSS software (version 23, SPSS, Chicago, IL, United States).

3.7 Chapter 7

Effects of pectin-encapsulated and unencapsulated tannins on gut microbiota composition and short chain fatty acid production



3.7.1 Materials

Pectin AGLUPECTIN LA-20P and gelatine from porcine skin were supplied from JRS Silvateam Food Ingredients S.r.l. (Italy) and Sigma-Aldrich (Ireland), respectively. Silvateam Spa kindly provided the tannin extract powder from quebracho (QUE) and chestnut (CHE), corresponding to Q2 and C2, previously described in **Chapter 2**.

Both extracts were obtained by hot water extraction from wood and bark. QUE is a phytocomplex characterised by the presence of condensed tannins, deriving from profisetinidin, with a degree of polymerization up to 6.25 (Pasch *et al*., 2001). CHE consists of an hydrolysable ellagitannins mix, of which 30% is constituted by castalagin and vescalagin (Pasch and Pizzi, 2002).

3.7.2 Preparation of the feed formulations

Pectin 2% (w/v) aqueous solution was prepared by magnetic stirring at room temperature and increasing concentrations of QUE and CHE with respect to the mass of pectin (0, and 20% w/v) were added and mixed until a homogeneous dispersion was obtained. The concentration of tannins to be added to the feed formulation was established based on the results of preliminary studies.

3.7.3 Preparation of the hydrogel capsules

All the pectin solutions were filtered through 0.8 μm pore syringe filters for aqueous media (Sartorius, Germany). Microbeads were produced according to the protocol of Gómez-Mascaraque *et al.* (2019), with slight modifications. Briefly, microbeads were produced with an InotechEncapsulator IER-50 (Inotech Biosystems Intl. Inc., Switzerland), by extrusion of the feed solutions through a 100 μm nozzle at a flow rate of 2.5 mL/min into the gelling bath (140 mm diameter) containing 250 mL of 0.1 M $CaCl_2$ solution. The gelling bath was located at a distance of 16 cm from the nozzle and maintained under constant agitation. The formation of pectin droplets and their breakup was performed with a nozzle vibration frequency of 1240 Hz and an applied voltage of 1.3 kV. The collection time was set at 4 min for each batch, subsequently followed by a curation within the gelling solution of 90 min. After that, microbeads were filtered and thoroughly washed with deionized water. The produced microbeads were stored at -80 ºC and freeze-dried with a FreeZone benchtop freeze drier (Labconco, USA), until further analysis.

3.7.4 *In vitro* digestion and fermentation

In order to study the effect of the microencapsulated tannins, a quantity of microbeads containing a total of 30 mg of tannin extracts was weighed (CHE and QUE). For comparison purposes 30 mg of tannin extracts were also submitted to digestion and fermentation. The *in vitro* digestion was performed following the protocol described by Pérez-Burillo *et al.* (2018a), with slight modifications. Briefly, the microbeads or tannin extracts were added to falcon tubes and subjected to three steps, resembling human oral, gastric and intestinal digestion. The oral phase was mimicked with α-amylase for 2 min, at pH 7. For the gastric phase, pepsin was added with an incubation of 2 h, at pH 2–3. The gastric phase was performed with bile salts and pancreatin for 2 h, at pH 7. All the incubations were performed under agitation and at a controlled temperature of 37 °C. A liquid soluble fraction and a solid fraction were separated from the digestion mixture, after a centrifugation at 1000 rpm for 10 min at 4 °C. 10% of the supernatant was added to the solid residue to mimic the fraction that is not readily absorbed after digestion, while the remaining part was stored at −80 °C until further analysis.

500 mg of the digested wet-solid residues derived from the digestion process, or the corresponding amount of undigested tannin extracts, were subjected to fermentation. *In vitro* fermentation was performed at 37 °C for 20 h, as described by Pérez-Burillo *et al.* (2021). Fecal samples from three healthy donors (mean body mass index = 21.2; no antibiotics taken for three months prior to the assay) were pooled together to account for interindividual variability. After a centrifugation (10 min, at 500 rpm), the supernatants were recovered for analysis. A control fermentation was performed using only the fecal inoculum in fermentation solution (containing peptone, cysteine, and resazurin). Fermentations were performed in triplicate. After *in vitro* digestion and fermentation, three fractions were obtained: two liquid fractions, one from digestion and one from fermentation, and one solid fermented fraction.

3.7.5 Tannin release and antioxidant capacity methods

The content of tannins in the microbeads was estimated using the Folin-Ciocalteu (quantification of phenolic content) assay (Moreno-Montoro *et al*., 2015; Pérez-Burillo *et al*., 2018). Their antioxidant activity was also assessed through the $TEAC_{FRAP}$ and $TEAC_{ABTS}$ assay (Pérez-Burillo *et al*., 2018). The determinations were conducted on the liquid fractions from both the digestion and the fermentation process. Results were expressed as mmol of gallic acid equivalent/ml of digested or fermented liquid fraction, for the Folin-Ciocalteu assay. For the $TEAC_{FRAP}$ and $TEAC_{ABTS}$ assays, results were expressed as mmol of Trolox equivalent/ml of digested or fermented liquid fraction.

3.7.6 DNA extraction

The bacterial suspensions obtained from the solid part deriving from *in vitro* fermentation were lysed with lysozyme at a final concentration of 0.1 mg/ml. Then, the extraction of genomic DNA was performed with the MagNA Pure LC JE379 platform (Roche) and DNA Isolation Kit III (Bacteria, Fungi) (REF 03264785001), following the manufacturer's instructions. Agarose gel electrophoresis (0.8 % w/v agarose in Tris-acetate-EDTA buffer) was used to determine DNA integrity, while the sample DNA was quantified with a Qubit 3·0 Fluorometer (Invitrogen). Finally, the DNA samples were stored at –20 °C until further processing.

3.7.7 High-throughput amplicon sequencing

12 ng of DNA were used as template for the amplification of the V3-V4 hypervariable region of the 16S rRNA gene. Following the protocol of Klindworth *et al. (*2013), the forward primer (5′-TCGT CGGC AGCG TCAG ATGT GTAT AAGA GACA GCCT ACGG GNGG CWGCA-G3′) and reverse primer (5′-GTCT CGTG GGCT CGGA GATG TGTA TAAG AGAC AGGA CTAC HVGG GTAT CTAA TCC3′) were used as PCR primers. The library construction was performed as described by the Illumina protocol for the small subunit ribosomal DNA gene (16S rRNA) Metagenomic Sequencing Library Preparation (Cod 15044223 RevA). Primers were fitted with adapter sequences added to the gene-specific sequences to make them compatible with the Illumina Nextera XT Index kit. Then, amplicon sequencing was carried out with an Illumina MiSeq sequencer, according to the manufacturer's instructions in a 2x300 cycles paired-end run (MiSeq Reagent kit v3).

3.7.8 Bioinformatic analyses

The DADA2 (v1.8.0) package from R (v3.6.0) was used for sequence processing, assembly, Amplicon Sequence Variants (ASVs) generation and annotation (Callahan *et al*., 2016). The filter and trimming parameters used were the following: maxN=0, maxEE=c(2,5), truncQ=0, trimLeft=c(17,21), truncLen=c(270,220) and rm.phix=TRUE. A minimum overlap of 15 nucleotides and a maximum mismatch of 1 were required for the merging process of the forward and reverse reads. The reads were then aligned using Bowtie2 (v2,3,5,1) against the human genome (GRCh38.p13) and matches were subsequently discarded (Langmead & Salzberg, 2012). The ASVs were generated by clustering sequences with 100% identity. Taxonomic annotation was assigned by comparison to the SILVA 138 reference database (Quast *et al*., 2013). Annotation was assigned at species level for 100%

similarity matches and for those matches that had a similarity of 97% or higher if there was a difference of at least 2% with the next highest match. Other sequences were annotated at the deepest possible taxonomic level.

3.7.9 Short chain fatty acids analysis

The production of SCFAs was assessed on the liquid fractions deriving from fermentation. One ml was centrifuged to remove solid particles, filtered through a 0.22 μm nylon filter, and finally transferred to a vial for UHPLC (Ultra High Performance Liquid Chromatography) analysis. The analysis of SCFAs was carried out on a 1290 Infinity II UHPLC (Agilent). The mobile phase was methanesulfonic acid 0.1 M pH 2.8/acetonitrile 99:1 v/v delivered at a 0.2 ml/min flow rate; the column used was an Accalim OA C18 reverse phase (Thermo Scientific) (150 × 2.1 mm, 3 μm), with a total run-time of 22 min. Detection was made at 210 nm with a UV–VIS PDA detector.

Three SCFAs were identified and quantified: acetic, propionic, and butyric acid. The respective standard solutions were quantified with concentrations ranging from 10,000 to 125 ppm. The results were expressed as mmol of SCFAs per ml of fermented soluble fraction.

3.7.10 Statistical analyses

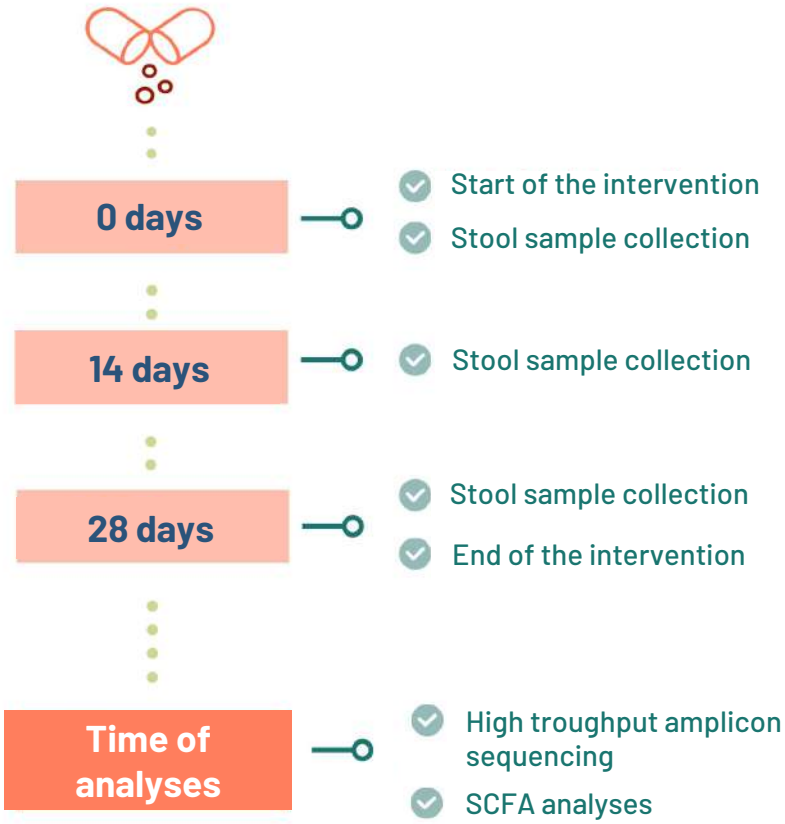
The Shannon diversity index, Chao1 estimator and ACE were obtained with Vegan (v2.5-2) (Oksanen *et al*., 2012). ASVs with less than 10 counts in total were discarded. The ASV count table was normalized by total-sum scaling (TSS). To assess the effect of tannins on the bacterial composition, the Bray-Curtis dissimilarity index between samples was calculated using Vegan (v2,5-2) and used for Principal Coordinate Analysis (PCoA). Wilcoxon signed-rank tests with adjustment for multiple comparisons were employed to evaluate differences in richness and diversity among samples. Analysis of the composition of microbiomes (ANCOM) was used to identify differentially abundant taxa among samples and significance was determined using the Benjamini-Hochberg procedure for false discovery rate control, as described by Kaul *et al.* (2017).

Results of antioxidant activity and SCFA production were expressed as mean values of triplicates (n = 3) ± standard deviation (sd). One-way ANOVA with Bonferroni post-test correction was performed with the SPSS software (version 23, SPSS, Chicago, IL, USA) to determine significant differences among mean values on all the measured parameters. All the graphs were obtained using R scripts.

Correlations between gut microbiota taxonomic groups and SCFAs and antioxidant activity were analysed using the network function in the mixOmics package (v6,10,9) from R, employing pair-wise similarity matrices that incorporate latent components obtained by sparse Partial Least Squares (sPLS) regression. The values in the similarity matrix can be seen as a robust approximation of the Pearson correlation (Rohart *et al*., 2017).

3.8 Chapter 8

Tannin supplementation increases diversity and short-chain fatty acid production in the gut microbiota of healthy subjects



3.8.1 Subjects and trial design

Eight healthy subjects were recruited at the University of Granada (Spain). Subjects were 25 - 45 years old and had a body mass index (BMI) within the normal range (18.5 – 24.99). The trial consisted of a 4-weeks longitudinal nutritional intervention. Subjects were asked not to consume probiotics nor prebiotics during 2 weeks before the start of the trial. After that, all the participants took one capsule of tannin extract (240 mg) per day and were asked to follow an isocaloric diet. The day before starting the intervention (T0), fecal samples were taken and the sample collection was repeated after 2 weeks and at the end of the supplementation period.

The trial complied with the principles of the declaration of Helsinki. The Ethics Committee of the University of Granada approved the trial protocol (1080/CEIH/2020) and informed consent was obtained from all participants.

3.8.2 Dietary supplement

Based on the results of preliminary studies (Molino *et al*., 2018, 2021), two different natural tannin extracts (Q2 and C2, described in **Chapter 2**) were chosen to formulate the dietary supplement. One of the extracts is obtained from quebracho colorado (*Schiopsis lorentsii spp.*), and is characterised by the presence of condensed tannins, in particular profisetinidins (Pasch *et al*., 2001). The second one is composed of hydrolysable gall oak tannins, mainly represented by gallic acid and its derivatives (Radebe *et al*., 2013).

Tannin capsules were provided by Silvateam Spa (San Michele di Mondoví, Italia), according to the suggested formulation (two thirds quebracho extract and one third gall oak extract). The extraction methods were all food grade.

3.8.3 DNA extraction

Genomic DNA was extracted from bacterial suspensions obtained from stool samples. Both the MagNA Pure LC JE379 platform (Roche), with DNA Isolation Kit III (Bacteria, Fungi) (REF 03264785001), and the eMAG Magnetic Extraction System (Biomerieux) (REF 418591) were used for DNA extraction, following the manufacturer's instructions, with a previous lysis with lysozyme at a final concentration of 0.1 mg/ml. These two DNA extraction methods were previously proven to produce reliable and comparable results in our laboratory. DNA integrity was determined by agarose gel electrophoresis (0.8 % w/v agarose in Tris-acetate-EDTA buffer) and DNA samples were quantified using a Qubit 3·0 Fluorometer (Invitrogen). All DNA samples were stored at –20 °C until further processing.

3.8.4 High-throughput amplicon sequencing

Total DNA (12 ng) was used as template for the amplification of the V3-V4 hypervariable region of the 16S rRNA gene. PCR primers were used as described by Klindworth *et al. (*2013), using the forward primer (5′-TCGT CGGC AGCG TCAG ATGT GTAT AAGA GACA GCCT ACGG GNGG CWGCA-G3′) and reverse primer (5′-GTCT CGTG GGCT CGGA GATG TGTA TAAG AGAC AGGA CTAC HVGG GTAT CTAA TCC3′). For library construction, we followed the Illumina protocol for the small subunit ribosomal RNA gene (16S rRNA) Metagenomic Sequencing Library Preparation (Cod 15044223 RevA). Primers were fitted with adapter sequences added to the gene-specific sequences to make them compatible with the Illumina Nextera XT Index kit. Amplicons were sequenced in an Illumina MiSeq sequencer according to the manufacturer's instructions in a 2x300 cycles paired-end run (MiSeq Reagent kit v3). The data for the present study were deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJEB46824.

3.8.5 Bioinformatic analyses

The sequence processing, assembly, Amplicon Sequence Variants (ASVs) generation and annotation were performed in the DADA2 (v1.8.0) package from R (v3.6.0) (Callahan *et al*., 2016). The filter and trimming parameters used were the following: maxN=0, maxEE=c(2,5), truncQ=0, trimLeft=c(17,21), truncLen=c(270,220) and rm.phix=TRUE. A minimum overlap of 15 nucleotides and a maximum mismatch of 1 were required for merging the forward and reverse reads. The reads were aligned using Bowtie2 (v2,3,5,1) against the human genome (GRCh38.p13) and matches were subsequently discarded (Langmead and Salzberg, 2012). The ASVs were generated by clustering sequences with 100% similarity. Taxonomic annotation was assigned by comparison to the SILVA 132 reference database using DADA2 (Quast *et al*., 2013). Annotation was assigned at species level for 100% identity matches and for those matches that had a similarity of 97% or higher if there was a difference of at least 2% with the next highest match. Other sequences were annotated at the deepest possible taxonomic level.

3.8.6 SCFA Analysis

The production of acetic, propionic, and butyric acids was directly assessed in feces from the healthy donors. 100 mg of feces were weighted and resuspended in 1 ml of Milli-Q water. After centrifugation (16000 x g, 2 min, 4 ºC), the supernatant was collected and filtered through a 0.22 µm nylon filter, and finally transferred to a

vial for UPLC (Ultra High Performance Liquid Chromatography) analysis. The samples did not require any further pre-treatment before injection. Standard solutions were quantified with concentrations ranging from 10,000 to 125 ppm. The analysis of SCFAs was carried out on a 1290 Infinity II UHPLC (Agilent). The mobile phase was methanesulfonic acid 0.1 M pH 2.8/acetonitrile 99:1 v/v delivered at a 0.2 mL/min flow rate; the column used was an Acclaim OA C18 reverse phase (Thermo Scientific) (150 × 2.1 mm, 3 μm), with a total run-time of 22 min.

Detection was made at 210 nm with a UV–VIS PDA detector. The results were expressed as mmol of SCFAs per Kg of feces.

3.8.7 Statistical analysis

The Shannon diversity index, Chao1 estimator and ACE were obtained with Vegan (v2.5-2) (Oksanen *et al*., 2012). ASVs with less than 10 counts in total were discarded. The ASV count table was normalized by total-sum scaling (TSS) in order to obtain relative taxonomic abundances. To assess the effect of tannins on the bacterial composition, the Bray-Curtis dissimilarity index between samples was calculated using Vegan (v2,5-2) and used for Principal Coordinate Analysis (PCoA).

Wilcoxon signed-rank tests with adjustment for multiple comparisons were employed to evaluate differences in richness and diversity among samples. Analysis of the composition of microbiomes (ANCOM) was used to identify differentially abundant taxa among samples and significance was determined using the Benjamini-Hochberg procedure for false discovery rate control, as described by Kaul *et al.* (2017). The Linear Discriminant Analysis (LDA) Effect Size (LEfSe) algorithm was applied to identify taxonomical biomarkers for the effects of tannins (Segata *et al*., 2011). It combines Kruskal-Wallis and pairwise Wilcoxon rank-sum tests for statistical significance assessment and feature selection. Default parameters were used for significance (p value < 0.05) and linear discriminant analysis threshold (<2.0).

Correlations between gut microbiota taxonomic groups and SCFAs were performed using the network function in the mixOmics package (v6,10,9) from R, employing pair-wise similarity matrices that incorporate latent components obtained by sparse Partial Least Squares (sPLS) regression. The values in the similarity matrix can be seen as a robust approximation of the Pearson correlation (Rohart *et al*., 2017).

Results of SCFA production are expressed as mean values of triplicates ($n = 3$) ± standard deviation (sd). Repeated measures ANOVA with Bonferroni post-test correction was performed with the SPSS software (version 23, SPSS, Chicago, IL, USA) to determine significant differences among mean values on all the measured parameters. All the graphs were obtained using R scripts.

Results and discussion

4.1 Chapter 1

Study of antioxidant capacity and metabolization of quebracho and chestnut tannins through *in vitro* gastrointestinal digestion-fermentation

4.1.1 Antioxidant capacity trough *in vitro* gastrointestinal digestion

Global antioxidant capacity (GAR+) has been established as a suitable method to evaluate total antioxidant capacity of fresh and processed foods (Pastoriza *et al*., 2011). The GAR+ was determined in the soluble fraction obtained after enzymatic digestion, as well as in the soluble and insoluble fractions of fermented QUE and CHE. As GAR+ is the total antioxidant capacity of the three fractions, it is expressed as a single value. **Table 4.1.1** and **Figure 4.1.1** present the results obtained by $TEAC_{FRAP}$ and $TEAC_{ABTS}$ assays. CHE had a higher reducing capacity (6.90 mmol Trolox/g) than QUE (5.07 mmol Trolox/g) with the $TEAC_{FRAP}$ assay (**Table 4.1.1**). Comparing these values with those reported by Pérez-Burillo *et al.* (2018a,b), both QUE and CHE have an antioxidant capacity 70-820 fold higher than regular foods (i.e. yoghurt, whole grain bread, lentils and peanuts). This potent reducing activity could be explained by the composition of the extracts, which are polyphenol concentrates. In particular, in the case of QUE, it was calculated by Venter *et al.* (2012) that 95% of the extract consisted of proanthocyanidins, while only the remaining 5% were soluble sugars.

Subsequently, the contribution to the global reducing capacity of the bioaccessible fractions (supernatants from digestion and fermentation) *vs.* the solid residue were compared (**Figure 4.1.1**). The antioxidant capacity of supernatants of both QUE and CHE was significantly higher ($p<0.05$) than that of the solid residues. This means that the tannin extracts could exert their reducing activity not only in the intestinal tract, but also at systemic level, possibly reaching several organs.

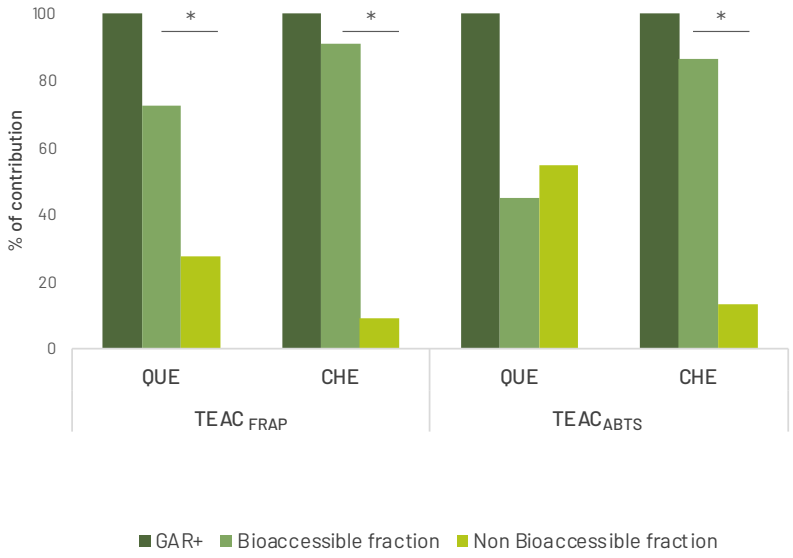


Figure 4.1.1 Contribution of bioaccessible fractions (supernatants from digestion and fermentation) and solid residue to the antioxidant capacity of foods.

The high contribution of QUE and CHE supernatants (95.7 and 99.2%, respectively) suggests that most of the reducing capacity is exerted by the soluble fractions, with a small reducing capacity released in the solid residue. The liquid fraction obtained after digestion of QUE was statistically (p<0.05) less antioxidant than that obtained after fermentation (**Table 4.1.1**), reinforcing the idea of the release of bioactive compounds (mainly polyphenols and their metabolites) due to the activity of microbiota enzymes. On the contrary, the 1.8-fold decrease of the reducing capacity of CHE after fermentation, indicates that this extract is better metabolized through intestinal enzymatic action.

Regarding the $TEAC_{ABTS}$ method, the global antioxidant capacity ranged from 6.7 (CHE) to 8.16 (QUE) mmol Trolox per g of sample (**Table 4.1.1**). The results are 39 – 146 times higher than those of fresh and processed foods reported by Pérez-Burillo *et al*. (2018a,b). Similarly to the $TEAC_{FRAP}$, the discrepancy with foods could be explained by the high content in phenolic compounds of the studied extracts.

The contribution of the bioaccessible fractions of CHE was statistically higher (p<0.05) than that of QUE, as they accounted for 98.1 and 79.9% of the total, respectively (**Table 4.1.1**). The outstanding result is the high level of scavenging activity against ABTS radical recorded for the solid residue of QUE, contrary to the reducing activity measured with the FRAP method. Thus, even if QUE presented the highest GAR+, less than half could be absorbed. Finally, the bioaccessible fractions

obtained after digestion were 1.6 and 2.5 times more antioxidant ($p<0.05$) than those released after microbial fermentation for QUE and CHE, respectively (**Table 4.1.1**). Thus, the metabolites released after digestion not only displayed an important reducing capacity but also and even stronger antiradical activity.

Table 4.1.1 Antioxidant capacity obtained from $TEAC_{FRAP}$ and $TEAC_{ABTS}$ assays. Results expressed as mmol Trolox equivalents per g of fresh sample. The data represent means ± standard deviation. Different lower-case letters within the same column and upper-case letters for the same antioxidant assay indicate statistically significant differences ($p < 0.05$).

	$TEAC_{FRAP}$		$TEAC_{ABTS}$	
	QUE	CHE	QUE	CHE
Supernatant from digestion	$2.33\pm0.02^{a,b,A}$	$4.40\pm0.05^{a,A}$	$3.29\pm0.16^{a,A}$	$5.70\pm0.16^{a,A}$
Supernatant from fermentation	$2.52\pm0.01^{a,B}$	$2.43\pm0.05^{b,B}$	$2.07\pm0.078^{b,B}$	$2.30\pm0.15^{b,B}$
Solid from fermentation	$0.22\pm0.001^{b,C}$	$0.056\pm0.001^{b,C}$	$1.35\pm0.22^{c,C}$	$0.16\pm0.02^{c,C}$
Total – GAR+	5.07	6.90	6.70	8.16

4.1.2 Potential prebiotic activity

The analysis of SCFAs is a good indicator of the effect of a foodstuff on the gut microbiota (Pérez-Burillo *et al*., 2018a). The high content of SCFAs evidences the fermentative activity, as they are by-products of the colonic microbiota metabolism. Some recent studies suggest that polyphenols and their metabolites could selectively stimulate some microbial metabolic pathways, including SCFA production (Tzounis *et al*., 2011; Bolca Van de Wiele& Possemiers, 2013). **Figure 4.1.2** depicts the levels of total SCFAs, acetate, propionate and butyrate released after fermentation of QUE and CHE extracts. CHE gave rise to a higher generation of total SCFAs in comparison to QUE (11.14 and 4.79 mmol/g, respectively).

When the production of individual SCFAs was analysed, statistically significant differences ($p<0.05$) were also observed between the two tannin extracts for the fermentation of acetic, propionic and butyric acids, being all higher for CHE (**Figure 4.1.2**). This difference might be explained by the different composition of these products; while QUE principally consists of a mixture of condensed tannins, CHE

possesses a high concentration of hydrolysable tannins, which probably are easily available for microbial fermentation.

Comparing these results with those of inulin fermentation, lower levels were obtained for QUE only for acetic acid. Propionic acid values were higher for both extracts (7.8 and 16-times higher for QUE and CHE, respectively). While the amount of butyrate obtained for QUE was almost the same as that of inulin, that of CHE was 2.6 times higher. It should be taken into account that inulin is a fructose polymer and the most common energy sources for the microbiota associated with SCFA production are carbohydrates that escape absorption in the small intestine. Therefore, given the composition of the analysed extracts (mainly polyphenols), the generation of SCFAs by the gut microbiota was surprisingly high.

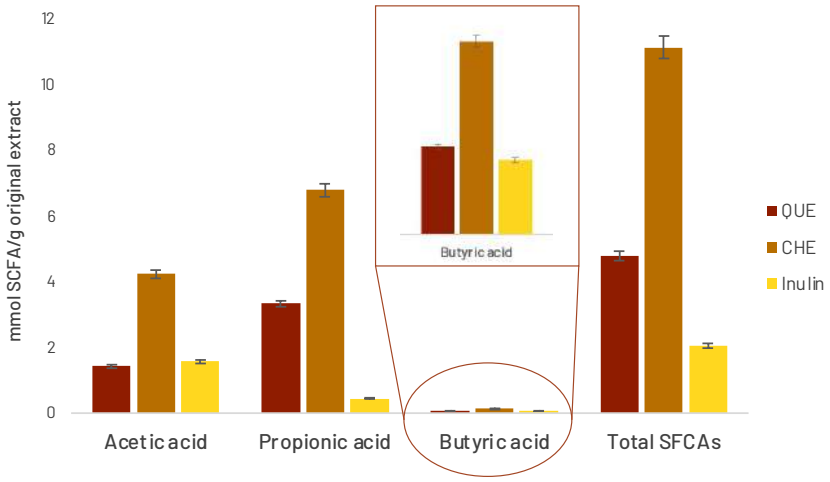


Figure 4.1.2 Release of SCFAs (mmol per g of original extract) produced from QUE and CHE after fermentation.

Aura *et al*. (2013) reported similar values for the production of total SCFAs by proanthocyanidins, while grape and wine were reported as higher SCFA releasers, probably due to the presence in the food matrixes of polysaccharides such as pectin. On the other hand, another study conducted on apple and ciders reported low levels of production of total SCFAs, despite the content of polysaccharides (Bazzocco *et al*., 2008). According to Gibson *et al*. (2017), tannins could be suggested as important prebiotic substrates, since they are fermented by the gut microbiota, producing SCFAs. It is noteworthy to mention that the relationship between gut microbiota and tannins is bi-directional, since tannins are metabolized by the gut

microbiota but also tannins act on different colonic pathways and processes due to their bacteriostatic-bactericidal activity (Duda-Chodak *et al*., 2015).

The relative proportions of SCFAs in the gastrointestinal tract can vary depending on (i) the fermented substrate, (ii) individuals, (iii) different life stages. The predominant SCFA in the human gut is acetic acid, followed by propionic and butyric acids (Verbeke *et al*., 2015). In this study, the main SCFA released after fermentation was propionic acid, for both QUE and CHE, followed by acetic and butyric acids. In support of these findings, Pereira-Caro *et al*. (2015a,b) reported that orange juice polyphenols determined the generation of acetate and propionate by the gut microbiota. More specifically, the bioavailability of orange juice polyphenols depends on the production of hydroxy- and methoxyphenyl propionic acids by the gut microbiota, metabolites that are readily absorbed.

4.1.3 Mass spectrometry

Quantifications by standard solutions of gallic acid, syringic acid, vanillic acid, and p-coumaric acid are shown in **Table 4.1.2**, while ellagic acid and quercetin were not quantifiable. **Table 4.1.3** shows the 18 compounds identified and **Table 4.1.4** provides semi-quantitative information of the analysis of the supernatant obtained from digestion-fermentation of both QUE and CHE.

Table 4.1.2 Quantification of single polyphenols (mg/kg of fresh tannin extract) in supernatants derived from digestion and fermentation of QUE and CHE. N.Q. – not quantifiable.

	QUE		CHE	
	Digested fraction	*Fermented fraction*	*Digested fraction*	*Fermented fraction*
Gallic Acid	8.92 ± 0.02	975.9 ± 2.53	N.Q.	91.6 ± 0.23
Syringic acid	180.1 ± 0.46	7,581.9 ± 20.4	1.27± 0.01	160.14 ± 0.45
Vanillic acid	497.2 ± 1.49	1,126.3 ± 2.82	96.8± 0.25	15.9 ± 0.05
p-Coumaric acid	162.2 ± 0.41	808.5 ± 2.18	71.4± 0.19	360.2 ± 0.83

The profile of CHE tannins has been previously described by Pasch & Pizzi (2002). In abundance order, tannin dimer>tannin trimer>tannin tetramer>catechin, were obtained from the supernatant derived from the digestion of CHE. Nevertheless, it should be noted that the amount of tetramer was higher in the bioaccessible fraction from digestion than that obtained after fermentation. Some of the characteristic molecules of CHE, such as pentagallyglucose, gallocatechin and epicatechin, were identified only in the digested bioaccessible fraction. After digestion, large compounds such as castalagin and vescalagin described by Pasch and Pizzi (2002) were not found, probably because of their hydrolysis during the acidic gastric phase.

After fermentation, the amount of dimer, trimer and catechin increased, probably due to the metabolization of the former compounds. As previously mentioned, microbial metabolism was responsible for the production of different compounds such as sinapinic acid and quercetin, or the increase of other metabolites like hydroxyphenyl acetic, p-coumaric, vanillic and ellagic acids. Ellagic acid produced urolithin A and urolithin B after microbial metabolization. The production of urolithins by the gut microbiota was deeply described by Tomás-Barberán *et al*. (2017), who also put a focus on the different metabotypes able to generate these metabolites.

The results obtained support the hypothesis of transformation of tannins during the gastrointestinal digestion and subsequent metabolization by the gut microbiota. Thus, new bioactive compounds are released, which in turn could determine health effects in human beings. For example, the production from ellagitannins and ellagic acid of urolithins (A and B) is interesting, since they are absorbed in the intestine and exert anti-inflammatory and anticancer effects, as well as antimicrobial activity (Espín Larrosa, García-Conesa, & Tomás-Barberán, 2013). Verbeke *et al*. (2015) reported also specific health effects for some of the detected microbial metabolites, i.e. antimicrobial activity of hydroxyphenyl acetic acid and oestrogenic activity for urolithin A and urolithin B. However, the physiological relevance of other compounds is still unknown and deserves further studies.

Table 4.1.3 Tannins and metabolites identified in supernatants derived from digestion and fermentation of QUE and CHE.

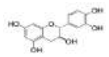
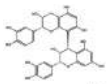
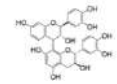
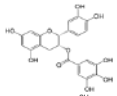
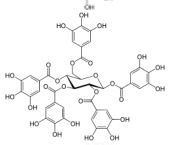
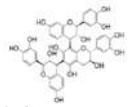
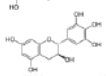
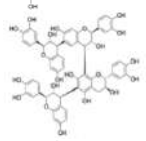
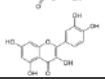
N.		Compound	Molecular formula	m/z [M-H]⁻	MS fragments	RT (min)
1.		Gallic acid	$C_7H_6O_5$	167.0137	125.0244	1.59
2.		Catechin	$C_{15}O_6H_{14}$	289.0712	271.0611; 151.04; 137.0244	2.91
3.		Procyanidin B1	$C_{30}O_{12}H_{26}$	577.135	289.0717; 141.04	3.31
4.		Tannin dimer	$C_{30}O_{11}H_{26}$	561.14	289.0712; 271.0611; 151.04	3.53
5.		Syringic acid	$C_9H_{10}O_5$	197.0450	169.0506; 153.0557	4.03
6.		Epicatechin gallate	$C_{22}O_{10}H_{18}$	441.08	303.05; 169.014	4.05
7.		Pentagalloyl glucose	$C_{41}O_{26}H_{32}$	939.1104	787.09; 169.0172	4.18
8.		Tannin trimer	$C_{45}O_{16}H_{38}$	833.2082	681.16; 561.14; 151.04	4.44
9.		Vanillic acid	$C_8O_4H_8$	167.034	123.00	4.52
10.		Gallocatechin	$C_{15}O_7H_{14}$	305.66	167.0414; 137.0211	4.53
11.		Tannin tetramer	$C_{60}O_{21}H_{50}$	1105.2766	833.2082; 681.16; 561.14	4.67
12.		*p*-Coumaric acid	$C_9O_3H_8$	163.039	-	5.06
13.		Sinapinic acid	$C_{11}O_5H_{12}$	223.0606	-	5.45
14.		Urolithin A	$C_{13}O_4H_8$	227.034	199.04; 183.04	5.64
15.		Urolithin B	$C_{13}O_3H_8$	211.039	183.04	5.98
16.		Hydroxyphenyl acetic acid	$C_8O_3H_8$	151.04	107.05	6.19
17.		Ellagic acid	$C_{14}O_8H_6$	300.998	257,009	6.78
18.		Quercetin	$C_{15}O_7H_{10}$	301.0353	283.0248; 273.04	6.79

Table 4.1.4 Semi-quantitative analysis of the soluble tannins and their metabolites in supernatants derived from digestion and fermentation of QUE and CHE.

		QUE				CHE			
		Digested		Fermented		Digested		Fermented	
N.	Compound	mDa	Area/g	mDa	Area/g	mDa	Area/g	mDa	Area/g
1.	Gallic acid	1.5	$1.72x10^{3}$	0.3	$6.56x10^{5}$	-	-	-0.4	$2.16x10^{4}$
2.	Catechin	2.8	$3.47x10^{5}$	-0.3	$2.12x10^{5}$	-1.5	$4.52x10^{3}$	0.7	$2.91x10^{4}$
3.	Procyanidin B1	4.6	$1.48x10^{4}$	-	-	-	-	-	-
4.	Tannin dimer	-0.4	$1.01x10^{6}$	4.1	$1.04x10^{6}$	4.6	$1.69x10^{5}$	2.7	$1.60x10^{5}$
5.	Syringic acid	2	$3.95x10^{4}$	0.7	$1.57x10^{6}$	0.2	$2.58x10^{3}$	1.2	$8.05\ x10^{4}$
6.	Epicatechin gallate	2.9	$1.54x10^{4}$	-2.5	$1.35x10^{4}$	4.9	$8.98x10^{3}$	-	-
7.	Pentagalloyl glucose	-	-	-	-	4.9	$1.81x10^{4}$	-	-
8.	Tannin trimer	0.3	$1.87x10^{6}$	-1.2	$1.17x10^{6}$	0.2	$1.03x10^{5}$	1.6	$1.52x10^{5}$
9.	Vanillic acid	0.1	$1.14x10^{5}$	-0.3	$3.00x10^{5}$	1.2	$4.00x10^{4}$	0.8	$3.50x10^{4}$
10.	Gallocatechin	0.5	$4.91x10^{4}$	5.1	$4.38x10^{4}$	5	$1.17x10^{4}$	-	-
11.	Tannin tetramer	-0.5	$3.84x10^{4}$	0.5	$4.85x10^{4}$	4.7	$1.47x10^{4}$	-1.5	$6.97x10^{3}$
12.	*p*-Coumaric acid	0.1	$2.85x10^{4}$	-0.3	$1.65x10^{5}$	0.2	$2.15x10^{4}$	0.3	$8.82x10^{4}$
13.	Sinapinic acid	-	-	2.1	$2.34x10^{5}$	-	-	0.5	$7.31x10^{4}$
14.	Urolithin A	-	-	1.3	$1.02x10^{5}$	0.5	$2.62x10^{4}$	0.7	$5.24x10^{5}$
15.	Urolithin B	-	-	-	-	1.5	$3.24x10^{4}$	0.3	$8.70x10^{4}$
16.	Hydroxyphenyl acetic acid	0.7	$2.20x10^{5}$	1	$6.55x10^{5}$	0.2	$7.59x10^{4}$	0.9	$1.07x10^{5}$
17.	Ellagic acid	0.7	$6.03x10^{3}$	0.2	$2.72x10^{4}$	-0.4	$4.87x10^{3}$	0.5	$1.21x10^{4}$
18.	Quercetin	-	-	1.3	$6.10x10^{4}$	-	-	-1.3	$9.83x10^{3}$

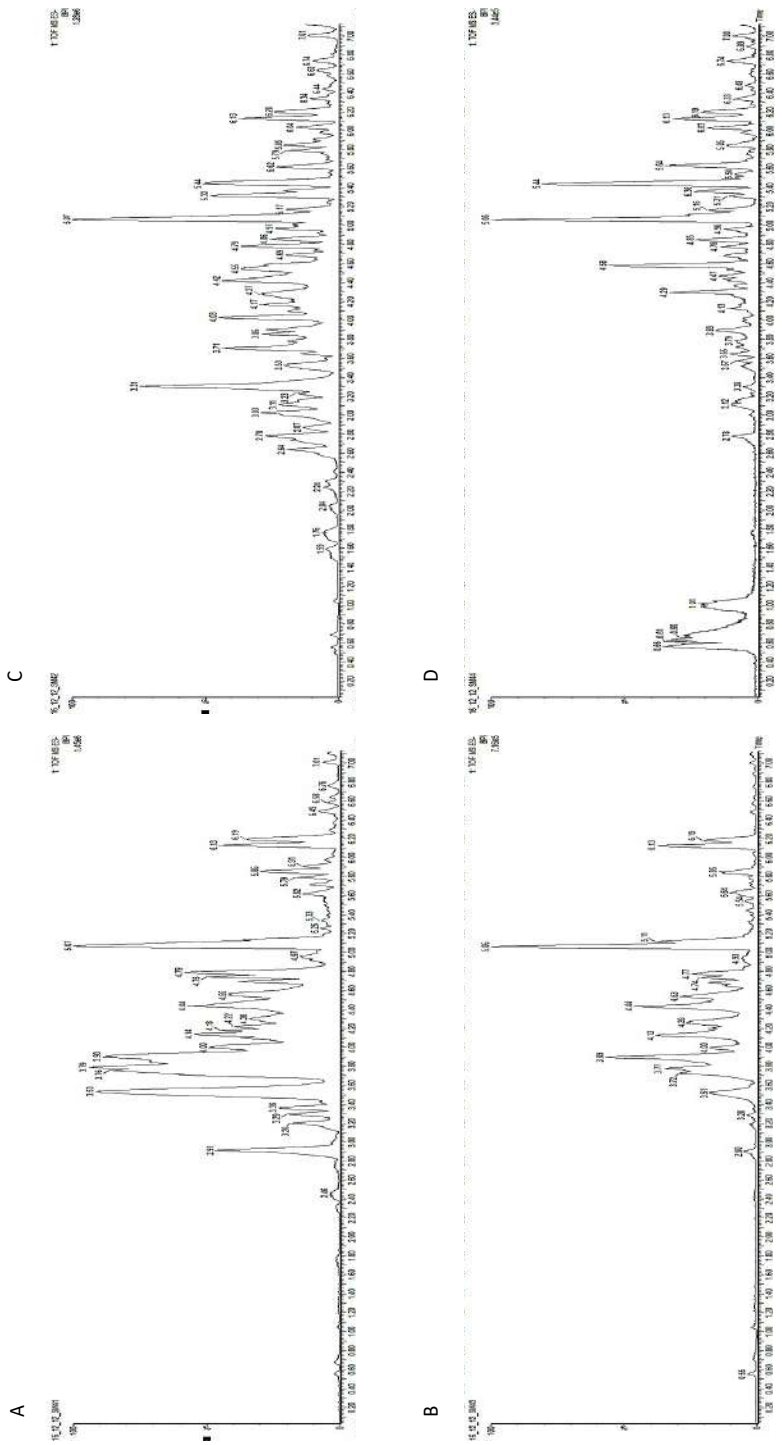


Figure 4.1.3 UPLC-MS total ion current (TIC) chromatograms of supernatants derived from digestion and supernatants derived from fermentation of QUE (A and B) and CHE (C and D).

