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VALORIZATION OF GRAPE BY- PRODUCTS THROUGH EXTRACTION AND ENCAPSULATION OF BIOACTIVE COMPOUNDS

Thesis submitted to *Universidade Católica Portuguesa* to attain the degree of PhD in Biotechnology, with specialization in Food Science and Engineering

Joana Ribeiro da Costa

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Under the supervision of Professor Maria Manuela Estevez Pintado and the co-supervision of Professors Lourdes Maria Correa Cabral and Lorenzo Miguel Pastrana-Castro

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This work was funded by People Marie Curie Actions through FP7 project BiValBi – Biotechnologies to Valorise the regional food Biodiversity in Latin America (PIRSES-GA-2013-611493) and by FCT – Fundação para a Ciência e Tecnologia through project UID/Multi/50016/2019. This work was also financially supported through MultiBiorefinery (POCI-01-0145-FEDER-016403) and MicroDigest (agreement 037716) projects, co-founded by FCT and FEDER – Fundo Europeu de Desenvolvimento Regional through COMPETE 2020 and Portugal 2020.

RESUMO

Atualmente existe uma tendência para a reutilização de subprodutos da indústria alimentar, com foco na extração de compostos bioativos e aplicação no desenvolvimento de novos ingredientes para nutrição humana e animal, cosméticos e nutracêuticos. A indústria vitivinícola não é exceção, pois gera anualmente até 7 milhões de toneladas de biomassa, desperdiçada na forma de bagaço de uva, descrito como sendo rico em compostos de alto valor, tais como polissacáridos neutros, proteínas estruturais e compostos fenólicos. O objetivo deste trabalho foi a valorização de um subproduto representativo da indústria vitivinícola, o bagaço de uva, através da produção de um extrato com elevado valor agregado para a indústria alimentar, a sua caracterização composicional e bioactiva, melhoria da biodisponibilidade através da libertação controlada no intestino e prova de conceito da sua aplicação numa bebida funcional.

A otimização da produção do extrato de bagaço de uva (EBU) foi realizada com base na extração de xilo-oligossacáridos (XOS), utilizando métodos convencionais (extrações ácida e alcalina) ou enzimáticos. Os métodos convencionais recuperaram entre 21.8 a 74.6% e entre 5.2 a 96.3% do total de XOS, para o tratamento ácido e alcalino, respetivamente. Os métodos enzimáticos permitiram extrair até 88.7 ± 0.12% do total de XOS. Tendo em conta o rendimento de extração, a qualidade do extrato e o fato de tratar-se de um processo verde, selecionou-se o extrato produzido enzimaticamente com xilanases de *A. niger* (EBU) para a continuação deste estudo.

O EBU foi caracterizado em termos da sua composição química e bioatividades, e submetido à simulação da digestão gastrointestinal para compreender como essas propriedades são afetadas pelo trato gastrointestinal. O EBU apresentou elevado teor de fibra (26.1 ± 1.59 $g.100g^{-1}$) e outros hidratos de carbono, incluindo XOS, minerais e polifenóis. O uso de 2% (m/v) de EBU provou ser uma potencial fonte de carbono, podendo ser fermentada por diferentes espécies de probióticos, mesmo após a digestão, confirmando o seu potencial prébiótico. O EBU também apresentou elevada atividade antioxidante e antimicrobiana contra diferentes microrganismos patogénicos. A simulação *in vitro* da digestão permitiu concluir que os XOS foram resistentes às condições gástricas, ao contrário dos compostos fenólicos, que foram determinantes para a sua atividade antioxidante e antimicrobiana.

Dessa forma, a fim de melhorar a sua biodisponibilidade, o EBU foi encapsulado em micropartículas (MPs) de quitosano ou alginato. O impacto da digestão gastrointestinal simulada sobre as propriedades biológicas e acessibilidade dos compostos core foi avaliado, bem como a permeabilidade de fenólicos e XOS através da co-cultura de células Caco-2/ HT29-MTX. Além disso, o quitosano foi modificado com uma sonda fluorescente para estudos de *uptake* celular. As MPs de alginato apresentaram uma dimensão de 523 nm, polidispersão de 0.112, potencial zeta de 15.0 mV e eficiência de associação de polifenóis de 68%. As MPs de quitosano apresentaram dimensão de 853 nm, polidispersão de 0.358, potencial zeta de 14.9 mV e eficiência de associação de polifenóis de 65%. Ambos os sistemas permitiram a libertação do EBU no intestino, aumentando a biodisponibilidade dos compostos fenólicos, bem como as atividades antioxidante e antimicrobiana. A permeabilidade das células intestinais dos XOS diminuiu de 45% para 7.9 e 15.7%, após a encapsulação em MPs de alginato ou quitosano, respetivamente. A modificação de MPs de quitosano com Cyanine 5.5 para ensaios de *uptake* celular não afetou a biocompatibilidade das MPs com EBU em relação às células intestinais e a análise de microscopia confocal confirmou a integridade das *tight junctions* após a internalização das MPs.

Como prova de conceito, foi desenvolvida uma bebida funcional à base de água de côco com o EBU encapsulado. A estabilidade química foi avaliada em duas condições de armazenamento, liofilizado à temperatura ambiente ou em estado líquido a 4 ºC, ao longo de 60 dias. O armazenamento à temperatura ambiente acelerou a taxa de degradação de fenólicos e antocianinas, comparando com o armazenamento a 4 ºC. Os potenciais antimicrobiano e prebiótico, após digestão da bebida, também foram testados, verificando-se uma redução no crescimento dos diferentes patogénicos e promoção no desenvolvimento das bifidobacterias e lactobacilos. A análise sensorial permitiu concluir que a incorporação das MPs não promoveu diferenças significativas na maioria dos atributos avaliados, relativamente à água de côco.

Os resultados deste trabalho poderão contribuir para a sustentabilidade da indústria vitivinícola num contexto de economia circular, através do desenvolvimento de ingredientes de alto valor agregado com impacto positivo na saúde humana.

Palavras-chave: bagaço de uva, enzimas, biodisponibilidade, encapsulação

ABSTRACT

There is a current trend for reutilization of food industry by-products, focused on the extraction of bioactive compounds and application in the development of new feed, food, cosmetic or nutraceutical ingredients. The wine industry is no exception and generates up to 7 million tons of wasted biomass in the form of grape bagasse, which is reported to be rich in high added value compounds such as neutral polysaccharides, structural proteins and phenolic compounds. This project aimed to valorize a major wine production by-product, grape pomace, through the production and characterization of a high valued extract for food industry, improvement of intestinal bioavailability through a gastrointestinal delivery system, and application in the development of a functional beverage as a proof-of-concept.

Optimization of grape pomace extract (GPE) was performed through the optimization of xylooligosaccharides (XOS) production, using conventional (acid and alkaline extraction) or enzymatic processes. Conventional methods recovered 21.8 to 74.6% and 5.2 to 96.3% of total XOS, for acid and alkaline processes, respectively. Enzymatic process extracted up to 88.7 ± 0.12% of total. Taking into account the extraction yield, the quality of the extract and the fact of being considered a green process, the enzymatically produced extract with *A. niger* xylanases (GPE) was selected to continue this study.

GPE was characterized for its chemical composition and biological activities, and submitted to the simulation of gastrointestinal digestion to understand how these properties are affected by the gastrointestinal environment. The extract presented high content of dietary soluble fiber (26.1 ± 1.59 $g.100g^{-1}$) and other carbohydrates, including XOS, minerals and phenolics. *In vitro* simulated digestion allowed to conclude that XOS were resistant to gastric conditions but phenolics were not. The use of 2% (w/v) of GPE proved to be a potential carbon source that could be fermented by different probiotics, even after digestion. GPE also exhibited strong antioxidant and antimicrobial activity against different pathogens, however, after digestion, these bioactivities were strongly reduced. These results allow to conclude that GPE had low intestinal bioavailability due to action of digestion enzymes and acidic pH.

Aiming at improving its bioavailabiliy, GPE was then encapsulated into chitosan or alginate microparticles (MPs) , in order to improve its bioavailability. MPs were characterized for

their size, polydispersity, zeta potential and total phenolics (TPC) association efficiency, and the simulation of gastrointestinal digestion was performed to evaluate the release profile of polyphenols, the antioxidant and antimicrobial activities, and permeability of phenolics and XOS across Caco-2/ HT29-MTX cell layer. Also, chitosan was modified with a fluorescent probe for cellular uptake studies. GPE-loaded alginate MPs presented size of 523 nm, polydispersity of 0.112, zeta potential of -15.0 mV and 68% of association efficiency of polyhenols. GPE-loaded chitosan MPs presented size of 853 nm, polydispersity of 0.358, zeta potential of 14.9 mV and 65% of association efficiency of polyphenols. Both systems allowed the delivery of GPE in the intestine, increasing the bioavailability of different polyphenols, the antioxidant and antimicrobial activities. Permeability of XOS across the intestinal cell layer decreased from 45% to 7.9 and 15.7%, after encapsulation in alginate or chitosan MPs, respectively. Modification of chitosan MPs with Cyanine5.5 for cellular uptake studies did not affect the biocompatibility with intestinal cells, and confocal microscopy analysis confirmed the integrity of these cells tight junctions.

As a proof of concept, it was developed a coconut beverage through the incorporation 2.5% (w/v) of the encapsulated GPE. Physicochemical stability under two storage conditions (freeze-dried at room temperature or liquid at 4 ˚C) was evaluated along 60 days. The antimicrobial and prebiotic potential after the digestion of the beverage were assessed using different microbial strains. Room-temperature storage accelerated the rate of degradation of phenolics, comparing to storage at 4 ˚C. Alginate and chitosan functional beverages decreased the growth of different pathogens and promoted the growth of different probiotics. Sensory analysis allowed to conclude that the incorporation of particles did not promoted significant differences in most of evaluated attributes. The results from this work will contribute for the sustainability of wine industry in circular economy context, through the development of value-added ingredients with positive biological impact.

Keywords: grape pomace, enzymes, bioavailability, encapsulation

ACKNOWLEDGMENTS

To Escola Superior de Biotecnologia da Universidade Católica Portuguesa, for accepting me as PhD student and for provide me the necessary conditions to develop this work.

To Ouro Verde Winery and Nuvi Fruits, for providing the grape pomace and the coconut water, indispensable to carry out the work.

This thesis is the outcome of an amazing and intense journey, which would never been possible to complete alone. Thereof, I would like to express my deep gratitude to the following people:

To Professor Manuela Pintado, thank you for the opportunity to grow as a better person and researcher, for your guidance, care and for trusting me. This venture was only possible for your standards of excellence, immense knowledge and creativity. To Lorenzo, Lourdes and Renata, thank you for the shared knowledge, advice and constant motivation, it was a pleasure working with you.

To Embrapa Agroindústria de Alimentos, for providing all the conditions, equipment and techniques, necessary to develop this thesis. I am very grateful to all the staff, particularly to my labmates, thank you for your kindness and receiving me so well, definitely improving this experience.

To my colleagues of 4th floor laboratory, who lightened this journey so much, thank you for all the laughs, joy and the sharing. To Tânia and Marta, for boarding with me on the silly "Veggy Flours" project that threw us out of the comfort zone called "lab". To "AmyrisValue" team, Sílvia, and Nelson for all the support, sharing and for being so kind and patient. It was a pleasure to work with all of you by my side.

To International Iberian Nanotechnology Laboratory, for providing the support necessary to carry out this work. To Miguel, Isabel and Catarina, for the collaboration and knowledge, smoothing the last steps of this work.

To my grandmother Marília, for all the love, sensitivity, kindness and laughs that you convey to those around you, and teaching me how to face the worst challenges with optimism, although I still have to go a long way to be up to you. Thank you for your endless smile!

To Hélder, for being by my side during this journey so full of ups and downs, calm my nerves and holding my hand through the all the challenges. Thank you for making me smile, you are my love, best friend and partner in life.

To my parents, for your unconditional support and guidance in every moment of my life. Thank you for continuously encourage me in every challenge, believe in me and for all you have done in shaping the person that I have become.

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ABBREVIATIONS

AAeq – Ascorbic acid equivalent

ABTS – 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)

AE – association efficiency

Alg - alginate

AX – arabinoxylan

BSA – bovine serum albumine

CFU – Colony-forming unit

CS – chitosan

Cy5.5 – cyanine 5.5

Cy5.5-CS – cyanine 5.5-modified chitosan

DAD – Diodo Array Detector

DAPI - 4', 6-diamidino-2-phenylindole, dilactate

DNS – 3,5 - dinitrosalysilic acid

DP – Degree of polymerization

DW – dry weight

FOS - Fructooligosaccharides

GAE – Gallic Acid Equivalent

GI - gastrointestinal

GID – gastrointestinal digestion

GP – grape pomace

GPE – grape pomace extract

GPE-Alg - grape pomace extract-loaded alginate particles

GPE-CS - grape pomace extract-loaded chitosan particles

DGPE – digested grape pomace extract

DP – degree of polymerization

HPLC - High Performance Liquid Chromatography

LMW – low molecular weight

MH – Mueller-Hinton

MIC – Minimal Inhibitory Concentration

MPs - Microparticles

MRS – De-Man, Rugosa and Sharp media

MRSA – methicillin-resistant *Staphylococcus aureus*

MSSA - methicillin-susceptible *Staphylococcus aureus*

MW – molecular weight

OD – Optical density

ORAC - Oxygen Radical Absorbance Capacity

PBS – phosphate buffered solution

PF – pomace flour

POS – pectin-dereived oligosaccharides

RID – Refractive Index Detector

RT – retention time

RTR – ready-to-reconstitute

S:L – solid to liquid mass ratio

SCFA – short chain fatty acid

SDF – soluble dietary fibre

TE – Trolox Equivalent

TEER – trans-epithelial electric resistance

TPC – Total phenolic compounds

TPP – Sodium tripolyphosphate

TRITC - tetramethylrhodamine B isothiocyanate

X_2 - xylobiose

X_3 - xylotriose

X_4 - xylotetraose

X_5 - xylopentaose

X_6 - xylohexaose

XOS – xylooligosaccharides

XTT - 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide sodium salt

SCOPE AND OUTLINE

This thesis is organized in four major parts, which are subdivided in chapters. The first part is composed of two chapters, Chapter 1 corresponding to a literature review on enzymatic processes applied to by-products valorization and Chapter 2 corresponding to the objectives of this thesis.

Parts II and III correspond to the experimental work. Part II contains chapters 3 and 4, focused on the optimization of xylooligosaccharides production (Chapter 3) and the chemical and bioactive characterization of the extracts (Chapters 3 and 4). Explicitly, Chapter 3 reports the optimization of xylooligosaccharides extraction from grape pomace using conventional and enzymatic methods, and Chapter 4 includes the simulation of gastrointestinal digestion of the enzymatic grape pomace extract, and how it affects the chemical characterization and the different biological properties, including the antioxidant capacity, antimicrobial activity and prebiotic potential.

Part III focus on the improvement of bioavalibility and is composed by chapters 5 and 6. Chapter 5 describes the development of polymeric microparticles to encapsulate the grape pomace extract and protect it along the gastrointestinal digestion. Chapter 6 describes the potential application of the encapsulated grape pomace extract into coconut water to produce a functional beverage, as proof of concept. Finally, part IV is composed by two chapters, corresponding to the general conclusions and future work.

The core of this thesis is composed of five papers published or submitted in international peer-reviewed journals, according to the following list:

Chapter 1

Costa, J.R., Tonon, R.V., Cabral, L.M.C., Pastrana, L., Pintado, M.E. Enzymatic and enzyme-assisted treatment of agricultural by-products: towards sustainable recovery of high-added value compounds. Submitted to *Sustainable Chemistry and Engineering.*

Chapter 3

Costa, J.R., Tonon, R.V., Gottschalk, L. M. F. , Santiago, M.C.P.A., Mellinger-Silva, C., Pastrana, L., Pintado, M.E., Cabral, L.M.C. 2018. Enzymatic production of xylooligosaccharides from Brazilian Syrah grape pomace flour: a green alternative to conventional methods for adding value to agricultural by-products. *Journal of the Science of Food and Agriculture* 99: 1250 - 1257.

Chapter 4

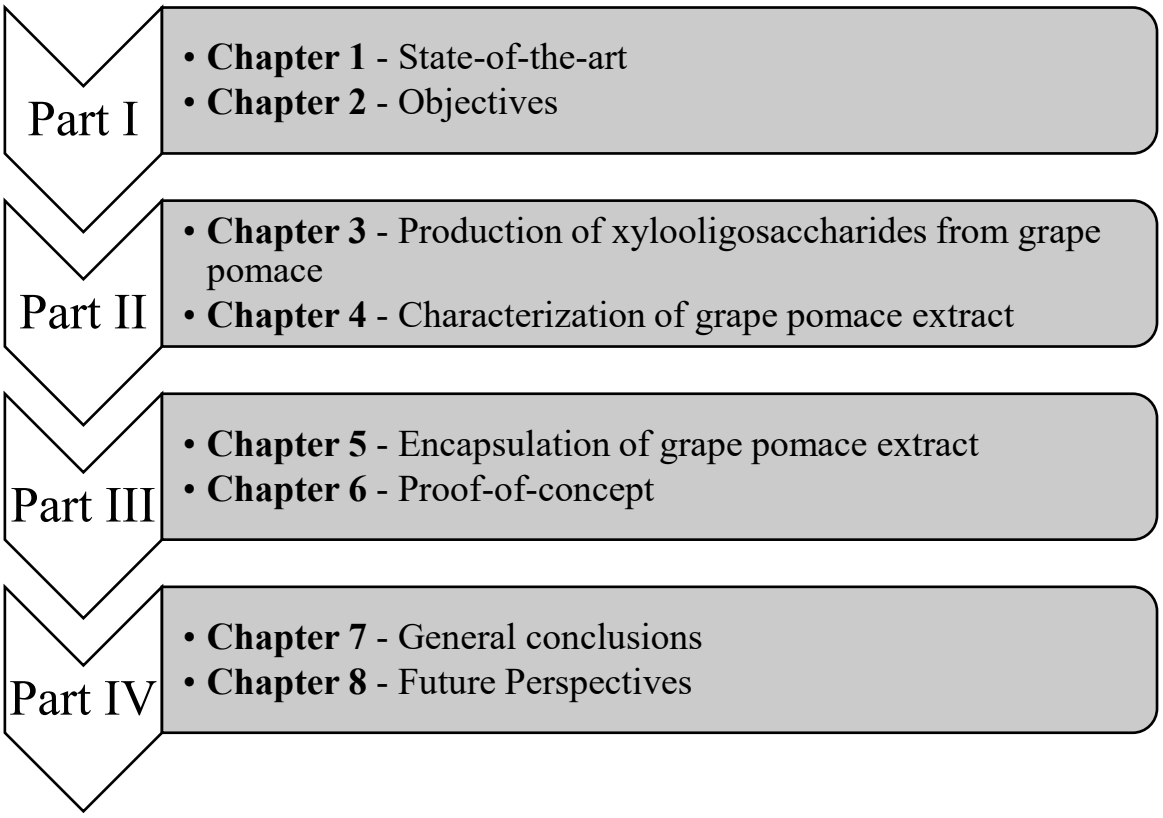
Costa, J.R., Amorim, M., Vilas-Boas, A., Tonon, R.V., Cabral, L.M.C., Pastrana, L., Pintado, M.E. 2019. Impact of in vitro gastrointestinal digestion on chemical composition, bioactive properties and cytotoxicity of a *Vitis vinifera* L. cv. *Syrah* grape pomace extract. *Food & Function* 10: 1856 – 1869.