4.2 Chapter 2

Profiling of eleven tannin extracts deriving from different sources through *in vitro* digestion and fermentation

In total, four condensed tannin extracts from quebracho wood (Q1, Q2, Q3 and Q4), four ellagitannin extracts from chestnut wood and bark (C1, C2, C3, C4), and three gallotannin extracts from both tara pods (T1) and gallnuts (G1 ad G2) were examined. All the products, obtained by different extraction methods were submitted to *in vitro* gastrointestinal digestion and fermentation to further study their bioactivity, by analysing the antioxidant capacity exerted and the SCFA production. Moreover, HPLC fingerprinting was used to identify any similarity among the chemical profile of the samples. The analyses were conducted on the original extracts, and on the samples obtained through both the digestion and the fermentation process.

4.2.1 HPLC fingerprint

Usually, powerful equipment and refined techniques, most often requiring the use of mass spectrometers, are used to characterize phytocomplexes such as those under investigation. In this respect, HPLC is probably not the most suitable technique, especially in the case of the original samples. However, in this study the intention was not to provide a detailed chemical analysis of the sample composition but to investigate possible similarities in the chromatographic profile of the different samples studied, searching for the presence of peak overlaps in terms of retention time and size (**Figures 4.2.1, 4.2.2, 4.2.3**). In fact, it is likely that similar profiles correspond to similar chemical compositions, in terms of quality and quantity. If the study of the original samples can help to understand their chemical organisation, chromatograms obtained from samples after the *in vitro* digestion and fermentation process give an idea of the extent to which these phytocomplexes are metabolised.

Starting with pure samples, they can be divided into three groups according to their chromatogram profiles. The first group includes condensed tannins extracted from quebracho wood (**Figure 4.2.1**), which presented two big areas, one at the very beginning, containing probably the elution front with all the molecules that could not be retained by the column. Conversely, the second area, between 12 mins and 25 mins retention time, comprised separate multiple peaks.

The samples extracted from the chestnut wood and bark showed a pattern similar to the previous one, although the profiles were not overlapping with those of quebracho. Therefore, a first large peak presented by the elution front was obtained, followed by a part of the chromatogram comprising separate peaks. Of interest is that sample C4 returned a chromatogram with much fewer peaks compared to the other samples (**Figure 4.2.2**). This is probably due to the fact that this is obtained from the recovery of the extraction waste from chestnut wood and bark.

The samples obtained from the extraction of gallotannins presented a characteristic profile, that differed greatly from both condensed tannins of quebracho and ellagitannins of chestnut. Chromatograms obtained at 280 nm wavelength (**Figure 4.2.3**) showed a poor peak separation for all three pure samples and three wide amalgamate peaks were found. Probably, an *ad hoc* protocol should be developed for these samples, to produce a better separation of the compounds of the phytocomplex. However, it is conceivable that the characteristic compounds of these extracts show better absorption at a different wavelength. In fact, at 340 nm it was possible to observe a clear peak separation (**Figure 4.2.4**).

After digestion, for quebracho and chestnut extracts a clear diminution of the big peak at the start, representing the elution front, was observed (**Figure 4.2.1 and 4.2.2**). As the method is designed to identify the presence of relatively small polyphenols, the high molecular weight compounds characterising the original extracts cannot be retained in the column and be properly separated. A decrease in the elution front therefore indicates that some of the large compounds present in the original extract have been degraded into smaller molecules. Thus, enzymatic digestion plays a partial role in the metabolization of these extracts.

Within each group of quebracho and chestnut extracts there are still similarities in the chromatographic profile after the *in vitro* digestion process, but with some variations of the different peak areas. This means that they have a qualitatively similar composition, but with different proportions of the individual compounds. As regards tara and gallnuts extracts, they again showed very different chromatograms. After the digestion process, the large, non-separated peaks disappeared, and a sparse profile of peaks remained (**Figure 4.2.3**). These results likewise confirm that the compounds present in T1, G1 and G2 extracts and their metabolites probably need their own method of analysis.

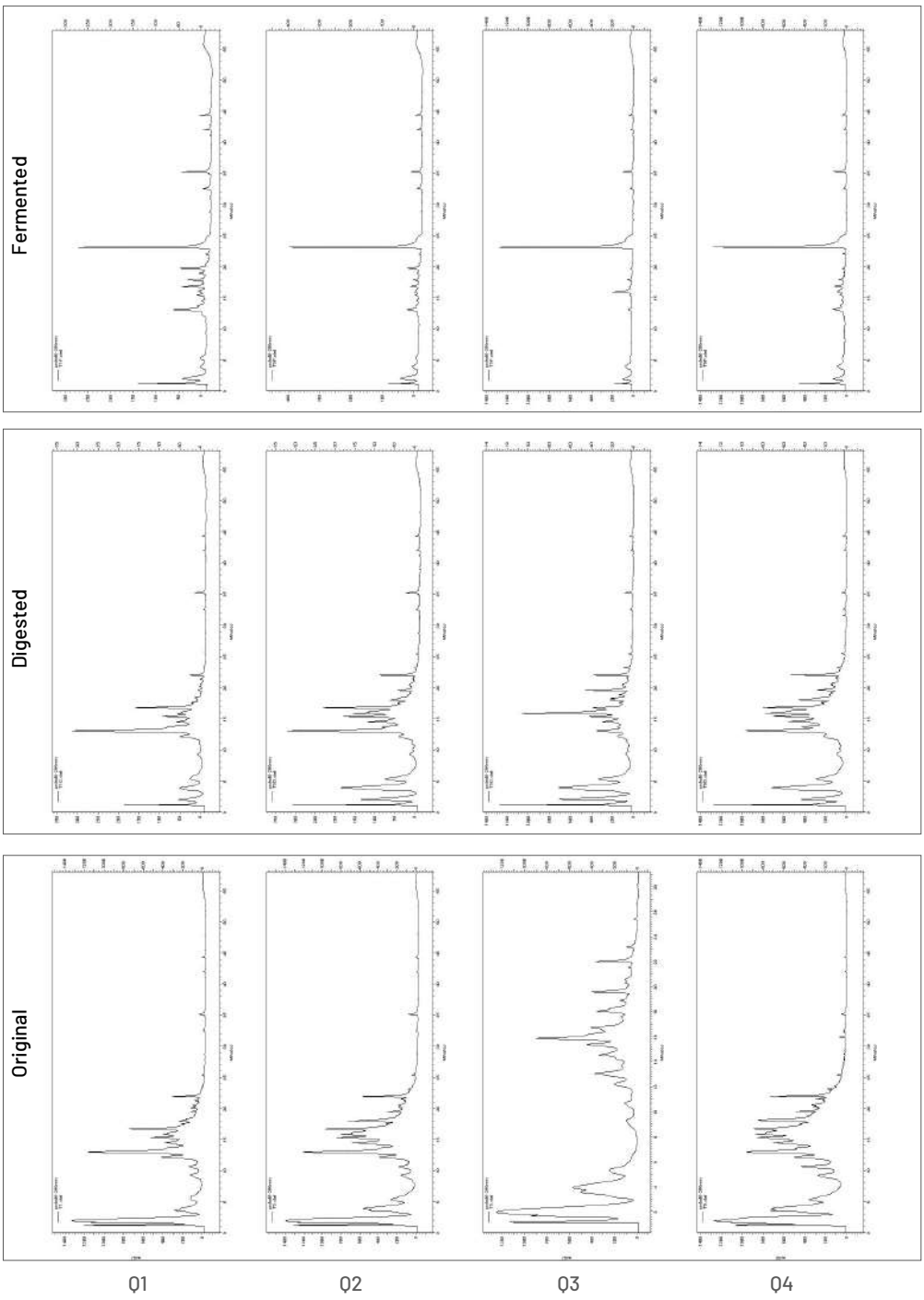


Figure 4.2.1 HPLC chromatograms of the original sample, supernatants after digestion and after fermentation of quebracho wood extracts (Q1, Q2,Q3 and Q4) at 280 nm.

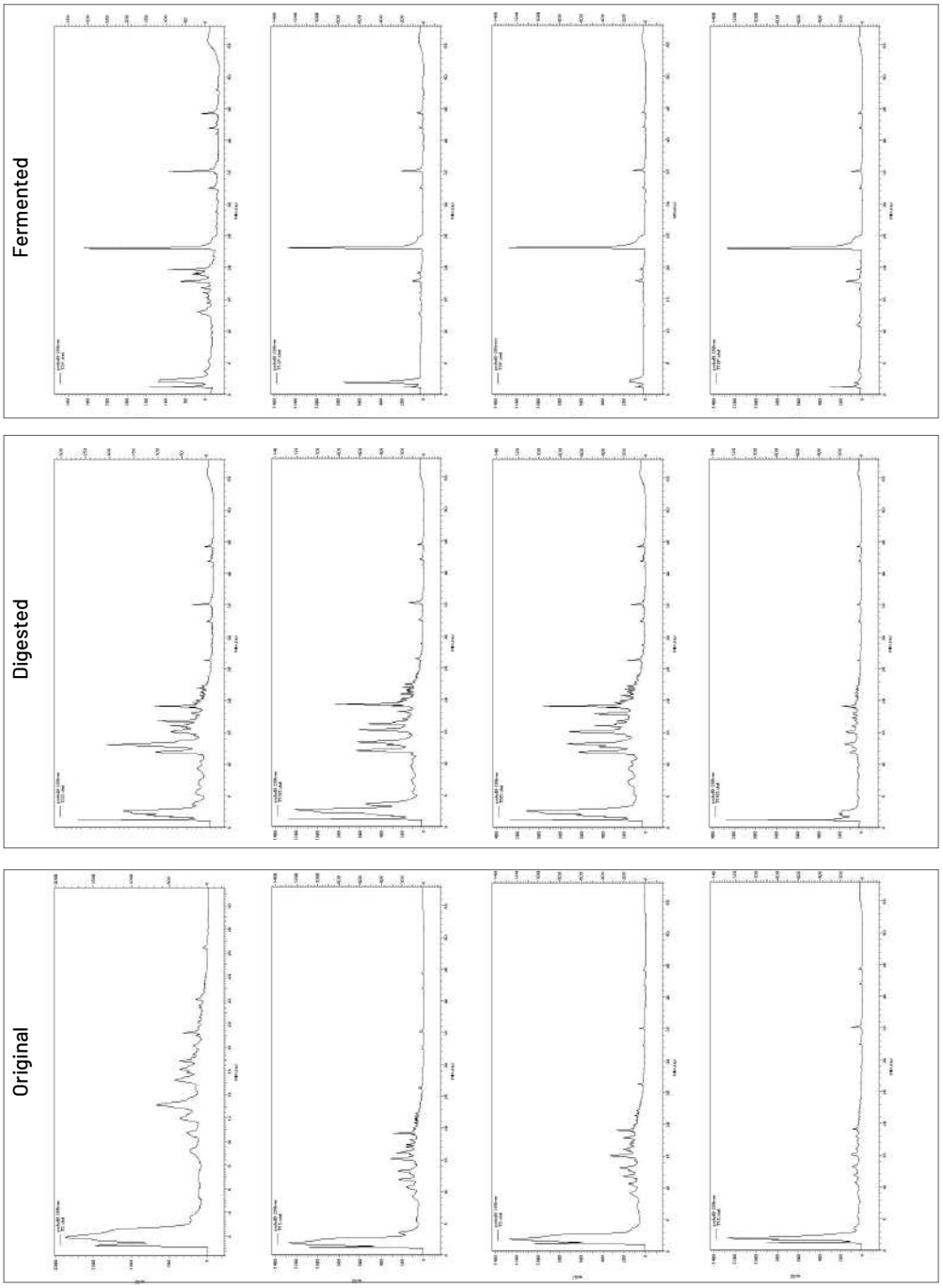


Figure 4.2.2 HPLC chromatograms of the original sample, supernatants after digestion and after fermentation of chestnut wood and bark extracts (C1, C2, C3 and C4) at 280 nm.

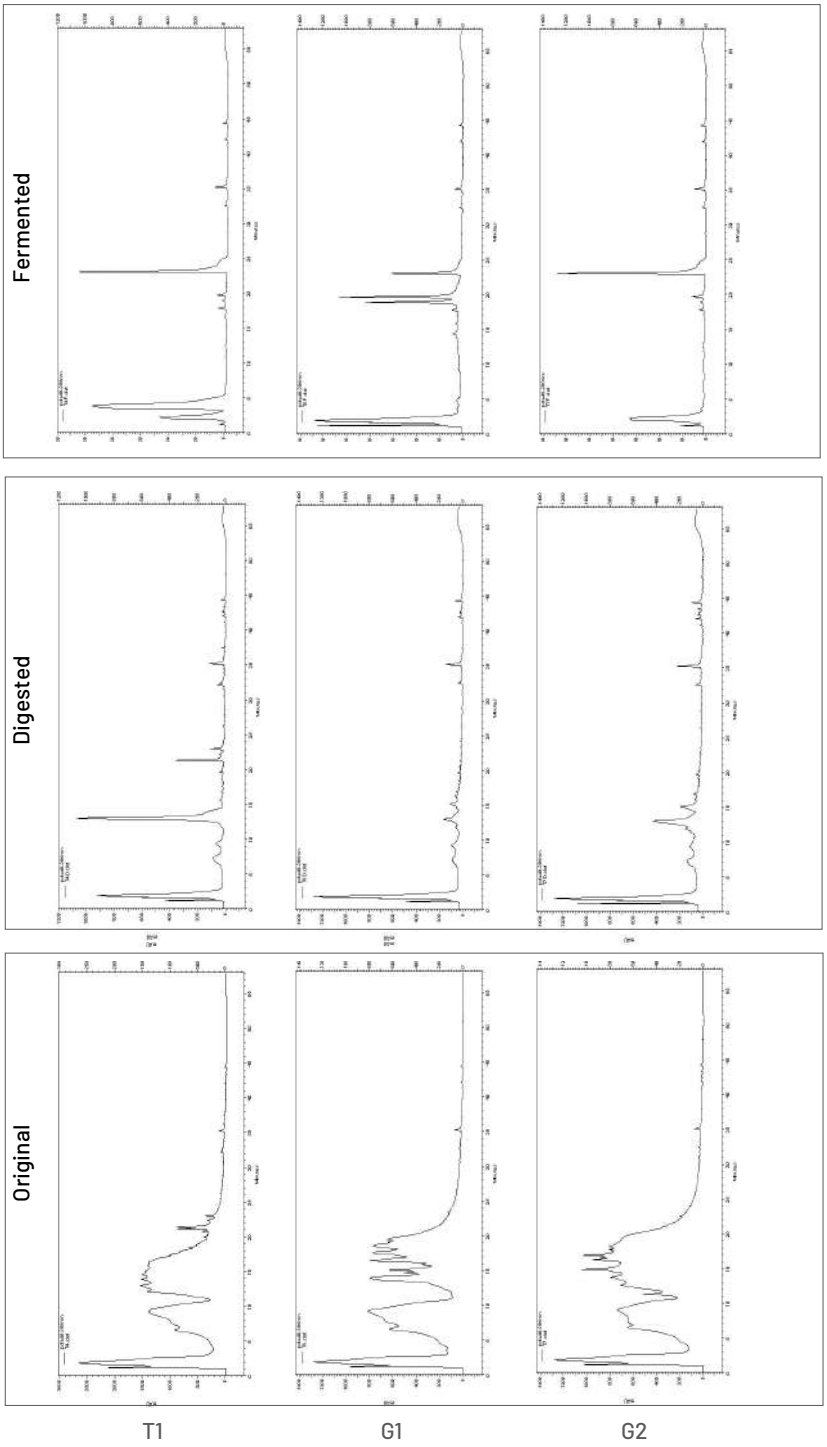


Figure 4.2.3 HPLC chromatograms of the original sample, supernatants after digestion and after fermentation of tara pods (T1) and gallnuts (G1 and G2) at 280 nm.

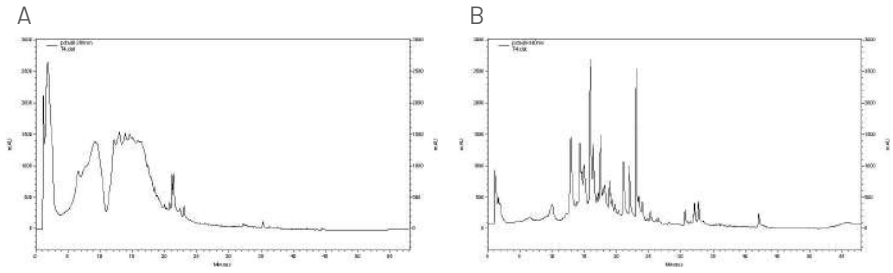


Figure 4.2.4 HPLC chromatograms of tara pods extract (T1), obtained at wavelength (A) 280 nm and (B) 340 nm.

The *in vitro* fermentation process led to a further change in the chromatographic profiles and probably the most interesting one. In fact, it is possible to observe how the compositions of the extracts determine a different interaction with the gut microbiota, resulting in a distinct metabolization for each sample. In particular, it can be observed that for both quebracho and chestnut extracts, the milder extraction technique applied, using only hot water, resulted in the greatest production of metabolites. This is reflected in the chromatograms of Q1 and C1 (**Figure 4.2.1 and 4.2.2**), which show a greater number of peaks and greater intensity, compared to those of fermented samples from the extracts obtained with more refined techniques (i.e. sulphation for quebracho and clarification for chestnut).

Chromatograms obtained from fermented tara and gallnut extract did not present any relevant aspect.

4.2.2 Antioxidant capacity

Tannin extracts are constituted by a mix of bioactive compounds, thus the best way to estimate their amount is by using spectrophotometric methods as those used for the present study. In particular, Folin-Ciocalteu, $TEAC_{FRAP}$ and $TEAC_{ABTS}$ were used to detect the total polyphenol content in solution, the reducing capacity and the scavenging activity, respectively.

More specifically, here the intention was to understand which of the samples exerted the greatest antioxidant capacity and how the processes of digestion and fermentation can affect this. Hence, the product as such (original), as well as the samples after digestion (digested) and fermentation (fermented) were analysed.

In general, a linear correlation was obtained by the Spearman method among the three methods (**Table 4.2.1**). The significant correlations found ($p < 0.01$) were positive, with values around $Rs = 0.85$.

Table 4.2.1 Linear correlations between the antioxidant capacity measures of tannin extract. **Indicates statistically significant correlation at the 0.01 level (2-tailed).

	Folin-Ciocalteu	$TEAC_{FRAP}$	$TEAC_{ABTS}$
Folin-Ciocalteu	1	0.776**	0.837**
$TEAC_{FRAP}$	0.776**	1	0.954**
$TEAC_{ABTS}$	0.837**	0.954**	1

Figure 4.2.5 illustrates the obtained results. As regards original products, among quebracho samples, Q4 showed higher values by Folin-Ciocalteu and $TEAC_{FRAP}$ than the other quebracho extracts, while for $TEAC_{ABTS}$ the inter-sample differences decreased. Apparently, the extraction process plays a critical role in the final performance of the product. Specifically, hot water extraction returned the lowest values (Q1), which then increased with the sulphation processes (Q2 and Q3), and then reached the highest values with refining (Q4).

Among pure chestnut samples, no major differences were recorded, only the double clarification process resulted in a significant change in the scavenging capacity for C3. Remarkably, C4 exhibited the lowest values among all the evaluated samples for this study. This is explained by the fact that C4 represents the residue normally discarded after the extraction of chestnut tannins. It is therefore not expected to be rich in polyphenols, nor to have a high antioxidant capacity.

T1, G1 and G2 were obtained by solvent extraction. In this case, the extraction method did not have a particular influence on the yield of the products, as in terms of polyphenol content the values are comparable with those extracts obtained with hot water (with or without further treatments). However, these three samples showed the highest antioxidant capacity measured by both methods $TEAC_{FRAP}$ and $TEAC_{ABTS}$. This means that the composition of T1, G1 and G2 is much more reactive than all the other samples. In fact, these three phytocomplexes share a similar chemical composition characterised by a particular presence of gallotannins, whereas the Q and C samples consist of condensed tannins and ellagitannins, respectively. In general, digestion led to a decrease in polyphenol content, although it was statistically significant ($p < 0.05$) only for Q1, C1 and G1. For some samples,

this decrement was not correlated to a reduction of the antioxidant capacity for $TEAC_{FRAP}$, $TEAC_{ABTS,}$ or both methods (**Table 4.2.2, 4.2.3, 4.2.4**). Some samples (Q2 and C3 for $TEAC_{FRAP}$ and Q2, Q3 and T1 for $TEAC_{ABTS}$) exerted an antioxidant activity almost identical antioxidant activity, at the expense of a reduction in the polyphenol content. Digested Q3, Q4 and C2 presented higher $TEAC_{FRAP}$ values than their respective original samples. The same increasing effect was recorded for digested Q4, measured with $TEAC_{ABTS.}$ This could be due to the fact that digestion leads to a partial degradation of the tannin extracts analysed and to a decrease in the total polyphenols measured. However, the remaining molecules have a high capacity to react with iron ions and or to scavenge radicals. On the other hand, the chemical conformation of the compounds in the original samples was such that they probably could not exert their full potential.

In all cases, the fermented samples showed a significantly lower polyphenol content ($p < 0.05$) and antioxidant activity than the original or digested samples. This is in accordance with the fact that gut microbial fermentation plays a critical role on food antioxidant capacity (Pérez-Burillo *et al*., 2018).

Some authors found that through fermentation the antioxidant activity values of various foods increase compared to their respective digested state (Navajas-Porras *et al*., 2020). They suggested that the intestinal fermentative activity helped to metabolize and release antioxidant compounds from the food matrices analysed. In this study, the opposite effect has been observed, with a decrease in the antioxidant activity of the fermented samples compared to the original and digested ones. Probably, as the antioxidant molecules were not protected by any matrix, they were more exposed to the degradation resulting from the fermentation process. These data confirm the results obtained in Chapter 1. The decrease in antioxidant activity does not necessarily have to be considered as a prejudicial finding. Indeed, it could mean that tannin extracts are metabolised to such an extent that they are not widely available after fermentation. In fact, as studied in **Chapter 1**, tannins are extensively used as a source for the production of SCFAs by the intestinal microbiota, exerting a prebiotic function.

It is of interest that, similarly to pure samples, fermented tannins constituted by gallotannins (T1, G1 and G2) showed a smaller decrease, through fermentation, in both polyphenol content and antioxidant activity compared to all other samples. It should be further investigated whether this minimal reduction is linked to a lower metabolization by the microbiota or to a greater resistance of the compounds themselves. What is certain is that these higher values indicate that the compounds left available after the fermentation process could exert their reducing activity not only in the intestinal tract, but also at systemic level, possibly reaching several organs.

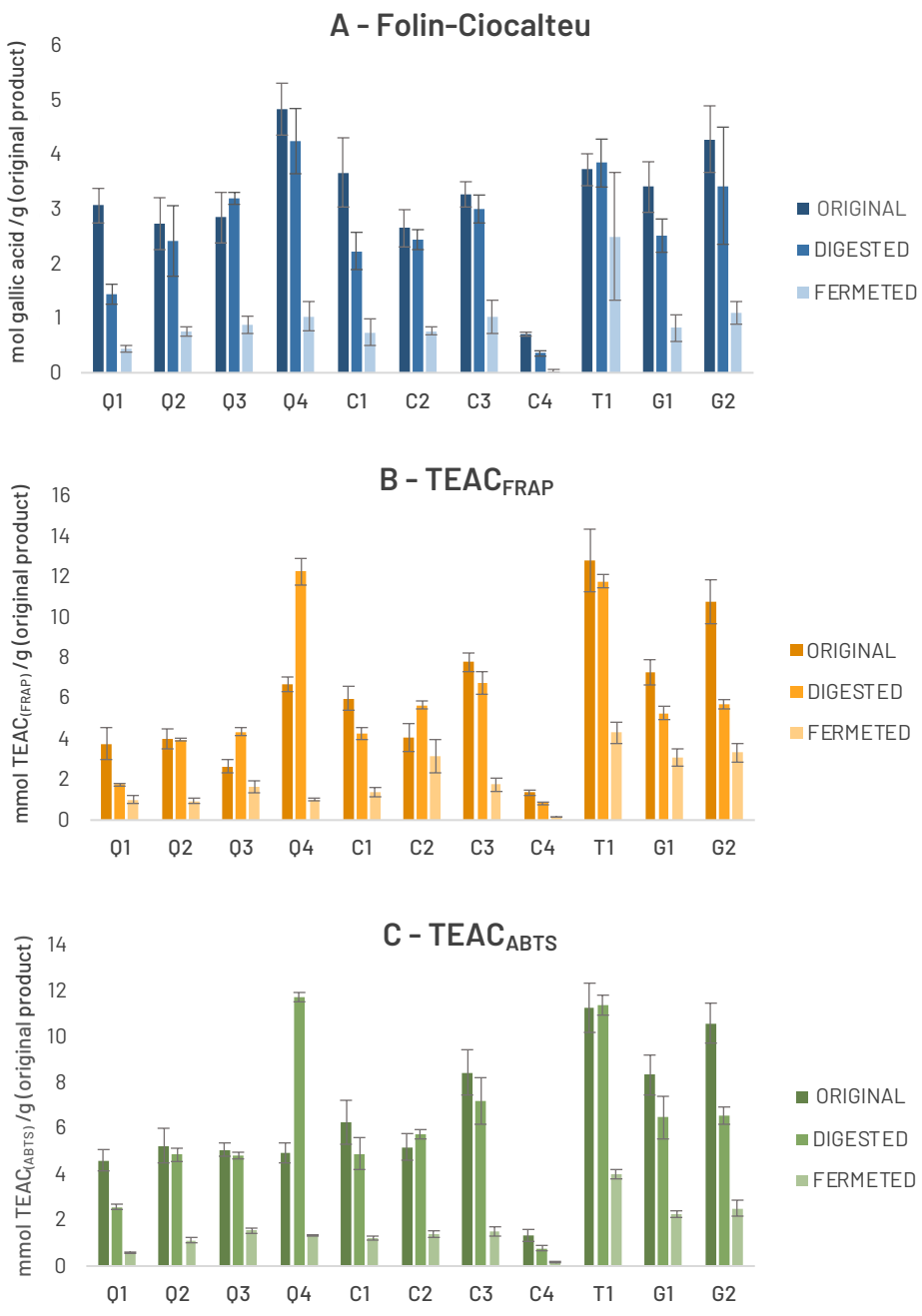


Figure 4.2.5 Total polyphenol content (A – Folin-Ciocalteu) and antioxidant capacity (B – TEACFRAP, C – TEACABTS) of original, digested and fermented samples.

Table 4.2.2 Total polyphenol content of pure, digested and fermented samples, measured by Folin-Ciocalteu. Results are expressed as mol of gallic acid/g of original product and reported as mean ± SD. Different letters indicate statistically significant differences by ANOVA and Bonferroni post-hoc test ($p<0.05$) among different fractions of the same sample.

	Q1	Q2	Q3	Q4	C1	C2	C3	C4	T1	G1	G2
Pure	3.08 ± 0.36^{q}	2.73 ± 0.47^{a}	2.85 ± 0.46^{a}	4.82 ± 0.47^{a}	3.67 ± 0.64^{a}	2.64 ± 0.30^{a}	3.26 ± 0.23^{a}	0.73 ± 0.05^{a}	3.72 ± 0.29^{a}	3.40 ± 0.48^{a}	4.28 ± 0.58^{a}
Digested	1.44 ± 0.19^{b}	2.41 ± 0.64^{a}	3.20 ± 0.11^{a}	4.31 ± 0.62^{a}	2.22 ± 0.34^{b}	2.43 ± 0.28^{a}	3.00 ± 0.25^{a}	0.36 ± 0.05^{b}	3.84 ± 0.43^{a}	2.51 ± 0.31^{b}	3.42 ± 1.07^{a}
Fermented	0.07 ± 0.02^{c}	0.09 ± 0.04^{b}	0.16 ± 0.05^{b}	0.26 ± 0.08^{b}	0.24 ± 0.08^{c}	0.16 ± 0.05^{b}	0.30 ± 0.10^{b}	0.02 ± 0.01^{c}	1.16 ± 0.48^{b}	0.24 ± 0.09^{c}	0.20 ± 0.06^{b}

Table 4.2.3 Reducing capacity of pure, digested and fermented samples, measured by $TEAC_{FRAP}$ assay. Results are expressed as mmol Trolox equivalents/g of original product and reported as mean ± SD. Different letters indicate statistically significant differences by ANOVA and Bonferroni post-hoc test ($p<0.05$) among different fractions of the same sample.

	Q1	Q2	Q3	Q4	C1	C2	C3	C4	T1	G1	G2
Pure	3.75 ± 0.79^{a}	4.00 ± 0.49^{c}	2.62 ± 0.33^{a}	6.66 ± 0.37^{a}	5.97 ± 0.60^{a}	4.07 ± 0.68^{a}	7.76 ± 0.46^{a}	1.34 ± 0.15^{a}	12.8 ± 1.54^{a}	7.25 ± 0.61^{a}	10.7 ± 1.08^{a}
Digested	1.74 ± 0.08^{b}	3.93 ± 0.08^{c}	4.34 ± 0.18^{b}	12.2 ± 0.67^{b}	4.24 ± 0.27^{b}	5.65 ± 0.22^{b}	6.75 ± 0.56^{b}	0.80 ± 0.06^{b}	11.8 ± 0.32^{a}	5.25 ± 0.34^{b}	5.67 ± 0.23^{b}
Fermented	0.98 ± 0.21^{c}	0.93 ± 0.11^{b}	1.63 ± 0.29^{c}	0.99 ± 0.07^{c}	1.33 ± 0.25^{c}	3.13 ± 0.84^{c}	1.72 ± 0.31^{c}	0.12 ± 0.01^{c}	4.28 ± 0.52^{b}	3.04 ± 0.43^{c}	3.31 ± 0.48^{c}

Table 4.2.4 Scavenging capacity of pure, digested and fermented samples, measured by $TEAC_{ABTS}$ assay. Results are expressed as mmol Trolox equivalents/g of original product and reported as mean ± SD. Different letters indicate statistically significant differences by ANOVA and Bonferroni post-hoc test ($p<0.05$) among different fractions of the same sample.

	Q1	Q2	Q3	Q4	C1	C2	C3	C4	T1	G1	G2
Pure	4.57 ± 0.47^{a}	5.22 ± 0.76^{a}	4.54 ± 0.48^{a}	4.89 ± 0.45^{a}	6.35 ± 0.87^{a}	5.15 ± 0.58^{a}	8.41 ± 0.97^{a}	1.30 ± 0.28^{a}	11.2 ± 1.06^{a}	8.31 ± 0.86^{a}	10.6 ± 0.88^{a}
Digested	2.55 ± 0.11^{b}	4.83 ± 0.27^{a}	4.82 ± 0.15^{a}	11.7 ± 0.21^{b}	4.88 ± 0.69^{b}	5.73 ± 0.21^{a}	7.16 ± 1.00^{a}	0.74 ± 0.12^{b}	11.4 ± 0.46^{a}	6.45 ± 0.92^{b}	6.54 ± 0.40^{b}
Fermented	0.56 ± 0.04^{c}	1.09 ± 0.11^{b}	1.52 ± 0.10^{a}	1.32 ± 0.03^{c}	1.20 ± 0.07^{c}	1.39 ± 0.15^{b}	1.50 ± 0.22^{b}	0.15 ± 0.04^{c}	3.99 ± 0.22^{b}	2.25 ± 0.16^{c}	2.49 ± 0.35^{c}

4.2.3 SCFA production

Short chain fatty acids are produced by the metabolism of the gut microbiota, which is capable of fermenting several different compounds deriving from digestion. Here are investigated the three most prevalent subtypes: acetate, propionate, and butyrate. **Figure 4.2.6** depicts the sum of the release of SCFAs induced by the tannin extracts.

Extracts characterised by the presence of gallotannins (T1, G1 and G2) resulted in significantly higher production ($p < 0.05$) than extracts characterised by the presence of condensed tannins or ellagitannins. Although chestnut tannins are hydrolysable, it seems that their performance is more similar to quebracho tannins (condensed) than to other hydrolysable tannins (gallotannins).

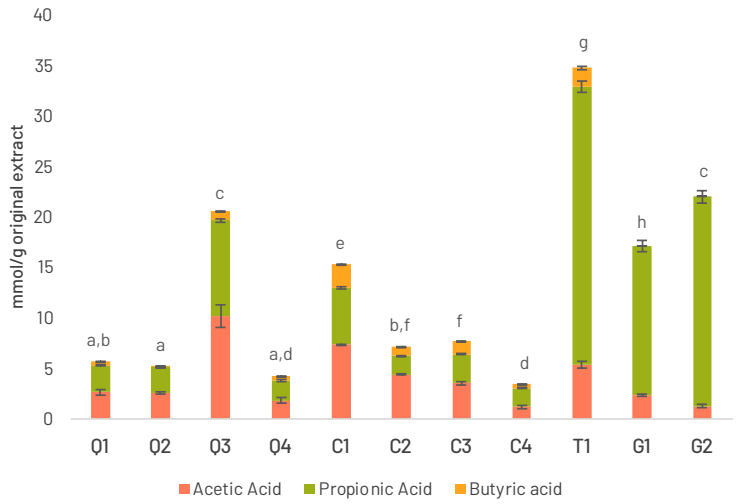


Figure 4.2.6 Release of total sum of SCFAs for different tannin extracts after fermentation. Different letters indicate statistical differences by ANOVA and Bonferroni post-hoc test ($p<0.05$) among samples.

Looking within each tannin extract group, it seems that the different extraction techniques applied have an impact on the production of SCFAs. Among quebracho tannins, Q4, which undergoes a further refining step after extraction in hot water, stands out with a high release (SCFA sum = 20.59 ± 1.30). On the contrary, it seems that for chestnut tannins the minimum treatment applied (only hot water extraction) leads to the highest production of SCFAs (C1 SCFA sum =15.29 ± 0.03). Then, C2 and C3 (subjected to a single and double clarification treatment, respectively) induce

a lower release, while the lowest production is attributed to C4 (SCFA sum =3.48 ± 0.25). As indicated above, this product consists of residues deriving from chestnut tannin extraction and is therefore characterised by a lower tannin content.

Quebracho and chestnut tannins produced in decreasing order, acetic acid, propionic acid, and butyric acid (**Table 4.2.5**). Just in a few cases (Q1, Q4, C4) propionate production was slightly higher than that of acetate, but this was not statistically significant. Once again, it should be emphasised that the SCFA production with a different profile to that of other chestnut extracts could be due to a distinct chemical composition of the initial sample. Remarkably, gallotannin-rich extracts (T1, G1 and G2) resulted in a completely distinct SCFA profile. Two relevant points emerged here: the very strong production of propionate and (only in the case of gall extracts) also the lack of release of butyric acid.

The importance of understanding whether a product is able to stimulate the release of SCFAs is strongly related to the numerous health beneficial properties exerted by these metabolites (Edwards *et al*., 2017; Arun *et al*., 2019). Moreover, these microbial by-products could be used as indicators of microbiota functionality: a high production indicates presence of the fermentative activity. Gibson *et al*. (2017) indicated the potential prebiotic activity of tannins, and this data was then corroborated by the study presented in the previous chapter (Molino *et al*., 2018). In particular, Q1 and C1 resulted in a higher induction of SCFA release than a commercial prebiotic fibre, inulin.

What is new in the present study is that a different tannin chemical composition can induce a different production of SCFAs. Thus, tannins can stimulate the growth of different taxa to different degrees. In this sense, it has been described that the abundance of specific bacterial taxa could vary upon different supplementation, explaining the changes in SCFA profiles. For example, Bacteroidaceae-Ruminococcaceae and Prevotellaceae-Ruminococcaceae dominated microbiota producing more butyrate (up to 96%) or propionate (up to 40%), respectively (Louis *et al*., 2007). Moreover, tannins could also act by selectively stimulating different microorganisms' metabolic pathways. In line with this, some studies suggested that polyphenols and their metabolites could differentially induce SCFA production (Tzounis *et al*., 2011; Bolca *et al*., 2013).

Further studies on the different modulation of microbiota composition by the different tannin extracts could help to interpret the present findings.

Table 4.2.5 Production of SCFAs through *in vitro* fermentation. Results are expressed as mmol of SCFA per g of original extract and reported as mean ± SD. Different letters indicates statistical differences by ANOVA and Bonferroni post-hoc test ($p<0.05$) among samples in the same group, for acetic acid, propionic acid and butyric acid, separately.

	Acetic acid	**Propionic acid**	**Butyric acid**
Q1	2.636 ± 0.237^{a}	2.655 ± 0.065^{a}	0.448 ± 0.011^{a}
Q2	2.609 ± 0.115^{a}	2.505 ± 0.069^{a}	0.193 ± 0.003^{b}
Q3	10.19 ± 1.110^{b}	9.487 ± 0.201^{b}	0.917 ± 0.006^{c}
Q4	1.801 ± 0.282^{a}	2.027 ± 0.086^{c}	0.412 ± 0.003^{d}
C1	7.336 ± 0.065^{a}	5.674 ± 0.077^{a}	2.281 ± 0.011^{a}
C2	4.408 ± 0.060^{b}	1.854 ± 0.055^{b}	0.888 ± 0.002^{b}
C3	3.572 ± 0.160^{c}	2.823 ± 0.045^{c}	1.303 ± 0.005^{c}
C4	1.188 ± 0.173^{d}	1.875 ± 0.081^{b}	0.416 ± 0.002^{d}
T1	5.339 ± 0.320^{a}	27.59 ± 0.512^{a}	1.870 ± 0.169
G1	2.351 ± 0.095^{b}	14.79 ± 0.555^{b}	-
G2	1.272 ± 0.185^{c}	20.76 ± 0.616^{c}	-

4.3 Chapter 3

Effect of different concentrations of tannin extracts on their bioactivity and microbiota modulation

Four tannin extracts (Q2, C2, T1, G2) were submitted to *in vitro* digestion and fermentation to test in what extent different concentration (A, B, C, D) could influence the bioactivity of these phytocomplexes. In view of this, the antioxidant capacity of all the samples was assessed after both the digestion and fermentation processes. Furthermore, the release of SCFAs through microbial fermentation was also examined.