Chapter 5

Costa, J.R.,, Xavier, M., Amado, I. R., Gonçalves, C., Castro, P. M., Tonon, R.V., Cabral, L.M.C., Pastrana, L., Pintado, M.E. Polymeric microparticles as oral delivery systems for a bioactive grape pomace extract: towards improvement of intestinal antimicrobial and antioxidant activities. Submitted to *Food Hydrocolloids*.

Chapter 6

Costa, J.R., Monteiro, M.J., Tonon, R.V., Cabral, L.M.C., Pastrana, L., Pintado, M.E. Development of a functional coconut beverage through incorporation of a grape by-product extract. Submitted to *Journal of Agricultural and Food Chemistry*.



Part I

Introduction

CHAPTER 1

Valorization of agricultural lignocellulosic plant by-products through enzymatic and enzyme-assisted extraction of high-added value compounds: a review

Abstract

Extraction of bioactive molecules have become a major trend in the valorization of food and agroindustrial by-products, allowing a sustainable production of novel ingredients with different industrial applications. Enzymatic and enzyme-assisted extractions are selective and eco-friendly methods that could be used to extract value added compounds from lignocellulosic plant byproducts, namely polysaccharides and polyphenols, with interesting biological properties. Nevertheless, a depth understand of their enzymatic hydrolytic properties and the interaction with the plant cell material is necessary to obtain better extracts with high yields. Therefore, this review is focused on enzymatic treatment of food lignocellulosic by-product in order to recover bioactive compounds such as polysaccharides and polyphenols.

1.1. Introduction

For many years, the waste from agro-food industries ended up as landfill, incineration or fertilizers, causing several environmental issues. Later, the use of vegetable by-products

in the production of animal feed appeared as a more sustainable solution, as it allowed the valorization of these wastes at the same time it reduced the environmental impact associated with animal feed production (Martin *et al*., 2016). Nevertheless, these solutions are predisposed to income losses for their valuable components, so the valorization of agroindustrial by-products throughout the recovery of ingredients with value for human nutrition, nutraceuticals and cosmetics rises as a novel solution, even though the inherent research and economic challenges (Rabetafika *et al*., 2014; Naziri *et al*., 2014).

Lignocellulosic agroindustrial by-products are considered promising renewable resources, as they are plentifully available around the world (Liguori and Faraco, 2016). Moreover, they are usually rich in compounds with interest for different industries, such as food or cosmetics, including digestible and indigestible fibers, polyphenols, proteins and amino acids, minerals and lipids. Most abundant agroindustrial by-products include ripped fruits and vegetables, pomaces, stems, peels and seeds (eg: from grapes, apples, oranges, tomatos, carrots, sugarbeet, coffee…), leaves, cereal bran and grains (eg: rice, oat, wheat), and spent yeast from fermentation processes, such as beer production.

The quality of the extracts produced from vegetable lignocellulosic by-products is directly dependent on the quality of raw biomass (composition, harvest, storage, microbiological contaminations, among others) but also on the applied extraction technique. Conventional extraction methods usually include a solid-liquid step, using aqueous or organic solvents, with or without heat application, although these methods usually require long extraction time, high quantities of expensive and toxic solvents, evaporation of the same solvents for extract concentration, and low compounds selectivity (Azmir *et al*., 2013). Linked to these disadvantages, the presence of different polysaccharides inside plants cell walls decreases the conventional extraction yields even

more, as they hinder the solvent penetration into the plant material, thus decreasing the solubility of entrapped compounds of interest, such as polyphenols and polysaccharides or even proteins (Nadar *et al*., 2018).

As alternatives, new extraction methods upraised to increase the cost/ benefit of this type of processes, in which are included the enzymatic and enzyme-assisted extractions, as well as the quality of the extracts. These techniques are eco-friendly, more cost-effective and highly specific for a wide-range of biomolecules, particularly to lignocellulosic plant materials. As enzymes have high capacity to destroy the structural components of plant cell walls, depending on their catalytic properties, and increase the membrane permeability, enzymes allow an enhanced extraction of the bioactive compounds from the cell (Puri *et al*., 2012a; Nadar *et al*., 2018; Sheldon and van Pelt, 2013).

This review provides an overall insight of enzymatic and enzyme-assisted extractions from plant tissues, focusing on the application of these sustainable methods towards the recovery of high value added molecules from agricultural and industrial lignocellulosic plant by-products, including polyphenols, polysaccharides, organic acids, carotenoids and flavors.

1.2. Enzymatic extraction – process and advantages.

The principle behind enzymatic technology is the use of enzymes as catalysts to hydrolyze the plant cell wall components through the binding of the desired substrate to the active site of the enzyme. This interaction happens when the enzyme changes its shape in order to fit the substrate on its active site and thus increasing the enzyme-substrate interaction that leads to the disruption of cell wall bonds (Sheldon and van Pelt, 2013; Nadar *et al*.,

2018a). However, to get potential results it is necessary to have a great knowledge about enzymatic reactions and the parameters influencing the process.

First, it should be comprehended the biochemical and morphological composition of the biomass that will be subjected to the enzymatic treatment, identify the compounds of major interest or value and then the selection of an adequate protocol. The selection of the enzyme based on their catalytic activity is one of the variables that causes more impact in the final result, either in the type of extracted compounds or in the overall yield. The most common enzymatic approach to extract bioactive compounds from lignocellulosic biomass is using hydrolases, enzymes that catalyze the hydrolysis of chemical linkages such as glycosidic bonds, so frequently found within the cell wall carbohydrates. Enzymes such as cellulases, amylases, hemicellulases or pectinases, solubilize the cell wall polysaccharides, accelerating the release of intracellular molecules (Nadar *et al*., 2018a). Table 1.1 resumes the most common enzymatic activities applied to lignocellulosic materials and their action, as well as some commercial names.

Activity	Types and Action	Commercial names	Applications
Cellulases	**endo-(1,4)-β-D-glucanase**: cellulose hydrolysis in oligosaccharides, cellobiose and glucose **exo-(1,4)-β-D-glucanase**: hydrolysis of β-1,4-glucosidic linkages, releasing cellobiose **β-glucosidases**: hydrolysis of cellobiose to glucose	Celluclast® 1.5L Carezyme 1000® Cellic®CTec2 Rohalase® CL Biotouch® DCC	Extraction of pectins from artichoke by-products, apple and kiwifruit pomaces and cauliflower by-products (Bhalla *et al*., 2013; Sabater *et al*., 2018; Wikiera *et al.*, 2015; Yuliarti *et al*., 2015). Extraction of polyphenols from grape seed and apple pomace, and flavonoids from Ginkgo biloba leaves (Chamorro *et al*., 2012; Chen *et al*., 2011b; Junjian *et al*., 2012). Extraction of polysaccharides from dandelion leaves and production of oligosaccharides from sugar beet pulp and apple pomace (Gullón *et al*., 2007; Martínez *et al*., 2009; Wang *et al*., 2019).
Amylases	**α-amylase or endoamylase**: hydrolysis of α-1,4 –glycosidic bonds **β-amylase or exoamylase:** hydrolysis of α-1,4 –glycosidic bonds **Debranching enzymes (pullulanase and glucoamylase)** hydrolysis of α-1,6 –glycosidic linkages	Promozyme™ 400L Promozyme® D2 Termamyl 120L® BAN™ 480L Fungamyl® 800L Duramyl®	Extraction of polysaccharides from pea byproducts, banana peel and bamboo shoot (Cheng *et al*., 2018; Chen *et al*., 2019; Marenda *et al*., 2019). Production of nanocellulose from potato peel (Raigond *et al.,* 2018). Maltose production from cassava bagasse (Araújo-Silva *et al.*, 2018).
Pectinolytic enzymes	**Depolymerases (eg: galacturunase):** hydrolysis of α-1,4 –glycosidic bonds **Esterases**: removal of methoxy esters **Lyases (or transeliminases)**: hydrolysis of 1,4-glycosidic linkage and hydrogen removal from C-5	Pectinase Multieffect FE® Pectinex ® Pectinex ® Ultra SP ROHAPECT® PTE XPECT®	Production of monosaccharides from grapefruit peel and pectic-oligosaccharides from sugar beet pulp and bergamot peel (Mandalari *et al*., 2006; Olmos *et al*., 2012; Wilkins *et al*., 2007). Extraction of rhamnogalacturonan I from potato waste (Byg *et al*., 2012). Extraction of flavour compounds from celery seeds (Sowbhagya *et al*., 2010).
Hemicellulases	**Xylanases:** endo-(1,4)-β-D-xylanase; exo-(1,4)-β-D-xylanase; β-D-xylosidases; Acetyl xylan esterase **Mannanases**: endo-β-(1,4)-mannanase; exo- β- (1,4)-mannanase; Acetyl mannan esterase **Arabinanases**: α-L-arabinofuranosidase; Endo-α-1,5-arabinanase **Galactanase**: Endo-galactanase; α-galactosidase	Pentopan Mono BG® Nutrase Xyla® Panzea® Mannaway® Biotouch® M7 Rohalase® SEP-VISCO Pulpzyme® RC 2500	Production of xylooligosaccharides from sugarcane bagasse, cotton stalks, oil palm frond fibres and kenaf stem (Akpinar *et al.,* 2007; Azelee *et al.*, 2016; Reddy *et al*., 2016; Sabiha-Hanim *et al.*, 2011;). Extraction of quercetin from onion skin (Choi *et al*., 2015). Extraction of steviosides from *Stevia rebaudiana* leaves (Puri *et al*., 2012b). Extraction of essential oils from bay leaves (Boulila *et al*., 2015).

Table 1.1 – Enzymes applied in hydrolysis of plant carbohydrates for extraction of biomolecules.

Table 1.1 (continued)

Activity	Types and Action	Commercial names	Applications
Enzyme mixtures	Combination of different enzymatic actions	Bioliva Cytolase 0 Laminex® Multiefect CX 13L Natuzyme Protizyme™ Rapidase LIQ Plus Shearzyme® Viscozyme®	Extraction of pectin from lemon peel and lime peel (Dominiak *et al*., 2014; Lim *et al*., 2012). Oligosaccharides from grape pomace and brewer's spent grain (Costa *et al*., 2018; Sajib *et al*., 2018). Extraction of polyphenols from winery by-products, unripe apples and cauliflower leaves (Camargo *et al*., 2016; Huynh *et al.*, 2014; Zheng *et al*., 2009). Extraction of carotenoids from tomato seeds and skins (Catalkaya and Kahveci, 2019). Extraction of flavors from cumin and celery seeds (Sowbhagya *et al*., 2010).

After the successful selection of enzyme, yields will rely on the solvent used, temperature and pH of the reaction, incubation time, enzyme loading and substrate availability (Nadar *et al*., 2018; Puri *et al*., 2012a; Yusoff *et al*., 2015). Although commercial available enzymes are functional at a wide range of temperatures and pH values, they have their own optimal temperature and pH of reaction, which can be slightly modified depending on the substrate of application. As pH values should be continuous along the incubation time, aqueous enzymatic treatment usually involves buffer salts as solvents, allowing to maintain the enzyme integrity and the extract stability. The reaction temperature should be mild, as high temperatures can cause gradual loss of enzyme activity due to denaturation, but not so low that could not be enough to activate the enzyme. In fact, these mild temperature conditions, ranging 40 to 60 ˚C, are the most inherent characteristic that makes enzymatic extractions an excellent approach in the recovery of temperature-sensitive molecules, such as polyphenols or volatiles (Nadar *et al*., 2012). Enzyme

loading and substrate availability are other critical parameters in an enzymatic process. Although the use of high enzyme concentrations can increase the interaction between enzyme and substrate, promoting an extended degradation of the cell wall, it is also reported that the extraction yields of biomolecules usually increase up to a given enzyme concentration and then, as the substrate gets saturated, it stabilizes and finally decreases, so not always higher enzyme or substrate concentrations lead to the best results (Yusoff *et al*., 2015). Also, the increase of incubation time can enhance the extraction yield but there has to be a compromise between the yield and the quality of the extract, as longer incubation times (longer than 24 h) can promote the degradation of the extract (Yusoff *et al.*, 2015). Furthermore, concerning polysaccharides, an extended extraction time can hydrolyse them into molecules with molecular weights smaller than the desired ones.

Besides the parameters directly involved in the enzymatic process, also the fractionation and the particle size of the raw biomass play important roles in the extraction yield, since the action of the enzymatic treatment is performed through the interaction with the cell wall (Chundawat *et al*., 2006; Yusoff *et al*., 2015). In some cases, it may be profitable to perform a pretreatment before the enzymatic extraction, allowing isolating a target fraction, and then performing a more specific treatment using enzymes. Common pretreatments include autohydrolysis, dilute acid or alkaline, organosolv, steam explosion, and ammonia fiber explosion (Zhao *et al*., 2009). A previously reduction of the substrate particle size improves its availability for the enzyme to bind, thus increasing the overall extraction yields, reducing pretreatments and making hydrolysis more cost-effective (Puri *et al*., 2012; Chundawat *et al*., 2006).

Enzymatic extractions present three major advantages regarding conventional methods: a) environmental, as these methods are usually aqueous-based, avoiding the use of toxic

and pollutant organic solvents, thus becoming a green and sustainable alternative; b) specificity, as enzymes are able to accurately extract specifically biomolecules of interest, achieving a purer extract with less contaminants; and c) superior quality, as the specificity and mild conditions of the treatment allows the separation of the bioactive compounds without changing its bioachemical structure and other biological properties, resulting in a final product of higher quality (Nadar *et al*., 2018; Yusoff *et al*., 2015).

Nevertheless, it has also been pointed out a critical limitation to this method, regarding economic issues, as at industrial scale, enzymes application and drying process after enzyme treatment can become relatively expensive (Puri *et al*., 2012a; Yusoff *et al*., 2015). However, as enzymatic extracts are products of superior quality, it is more likely for them to be used by more refined industries such as cosmetic or pharmaceutical that generally are able to pay for more differentiated premium ingredients. In addition, a potential scale up could result in loss of stability due to the difficulty in controlling pH value and temperature at industrial scale (Nadar *et al*., 2018). A well-known strategy to control these parameters is the enzyme immobilization, which improves the catalytic properties of the enzyme within larger ranges of pH value or temperature, and avoids losses of enzymes, allowing their reutilization thus reducing the costs inherent to the enzymatic process (Nadar *et al*., 2018).

1.3. Synergic biomass treatment using enzymes and other green techniques

In some cases, enzymatic extraction disrupts the plant cell wall material but the bioactive compounds of interest do not solubilize adequately to allow their extraction from the tissues. Ocasionally, enzymes can work as a first pretreatment and the further extract can be achieved using an additional extraction method, or can be carried out as an extraction

extension. Enzymes can be used in synergy with conventional methods but also with other greener approaches, such as microwave digestion, ultrasound application, high pressure processing, or use of deep eutectic solvent, allowing to reduce the reaction time or the solvents concentration. In these cases, the extraction process is referred as enzyme-assisted extraction (Özkan and Bilek, 2015).

Ultrasound extraction of plant molecules occur through the generated cavitation that causes the swelling and/or disruption of the cell wall, resulting in higher diffusion rate of molecules across the wall that can enhance the enzymatic extraction (Khan *et al*., 2010). In enzymatic assisted ultrasound extraction, besides the conditions affecting the enzymatic treatment, properties as ultrasound power and frequency, ultrasound temperature and time also affect the extraction yield and possibly the molecule chemical structure and molecular weight (Nadar *et al*., 2018). An enzyme-assisted ultrasound extraction of polyphenols from *Ulmus pumila L.* barks, using a mix of cellulase, pectinase and β-glucosidase allowed to increase the extraction of rutin, catechin, epicatechin and quercetin, thereof increase the antioxidant capacity, when compared with those obtained through ultrasound-assisted and conventional extractions (Zhou *et al*., 2017).

Microwave-assisted extraction is a green technique that combines microwaves with conventional solvents, as well as enzymes, where a generated electromagnetic field increases kinetic energy of molecules, reducing solvent and energy required, with high reproducibility (Zhang *et al*., 2013a; Li *et al*., 2013). Corilagin and geraniin were extracted from *Geranium sibiricum* Linne using combined microwave irradiation and cellulase treatment, simultaneously, for 9 minutes, allowing increasing extraction yields by 64 and 72%, for corilagin and geraniin, respectively, when compared to conventional extraction with acetone, without chemical decomposition (Yang *et al*., 2010). Both treatments can also be applied separately, for instance for the oil extraction from yellow

horn seed kernels, where enzymatic treatment with an optimal mixture of cellulase, hemicellulase and pectinase was used as pretreatment, followed by microwave irradiation for 45 min (Li *et al*., 2013). To increase the extraction yields, microwave pretreatment for 2 min was used to increase the extraction of polyphenols from waste peanut shells, followed by enzymatic extraction with cellulase for 2 h (Zhang *et al*., 2013).

Supercritical fluids have properties similar to gases, including diffusion, viscosity and surface tension, characteristics that are suitable to achieve high yields of bioactive compounds extraction in reduced periods of time (Azmir *et al*., 2013). Unlike the other described enzyme-assisted methodologies, it is not described the use of a real enzyme-assisted supercritical extraction but instead the application of supercritical extraction as a pretreatment before the enzymatic extraction. Commonly, supercritical fluids are used to isolate the biomass lipophilic fraction, followed by the specific extraction through enzymatic treatment but the reverse order of application is also described. Sequential supercritical carbon dioxide extraction and an enzymatic extraction with Viscozyme and CelluStar XL were used to improve the extraction of polyphenols from blackcurrant pomace, isolating higher polarity molecules with increased extraction yield increased by almost 40% (Basegmez *et al*., 2017). Different enzymatic cocktails, including Viscozyme, Pectinex, Alcalase or Kemzyme were used as pretreatment of pomegranate peel before CO_2 supercritical extraction of polyphenols. The combination of both methods allowed to extract significantly more phenolic compounds than enzymatic extraction by itself (Mushtaq *et al*., 2015).

Other novel green extraction techniques such as pulsed-electric field, ohmic heating, high pressure or the use of deep eutectic solvents could potentially be used in synergy with enzymes, although the application of these methods in the valorization of agricultural lignocellulosic plant by-products is not fully explored yet. As enzymes commercially

available are not able to fully hydrolyze vegetal cell walls and extract all of the bioactive molecules entrapped within them, the co-adjuvant application of enzymes with one or more of the clean technologies described above can become a strategy to overcome the disadvantages of each method. Nonetheless, the selection of an appropriate methodology, using only enzymes or in combination with other green methods, depends on the biochemical nature of the compound of interest.

1.4. Polysaccharides

Polysaccharides are the core structures of different lignocellulosic biomasses, including food and agricultural by-products (Donato *et al*., 2014). According to the chemical structure and chain conformation, polysaccharides can have different bioactivities such immunomodulatory, antitumor, antiviral, regulation of blood glucose and triglyceride, or prevention of hyperglycemic activities (Yang and Zhang, 2009; Giavasis, 2014). Some non-digestible carbohydrates, such as β-glucans, pectins or oligosaccharides are well-known for their health benefits, particularly in the gastrointestinal tract, where they are available to be fermented by colon microbiota and produce short-chain fatty acids (Elleuch *et al.*, 2011; Fernández *et al*., 2015).

Conventional extraction of carbohydrates is performed by hot water extraction and is considered a sustainable method. In this method, the recovery yields are mainly affected by temperature and extraction time, and the use of high temperatures and long incubation periods usually degrades part of the polysaccharide structure and other associated bioactive compounds (Nadar *et al*., 2018). The use of enzymes to extract carbohydrates from lignocellulosic biomass increases the solubility of hemicelluloses and pectins by hydrolyzing these molecules into smaller fragments, as enzymes can be particularly specific for the target polysaccharide (Nadar *et al*., 2018; Janardhnan and Sain, 2006).

Table 1.2 summarizes the most recent works using commercial enzymes for extraction and recovery of polysaccharides from agricultural lignocellulosic plant by-products.

Table 1.2 – List of bioactive polysaccharides obtained by enzyme-assisted extraction from industrial by-products

Polysaccharide	Source	Enzyme used	Conditions	Yield (%)	Reference
Pectin Pectin-derived oligosaccharides (POS)	Lemon peel	Promod 24L + Cellulyve	Sodium acetate buffer (50 mM) at pH 5.5, 4 h, 50 ˚C	12.6%	Zykwinska *et al.* 2008
	Sugarbeet pulp	Promod 24L + Cellulyve Rohapect DA6L/ Macer 8 FJ + amyloglusidase + Trypsin T069P	Acetate buffer (50 mM) at pH 5.5, 4 h at 50 ˚C Acetate buffer: pH 4.0, 15 h, 50 ˚C + pH 4.8, 3 h, 55 ˚C + pH 8, 18 h, 37 ˚C	86.4% 95% of POS	Zykwinska *et al.* 2008 Olmos & Hansen, 2012
	Passion fruit peel	Polygalacturonase from *Geotrichum klebahnii* Celluclast 1.5L	Citrate buffer pH 5, 30 ˚C, 6 h	25% 9%	Vasco-Correa and Zapata, 2017
	Prickly pear cladodes	Cellulase from *Aspergillus niger* + Bakezyme HSP 6000 BG	pH 5.5, 50 ˚C, 6 h	17.9%	Bayar *et al*., 2018
	Orange peel	Cellulase + xylanase from *Aspergillus japonicus* PJ01	45 ˚C, 6 h	34.6% and MW < 3kDa	Li *et al*., 2016
Xylooligosaccharides (XOS)	Kenaf stem	*Xylanase and arabinofuranosidase from Trichoderma reesei*	Acetate buffer (50 mM) pH 4, 40 ˚C, 48 h	350 mg XOS/ g biomass	Azelee *et* al., 2016
	Coconut husk	Commercial xylanase	Citrate buffer 5.0, 55 ˚C, 18 h	93%	Jnawali *et al*., 2018
	Cotton stalks	Veron 191	Citrate buffer (50 mM) pH 5.4, 40 ˚C, 24 h	53% with DP 2-7	Akpinar *et al*., 2007
	Rice husk	Pentopan™ Mono BG	Citrate buffer (50 mM) pH 6, 50 ˚C, 9 h	17.35 mg/ mL	Khat-udomkiri *et al*., 2018
	Grape stalk Apple pomace	Buzyme 2511	Phosphate buffer (50 mM) pH 6, 39 ˚C, 30 min	6.5 g/ L 3.7 g/ L	Mazzaferro *et al.,* 2011
	Sugarcane bagasse	Xylanase and feruloyl esterase from *Clostridium thermocellum*	Phosphate buffer (0.1 M) pH 6.4, 50 ˚C, 5 h	14.8% with DP 2 -4	Mandelli *et al*., 2014
Arabinoxylans	Barley husk	Termamyl Ultra 300 L	pH 6, 80 ˚C, 45 min + 60 ˚C, 45 min	55% with MW 35 to 45 kDa	Höije *et al*., 2005
	Spelt bran Spelt hull	Belfeed + Celluclast 1.5 L Ultraflo L	Acetate buffer (25 mM) pH 5, 55 ˚C, 24 h Acetate buffer (25 mM) pH 4.7, 40 ˚C, 24 h	70%, DP 1 - 1164 6.5%, DP 2 - 17	Escarnot *et al*., 2012
Heterogeneous polysaccharides	Soybean by-product	Alcalase® + Viscozyme L	Phosphate buffer pH 8, 60 ˚C, 4 h + Citrate buffer pH 4.5, 60 ˚C, 2 h	29.48 %	Rovaris *et al*., 2012
	Pea by-product	Viscozyme L + commercial amylase	pH 6.5, 50 ˚C, 1 h + pH 5.0, 50 ˚C	9.4%	Cheng *et al*., 2018

1.4.1. Pectin and pectic - oligosaccharides

Pectin is an extremely complex polysaccharide mainly due to the high diversity of monosaccharides present in its composition, which includes D-galacturonic acid (GalA), L-rhamnose, arabinose and galactose monomers (Mamma and Christakopoulos, 2014; Vasco-Correa and Zapata, 2017; Adetunji *et al*., 2017). The most common class of pectins in the cell wall are homogalacturonan, rhamnogalacturonan-I and rhamnogalacturonan-II but there are other classes of pectins. Pectins are widely used in food industry as jellifying, emulsifier and stabilizer agents, and also as outstanding prebiotics, or pectin-derived oligosaccharides (POS), as they are able to reach the distal part of the colon and modulate its microbiota preventing diseases as colon cancer and ulcerative colitis (Mamma and Christakopoulos, 2014; Gullón *et al.*, 2007).

The high pectin content in fruit and vegetable peels and pomace, especially citric fruits, made this polysaccharide one of the first bioactive molecules to be recovered from by-products. There are two common approaches to extract pectin from plants. The first one is the use of enzymes to destroy the other cell wall components, namely cellulases, hemicellulases and proteases with minimal pectinolytic activity, aiming the isolation of pectins. For instance, Dominiak and co-workers used commercial Laminex C2K, Multiefect B and Validase TRL mixtures in lime peel, recovering high yields of pectin with 80% purity (Dominiak *et al*., 2014). The second approach is the use of enzymes with pectinolytic activity, such as endopolygalacturonases and pectinesterases, in order to degrade pectin, isolating its monomers (Adetunji *et al.*, 2017). The use of polygalacturonases and commercial mixes of cellulases and proteases have been reported to extract up to 18% of pectin from lemon peels (Donaghy and McKay, 1994; Contreras-Esquivel *et al.*, 2006; Lim *et al*., 2012).

Apple pomace is another major source for pectin extraction, and the use of endo-xylanase and endo-cellulase were reported to recover around 20% of this polysaccharide, with 70% purity, very similar to the pectin extraction and GalA units yields obtained using commercial Celluclast 1.5L for 18 h (Wikiera *et al*., 2015; Wikiera *et al*., 2016).

Celluclast 1.5 L was also used in the pectin extraction from gold kiwifruit pomace, sisal waste, and artichoke by-products, with yields of 4.5%, 9.4% and 20%, respectively (Yuliarte *et al*., 2015; Yang *et al*., 2018; Sabater *et al*., 2018). Although these differences in recovery yields are inherent to the raw biomass composition used to extract pectins, it is also inquisitive to observe the differences in incubation times applied, 30 min for kiwifruit, 18 h for sisal and 27 h for artichoke. These differences are clearly related to the total cellulose content present in each biomass and the time necessary to destroy it in order to recover the pectin. Overall, is quite outward that the enzymatic treatment allows the improvement of highly pure pectins, low-methylated and with high concentration of galacturonic units.

Enzymatic production of pectic oligosaccharides is also widely studied, especially for their prebiotic potential. The application of Celluclast 1.5L, Viscozyme L and Pectinex Ultra SP-L, followed by membrane filtration was described to produce pectin-derived oligosaccharides from lemon peels, with degrees of polymerization (DP) in the range 2–10 and a recovery yield of 13.1 kg per 100 kg of citrus peel (Gómez *et al*., 2014). These same enzyme mixtures were also applied to orange peels, after removal of monosaccharides and other impurities, producing ca. 30 g per 100 g of raw biomass (Sabajanes *et al*., 2012). In the case of oligosaccharides production, enzymes may allow to achieve an optimal mixture with different molecular weight or DP, which could be

fermented in the proximal or the distal colon, according to their molecular weight (Gómez *et al*., 2014).

1.4.2. Hemicelluloses

Hemicelluloses are heterogeneous polysaccharides composed by β-(1-4)-linked monomeric units of D-xylose, L-arabinose, D-glucose, D-galactose, 4-*O*-methylglucuronic acid, D-galacturonic and D-glucoronic acids (Mellinger *et al*., 2005; Scheller and Ulvskov; 2010). Most common hemicellulose polysaccharides include xylans, arabinoxylans and glucoarabinoxylans (β-D-(1,4) xylose), xyloglucans (β-D-(1,4) glucose), glucomannans and galactoglucomannans (β-D-(1,4) mannose and β-D-(1,4) glucose); and galactomannans (β-D-(1,4) glucose) (Scheller and Ulvskov; 2010; Elleuch *et al*., 2011). Conventional methods for recovery of hemicelluloses usually involves hot water or alkaline treatments, followed by ethanol precipitation, and produce high molecular weight xylans, well-known for their beneficial effects for the gastrointestinal tract (Otieno and Ahring, 2012). However, these methods require long incubation times and are not specific, recovering other undesired molecules, such as lignin. In this sense, the use of enzymes may aid in the hemicellulose recovery, reducing the extraction time and increasing the purity of the recovered molecules.

It is described the use of endoxylanases to recover xylan from wheat straw, alone or combined with ammoniac pretreatment, achieving higher production yields with the enzymatic treatment alone, with less concentration of glucans, resulting in a more pure xylan molecule (Rémond *et al*., 2010). The use of cellulases directly in raw materials can also be an alternative for xylan extraction, and release of 50% of total xylan from wheat bran by this method was reported by Zhao and Dong (2016).

Besides the extraction of xylan, enzymatic treatments are widely used for the production of xylo-oligosaccharides (XOS) from lignocellulosic plant by-products, after an alkali

pretreatment or directly. For this purpose, the enzyme should have the minimal exoxylanase activity, in order to decrease the xylose produced during the extraction. Straight production of XOS from raw biomass is also equally reported using xylanases and feruloyl esterases from different microrganisms (Gupta *et al*., 2017; Costa *et al*., 2019). The major advantage of direct enzymatic hydrolysis is the synergic recovery of other bioactive molecules, such as polyphenols or minerals, as they are not degraded or solubilized by the conventional pretreatments. However, this lack of selectivity may be negative if the goal is the production of pure molecules, as further separation and purification processes are required, increasing the production costs.

Arabinoxylans (AX) have been mostly recovered from cereal grains by-products. High molecular weight AX (ca. 350 kDa) can be recovered through hot water pretreatement followed by the use of xylanases or α-amylases (Zhou *et al*., 2010; Wang *et al.,* 2014). Termamyl Ultra 300 L was applied to barley husks, recovering 55% of AX with medium molecular weight ranging from 35 to 45 kDa (Höije *et al*., 2005). Spelt bran and hull were hydrolyzed with different endo-xylanases, Celluclast 1.5L and other enzymatic cocktails (Shearzyme 2x, Shearzyme Plus, Pentopan Mono BG, Ultraflo L and Rohalase WL), releasing up to 70% of AX from spelt bran, but only 6.5% of spelt husk AX, with DP ranging from 2 to 71 (Escarnot *et al*., 2012). These AX can be interesting as a potential prebiotic ingredient, as smaller fragments can be fermented by the intestinal microbiota, and larger non-fermentable fragments also plays a role in the modulation of gut microbiota, as well as other interesting biological properties such as anti-inflammatory and even suppressing of autoimmune neurological disease development (Berer *et al*., 2018). Furthermore, low to medium molecular weight AX, only produced via enzymatic treatment, may have other applications of interest in cosmetics, as they are described as inhibitors of melanin production and to have moisturizer properties (WO2011/157968).

When enzymes with endo-xylanase activity are applied to AX, the xylan backbone is hydrolysed and arabinoxylo-oligosaccharides (AXOS) are produced: Multifect® GC-Extra was used to produce high molecular weight oligosaccharides from corn bran and the xylanases to produce AXOS from brewer's spent grain with DP between 1 and 5 (Kale *et al*., 2018; Sajib *et al*., 2018).

Galactomannans are more frequently present in legumes and have been recovered from *Caesalpinia pulcherrima* seeds using commercial cellulases from *A. niger,* as well as from spent coffee ground using cellulase cocktail combined with β-mannanases, after a steam explosion pretreatment (Buriti *et al*., 2014; Chiyanzy *et al*., 2014). Treatment of *Cassia obtusifolia* seeds, a leguminous with medical interest, with commercial endo-1,4-β-mannanase from *Cellvibrio japonicas* after hot water extraction allowed to purify an homogeneous glucoronoxylan with MW of 29 kDa (Feng *et al*., 2018).

For last, β-glucans are one of the polysaccharides with more studied bioactive properties, as they do not only have benefits for gastrointestinal health, but also for systemic and immune systems, as they modulate the blood cholesterol and the glycemic load (Aguiló-Aguayo *et al*., 2017). These molecules can also provide food ingredients with functional properties as thickening agent, water-holding, oil-binding agent and emulsifying stabilizer (Ferreira *et al*., 2010). β-glucans from brewery's spent yeast was also recovered after autohydrolysis followed by enzymatic treatment with different proteases, including Protamex, FM2.OL, Neutrase and Protease 1398. Although with a reduced yield (11 %) they have maintained the β-glucan original structure well preserved, in contrast to traditional techniques involving hot alkaline solvents that degrade its structure (Liu *et al*., 2008). Enzymatically extracted β-glucans are known for their higher purity degree that enables their use as immunomodulatory in cosmetic and biomedical applications, unlike

the aggressive traditional methods that destroy part of the molecule structure, thus producing β-glucans with low purity that cannot be applied in these industries.

1.4.3. Cellulose

Cellulose is one of the most abundant structural polysaccharide, composed by β-1,4 – linked glucose monomers arranged in microfibrils that compose the cell wall (Brigham, 2018). Due to its natural origin and characteristics similar to synthetic polymers, it could be used as a feedstock to produce plastic alternative materials or as base for different materials (Tibolla *et al*., 2014). Although direct cellulose does not have much interest for food and cosmetic industries, these fibers are extremely important for the textile and paper industries, and enzymatic treatments of lignocellulosic by-products have also been performed. On the other hand, cellulose nano or microfibers are also of great interest for their mechanical strength, texture and also for their high crystallinity, so they may have more broad range of applications in food and cosmetic industries, such as thickener agents, absorbants or emulsifiers (Tibolla *et al*., 2014). Cellulose nanofibers have to suffer an alkali pretreatment, usually followed by xylanase or xylanase-cellulase combo in order to cleave the β-1,4-glycosidic bonds and isolate cellulose fibers (Tibolla *et al*., 2014). This approach was used to recover cellulose from banana peel, rice straw and cornhusks (Reddy and Yang, 2005; Reddy and Yang, 2006).

1.4.4. Other Polysaccharides

Other heterogeneous polysaccharides with bioactive properties have also been enzymatically recovered from agroindustrial by-products. For instance, sequential enzymatic treatment of banana peel with Termamyl, followed by Amyloglucosidase 300L and neutrase, allowed to recover pectin and other polysaccharides containing glucose,

arabinose and xylose monomers (Marenda *et al*., 2019). Dietary fibre of deoiled cumin, a waste biomass from cumin essential oil extraction, was hydrolyzed with Alcalase®, achieving high extraction yields (85% of total dietary fibre) with high technological capacities, such as oil and sugar absorption capacity (Ma *et al*., 2015). Other soluble dietary fibers (SDF) from agricultural by-products have also been enzymatically extracted: α-amylase, cellulase, and amyloglucosidase were used to recover SDF from *Canna edulis* Ker by-products and grape pomace; crude enzyme extracted from edible snail, with cellulase and β-glucosidase activities was applied in the extraction of SDF from carrot pomace (Alía *et al*., 2003; Zhang and Wang, 2013; Yoon *et al*., 2005). Amyloglucosidase and trypsin were used to extract and characterize the insoluble fraction of coconut lignocellulosic by-products, composed by ca. 70% of cellulose, and cellulases were used to extract SDF from apple pomace (Ng *et al*., 2010; Li *et al*., 2014). Papain and amylase were used together to extract SDF from pods (Chen *et al*., 2011).

Different enzymes and enzymatic cocktails and mixtures have been used in the recovery of polysaccharides from agroindustrial by-products, with potential claim in food, pharmaceutical or biomedical fields due to their improved biological activities promoted by molecules with higher purity degrees. The application of enzymes in the extraction of polysaccharides can also increase the yields and, at the same time, reduce the ethanol necessary for the polysaccharide precipitation process, thereof decreasing the ecological impact of the process.