4.3.1 Antioxidant capacity

Three different spectrophotometric techniques were applied to determine the antioxidant capacity of the samples. Since there is no absolute technique for measuring the antioxidant capacity of a phytocomplex (Pérez-Burillo *et al*., 2015), the evaluation of the antioxidant activity measured in different modalities gives an overall view of a product performance. Thus, Folin-Ciocalteu was applied to quantify the total polyphenol content, $TEAC_{FRAP}$ to detect the reducing capacity against ferric ions and $TEAC_{ABTS}$ to evaluate the scavenging ability against radicals. A certain degree of similarity was observed between the three methods; thus, a significant positive correlation ($p < 0.01$) was calculated by the Spearman method, with values around $Rs = 0.87$ (**Table 4.3.1**).

In particular, in this chapter the objective was to study whether tannin extracts taken at different concentrations could have differing degrees of antioxidant power.

Figure 4.3.1 depicts the results after digestion and fermentation for each sample. For all assessed techniques, the ANOVA performed by grouping the samples by type of extract showed that the antioxidant capacity was exerted in descending order by T1 > G2 > C2 > Q2.

Table 4.3.1 Linear correlations between the antioxidant capacity of tannin extract. **Indicates statistically significant correlation at the 0.01 level (2-tailed).

	Folin-Ciocalteu	$TEAC_{FRAP}$	$TEAC_{ABTS}$
Folin-Ciocalteu	1	0.890**	0.890**
$TEAC_{FRAP}$	0.890**	1	0.823**
$TEAC_{ABTS}$	0.890**	0.823**	1

These results are in line with those found in the previous chapter, in which the extracts obtained from tara and gall with solvents presented greater antioxidant activity than those obtained with hot water, as in the case of both quebracho and chestnut. However, here the new finding highlights that regardless of the amount of extract submitted to digestion, the samples exert the same antioxidant activity, reported per gram of original product. In fact, although the results show some variations within each group, these are not statistically significant in any case (**Table 4.3.2**).

Similarly to the digested samples, by grouping samples independently of the concentration, T1 was found to have the highest antioxidant activity compared to the other products after fermentation. Then, in decreasing order, G2, C2 and Q2 were recorded (**Figure 4.3.1**). Within each extract, lower concentrations submitted to fermentation led to a decrease in the content of total polyphenols and also to a reduction in antioxidant capacity. In particular, the largest drop was detected between concentrations A and B, while between B, C and D a smaller decrease was measured. For the smallest concentrations (C and D), no significant differences were recorded for any of the extracts studied (**Table 4.3.2**).

It is important that a balance between the food and the microbiota would be maintained. As we reported in the previous chapter, fermentation of food by gut microbiota has a major influence on its antioxidant capacity (Pérez-Burillo *et al*., 2018). However, tannins, at certain concentrations, can exert a powerful bacteriostatic or even bactericidal effect (see **Section 1.4.3**, Antimicrobial properties). In this view, excessive amounts of tannins may partially impair the composition of the microbiota or its functionality. Thus, this would lead to less metabolization of the phytocomplexes, which would continue to exert a relatively high antioxidant effect.

Supporting this hypothesis, some authors reported that the presence of galloyl groups in hydrolysable tannins results in a higher antibacterial action than that

exerted by condensed tannins. Among hydrolysable tannins, gallotannins seem to be more effective than ellagitannins (Funatogawa *et al*., 2004; Ekambaram *et al*., 2016; Shimozu *et al*., 2017). In accordance with this hypothesis, T1 and G2 would be the products with the highest antibacterial activity. These two samples showed the greatest drop in antioxidant activity as the concentration from A to B decreased. This result could mean that high doses of tannins may be detrimental to the gut microbiota, but a decrease in their content may lead to a more balanced equilibrium between these phytocomplexes and the microbiota ecosystem.

Therefore, the decrease in antioxidant activity of fermented samples should not be taken as a detrimental result. It could reflect an increased fermentative activity by the microbiota. Thus, an increased metabolization of the phytocomplexes could lead to their transformation into much smaller compounds that do not exert the same antioxidant capacity as their parent compounds.

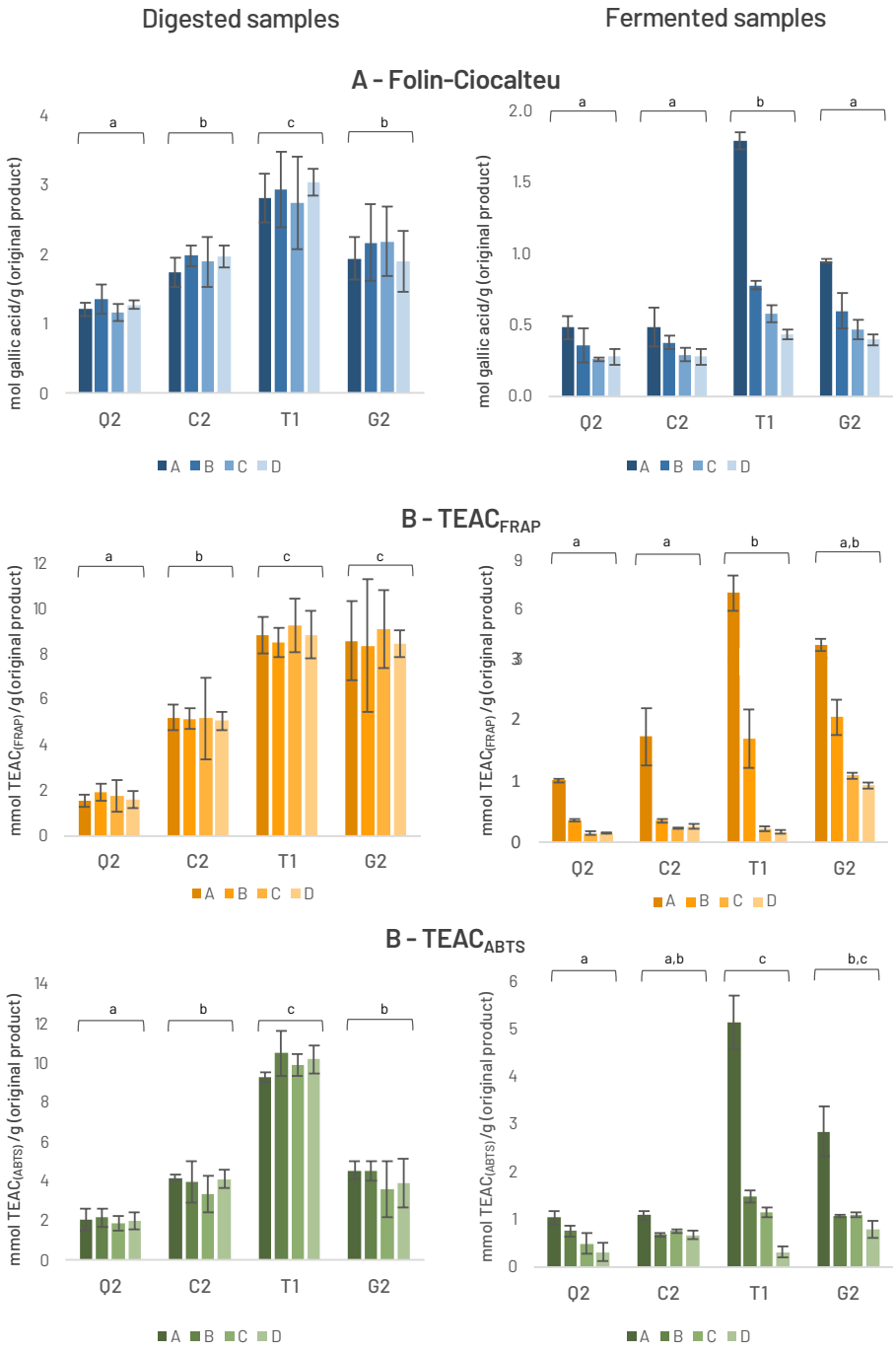


Figure 4.3.1 Total polyphenol content (A-Folin-Ciocalteu) and antioxidant capacity (B – TEAC$_{FRAP}$, C – TEAC$_{ABTS}$) of digested and fermented samples. Different letters indicate statistically significant differences by ANOVA and Bonferroni post-hoc test ($p<0.05$) among different tannin extracts.

Table 4.3.2 Antioxidant capacity of digested and fermented samples measured by Folin-Ciocalteu, TEACFRAP and TEACABTS. Folin-Ciocalteu results are expressed in mol gallic acid/g of original extract; TEAC (FRAP and ABTS) are expressed in mmol Trolox/ g of original extract. Data are reported as mean ± SD.

		Folin-Ciocalteu		$TEAC_{FRAP}$		$TEAC_{ABTS}$	
		Digested	Fermented	Digested	Fermented	Digested	Fermented
O2	A	1.21 ± 0.10	0.48 ± 0.08^{a}	1.54 ± 0.27	1.01 ± 0.03^{a}	2.04 ± 0.56	1.03 ± 0.13^{a}
	B	1.35 ± 0.21	0.36 ± 0.12^{a,b}	1.90 ± 0.38	0.36 ± 0.01^{b}	2.14 ± 0.48	0.76 ± 0.12^{b}
	C	1.16 ± 0.13	0.26 ± 0.01^{b}	1.75 ± 0.69	0.15 ± 0.03^{c}	1.86 ± 0.34	0.49 ± 0.22^{c}
	D	1.27 ± 0.06	0.28 ± 0.05^{b}	1.60 ± 0.37	0.15 ± 0.01^{c}	2.01 ± 0.43	0.32 ± 0.20^{c}
C2	A	1.73 ± 0.21	0.48 ± 0.14^{a}	5.21 ± 0.56	1.72 ± 0.47^{a}	4.17 ± 0.14	1.09 ± 0.09^{a}
	B	1.98 ± 0.15	0.38 ± 0.05^{a,b}	5.15 ± 0.47	0.35 ± 0.03^{b}	3.96 ± 1.05	0.69 ± 0.04^{b}
	C	1.89 ± 0.36	0.29 ± 0.05^{b}	5.17 ± 1.79	0.23 ± 0.02^{b}	3.34 ± 0.95	0.76 ± 0.03^{b}
	D	1.97 ± 0.16	0.28 ± 0.06^{b}	5.06 ± 0.42	0.26 ± 0.04^{b}	4.11 ± 0.48	0.68 ± 0.09^{b}
T1	A	2.81 ± 0.35	1.79 ± 0.06^{a}	8.83 ± 0.81	7.01 ± 1.06^{a}	9.27 ± 0.23	5.14 ± 0.57^{a}
	B	2.94 ± 0.54	0.78 ± 0.03^{b}	8.51 ± 0.62	1.69 ± 0.47^{b}	10.5 ± 1.14	1.49 ± 0.13^{b}
	C	2.74 ± 0.67	0.58 ± 0.06^{c}	9.26 ± 1.19	0.22 ± 0.04^{c}	9.91 ± 0.55	1.15 ± 0.10^{c}
	D	3.04 ± 0.20	0.43 ± 0.03^{d}	8.86 ± 1.04	0.17 ± 0.03^{c}	10.2 ± 0.69	0.31 ± 0.12^{d}
G2	A	1.94 ± 0.31	0.95 ± 0.01^{a}	8.58 ± 1.75	3.86 ± 0.36^{a}	4.55 ± 0.48	2.84 ± 0.53^{a}
	B	2.17 ± 0.56	0.60 ± 0.13^{b}	8.37 ± 2.91	2.04 ± 0.28^{b}	4.51 ± 0.50	1.07 ± 0.03^{b}
	C	2.18 ± 0.50	0.47 ± 0.07^{b,c}	9.12 ± 1.71	1.09 ± 0.05^{c}	3.57 ± 1.41	1.09 ± 0.05^{b}
	D	1.90 ± 0.44	0.40 ± 0.04^{c}	8.47 ± 0.60	0.93 ± 0.05^{c}	3.90 ± 1.23	0.79 ± 0.18^{b}

4.3.2 SCFA production

In this chapter, the quantification of SCFA release was used to evaluate the fermentative activity of the microbiota in the presence of different concentrations of the four tannin extracts Q2, C2, T1 and G2. Thus acetic, propionic and butyric acid were identified and quantified in the supernatants derived after the *in vitro* fermentation process. **Figure 4.3.2** represents the sum of the release of SCFAs determined by each evaluated sample.

Some similarities were recorded for Q2 and C2. The three SCFAs were produced in the same proportion, in decreasing order acetate > propionate > butyrate (**Table 4.3.3**). Interestingly, a decrease of the concentration (from A to D) determined a statistically significant increase in the production of total SCFAs.

Table 4.3.3 Production of short chain fatty acids (SCFAs) of samples after *in vitro* fermentation. Results are expressed as mmol of SCFA per g of original extract and reported as mean ± SD. Different letters indicate statistical differences by ANOVA and Bonferroni post-hoc test ($p<0.05$) among samples for acetic acid, propionic acid and butyric acid, separately.

		Acetic acid	Propionic acid	Butyric acid
Q2	A	3.877 ± 0.453^{a}	2.924 ± 0.139	0.039 ± 0.011^{a}
	B	$4.475 \pm 0.146^{a,b}$	2.909 ± 0.397	0.692 ± 0.134^{b}
	C	5.268 ± 0.306^{b}	2.828 ± 0.157	1.178 ± 0.200^{c}
	D	6.687 ± 0.086^{c}	2.605 ± 0.200	1.692 ± 0.210^{d}
C2	A	4.817 ± 0.201^{a}	2.659 ± 0.094^{a}	0.775 ± 0.220^{a}
	B	5.666 ± 0.199^{b}	3.559 ± 0.242^{a}	$1.044 \pm 0.056^{a,b}$
	C	9.610 ± 0.306^{c}	5.104 ± 0.372^{b}	1.420 ± 0.171^{b}
	D	11.12 ± 0.251^{d}	6.072 ± 0.560^{b}	2.390 ± 0.254^{c}
T1	A	2.663 ± 0.123^{a}	15.49 ± 0.430^{a}	1.515 ± 0.020^{a}
	B	3.251 ± 0.208^{b}	14.45 ± 1.912^{a}	$2.807 \pm 0.335^{a,b}$
	C	5.579 ± 0.213^{c}	10.34 ± 0.397^{b}	2.912 ± 0.363^{b}
	D	8.230 ± 0.195^{d}	3.068 ± 0.212^{c}	2.807 ± 0.250^{c}
G2	A	1.614 ± 0.025^{a}	3.670 ± 0.585^{a}	-
	B	3.392 ± 0.096^{a}	23.00 ± 3.132^{b}	-
	C	7.159 ± 0.387^{b}	19.87 ± 1.496^{b}	1.725 ± 0.231
	D	12.54 ± 2.003^{c}	8.148 ± 0.412^{c}	2.527 ± 0.250

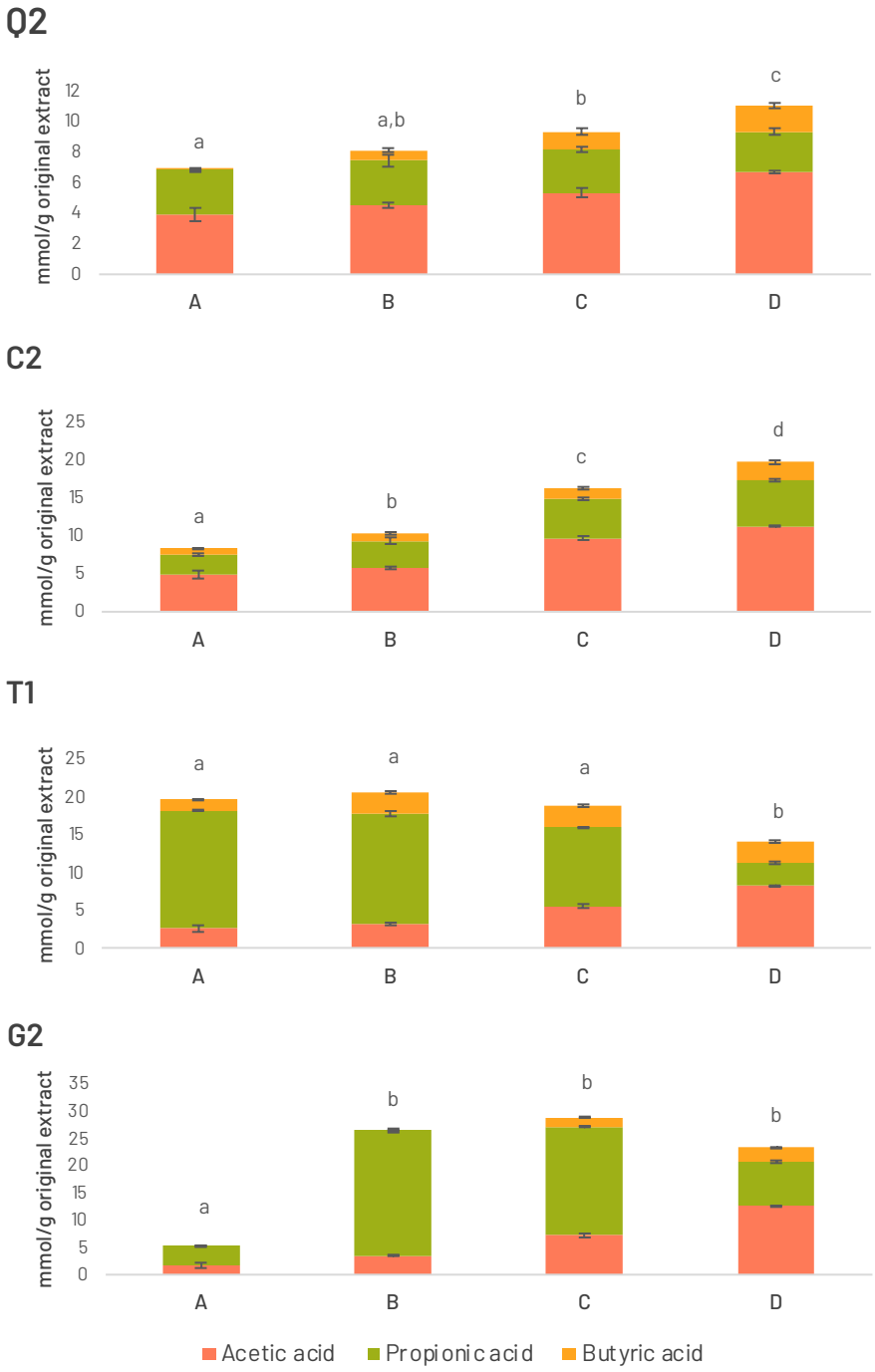


Figure 3.3.2 Release of SCFAs for different concentrations of tannin extracts (A, B, C and D) after fermentation. Different letters indicate statistical differences by ANOVA and Bonferroni post-hoc test ($p<0.05$) of total sum of SCFAs among concentrations (A-D).

As regards T1, the production of total SCFAs showed no significant difference between concentrations A (SCFA sum = 19.67 ± 0.35), B (SCFA sum = 20.51 ± 1.54) and C (SCFA sum = 18.83 ± 0.19), and decreased with concentration D (SCFA sum = 14.11 ± 0.54). Looking at the individual SCFAs, concentration A induced proportionally more propionic acid production, then less acetic and finally butyric acid. Then, as the concentration of fermented T1 was gradually reduced, propionate decreased while acetate and butyrate increased.

The highest fermented concentration of G2 was found to result in the lowest production of SCFAs overall (SCFA sum = 5.28 ± 0.61), with no production of butyrate. The sum of total SCFAs produced by microbial metabolization of concentrations B (SCFA sum = 26.39 ± 3.10), C (SCFA sum = 28.76 ± 1.57) and D (SCFA sum = 23.22 ± 2.60) showed no significant differences. However, the composition varied greatly, depending on the concentration of fermented G2. In B, the presence of butyrate could still not be detected, and the concentration of propionate was much higher than that of acetate. By progressively decreasing the concentration (C and D), the proportions changed until decreasing amounts of acetic acid > propionic acid > butyric acid were detected.

In general, from this study it emerges that the different chemical compositions of the evaluated extracts and their different concentrations result in a differing response in metabolization by the gut microbiota. Although high concentrations of Q2 and C2 induce generally high production of SCFAs, the results of this study support the hypothesis mentioned above that excessive amounts of tannin extracts may reduce rather than increase microbiota functionality.

According to chapter 2, high concentration of gallotannin-rich extracts (T1 and G2) resulted in a completely distinct SCFA profile compared to Q2 and C2. Here the different modulation of the microbiota composition due to the chemical composition of the tannin extract and their concentration is apparent. For instance, an excessive amount of G2, as in the case of concentration A, could be detrimental to the microbiota, even reducing its functionality, although this should be investigated further with metagenomic studies.

The absence of production of butyrate by fermenting higher amounts of G2 could be related to the relative abundance of specific fermenting taxa. Indeed, some authors showed that some prebiotic fibres, by increasing luminal pH, could induce the almost complete disappearance of the butyrate-producing bacteria, while the acetate- and propionate-producing Bacteroides-related bacteria become dominant (Walker *et al.*, 2005).

Even though both T1 and G2 resulted in a large production of total SCFAs (excluding concentration A of G2), this was determined by a great release of propionic acid. Propionic acid, similarly to the other SCFAs, has been reported to play a crucial role in maintaining host physiology. However, an excessive production

of propionate has been related with potential detrimental effects for the organism (Al-Lahham *et al*., 2010). Moreover, it has been stated that, in the colon and stool of healthy individuals and animals, acetate, propionate, and butyrate are approximately represented by the molar ratio of 60:20:20 (Den Besten *et al*., 2013). In this sense, the progressive decrease in the concentration of fermented T1 and G2 resulted in a SCFA production closer to that of physiological conditions.

Once more, the presence of tannins at different concentration could have modulated the microbiota to various extents, leading to varying SCFA profiles. Indeed, propionate is mainly produced by *Bacteroides* species, as well as acetate, while many species of the Firmicutes phylum have butyrate as their primary metabolic end product (Adamberg *et al*., 2018).

Further studies on the different modulation of microbiota composition by the different tannin extracts could help to interpret the present findings.

4.4 Chapter 4

Enrichment of food with tannin extracts promotes healthy changes in the human gut microbiota

To assess the bioactivity of tannin wood extracts of different origin and chemical composition, we chose three extracts representative of different classes of tannins: condensed tannins (QUE), ellagitannins (CHE) and gallotannins (TE). We evaluated the effect on the gut microbiota by adding the extracts to 8 different sources of food grouped in three food types: cereal-based foods (breakfast cereals (C), breakfast cereals with sugar (CS), bread (B)), meat (meat (M) and meat with 30% fat (MF)) and dairy products (milk (L), low fat yogurt (Y) and full fat Greek yogurt (YG)). All the samples were subjected to an *in vitro* digestion-fermentation process designed to mimic natural digestion in the human oral, gastric, and intestinal chambers. The bioactivity was measured as the capacity to modify the gut microbiota in terms of taxonomic composition and SCFA production.

4.4.1 Effect on microbiota composition

The gut microbiota has a determinant role in maintaining human health. Specific foods and products containing prebiotics interact directly or indirectly with the microbiota, driving its composition and function (Laitinen and Gueimonde, 2019). In this context, tannins can modulate gut microbial composition and function, selectively inhibiting pathogens and promoting the growth of beneficial bacteria (Ozdal *et al*., 2016). Tannins are known also for their capacity to interact with proteins and carbohydrates, among other compounds. The addition of tannin to the different food matrices determined a general trend of increase of the sample richness and diversity, as estimated by different estimator indexes: phylogenetic diversity, Chao1 and ACE and Shannon's diversity index (**Table 4.4.1**). As an exception, microbiota richness and diversity rather decreased in cereal-based foods supplemented with CHE.

For most food matrices and tannins tested, tannin enrichment resulted in a change in the composition of the microbiota, compared with the fermentations employing the foods alone. **Figure 4.4.1** shows the increase in diversity detected for QUE and TE when all food matrices are considered together (p=0.016, adjusted p=0.023, and p=0.008, adjusted p=0.023, respectively), and the larger variability observed with the addition of CHE.

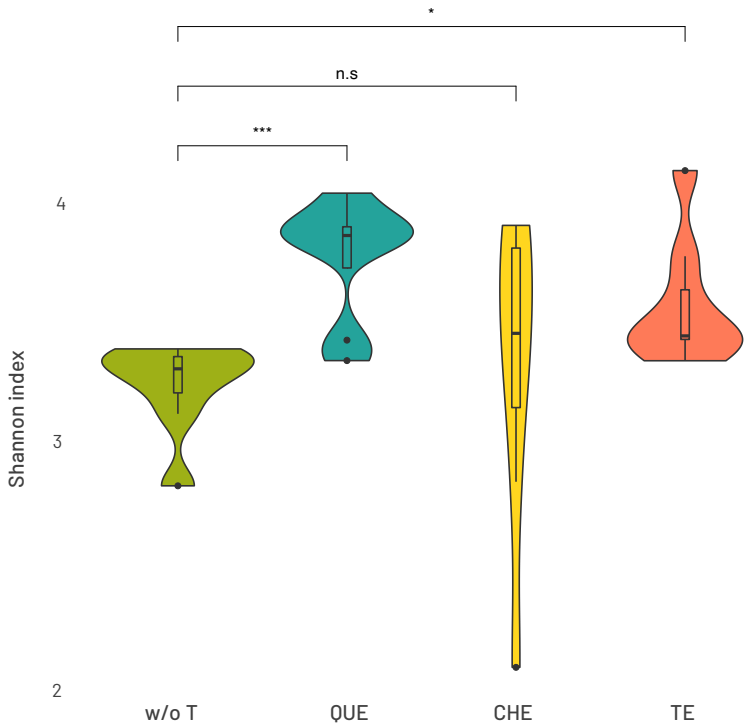


Figure 4.4.1 Microbiota diversity in fermentations of food matrices with and without tannin extracts. Diversity increases for QUE and TE when all food matrices are considered together (p=0.016, adjusted p=0.023, and p=0.008, adjusted p=0.023, respectively). w/o T: w without tannins, QUE: quebracho tannins extract, CHE: chestnut tannins extract, TE: tara tannins extract.

Table 4.4.1 Indexes of α diversity.

Food	Tannin	Diversity indexes					
		Phylogenetic diversity	Shannon	Chao1	SE.Chao1	ACE	SE.ACE
Milk	w/o T	7.79	3.29	219.00	4.13	218.25	7.03
	QUE	9.79	3.96	287.50	5.41	286.86	8.10
	CHE	10.20	3.91	302.80	4.53	303.45	8.46
	TE	10.35	4.16	305.00	4.54	304.23	8.12
Yogurt	w/o T	6.73	3.41	172.00	1.82	171.63	5.79
	QUE	9.65	3.85	269.00	4.31	267.24	7.68
	CHE	10.08	3.93	297.21	7.43	292.75	8.13
	TE	8.46	3.80	261.00	4.13	259.78	7.70
Greek yogurt	w/o T	6.38	3.35	139.00	0.12	139.17	4.94
	QUE	7.00	3.90	159.00	0.10	159.22	5.15
	CHE	8.97	3.80	227.25	0.74	227.39	6.58
	TE	8.10	3.36	216.88	1.25	218.35	6.91
Cereals	w/o T	8.53	3.25	311.17	17.56	293.07	8.37
	QUE	9.91	3.87	307.79	8.35	303.48	8.49
	CHE	6.41	3.26	167.00	4.31	166.10	6.28
	TE	8.74	3.45	271.77	10.02	264.40	7.87
Cereals with Sugar	w/o T	6.78	3.13	165.67	1.15	166.13	5.93
	QUE	10.05	3.91	325.53	7.25	328.39	8.95
	CHE	5.95	2.85	146.50	3.92	146.89	5.73
	TE	8.54	3.46	261.93	6.85	259.30	7.74
Bread	w/o T	8.28	3.38	230.40	1.62	231.95	7.47
	QUE	9.92	3.44	290.81	8.71	286.05	8.13
	CHE	5.36	2.07	108.14	0.49	108.58	4.94
	TE	8.79	3.46	263.22	3.69	264.45	7.93
Meat	w/o T	7.72	2.83	212.33	2.42	212.42	7.08
	QUE	9.69	4.06	281.77	2.68	282.23	7.65
	CHE	8.56	3.50	258.13	3.36	258.58	7.60
	TE	8.29	3.61	231.33	2.55	231.10	6.98
Meat with 30% fat	w/o T	7.89	3.37	214.06	0.28	214.52	7.13
	QUE	6.87	3.36	181.60	1.19	181.73	6.08
	CHE	7.90	3.45	231.88	3.19	233.51	7.24
	TE	7.41	3.43	203.50	3.92	203.21	6.43

A PCoA based on weighted UniFrac distances was performed to establish whether samples separate into clusters. As seen in **Figure 4.4.2**, PCo1 and PCo2 accounted for 61.47 and 22.8%, respectively, of the total variation. All samples containing QUE and CHE clustered away from non-enriched foods, as did the milk and meat samples enriched with TE. Thus, the results indicate that in most cases the microbiota communities resulting from foods enriched with these tannin extracts are different from those of the food matrices alone.

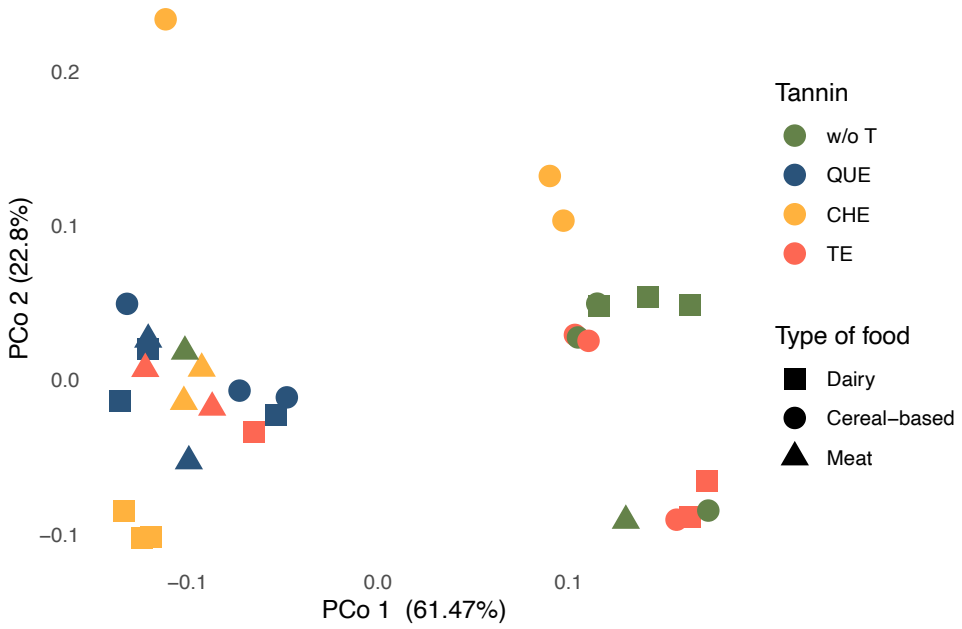


Figure 4.4.2 Principal coordinate analysis (PCoA) plot of total variation based on weighted UniFrac distances of microbial genus abundance among all profiled samples. The food sources were grouped in three food types: cereal-based foods (breakfast cereals, breakfast cereals with sugar, bread), meat (meat and meat with 30% fat) and dairy products (milk, low fat yogurt and full-fat Greek yogurt). w/o T: without tannins, QUE: quebracho tannins extract, CHE: chestnut tannins extract, TE: tara tannins extract.

More in depth, the obtained pattern of sample clustering does not reflect exclusively the tannin or the associated food matrix, but is rather influenced by both. Among the evaluated tannin extracts, QUE clustered most homogeneously, with all samples present in a single cluster independently of the matrix to which QUE was associated. In contrast, for CHE and TE, microbial composition depended on the food matrix. In the case of CHE, although all samples separated well from non-enriched foods, dairy and meat samples clustered with the QUE samples, whereas cereal-based foods clustered apart from the rest. In the case of TE, the yogurt and cereal-based samples did not separate from non-enriched foods, whereas the milk and meat samples were located in the cluster containing all the QUE samples and the dairy and meat CHE samples. This suggests that TE only has an effect on the gut microbiota when added to meat or milk.

At the phylum level, the gut microbiota after *in vitro* fermentation of most samples was similar and dominated by Firmicutes and Bacteroidetes, followed by Proteobacteria, Verrucomicrobia and Actinobacteria, with some exceptions (**Figure 4.4.3** and **Table 4.4.2**). Indeed, for meat (M and MF) enriched with tannin extracts the relative abundance of Proteobacteria was higher ($p=0.00084$, adjusted $p=0.0051$), while that of Bacteroidetes was lower ($p=0.027$, adjusted $p=0.053$) compared to meat samples not enriched with tannins.

To identify the bacteria most generally affected by the addition of each of the tannins, Wilcoxon signed-rank tests were applied to compare bacterial relative abundances in all samples enriched with a given tannin vs all samples containing no tannins. These comparisons identified a large number of genera for which abundance after tannin addition differed from that of non-enriched foods at nominal significance level, but only differences in QUE were still significant after adjusting for multiple comparisons (data not shown).

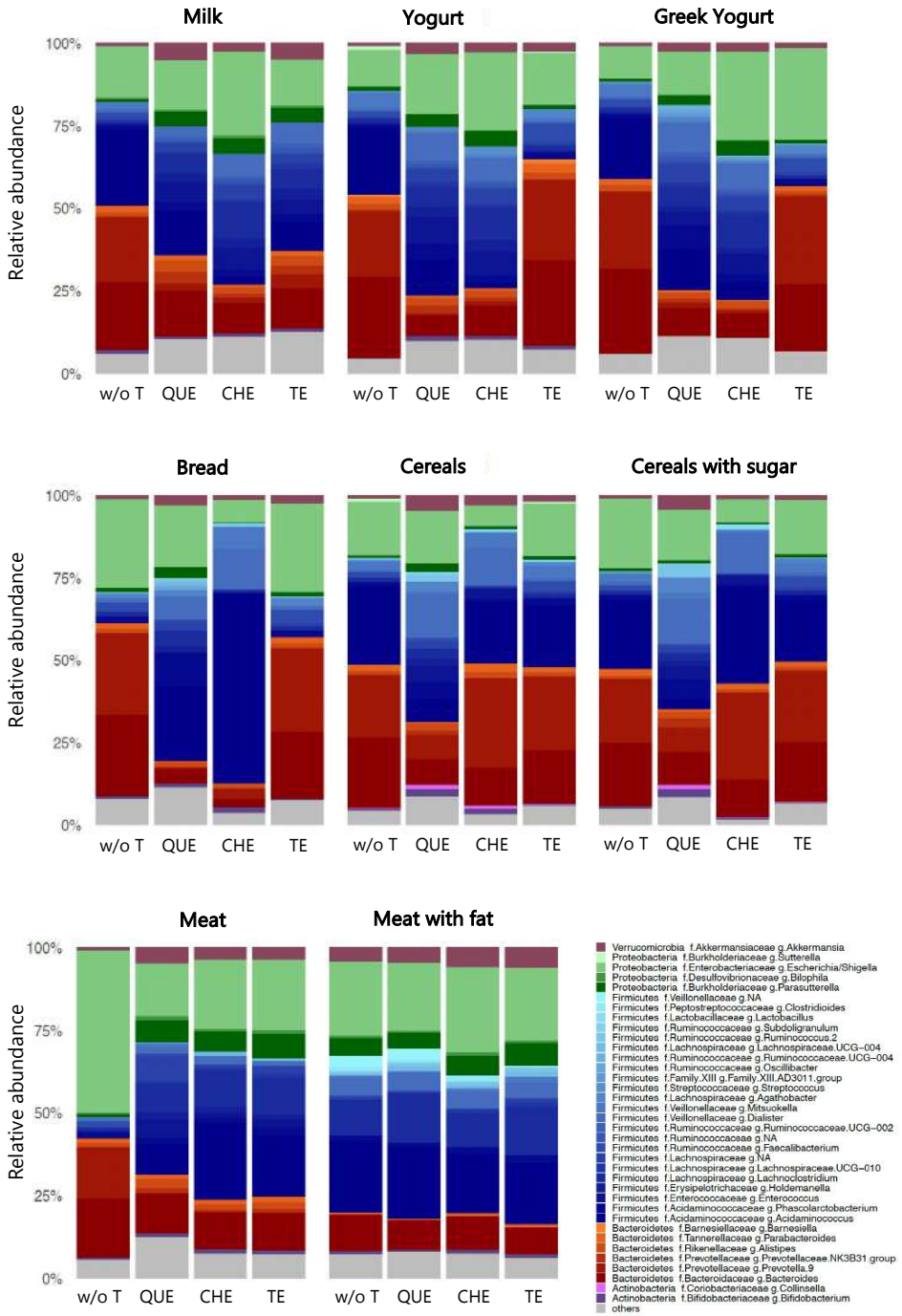


Figure 4.4.3 Barplot of gut microbial community at genus level. w/o T: without tannins, QUE: quebracho tannin extract, CHE: chestnut tannins extract, TE: tara tannin extract. Relative abundances were obtained by TSS abundance table. "Others" include genera with relative abundance lower than 1% for all conditions.

Table 4.4.2 Relative abundance of phyla.

Food	Tannin	Actinobacteria	Bacteroidetes	Firmicutes	Proteobacteria	Verrucomicrobia	Other
Milk	w/o T	1.11	43.87	31.33	16.77	1.14	5.79
	QUE	0.66	24.64	39.25	19.99	5.21	10.25
	CHE	1.18	14.60	39.79	30.91	2.69	10.83
	TE	0.98	23.57	38.92	19.06	5.08	12.39
Yogurt	w/o T	0.24	49.33	31.71	13.48	0.95	4.30
	QUE	1.33	12.20	51.24	22.11	3.42	9.69
	CHE	0.95	14.54	43.07	28.53	2.84	10.07
	TE	1.08	56.36	15.40	17.39	2.68	7.09
Greek yogurt	w/o T	0.00	52.78	29.43	10.83	1.03	5.93
	QUE	0.00	13.89	56.27	16.11	2.65	11.08
	CHE	0.00	11.41	43.72	31.47	2.70	10.70
	TE	0.00	50.05	12.93	28.80	1.71	6.51
Cereals	w/o T	0.78	43.36	32.49	18.07	1.08	4.21
	QUE	3.31	19.29	45.37	18.64	4.78	8.60
	CHE	2.48	43.25	40.85	7.21	3.14	3.07
	TE	0.68	41.58	32.56	17.52	2.07	5.58
Cereals with Sugar	w/o T	0.75	41.65	29.64	22.16	1.04	4.76
	QUE	3.49	23.03	44.16	16.56	4.35	8.40
	CHE	0.98	40.29	48.53	7.59	1.24	1.36
	TE	0.36	42.57	31.64	17.49	1.47	6.47
Bread	w/o T	0.84	52.53	9.43	28.17	1.30	7.73
	QUE	1.00	6.93	55.44	22.02	3.24	11.36
	CHE	1.61	7.25	78.84	7.27	1.42	3.61
	TE	0.52	49.10	12.44	28.19	2.49	7.26
Meat	w/o T	0.54	36.19	6.35	50.40	1.10	5.42
	QUE	1.02	17.80	39.98	23.99	4.84	12.36
	CHE	1.28	15.00	44.87	27.81	3.78	7.26
	TE	1.16	16.46	41.55	30.08	3.73	7.03
Meat with 30% fat	w/o T	0.85	11.71	47.49	28.44	4.44	7.07
	QUE	0.67	9.45	51.54	25.93	4.74	7.67
	CHE	1.02	11.20	41.57	32.78	6.03	7.41
	TE	0.97	9.07	48.05	29.35	6.41	6.13

Remarkably, with every tannin tested *Bacteroides* decreased (QUE: p=0.008, adjusted p=0.048; CHE: p=0.008, adjusted p=0.11; TE: p=0.023, adjusted p=0.14) (**Figure 4.4.4 A**). *Bacteroides* is known to be implicated in proteolytic fermentation in the gut, so that the binding of proteins by tannins may be responsible for the decrease of these bacteria by rendering protein molecules unavailable for digestion (Smith and MacFarlane, 1998). Inhibitory effects of tannins on proteolytic bacteria and proteolytic enzyme activity have been proposed, likely due to coating of the protein surface leading to interference with the interaction of enzyme and substrate (McManus *et al*. 1981; Patra, Min, and Saxena 2012).