1.5. Phenolic compounds

Plant-derived polyphenols are well known for their antioxidant capacity and other health benefits, such as protection against cardiovascular diseases, with interest to food and nutraceutical industries (Mushtaq *et al*., 2017). The phytochemical compounds are

usually present in higher concentration in fruit and vegetables wastes, such as seeds and skins, than in the product itself. Phenolic compounds can be present in the cell wall, mainly bound to other molecules such as fatty acids or to cell wall structural carbohydrates by hydrophobic interactions, hydrogen and covalent bonds, or they can be present in the nucleus or vacuoles of plant cells, mainly in free form (Xu *et al*., 2014; Shahidi and Yeo, 2016). The covalent bonds that link polyphenols to cell wall carbohydrates boost the rigid structure of the wall matrix, and the polyphenols improve the cell protection against pathogens, fungi and UV radiation, which difficult the extraction of these bound phenolics (Shahidi and Yeo, 2016).

Enzymatic and enzyme-assisted treatments are ideal for the polyphenols extraction, as they do not involve high temperatures or solvents that could potentially damage these compounds, and are much efficient to recover the bound phenolics due to the interaction with the cell wall polysaccharides (Camargo *et al*., 2016). Cellulolytic enzyme mixtures are quite exploited for the extraction of phenolics, regardless of its classification, as these enzymes weakened the integrity of the cell wall. Celluzyme MX, Celluzyme CL and Kleepase AFP were used to extract polyphenols from citrus peels such as lemons, mandarin, sweet orange and grapefruit; Viscozyme L and CeluStar XL showed to increase total phenolics extraction from chokeberry pomace up to 40% (Kitrytė *et al*., 2017).

However, there are other enzymes more specific for the extraction of polyphenols, such as tannases, which are able to hydrolyze the ester and depside bonds of gallotannins, reacting with any phenolic hydroxyl group and releasing glucose and gallic acid. Polyphenol esterases, such as feruloyl esterase, are also able to hydrolyze the ester bonds (Rodrigues, 2016). Tannase from *Paecilomyces variotti* and other commercial enzymes, including β-glucosidase, Celluclast 1.5L and pectinase Novozyme 33095, are described

as enhancers of polyphenols extraction from lignocellulosic by-products, as they can convert the glycoside-linked phenolics into their respective aglycone form (Ruviaro *et al*., 2018). Table 1.3 resumes the most recent works using commercial enzymes for extraction and recovery of polyphenols from agricultural lignocellulosic by-products.

Table 1.3 - List of polyphenols obtained by enzyme-assisted extraction from industrial by-products

Source	Enzyme used	Conditions	Extract composition	Reference
Grape pomace	Cellulase Tannase	Acetate buffer (50 mM) at pH 5, 45 ˚C, 2h	0.74 – 0.76 g of GAE/ 100 g of total phenolics composed by gallic, ρ-coumaric and syringic acids; (+)-catechin, malvidin-3-*O*-glucoside	Meini *et al*., 2019
	Laminex + Pektozyme + Tannase	Acetate buffer at pH 5.5, 35 ˚C, 24 h	0.11 – 0.14 g of GAE/ 100 g of total phenolics composed by gallic acid, gallocatechin, epigallocatechin, catechin, epicatechin, procyanidin, epicatechin-*O*-gallate	Chamorro *et al*., 2012
Unripe apple	Viscozyme L	pH 3.5, 50 ˚C, 12 h	163. 43 mg of GAE/ 100 g of total phenolics composed by procyanidin, (+)-catechin, (-)epicatechin, chlorogenic acid, caffeic acid, *p*-coumaric acid, ferulic acid, quercetin, phloridzin, phloretin	Zheng *et al*., 2009
Cranberry pomace	B-glucosidase	pH 5.5, 60 ˚C	gallic acid, chlorogenic acid, p-hydroxybenzoic acid, p-coumaric acid	Zheng and Shetty, 2000
Onion skin	Celluclast 1.5 L + Pectinex SP-L	Citrate buffer pH 4.8, 45 ˚C, 48 h	Quercetin	Choi *et al*., 2015
Cauliflower leaves	Viscozyme L Rapidase	pH 4.0, 35 ˚C, 24 h	429 - 650 mg GAE/ 100 g, mainly composed by kaempferol-3-feruloyldiglucoside	Huynh *et al*., 2014
Pigeonpea leaves	Celluclast 1.5 L Novozym 188	7.0, 25 ˚C, 24 h	0.17 mg/ g of luteolin and 0.08 mg/ g of apigenin	Fu et al., 2008
Geranium sibiricum linne	Celluclast 1.5 L	35 ˚C, 6 h	6.8 mg of corilagin and 19.8 mg geraniin	Yang *et al*., 2010

GAE – gallic acid equivalent

1.5.1. Phenolic acids

Phenolic acids are non-flavonoid polyphenols derived from benzoic or cinnamic acids backbones. Phenolic acids form ether linkages with lignin through their hydroxyl groups in the aromatic ring and ester linkages with structural carbohydrates and proteins through their carboxylic group, and can be released by conventional techniques using acid or alkali extraction, or alternatively, using enzymes (Tsao, 2010; Acosta-Estrada *et al*., 2014). In fruits and vegetables, most of phenolic acids are usually free, while in grains and seeds, the majority are bound to the cell wall, and in this case, enzymatic hydrolysis presents a major advantage in their extraction (Tsao, 2010).

The combined use of commercial cellulase, pectinase and β-glucosidase for polyphenols extraction from winery by-products, modifies the galloylated polyphenols to low molecular weight phenolics, such as the galloylated form of catechin that release gallic acid, and thus increasing the antioxidant capacity (Chamorro *et al*., 2012). Furthermore, the use of enzymes allows reducing the incubation time, thereof reduce molecules degradation and improving the quality (Xu *et al*., 2014). The synergic use of pectinase Pectinex Ultra SP, commercial cellulase and tannase also allowed reaching higher extraction yields than the enzymes separately, with higher antioxidant capacity, but without differences in the recovered compounds, mostly gallic acid, caffeic acid, quercetin and trans-resveratrol (Fernández *et al*., 2015; Martins *et al*., 2016). Besides the bound phenolics, enzymatic hydrolysis could also be a powerful tool in the recovery of soluble phenolics. For instance, the application of Viscozyme® to winemaking by-products increased the amount of soluble phenolics and thus the ratio soluble-insoluble phenolics (Camargo *et al*., 2016).

Coffee pulp is an abundant industrial by-product from wet pulping of coffee beans and was used as substrate to recover more than 5g.kg^{-1} of dry matter of ferulic, caffeic, *p*-coumaric and chlorogenic acids, using commercial pectinase and an enzymatic cocktail with feruloyl

activity (Torres-Mancera *et al.*, 2011). Yuan *et al.* (2015) tested different commercial mixtures of cellulase, hemicellulase, tannase, β-glucosidase and amylase to extract oleuropein from olive leafs and concluded that enzymes with the highest hemicellulase activity generated the more pure oleuropein extract and also facilitate the further hydrolysis of these polyphenols into hydroxytyrosol and elenolic acid.

The use of enzymatic cocktails or enzyme combinations is transversal to most of the works focused on the enzymatic extraction of phenolic acids. In fact, the synergy between enzymes can improve the extraction yields due to the larger action spectrum that allow the hydrolysis of different components of cell wall, as well as the release of free phenolic acids (Nadar *et al*., 2018).

Overall, further than the yield improvement, application of food-grade enzymes in the recovery of phenolic acids from biomass can hold their nutraceutical properties, which can be smoothly adapted by food industry, allowing to obtain these antioxidant nutrients from renewable sources and at low cost.

1.5.2. Flavonols and Tannins

Flavonols are low molecular weight compounds known for their antioxidant, anti-inflammatory, antimutagenic and anti-carcinogenic properties (Panche *et al*., 2016). They are usually linked to different cell wall polysaccharides but have more affinity with pectins and hemicelluloses than with cellulose, reason why pectinases are the most common enzymes applied in the extraction of flavonols (Pinelo *et al*., 2006). For grape pomace, it is described that the use of pectinolytic Novoferm 106 and cellulolytic Cellubrix L mix, after aqueous pretreatment, can recover more than 90% of flavonoid compounds, which is similar to the results from organosolv treatment (Maier *et al.*, 2008). Other approaches are also effective, for instance cellulase was used to extract flavonoids from *Gingko biloba* leaves,

as besides the cell wall degradation activity, this enzyme can transglycosylate flavonol aglycones into more polar glycosides through the introduction of more hydrophilic groups into flavonoids, increasing their solubility (Chen *et al*., 2011a). Hesperetin is a trihydroxyflavanone commonly found in citrus fruits, mainly in peels, pulp and seeds, and can be extracted by enzymatic treatment for 24 h with β-glucosidase or α-L-rhamnosidase, together with other similar flavonoids such as naringenin or neohesperidin (Ruivaro *et al*., 2018; Céliz *et al*., 2015).

Tannins are usually present in fruit peels, seeds coating and in grains bran or barks (Pinelo *et al.*, 2006). They can be located in the plant vacuole or associated with cell wall elements, where they can be esterified in certain positions with gallic acid or, not often, with sugars (Pinelo *et al.*, 2005). Cellulolytic enzymes and tannases are the most common enzymatic treatments applied to the extraction of this bioactive compound. Pectinex Ultra SP-L and commercial cellulase and tannase, separately or mixed, were applied to grape seeds and skins for 3 h, and high yields of (+)-catechin and epicatechin gallate were extracted from grape skins, as well as (+)-catechin and (-)-epicatechin from the grape seeds (Fernández *et al*., 2016).

Besides the evident ecological benefits, the application of enzymes in the recovery of phenolic compounds presents a major advantage regarding the conventional methods using aggressive solvents, such as dichloromethane or methanol, which is the increase of the extract solubility in aqueous media that can allow a future application in a food or cosmetic matrix.

1.5.3. Anthocyanins

Anthocyanins are glycosylated hydrophylic colored pigments, known for antidiabetic, anticancer, anti-inflammatory, antimicrobial, anti-obesity effects, prevention of

cardiovascular diseases and strong antioxidant activity (Khoo *et al*., 2017). Anthocyanins color is influenced by the pH due to its ionic structure, which confers a red color at acidic pH, purple at neutral pH and blueish color at alkaline pH, reason why enzymatic tretaments at neutral pH presents a major advantage regarding conventional methods, as they have greater capacity to preserve anthocyanins structure (Khoo *et al*., 2017). Furthermore, incubation temperatures for enzymatic treatments are usually below than 50 ˚C, which is another important advantage, as anthocyanins are also vulnerable to high temperatures (Khoo *et al*., 2017). As anthocyanins are located within the cell vacuole, solvents must cross the cell wall, membrane, cytoplasm and vacuolar membrane, thus the use of cellulolytic enzymes able to disrupt the cell wall might increase the extraction of anthocyanins through a green process (Silva *et al*., 2017).

Different commercial proteases and pectinases (Grindamyl, Macer 8, Pectinex BE and Novozyme 89) were applied to recover anthocyanins and other polyphenols from black currant pomace, and concluded that Macer enzymes reduced the extraction yield of anthocyanins, possible because these enzymes hydrolyze the polyphenol to its aglyconated form (Landbo and Meyer, 2001). Furthermore, authors concluded that seedless pomace presented higher extraction yields than the pomace with the seeds (Landbo and Meyer, 2001). Nine different combinations of cellulases, hemicellulases and pectinases produced by *Aspergillus* or *Trichoderma* spp. were used to extract anthocyanins from blueberry juice by-products, mostly blueberry skins, and conclude that the enzyme mix with cellulase and pectinase allowed to achieve the highest recover yield, as these enzymes are well correlated with the cell wall breakdown (Lee and Wrolstad, 2004). Pectinex mixture was also able to extract up to 7 mg of anthocyanins per gram of saffron tepals, representing 40% more than the anthocyanins extracted with ethanol and with less degradation and

color loss (Lotfi *et al.*, 2015). Overall, enzymatic methods allow to obtain natural extracts from lignocellulosic by-products, rich in antioxidants and natural pigments.

1.6. Other bioactive compounds

1.6.1. Organic acids

Organic acids are weak acids with a wide range of applications, from acidity regulator to preservative or excipient, but also they play an important role in gastrointestinal health, regulating diseases such as dysbiose or inflammatory bowel disease (Leung *et al*., 2012; Connors *et al*., 2019). Organic acids can be produced by enzymatic hydrolysis of food by-products. Fermentation of *Aspergillus awamori* and *Aspergillus oryzae* produced a cocktail rich in amylolytic and proteolytic enzymes that was then used to produce succinic acid from waste bread, with concentration of almost 50 $g.L^{-1}$ (Leung *et al.*, 2012). Other enzymatic cocktail produced by the same fungi was also used to produce a similar concentration of succinic acid from wheat bran, and also glucose and maltose in high concentrations (Dorado *et al*., 2009).

1.6.2. Pigments

Colors are key factors in food and cosmetic industries as they have significant influence in their products acceptance (Swer *et al*., 2016). As synthetic pigments are manifestly harmful for health and environment, the search for plant-derived pigments has increased (Swer *et al*., 2016; Özkan and Bilek, 2015). The most abundant pigments, besides anthocyanins that can also be classified as a polyphenol, are carotenoids (yellow/ red/ orange), betalains (yellow/ red), and chlorophylls (green) (Swer *et al*., 2016). Due to their commonly lipophilic nature, conventional extraction of pigments includes the use of organic solvents such as ethanol, hexane or dichloromethane that are not always efficient in penetrating the plant tissue to

solubilize these molecules (Strati *et al*., 2015). Enzymatic treatment rises as a more specific and eco-friendly alternative for the extraction of carotenoids, as cellulases and pectinases disrupt the cell wall integrity and release the pigments that are entrapped, usually attached to proteins that prevent the pigment oxidation (Çinar *et al*., 2005). Further extraction process using organic solvents can be also necessary, although the enzymatic pretreatment helps to reduce the amounts of solvents and incubation time. However, enzymatic pretreatment also increases the extraction of nonpolar molecules that will decrease the porosity of the biomass and difficult the penetration of these organic solvents (Strati and Oreopoulou, 2014).

Carotenoids are colored lipophilic pigments produced by plants and some microorganisms, with antioxidant capacity that can protect the human organism against some diseases promoted by oxidative stress, especially in liver injuries, where this compound is accumulated but also have benefits in cardiovascular and ocular health (Sugiura, 2013). Among the carotenoids, carotenes, zeaxanthin and lycopene are the most abundant in plants. Crustaceous industries produced tons of by-products in the form of shrimps heads and skins, which are rich in chitin, a bioactive polymer widely used, and carotenes (Babu *et al*., 2008). Carotene-protein complex can be extracted through enzymatic treatment with trypsin, papain or pepsin, followed by enzyme precipitation to separate the pigments, although trypsin presents higher extraction yields of astaxanthin and other carotenoids as β-carotene, lutein or zeaxanthin (Babu *et al*., 2008). One common source of carotenoid–rich by-products is the tomato processing industry, which generates tons of skins and seeds (Strati *et al*., 2014; Çinar, 2005). Viscozyme and Celluclast were used to hydrolyze cell wall polysaccharides from tomato by-product and make lycopene more available for further ethyl acetate extraction, increasing the lycopene concentration and antioxidant activity (Catalkaya and Kahveci, 2019). A mixture of Peclyve PR and Cellulyve 50LC can increase by 8 to 18-fold the lycopene extraction yield from tomato skins, and an optimization with alkaline

pretreatment followed by extraction with the same enzymes separately, could increase the yield up to 20 or 30-fold (Zuorro *et al*., 2011; Cuccolini *et al*., 2013). A crude enzymatic extract produced by *Fusarium solani* with pectinolytic, cellulolytic and cutinolytic activities, followed by ethanol treatment, was able to extract up to 3 g of lycopene per 100 g of tomato by-product, in an extract that is also rich in phenolic compounds and has high antioxidant capacity (Azabou *et al*., 2016). It is possible to conclude that enzymatic treatment can be a green approach in the recovery of carotenoids from agro-industrial by-products with interesting biological activities, including antioxidant capacity.

1.6.3. Flavors

Flavors are another important factor in the selection or consumption of a food ingredient and spices are among the plants with highest flavor due to the high concentration of volatile compounds (Nadar *et al*., 2018). Enzymatic treatments have been used for the extraction of flavor compounds as the have shown improved recover of aroma volatiles (Sowbhagya and Chitra, 2010). Cumin seeds were treated with cellulase, protease, pectinase or Viscozyme, individually, and the extraction yield of flavor compounds increased approximately 20% (Sowbhagya and Chitra, 2010). These enzymes also increased the recover yield of volatiles from celery seeds in almost 30% (Sowbhagya *et al*., 2010). Cellulase, hemicellulase and xylanase were used separately to improve the extraction yield of volatile compounds from bay leaves, and no significant differences were found between them, with extraction yields of 25%, approximately, but the mix of them significantly decreased the yield to 0.5%, suggesting a competitive adsorption of enzymes to the cell wall that negatively influences the breakdown of cell wall components (Boulila *et al*., 2015). Hemicellulase also allowed to achieve higher recovery yields of stevioside from *Stevia rebaudiana* leaves, compared to

cellulase or pectinase, but all increased 35 times the extraction of this sweetener compared to a conventional control (Puri *et al*., 2012).

1.7. Future perspectives

Valorization of agro-industrial lignocellulosic by-products is an increasing trend and the future of biotechnology, as they are loaded with countless bioactive compounds with enormous potential for the field of food, cosmetics, nutraceuticals and even health. The recovery of these compounds is only valid if it is economically and environmentally sustainable, so to guarantee their full potential it is very important to use clean and cost-effective extraction techniques.

Among the leading eco-friendly extraction methods, enzymes can be used to selectively recover a wide range of bioactive molecules from lignocellulosic biomass such as plant-derived by-products. Although the extraction process is relatively simple, it is necessary to understand the biochemical composition of the plant tissue, enzyme catalytic properties as well as the substrate-enzyme interaction, to achieve the maximum yield of the desired compound. The use of enzymatic cocktails with different hydrolytic actions should also be considered for maximum extraction yields, with reduced incubation times, temperatures, solvents and energy required, therefore achieving a more cost-effective process. Although there are a wide range of bioactive molecules that can be extracted through enzymatic treatment, there are still other to be exploited, such as chlorophylls or vitamins. The synergic enzymes application with other novel extraction methods can also be an option for the recovery of more pure extracts, nevertheless further studies would be necessary particularly regarding the latest cutting-edge technologies such as ohmic heating or pulsed electric field.

CHAPTER 2

Objectives

In the last decade, environmental and economic sustainability are subject of great attention from society. Sustainable production and processing of food ingredients are proposed as solutions to significantly reduce the waste of raw biomass and minimize pollution, becoming compulsory mechanisms of novel business models. Indeed, by adopting the Paris Agreement on climate change and the UN 2030 Agenda for Sustainable Development, the European Commission is leading towards a more sustainable planet. Sustainability and the transition to a low-carbon, more resource-efficient and circular economy are key in ensuring long-term competitiveness of the european economy. This agenda also encourages industries for innovation opportunities. Sustainable production is, hence, a challenge and an opportunity, allowing exploit the business potential for crack environmental tasks into economic opportunities. The capacity to reduce the environmental impacts, in association with the capacity to re-introduce underrated by-products in the production chain can be the assurance of balance between economics, politics and environment. Thus, cope value added products such as winery and fruit by-products, introducing them in the chain of active ingredients used in food industry, could be an added value for green chemistry and environmental ecology.

This project aims to valorize a major wine production byproduct – the grape pomace - by recovering high added value molecules, such as oligosaccharides, and developing a

gastrointestinal delivery system that allows a controlled release of these molecules. Furthermore, this system should be incorporated into a food matrix that beneficially promote the intestinal microbiota and other biological functions.

The main objectives undertaken on the present thesis include:

(i) Characterization of grape pomace composition and evaluation of oligosaccharides conventional extraction methods;

(ii) Production of xylanases and enzymatic treatment of grape pomace;

(iii) Characterization of chemical and biological properties of the obtained extract and simulation of gastrointestinal digestion;

(iv) Optimization of biocompatible nanocapsules to protect the extract from gastric digestion and improvement of bioavailibility throughout controlled release;

(v) Development of a functional coconut beverage using the encapsulated extract, as a proof of concept of this work.

Part II

Production and characterization of grape pomace extracts

CHAPTER 3

Enzymatic production of xylooligosaccharides from Brazilian Syrah grape pomace flour: a green alternative to conventional methods for adding value to agricultural by-products

Abstract

The aim of this work was to determine the most favorable conditions for the production of XOS from Brazilian Syrah GP. Chemical processes were performed using a rotatable central composite design, where the concentration of sulfuric acid or concentration of sodium hydroxide and GP flour: solvent mass ratio were the dependent variables. Enzymatic production was also evaluated using xylanase produced by *Aspergillus niger* 3T5B8 and Viscozyme® enzymatic commercial cocktail.

Chemical extraction allowed to recover 21.8 to 74.6% and 5.2 to 96.3% of total XOS for acid and alkaline processes, respectively. Enzymatic production using xylanase extracted up to 88.68 ± 0.12% of total XOS and up to 84.09 ± 2.40% with Viscozyme®.

The present study demonstrated different feasible methods to produce high added value molecules, the xylooligosaccharides, from *Syrah* GP flour, valorizing this major by-product. The use of enzymatic cocktails demonstrated to be an alternative to the conventional methods, allowing to obtain an eco-friendly and sustainable grape pomace extract.

3.1. Introduction

Grapes are one of the most cultivated fruit crops worldwide, around 67 million tons annually, from which more than 70% is intended to wine industry that generates up to 20% of wasted biomass in the form of grape skin, seeds, stems and residual pulp, known as grape pomace (GP) (FAO, 2016; Spanghero *et al*., 2009; Corbin *et al*., 2015). Syrah is a vigorous grape variety with a spreading growth habit and a tendency to produce long, trailing shoots. Growth can be excessive on deep, fertile soils and with high-vigor rootstocks. A versatile variety, Syrah is well adapted to a wide range of vinicultural temperature regions, winery uses, and wine styles (Christensen, 2003).

As a lignocellulosic feedstock, GP is mainly constituted by polysaccharides, arranged as hemicellulose and cellulose cross-linked to lignin, but there are also other components such as proteins, fat and ash (Lee *et al*., 2007; Beres *et al*., 2016). GP is reported to be rich in high added value compounds such as non-digestive polysaccharides that constitute the dietary fiber, structural proteins and phenolic compounds (Beres *et al*., 2016; Chamorro *et al*., 2012).

More recently, the use of different solvents and enzymes to partially hydrolyze the polysaccharides into oligosaccharides is in evidence due to their diverse application and market evaluation. The composition and the structure of XOS depend on the source and the production process (Akpinar *et al*., 2010; Garcia *et al*., 2001). XOS can be prepared from different vegetal sources, such as from xylan rich agricultural residues by autohydrolysis process, which does not eliminate undesirable components such as soluble lignin and monosaccharides, generates oligosaccharides with a high degree of polymerization and it requires extensive purification processes (Akpinar *et al*., 2010). Alternatively, as an improvement from the method, acid or alkaline solvents or enzymes can be added aiming better results in terms of oligosaccharides recovery (Moure *et al*., 2006) Enzymatic

hydrolysis is attractive because it does not produce undesirable byproducts, results in lower production of monosaccharides and its hydrolytic action is specific for each biochemical group present in the biomass (Chapla *et al*., 2012). Production of XOS is improved by endoxylanases with less or no side β-xylosidase activity. While endoxylanases produce XOS from xylan, β-xylosidase degrades XOS to xylose (Chapla *et al*., 2012; Vasquez *et al*., 2010; Reddy and Krishnan, 2016).

Recently, production of XOS from lignocellulosic feedstocks using alternative methods has become very common. Acidic and enzymatic extractions were applied to obtain XOS from tobacco stalk, corn cob and wastewaters of viscose fiber mills (Akpinar *et al*., 2010; Zhang *et al*., 2016; Gowdhaman and Ponnusami, 2015). Enzymatic treatment using xylanases have been the most common method to produce XOS from widely different biomasses, such as corn cob, sugarcane bagasse, oil palm fronds, cotton stalk, sunflower stalk, wheat straw or rice hull (Reddy and Krishnan, 2016; Chapla *et al*., 2013; Sabiha-Hanim *et al*., 2011; Kiran *et al*., 2013). Nevertheless, there are no works referring to production of XOS from GP.

Therefore, the aim of this work was to valorize a major agricultural by-product through the production of XOS from the Brazilian *Syrah* GP by the use of different methods, namely chemical and enzymatic hydrolysis.

3.2. Material and Methods

3.2.1. Raw Material

Syrah GP (*Vitis vinifera L. cv. Syrah*) from red sparkling production, harvested in January 2016, was provided by Ouro Verde Winery (Miolo Wine Group), located at Vale do São Francisco, Bahia, Brazil. The GP was oven-dried at 45 ºC for 24 h and the flour was obtained by milling and sieving the dried GP in a Bonina 0.25 df depulper (Itametal, Itabuna, Bahia,

Brazil). Since the seeds already have a well-established technological route for recovery of grape seed oil, which is a well-established value-added ingredient, they were retained in the sieve and the pomace flour (PF) was packed under vacuum and stored in a desiccator, at room temperature, until use.

PF was analyzed, in triplicate, for moisture content, ash, protein and total dietary fiber through AOAC methods (2007, 2010a, 2010b), and for fat content through AOCS method (2009). Determination of cellulose and hemicellulose content was carried out according to NREL methodology (2012).

3.2.2. Chemical production of XOS

Chemical production of XOS were performed in an autoclave at 120 ºC for 90 min, using 5 g of PF. Sulfuric acid (acid extraction) or sodium hydroxide (alkaline extraction) were used as solvents at different concentrations. After cooling at room temperature, samples were filtered under vacuum with Whatman filter paper No.1.

The best parameters of acid and alkaline extractions were determined by response surface methodology, according to a 2^2 central composite design, using the XOS extract yield as response. Two factors were analyzed as independent variables: S:L ratio (X_1) and concentration of sulfuric acid or sodium hydroxide (X_2), being evaluated in five levels, according to Tables 3.1 and 3.2. The following polynomial equation was fitted to data:

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{12} x_1 x_2 \quad (3.1)$$

Where βn are constant regression coefficients, y is the response (XOS yield), and x_1 and x_2 are the coded independent variables (S: L ratio and H_2SO_4 or NaOH concentration, respectively).

Table 3.1 – Coded variables studied in the XOS produced by acid hydrolysis.

Coded variables	**-1.41**	**-1.0**	**0**	**+1.0**	**+1.41**
S : L ratio	1 : 8	1 : 10	1 : 14	1 : 18	1 : 20
H_2SO_4 concentration (%)	0.1	0.8	2.5	4.2	4.9

After the definition of the best production conditions, the kinetics of chemical extraction was determined for 120 min.

Table 3.2 – Coded variables studied in the XOS produced by alkaline hydrolysis.

Coded variables	**-1.41**	**-1.0**	**0**	**+1.0**	**+1.41**
S : L ratio	1 : 8	1 : 10	1 : 14	1 : 18	1 : 20
NaOH concentration (%)	0.2	1.6	5.0	8.4	9.8

3.2.3. Enzymatic production of XOS

Enzymatic extractions were performed as alternative to chemical treatment, using an enzymatic cocktail with xylanase activity, produced at our lab, and commercial Viscozyme® L cellulolytic enzyme mixture from Novozymes (Bagsvaerd, Denmark).

3.2.3.1. Xylanase production

Aspergillus niger 3T5B8 conidia, stored in sterile soil at -18 °C, were transferred for agar medium and incubated at 32 °C for 7 days. Conidia were then removed with 20 mL of

sterilized Tween 80 0.3% and 1 mL of spore suspension was transferred for inoculation in 4.6 g of corn cob enriched with 6 mL of aqueous solution containing: 22.8 µL $KHPO_4$ 20% (w/w), 0.118 mg $ZnSO_4$, 0.138 mg $Fe_2(SO_4)_3$, 0.3 µg $MnSO_4$, 0.0015 µL H_2SO_4 95% and 336 mg peptone, and finally incubated at 32 °C for 5 days. The initial moisture content, determined gravimetrically, was adjusted to 60%. All liquid added to the flask was taken into consideration for calculating the moisture content.

Solid-state fermentation was conducted in columns, using wheat bran as substrate. Briefly, 100 g of substrate were supplemented with 60 mL of $(NH_4)_2SO_4$ 0.91% in HCl 0.1 M, sterilized and conidia were inoculated at final concentration of 10^7 spores.g^{-1}. Inoculated media was transferred to the fermentation columns, which were incubated in a fermentation bath at 32 °C with adjusted aeration.

To extract the enzyme, fermented matter was mixed with citrate buffer (0.05 M, pH 4.8) at a S:L ratio of 1 g initial dry substrate to 2.5 ml buffer. The mixture was incubated at 32 °C for 60 min. Subsequently, solids were separated from the extract by centrifugation at 13,500 ×*g* for 15 min. The supernatant was filtered through Whatman No. 1 filter paper to obtain a clear extract and assayed for xylanase activity by the method of Gomes *et al*. with slight modifications (Gomes *et al*., 2002).

3.2.3.2. Determination of enzyme activity

The release of reducing sugars in 30 min, at 50 °C and pH 5.0 (0.2 M sodium acetate buffer), was measured as xylose equivalents using dinitrosalysilic acid (DNS) method, using 1% birchwood xylan solution as substrate (Gomes *et al*., 2002; Miller, 1959). The method was performed in triplicate. One unit of xylanase activity (U) is defined as the amount of enzyme liberating 1 µmol of xylose.min^{-1}, under assay condition.

3.2.3.3. Production of XOS

Enzymatic production of XOS was performed with the enzymatic cocktail obtained in 3.2.3.1 section and with Viscozyme®, with final xylanase activities of 10 and 100 UI.g^{-1} each, following the methods described by Sabiha-Hanim *et al*. (2011) and Goméz-García *et al*. (2012). Briefly, the enzyme mix was diluted in sodium acetate 0.2M buffer, in the desired enzymatic activity, and added to 100 mg of GP, with S: L ratio of 1:18. The pH was adjusted to 5.0 and incubated at 40 °C, with shaking at 200 rpm for up to 6 h. The reaction was stopped by heating the test tubes to 100 °C for 5 min and the supernatant was filtered through Whatman No.1 filter paper to obtain a clear extract.

3.2.4. Determination of total reducing sugars

Reducing sugars were analyzed through DNS method (Miller, 1959). DNS was added to 200 mL of NaOH 2N solution with a final concentration of 50 g.L^{-1}, homogenized and then 500 mL of Rochelle salt 0.6 g.mL^{-1} were added. The mixture was heated at 40 ºC and diluted to final volume of 1000 mL.

3.2.5. Quantification of monomeric and oligomer sugars: HPLC

Prior HPLC analysis, the acidic and alkaline extracts were neutralized with sodium hydroxide 20 M or sulfuric acid 0.01 M, respectively, and all samples were re-filtered with Millipore Millex syringe filter 0.22 µm. Identification and quantification of xylan-derived sugars (xylose, xylobiose (X_2), xylotriose (X_3), xylotetraose (X_4) and xylopentose (X_5)) were performed in Waters 600 HPLC equipped with Refractive Index Detector (RID) at 45 ºC (Waters Corporation, Milford, MA, USA), with an Agilent Carbohydrate 5 µm (4.6 x 250 mm) column at 30 ºC (Agilent, Santa Clara, USA), and based on the isocratic method described by Macrae (1998): the mobile phase was established by acetonitrile 70% with a

flow rate of 1 mL.min^{-1}. Compounds were identified by the comparison to the retention times of pure standards, as for xylose (Sigma, St. Louis, USA), and X_2, X_3, X_4 and X_5 (Megazymes, Wicklow, Ireland), and quantified through external standard calibration.

Percentage of XOS extraction yield was determined using equation 3.2., where 9.61 is the total xylan content in grape pomace flour.

$$\text{Production of XOS (\%)} = \frac{\text{Concentration of XOS } (\frac{\text{g}}{100\ \text{g}})}{\text{Total xylan in GP flour } (9.61 \frac{\text{g}}{100\ \text{g}})} \text{ x } 100 \qquad (3.2)$$

3.2.6. Statistical analysis

Statistical analysis was performed with IBM SPSS statistic program v 23.0 (Illinois, USA), using t-student test for independent samples and analysis of variance (ANOVA) with Bonferroni post hoc test. Differences were considered to be significant at a level of $p < 0.05$. For the central composite design, the analysis of variance (ANOVA), test for the lack of fit, determination of the regression coefficients and the generation of surface responses were carried out using the Statistica 7.0 software (StatSoft, Tulsa, USA).

3.3. Results and Discussion

3.3.1. Characterization of *Syrah* PF

Syrah GP was oven-dried at 45 ºC for 24 h and seeds were separated from the GP during the milling and sieving processes to obtain a PF with a particle size inferior than 300 µm, which was further analyzed for moisture content (95.9 g.kg^{-1}), ash (38.0 g.kg^{-1}), total protein (38.5 g.kg^{-1}), fat (13.4 g.kg^{-1}), sugars (543.0 g.kg^{-1}) and dietary fiber (271.2 g.kg^{-1}).

Structural carbohydrates were determined through acidic hydrolysis (NREL, 2012) and revealed high contents of glucose and xylose (119.5 and 84.6 $g.kg^{-1}$ of GP, respectively), a low concentration of arabinose (5.8 $g.kg^{-1}$ of GP) and no traces of mannose or galactose. As a lignocellulosic feedstock, GP was also analyzed, through the NREL method (2012), for its cellulose, hemicellulose and lignin content which were 132.7, 102.6 and 307.7 $g.kg^{-1}$, respectively. GP is usually rich in different classes of polysaccharides, and consists of 30% of neutral polysaccharides, including glucan, xylan, galactan, and mannans, depending on the grape variety (González-Centeno *et al*., 2010; Apolinar-Valiente *et al*., 2015). It also comprises ca. 20% of acidic polysaccharides, such as rhamnogalacturonans, arabinogalactans and pectins (González-Centeno *et al*., 2010). Due to the absence of mannose and galactose in the monosaccharide profile, and to the insignificant concentration of arabinose all the observed xylose was considered to be related to the xylan structure. Therewith, total xylan was determined based on xylose concentration and adjusted, corresponding to 96.1 $g.kg^{-1}$ of GP.

The carbohydrates profile is in accordance with the results reported by Zheng *et al*. (2012) for red GP (cellulose 14.5 wt % and hemicellulose 10.3 wt %), but lower than the values reported by Mendes *et al.* (2013) for *Touriga Nacional* (cellulose 20.8% and hemicellulose 12.5%). The protein content is in accordance with different pomace varieties such as *Cabernet Sauvignon* (31 $g.kg^{-1}$), *Callet* (27 $g.kg^{-1}$), *Manto Negro* (32 $g.kg^{-1}$), *Merlot* (38 $g.kg^{-1}$) and Syrah (33 $g.kg^{-1}$) (Mendes *et al*., 2013). Fat content in GP is mainly provenient from seeds oil. In this study, once the seeds were separated from the pomace using a depulper, a low fat composition was found, in accordance with previously reported data (Beres *et al.*, 2016). Dietary fiber and carbohydrate content had higher values than the other macromolecules, as expected for a vegetal matrix.

3.3.2. Chemical production of XOS

Total sugars present in the extracts obtained by chemical extraction varied from 174.4 to 825.7 $g.kg^{-1}$ of GP for acid extraction and from 96.8 to 361.2 $g.kg^{-1}$ of GP for alkaline extraction. Regarding XOS production, the extraction yields ranged from 21.83% to 74.58% for acid treatment and from 5.15 to 96.28% for alkaline treatment, as shown in Table 3.3. Strong acids, such as sulfuric acid used in the experiment, allow higher degree of polysaccharide hydrolysis and therefore, more simple sugars are produced. However, this hydrolysis is dependent on the acid concentration, which explains the high variable concentration of sugars present in the acid extracts when compared to the alkaline extracts.