In contrast, *Akkermansia* increased in all cases (QUE: p=0.008, adjusted p=0.048; CHE: p=0.008, adjusted p=0.11; TE: p=0.008, adjusted p=0.079) (**Figure 4.4.4 B**). The increase of the genus *Akkermansia* was one of the most general results of this study. These are mucin-degrading bacteria, living in the mucus layer, recognized as markers of a healthy gut. In fact, several studies have highlighted their anti-inflammatory properties, and the ability to increase insulin sensitivity, and boost gut barrier function (Masumoto *et al*. 2016; Cires *et al*. 2017; Rinninella *et al*. 2019).These properties are also related to their production of propionate and butyrate (Venegas *et al*., 2019). Furthermore, some authors have proposed *Akkermansia* as a key player in the breakdown of phenolic compounds in the intestine (Li *et al*. 2015), suggesting a likely reason for the observed increase of this genus with the addition of tannins.

Wilcoxon signed-rank tests at family level (**Figure 4.4.5**) then confirmed that Bacteroidaceae decreased (QUE: p=0.008, adjusted p=0.0313; CHE: p=0.008, adjusted p=0.049; TE p=0.023, adjusted p=0.103) and Akkermansiaceae increased (CHE: p=0.008 adjusted p=0.048; QUE: p value=0.008, adjusted p=0.031; TE p=0.008, adjusted p=0.068) with each of the tannins, although, in this case, only the differences with QUE and CHE were significant after adjustment for multiple comparisons. In addition, several abundance changes were significant only with QUE, including decreases of the Bacteroidales families Prevotellaceae (p=0.008, adjusted p=0.0313), Barnesiellaceae (p=0.008, adjusted p=0.031) and Tannerellaceae (p=0.008, adjusted p=0.031) and increases of the Rickenellaceae (Bacteroidales) (p=0.016, adjusted p=0.047) and several Clostridiales families (Ruminococcaceae (p=0.008, adjusted p=0.031), Lachnospiraceae (p=0.008, adjusted p=0.031), Christensenellaceae (p=0.016, adjusted p=0.047) and Family XIII (p=0.008, adjusted p=0.031)). On the other hand, Peptostreptococcaceae (Clostridiales) (p=0.008, adjusted p=0.049) and the actinobacterial families Coriobacteriaceae (p=0.008; adjusted p=0.048) and Bifidobacteriaceae (p=0.008, adjusted p=0.048) augmented significantly only with CHE.

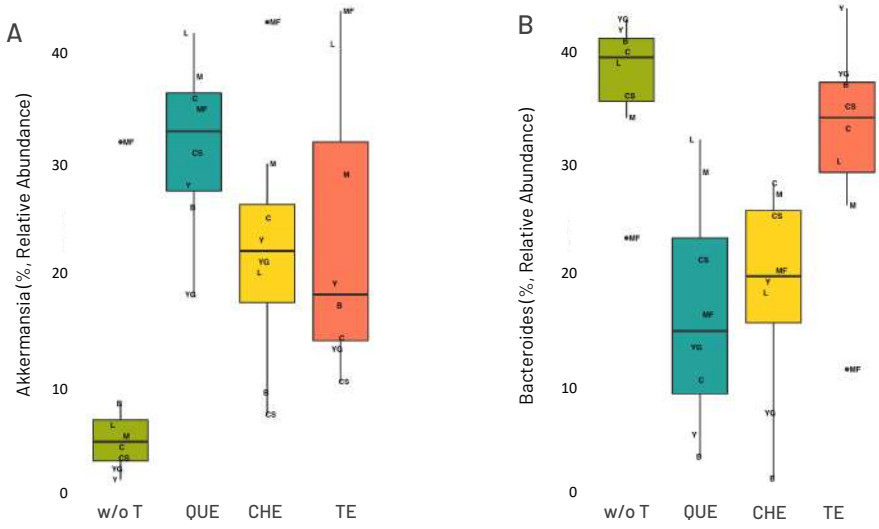


Figure 4.4.4 Changes in microbiota composition with tannin addition in all foods. (A) Decrease of *Bacteroides* (QUE: p=0.0078; CHE: p=0.0078; TE: p=0.023) and (B) increase of *Akkermansia* (QUE: p=0.0078; CHE: p=0.0078; TE: p=0.0078) with every tannin tested. C: breakfast cereals, CS: breakfast cereals with sugar, B: bread, M: meat, MF: meat with 30% fat, L: milk, Y: low fat yogurt and GY: full-fat Greek yogurt).

These results suggest that the interactions between tannins and food macromolecules may be different depending on the food matrices. Binding mechanisms between tannins and proteins or carbohydrates occur in a specific and selective way, mediated by hydrogen bonds and hydrophobic interactions (McManus *et al*., 1985; de Freitas and Nuno, 2012). Some factors related to these macromolecules may influence such interactions: size, charge, side chains, and conformation (Molino *et al*., 2019). Therefore, we expected that some effects of tannins would be dependent on the food matrix to which they were added.

LEfSe analyses allowed to identify the specific biomarkers that best characterize the microbiota changes generated by the addition of tannins within each of the three food groups (cereal-based foods (C, CS, B), meat (M, MF) and dairy products (L, Y, YG)). The only group where significant differences were not detected between tannin-enriched and non-enriched matrices was that of meats. **Figures 4.4.6** and **4.4.7** depict the over- and underrepresented genera (LDA score >3 and $p<0.05$) in dairy sources and cereal-based foods, respectively, enriched with the different tannin extracts, QUE, CHE and TE.

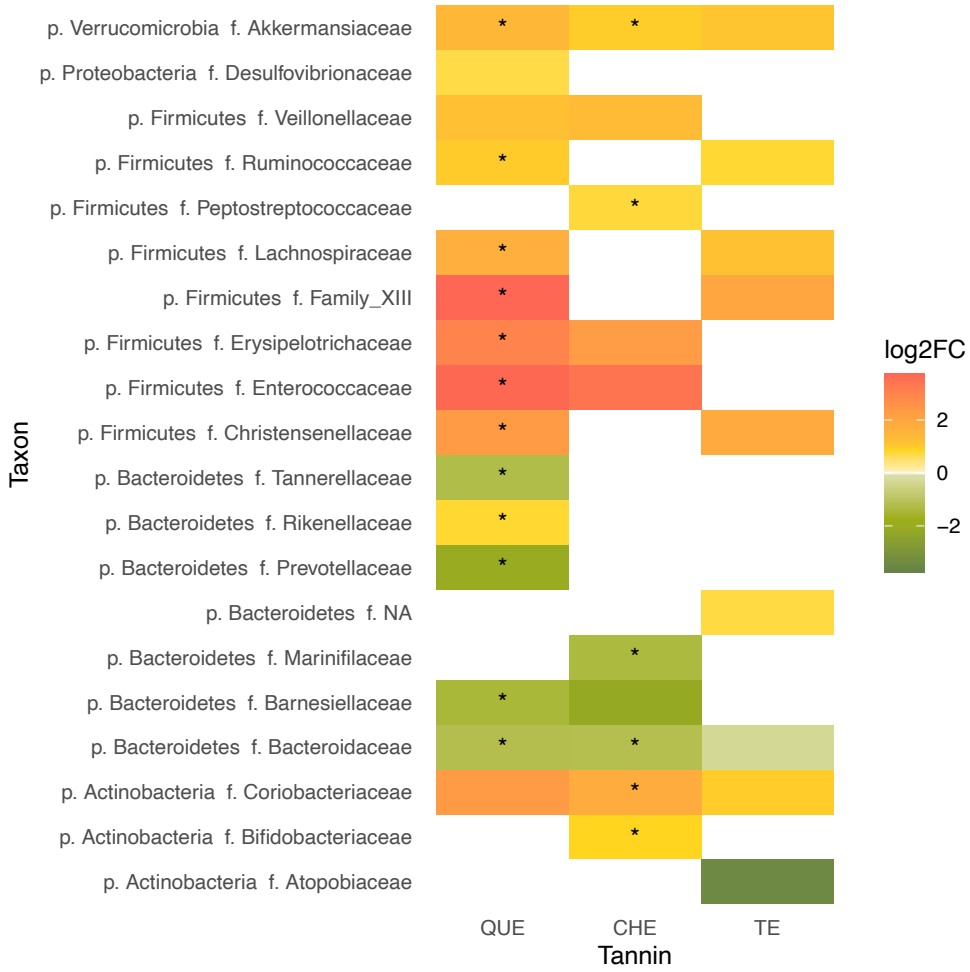


Figure 4.4.5 Fold-changes in the relative abundance of bacterial families with each tannin. All changes significant at nominal level by Wilcoxon signed-rank tests are shown ($p<0.05$). *Indicates statistically significant differences ($p<0.05$) after correction for multiple testing.

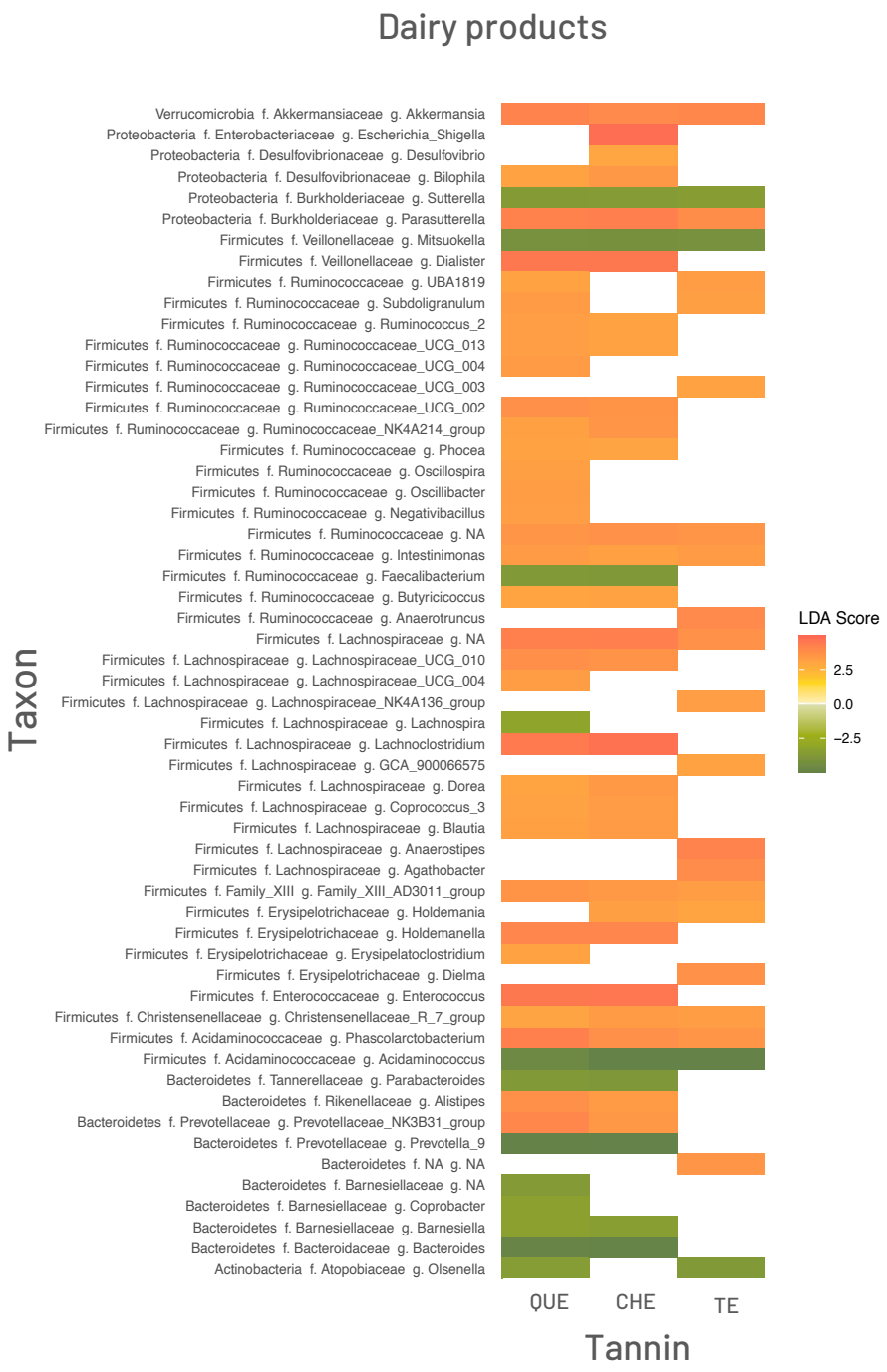


Figure 4.4.6 Genera responsible for the main differences in gut microbiota composition due to the addition of tannin extracts to dairy, detected by Linear Effects Size (LeFSe). Differences are represented as LDA score (>3) by colour gradient. All represented biomarkers are significant at $p<0.05$. NA taxon refers to unclassified ASVs at f. (family) or g. (genus) level.

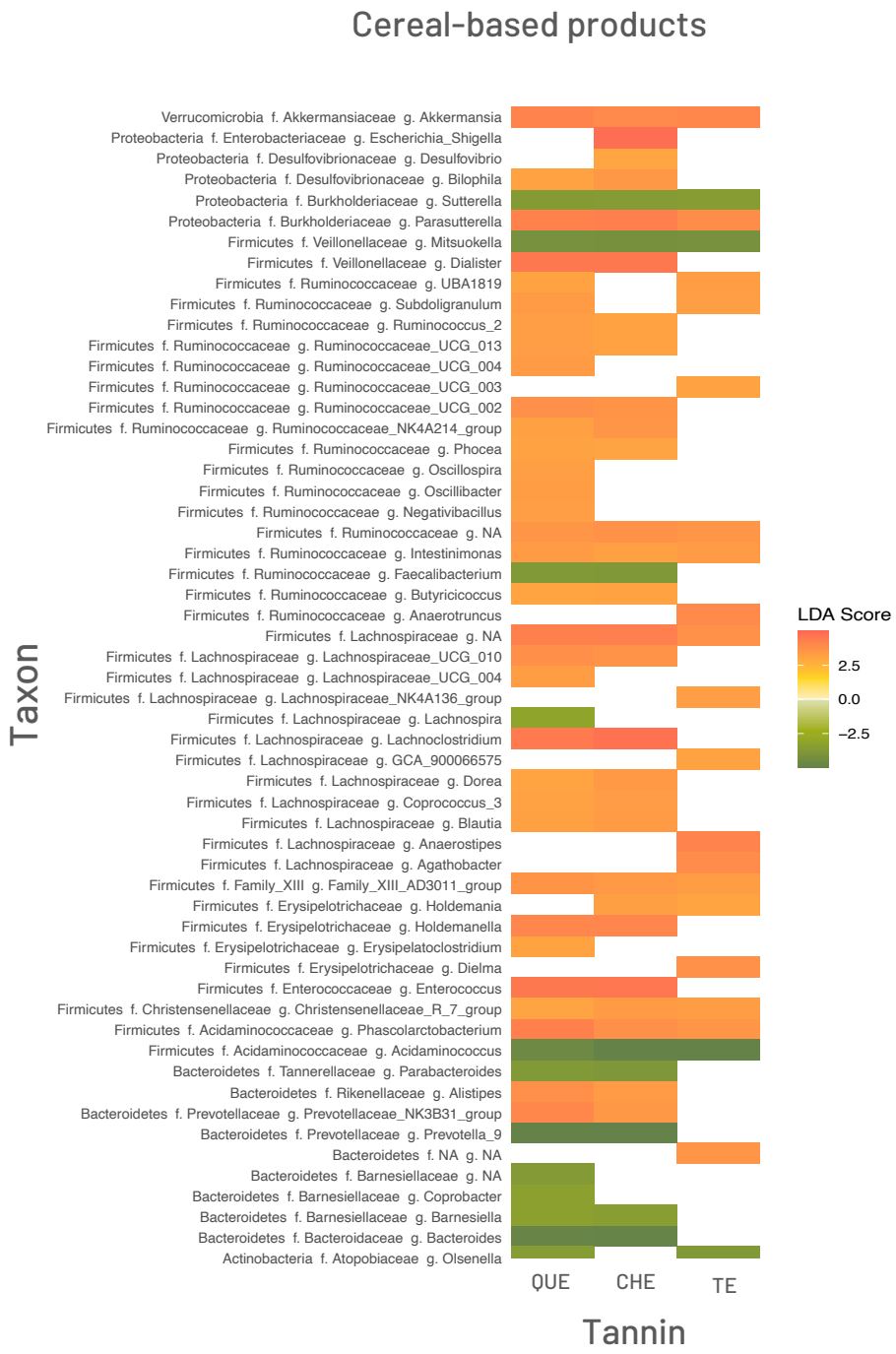


Figure 4.4.7 Genera responsible for the main differences in gut microbiota composition due to the addition of tannin extracts to cereal-based products, detected by Linear Effects Size (LeFSe). Differences are represented as LDA score (>3) by colour gradient. All represented biomarkers are significant at $p<0.05$. NA taxon refers to unclassified ASVs at f. (family) or g. (genus) level.

In most cases, genera were detected to increase rather than decrease significantly with the addition of tannins, except in the case of CHE added to cereal-based foods, where several members of the Proteobacteria, Bacteroidetes, and Firmicutes were found to decrease, including various genera of the Ruminococcaceae and Lachnospiraceae families. *Sutterella* was the only genus that decreased in both food matrices with all of the tannins tested. No genus was overrepresented in both food matrices with all of the tannins, but *Akkermansia*, *Intestinimonas*, *Phascolarctobacterium* and various unassigned Lachnospiraceae and Clostridiales Family XIII genera did increase in all cases except when cereal-based foods were enriched with CHE. It is interesting to note that several of the genera that decreased when CHE was added to carbohydrates actually increased when CHE was added to dairy (*Escherichia, Bilophila,* or *Lachnoclostridium*).

Finally, the differences generated by the addition of each tannin extract to each individual food matrix are represented as fold changes in Supplementary Figure S2, although further repetitions of each fermentation would be needed to assess their significance.

Several authors reported an increase of the abundance of bacteria belonging to the Lachnospiraceae and Ruminococcaceae families when tannins were added to animal feed (Choy *et al*., 2014; Díaz Carrasco *et al*., 2018). Lachnospiraceae and Ruminococcaceae belong to the order Clostridiales, which encompasses mostly beneficial bacteria, including members that have been associated with the modulation of physiologic, metabolic and immune processes in the gut and with prevention of inflammatory bowel disease. Furthermore, numerous genera of these families have been attributed the capacity to produce SCFAs (Lopetuso *et al*., 2013; Louis and Flint, 2017).

Diaz Carrasco *et al.* (2018) observed the same trend of increment of the aforementioned Clostridiales families in chicken supplemented with a mix of QUE and CHE. In particular, they detected an increment of the genus *Faecalibacterium*, among others. In these analyses, *Faecalibacterium* rather decreased with addition of QUE and CHE to cereal-based and dairy products (**Figure 4.4.8, 4.4.9** and **4.4.10**). However, addition of TE did determine a large increase of *Faecalibacterium* in C, CS, Y and GY (Figure S2), although *Faecalibacterium* increases did not reach significance when all cereal-based or dairy foods were considered together. This finding suggests that it will be interesting to analyse these individual food matrices supplemented with TE in further experiments to assess the significance of the *Faecalibacterium* increases independently in each of them.

Besides not showing an increase in Lachnospiraceae and Ruminococcaceae (**Figure 4.4.6**), cereal-based foods supplemented with CHE clustered far from the other samples (**Figure 4.4.2**) and, unlike other tannin-food combinations, showed a decrease in microbiota richness and diversity (**Table 4.4.1**). These results could be

related to a specific interaction between the hydrolysable ellagitannins contained in the chestnut wood extract and the fibre present in the evaluated food matrices. The inhibitory effects of ellagitannins on the activity of carbohydrate digestive enzymes, reported in the systematic review of Prpa *et al.* (2020), could also contribute to explain these differences.

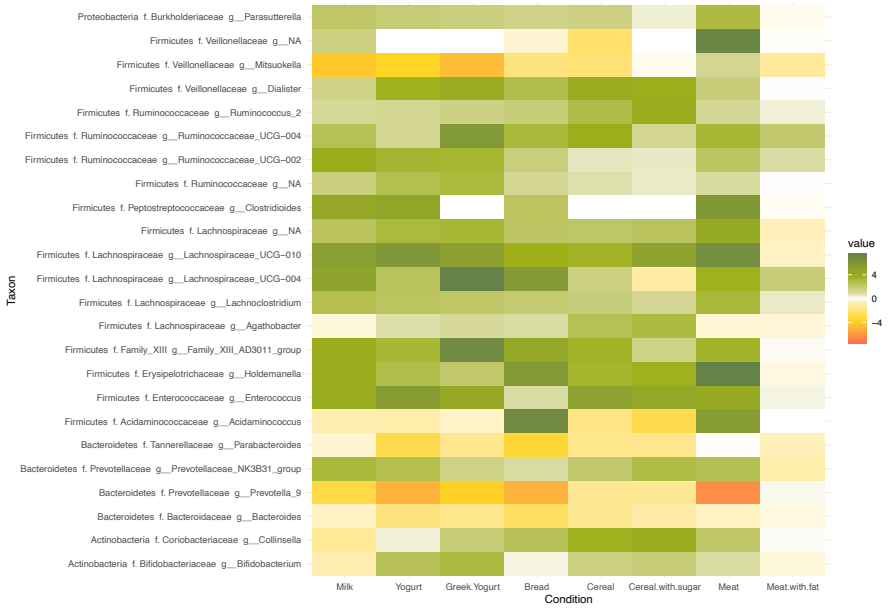


Figure 4.4.8 Heatmap of relative abundance fold changes of genera induced by QUE (quebracho tannin extract). The graph reports the genera with an abundance > 1% with a fold change > 2.5.

Figure 4.4.9 Heatmap of relative abundance fold changes of genera induced by CHE (quebracho tannin extract). The graph reports the genera with an abundance > 1% with a fold change > 2.5.

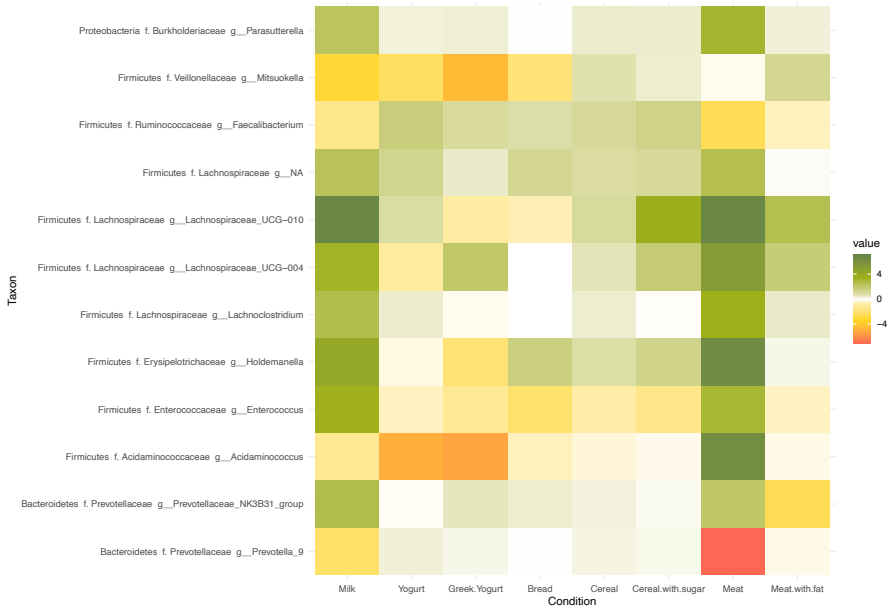


Figure 4.4.10 Heatmap of relative abundance fold changes of genera induced by CHE (quebracho tannin extract). The graph reports the genera with an abundance > 1% with a fold change > 2.5.

4.4.2 Production of SCFAs

Acetate, propionate, and butyrate are the principal SCFAs produced by gut microbial fermentation of carbohydrates, dietary fibres, proteins, and resistant starch. **Figure 4.4.12** depicts the output of the PCA of SCFA abundance. It evidences that SCFA production is similar in fermentations of the same food group independently of the presence of the tannin extracts.

As expected, cereal-based foods released the highest amount of SCFAs (**Figure 4.4.13**), producing, in decreasing order, acetic acid, propionic acid and butyric acid. In foods with a higher protein content, the ratio between acetic and propionic acid decreased, or even reversed, as in the case of M (**Table 4.4.3**). In most cases, the addition of tannins did not alter the relative production of the different SCFAs, but resulted in a booster effect (**Table 4.4.3**).

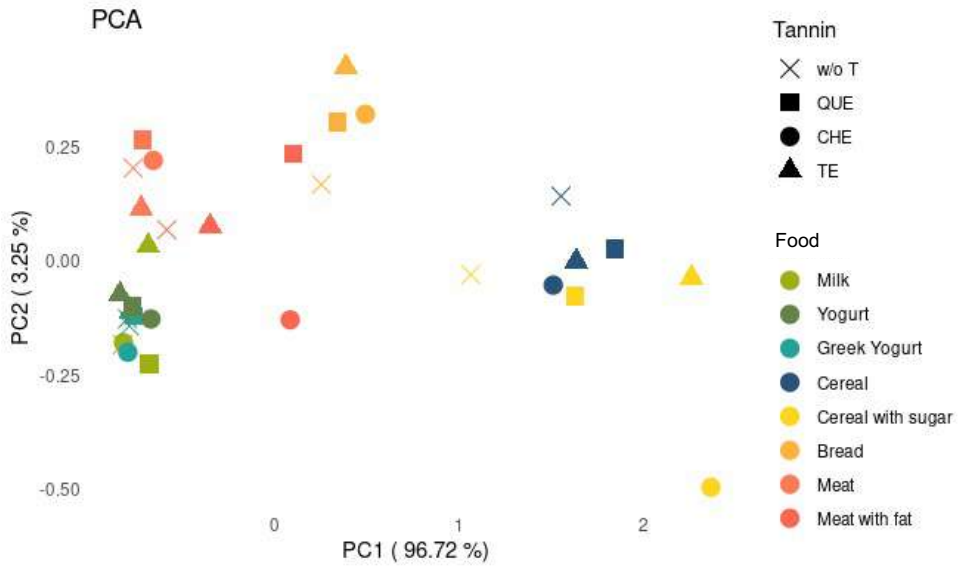


Figure 4.4.11 SCFAs released after *in vitro* fermentation of the food matrices with and without tannins. Principal Component Analysis (PCA) based on Euclidean distance. w/o T: without tannins, QUE: quebracho tannins extract, CHE: chestnut tannins extract, TE: tara tannins extract.

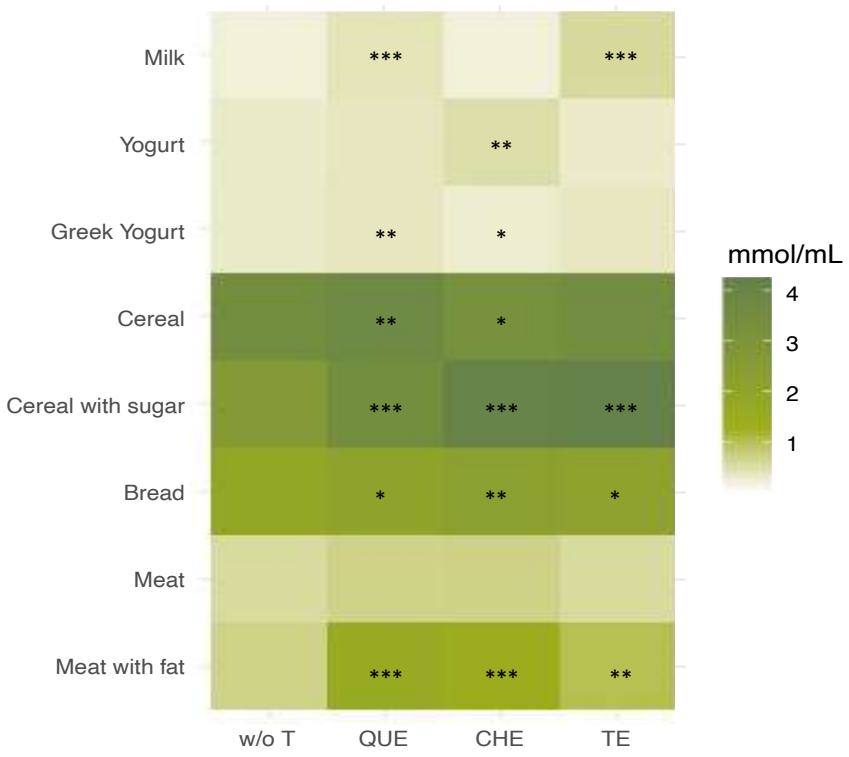


Figure 4.4.12 Sum of the concentration of SCFAs (in mmol/mL) per each sample, represented by colour gradient as shown next to the heatmap. w/o T: without tannins, QUE: quebracho tannins extract, CHE: chestnut tannins extract, TE: tara tannins extract. * Indicates statistically significant differences by ANOVA and Bonferroni post-hoc test: *p<0.05, ** p<0.01, ***p<0.001 within each food matrix.

Looking at the production of total SCFAs (**Figure 4.4.12**), QUE, CHE and TE nearly always resulted in a significant increase, particularly when they were combined with CS (QUE: p= 0.000014, CHE: p=0.00000012, TE: p=0.00000008), and B (QUE: p=0.027, CHE: p=0.0026, TE: p=0.009) and MF (QUE: p=0.000017, CHE: p=0.000053, TE: p=0.0015). On the contrary, when added to M, none of the three tannin extracts produced a statistically significant increase of total SCFAs. However, the addition of QUE, CHE or TE to M induced a significant increase in the production of propionic acid compared to M without tannins, suggesting that they were exerting a booster effect on specific propionate-producing bacteria (**Table 4.4.3**).

Table 4.4.3 Short Chain Fatty Acids (SCFAs) produced after the fermentation of the food source enriched or not with tannin extracts. * Indicates statistically significant differences ($p<0.05$) by ANOVA and Bonferroni post-hoc test within each food matrix.

		Acetic acid	Propionic acid	Butyric acid
Milk	w/o T	0.117 ± 0.006	0.055 ± 0.000	0.047 ± 0.001
	QUE	0.269 ± 0.020*	0.063 ± 0.021	0.077 ± 0.000*
	CHE	0.120 ± 0.001	0.060 ± 0.001*	0.042 ± 0.004
	TE	0.174 ± 0.004*	0.302 ± 0.002*	0.107 ± 0.024*
Yogurt	w/o T	0.124 ± 0.009	0.115 ± 0.000	0.081 ± 0.008
	QUE	0.138 ± 0.003	0.149 ± 0.000*	0.091 ± 0.001
	CHE	0.244 ± 0.013*	0.158 ± 0.006*	0.098 ± 0.001*
	TE	0.067 ± 0.019*	0.151 ± 0.001*	0.092 ± 0.003
Greek Yogurt	w/o T	0.139 ± 0.008	0.104 ± 0.001	0.088 ± 0.002
	QUE	0.152 ± 0.020	0.133 ± 0.010*	0.092 ± 0.000
	CHE	0.151 ± 0.002	0.047 ± 0.002*	0.087 ± 0.000
	TE	0.133 ± 0.006	0.133 ± 0.010*	0.090 ± 0.001
Cereals	w/o T	2.232 ± 0.033	1.181 ± 0.010	0.059 ± 0.000
	QUE	2.545 ± 0.027*	1.173 ± 0.009	0.068 ± 0.003
	CHE	2.259 ± 0.010	0.985 ± 0.038*	0.042 ± 0.005*
	TE	2.359 ± 0.009*	1.076 ± 0.011*	0.064 ± 0.001
Cereals with Sugar	w/o T	1.832 ± 0.002	0.853 ± 0.006	0.016 ± 0.001
	QUE	2.379 ± 0.023*	1.002 ± 0.029*	0.075 ± 0.003*
	CHE	3.214 ± 0.018*	0.865 ± 0.008	0.047 ± 0.001*
	TE	2.957 ± 0.028*	1.259 ± 0.004*	0.054 ± 0.003*
Bread	w/o T	1.006 ± 0.005	0.754 ± 0.012	0.093 ± 0.010
	QUE	1.043 ± 0.022	0.912 ± 0.022*	0.122 ± 0.002*
	CHE	1.177 ± 0.025*	0.980 ± 0.013*	0.112 ± 0.003
	TE	1.041 ± 0.046	1.043 ± 0.001*	0.060 ± 0.002*
Meat	w/o T	0.039 ± 0.001	0.434 ± 0.026	0.104 ± 0.005
	QUE	0.068 ± 0.000*	0.510 ± 0.014*	0.115 ± 0.002
	CHE	0.134 ± 0.003*	0.488 ± 0.033	0.088 ±0.000
	TE	0.110 ± 0.003*	0.367 ± 0.038*	0.093 ± 0.005
Meat with 30% fat	w/o T	0.255 ± 0.004	0.372 ± 0.019	0.069 ± 0.000
	QUE	0.842 ± 0.015*	0.765 ± 0.029*	0.074 ± 0.001
	CHE	0.951 ± 0.025*	0.419 ± 0.017*	0.075 ± 0.002
	TE	0.473 ± 0.002*	0.460 ± 0.021*	0.089 ± 0.001*

Tannins can act locally at intestinal level (especially non-absorbable tannins of high molecular weight), reaching the colonic gut microbiota, and exhibiting a prebiotic effect. Herein, these compounds could be used by microorganisms, resulting in metabolites with different bioavailability, activity, or functional effect compared to the parent molecule (Serrano *et al*., 2009). On the other hand, it is well known that carbohydrates exert a considerable prebiotic effect because polysaccharides act as a nutrient for the gut microbiota (Aguirre *et al*., 2016). Their metabolization by the microbiota results in the production, in decreasing order of proportion, of acetate, propionate and butyrate. Generally, proteins are not a good source of prebiotics and they alter the profile of SCFA production. Increased fermentation of amino acids results in an elevated production of propionate, together with branched SCFAs, and some potentially harmful molecules such as amines or hydrogen disulfide (Diether and Willing, 2019).

Tannins have been proposed as prebiotic substrates for gut microbes as these molecules favour SCFA production, have growth-promoting effects for beneficial bacteria, and/or could activate their metabolic functions. In Chapter 2, it was shown that a high production of SCFAs follows *in vitro* digestion and fermentation of QUE and CHE (Molino *et al*., 2018). Now, the combination of tannin extracts (QUE, CHE and TE) with the food matrices was responsible for a further increment of total SCFA production (**Figure 4.4.13**).

The increased SCFA production by the food matrices in presence of tannins may be potentially related to the synthesis of these compounds using the tannins or their metabolized products as substrates, or to the increased relative abundance and/or activity of gut microbiota species that ferment carbohydrates. Indeed, the increment in the relative abundances of both potential tannin metabolizers such as *Akkermansia* and of carbohydrate fermenters/SCFA producers such as the Ruminococcaceae and Lachnospiraceae families suggests that both processes are likely taking place.

As illustrated in **Figure 4.4.12**, the clustering of the different samples depended on the food sources (dairy products, meat and cereal-based foods). So, the factor driving the clustering of SCFA profiles was the different nutritional composition of different food sources, while the tannin enrichment didn't alter the SCFA profile compared to the original foods. In this sense, we can assume that tannins acted as a booster for the production of the specific SCFAs favoured by each food matrix, without affecting the expected profile. The only case where tannins did not lead to any significant effect was when combined with M. The strong protein-binding capacity is one of the distinguishing properties of tannins. This may have affected the potential interaction of the new-formed tannin-protein complexes with the microbiota, probably making the proteins less digestible and the tannins less available to exert their probiotic action. The presence of a higher percentage of fat in MF may have reduced the formation of complexes between proteins and tannins,

thus allowing greater interaction between tannins and the microbiota, resulting then in a greater production of SCFAs.

Finally, our investigation did not reveal any relevant difference in modulation of SCFA production based on the chemical origin among condensed tannins (QUE), ellagitannins (CHE) and gallotannins (TE). It is well known that the interaction between macromolecules and the different classes of tannins could differ (McManus *et al*., 1985; Baxter *et al*., 1997; Carn *et al*., 2012). In the present study the different complexation mechanisms did not seem to play a relevant role, although it is possible that longer fermentation times might be needed to obtain significant differences in the effect of different tannins on SCFA production.

This study may suffer some limitations related to the *in vitro* digestion and fermentation system. Indeed, some bacteria could need longer times for growth, so that the length of the fermentation experiment would not be sufficient to observe an increment of their relative abundance and/or their metabolic activity reflected in the production of SCFAs. An *in vivo* study could be more reliable because factors such as the interaction between the tannins and the gastrointestinal system could come into play. Furthermore, a long-term interventional study could allow investigating gradual changes in the composition of the intestinal microbiota and its functions. On the other hand, an *in vivo* study could introduce many confounding variables resulting in a disadvantage for the study of the interaction of tannins with specific food matrices.

4.5 Chapter 5

Tannin-rich extracts improve the performance of amidated pectin as an alternative microencapsulation matrix to alginate

4.5.1 Rheological properties of the feed formulations

The viscosity of the different solutions prepared with alginate (AI) or pectin (PI), and different concentrations (10 and 20 % w/w with respect to the mass of the different hydrocolloids) of quebracho tannin extract (AQ10I, AQ20I, PQ10I, PQ20I) or chestnut tannin extract (AC10I, AC20I, PC10I, PC20I) was measured. The rheological behaviour of biopolymer systems is the result of the interactions among the macromolecules present within them when subjected to mechanical stress, and therefore provides relevant insights about their molecular structure and the interactions between components. The flow behaviour of all the evaluated solutions was Newtonian, with a linear relationship between the shear stress and the shear rate. Thus, the viscosity was calculated from the slope of the shear stress *vs*. shear rate curves (**Table 4.5.1**) (Gunness *et al*., 2021).

The difference in viscosity was statistically significant ($p<0.05$) between the alginate- and the pectin-based feed formulations. This was expected given their different molecular structures. In particular, even though pectin was used in higher concentrations than alginate (2% w/v and 1.5% w/v respectively), its solutions turned out to be significantly less viscous (PI 0.104 ± 0.009 Pa·s; AI 0.130 ± 0.014 Pa·s).

The addition of the tannin extracts, QUE and CHE, regardless of their different composition, did not result in significant viscosity changes. However, when tannins were added in the 20% w/w ratio to the alginate solutions (AQ20I, AC20I), a frequent obstruction of the nozzle was observed, which resulted in a greater difficulty to obtain microbeads by extrusion. For this reason, AQ20I and AC20I were discarded for the purposes of microbeads production.

Table 4.5.1 Viscosity of the feed formulations. Results are expressed as Pa.s. Different letters indicate statistically significant differences by ANOVA and Bonferroni post-hoc test ($p<0.05$).

	Viscosity (Pa•s)
AI	$0.130^{A} \pm 0.014$
AC10I	$0.128^{A} \pm 0.008$
AQ10I	$0.128^{A} \pm 0.018$
AC20I	$0.129^{A} \pm 0.009$
AQ20I	$0.136^{A} \pm 0.006$
PIF	$0.104^{B} \pm 0.009$
PC10I	$0.109^{B} \pm 0.003$
PQ10I	$0.109^{B} \pm 0.003$
PC20I	$0.101^{B} \pm 0.005$
PQ20I	$0.101^{B} \pm 0.005$

4.5.2 Morphology

Figure 4.5.1 shows optical micrographs of the obtained microbeads. In the case of alginate, both with and without the addition of tannins, spherical microbeads with no defects were obtained, as expected for the polysaccharide most widely used for microencapsulation purposes. On the contrary, the use of pectin alone (P) led to the formation of microbeads with a very irregular shape or a small vermiform appendix, and often the microbeads appeared broken or non-spherical (highlighted with arrows).

These defects could have been partially due to the lower viscosity of pectin solutions compared to that of alginate. As several authors report, if the viscosity is too low, when the droplet impacts on the gelling bath surface tension forces are too weak to counteract the effect of the impact an drag forces in the gelling bath, with a consequent deformation (Chan *et al*., 2009; Davarcı *et al*., 2017). Moreover, as discussed above, pectins and alginates have a different gelling mechanism, the former yielding weaker gels. It has to be taken into account that usually pectin is employed as excipient to improve the microstructure of other hydrogel network systems, in terms of size, compactness as well as their interconnectivity (Mitrevej *et al*., 2001; Díaz-Rojas *et al*., 2004; Aguirre Calvo *et al*., 2019), rather than on its own.