Table 3.3 - XOS produced (%) in the acid and alkaline extractions

Acid hydrolysis				**Alkaline hydrolysis**			
Trial	**S : L**	**H_2SO_4 conc. (%)**	**XOS produced (%)**	**Trial**	**S : L**	**NaOH conc. (%)**	**XOS produced (%)**
1	1 : 10	0.8	49.49	**1**	1 : 10	1.6	21.12
2	1 : 18	0.8	74.58	**2**	1: 18	1.6	89.95
3	1 : 10	4.2	40.92	**3**	1 : 10	8.4	33.94
4	1 : 18	4.2	60.10	**4**	1 : 18	8.4	96.28
5	1 : 8	2.5	21.83	**5**	1 : 8	5.0	10.57
6	1 : 20	2.5	48.49	**6**	1 : 20	5.0	58.60
7	1 : 14	0.1	73.97	**7**	1 : 14	0.2	5.17
8	1 : 14	4.9	65.14	**8**	1 : 14	9.8	73.76
9	1 : 14	2.5	67.27	**9**	1 : 14	5.0	76.47
10	1 : 14	2.5	66.73	**10**	1 : 14	5.0	78.22
11	1 : 14	2.5	65.95	**11**	1 : 14	5.0	75.48

These results are in accordance with the results obtained by Chapla *et al.* (2012), who reported that dilute alkali extraction method was the most suitable method for the production

of xylan from raw corncobs. Alkali causes the swelling of lignocellulosic feedstocks, leading to a decrease in the degree of polymerization and crystallinity, separation of structural linkages between lignin and carbohydrates, and final disruption of lignin, helping to achieve a simple recovery of xylan from lignocellulosic feedstocks (Gokhale *et al*., 1998; Okeke and Obi, 1995). Dilute acid method extracted relatively less amount of xylan when compared to alkaline process.

Table 3.4 - Coded second-order regression coefficients for encapsulation efficiency and bulk density

Coefficient	XOS produced by acid hydrolysis	XOS produced by alkaline hydrolysis
β_0	66.63	72.47
β_1	-4.45	13.36
β_2	10.26	24.18
β_{11}	2.47	--
β_{22}	-14.82	--
β_{12}	-1.48	-10.23
R^2	0.980	0.966
F	**48.76**	**28.67**

Table 3.4 shows the regression coefficients for the coded polynomial equations, the F values and the determination coefficients (R^2). Some non-significant terms were eliminated and the resulting Modelling and analyzing the surface response for XOS recovery allowed to conclude that the most adequate operational conditions was achieved with NaOH at concentration of 8.4% and a S:L ratio of 1:18, with a yield of 96.28% for total extraction of XOS. Equations were tested for adequacy and fitness by the analysis of variance (ANOVA).

The fitted models were suitable, showing significant regression, low residual values, no lack of fit and satisfactory determination coefficients.

According to Figure 3.1, GP (solute) and solvent mass ratio was the parameter with the highest impact on the XOS production yield, for both acid and alkaline extractions. The use of higher volumes of solvent involves higher mass transfer gradient, resulting in higher production of XOS. In the case of alkaline extraction, XOS production showed a linear behavior and, thus, the axial points were not used in the model. In contrast, the acid extraction showed a significant curvature and therefore, in this case, the axial points were considered.

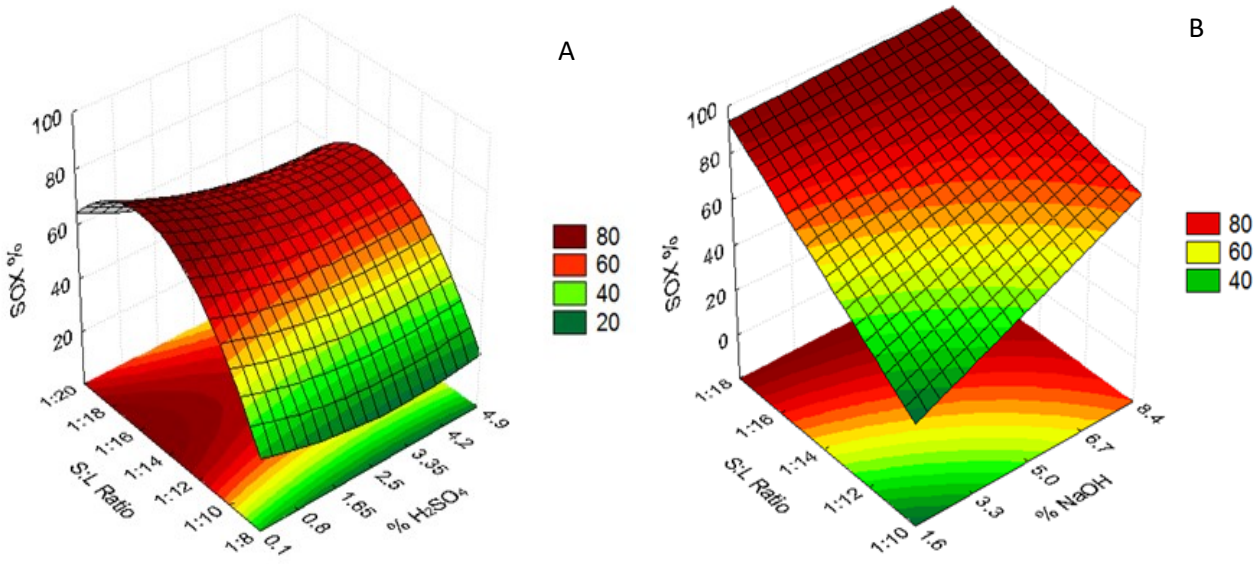


Figure 3.1 – Surface response for extraction through conventional methods: A) acid and B) alkaline.

Acid concentration showed a slight effect on XOS production (Figure 3.1A), while the increase of NaOH concentration led to the increase on the amount of recovered XOS, which can be a result from the more intense hydrolysis. This result could be explained by the concentration range of NaOH used in the alkaline hydrolysis (0.2 – 9.8%, w/v), which was higher than the concentration range of H_2SO_4 used for acid hydrolysis (0.1 – 4.9%, w/v),

resulting in more pronounced effects on the XOS production. The results from acid hydrolysis are in accordance with the results obtained by Akpinar and co-workers, who produced higher amounts of XOS with lower concentrations of H_2SO_4 (0.125 and 0.250 $mol.L^{-1}$) than with 0.5 $mol.L^{-1}$ H_2SO_4 (Akpinar *et al*., 2010). The results obtained by Sun and colleagues, who extracted XOS from perennial shrub using alkaline solvent, KOH, are in accordance with our results, as they produced more XOS when using higher concentrations of alkali (Sun *et al*., 2011).

The specific extraction of each XOS is shown in Figure 3.2. Chemical extraction only allowed the production of X_4 and X_5 from the pomace flour and an overlook to the results indicates that both methods extracted a majority of X_4 and X_5. In the acid extraction, trials 7, 8 and 9 (trial 9 corresponding to the average of extractions of XOS in the central point) had a significantly higher ($p < 0.05$) extraction of X_4, indicating that a middling mass ratio (1:14 in these cases) may be useful to control the degree of hydrolysis of xylan. On the other hand, trials 1 to 4 produced more X_5 but no significant differences were found between them, except for trial 2, which significantly produced more X_4 ($p < 0.05$). Alkaline extraction presented a more heterogeneous XOS recovery: trials 2, 4, 8 and 9 produced more X_5, while trial 6 extracted more X_4 ($p < 0.05$).

Although it is known that degree of polymerization of XOS affects their prebiotic effect, to be studied in the near future it is not so well studied how specific strains degrade and use XOS as carbon source. Efficient degradation of XOS by bacteria requires different enzymes, including β-xylosidase, α-glucuronidase, α-L-arabinosidase, or acetyl xylan esterase, thus degradation of XOS vary between strains (Sun *et al*., 2011). Gullón and co-workers studied the use of XOS by different Bifidobacteria and concluded that X_3 was the most consumed, followed by X_2, X_4 and X_5 (Gullón *et al.*, 2008). However, many authors use mixtures of XOS with different DP (2 – 6) as carbon source for *in vitro* fermentations of Bifidobacteria

(Okazaki *et al.*, 1990; Hopkins *et al.*, 1998). Considering all these variables, the best extraction condition was considered the one that extracted more amount of total XOS and not a specific xylooligosaccharide.

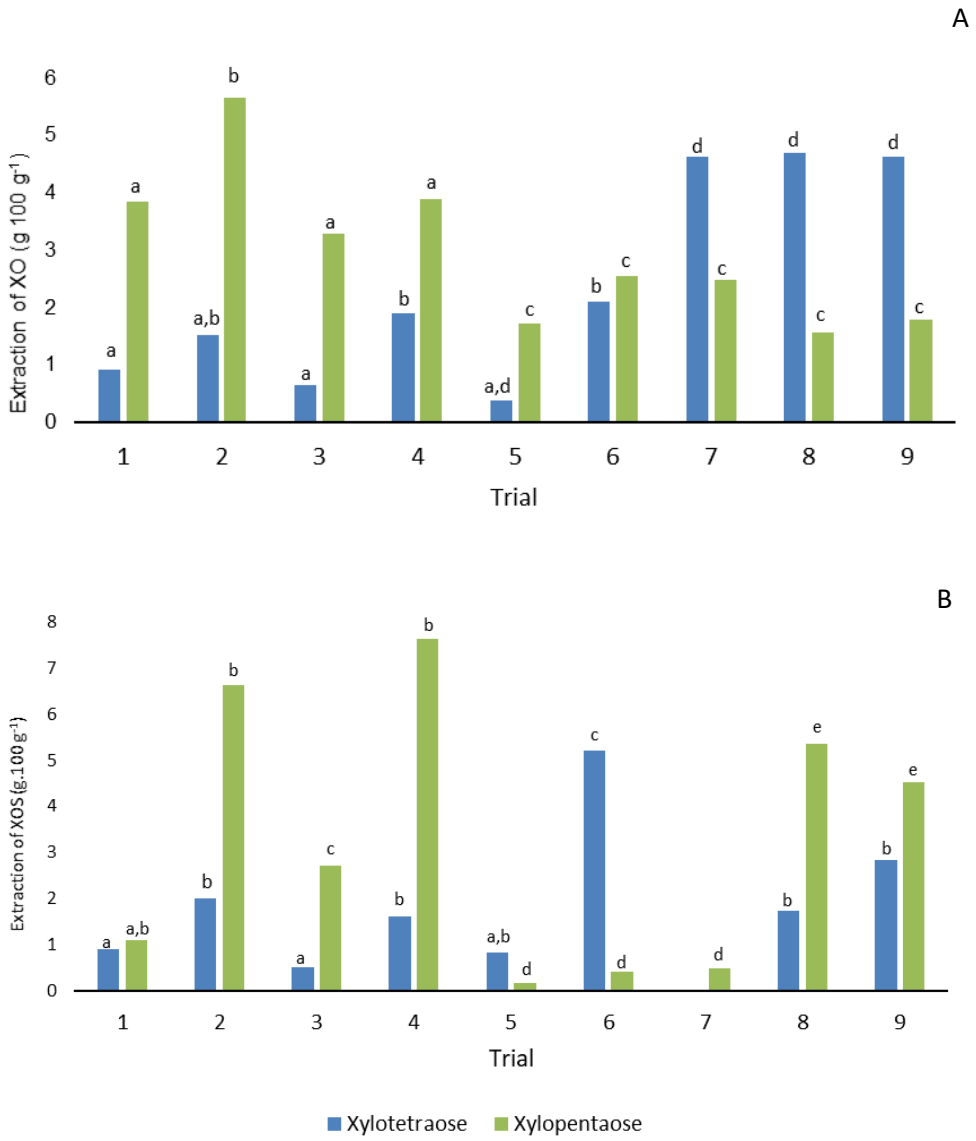


Figure 3.2 – Total extraction of each xylooligosaccharide for both conventional treatments: A) acid extraction and B) alkaline extraction.

As all extractions were performed for 90 minutes, an additional kinetics assay was performed in the selected best extraction condition (8.4% NaOH and S:L ratio of 1:18), in order to analyze the influence of extraction time on XOS produce. Results are presented in Figure 3.3, which shows that XOS extraction continuously increased up to 90 minutes and did not

show significant increase after 120 min of extraction ($p > 0.05$). This means that the optimal time of extraction would be 90 minutes, as used in the factorial planning, and the best condition for XOS extraction was 8.4% NaOH and 1:8 of S:L ratio.

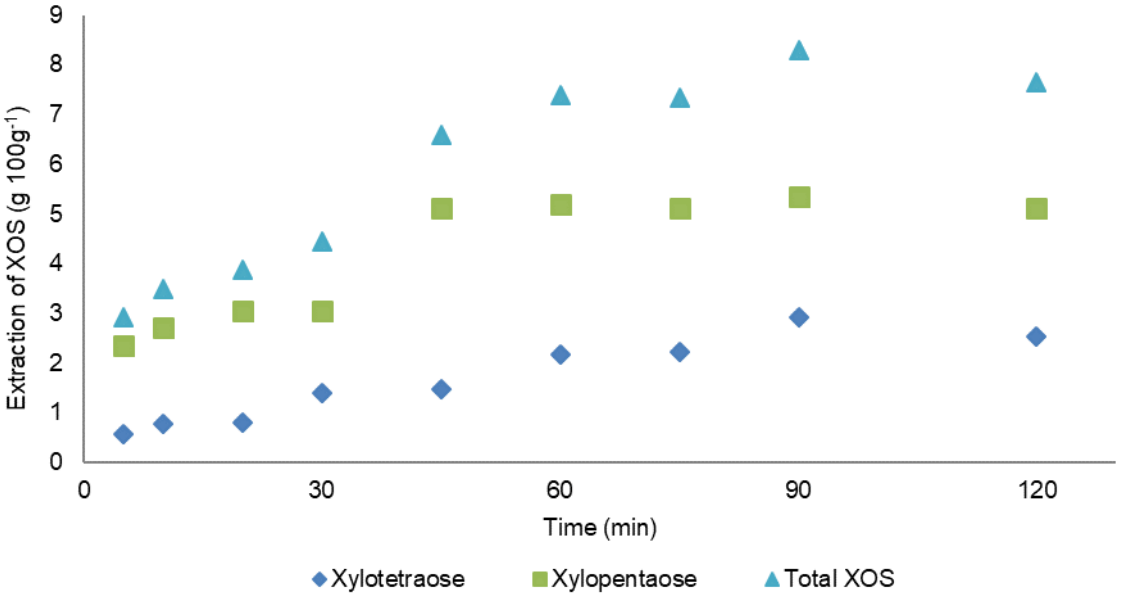


Figure 3.3 – Kinetics of conventional extraction of XOS using NaOH.

Although chemical treatments at high temperatures are efficient in breaking the ultrastructure of the cell wall, generating partial hydrolysates of polysaccharides, there are also undesirable reactions, such as the formation of monosaccharides that can easily originate toxic compounds like furfural and 5-hydroxymethylfurfural, requiring purification steps to remove them, and lately increasing the process costs (Okazaki *et al*., 1990; Akpinar *et al*., 2007) Thus, another alternative for obtaining XOS from plant material is the enzymatic treatment. Enzymatic hydrolysis using endoxylanases, in turn, prevents formation of toxic byproducts due to low temperature and high specificity, so it has been considered a good alternative for XOS production (Reddy and Krishnan, 2016; Akpinar *et al*., 2007; Yang *et al*., 2005).

2.3.3. Enzymatic extraction

2.3.3.1. Production of xylanases and determination of enzymatic activity

Production of XOS from various sources of xylan, such as sugarcane bagasse or cotton stalks, using commercial xylanases have been reported in different works (Reddy and Krishnan, 2016; Akpinar *et al*., 2007). However, fairly few attempts have been made for production of XOS using indigenously produced xylanases. In order to make the process cost effective and economic, xylanase used under the present study was produced with a low cost technique under optimized conditions using wheat bran as a substrate under solid state fermentation, as mentioned (Gomes *et al.*, 2002).

Viscozyme® (Novozymes, Bagsvaerd, Denmark) is an enzymatic cocktail with (endo-1,3(4)-) beta-glucanase, xylanase, cellulase and hemicellulase activities, produced by *Aspergillus aculeatus*. It was chosen as control for its hemicellulase and xylanase activities. Xylanase activity determined for the xylanase produced from *A. niger* 3T5B8 and for Viscozyme® were 28.77 ± 0.79 and 116.41 ± 4.27 $IU.mL^{-1}$, respectively. Data sheet of Viscozyme reports an activity of ca. 100 $U.g^{-1}$, which is in accordance with the results obtained. Although the produced enzyme is suitable for the extraction of XOS, it has a significantly lower xylanase activity than the enzymes produced by Chapla *et al.* (2009), 9200 ± 78.5 $IU.mL^{-1}$, who used *Aspergillus foetidus* MTCC 4898 instead of *A. niger* and applied additional steps for enzyme purification, including ammonium sulfate precipitation and dialysis, achieving a pure enzyme with high xylanase activity but also with additional costs of production (Chapla *et al*., 2013)

2.3.3.2. Enzymatic extraction

Enzymatic extraction was performed using the produced xylanase from *A. niger* 3T5B8 and Viscozyme®, with enzyme load of 10 and 100 IU.g^{-1}. Extraction times were 1, 2, 4, and 6 hours. Results of total extraction of XOS and xylose are presented in Figure 3.4.

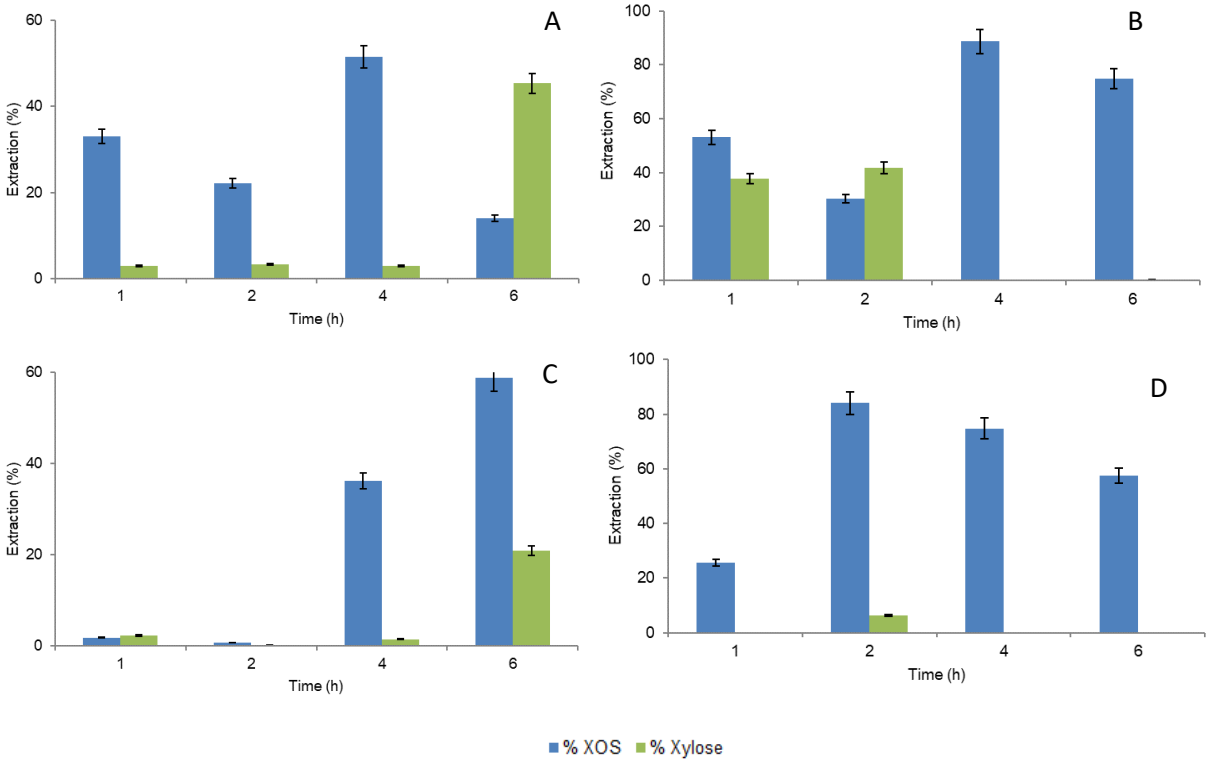


Figure 3.4 – Enzymatic extraction of XOS and xylose from grape pomace, using a mixture of enzymes from *A. niger* with final activity of (A) 10 IU.g^{-1} and (B) 100 IU.g^{-1}, and commercial Viscozyme with final activity of (C) 10 IU.g^{-1} and (D) 100 IU.g^{-1}.