The pectin used in this work was characterised by a certain level of amidation. This chemical modification of pectin is normally carried out to improve its gelling

properties, without requiring the addition of other ingredients, such as sugars, in certain cases (Chan *et al*., 2017). As commented on above, despite the amidation, pectin alone did not result in good microbead formation. However, the addition of tannins improved the shape of the microbeads considerably.

Although PC10 and PQ10 microbeads still presented some imperfections, marked with arrows in **Figure 4.5.1**, a further increase in tannin concentration allowed obtaining more spherical microbeads almost free of defects. In particular, PC20 had the most regular spherical shape among the pectin microbeads. The improved microbead formation was therefore attributed to the presence of tannins, and their interactions with pectin.

It is understood that the presence of amide groups in proteins plays a role in their binding to tannins. Some authors also reported that tannins can react with non-protein organic N compounds similarly to their reaction with proteins, These interactions, that are influenced by the concentration and chemical structure, are facilitated by the ability of amide groups to form multiple hydrogen bonds with negatively charged carboxylate groups and their free electron pair (Adamczyk *et al*., 2011; Buchweitz *et al*., 2013). This mechanism by which amide-containing compounds form complexes with tannins would explain the improved hydrogel network formation in the pectin-based microbeads. To our knowledge, this is the first study that describes the successful production of pectin microbeads obtained without blending with other biopolymers, aided by the addition of tannin-rich extracts.

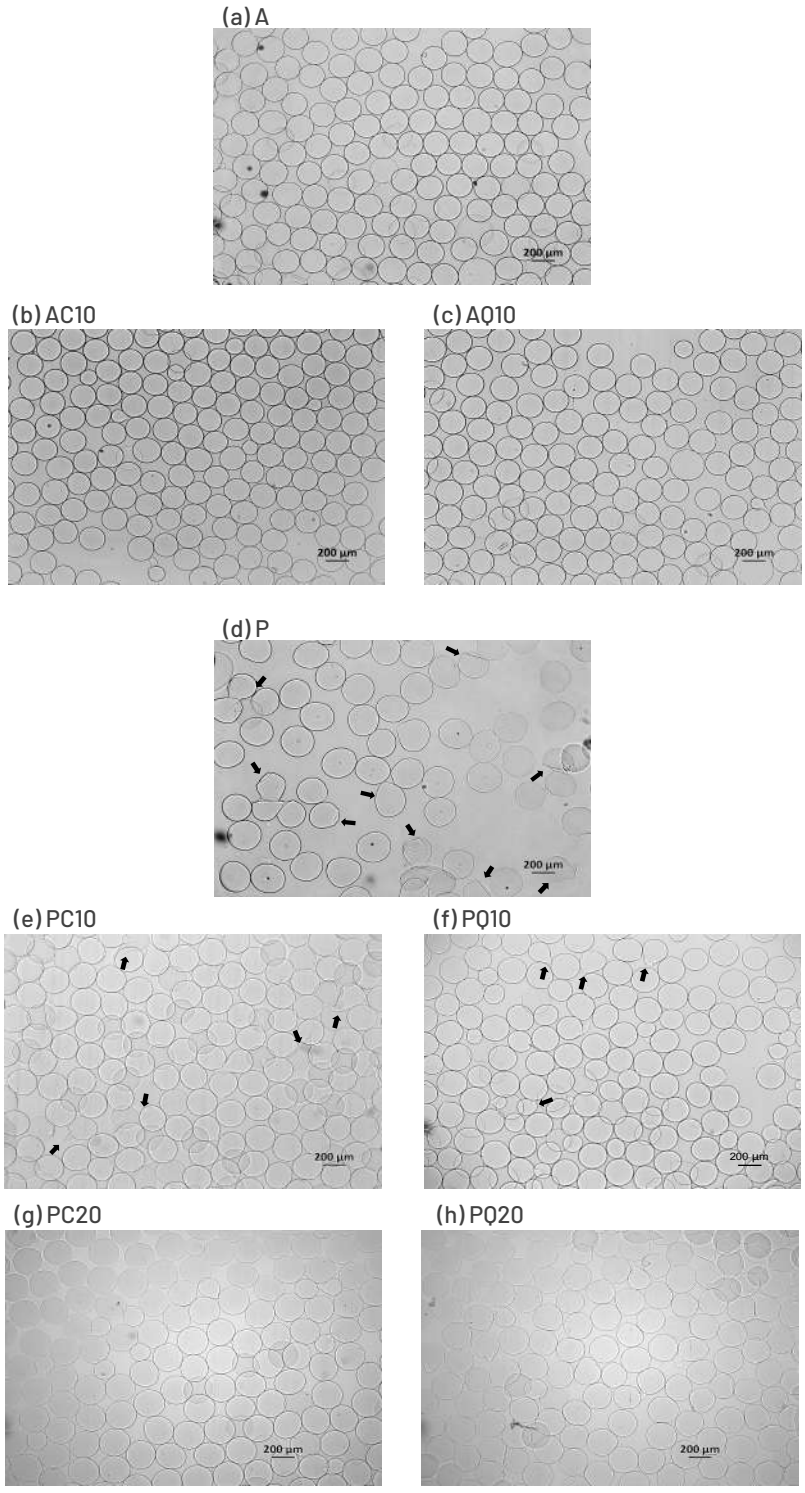


Figure 4.5.1 Micrographs of the different systems of microbeads, at 4x magnification. Arrows in the images indicate bead defects.

4.5.3 Size and water content of microbeads

Figure 4.5.2 reports the size distribution and water content of the microbeads produced through the extrusion-external gelation method.

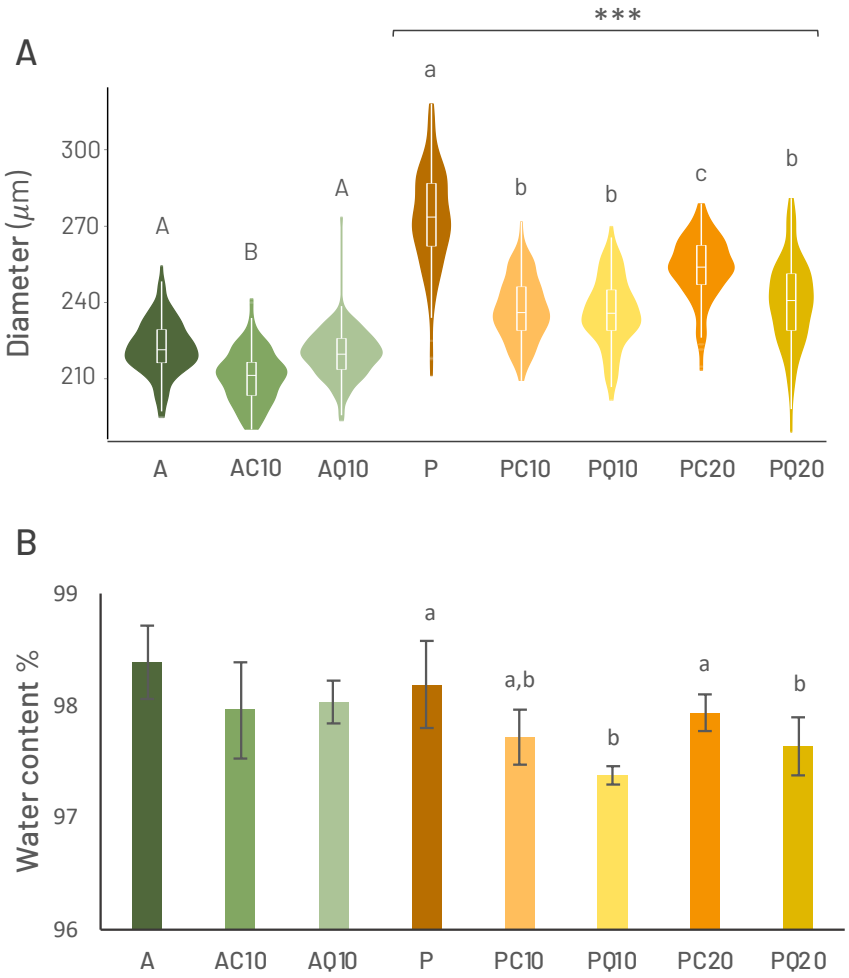


Figure 4.5.2 Size distribution and water content of the microbeads. (A) Size distribution reported as diameter (μm). *** Indicate statistically significant differences ($p<0.001$) by Student's t-test between alginate- and pectin-based microbeads. Capital and small letters indicate statistically significant differences by ANOVA and Bonferroni post-hoc test ($p<0.05$) among alginate- and pectin-based microbeads. **(B)** Water content of the microbeads. Different letters indicate statistically significant differences by ANOVA and Bonferroni post-hoc test ($p<0.05$) among alginate- and pectin-based microbeads.

Alginate microbeads were significantly smaller than pectin microbeads ($p<0.001$). In general, when vibrating nozzle technologies are used for bead production, greater viscosities result in greater droplet sizes (Del Gaudio *et al*., 2005). Accordingly, given the higher viscosity of alginate-based feed formulations, bigger sizes could have been expected. However, other factors play a role in determining the size, such as the different gelling mechanisms of the polymers.

The hydrogel networks that pectins form through the egg-box mechanism have been described to entail more defects than those of alginates (Fang *et al*., 2008), which explains the greater extent of shrinkage that the alginate droplets experienced upon crosslinking to form hydrogels. In fact, pectin microbeads without added tannins were the largest and most polydisperse among all (D = 273 ± 19 μm). Interestingly, the addition of tannins resulted in a statistically significant reduction ($p<0.05$) in size (PC10 D = 237 ±12 μm; PQ10 D = 236 ± 13 μm; PC20 D = 254 ± 12 μm; PQ20 D = 241 ± 16 μm) compared to those obtained with pectin alone.

The size of alginate microbeads also decreased slightly with the addition of tannins (A D = 222 ± 11 μm; AC10 D = 211 ± 10 μm; AQ10 D = 220 ± 9 μm), but to a lesser extent, which was not even significant in the case of quebracho tannins. Since no significant changes in viscosity were observed upon addition of the tannins, the decrease in size in the case of pectin was attributed to the interactions between the polysaccharide and the tannins discussed previously. These likely acted as cross-linkers, resulting in an improved hydrogel network formation and therefore less swelling, which was also confirmed by a lower water content in these microbeads.

Previously, it has been described that tannic acid could act effectively as a cross-linking agent to harden the microbead structure, when obtained through complex coacervation (Zhang et al., 2011). Moreover, Mamet *et al.* (2017) reported that persimmon condensed tannins with a low degree of polymerization (DP 5), similar to those used in the present study, improved pectin gelling properties (Mamet *et al*., 2017). In fact, these had a greater impact than those with a higher degree of polymerization, as they could penetrate into the junction zones and stabilize them through hydrogen bonds, while the latter could not get into the junctions due to steric hindrance. In view of these results, tannins can not only be used as bioactive compounds to be delivered with the microbeads, but also as cross-linkers to enhance pectin microbead formation.

These findings are confirmed by the water content results. The presence of tannins resulted in a lower water content, thus the generation of smaller and denser microbeads. In the case of alginate the greatest changes were induced by the presence of chestnut extract, while when pectin was used, the smallest microbeads were obtained with the quebracho extract. This could be attributed to the different chemical structure of the tannins in both extracts, which seemed to play a role in the extent of interactions with the two biopolymers. As reported in **Section 2.1**, the two

tannin extracts are characterised by different chemical compositions, in particular CHE contains hydrolysable tannins while QUE is characterized by the presence of condensed tannins. This suggests that several factors, such as tannin molecular weight and degree of galloylation, may play a critical role in the affinity for pectin, and resulted in one extract performing better than the other (Molino *et al*., 2019).

4.5.4 Microencapsulation efficiency

There is no universal method to quantify a phytocomplex such as those examined in this study. Therefore, the capacity of retention of tannins (CHE or QUE) by the different microencapsulation systems was tested using three different spectrophotometric techniques, i.e. UV 280 nm, ABTS and Folin-Ciocalteu assays, to provide an overview by evaluating different aspects: the presence of tannins in solution, as these compounds exhibit strong absorption at 280 nm, the scavenging capacity and the total polyphenol content, respectively.

In order to reflect as precisely as possible the amount of tannins contained in the samples examined, reference calibration curves were generated with CHE or QUE for samples containing the respective extracts, and the obtained results were expressed as mg of tannins (CHE or QUE) per mg of encapsulation matrix (**Table 4.5.2**). Finally, the ME% was calculated as the percentage of the tannin content in the original feed solutions that was detected in the microbeads (**Table 4.5.3**).

Although with slight variations in the absolute values, the results showed the same trends for the three techniques. Pectin microbeads showed significantly better encapsulation efficiencies compared to alginate microbeads.

Alginate is generally regarded as the polymer of choice for microencapsulation through extrusion-external gelation. Its success is due to the simplicity of the method to gel it, low cost and good biocompatibility. However, one of its main disadvantages is related to the high porosity of the microbeads, which is responsible for a final low encapsulation efficiency for small compounds. Indeed, the ME% for all the alginate-tannin systems studied in this work was very low (<16%). For this reason, alginate is mainly used as a delivery vehicle for large bioactive agents such as cells or probiotics, although in some cases this polymer has again proved to be scarcely efficient (Krasaekoopt and Bhandari, 2012). To mitigate these defects, the blending with other compounds or coating the microbeads is often necessary, especially when trying to entrap water-soluble and small bioactive compounds (Krasaekoopt and Bhandari, 2012; Mohammadi *et al*., 2018; Aguirre Calvo *et al*., 2019).

Table 4.5.2 Tannin amount retained in the microbeads expressed as mg of tannins (CHE or QUE) per mg of microbeads, measured with three different methods: UV 280 nm, ABTS and Folin-Ciocalteu assay and expressed as a percentage. Data are reported as means ± SD. QUE, quebracho tannin extract; CHE chestnut tannin extract.

		UV 280 nm	Folin-Ciocalteu	ABTS
CHE	AC10I	6.939 ± 0.690	5.154 ± 0.137	7.570 ± 0.122
	PC10I	6.674 ± 0.046	8.836 ± 0.245	11.05 ± 0.252
	PC20I	13.72 ± 0.908	10.896 ± 0.269	18.51 ± 0.440
	AC10	1.049 ± 0.737	0.321 ± 0.028	0.705 ± 0.045
	PC10	3.390 ± 0.836	1.936 ± 0.094	4.434 ± 0.512
	PC20	4.407 ± 0.815	2.959 ± 0.226	4.828 ± 0.361
QUE	AQ10I	8.006 ± 0.345	4.623 ± 0.273	8.156 ± 0.418
	PQ10I	9.228 ± 0.246	3.961 ± 0.108	8.207 ± 0.641
	PQ20I	18.08 ± 0.996	9.093 ± 0.873	16.50 ± 0.431
	AQ10	0.615 ± 0.481	0.225 ± 0.040	0.732 ± 0.016
	PQ10	3.607 ± 0.014	1.1.50 ± 0.167	4.523 ± 0.352
	PQ20	3.978 ± 0.282	6.638 ± 0.519	4.354 ± 0.475

Table 4.5.3 Microencapsulation efficiency (ME %) measured with three different methods: UV 280 nm, ABTS and Folin-Ciocalteu assay and expressed as a percentage. Data are reported as means ± SD. QUE, quebracho tannin extract; CHE, chestnut tannin extract. Different capital letters indicate statistically significant differences within the same extract and method of analysis ($p<0.05$) by ANOVA and Bonferroni post-hoc test.

		UV 280 nm	Folin-Ciocalteu	ABTS
CHE	AC10	15.19 ± 0.794^{A}	6.217 ± 0.433^{A}	9.311 ± 0.544^{A}
	PC10	48.42 ± 12.20^{B}	33.20 ± 1.838^{B}	40.11 ± 4.593^{B}
	PC20	32.20 ± 1.318^{C}	27.53 ± 2.121^{C}	26.07 ± 1.593^{C}
QUE	AQ10	7.710 ± 0.785^{A}	4.889 ± 0.957^{A}	8.998 ± 0.571^{A}
	PQ10	38.37 ± 1.174^{B}	29.06 ± 4.451^{B}	53.73 ± 5.637^{B}
	PQ20	22.19 ± 1.177^{C}	28.11 ± 3.319^{B}	24.38 ± 3.709^{C}

Pectin has received considerably less attention as a basic material for microencapsulation because of its more inefficient gelling mechanism which, as discussed in the previous section, results in microbeads with a greater extent of defects. The results in **Table 4.5.3**, however, show that the formation of interactions between pectin and tannins previously discussed not only results in microbeads with improved morphological characteristics, but also in a better encapsulation efficiency compared to alginate. The extent of interactions between the tannins and pectin through hydrogen bonds thus seemed to be greater compared to those with alginate, presumably enhanced by the presence of amide groups. It is worth mentioning that the pectin used for the study also contained a small amount of protein impurities (0.5%). The well-known ability of tannins to bind proteins, by establishing cross-links with the implication of a different nature of bonds, such as hydrophobic interactions and hydrogen bonds (Molino *et al*., 2019), might also have partially contributed to the improvement in ME%.

As expected, the tannins content of microbeads PC20 and PQ20 was the highest, since a greater amount of tannins was added to the feed formulations used to produce these samples. However, their encapsulation efficiencies were lower than those of samples PC10 and PQ10, which showed the best results. The microencapsulation efficiency generally decreases as the ratio matrix-to-encapsulated compound decreases (Gómez-Mascaraque and Lopez-Rubio, 2019). Indeed, by increasing the concentration of bioactive compound in PC20 and PQ20, the amount of pectin will be proportionally lower compared to PC10 and PQ10, leading to a lower retention by the matrix. In this particular case, the interactions in the pectin-tannin system seemed to reach a saturation point, beyond which further quantities of the bioactive compounds cannot be retained.

4.5.5 Tannin retention during storage

The changes in the content of bioactive compounds during storage is an important factor to evaluate when working with products intended for functional food applications, to ensure that the activity of the compounds of interest is maintained during the shelf-life of the products. In the specific case of water soluble compounds encapsulated within hydrogel microbeads, it is of particular interest that the diffusion of these compounds out of the microbeads is as limited as possible.

To assess the retention of tannins from the prepared microbeads during storage, the alginate and pectin systems containing the greatest tannins content, i.e. alginate AC10 and AQ10 microbeads and pectin PC20 and PQ20 microbeads, were selected, and their content of tannins was monitored over a period of 14 days. Despite exhibiting a lower microencapsulation efficiency than PC10 and PQ10, PC20 and PQ20 were selected among the pectin-based systems for two reasons: their improved

morphology and the greater amount of tannins they were able to carry compared to PC10 and PQ10.

Figure 4.5.3 illustrates the results obtained through three different methods (UV 280 nm, ABTS and Folin-Ciocalteu assays). In general, a more sustained release was observed for pectin-based microbeads. In the case of alginate-based microbeads, a gradual and continuous release of the tannin extracts was observed over time, for both CHE and QUE. As reported by Goh *et al.* (2012), the release of water soluble compounds from alginate occurs by diffusion, and it mainly depends on the porosity of the microbeads, and in particular the pore size. Some approaches such as drying have been proposed to reduce the matrix porosity and thus limit the loss of bioactive compounds from the microbeads (Goh *et al*., 2012). While drying is a feasible approach to preserve ingredients, including in microbeads, once incorporated within the final food products the diffusion towards the aqueous phase would resume, given that most foods have a high water content.

Pectin microbeads showed a reduced loss of tannin content, compared to alginate, especially as measured through the Folin-Ciocalteu method, for which the loss of tannin content over the 14 days was AC10 = 77%, AQ10 = 81%, PC20 = 29%, PQ20 = 15%. These results again confirm the greater extent of interactions between tannins and pectin that allowed not only a greater encapsulation efficiency than for alginate-based systems, but also a higher retention of the tannins and therefore a greater stability of the product over time.

The differences in retention patterns were less obvious when monitored using the UV 280nm method, and a similar trend was observed for all encapsulation systems through the ABTS assay. The differences between the results obtained by the three methods are due to the fact that they evaluate different parameters. Not all the phenolic compounds in a complex extract exhibit the same absorbance at 280 nm, nor do they have the same radical scavenging activity. On the contrary, not only is each individual phenolic compound unique in its chemical structure and functionality, but the antioxidant activity varies considerably depending on a number of factors, including the oxidation and oligomerization extent, and the interactions with other compounds (Gómez-Mascaraque *et al*., 2016; Falcó *et al*., 2018).

As a result, although for pectin the decrease of tannins content is comparable to that of the radical scavenging activity, for alginate the great loss in phenolics content over the 14 days of storage was not reflected in a similar loss in radical scavenging capacity. This suggests that the compounds that are still entrapped in the alginate network after day 14 are also the main contributors to antioxidant capacity of the extracts.

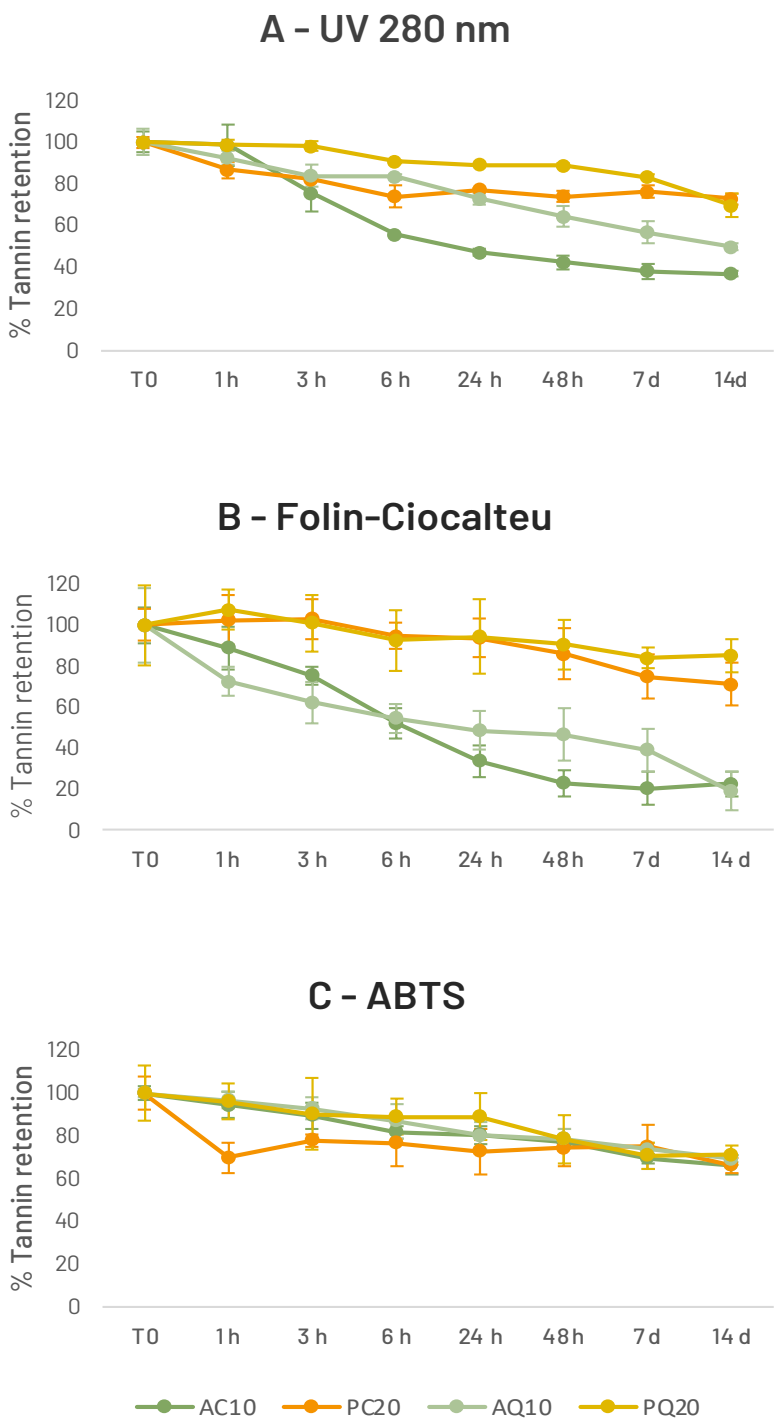


Figure 4.5.3 Effect of long-term storage on ME% of fresh microbeads, evaluated with three different methods: (a) UV 280 nm, (b) Folin-Ciocalteu and (c) ABTS assays. Tannin retention is expressed as percentage of the tannins initially encapsulated (ME%) remaining in the microbeads after each selected time period.

4.6 Chapter 6

Impact of gelatine coating on the performance of tannin-loaded pectin microbeads obtained through external gelation

4.6.1 Morphology

Figure 4.6.2 shows optical micrographs of the microbeads, of the various microbeads systems produced in the study, providing information on the morphology and size distribution.

Microbeads generated with pectin, without the addition of tannins nor gelatine (P) showed mostly a designless (not spherical) shape, with the presence of small vermiform appendices, and broken or non-spherical microbeads (highlighted with arrows). Pectins alone generally are not considered good matrices because, due to their low viscosity, they do not form gels fast enough resulting in morphological distortions (Chan, Lee, Ravindra, & Poncelet, 2009; Davarcı, Turan, Ozcelik, & Poncelet, 2017). For this reason, as pectins are considered to form relatively weak gels, they are frequently exploited as excipient to improve the microstructure of other hydrogel network systems, in terms of size, compactness as well as their interconnectivity (Aguirre Calvo, Santagapita, & Perullini, 2019; Díaz-Rojas et al., 2004; Mitrevej, Sinchaipanid, Rungvejhavuttivittaya, & Kositchaiyong, 2001), rather than on its own. The partial formation of microbeads observed for the pectin chosen for this study, could be due to the partial amidation of the hydrocolloid. Indeed, this chemical modification is reported to enhance pectin gelling properties, without requiring the addition of other ingredients, such as sugars, in certain cases (Chan et al., 2017).

The presence of tannins noticeably contributed to an improvement of the morphology of the microbeads. Tannin-pectin interaction could be promoted by the capacity of pectin amide groups to establish multiple hydrogen bonds with negatively charged carboxylate groups and their free electron pair of tannins (Adamczyk, Adamczyk, Smolander, & Kitunen, 2011; Buchweitz, Speth, Kammerer, & Carle, 2013). Even though PC10 and PQ10 microbeads still presented some imperfections, a further increase in tannin concentration (PC20, PQ20) allowed

obtaining more spherical microbeads almost free of defects. However, it has to be taken into account that an excess of the tannin content could be detrimental. In our preliminary studies, a tannin content higher than 20% (w/w) meant that the feed formulation could not be pumped through the 100 μm nozzle. Moreover, previous work reported that the incorporation of tannic acid 30% (w/w) within gelatine-coated ι-carrageenan microbeads resulted in great structural changes, with a rigid and fragile skin-like surface, that also incorporated a lower amount of gelatine on the surface. The authors suggested that the high reactivity of tannic acid with gelatine in the gelling bath might have competed with the interactions between ι-carrageenan and gelatine, resulting in a lower protein uptake (Gómez-Mascaraque et al., 2019).

Similarly to the tannins, the presence of the outer gelatine layer also contributed to the improvement of the morphological characteristics of the microbeads. This can be readly observed in the improved morphology with well-formed spherical microbeads of PG compared to P (**Figure 4.6.1 A** and **B**).

CSLM was used to also investigate the presence of a gelatine layer around the microbeads by selective staining with Fast Green FCF, a dye commonly used for protein staining (Jiménez-Munoz, Brodkorb, Gómez-Mascaraque, & Corredig, 2021). Surprisingly, all the produced microbeads, even those prepared without gelatine, showed a full surface greenish colour. This colouration may be due to the presence of a small protein content in the product (0.5%), as specified in the technical sheet. Moreover, the amide groups of pectin used might have interacted to some extent with the dye.

CSLM investigations highlights how influential the presence of tannins is in the formation of the gelatine coating. In PG (**Figure 4.6.1**), there is no appreciable green border to indicate the presence of gelatine attached to the microbeads. In contrast, by increasing the tannin concentration (PC10G, PQ10G, PC20G, PQ20G), an outline could be observed to a greater and greater extent. It could be suggested then that there is a relationship between a higher tannin content and a higher amount of gelatine attached due to their interaction. As it was previously described, tannin-protein interactions depend, among other factors, on the concentration (Molino et al., 2019).

Further topographical information can be found in the SEM images (**Figure 4.6.3**) in which the presence of fibrous appendages distinguishes (more in the case of PC20G and PQ20G) the microbeads produced with a gelatine coat from those without. However, these morphological data obtained with CLSM will be confirmed by quantitative analyses with LECO.

From these morphological evaluations, apparently there are no noticeable differences due to the use of condensed (quebracho) or hydrolysable (chestnut) tannins.

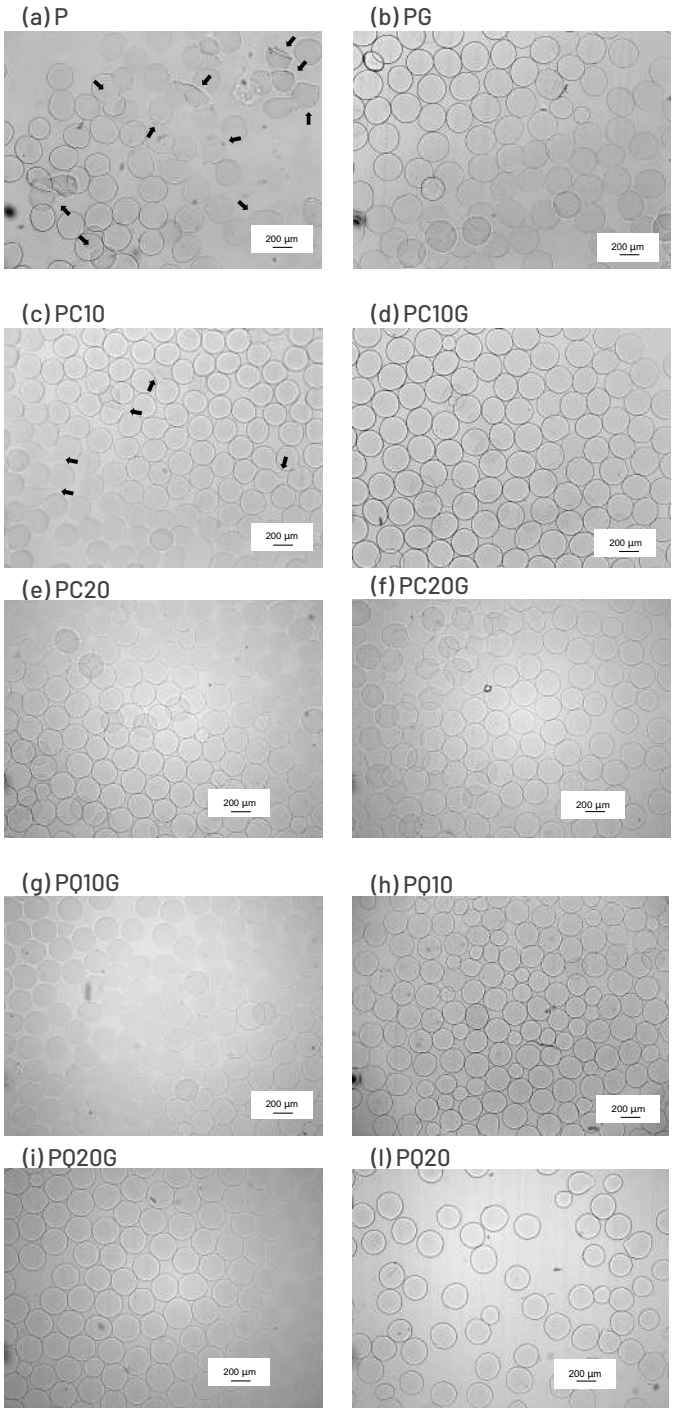


Figure 4.6.1 Micrographs of the different microbeads systems, at 4x magnification. Arrows in the images indicate bead defects. (a) P, (b) PG, (c) PC10, (d) PC10G, (e) PC20, (f) PC20G, (g) PQ10, (h) PQ10G, (i) PC20, (l) PC20G.

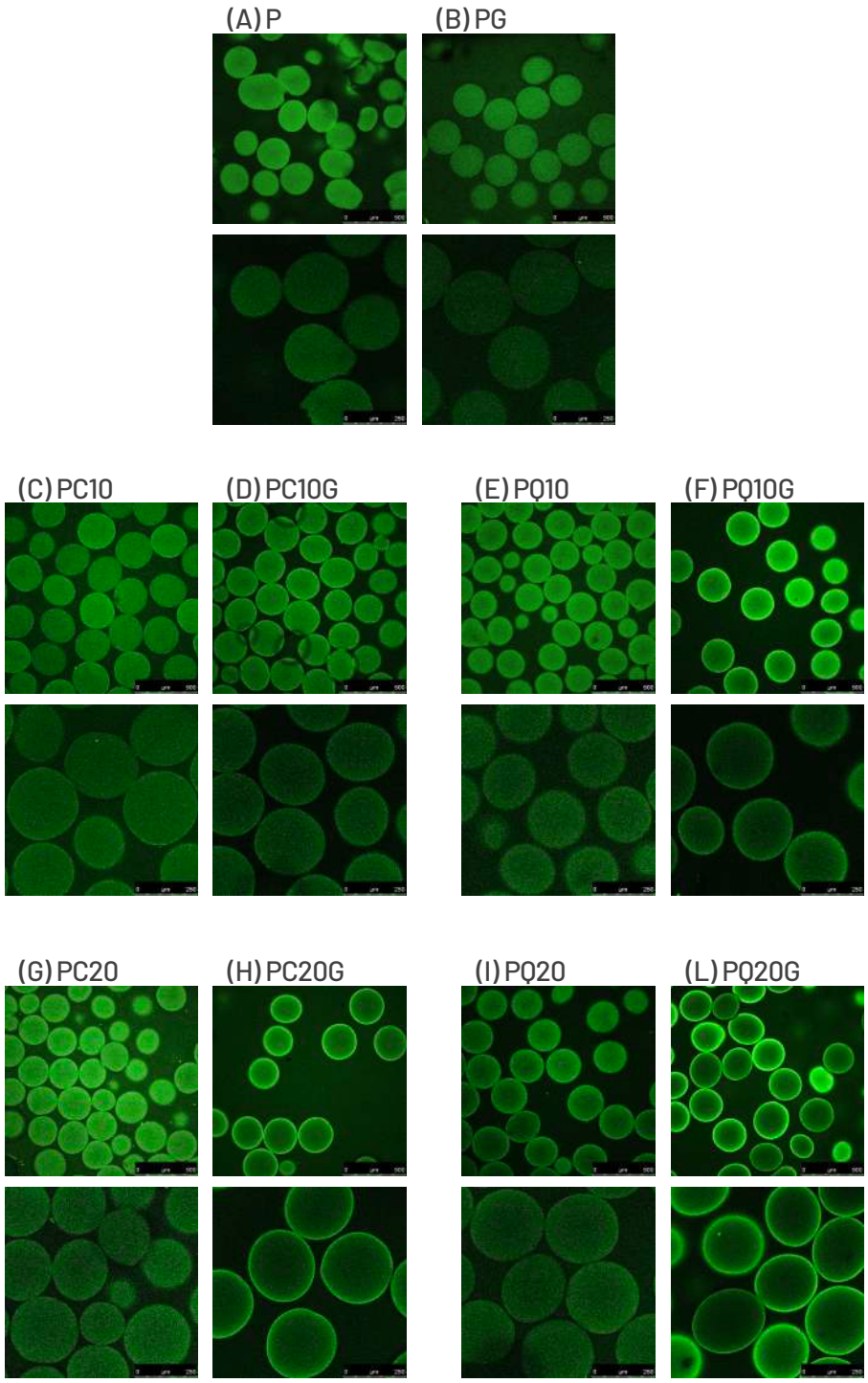


Figure 4.6.2 Confocal laser scanning microscopy (CSLM) images of the different systems of microbeads, at two different magnifications (10x and 20x). (A) P, (B) PG, (C) PC10, (D) PC10G, (E) PQ10, (F) PQ10G, (G) PC20, (H) PC20G, (I) PQ20, (L) PQ20G.

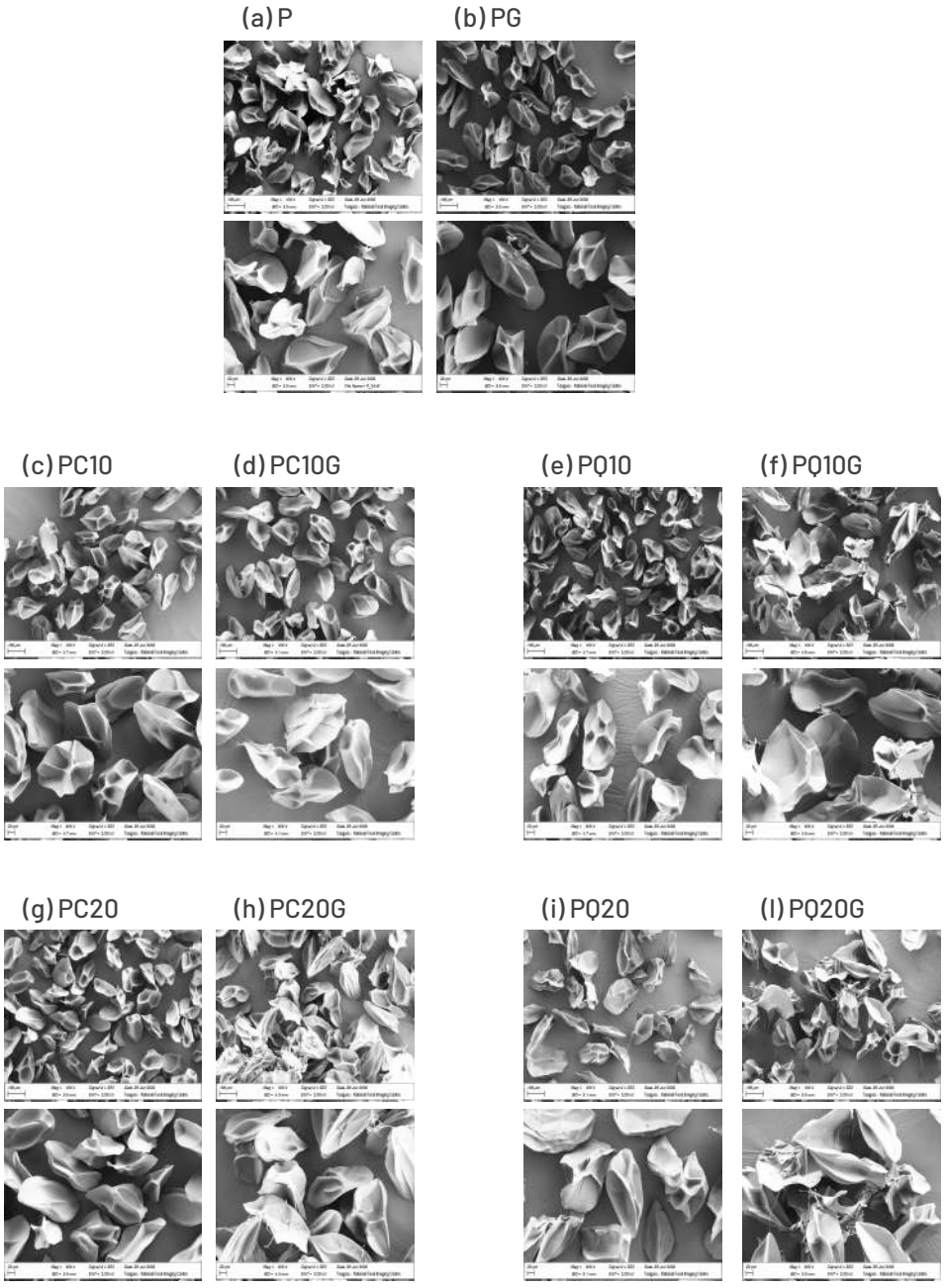


Figure 4.6.3 Scanning electron microscope (SEM) images of the different microbeads systems, at two different magnifications (100x and 200x). (a) P, (b) PG, (c) PC10, (d) PC10G, (e) PQ10, (f) PQ10G, (g) PC20, (h) PC20G, (i) PQ20, (l) PQ20G.