Extraction of XOS using the mixture of enzymes produced by *A. niger* 3T5B8 with activity of 10 IU.g^{-1} for 1 and 4 hours, allowed to extract from 22.20 ± 0.87 and 51.52 ± 0.70% of XOS, respectively. After 6 hours of extraction, the amount of xylose extracted (45.39 ± 6.25 %) was much higher than the amount of XOS (14.09 ± 3.17%). The same mixture of enzymes with final concentration of 100 IU.g^{-1} allowed to produce the maximum amount of XOS (88.68 ± 0.13%) after 4 h of extraction, without producing xylose monomers. The other

extraction times allowed to produce from 13.00 ± 0.38 to 74.83 ± 3.89% of XOS. While the extraction of 4 and 6 h produced no xylose monomers, the other extraction times produced higher amount of xylose, not being suitable for XOS extraction.

The use of 10 $IU.g^{-1}$ of Viscozyme® had a slight produce of XOS within 1 and 2 h. Extraction at 4 h produced 36.26 ± 0.13% of total XOS with minimal concentration of xylose. Extraction for 6 h had superior extraction of XOS but also a superior production of xylose: 20.88% of sugar monomers. At last, the use of 100 $IU.g^{-1}$ of Viscozyme® allowed to recover from 25.60 to 84.09 ± 2.40% of total XOS, without production of any xylose monomers. This data is in accordance with the results obtained by Akpinar and co-workers, who used a commercial xylanase (Veron 191 from *A. niger*, AB Enzymes, Germany) to extract XOS from cotton stalks, and produced ca. 53% of total XOS (Akpinar *et al*., 2009). However, these authors produced a majority of X_6 and X_5 instead of the X_4 recovered in this work (Table 3.5).

Although extractions with Viscozyme® produced more quantity of XOS ($p = 0.050$), the use of our enzyme (condition of 100 $IU.g^{-1}$ for 4 h) allowed to extract XOS with a minimum amount of xylose monomers. This capacity could be due to the substrate composition or due to other enzymatic activities associate with our enzymatic cocktail that were not analyzed (hemicellulolytic activity, for instance). Extractions performed with 100 $IU.g^{-1}$ of enzyme allowed to produce more quantity of XOS than extractions with 10 $IU.g^{-1}$ ($p < 0.05$), in accordance with the results obtained by Akpinar *et al.,* who concluded that xylan hydrolysis yield increases with enzyme concentration as production of XOS using lower enzyme concentrations is too slow (Akpinar *et al*., 2007). The use of both enzymes for enzymatic treatment of GP allowed to produce high amounts of X_6 but also some X_5, depending on the quantity of enzyme used and time of incubation. Detailed data is show in table 3.5.

Table 3.5 - Content ($g.kg^{-1}$ of grape pomace) of each xylooligosaccharide present in the enzymatic extracts of grape pomace

	3T5B8		**Viscozyme**	
	10 UI.g^{-1}	**100 UI.g^{-1}**	**10 UI.g^{-1}**	**100 UI.g^{-1}**
1 h	$X_4 = 31.8$	$X_4 = 22.2$ $X_5 = 28.9$	-	$X_4 = 49.2$
2 h	$X_4 = 21.3$	$X_4 = 10.6$ $X_5 = 18.5$	-	$X_4 = 80.8$
4 h	$X_4 = 49.5$	$X_4 = 85.2$	$X_4 = 34.8$ $X_5 = 0.1$	$X_4 = 71.9$
6 h	$X_4 = 13.5$	$X_4 = 71.9$	$X_4 = 55.9$ $X_5 = 0.7$	$X_4 = 55.3$

In conclusion, enzymatic extractions proved to be as efficient as acid or alkali, without the need of using environmental unfriendly solvents, and should be used in the future for studies on bioactivities.

3.4. Conclusion

The present study demonstrated different feasible methods to produce high added value molecules, the xylooligosaccharides, from *Syrah* GP flour, as an alternative to valorize this major by-product. Enzymatic XOS production is affected by the enzyme type and enzyme loading: xylanases mixture produced by *A. niger* 3T5B8 at 100 $UI.g^{-1}$ was found to be the most suitable condition. The use of enzymatic cocktails demonstrated to be an alternative to the conventional methods, as they allowed to obtain similar yields of XOS, but within an eco-friendly and sustainable grape pomace extract. Also, in both chemical and enzymatic extracts, produced XOS were mainly composed by X_4 and X_5. The biological activities of XOS, particularly their described biological activities with impact on gastrointestinal health, allow to conclude that these enzymatic GP extracts can be a potential candidate to be used

in the development of a new functional ingredient. The development of such product requires further studies on biological activities, specifically the demonstration of prebiotic activity and absence of toxicity.

CHAPTER 4

Impact of in vitro gastrointestinal digestion on chemical composition, bioactive properties and cytotoxicity of a *Vitis vinifera* L. cv. *Syrah* grape pomace extract

Abstract

Grape pomace is well-known for its bioactive compounds, such as fibers and polyphenols that are popular for their impact upon human health, including gastrointestinal health. The objective of this work was to evaluate the chemical composition and biological activities of an enzymatic GPE, and to investigate how the GID modulates these properties. GPE was previously produced using an enzymatic cocktail with xylanase activity and was then exposed to simulated conditions of GID, characterized for its chemical composition and screened for antimicrobial, prebiotic and antioxidant activities. The safety of this ingredient after GID was also assessed. GPE presented high content of dietary fibre and other carbohydrates, including XOS, besides minerals and phenolic compounds. *In vitro* simulated GID allowed to conclude that X_2 was resistant to gastric conditions, unlike polyphenols. The use of 2% (w/v) of this ingredient proved to be a potential carbon source that could be fermented by *Lactobacillus* and *Bifidobacterium* spp, even after GID. The GPE also exhibited strong antioxidant and antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*, however, after GID, the antioxidant capacity

decreased, and the antimicrobial capacity was strongly reduced or lost. Furthermore, the extract safety was also guaranteed on Caco-2 intestinal cells. This novel and green GPE proved to be composed by relevant bioactive molecules, including XOS, polyphenols, organic acids and minerals, which provided different biological properties, with potential application in food industry to be used as an ingredient in the development of new functional foods.

4.1. Introduction

Circular Economy has been receiving increasing attention over the last decade, as it brings together both agroindustrial sustainability and social-economic concerns, throughout the looking for innovative solutions for waste disposal, taking advantage of renewable resources, while improving both anthropological and environmental health (Ghisellini *et al*., 2016; Liguori and Faraco, 2016; Geissdoerfer *et al*., 2017). Agriculture and food processing industries produce the most significant and promising by-products, which, despite all their biological potential for food, cosmetic or nutraceutical applications, are being used as plant fertilizers or animal feed, or simply incinerated (Oliveira *et al*., 2013).

Grapes are one of the most cultivated fruit crops worldwide, with ca. 67 million tons produced annually, from which more than 70% is intended to wine industry, which generates up to 20% of wasted biomass in the form of grape skin, seeds, stems and residual pulp, known as GP (FAO, 2016; Spanghero *et al*., 2009; Corbin *et al*., 2015). GP is a lignocellulosic biomass, rich in neutral polysaccharides and bioactive compounds that has been exhaustively studied for the extraction of its bioactive phenolic compounds, but slight studied concerning the potential of its dietary fiber and polysaccharides (Bonilla *et al*., 1999; Negro *et al.*, 2003; Pinelo *et al*., 2005; Spigno *et al*., 2007; Vatai *et al*., 2009; Llobera and Cañellas, 2007; Beres *et al*., 2016).

In 1998, Saura-Calixto introduced the concept of antioxidant dietary fibre, a natural compound rich in both dietary fiber and polyphenols, which has been reported as beneficial for human health, as it stimulates colonic fermentation and regulates the blood glucose and insulin response, obesity and cardiovascular diseases (Elleuch *et al*., 2011; Roberfroid *et al*., 2010). Among other molecules found in GP, oligosaccharides from the hemicellulose fraction are recognized for their prebiotic activity due to their resistance to gastric acidity and to mammalian enzymatic hydrolysis, and their capacity to selectively stimulate the gut microbiota growth. This property, in combination with other reported bioactivities, makes GP a promising by-product that can be converted into a high added value ingredient with potential food application (Roberfroid *et al*., 2010; Broekaert *et al*., 2011).

Another bioactivity reported for GP and other alcohol-free winery products is its antimicrobial capacity, especially against the microorganisms related to food spoilage and intestinal infections, including *Staphylococcus aureus*, *Escherichia coli*, *Salmonella infantis* and *Candida albicans* (Oliveira *et al*., 2013) The antimicrobial activity of grapes, grape byproducts and wine is usually associated to the presence of polyphenols such as resveratrol and anthocyanins, or even to phytoalexins produced by the grapevine under stress conditions including fungal attack or high UV exposure (Oliveira *et al*., 2013).

In order to assure the feasibility of the by-product valorization process, it is necessary an environment friendly method to obtain fiber-rich extracts with potential bioactive properties. In this sense, enzymatic hydrolysis is one of the mildest processes to achieve a final extract with the desired chemical and biological characteristics (Binod and Pandey, 2015; Chamorro *et al*., 2012). Using such methods, it has previously been developed a novel enzymatic grape pomace extract, which composition rich in fibers and XOS indicates a potential biological activity, although it was not further studied for its chemical composition that could grant these properties (Costa *et al*., 2018).

So, the objective of this work was to perform an extensive chemical characterization of an enzymatic GPE and evaluate a group of important bioactive properties, including antioxidant, antimicrobial, and prebiotic activities, trying to relate chemical properties to its biological activity. Furthermore, evaluation of these bioactive properties upon *in-vitro* GID was also performed to understand the stability and bioaccessibility of the main bioactive compounds present in this multifunctional extract.

4.2. Material and Methods

4.2.1. Raw Material

Syrah grape pomace (*Vitis vinifera L. cv. Syrah*) from red sparkling production was provided by Ouro Verde Winery (Miolo Wine Group), located at Vale do São Francisco, Bahia, Brazil. The pomace was oven-dried at 45 ºC for 24 h, milled and sieved to obtain a powder.

4.2.2. GPE production

GPE was obtained by enzymatic extraction using the enzymes cocktail produced at our lab by solid state fermentation as previously described. Briefly, the enzymatic cocktail, with final xylanase activity of 100 $IU.g^{-1}$, was added to 50 g of grape pomace in sodium acetate buffer 0.2 M at S:L ratio of 1:18, pH was adjusted to 5.0, and the mixture was incubated at 40 °C with shaking at 200 rpm for 4 h. The reaction was stopped by heating the test tubes to 100 °C for 5 min, and the supernatant was vacuum-filtered using Whatman No.1 filter paper. The soluble fraction (GPE) was then freeze-dried (Telstar LyoQuest, Spain).

GPE was characterized for moisture content, ashes, total dietary fiber and total protein using AOAC methods (2007, 2010a, 2010b), and for its bioactive properties (antioxidant, antimicrobial, and prebiotic activities), as described later.

4.2.3. *In vitro* simulation of GID

Simulation of the effect of digestive tract upon GPE was performed following the method described by Madureira *et al.* with slight modifications, by dissolving 900 mg of the lyophilized GPE into 20 mL of ultra-pure water (Madureira *et al*., 2011).

Mouth Digestion: The pH was adjusted to 6.9, using HCl 1 M. Artificial saliva was simulated by using α-amylase (Sigma-Aldrich Chemistry, St. Louis, Missouri, USA) at 100 $U.mL^{-1}$, and added at a rate of 0.6 $mL.min^{-1}$ of digestion. Incubation was made for 2 min at 37 ˚C and 200 rpm.

Stomach Digestion: The pH was adjusted to 2.0 using HCl 1 M. Gastric juice was simulated by dissolving pepsin (Sigma-Aldrich Chemistry, St. Louis, Missouri, USA) 25 $mg.mL^{-1}$, and added at a ratio of 0.05 $mL.mL^{-1}$ of sample. Incubation lasted 120 min (long digestion), at 37 ˚C and 130 rpm.

Gut digestion: Simulation of gut conditions was performed by initial adjustment of pH to 6.0 using $NaHCO_3$ 1 M. The intestinal juice was simulated by dissolving 2 $g.L^{-1}$ of pancreatin (Sigma-Aldrich Chemistry, St. Louis, Missouri, USA) and 12 $g.L^{-1}$ bile salts (Oxoid™, Hampshire, UK). This solution was then added at a concentration of 0.25 $mL.mL^{-1}$ of sample. All samples were incubated during 1 h, at 37 ˚C and 45 rpm.

After gut digestion, enzymes were removed using 3 kDa cut-off filters and the sample was freeze-dried. This digested GPE (DGPE) was characterized for its bioactive properties (antioxidant, antimicrobial, and prebiotic activities).

All assays were performed in triplicate. After each stage the monosaccharide and oligosaccharide profiles were assessed by HPLC-RID, as described above.

4.2.4. Carbohydrates and organic acids composition

Identification and quantification of carbohydrates and organic acids were performed by High-performance liquid chromatography – HPLC, coupled to RI and diode array (DAD) detectors. Before HPLC analysis, lyophilized samples were re-suspended in ultra-pure water and re-filtered with Millipore Millex syringe filter of 0.22 μm.

4.2.4.1. Identification and quantification of monosaccharides

Monosaccharides were analyzed by HPLC-RID, using a Beckman Coulter System Gold HPLC with RID (Knauer, Berlin, Germany). Identification and quantification were performed with an Aminex HPX-87P (Bio-rad, Berkeley, USA) column, using ultrapure water as mobile phase and an isocratic flow rate of 0.5 $mL.min^{-1}$ (Zappa *et al*., 2001). All compounds were identified and quantified by comparison to the retention times of pure standards (xylose, fructose, mannose, and glucose) (Sigma Aldrich, St. Louis, USA), and using a calibration curve in the range of concentrations of 0.2 - 2.0 $mg.mL^{-1}$.

4.2.4.2. Quantification of XOS and identification of polysaccharides

Oligosaccharides and polysaccharides were also analyzed by HPLC-RID, using a Waters Ultrahydrogel 120 (7.8 x 300 mm) and a Ultrahydrogel 250 (7.8 x 300 mm) columns, with ultrapure water as mobile phase, and an isocratic flow rate of 0.5 $ml.min^{-1}$ (Gullón *et al*., 2014a). XOS were identified by comparison to the retention times of pure standards: xylobiose (Sigma, St. Louis, USA), xylotriose, xylotetraose, and xylopentose (Megazymes, Wicklow, Ireland), using a calibration curve in the range of concentrations of 0.1-2 $mg.mL^{-1}$.

High molecular weight polysaccharides were identified by comparison to the retention times of Shodex (Showa Denko K. K., Tokyo, Japan) Pullulan P-82 standards, ranging from P-5 (5.9 kDa) to P-800 (708 kDa).

4.2.4.3. Identification of organic acids

Organic acids were analyzed in a Beckman Coulter System Gold HPLC equipped with a DAD detector (System Gold 168 Detector, Beckman Coulter) at wavelength of 220 nm, using an Aminex HPX-87H (Bio-rad, Berkeley, USA) column with sulfuric acid 13 mM as mobile phase and an isocratic flow rate was 0.5 $mL.min^{-1}$ (Zappa *et al*., 2001). All organic acids were identified by comparison to the retention times of pure standards: citric, tartaric, malic, succinic, lactic, formic and acetic acids (Sigma-Aldrich, St. Louis, USA), using a calibration curve in the range of concentrations of 0.1 – 2 $mg.mL^{-1}$.

4.2.5. Mineral Analysis

The digestion of GPE and DGPE for mineral analysis was performed following the method described by Roriz *et al.*, with slight modifications (2014). Briefly, 250 mg of samples were mixed in a Teflon vessel with 6 mL of HNO_3 65% and 1 mL of H_2O_2 30% and heated in a microwave system (Berghof, Eningen, Germany). Digestion procedure was conducted in five steps: 130 °C for 5 min; 170 °C for 10 min; 200 °C for 15 min; 100 °C for 2 min; and 100 °C for 2 min. After microwave digestion, samples were cooled down to room temperature and diluted to a final volume of 50 mL.

Microwave-digested samples were then analyzed through Inductively Coupled Plasma Atomic Emission Spectrometry (Perkin Elmer, Massachusetts, USA) and quantify through internal standard calibration. All analysis were performed in triplicate.

4.2.6. Determination of Phenolic Compounds

4.2.6.1. Total Phenolics

Concentration of total phenolic compounds (TPC) was determined colorimetrically by Folin–Ciocalteu method (Singleton and Rossi, 1965). GPE and DGPE were dissolved in ultra-pure water, at concentration of 200 $mg.mL^{-1}$. Quantification was done at 750 nm (UV mini 1240, Shimadzu, Tokyo, Japan) with gallic acid as standard in the range of 0.015–1.00 $mg.mL^{-1}$.