4.6.2 Size and water content of microbeads

Size distribution and water content of the microbeads produced through the extrusion-external gelation method are depicted in **Figure 2**. With regards to non-coated microbeads (without gelatine), P resulted to be the largest (P D = 274 ± 19 μm). Then the incorporation of tannins determined a statistically significant reduction ($p<0.05$) in microbead size in comparison to those obtained in absence of tannins. As some authors already reported, tannins can improve pectin gelling properties, acting as crosslinkers and improving the hydrogel network formation (Mamet *et al*., 2017). More specifically, condensed tannins with a relatively low degree of polymerisation (DP 5), similar to that of the tannins used in this study, were shown to penetrate into the junction zones and stabilize them through hydrogen bonds with pectin. In this sense, fewer interactions between chestnut tannins and pectin could correspond to the minor efficiency in the formation of a structured network, resulting in more swelling (bigger sizes).

It is worth mentioning that, among tannin-loaded microbeads, PC20 presented the highest size ($p<0.05$, PC20 D= 253 ± 12 μm) compared to the others. Within the gelatine-coated group, again the absence of tannins determined the highest microbead size for PG (PG D = 288 ± 17 μm).

Comparing the two different groups of microbeads, i.e. non-coated vs. gelatine-coated, the differences in their size distribution were not statistically significant. However, in general, the attachment of an external layer of gelatine resulted in an increase in the size compared to uncoated microbeads, and in a significant manner for PQ10G ($p=0.011$) and PQ20G ($p=0.004$).Whereas it is possible that the total volume of the microbeads could be increased by means of a gelatine coating, it has to be taken into account that further factors may interplay.

These findings were also reflected in the water content results. In both groups, the presence of tannins resulted in a decrease in microbeads water content, with the production of smaller and denser microbeads. Even though no statistical differences were found between groups (i.e., gelatine-coated and uncoated microbeads), in general gelatine-coated microbeads presented a lower water content than the non-coated ones, and in a significant manner for PG ($p=0.031$) and PC20G ($p=0.001$). As for the size distribution, PC20G showed the lowest values, confirming that different chemical composition and concentration play a significant role in the extent of interactions with the two biopolymers, as discussed above.

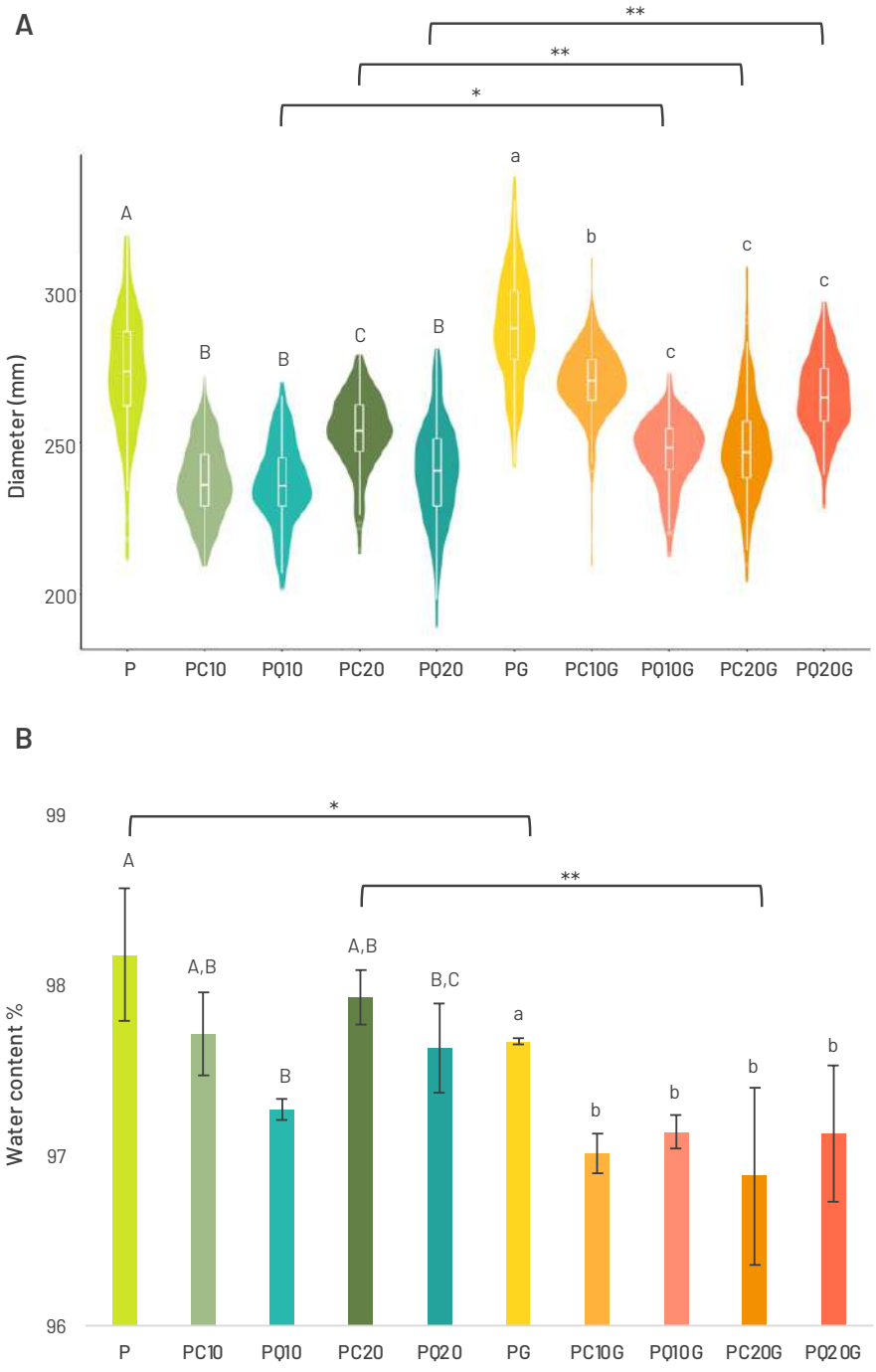


Figure 4.6.4 (A) Size distribution reported as diameter (μm) and (B) water content of the microbeads. * Indicate statistically significant differences by Student's t-test between uncoated and coated microbeads: *p < 0.05, **p < 0.01. Capital and small letters indicate statistically significant differences by ANOVA and Bonferroni post-hoc test (p<0.05) among uncoated and coated microbeads.

4.6.3 Protein content

In order to estimate the amount of gelatine adhered to the coated microbeads, we quantified the protein content (**Table 4.6.1**). As we mentioned earlier, the commercial pectin used for the present study was amidated and the microbeads without gelatine contained a certain amount of nitrogen *per se*. Therefore %N due to the presence of pectin for each microbead system was subtracted (**Table 4.6.1**). To calculate the % of gelatine attached, the % of N in the commercial gelatine used for this study was measured and used to estimate the nitrogen-to-protein conversion factor (6.17) for gelatine.

Even though the gelatine coating in PG could not be observed in the CLSM images, a certain amount of gelatine was detected (% 7.3 ± 0.6). This indicates that there is an interaction between pectin and gelatine. Some authors exploited the attraction between these materials, with their complexation, to perform microencapsulation by complex coacervation for drug delivery (Saravanan and Rao, 2010).

Table 4.6.1 Content of nitrogen (%N) and amount of gelatine attached to the microbeads. Different letters indicate statistically significant differences by ANOVA and Bonferroni post-hoc test ($p<0.05$).

	%N		% N	% gelatine
P	1.698 ± 0.019	PG	2.835 ± 0.090^{A}	7.278 ± 0.557^{A}
PC10	1.649 ± 0.014	PC10G	3.728 ± 0.021^{B}	12.83 ± 0.311^{B}
PQ10	1.538 ± 0.067	PQ10G	3.716 ± 0.216^{B}	13.44 ± 1.335^{B}
PC20	1.785 ± 0.062	PC20G	3.747 ± 0.382^{B}	12.11 ± 2.355^{B}
PQ20	1.711 ± 0.077	PQ20G	3.755 ± 0.105^{B}	12.62 ± 0.648^{B}

However, the amount of gelatine present in the coated microbeads without tannins (PG) was significantly lower ($p<0.05$) than that in the tannin-loaded microbeads. Conversely, no statistically significant differences were detected among the different gelatine-coated microbeads loaded with tannins. This indicated that the ability of tannins to bind to proteins facilitated the uptake of additional gelatine beyond the amount that could be bound due to pectin-gelatine polyelectrolyte interactions only. Supporting these data, Sun *et al*. (2020) found that the addition of tannic acid to pectin/gelatine coacervate helped in improving its properties, leading to the production of better performing microbeads.

A different chemical composition, such as molecular weight and degree of galloylation, could determine a distinctive interplay between tannins and gelatine (de Freitas and Nuno, 2012; Molino *et al*., 2019). However, gelatine–tannin interaction can also be limited by the interference of other molecules, pectin among them, which could compete with proteins for binding tannins. This issue is not yet totally understood because, some authors showed that the presence of polysaccharides, in particular mannoproteins, can inhibit the evolution of tannin aggregate particle size but not their generation (Riou *et al*., 2002). The different affinity for pectin and/or gelatine could be the reason for a different intra-group size distribution.

In a previous work, gelatine-coated ι-carrageenan microbeads incorporating 30% tannic acid, which, similarly to chestnut extract, belongs to the hydrolysable tannins, were found to exhibit a much lower protein content than those measured in this study, despite the fact that the control microbeads in the absence of tannic acid, and those containing other types of phenolic compounds, took up to 40% gelatine (Gómez-Mascaraque *et al*., 2019). The authors attributed the lower gelatine adherence to the competing interaction of tannic acid with gelatine rather than ι-carrageenan. In our systems, instead of hindering the attachment of gelatine, the presence of tannins helped the formation of the gelatine coating, adhering more protein than the control (PG). This is evidence of the complexity involved in the interactions in these ternary systems, where the type and concentration of polyphenols and biopolymers will determine whether the tannins have a synergistic effect towards the gelatine uptake or not.

4.6.4 Microencapsulation efficiency

A phytocomplex such as those examined in this study could not be quantified by a single universal method. For this reason, three different spectrophotometric techniques (UV 280 nm, Folin-Ciocalteu and ABTS assays) were used to estimate the capacity of tannin retention (CHE or QUE) by the different microencapsulation systems. More specifically, the three selected techniques provided information on different aspects of the extracts: the presence of tannins in solution, thanks to the high absorption at 280 nm exhibited by tannins, the total polyphenol content, and the radical scavenging capacity.

To calculate the tannin amount in the samples as accurately as possible, CHE or QUE were used to produce calibration curves as reference for samples containing the respective extracts. Thus, results could be reported as mg of tannins (CHE or QUE) per mg of encapsulation matrix (**Table 4.6.2**).Finally, the ME% was calculated as the percentage of the tannin content in the original feed solutions that was detected in the microbeads (**Table 4.6.3**). In general, the three techniques applied generated similar trends, with some slight variations.

Table 4.6.2 Tannin amount retained in the microbeads expressed as % mg of tannins (CHE or QUE) per mg of microbeads, measured with three different methods: UV 280 nm, ABTS and Folin-Ciocalteu assay and expressed as a percentage. Data are reported as means ± SD. QUE, quebracho tannin extract; CHE chestnut tannin extract.

		UV 280 nm	Folin-Ciocalteu	ABTS
CHE	PC10	2.534 ± 0.178	1.227 ± 0.765	3.188 ± 0.094
	PC20	4.809 ± 0.122	2.176 ± 0.437	5.223 ± 0.393
	PC10G	3.775 ± 0.233	1.886 ± 0.078	4.641 ± 0268
	PC20G	8.166 ± 0.253	5.052 ± 0.882	7.688 ± 0.358
QUE	PQ10	3.529 ± 0.006	0.821 ± 0.270	2.823 ± 0.583
	PQ20	6.882 ± 0.319	2.542 ± 0.064	6.099 ± 0.583
	PQ10G	5.464 ± 0.439	1.288 ± 0.064	4.535 ± 0.413
	PQ20G	10.05 ± 0.096	4.138 ± 0.251	10.10 ± 0.775

Table 4.6.3 Microencapsulation efficiency (ME %) measured with three different methods: UV 280 nm, Folin-Ciocalteu and ABTS assays and expressed as a percentage. Data are reported as means ± SD. QUE, quebracho tannin extract; CHE, chestnut tannin extract; Different capital letters indicate statistically significant differences within the same extract and method of analysis ($p<0.05$) by ANOVA and Bonferroni post-hoc test.

		UV 280 nm	Folin-Ciocalteu	ABTS
CHE	PC10	37.97 ± 6.518^{A}	21.02 ± 3.207^{A}	32.14 ± 2.089^{A}
	PC20	35.05 ± 0.217^{A}	19.96 ± 0.979^{A}	28.60 ± 7.554^{A}
	PC10G	56.65 ± 8.550^{B}	32.31 ± 3.277^{B}	43.10 ± 4.994^{B}
	PC20G	59.51 ± 4.253^{B}	46.36 ± 1.982^{C}	44.42 ± 6.596^{B}
QUE	PQ10	40.05 ± 2.702^{A}	20.73 ± 1.671^{A}	42.69 ± 12.69^{A}
	PQ20	37.99 ± 0.406^{A}	$27.39 \pm 1.965^{A,B}$	33.01 ± 7.525^{A}
	PQ10G	66.58 ± 1.311^{B}	32.51 ± 3.937^{B}	58.34 ± 11.06^{B}
	PQ20G	60.89 ± 1.432^{C}	45.50 ± 6.76^{C}	62.20 ± 9.250^{B}

As discussed previously, the incorporation of tannins could improve the structure of pectin microbeads, which was attributed to the interactions between both components. The presence of amide groups and a small amount of protein impurities (0.5%) in the commercial pectin used for this study likely led to hydrogen bonding with tannins, which contributed to their retention within the microbeads to some extent (**Table 4.6.3**), considering the fact that they are relatively small water-soluble molecules.

The inclusion of the highest amount of tannins in the feed formulations (20% w/w) resulted in PC20 and PQ20 being the microbead systems with the highest tannin content. Even though PC10 and PQ10 showed slightly better values of ME%, these were not statistically significant. Gómez-Mascaraque & Lopez-Rubio (2019) reported that the reduction of the ratio matrix-to-encapsulated compound could result in a decrease of the ME%.

In contrast, when microbeads were coated with gelatine, the systems produced with the feed formulations with the highest concentrations of tannins (PC20G and PQ20G) were matched by highest %ME. The well-known ability of tannins to bind proteins (Molino et al., 2019) might have played a fundamental role in the improvement of their encapsulation. In this sense, some of the excess tannins that could not interact with pectin and diffused out to the gelling bath were able to bind the gelatine being absorbed on the surface of the microbeads, being retained and avoiding their loss.

Moreover, the addition of a gelatine layer resulted in a significant improvement of the ME%, in some cases even doubling the values, compared to the values reported by non-coated microbeads.

4.6.5 Tannin retention during storage

For the development of products intended for functional food application, it is important to ensure a stable content of the bioactive compounds along the shelf-life of the products. Thus, the potential diffusion of water soluble compounds, when they are encapsulated within hydrogel microbeads, should be prevented as much as possible. To evaluate this, the retention of tannins from both microbead systems (with and without gelatine coating) was monitored over a storage period of 14 days. The microbeads with the greatest tannins content (PC20, PQ20, PC20G, PQ20G) were selected for this study. **Figures 4.6.5 A, B** and **C** show the results generated by UV 280 nm, Folin-Ciocalteu and ABTS assays, respectively.

Similar trends were observed through the three methods, so there was a correspondence between the measured tannin content and the antioxidant capacity exerted. In general, all systems presented over time a slow and gradual release of the tannin extracts, demonstrating a good retention ability over the 14-day study period.

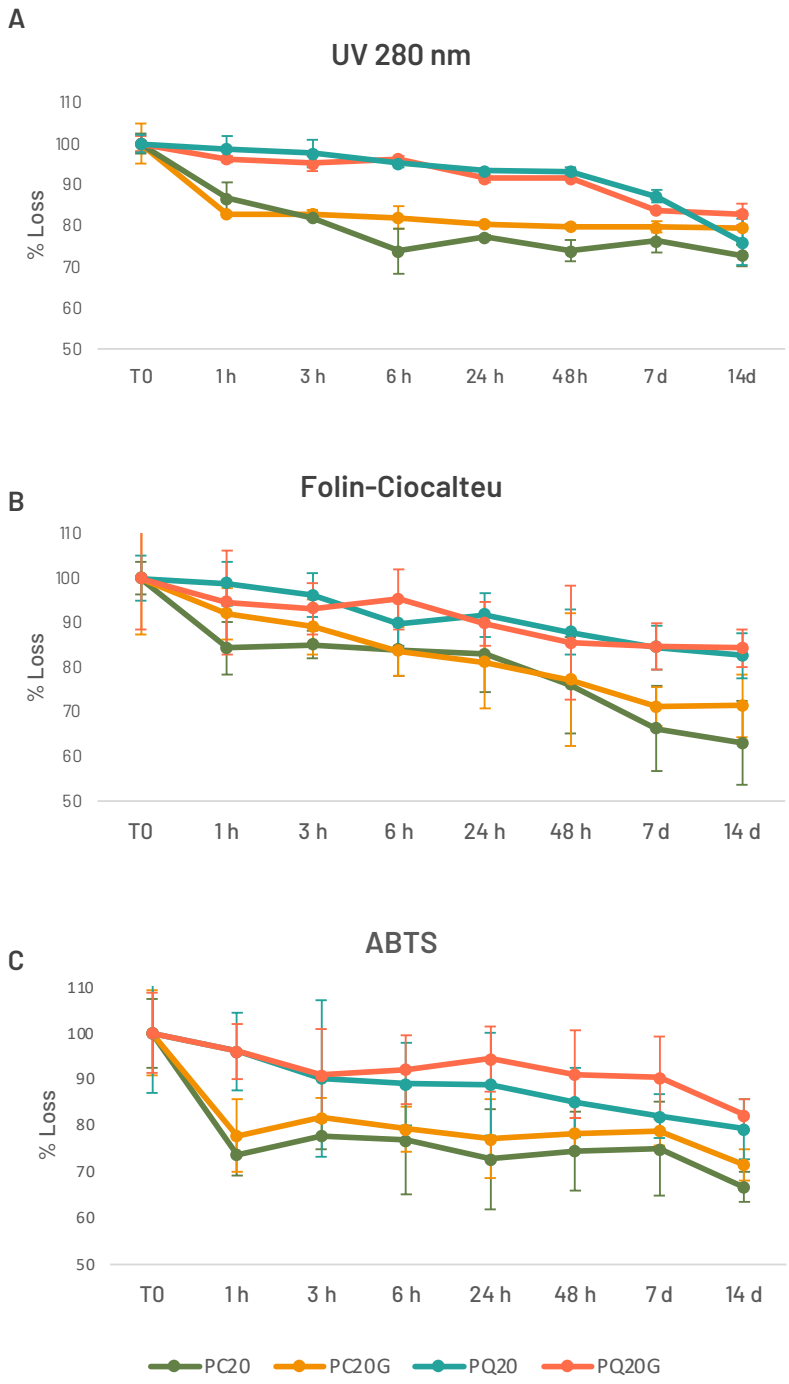


Figure 4.6.5 Effect of storage on tannin retention of fresh microbeads, evaluated with three different methods: (A) UV 280 nm, (B) Folin-Ciocalteu and (C) ABTS assays. Tannin retention is expressed as percentage of the tannins initially encapsulated (ME%) remaining in the microbeads after each selected time period.

The release of tannins from the gelatine-coated microbeads exhibited the same trend as their uncoated-counterparts, indicating that the additional protein layer did not affect to a large extent the release kinetics of the systems.

The incorporation of quebracho extract in PQ20 and PQ20G determined a greater retention ability, resulting in a lower loss of bioactive compounds during storage, compared to PC20 and PC20G. Interestingly, the microbeads containing quebracho tannin extract also gave higher values of microencapsulation efficiency than the respective counterpart with chestnut tannins . This means that in PQ20 and PQ20G not only more tannins are incorporated, but also fewer are lost during storage. Taking these data together, it can be assumed that there may be a greater interaction of condensed tannins with pectin than with hydrolysable tannins. Various factors including the nature of the bonds formed with pectin could interplay. However, the different chemical conformation could also be relevant, considering that condensed tannins have a relatively flat structure while chestnut ellagitannins are characterised by a more complex structure that could play a disadvantage by creating steric hindrance (Radebe, Rode, Pizzi, Giovando, & Pasch, 2013; Radebe, Rode, Pizzi, & Pasch, 2013).

The slight loss of tannins during the 14-days storage, once again, support that the interactions between tannins and pectin play an important role in the creation of a network, capable of efficiently maintaining tannins trapped. However, despite the gelatine-coating can improve the ME% in a statistical significantly manner compared to non-coated microbeads, it is unable to make a great improvement in tannin retention over time. In this sense, it has to be highlighted that uncoated microbeads already gave good results in this respect.

4.7 Chapter 7

Effects of pectin-encapsulated and unencapsulated tannins on gut microbiota composition and short chain fatty acid production

Microencapsulation of CHE and QUE in pectin microbeads by extrusion was successfully achieved. The microencapsulation efficiency values ranged between 29–35% and 33–38% for CHE and QUE, respectively, depending on the different assays used (data not shown).

To determine the best form of administering natural tannin extracts as a supplement to achieve beneficial effects, both tannins *per se* and encapsulated tannins were subjected to *in vitro* digestion and fermentation. In order to consider also the partial effect of enzymatic digestion on tannins, samples of QUE and CHE extracts were subjected directly to fermentation (QUE-F, CHE-F, respectively), for comparison purposes.

The bioactivity was then measured as the antioxidant capacity exerted after *in vitro* digestion and fermentation, or fermentation only, and as the capacity for modifying the gut microbiota in terms of taxonomic composition and SCFA production.

4.7.1 Tannin release/antioxidant capacity

One of the required characteristics of the microbead matrix is that it dissolves at a certain point after ingestion, in order to release its content of bioactive compounds. So, quantifying the amount of tannins released during digestion and fermentation can be indicative of how well the microbeads are liberating their content during the human digestive process.

Since there is no single method to quantify a phytocomplex such as those used in the study (QUE and CHE), their release through *in vitro* gastrointestinal digestion and fermentation was tested using three different spectrophotometric techniques, i.e. Folin-Ciocalteu, $TEAC_{FRAP}$ and $TEAC_{ABTS}$ assays. By combining the results obtained from the three different methods it is possible to obtain an overview that

evaluates different aspects: the total polyphenol content, the reducing capacity and the scavenging capacity, respectively. Both liquids from digestion and fermentation were tested and, although some variations in the absolute values were found, the results showed the same trends for the three techniques.

As regards the liquid part derived from the *in vitro* digestion, the lowest values were returned by the pectin microbeads (P). The addition of tannins (PC and PQ) resulted in an increase in antioxidant capacity in the liquid from digestion, resulting in a significant difference only in terms of total polyphenol content as detected by the Folin-Ciocalteu assay (**Figure 4.7.11 A**).

When the non-encapsulated extracts (CHE and QUE) were digested, the digestion liquid showed a higher antioxidant capacity than that of the pectin microbeads with (PC and PQ) or without (P) tannins. However, the clear difference between pectin microbeads (P, PC and PQ) and pure digested extracts (QUE and CHE) that emerged from $TEAC_{FRAP}$ and Folin-Ciocalteu assays was not so marked for $TEAC_{ABTS}$. As stated before, a phytocomplex or a compound can act in a distinct way depending on its composition and the type of oxidation process it undergoes. Apparently, P (4.337 ± 0.339 μmol/ml) showed a high activity against $ABTS^{*+}$ radicals, getting closer to the values shown by QUE (5.863 ± 1.096 μmol/ml) and CHE (5.589 ± 0.784 μmol/ml) (**Figure 4.7.1 E**). This means that the minimal release of tannins during digestion of PQ and PC resulted in a total antioxidant effect almost reaching that exerted by the digested pure extracts.

After the *in vitro* fermentation process, only the Folin-Ciocalteu method allowed to highlight the differences between microbeads and pure extracts. In fact, similarly to digestion, P once again showed the lowest values of total polyphenol content (0.373 ± 0.036 μmol/ml). The quantification of tannins in the liquids derived from the fermentation of PQ (0.6 ± 0.12 μmol/ml) and PC (0.5833 ± 0.138 μmol/ml) showed a slight release of polyphenols. However, these values were lower in a statistically significant manner than those of the individual extracts (**Figure 4.7.1 B**).

This trend was not observed for the fermented fractions when testing the antioxidant capacity against ferric ions. Indeed, it seems that the bacterial fermentative action on pectin resulted in the release of compounds that have a relatively high antioxidant capacity, comparable to that of tannin extracts. A similar effect resulted from the $TEAC_{ABTS}$ assay, although P had significantly lower values than QUE ($p = 0.007$) and CHE ($p = 0.007$). However, the presence of a slight release of tannins meant that the antioxidant activity (for both $TEAC_{ABTS}$ and $TEAC_{FRAP}$) exerted by PQ and PC fermented liquid equalled that of the individual extracts (**Figure 4.7.1 D, E**).

These data suggest that there was little degradation of the encapsulating matrix (pectin) during *in vitro* digestion, resulting in a very limited release of their tannin content.

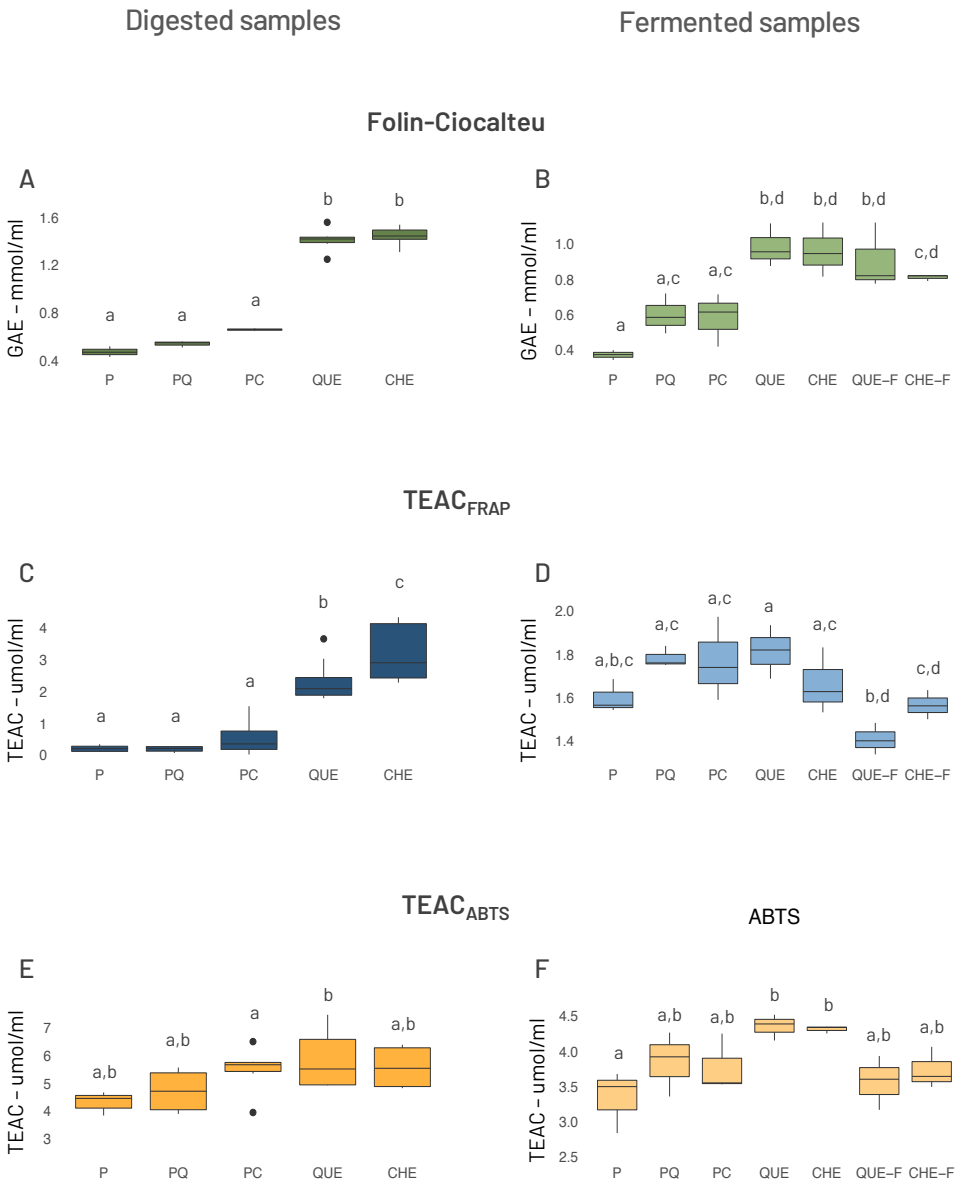


Figure 4.7.1 Total polyphenol content (by Folin-Ciocalteu) and antioxidant capacity (by TEACFRAP and TEACABTS) from digested (A, C, and E) and fermented (B, D, and F) fractions for pectin microbeads and tannin extracts. (A) and (B) show TEACFRAP, (C) and (D) TEACABTS, and (E) and (F) TEACAAPH. P: pectin microbeads; PQ: Pectin microbeads with quebracho; PC: Pectin microbeads with chestnut; QUE: quebracho extract; CHE: chestnut extract; QUE-F: quebracho extract just fermented; CHE: chestnut extract just fermented. Median ± standard deviations values are reported. Different letters indicate significant differences ($p < 0.05$) among samples calculated by ANOVA with Bonferroni post-hoc test.

Although there was a slight release of QUE or CHE from the microbeads in the soluble fraction of digestion, the values were much lower compared to pure digested extracts. Moreover, the results showed that even after the fermentation process, there was no significant release of tannins into the fermentation liquid.

The combination of pectin and tannins to generate microbeads was chosen because of the high binding affinity of these molecules. Tannins have been previously described as potential powerful cross-linkers, improving pectin gelling properties (Mamet *et al*., 2017). But in this case the bonds formed between pectin and tannins during the microencapsulation process were so strong that neither digestion nor the action of the microbiota could degrade them.

Aguirre *et al.* (2021) investigated different encapsulation systems for beet waste extracts and, similarly to the present study, the products were subjected to *in vitro* digestion and fermentation (Aguirre-Calvo *et al*., 2020). In this case, the encapsulated extracts showed a much higher antioxidant capacity than both non-loaded microbeads and non-encapsulated extracts. It should be noted that the extracts used by Aguirre and co-workers were of a different chemical nature and the combination with a distinct formulation of the microbead matrix allowed for a more massive release of content and thus a combined antioxidant action.

As regards QUE and CHE, no significant differences were found between the two extracts. Similarly, the same trend was reported by Molino *et al*. (2018) when the pure extracts were submitted to *in vitro* digestion and fermentation (Molino *et al*., 2018). With the present study, it further emerged that, although by-passing the digestion step, CHE-F did not show significant differences with the digested and fermented CHE. Thus, the digestion process likely did not affect this extract in terms of polyphenol content or antioxidant capacity. Conversely, a significant difference ($p < 0.001$) was recorded between QUE and QUE-F with regard to reductive capacity ($TEAC_{FRAP}$), suggesting that direct fermentation of this extract may result in a partial loss of the antioxidant capacity that the extract could alternatively exert if it were subjected to prior digestion.

4.7.2 Effect on microbiota composition

Figure 4.7.2 represents the distribution of beta diversity among the samples, calculated as Bray–Curtis dissimilarity, where PCo1 and PCo2 respectively contributed 56.16 % and 18.35 % of the total variation. The plot illustrates the differences in relative abundance of taxa and shows a clustering of samples based on the type of material. This grouping indicates that the *in vitro* fermentation system did not randomly influence the composition of the microbiota.

In terms of composition, the analysed microbiota after *in vitro* fermentation was globally similar for all samples and dominated by Firmicutes and Bacteroidota,

followed by Proteobacteria and Verrucomicrobia (**Figure 4.7.3** and **Table 4.7.1**). The presence of a relatively high abundance of Bacteroidota was already detected in the original inoculum, used to ferment the samples (**Figure 4.7.3**).

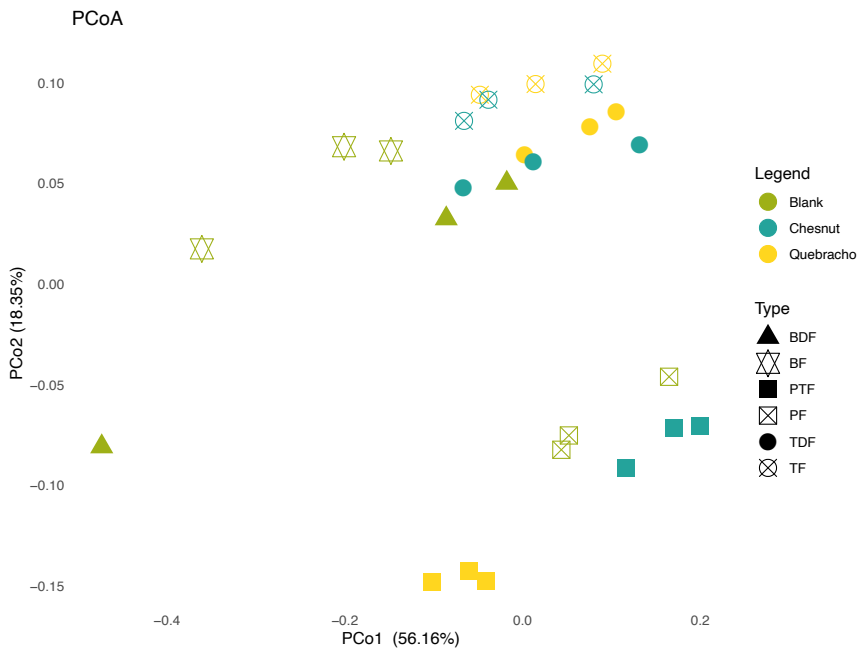


Figure 4.7.2 Principal coordinate analysis (PCoA) plot of total variation based on Bray-Curtis dissimilarity of microbial genera abundance among all profiled samples. The samples cluster by sample type.

Sample richness and diversity were evaluated by different estimator indexes, i.e. Chao1, ACE and Shannon's diversity index (**Table 4.7.2**). When analysed individually, differences were small and not significant. This may be due to a low test strength as the samples were only represented in triplicates. If the samples are grouped regardless of the type of tannin, it can be observed that digested and fermented or fermented-only tannin extracts induce a significantly greater increase in diversity than when they are encapsulated (**Figure 4.7.4**). This suggests that: (i) tannins are not totally released from the microbeads during the digestion and fermentation process; (ii) although the amount of pectin is significantly larger than that of tannins, it is not able to elicit a change of equal intensity.

ANCOM tests were applied to identify which bacterial taxa were responsible for a significant difference among samples. Based on the previous findings, the data were analysed separately in order to study (i) the effect of microencapsulation of tannin extracts vs. the use of pure extracts, and (ii) the effect of the digestion and fermentation process vs. the fermentation process alone on the tannin extracts.

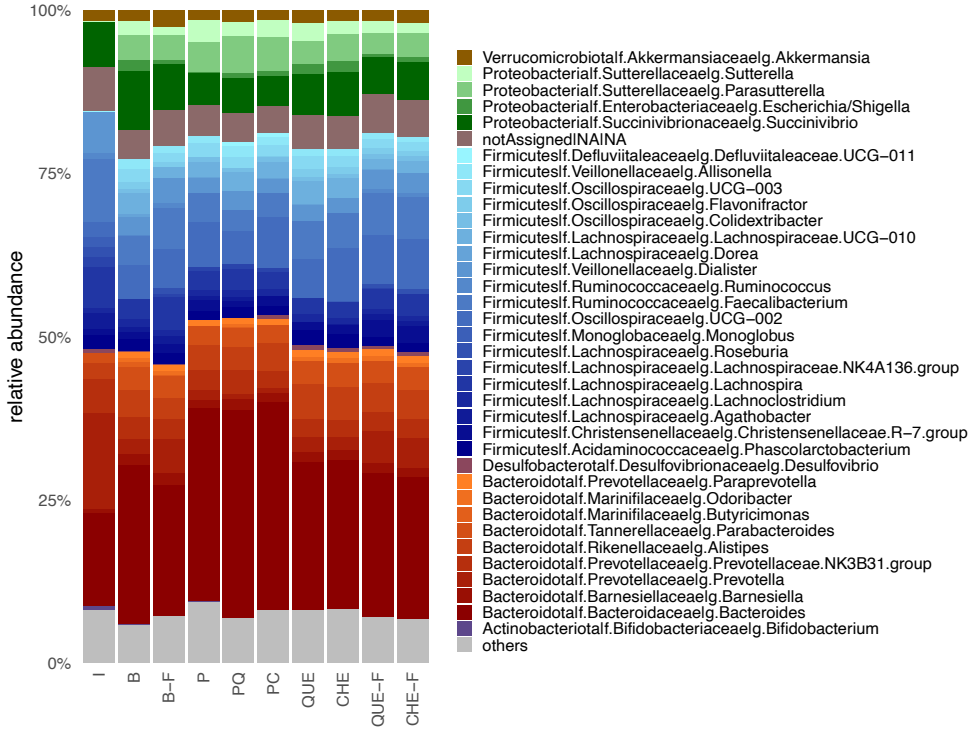


Figure 4.7.3 Barplot of gut microbial community structure at genus level. I: original inoculum; B: blank digested and fermented; B-F: blank just fermented; P: pectin microbeads w/o tannins; PQ: pectin microbeads with quebracho; PC: pectin microbeads with chestnut; QUE: quebracho extract digested and fermented, CHE: chestnut extract digested and fermented; QUE-F (quebracho extract just fermented); CHE -F (chestnut extract just fermented). Relative abundance obtained by total-sum scaling (TSS) from genus-level abundance table. "Others" include genera with relative abundance lower than 1% for all conditions.

Table 4.7.1 Relative abundance of phyla.

Sample	Actinobacteriota	Bacteroidota	Firmicutes	Proteobacteria	Verrucomicrobiota	Others
IO	0.826	40.24	48.07	8.017	1.67	0.230
BD	0.359 ± 0.139	42.46 ± 1.063	38.02 ± 1.153	16.776 ± 1.184	1.615 ± 0.516	0.151 ± 0.028
B-F	0.405 ± 0.335	40.11 ± 1.955	43.19 ± 2.162	12.94 ± 0.427	2.479 ± 0.346	0.172 ± 0.025
P	0.416 ± 008	44.60 ± 0.466	38.92 ± 0.550	13.61 ± 0.405	1.55 ± 0.146	0.178 ± 0.026
PQ	0.204 ± 0.038	46.90 ± 0.774	36.24 ± 0.705	14.01 ± 0.934	1.84 ± 0.237	0.156 ± 0.028
PC	0.389 ± 0.070	45.90 ± 0.669	37.89 ± 1.291	13.23 ± 0.820	1.41 ± 0.129	0.231 ± 0.028
QHE	0.531 ± 0.054	41.00 ± 0.485	40.81 ± 0.941	14.26 ± 0.501	1.918 ± 0.120	0.292 ± 0.016
CHE	0.502 ± 0.049	40.55 ± 0.595	41.41 ± 1.279	14.52 ± 0.925	1.703 ± 0.221	0.260 ± 0.021
QUE-F	0.609 ± 0.031	42.77 ± 0.420	42.62 ± 0.091	11.32 ± 0.181	1.716 ± 0.053	0.189 ± 0.032
CHE-F	0.627 ± 0.083	41.77 ± 0.875	42.68 ± 0.787	11.982 ± 0.059	1.899 ± 0.049	0.205 ± 0.037

Table 4.7.2 Indexes of α diversity.

	Diversity indexes				
Sample	Shannon	Chao1	SE.Chao1	ACE	SE.ACE
I0	4.578	293.0	0.499	293.1	5.310
BD	4.549 ± 0.161	265.8 ± 72.01	1.478 ± 1.327	265.5 ± 71.64	6.096 ± 0.647
B-F	4.665 ±0.088	266.0 ± 54.62	0.097 ± 0.087	266.1 ± 54.71	5.981 ± 1.126
P	4.396 ± 0.030	283.3 ± 5.859	0.222 ± 0.254	283.4 ± 5.878	6.612 ± 0.102
PQ	4.543 ± 0.016	359.9 ± 8.125	2.531 ± 3.111	358.6 ± 6.327	6.915 ± 0.268
PC	4.579 ± 0.034	347.8 ± 27.04	0.942 ± 0.867	347.7 ± 27.27	7.024 ± 0.179
QHE	4.756 ± 0.018	349.8 ± 13.01	0.998 ± 0.786	349.6 ± 12.66	6.957 ± 0.270
CHE	4.721 ± 0.009	346.7 ± 25.93	1.645 ± 0.852	346.3 ± 25.60	7.201 ± 0.193
QUE-F	4.829 ± 0.022	370.5 ± 28.02	1.736 ± 1.299	370.0 ± 27.26	7.214 ± 0.252
CHE-F	4.784 ± 0.015	343.2 ± 20.25	1.043 ± 0.701	343.1 ± 20.07	6.735 ± 0.283

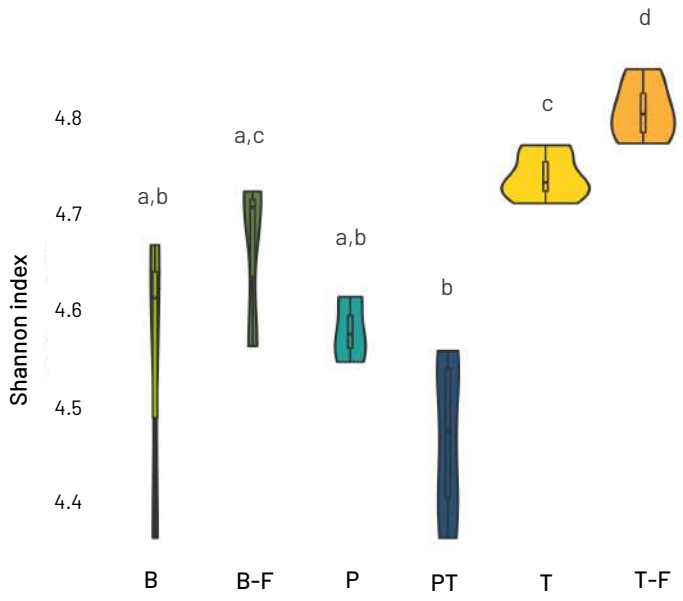


Figure 4.7.4 Microbiota diversity measured as Shannon index in B (blank digested and fermented), B-F (blank just fermented), P (pectin microbeads w/o tannins), PT (pectin microbeads with tannins), T (tannin extracts digested and fermented), T-F (tannin extracts just fermented). Different letters indicate significant differences among samples (adjusted $p < 0.05$) by Wilcoxon test.

Effect of microencapsulation

PC and P shared a very similar behaviour when compared to the blank that was submitted to digestion and fermentation (BDF). This suggests that there was no significant contribution from the extract of hydrolysable tannins, so that the interaction with the microbiota is attributable solely to pectin. Among Firmicutes, several taxa increased after fermentation of both PC and P, belonging to the families Ruminococcaceae (UBA1819), Clostridiaceae (*Anaerotruncus*), Oscillospiraceae (UCG 007) and Lachnospiraceae (NK4A136.group and *Hungatella*). Bang and co-workers (2018) conducted *in vitro* fermentation of pectin to investigate possible changes in the gut microbiome and SCFA production. The hydrocolloid fermentation produced mainly increases of taxa belonging to Clostridium cluster XIV within the Lachnospiraceae family (Bang *et al*., 2018).

The fermentation of P and PC also led to an increase of *Collinsella*, belonging to the Actinobacteriota. Regarding this genus, the information in the literature is controversial. Adamberg and co-workers (2020) correlated *Collinsella* to fibre-deficient diets, but the *in vitro* fermentation of Carlson *et al.* (2017) using a whole fibre diet (rich in pectin among others) promoted the growth of *Collinsella* comparing the 24 h samples to the 0 h samples (Carlson *et al*., 2017; Adamberg *et al*., 2020).

The only common finding between the three microbead samples was a considerable increase in Defluviitaleaceae UCG-011. This is a newly discovered taxon, which has been recently associated with colitis induced by dextran sulfate sodium (Zha *et al*., 2020). Apart from Defluviitaleaceae UCG-011, PQ was not able to induce any effect on the composition of the microbiota. This may be due to the strong interaction between pectin and quebracho extract molecules (Mamet *et al*., 2017). Their association could possibly lead to the generation of complexes that don't allow any interaction of pectin and quebracho extract molecules with the microbiota due to steric hindrance.

The fact that tannins do not play an important role in modulating the microbiota when administered through microencapsulation with pectin, was further highlighted by comparing the extract (QUE or CHE) with the corresponding microencapsulated sample (PQ or PC). It quickly becomes evident that there were several differences (**Figure 4.7.5**). Compared to the microbeads, the extracts showed a substantially lower abundance of Defluviitaleaceae UCG-011 and of many of the earlier-mentioned Clostridiales (i.e. Ruminococcaceae UBA1819, *Anaerotruncus*, Lachnospiraceae NK4A136.group and *Hungatella*), confirming that it is the microbeads' pectin rather than the tannins that induces the increase of these bacterial groups. The bacteria induced by the fermentation of tannins are discussed below in the context of the comparison of fermentations with and without a previous digestion step.

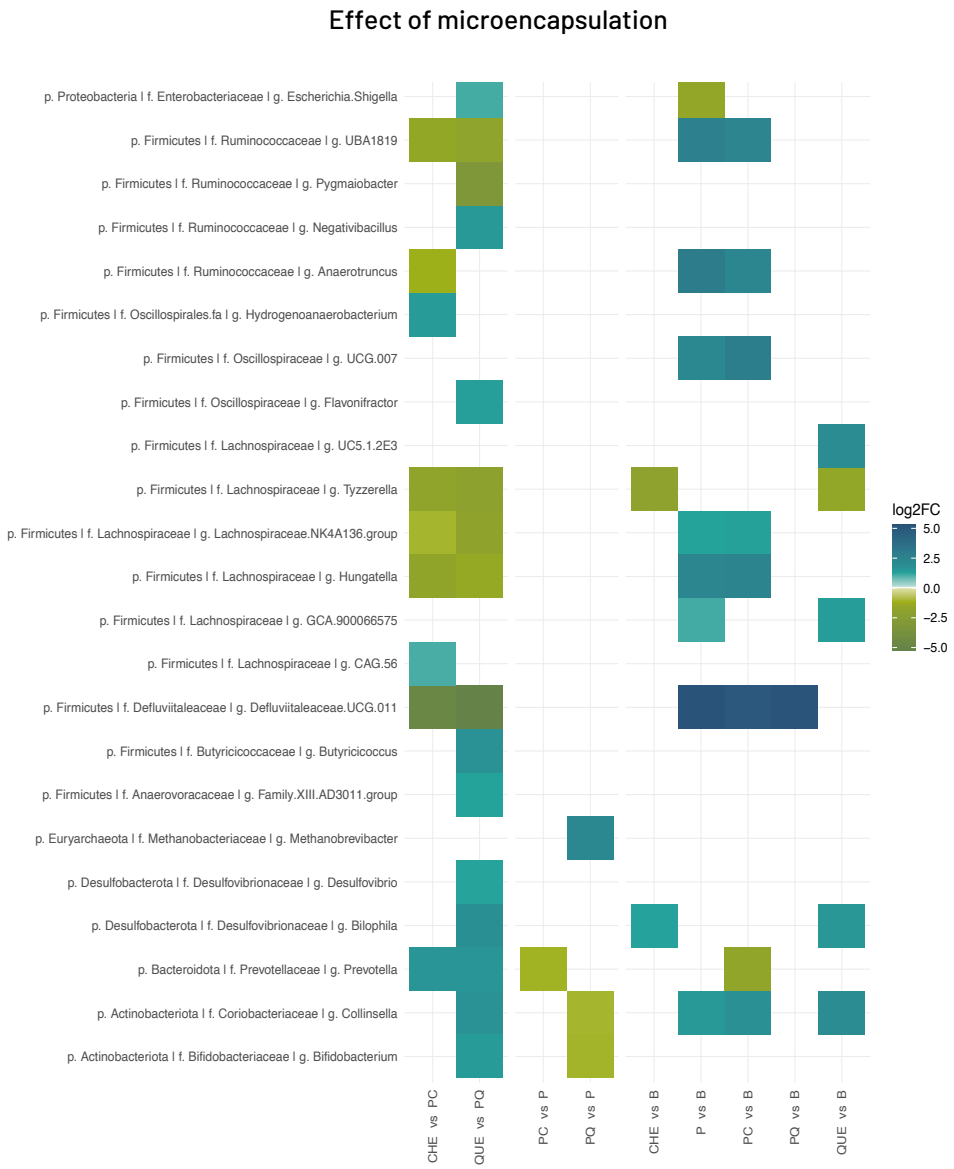


Figure 4.7.5 Effect on gut microbiota composition induced by tannins through microencapsulation. The heatmap represents the fold-changes in the relative abundance at genus level. All changes significant for adjusted p < 0.05 and filtered with LOG2FC>1 at nominal level by ANCOM tests are shown.

Effect of digestion

To investigate whether digestion had an impact on the compounds that will come into contact with the microbiota in the large intestine, QUE and CHE were submitted to both *in vitro* digestion and fermentation, and also just to fermentation (QUE-F, CHE-F). Many microencapsulation systems are used to preserve a compound from degradation that can occur along the digestive process. This analysis therefore highlights whether it is possible to dispense tannins with microencapsulation and still allow them to reach the intestine in order to produce an effect on the gut microbiota. Some studies report that tannins can pass through the digestive process almost intact. However, the two extracts under consideration show very distinct characteristics and enzymatic digestion may affect the chemical structure of these two extracts differently (**Figure 4.7.6**).

For chestnut extract, CHE and CHE-F induce different changes when compared to their respective blanks (BDF and BF). CHE-F results in an increase of a Ruminococcaceae Incertae Sedis and the decrease of *Methanobrevibacter*, whereas the digested CHE induces an increase in Bilophila and a decrease in *Tyzzerella*. In addition, the direct comparison of CHE and CHE-F indicates that *Escherichia* is more abundant in the first and *Prevotella* is more abundant in the latter, indicating that previous digestion of the extract also results in a different effect on these genera. Several studies reported that both condensed and hydrolysable tannins are particularly effective in reducing *Prevotella*, at times correlated to inflammatory outcomes (Díaz Carrasco *et al*., 2017; Kalantar-Zadeh, Ward, Kalantar-Zadeh, & El-Omar, 2020; Sánchez-Patán *et al*., 2015). Our results suggest that digestion increases this effect in the case of CHE.

As can be seen in **Figure 4.7.6**, QUE induced more changes than QUE-F when compared with the respective blanks, suggesting that the chemical action of digestion may render the large characteristic structures of quebracho tannins more accessible to microbial fermentation. Digestion and fermentation of QUE led to an increase in Lachnospiraceae UC5.1.2E3, Lachnospiraceae GCA.900066575, *Bilophila* and *Collinsella* and a decrease in *Tyzzerella*. Of these, only *Collinsella* also increased in the case of QUE-F, which showed in addition an increase of Ruminococcaceae Incertae Sedis and a decrease of *Methanobrevibacter*, also seen with CHE-F. The direct comparison of QUE and QUE-F highlights numerous further differences that reinforce the marked effect of digestion on quebracho extract. QUE presented a higher abundance of *Escherichia*, *Lachnoclostridium* UCG 010, *Butyricicoccus*, *Methanobrevibacter* and *Bilophila*, and a lower abundance of *Mitsuokella*, Lachnospiraceae groups ND3007 and AC2044, *Agathobacter*, *Prevotella* and *Coprobacter*.

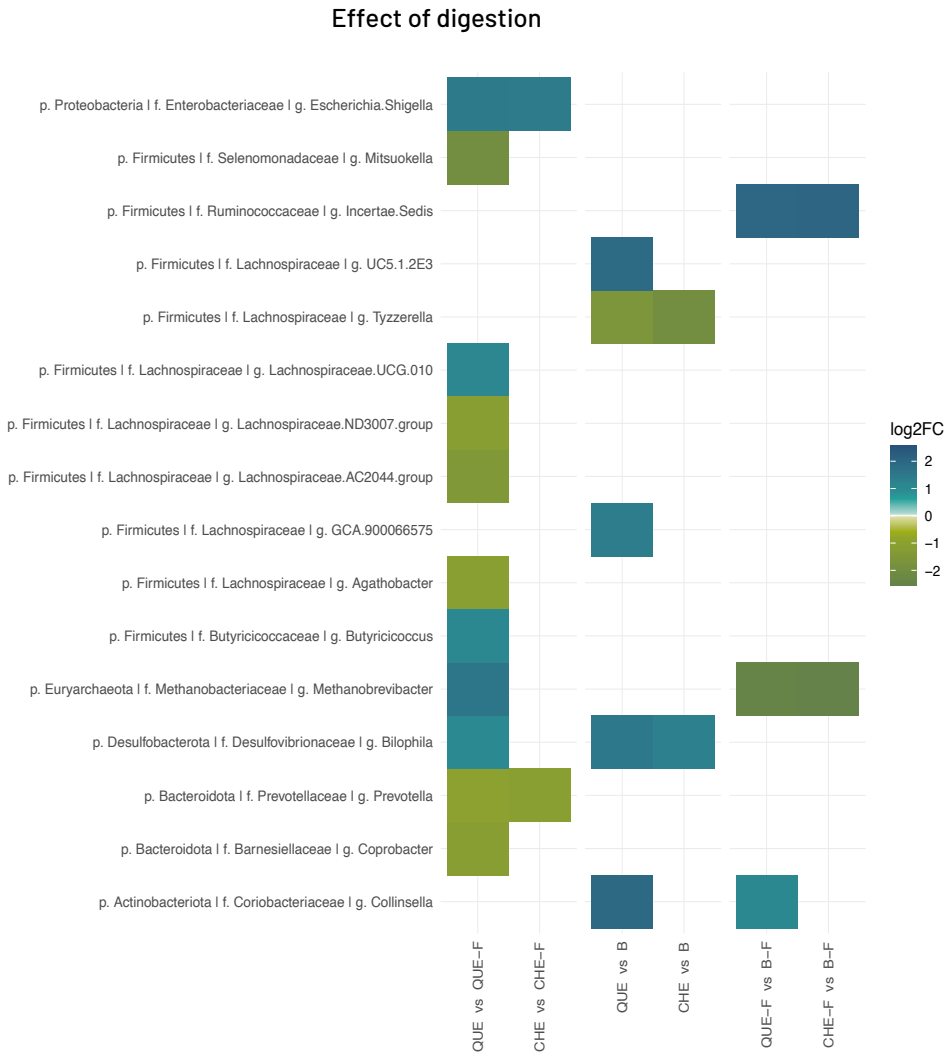


Figure 4.7.6 Effect on gut microbiota composition induced by tannins through digestion - fermentation. The heatmap represents the fold-changes in the relative abundance at genus level. All changes significant for adjusted p < 0.05 and filtered with LOG2FC>1 at nominal level by ANCOM tests are shown.

It is interesting to note that both the undigested CHE-F and QUE-F induced an increase in Ruminococcaceae Incertae Sedis and a decrease in *Methanobrevibacter* compared to the fermented blank (BF) that was not observed with digested extracts. *Methanobrevibacter* is a methanogenic bacteria for which both an excessive abundance as well as a total absence have been associated with several pathologies. Therefore some authors suggest using its relative abundance as an indicator of a healthy intestinal tract (Djemai, Drancourt, & Tidjani Alou, 2021). The capacity of undigested tannins to modulate the abundance of *Methanobrevibacter* is therefore of interest, and it is important to know that their digestion will remove such capacity.

The difference in the effect of digestion on the two extracts probably lies in their chemical composition. Indeed, hydrolysable tannins such as those present in chestnut extracts are identified as such because they can be fractionated hydrolytically into their components (Khanbabaee, van Ree, & Ree, 2001). This pronounced susceptibility to breakdown may mean that, even if they arrive intact in the intestinal environment, metabolization can occur in much the same way as when the extract is also subjected to prior digestion.

4.7.3 SCFA production

Non-digestible fibres are recognised to be great producers of SCFAs (acetate, propionate, and butyrate). However, other substances, tannins among them, have recently also been attributed the ability to stimulate the fermentative activity of the intestinal microbiota (Bolca *et al*., 2013; Kawabata *et al*., 2019). Testing SCFA production is not only important to verify the stimulation of microbiota activity but also because these compounds exert various health promoting effects (Koh *et al*., 2016b; Edwards *et al*., 2017).

Figure 4.7.7 illustrates the sum of SCFAs (i.e., acetate, propionate and butyrate) released after the fermentation of pectin-based microbeads containing tannin extracts, as well as after the fermentation of the extracts alone (CHE, QUE, CHE-F, QUE-F). All the tested samples showed a high production of SCFAs, compared to the blank, confirming once again the prebiotic potential of tannins. Subgrouping the samples into three groups (i.e. microbeads, digested and fermented tannin extracts and fermented tannin extracts), microbeads showed a higher release of SCFAs ($p<0.001$) compared to extracts. When analysing the different types of microbeads, those containing tannins (PC and PQ) did not differ significantly from non-loaded microbeads (P). This suggests that when microbeads were fermented, only the contribution of pectin was crucial in the production of SCFAs, whereas tannins did not seem to play a role. Thus, once again it seems likely that no appreciable amount of tannins was released from the microbead matrix.

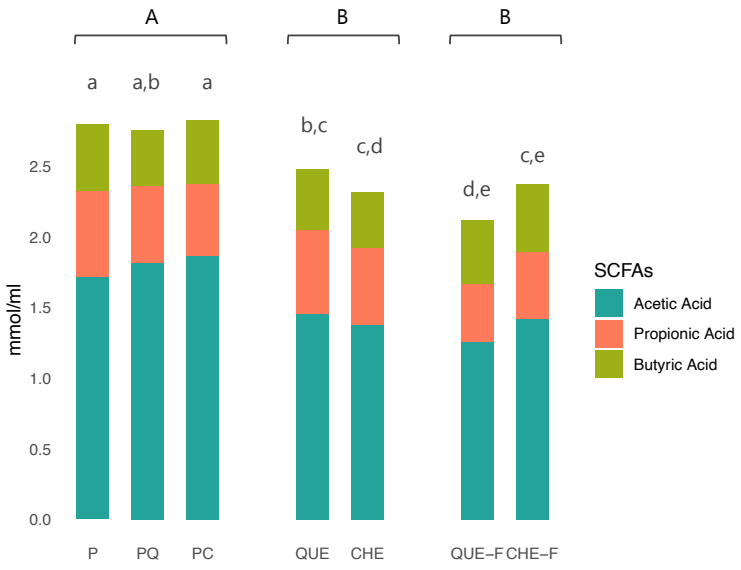


Figure 4.7.7 Release of SCFAs (mmol per ml of fermented liquid fraction) Mean ± standard deviation values are reported. Different capital letters indicate significant differences ($p < 0.05$) among groups of samples (microbeads, digested and fermented tannin extracts, just fermented tannin extracts) while lower-case letters indicate significant differences among all samples ($p < 0.05$), by ANOVA and Bonferroni post hoc test.

Although the individual tannin extracts stimulated a lower production of total SCFAs in comparison to pectin microbeads, it should be borne in mind that the amount subjected to *in vitro* digestion and fermentation was been calculated so as to equal their content within the microbeads (*circa* 4.5% w/w). Therefore, we are observing that an amount of tannins 22-fold lower than that of pectin is able to stimulate a similar production of SCFAs. In support of the high prebiotic activity of these two extracts, when Molino *et al.* compared equal amounts of tannin extracts (both CHE and QUE) with a known commercial fibre (inulin), the extracts presented much higher values of SCFA production (Molino *et al*., 2018).

With regard to tannin extracts, no significant differences were found between CHE and CHE-F, whereas QUE-F had a significantly lower ($p=0.025$) SCFA release than QUE. As suggested by 16S rRNA sequencing data, again it appears that chestnut hydrolysable tannins can be easily metabolised directly by the gut microbiota in the absence of previous digestion. On the other hand, quebracho extract was characterised by a more complex chemical structure (condensed tannins), so that the

preliminary step of enzymatic digestion seems to be essential to facilitate its microbial fermentation.

Regarding the production of the individual SCFAs (**Table 4.7.4**), microbeads resulted in a higher release of acetate compared to tannin extracts, independently of whether they were digested and fermented or just fermented. The fermented-only extracts showed a much lower propionate production than the other two sample groups (microbeads and digested-fermented extracts). In particular, QUE and QUE-F showed the largest difference, in that bypassing the enzymatic digestion process of the quebracho extract resulted in a significant decrease ($p<0.001$) in the release of propionic acid. In contrast, QUE-F and CHE-F were the samples that resulted in the greatest release of butyric acid. However, the difference between QUE and QUE-F was not statistically significant, whereas the increase presented by CHE-F, compared to CHE, was considerably greater ($p<0.05$).

Table 4.7.3 Short Chain Fatty Acids (SCFAs) produced after *in vitro* fermentation. Different letters indicate statistically significant differences ($p<0.05$) by ANOVA and Bonferroni post-hoc test among samples, within each SCFA.

Sample	Acetic acid	Propionic acid	Butyric acid
BD	0.129 ± 0.020^{a}	0.084 ± 0.024^{a}	0.052 ± 0.004^{a}
B-F	0.123 ± 0.013^{a}	0.073 ± 0.011^{a}	0.054 ± 0.008^{a}
P	$0.574 \pm 0.021^{b,c}$	0.204 ± 0.004^{b}	0.158 ± 0.003^{b}
PC	0.624 ± 0.048^{b}	$0.169 \pm 0.013^{b,c}$	0.149 ± 0.024^{c}
PQ	0.606 ± 0.101^{b}	$0.183 \pm 0.015^{c,d}$	$0.130 \pm 0.013^{b,c}$
QUE	$0.484 \pm 0.029^{b,c,d}$	$0.198 \pm 0.037^{b,d}$	$0.145 \pm 0.010^{b,c}$
CHE	0.460 ± 0.031^{d}	$0.181 \pm 0.008^{b,d,e}$	0.133 ± 0.013^{c}
QUE-F	0.418 ± 0.013^{d}	0.137 ± 0.006^{f}	$0.154 \pm 0.009^{b,c}$
CHE-F	0.476 ± 0.040^{d}	$0.159 \pm 0.011^{d,e,f}$	0.161 ± 0.012^{b}

4.7.4 Correlations

The previous results showed that when microbeads were analysed, pectin had a predominant effect compared to tannins on modulating the microbiota composition and functionality. As the interest of this study is mainly in studying the effect of tannins on the intestinal ecosystem, we investigated the possible correlations between the antioxidant activity techniques, the production of short-chain fatty acids and the specific taxa involved only for unencapsulated tannins.

The mixOmics network function for (sPLS) provided a great amount of information (**Figure 4.7.8**). First of all, it should be noted that the dendrogram grouped the techniques for measuring antioxidant activity ($TEAC_{ABTS}$ and $TEAC_{FRAP}$) with Folin-Ciocalteu. In fact, in the heatmap a close match among the three methods can be observed, showing that antioxidant activity and tannin content correlate with the abundance of the same bacterial taxa. Next to these, clustered the production of acetic acid, butyric acid and lastly propionic acid. This indicates that the bacteria that were most sensitive to the presence of tannins had a strong effect on the production of acetic acid and smaller effects on the other SCFAs.

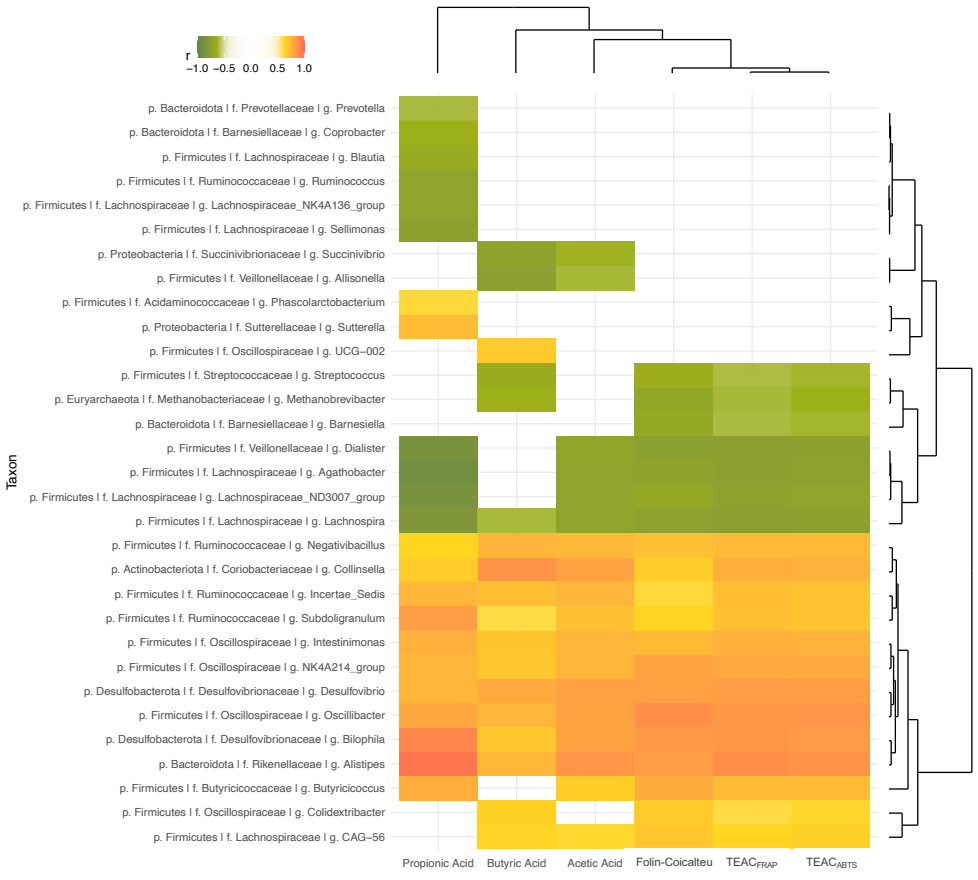


Figure 4.7.8 Heatmap of the correlation between microbial relative abundance and SCFA production or antioxidant capacity, calculated with the sPLS-based approach implemented in the mixOmics network function (correlation coefficient > 0.6).

Figure 4.7.8 shows that the presence of tannins increased the relative abundance of *Oscillibacter*, *Subdoligranulum*, Ruminococcaceae Incertae Sedis, *Negativibacillus*, *Collinsella*, *Bilophila*, *Alistipes*, *Intestinimonas*, Oscillospiraceae NK4A214 group and *Desulfovibrio.* Along with these, the release of SCFAs was also increased. Of interest, several of the mentioned taxa could be involved in tannin degradation. Indeed, *Collinsella* is well known for its potential for ring cleavage, dehydroxylation and hydrogenation in polyphenols (Medawar *et al*., 2021). Analogously, *Intestinimonas* is a relatively recently described genus, phylogenetically related to members of the genus *Flavonifractor,* which plays a key role in proanthocyanidin catabolism (Kutschera *et al*., 2011; Kläring *et al*., 2013).

Conversely, the relative abundance of *Dialister*, *Agathobacter*, Lachnospiraceae ND3007 group, *Lachnospira*, *Methanobrevibacter*, *Streptococcus* and *Barnesiella* showed a negative correlation with the presence of tannins, and also with the production of all or some SCFAs. Tannins have been widely investigated for their potential to affect the level of methane in ruminants, through the modulation of methanogenic genera, and *Methanobrevibacter* among them (Saminathan *et al*., 2016). The negative correlation with this taxon suggests that supplementation with tannins may help reduce methane-related bloating discomfort, also in human beings.

Spearman correlations further emphasize that the changes in some bacterial abundances that correlate with SCFA production were closely dependent on the presence of tannins. As an example, **Figure 4.7.9 A** and **B** illustrate how the abundances of *Collinsella* and *Methanobrevibacter*, which show strong positive and negative correlations, respectively, with acetic acid production, vary importantly between the blanks and the tannin fermentations.

Finally, in the case of some other taxa, their abundance correlated positively (i.e. *Sutterella*, *Phascolarcobacterium*, Ruminococcaceae UCG002) or negatively (i.e. Lachnospiraceae NK4A136 group, *Ruminococcus, Sellimonas, Coprobacter, Prevotella, Blautia, Allisonella*, *Succinivibrio*) with the production of short-chain fatty acids, but the variation of these taxa was not affected by the abundance of tannins (**Figure 4.7.8**).

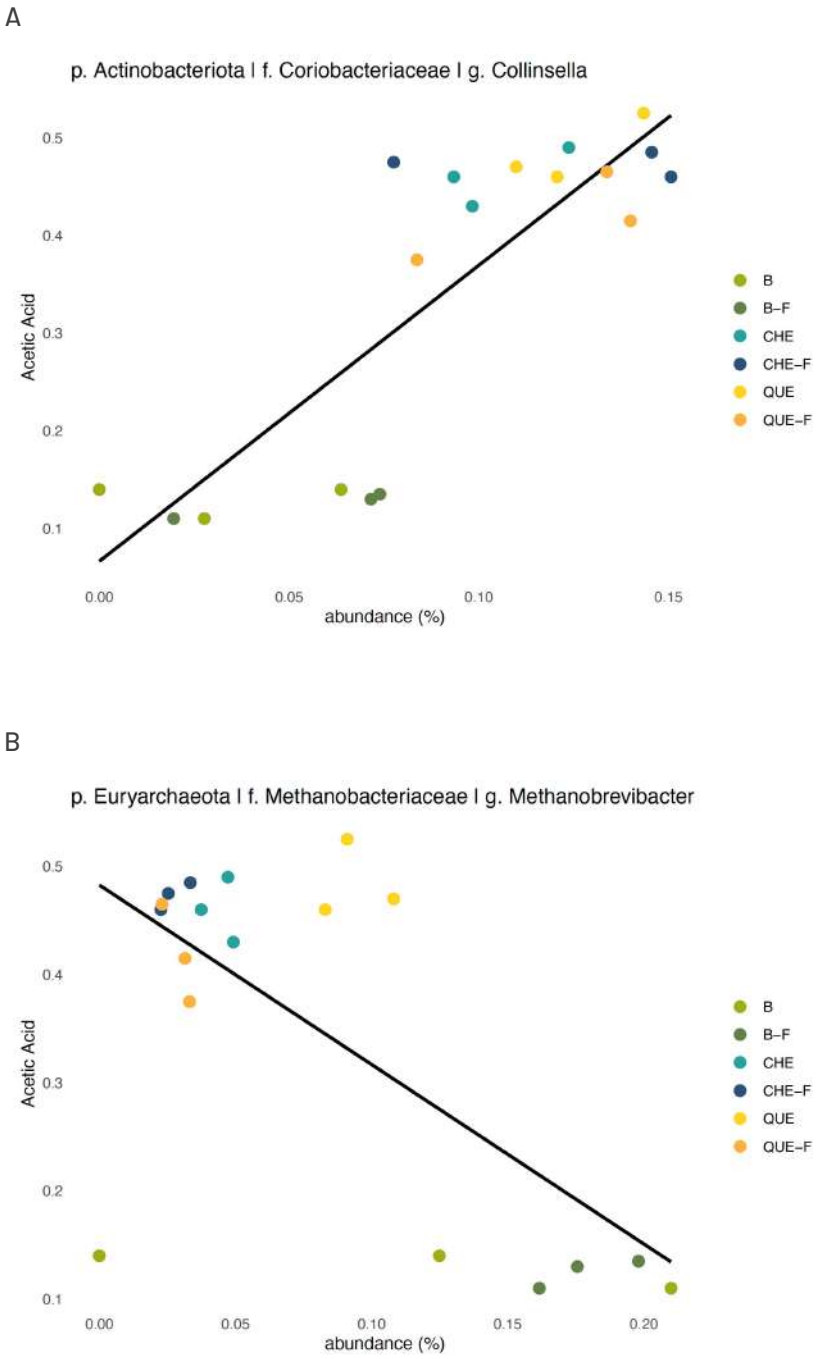


Figure 4.7.9 Spearman correlation between (A) acetic acid and *Collinsella* and between (B) acetic acid and *Methanobrevibacter*.

4.8 Chapter 8

Tannin supplementation increases diversity and short-chain fatty acid production in the gut microbiota of healthy subjects

Eight healthy volunteers completed a tannin supplementation, taking a microbead per day over a period of 4 weeks, with a high compliance, and there were no dropouts. No adverse event was reported during the study period. To study the effect of the tannin blend on the gut microbiota, comparisons were performed against the baseline T0, as the study did not include any placebo supplementation.

4.8.1 Effects of tannins on gut microbiota diversity

Sequenced profiles were employed to analyse microbiome alpha diversity for each sample, based on richness estimators (Chao1 and ACE), phylogenetic diversity and the Shannon diversity index (**Figure 4.8.1, Table 4.8.1**). A general upward trend over the 4-week supplementation period, as estimated by all three different indexes, was registered. However, only the Shannon index showed a statistically significant difference ($p=0.0078$, adjusted $p=0.016$) between T0 and T28d.

Furthermore, the beta diversity among the samples was investigated, calculated as the Bray–Curtis dissimilarity, which describes differences in relative abundance of taxa. In the PCoA based on the Bray-Curtis dissimilarity index, PCo1 and PCo2 respectively contributed 21,29 and 14,04% of the total variation (**Figure 4.8.2**). The results did not show a separation of the samples into clusters reflecting the intervention time, rather the samples tended to group by individual. More in detail, among the different volunteers, some showed a greater difference between the samples analysed over time (individuals 2, 4, 5, 6 and 11), while others showed almost no difference (individuals 3, 10 and 13).

Overall, the effects of the intervention on gut microbiota composition were moderate, since only the Shannon diversity index was found to increase significantly by T28d. However, it is important to consider that all participants in the intervention

were healthy and lean at T0, so that their initial microbiota was likely configured in a eubiotic state. Therefore, a dietary supplementation would not be expected to produce large changes, only minor improvements. Furthermore, it must be taken into account that the supplementation lasted for a relatively short period of time, further limiting the possibility of large changes. Probably for this reason, beta diversity, calculated as Bray-Curtis dissimilarity, presented a clustering mostly reflecting interindividual variability. Nevertheless, we detected some tendencies towards increases or decreases of certain taxa, mostly starting already in the first two weeks, and continuing to a lesser extent until the end of the intervention.

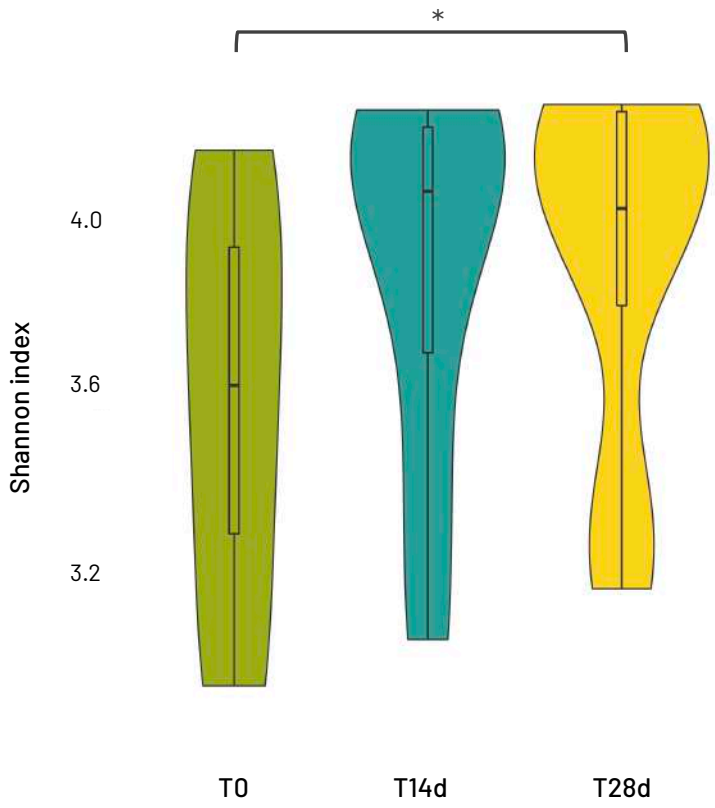


Figure 4.8.1 Variation of the Shannon index of microbiota diversity throughout the intervention. Diversity increases significantly only at T28d (p=0.0078, adjusted p=0.016).

Table 4.8.1 Indexes of α diversity.

		Diversity indexes					
Volunteer	**Tannin**	Phylogenetic diversity	Shannon	Chao1	SE.Chao1	ACE	SE.ACE
2	T0	2.95	165	0.25	165.2	4.80	2
	T 14d	3.79	172	0	172	3.70	8.10
	T 28d	3.36	145	0	145	4.48	8.12
3	T0	4.15	219	0	219	5.23	3
	T 14d	4.19	230	0	230	5.45	7.68
	T 28d	4.23	259.5	1.3	259.3	5.65	7.70
4	T0	3.77	156	0	156	3.99	4
	T 14d	4.24	186	0	186	3.48	6.58
	T 28d	3.95	160	0	160	3.69	6.91
5	T0	3.87	185	0.5	185.2	4.10	5
	T 14d	3.42	173	0.25	173.2	4.74	6.28
	T 28d	4.08	178	0	178	4.39	7.87
6	T0	3.48	152	0	152	4.78	6
	T 14d	4.10	216	0	216	4.94	5.73
	T 28d	3.95	217	0.25	217.2	4.76	7.74
10	T0	3.01	104	0	104	3.26	10
	T 14d	3.06	110	0	110	3.15	4.94
	T 28d	3.17	128	0	128	3.17	7.93
11	T0	4.12	207	0	207	4.92	11
	T 14d	4.23	213	0.25	213.2	4.98	7.60
	T 28d	4.25	242.3	0.93	242.5	5.30	6.98
13	T0	3.39	168	0	168	4.37	13
	T 14d	4.01	195	0	195	4.75	7.24
	T 28d	4.25	212	0	212	4.93	6.43

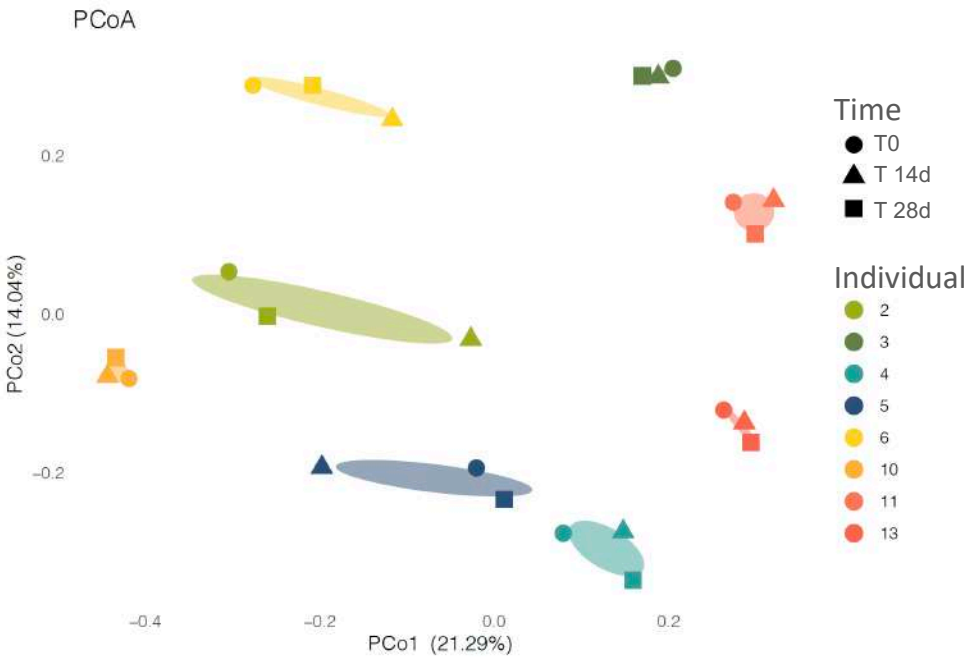


Figure 4.8.2 Principal coordinate analysis (PCoA) plot of total variation based on Bray-Curtis dissimilarity of microbial genera abundance among all profiled samples. The samples cluster by individual.

4.8.2 Tannins produced a shift in microbiota composition

At phylum level, the microbiota was dominated by Firmicutes and Bacteroidota, followed by Verrucomicrobia, Proteobacteria and Actinobacteria in all the evaluated samples. The supplementation determined a slight progressive modification in the proportions of these phyla over time, where the Firmicutes and the Proteobacteria decreased while the Bacteroidota increased, yet this trend was not significant (**Table 4.8.2**, **Figure 4.8.3**).

ANCOM tests were employed to determine if individual bacterial taxa changed in relative abundance during the 4-weeks intervention. Various genera and species were identified, which changed at a nominal level of significance, yet in no case were there statistically significant differences after adjusting for multiple comparisons.

Table 4.8.2 Relative abundance of phyla.

Individual	Time	Actinobacteria	Bacteroidota	Firmicutes	Proteobacteria	Verrucomicrobia	other
2	T0	1.61	17.21	35.59	9.36	35.63	0.60
	T 14d	2.14	11.35	78.63	0.16	3.67	4.05
	T 28d	0.14	23.90	47.11	1.20	26.29	1.37
3	T0	0.22	31.16	63.40	1.69	0.00	3.52
	T 14d	0.83	37.04	55.20	2.51	0.03	4.38
	T 28d	0.67	23.89	70.64	1.73	0.00	3.06
4	T0	3.29	13.31	77.21	2.09	3.85	0.26
	T 14d	0.84	35.96	61.57	1.22	0.36	0.05
	T 28d	6.21	29.34	60.08	4.29	0.07	0.01
5	T0	3.22	9.27	75.30	3.22	7.12	1.87
	T 14d	1.01	22.04	51.87	0.67	23.78	0.63
	T 28d	0.50	38.09	54.60	1.22	5.24	0.36
6	T0	0.31	17.77	62.28	2.94	15.26	1.44
	T 14d	1.48	22.38	71.18	0.73	3.48	0.74
	T 28d	0.28	27.15	57.82	2.42	11.50	0.83
10	T0	0.73	40.80	37.38	1.27	19.74	0.08
	T 14d	0.95	41.60	35.01	2.07	20.15	0.21
	T 28d	0.54	40.96	35.58	3.35	19.19	0.39
11	T0	2.16	19.88	66.73	11.10	0.02	0.11
	T 14d	1.48	37.43	59.16	1.60	0.08	0.25
	T 28d	2.08	40.96	54.72	0.86	1.28	0.09
13	T0	2.73	24.42	71.95	0.50	0.00	0.40
	T 14d	2.97	31.91	61.64	3.15	0.00	0.33
	T 28d	2.16	34.86	62.26	0.35	0.00	0.37

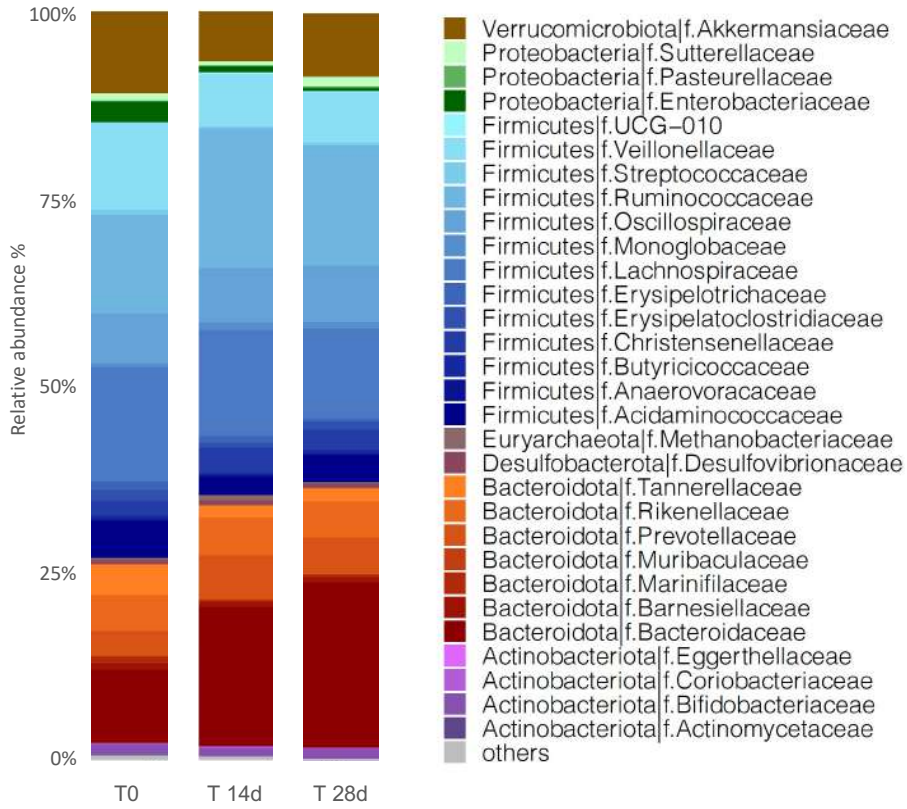


Figure 4.8.3 Barplot of gut microbial community structure at family level, at three different times of the intervention (T0, T14d and T28d). Relative abundances were obtained by total-sum scaling (TSS) from the genus-level abundance table.

Within the Firmicutes, two genus-level groups of the Lachnospiraceae family showed a nominal increase in abundance in comparisons of T0 to both time points T14d and T28d: Lachnospiraceae UCG 010 (T14d unadjusted p=0.043, T28d unadjusted p=0.045) and Lachnospiraceae NK4A136 group (T14d unadjusted p=0.044, T28d unadjusted p=0.032) (**Figure 4.8.4**). Since there was no change between times T14d and T28d, the trends towards increase in these genus-level groups occurred during the first 14 days. Other taxa, such as Lachnospiraceae UCG 001, as well as the genus *Ruminococcus*, the species *R. bicirculans* and one of the ASVs of *Faecalibacterium prausnitzii*, showed trends towards increased abundance only by the end of the intervention (T28d unadjusted p values p=0.041, p=0.027, p=0.005 and p=0.026, respectively).

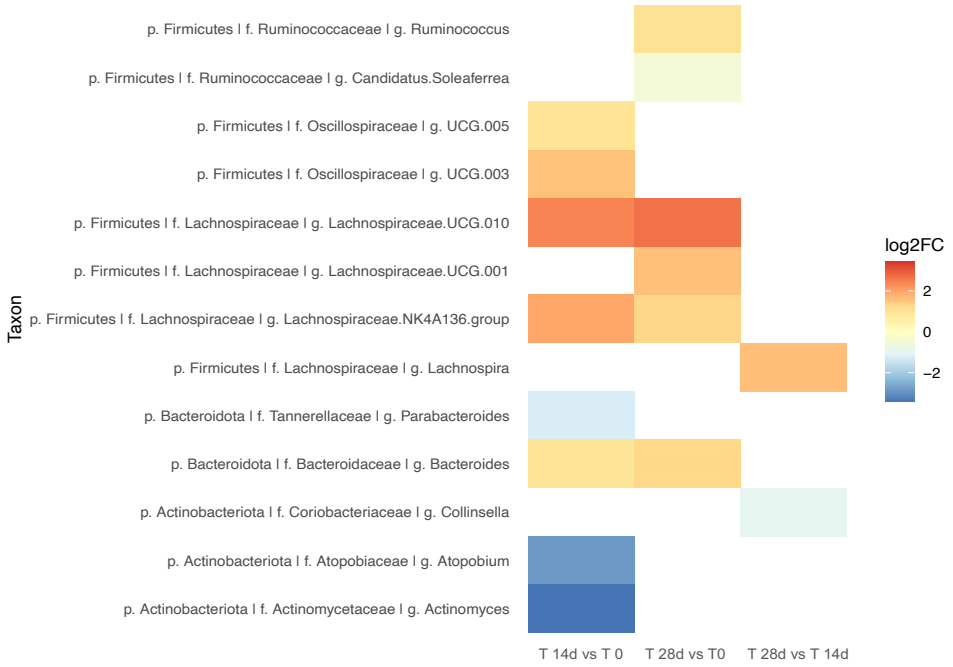


Figure 4.8.4 Effect of tannin supplementation on faecal microbiota. Fold-changes in the relative abundance at genus level. All changes significant (unadjusted $p < 0.05$) at nominal level by ANCOM tests between T0 vs T14d, T0 vs T28d, and T14d vs T28d are shown.

Oscillospiraceae UCG 005 and UCG 003 tended to increase slightly at T14d (unadjusted p=0.033, unadjusted p=0.038, respectively), but these effects were no longer present by T28d, suggesting a transitory modification in reaction to the initial exposure to the tannins.

Within the Bacteroidota, the family Bacteroidaceae (T14d unadjusted p=0.041, T28d unadjusted p=0.023) and the genus *Bacteroides* (T14d unadjusted p=0.034, T28d unadjusted p=0.013) also tended to have increased already by T14d (**Figure 4.8.4**). Among all *Bacteroides* species, the abundance of *B. thetaiotaomicron* was the highest at T28d, although the difference remained not statistically significant, likely due to the presence of an outlier sample.

Within the Actinobacteria, the Actinomycetales showed a tendency to decrease in comparisons of T0 to both T14d and T28d (unadjusted p=0.02 and unadjusted p=0.048, respectively). Other taxa showed a trend towards decreasing only in the T0-T14d comparison, but not when T0 was compared to T28d. This was the case for *Actinomyces* (T14d unadjusted p=0.034, T 28d unadjusted p=0.058) and *Atopobium*

(T14d unadjusted p=0.025, T28d unadjusted p=0.072), as well as for the family Atopobiaceae (T14d unadjusted p=0.02, T28d unadjusted p=0.068). Although this might suggest that a reversed trend towards increased abundance occurred between T14d and T28d, it should be emphasized that the above non-significant comparisons between T0 and T28d are likely due to the presence of only one outlier in the sample group.

To further characterize the shifts of microbiota composition, we also conducted LEfSe analyses to identify taxa that exhibit significant differential abundance ($p < 0.05$, logarithmic LDA score ≥ 2) (**Figure 4.8.5 A** and **B)**. These analyses further supported the increase of *Bacteroides* and of Lachnospiraceae family members in comparisons of T0 to both T14d and T28d, as well as the increase of *R. bicirculans* by T28d. In particular, LEfSe detected the increase of the Lachnospiraceae species *Lachnoclostridium edouardi*. In addition, the comparison between T0 and T14d detected that *Solobacterium moorei* was overrepresented in the T0 group.

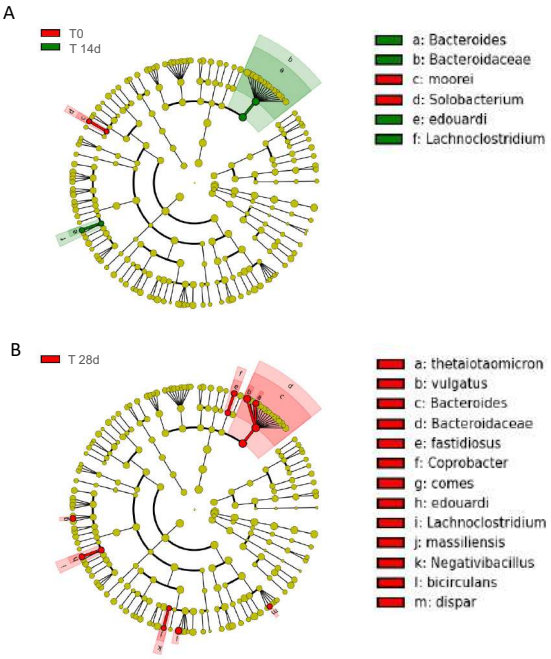


Figure 4.8.5 Taxonomic biomarkers characterizing the differences between (B1) T0 and T14d, and (B2) T0 and T28d according to LEfSe and represented with a cladogram visualization ($p < 0.05$, logarithmic LDA score ≥ 2).

Despite the lack of significance after correction for multiple comparisons in ANCOM analyses, some trends of change in the composition of the microbiota were detected, as stated above. Among the Firmicutes, we detected that the tannin supplementation promoted trends towards the increase of several genera belonging to the families Lachnospiraceae, Ruminococcaceae and Oscillospiraceae. All these families belong to the order Clostridiales, and include numerous bacteria involved in the regulation of the immune system and of numerous physiological metabolic functions (Raimondi *et al*., 2021).

The capacity of tannins for increasing the amount of the Lachnospiraceae and Ruminococcaceae families has been previously described by several authors (Díaz Carrasco *et al*., 2018; Molino *et al*., 2021). Notably, Diaz Carrasco *et al.* (2018) identified an increment of the genus *Faecalibacterium,* through a 30-day tannin supplementation in broilers. *Faecalibacterium* is recognised as a major source of butyrate (Louis and Flint, 2017). Analogously, after the 4-week intervention, we also detect a trend towards higher abundance of one ASV of *Faecalibacterium prausnitzii*. More in general, many taxa of the above-mentioned families are known for their ability to release SCFAs (Lopetuso *et al*., 2013; Delgado-Andrade *et al*., 2017; Louis and Flint, 2017; Maya-Lucas *et al*., 2019), including some of the species that we detected as markers of the microbiota shifts due to tannin supplementation, such as *R. bicirculans* and *L. edouardi* (Wegmann *et al*., 2014; Adamberg *et al*., 2018). Some of these bacteria have been also recognised to exert an anti-inflammatory effect. This is the case of the Lachnospiraceae NK4A136 group, which was detected to increase following polyphenol treatment in ethanol-treated mice, alleviating gut-derived LPS-mediated inflammation (Xia *et al*., 2021). Similarly, butyrate production by the Lachnospiraceae NK4A136 group was related to a reduction of intestinal inflammatory responses and an improvement of intestinal permeability in diet-induced obese mice (Ma *et al*., 2020a).

Together with Lachnospiraceae, Oscillospiraceae have been identified as markers of a healthy gut, since they are protein degraders, and they may take part in mucin breakdown. This process is the result of cooperation and cross-feeding among several species, which exhibit diverse metabolic capabilities (Raimondi *et al*., 2021). Moreover, a positive association has been reported between the Oscillospiraceae family and the amelioration of stool consistency, as well as a negative correlation with extraintestinal pain severity in irritable bowel syndrome patients (Hollister *et al*., 2020).

Regarding the role of Bacteroidota, it is still not clear whether an increase of the relative abundance of this phylum is associated to Western diet and obesity or to a lean phenotype and weight loss (Fabersani *et al*., 2021). Nevertheless, this uncertainty is likely due to the oversimplification of analysing phylum level changes (De Filippis *et al*., 2016). In our study, *Bacteroides spp.,* and *B. thetaiotaomicron* in

particular, tended to increase after the tannin supplementation. These bacteria have been described to metabolize a large variety of oligo- and polysaccharides from plants and to produce SCFAs, inducing satiety and regulating glucose metabolism. Moreover, *B. thetaiotaomicron* has also been shown to exhibit immunomodulatory properties, attenuating intestinal inflammation and reinforcing the intestinal barrier (Béchon *et al*., 2020). In fact, gut inflammation has been correlated with a reduction in the abundance of *B. thetaiotaomicron* and other *Bacteroides* species (Béchon *et al*., 2020), reinforcing the importance of a well-balanced gut microbiota composition for human health.

Apart from trends towards promoting beneficial bacteria, tannin supplementation also led to a reduction in potentially pathogenic taxa belonging to the phylum Actinobacteria. *Atopobium parvulum* has been identified as a marker and possible therapeutic target in paediatric inflammatory bowel disease patients, since it is also responsible of inducing inflammation (Muehlbauer *et al*., 2013). Similarly to *Atopobium, Actinomyces* is part of the resident gut microbiota. However, its increment has been associated with obesity in adolescents and with colorectal cancer (Peters *et al*., 2016; Chierico *et al*., 2018). Finally, the Firmicutes species *Solobacterium moorei*, an H_2S producer that has been associated with halitosis (Haraszthy *et al*., 2008), also decreased with the intervention.

4.8.3 Tannins increased colonic SCFA production

The three main SCFAs produced in the human intestine by gut microbial fermentation, i.e. acetic acid, propionic acid and butyric acid, were quantified. According to the repeated measures ANOVA, all three SCFAs showed an increasing trend over the intervention period, but the original relative proportions among the different SCFAs present in the basal level T0 were maintained. In all cases, we found that SCFAs were released in the following order of descending abundance: acetate>propionate>butyrate (**Figure 4.8.6**).

Specifically, the Bonferroni *post hoc* test evidenced that in the first two weeks the increment in the production of the different SCFAs was not statistically significant. However, the comparisons between T0 and T28d showed a statistically significant increase for acetate, propionate and butyrate ($p=0.001$, $p=0.001$, $p=0.002$, respectively). Acetate and butyrate presented also a statistically significant difference between T14d and T28d ($p=0.001$, $p<0.001$, respectively).

The search for evidence of SCFA production is of great relevance because these compounds exert several health-promoting functions, such as contributing to modulate cellular metabolism and immune responses (Koh *et al*., 2016a). Acting as prebiotic fibres, tannins have a booster effect on SCFA release (Kawabata *et al*., 2019). In particular, both hydrolysable and condensed tannins have been proved *in*

vitro to stimulate the production of these microbial metabolites by promoting the growth of beneficial bacteria and their metabolic functions (Molino *et al*., 2018, 2021). In this study, the same phenomenon could be confirmed *in vivo*, recording a significant increase of acetate, propionate and butyrate, due to the tannin supplementation. Remarkably, SCFA production correlated with some of the taxa that increased after tannin supplementation, suggesting which taxa are actually induced by tannins to increase the production of these molecules.

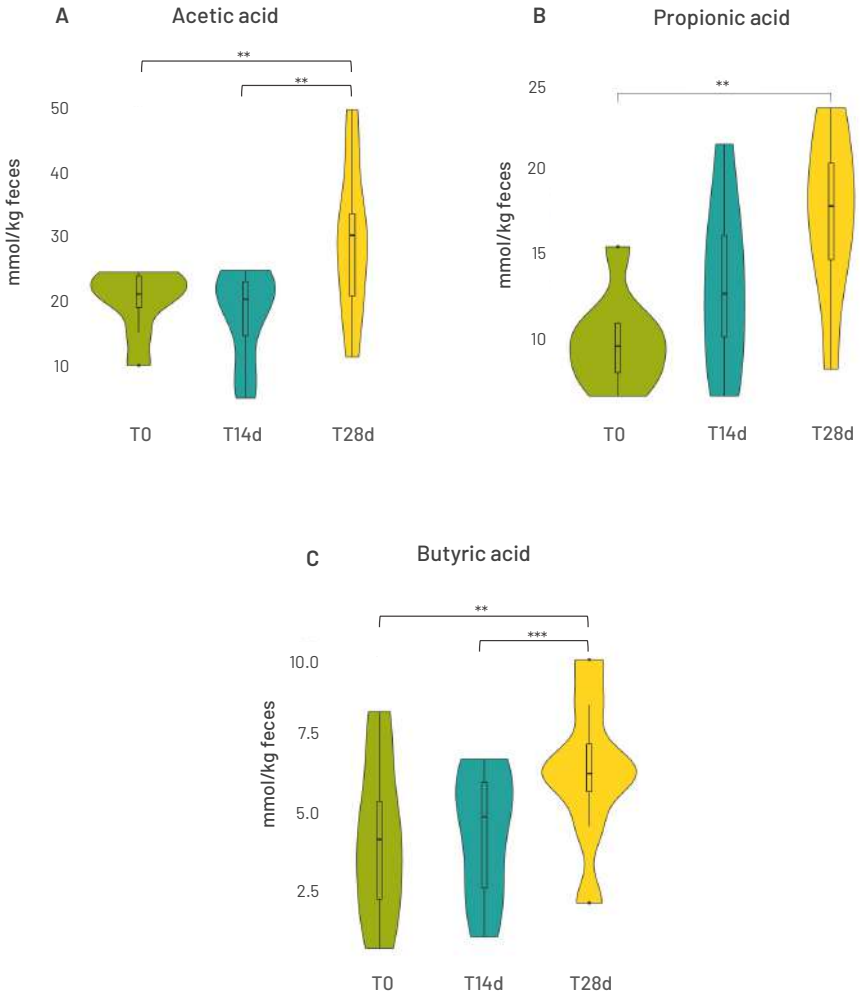


Figure 4.8.6 Violin plot of the short chain fatty acids (SCFAs) released during the tannin intervention, at different timepoints: T0, T14d and T28d. *Indicates statistically significant differences by repeated measures ANOVA and Bonferroni post-hoc test: **p < 0.01, ***p < 0.001.

4.8.4 Correlations between SCFA production and bacterial abundance

The mixOmics network function for sPLS regression was used to explore the potential correlation between the gut microbiota shifts and SCFA production (**Figure 4.8.7**). *Alistipes ihumii* and *Phascolarcobacterium faecium* were detected to negatively correlate with propionic acid and acetic acid, respectively (correlation coefficients of -0.666 and -0.614). Lachnospiraceae GAM79, belonging to the Lachnospiraceae NK4A136 group, positively correlated with acetic acid (0.600), while *L. edouardi* and *B. uniformis* positively correlated (0.648 and 0.653, respectively) with butyric acid. Finally, the increments of *R. bicirculans* and *B. thetaiotaomicron* were associated to an augmented release of both acetate (0.6483 and 0.683, respectively) and butyrate (0.765 and 0.651, respectively).

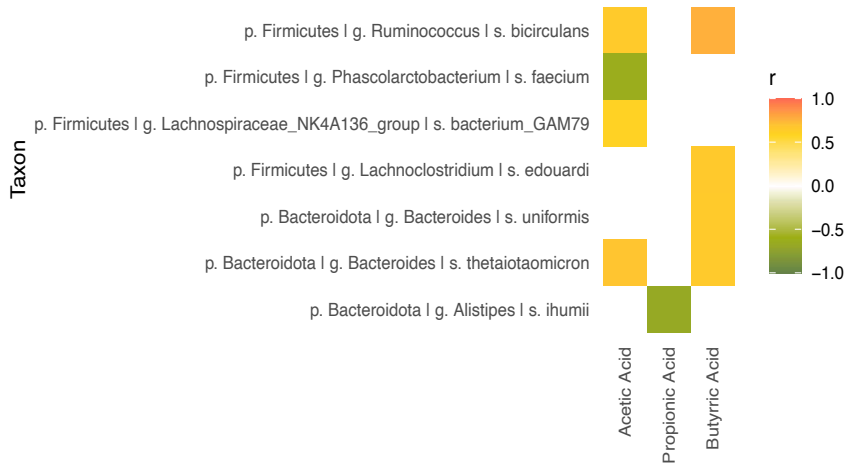


Figure 4.8.7 Heatmap of the correlation between microbial relative abundance and SCFA production, calculated with the sPLS-based approach implemented in the mixOmics network function (correlation coefficient > 0.6).

R. bicirculans and *B. thetaiotaomicron* were related to an increase of both acetate and butyrate. It has been described that these bacteria are acetic acid producers (Wegmann *et al*., 2014; Porter *et al*., 2018), but so far there is no evidence of butyrate production in these species. However, the production of butyric acid is likely to proceed mainly from acetate in the human gut, through the CoA-transferase route. In this process butyrate is formed by transfer of the CoA-moiety from acetic acid thanks to butyryl-CoA : acetate CoA-transferase (Louis and Flint, 2009).

Recently, *B. thetaiotaomicron* has also been investigated for its interaction in coculture with *Phascolarcobacterium faecium* (Ikeyama *et al*., 2020). Even though *P. faecium* abundantly colonizes the human gut, its functional role is still to be determined. This taxon is unlikely to use carbohydrates for its development and rather uses succinate as a substrate, which is abundantly produced by *B. thetaiotaomicron*. Succinate is a known intermediate of SCFA production in the intestine and the utilization of succinate by *P. faecium* in coculture with *B. thetaiotaomicron* resulted in the production of propionate. However, in our analyses *P. faecium* rather correlated negatively with acetate.

In the case of *B. uniformis*, a positive correlation was detected only with butyrate, which has also been recently demonstrated by Fabersani *et al.* (2021). Moreover, these authors proposed a direct effect on the increase of TRL5 expression. The activation of TLR5 contributed to curbing the spread of the inflammatory cascade from the intestine towards peripheral tissues in obese mice (Fabersani *et al*., 2021).

On the other hand, we found that the Lachnospiraceae bacterium GAM79 positively correlated with the release of acetic acid, although the Lachnospiraceae NK4A136 group, to which GAM79 belongs, has previously been correlated with the production of butyric acid and with anti-inflammatory effects (Ma *et al*., 2020; Xia *et al*., 2021). To our knowledge no specific effects on human health have yet been described for Lachnospiraceae bacterium GAM79.

Remarkably, the suppression of *Alistipes ihumii* by tannin supplementation was inversely correlated with the release of propionic acid. *Alistipes* is a recently described genus and its species have been mostly isolated from patients suffering from some intestinal-related pathologies. It is not yet clear whether this taxon has a leading role rather than just a bystander or co-inducer role in the observed clinical phenotypes. What is quite certain is that *Alistipes* abundance is closely related to gut dysbiosis (Parker *et al*., 2020).

General discussion

General discussion

Tannins are phytocomplexes largely applied in different sectors, thanks to their multiple properties. Given the great success in the animal field for their various beneficial health effects, in recent years the attention has shifted towards application in human beings. However, regarding the use of tannins as a dietary supplement, various aspects still need to be investigated in depth. In view of that, the present PhD thesis started with a pilot study exploring two types of tannins (condensed and hydrolysable) to understand if they could exert also in humans the effects previously described in animals. Therefore, a first approach to the study of the metabolization of quebracho (QUE) and chestnut (CHE) tannin extracts was carried out, in **Chapter 1**.

Through *in vitro* gastrointestinal digestion and fermentation, it was possible to investigate the production of SCFAs, the antioxidant capacity and the evolution of the polyphenols profile. The global antioxidant response obtained from two assays indicated a higher reducing capacity of CHE, while QUE showed higher antiradical activity. Tannins have been proven to be important substrates for microbial fermentation and production of SCFAs. In particular, the supernatant derived from the fermentation of CHE had the highest concentration of SCFAs. The UPLC-MS analysis represents the first tentative identification and a semi-quantitative analysis of the polyphenolic content of QUE and CHE, allowing a preliminary investigation of their metabolization during digestion and colonic fermentation. This study showed that the two types of extracts interacted differently with the human gut microbiota, giving different responses in terms of bioactivity and metabolite production. Therefore, a more in-depth study of different tannin extracts from a variety of sources and obtained by several methods of extraction was considered necessary.

Eleven tannin extracts were investigated in **Chapter 2**. In particular, four condensed tannin extracts from quebracho wood (Q1, Q2, Q3 and Q4), four ellagitannin extracts from chestnut wood and bark (C1, C2, C3, C4), and three gallotannin extracts obtained from tara pods (T1) and gallnuts (G1 ad G2) were examined. The HPLC analysis showed that the samples can be grouped according to their chemical composition due to a prevalent presence of condensed tannins, ellagitannins and gallotannins, in the samples extracted from quebracho wood,

chestnut wood and bark, and finally tara pods and gallnuts, respectively. The extraction method had a particular influence on the chemical composition of the original extracts, and resulted in the generation of small changes in the chromatographic profile. These differences were further evidenced in the chromatograms obtained from the analysis of samples subjected to *in vitro* digestion and fermentation. Interestingly, samples extracted with a mild treatment resulted in a higher production of metabolites. Due to the peculiar chemical composition of the extracts from tara and gall, it was not possible to obtain chromatograms with well separated peaks using the same method as for the other samples.

The analysis of the bioactivity of the samples again allowed to group the samples according to their chemical origin, and the extraction method played an important role in the antioxidant capacity and the production of short-chain fatty acids. All the extracts studied proved to be potential functional ingredients for food application due to both the elevated antioxidant activity exerted after digestion-fermentation, and the interaction with gut microbiota leading to a high release of SCFAs. The only sample with a low bioactivity was C4, which is obtained from the sedimentation of residues after the extraction of chestnut tannins. In itself, this sample has a lower tannin content as most extracted tannins remain in the aqueous phase.

Confirming the results obtained in chapter 1, the fermentation process of the samples led to a reduction in the content of polyphenols and antioxidant activity. However, the overall effects given by each formulation must also be taken into account when considering the bioactivity of a compound. In fact, the interaction of the compounds generated after digestion with the gut microbiota determined a large release of SCFAs, confirming the results obtained in the previous chapter. The samples that showed the highest bioactivity in terms of antioxidant activity and SCFA production were the extracts derived from tara and gall. This study lays the groundwork for choosing among all the available extracts the ones which are best suited for further in-depth analysis.

The next step of this thesis was to investigate the ideal amount of tannin extracts to achieve the greatest beneficial effect. Thus, **Chapter 3** was dedicated to identifying the best concentration of tannin extracts, depending also on their different chemical composition, to create dietary supplements beneficial for both the human organism and the gut microbiota. In view of this, four different tannin extracts, representing four groups of tannins (Q2, C2, T1 and G2) were selected and submitted to *in vitro* digestion and fermentation in decreasing concentration (A 1.5 g/12 ml, B 0.6 g/12 ml, C 0.1 g/12 ml and D 0.03 g/12 ml).

The analysis of the antioxidant capacity with different techniques revealed that independently of the concentration submitted to digestion, samples belonging to the same extract group still exerted the same effect. Once more, data confirmed the results obtained in the previous chapter, with T1 being the most antioxidant,

followed by G2, C2 and finally Q2. Thus, also in this case it emerged that the chemical composition of the different extracts plays a key role in their bioactive properties.

The same situation did not occur after fermentation, in which a progressive decrease of the amount of fermented extracts lead to a decrement of their antioxidant performance. This result led to the hypothesis that excessive amounts of tannins may lead to partial inhibition of the microbiota, and thus to the inability of the extracts to be fermented. Conversely, low concentrations of the products do not hamper the functionality of the microbiota, which can effectively metabolise them into smaller compounds that probably do not have the same antioxidant capacity as their parent compounds.

SCFA analysis was an important tool to understand how different chemical compositions of tannin extracts and their distinct concentrations can have a strong influence on the composition and function of the microbiota. Moreover, if the fermentation of the highest concentrations of tannin extracts induced a great release of SCFAs, lower concentrations led to a better-balanced proportion among the different SCFAs and in some cases also to an increment of their production. The results of **Chapter 3** indicated that lower amounts of these powerful bioactive compounds could establish a more profitable interaction with the gut microbiota, resulting in greater positive effects for both the host and the gut microbial environment.

Once the most effective extracts (chapter 2) and the ideal proportions (chapter 3) to be used as bioactive components in foods were identified, **Chapter 4** was dedicated to study the interaction of tannin extracts with complex food matrices. The enrichment of foods with different composition (dairy products, cereal-based foods and meat) with tannin extracts obtained from quebracho, chestnut and tara evidenced the potential to influence host physiology through the modulation of the composition and the functionality of symbiotic bacteria in the gut. In chapter 2, it was shown that a great production of SCFAs follows the *in vitro* digestion and fermentation of quebracho and chestnut tannins. Now, the combination of tannin extracts with the food matrices was responsible for a further increment of total SCFA production. This shift to a healthier environment in the colonic ecosystem plays a key role in the digestion and absorption of nutrients, but also in protecting the gastrointestinal system against pathogens.

These preliminary results highlighted that tannin extracts, produced in a sustainable way, could be a new cheap promising natural supplement to be exploited for their prebiotic effect, acting by boosting the production of SCFAs. Depending on the matrix to which they are added, the new products could determine a different modulation of the composition of the gut microbiota. In particular, quebracho extract generated the most homogeneous response across foods, whereas the effects of

chestnut and tara extracts were more highly dependent on the food matrix. These advantageous effects make tannins likely candidates for a potential dairy food supplement.

Even though tannins resulted to be appealing bioactive ingredients for designing functional foods, the biggest challenge related to their direct incorporation into food is their unpleasant bitter and astringent taste. Furthermore, the great ability of these bioactive ingredients to bind proteins means that the addition of excessive quantities can lead to detrimental changes in food structure. A potential strategy for introducing tannins into food is represented by microencapsulation, which could mask the off-flavours and avoid contacts with food. Thus, **Chapters 5** and **6** were designed to develop innovative microencapsulation systems, through extrusion.

In **Chapter 5** amidated pectin was proposed as an alternative encapsulation matrix to the most widely used biopolymer alginate, for the microencapsulation of tannin extracts through external gelation. In general, pectin-based microbeads were found to exhibit better performance than their alginate-based counterparts, which was attributed to the greater extent of interactions between the bioactive compounds and this amidated polysaccharide.

Most of the tannin content was lost during the encapsulation process for the alginate-based microbeads, as expected for water-soluble compounds with a relatively low molecular weight. However, the interactions between tannins and pectin limited the loss of bioactive compounds from this matrix, resulting in a significant increase in encapsulation efficiency, from 2-fold to almost 6-fold depending on the tannin extract and the method of analysis used. Being less viscous, pectin also allowed to incorporate a greater amount of tannins in the feed formulations, which, combined with the better encapsulation efficiency, resulted in a greater tannin load in the final microbeads. Pectin microbeads loaded with 10% tannin extract (w/w with respect to pectin) yielded the best encapsulation efficiency, although the final tannin content could be further increased by adding a 20% (w/w) concentration of the extracts.

As the microbeads are intended for food use, the release of the tannins during storage was assessed over a period of two weeks. The results revealed that only a slight loss of tannins occurred during this period for the pectin-based microbeads, suggesting that the proposed encapsulation system would be a better alternative to alginate for incorporating phenolic compounds into fresh foods with a high water content.

Not only did the amidated pectin achieve greater microencapsulation efficiencies and loading capacities for both tannin extracts used in this work, and a more sustained release of the phenolic compounds, but incorporating tannins within the pectin matrices also proved to enhance the structural properties of the microbeads

themselves, achieving improved morphologies, smaller sizes and reduced extent of swelling.

These promising results laid the basis for exploring strategies to further improve the microencapsulation efficiency, in **Chapter 6**. To the amidated pectin microbeads previously studied, an additional coating was added by including gelatine in the gelling. This approach proved to be a good technique to improve the morphology of pectin microbeads. Moreover, this 1-step approach for microbead formation and coating considerably improved the microencapsulation efficiency of the systems due to the interaction of the tannins with gelatine, which also resulted in greater amounts of the protein attaching to the microbeads.

The release of tannins from the fresh microbeads over a 14-days period was low, ensuring a minimum loss of bioactive compounds during storage in all the systems evaluated. Although the gelatine coating did not significantly contribute to preventing tannin release, it was a successful approach to achieve a higher tannin loading in the microbeads. These encouraging results suggest that the microencapsulation systems developed in the last two chapters, in particular those including gelatine-coating, could be applied to embed tannins into fresh foods with a high water content.

Basic features required from microbeads are to be resistant to food processing and minimise the release of bioactive compounds into the food matrix. On the other hand, these must be able to liberate their encapsulated ingredients through digestion, allowing them to be bioaccessible in the gastrointestinal tract and thus exert their beneficial effects. In order to reach a global overview for food applications, **Chapter 7** investigated the structural changes that the encapsulation systems developed in chapter 5 undergo during human gastrointestinal digestion, and the consequent release of the bioactive compounds. Amidated pectin microbeads were submitted to *in vitro* gastrointestinal digestion, in comparison to tannin extracts as such. Surprisingly, the complex structure formed between pectin and tannins during the microbeads gelation process meant that there was not a sufficient release of tannins after *in vitro* digestion and fermentation.

Pectin, a well-known fibre, induced a modulation of the microbiota distinct from that of tannins, promoting the growth of different taxa. Given the prebiotic activity of both types of compounds (pectin and tannins), a similar high production of SCFAs was recorded. However, it must be emphasized that for this study the amount of tannins analysed as such was about 22-times less than that of pectin, as it was referred to the tannin content present in the microbeads. These results showed that the binding pectin-tannins is so strong that although microbeads are efficiently generated, they cannot be used to deliver these bioactive compounds into the human body. Therefore, alternative encapsulation materials that do not interact as strongly with

tannins should be tested in order to meet the basic requirement of releasing the transported molecules.

On the other hand, this study showed that tannins, particularly those extracted from quebracho, interact differently with the intestinal microbiota depending on whether they undergo prior digestion or not. The antioxidant activity exerted and the SCFAs produced are greater in tannins submitted to both *in vitro* digestion and fermentation. From this chapter, therefore, it emerged that it would be preferable to take tannins directly. In the case of trying to mask the flavour through microencapsulation, it would be necessary to find a solution that releases tannins directly into the stomach to maximize their bioactive effect.

The gut microbiota is the most mouldable and adaptable component of the human ecosystem and diet is one of the most powerful instruments to drive it. Indeed, a balanced diet, reflected in gut eubiosis, plays a fundamental role in preserving human well-being. However, current bad dietary habits increase the risk for inflammation and various chronic pathological conditions. From the results obtained from the previous chapters, it emerged that introducing tannin supplements into the diet may help to re-establish a balance in the microbiota. Thus, **Chapter 8** was dedicated to carrying out a small nutritional intervention, by supplementing eight healthy volunteers with microbeads containing a tannin blend (including chestnut and gallnut tannins), in order to assess whether the supplementation could drive healthy modifications in gut microbiota composition and functionality, over a 4-week period.

Consistent with the hypothesis, the obtained results suggested that tannin supplementation could induce beneficial shifts in the gut microbiota. In a short period of 28 days, a very large change in the microbiota was not expected, as the supplemented subjects were healthy and lean. The goal was rather to try to influence the environment in the colonic ecosystem in a beneficial direction, conducive to limiting the collateral damage from the Western diet, such as the onset of low-grade chronic inflammation. In particular, it was found that the tannin blend increased alpha diversity and induced beneficial shifts in the microbiota, partially reducing potential pathogenic taxa, correlated with IBD or obesity and colorectal cancer. On the other hand, the tannin mixture boosted the growth of beneficial bacteria, which can attenuate the adverse impact of inflammation through immune regulatory effects. The most interesting result was that some of the taxa, such as *Ruminococcus bicirculans*, *Bacteroides thetaiotaomicron* and *Bacteroides uniformis*, that characterised the shift to a healthier status were positively correlated with increased production of SCFAs, which are crucial in maintaining the well-being of the host. The present study may have the limitation that there was no control group, but it provides important preliminary insights for future larger studies.

Conclusions

General conclusions

Tannins proved to be promising bioactive components to be exploited both to functionalize foods and to create new dietary supplements. The results of this thesis have shown that tannins administered in appropriate doses can exert a strong prebiotic activity as well as a high antioxidant capacity, thus being beneficial to the human organism at the intestinal level. Through the modulation of the gut microbiota and the production of metabolites, tannins could also determine effects at the systemic level.

The administration of these phytocomplexes can take place in different ways (within food matrices, microencapsulated or in the form of microbeads) but always taking into account that their positive effects are maximized when they face the entire process of digestion and fermentation.

Specific conclusions

- Conclusion **1**

Chestnut and quebracho tannins are important substrates for microbial fermentation with a great production of direct and indirect metabolites.

- Conclusion **2**

The source, chemical composition and extraction method have a crucial impact on the bioactivity of different tannin extracts. Extractions with mild treatment determine the highest production of metabolites, after *in vitro* digestion and fermentation. Although all the extracts studied proved to be potential functional ingredients, tara and gall tannin extracts present the highest bioactivity in terms of antioxidant capacity and short chain fatty acids (SCFAs) production.

- Conclusion **3**

Different chemical compositions and concentrations of tannins lead to different interactions with the microbiota in terms of composition and functionality. In particular, lower tannin amounts provide a more profitable interaction with the gut microbiota, resulting in greater positive effects for both the host and the gut microbial environment.

- Conclusion **4**

The enrichment of food with tannin extracts determines a shift to a healthier environment in the colonic ecosystem, with the potential of playing a key role in the digestion and absorption of nutrients, but also in protecting the gastrointestinal system against pathogens. Moreover, the combination results in a booster effect on the production of SCFAs, without altering the profile given by the foods alone.

- Conclusion **5**

Microencapsulation of tannins with amidated pectin results in a good microencapsulation efficiency and a low release over time. The strong interaction between tannins and pectin also improves the morphology of the microbeads, in terms of shape, size and swelling.

- Conclusion **6**

The pectin-tannin microbead system could be improved by the addition of a one-step gelatine-coating. The interaction of tannins with gelatine results in greater amounts of the protein attaching to the microbeads, with a further enhancement of the morphology and microencapsulation efficiency.

- Conclusion **7**

Pectin microbeads are not able to release their tannin content, keeping tannins trapped after the digestive process. Going through both digestion and fermentation steps is a fundamental requirement in order to maximize tannin bioactive effects, especially with regard to condensed tannins.

- Conclusion **8**

Tannin supplementation of healthy people during a 4-weeks period can help to maintain a healthy eubiotic state, through positive changes in the composition and functionality of the microbiota starting from the first two weeks onwards.

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