4.2.6.2. Identification and quantification

Qualitative and quantitative profiles of polyphenols were carried out by HPLC-DAD, according to the method described by Oliveira *et al* (2015 b). Analysis was conducted on a Waters Liquid Chromatograph (Waters Alliance, Mildford MA, USA), using a C18 guard column (Symmetry® C18) and an Alltech adsorbosil C18 reversed-phase packing column (250 x 4.6 mm i.d. 5 μm particle size and 125 Å pore size. Mobile phase: Solvent A: acetonitrile 100% with 0.2% TFA; Solvent B: acetonitrile/ water 5:95 (v/) with 0.2% TFA; flow rate = 1 $mg.mL^{-1}$. For HPLC analysis, GPE and DGPE were diluted in ultra-pure water at final concentration of 200 $mg.mL^{-1}$ and filtered using 0.45 μm syringe filters (Oliveira *et al*., 2015).

All compounds were identified and quantified by external calibration curve by comparison to pure standards (in the range of concentrations of 7 - 250 $\mu g.mL^{-1}$): caffeic acid, (+) cathechin, p-coumaric acid, rutin, syringic acid, vanillic acid, cyanidin-3-galactosidase, cyanidin–3-glucoside, peonidin-3-o-glucoside, and delphinidin-3-glucoside (Sigma Aldrich, St. Louis, USA); luteolin-7-o-glucoside and neochlorogenic acid (Extrasynthese, France).

4.2.7. Antioxidant capacity

4.2.7.1. ABTS Radical Scavenging Activity

The free radical-scavenging activity was measured using the $ABTS^+$ radical cation decolorization assay (Gião *et al*., 2007). Briefly, $ABTS^+$ solution was produced by reacting ABTS (7 $mmol.L^{-1}$) filtered using a 0.45 μm filter (Macherey-Nagel, Düren, Germany) with potassium persulfate (2.45 $mmol.L^{-1}$) in the ratio 1:1 (v/v). The ABTS+ solution was diluted with redistilled water to an absorbance of 0.700 ± 0.02 at 734 nm (Shimadzu 1240 UV–visible spectrophotometer). After addition of 1.0 mL of diluted ABTS+ solution to 10 μL of GPE and DGPE (dissolved in ultra-pure water at 200 $mg.mL^{-1}$), the absorbance reading was done exactly 6 min after initial mixing. Ascorbic acid was used as a standard to prepare a calibration curve in the range of 0.02 – 0.50 $mg.mL^{-1}$.

4.2.7.2. Oxygen Radical Absorbance Capacity (ORAC)

ORAC assay was performed according to the method described by Contreras *et al*. (2011). Briefly, the reaction was carried out at 40 ºC in 75 mM phosphate buffer saline (PBS, pH 7.4) and the final assay mixture contained 120 μL of fluorescein (70 mM), 60 μL of AAPH (14 mM) and 20 μL of antioxidant [Trolox (6.25–200 μM) or sample (GPE and DGPE (200 $mg.mL^{-1}$) also diluted (1:500 – 1:3200) in PBS]. The fluorescence was recorded during 137 min (104 cycles), using black polystyrene 96-well microplates, in a FLUOstar OPTIMA plate reader (BMG Labtech, Offnburg, Germany), with 485 nm excitation and 520 nm emission filters. AAPH and Trolox solutions were prepared daily and fluorescein was diluted from a stock solution (1.17 mM) in 75 MM PBS (pH 7.4). All reaction mixtures were prepared in duplicate and three independent runs were performed for each sample. The final ORAC values were expressed as μmol of Trolox equivalent per gram of GPE or DGPE (μmol $TE.g^{-1}$).

4.2.8. Antimicrobial activity

Antimicrobial activity of GPE and DGPE was determined against two Gram-positive bacteria - methicillin-Susceptible *Staphylococcus aureus* (MSSA) ATCC 25923, Methicillin-Resistant *Staphylococcus aureus* (MRSA) CCUG 60578, and two Gram-negative - *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 10145.

4.2.8.1. Determination of Minimal Inhibitory Concentrations (MICs)

MICs were determined using a microdilution assay, following the standards for antimicrobial susceptibility testing provided by the Laboratory Standards Institute (2012)). Inocula were prepared by suspending each bacterial colony, previously grown in nutrient agar media for 24 h, in calcium-adjusted Muller-Hinton (MH) broth in order to achieve a turbidity equivalent to a 0.5 McFarland standard (1×10^8 CFU.mL^{-1}). Inocula were then diluted in the same medium to reach a bacterial concentration of 10^6 CFU.mL^{-1} in each well of a 96-well microplate. MICs were determined by observing the lowest concentration of extract that completely inhibited bacterial growth. Sterile MHB was used as negative control. All assays were done in triplicate.

4.2.8.2. Growth inhibition curves

For determination of growth inhibition curves, inocula were prepared by suspending each bacterial colony into MH broth, with a final concentration of ca. 10^8 CFU.mL^{-1}. Twenty microliters of each inocula were transferred to a 96-well microplate and every well was fulfilled (to final volume of 200 µL) with 2% (w/v) of GPE and DGPE diluted in MH broth. Microplate was incubated in a microplate reader (Multiskan GO, Thermo Scientific) at 37 °C for 24 h, with absorbance measurements at 620 nm registered every hour. Three controls were also performed: the first one containing inoculum and MH broth (positive control), the second one containing the solubilized extracts (negative control) and the third one containing only MH broth. All assays were done in triplicate.

4.2.9. Prebiotic potential

The GPE and DGPE were tested for potential of prebiotic activity using two different *in vitro* methods using pure probiotic, the first one through the evaluation of growth curves using microplate assay and the other through *in vitro* fermentation assay to assess the metabolic activity using.

4.2.9.1. Growth curves *via* microplate assay

Potential prebiotic effect of GPE was determined for *Bifidobacterium animalis* Bo (CSK, Ede, Netherlands), *Bifidobacterium longum* BG3 (Cell Biotech, Hellerup, Denmark), *Bifidobacterium animalis* spp. *lactis* Bb12, *Lactobacillus casei 01* (Chr. Hansen, Hørsholm, Denmark), and *Lactobacillus rhamnosus R11* (Lallemand, Montreal, Canada). Strains were stored at −80 °C in de Man–Rogosa–Sharpe (MRS) broth (Biokar Diagnostics, Beauvais, France) with 30% (v/v) glycerol.

L. casei 01 and *L. rhamnosus R11* inocula were prepared by suspending each bacterial colony into MRS broth, achieving a turbidity equivalent to 0.5 McFarland standard, and then diluting to reach the recommended concentration of probiotic bacteria in wells, $5x10^5$ $CFU.mL^{-1}$. Twenty microliters of each inocula were transferred to a 96-well microplate and every well was fulfilled (to the final volume of 200 µL) with the GPE diluted in basal MRS broth without glucose, at concentrations of 1 and 2% (w/v). Microplate was incubated (Multiskan GO, Thermo Scientific) at 37 °C for 24 h with agitation.

B. animalis Bo, *B. longum* BG3 and *B. lactis* BB12 inocula were prepared under anaerobic atmosphere, by suspending each bacterial colony into MRS broth supplemented with 0.05% (v/v) L-cysteine-HCl, achieving a final turbidity equivalent to 0.5 McFarland standard ($1x10^8$ $CFU.mL^{-1}$), and then diluted to reach the recommended concentration of probiotic bacteria in wells, $5x10^5$ $CFU.mL^{-1}$. Twenty microliters of each inocula were transferred to a

96-well microplate and every well was fulfilled (to final volume of 200 µL) with the GPE, diluted in basal MRS broth without glucose at concentrations of 1 and 2% (w/v). Microplate was sealed with paraffin and incubated at 37 °C for 48 h with agitation, with absorbance measurements at 620 nm registered every hour.

Three controls were also performed: the first one containing inoculum and MRS broth (positive control), the second one containing the solubilized extracts in MRS broth without glucose (negative control) and the third one containing only MRS broth.

4.2.9.2. *In vitro* fermentation assay

In vitro fermentation of DGPE was assessed using only two of the previous strains *Bifidobacterium animalis* spp. *lactis* BB12 and *Lactobacillus casei* 01. Before the assays, all strains were grown in MRS broth at 37 °C for 16 h. Anaerobic conditions were used for *B. animalis* Bb12, whereas aerobic atmosphere was used for *L. casei* 01.

Fermentation assays were carried out with ca. 10^6 CFU.mL^{-1} for each strain, in sterilized media (121 °C and 15 min) containing MRS-medium without glucose supplemented with 2% (w/v) of DGPE, 2% (w/v) fructooligosaccharides (FOS, used as reference) and MRS without carbon source (negative control) at 37 °C for 48 h. All experiments were carried out in duplicate.

Samples of the fermentation broths were withdrawn at 0, 4, 8, 24 and 48 h for metabolites analysis, throughout determination of the pH values and organic acids in the culture media. The pH was evaluated using a digital potentiometer (Crison Instruments, Spain), and organic acids were quantified by HPLC as described in section 3.2.3.3. Viable cells were quantified by plating on MRS agar (supplemented with cysteine hydrochloride in the case of *Bifidobacterium*) and incubation at 37 °C for 48 h (anaerobic and aerobic conditions were used for *Bifidobacterium* and Lactobacilli strains, respectively).

4.2.10. Evaluation of cytotoxicity

Cytotoxicity of GPE and DGPE was assessed in Caco-2 cells through XTT assay. For the assays the 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide sodium salt (XTT) solution was prepared as follows. Briefly, a 10 mM of Phenazine Methosulfate solution (Sigma-Aldrich, St. Louis, USA) was prepared in PBS (0.01 M; pH 7.4) and a 1 $mg.mL^{-1}$ XTT solution was prepared in Dulbecco's Modified Eagle's Medium (DMEM), previously warmed to 37 °C. Both solutions were sterilized using a 0.22 µm membrane filter (Millipore, Billerica, USA), mixed (2.5 µL of Phenazine Methosulfate per mL of XTT solution) and aliquots of XTT working solution were frozen until usage.

4.2.10.1 Cell line growth conditions

Human intestinal cell line (Caco-2) was obtained from Cell Line Services (Appenheim, Denmark). The cells were cultured at 37 °C in a humidified atmosphere of 95% air and 5% CO_2, as monolayers using DMEM with 4.5 $g.L^{-1}$ glucose, L-glutamine without pyruvate (Lonza, Verviers, Belgium) containing 10% (v/v) fetal bovine serum (FBS, Biowest, Nuaillé, France) and 1% (v/v) Penicillin-Streptomycin-Fungizone (Lonza, Verviers, Belgium).

4.2.10.2. Biocompatibility assay

GPE and DGPE biocompatibility was assessed upon the Caco-2 cell line using the XTT colorimetric assay. Briefly, Caco-2 cells were seeded at 1 x 10^5 cells/well in a 96-well microplate and allowed to adhere. After 24 h, the media was removed and the cells were washed with PBS. Then, this media was added with GPE or DGPE at two different concentrations (10 and 20 $mg.mL^{-1}$). After 24 h, 25 µL of XTT working solution were added to each well and the cells were incubated, in the dark, for 2 h. The optical density at 485 nm

was then measured using a microplate reader (FLUOstar, OPTIMA, BMG Labtech, Ortenberg, Germany). All assays were performed in quintuplicate.

4.2.11. Statistics

Statistical analysis was performed with IBM SPSS statistic program v 23.0 (Illinois, USA), using t-student test for independent samples and analysis of variance (ANOVA) with Tukey post hoc test. Differences were considered to be significant at a level of $p < 0.05$.

4.4. Results and Discussion

4.3.1 GPE characterization

The nutritional composition of GPE extract is shown in Table 4.1. It is possible to observe that GPE is rich in soluble fiber, other carbohydrates and also in minerals. Glucose and fructose are the simple sugars present in the GPE, at similar concentrations ($p > 0.05$), with 14.90 ± 0.73 g.100g^{-1} of glucose and 11.91 ± 0.43 g.100g^{-1} of fructose. The GPE also presented a high concentration of XOS, especially X_2 at concentration of 5.57 ± 0.02 g.100g^{-1}. X_3 and X_4 were also present but at concentrations that could not be quantified by HPLC – RID (< 0.1 mg.mL^{-1}). The amount of extracted X_2 is in accordance with the results obtained by Kiran and co-workers, who produced XOS from cotton stalk, rice hull, wheat straw, corn cob and sunflower stalks mixture, and obtained yields of 6.94 and 7.22 g.100g^{-1} xylan (Kiran *et al*., 2013). The presence of other polysaccharides with molecular weights between 5 - 10 kDa and near 100 kDa was also identify through HPLC-RID.

Table 4.1 – Nutritional composition of grape pomace enzymatic extract.

	CONCENTRATION g.100 g^{-1}
Energy	929.7 kJ/ 222.2 kcal
Moisture	15.13 ± 0.30
Ashes	11.65 ± 0.38*
Protein	3.28 ± 0.03
Fat	6.73 ± 0.06
Fibre	26.08 ± 1.59
Carbohydrates	37.13 ± 1.74

*Buffer composition includes mineral concentration of 8.15 ± 0.03 g.100 g^{-1}

Some organic acids were also found in the GPE, being the acetic acid the most significant one, with a concentration of 2.24 ± 0.13 g.100g^{-1} of extract. Citric, tartaric, malic, and succinic acids are also present in the extract at concentrations of 1.95 ± 0.08, 0.34 ± 0.04, 0.30 ± 0.01 and 1.90 ± 0.11 g.100g^{-1}, respectively. While all these acids are characteristic of GP composition, acetic acid is present at higher quantity due to the enzymatic hydrolysis, as it was used for the acetate buffer preparation. The concentration of ashes present in GPE was also high and can be explained by the amount of salts used to prepare the buffers (sodium citrate) for the enzymatic extraction, which are present at a concentration of 8.15 ± 0.03 g.100g^{-1} of dry buffer. Nevertheless, this result indicates that almost all the minerals present in the grape pomace flour (38 g.kg^{-1} flour) were recovered.

4.3.2. Digestion of carbohydrates

In vitro gastrointestinal digestion (GID) models based on human physiology are important tools in assessment of polysaccharides during gastric and intestinal digestion, particularly because of their simplicity, low cost and bio-accessibility of nutrients (Hu *et al*., 2013). The

carbohydrate functions that affect bioavailability and health are determined by the chemical composition and the food matrix where are incorporated, and both of these aspects must be taken into account. As it is difficult to measure directly the biological function associated with different carbohydrate properties, *in vitro* methods provide rapid and reproducible measures (Englyst and Englyst, 2005).

The digestion of GPE was monitored through HPLC-RID for quantification of glucose, fructose and X_2 along the digestion. Results are presented in Figure 4.1 and indicate the mass (mg) of each sugar present in the initial extract and how they varied along the GI digestion. Initial amount of glucose was ca. 124 mg and no significant losses were observed during the mouth or stomach digestions ($p > 0.05$). The major increase was observed after the simulation of intestinal digestion, when the final mass of glucose was 155.01 mg, probably due to the use of pancreatin enzyme, mainly composed by amylases and proteases that could release some glucose monomers from polysaccharides with higher molecular weights.

As expected, xylobiose did not show any losses during upper gastrointestinal digestion and the initial amount of ca. 50 mg maintained until gut digestion. This result confirms its potential to be used as prebiotic, including the microbiota fermentation, as described in sections 4.3.7 and 4.3.8.

Furthermore, it is possible to observe from HPLC-RID chromatogram that the peak with retention time (RT) of 21.42 min, corresponding to the polysaccharide present in the range of 5-10 kDa, was hydrolyzed during the *in vitro* GID, especially during gastric digestion (RT = 26.39 min), achieving a molecular weight (MW) around 800 Da after gut (retention time 26.47 min). We can also observe that the peak for the substances present in the gut compartment, such as bile salts and pancreatin, appeared very late in HPLC-RID chromatogram, indicating that it did not contain high molecular weight polymers and thus, compounds used to simulate gut digestion did not affect determination of X_2 nor the MW of

GPE polysaccharide (Hu *et al*., 2013). Starch was reported to be degraded by gastric and intestinal media to oligosaccharides, and could be digested by amylase in small intestine to sugars that can be absorbed by the intestine (Heaton *et al*., 1998).

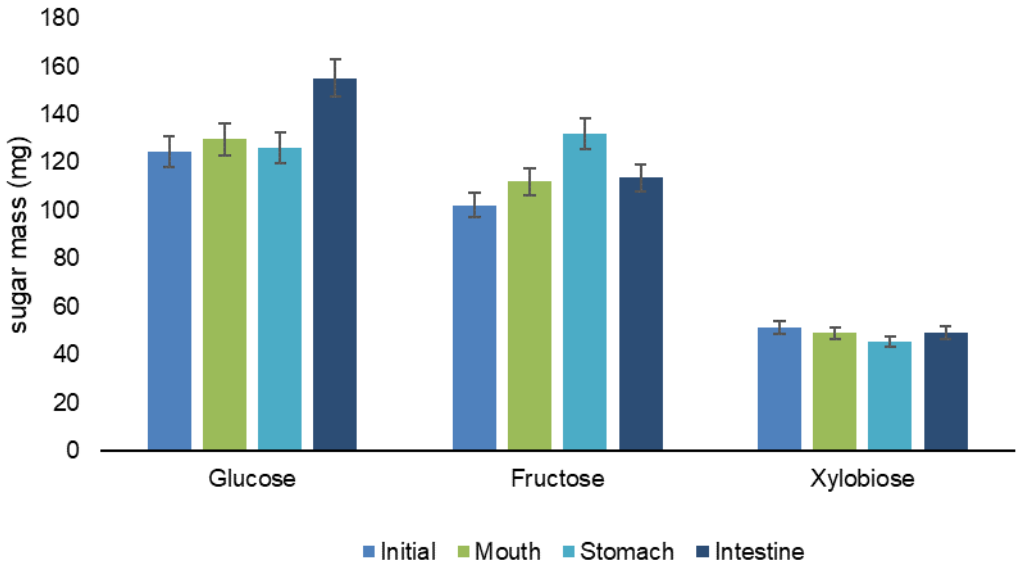


Figure 4.1 – Quantification of monosaccharides and oligosaccharides from GPE during gastrointestinal digestion simulation, expressed in mg.

4.3.3. Minerals

Owing to the high concentration of ashes in GPE, ICP-OEP analysis was performed in order to understand what elements were present. As expected, sodium (Na) was the element present in the highest concentration (9.97 ± 0.46 g.100g^{-1}), followed by potassium (K) (1.59 ± 0.33 g.100g^{-1}), as presented in Figure 4.2. Phosphorous (P), Boron (B), Magnesium (Mg) and Calcium (Ca) are also present at very low concentrations: 5.87 ± 0.42, 0.035 ± 0.02, 0.31 ± 0.27 and 1.58 ± 0.84 mg.g^{-1} sample, respectively.

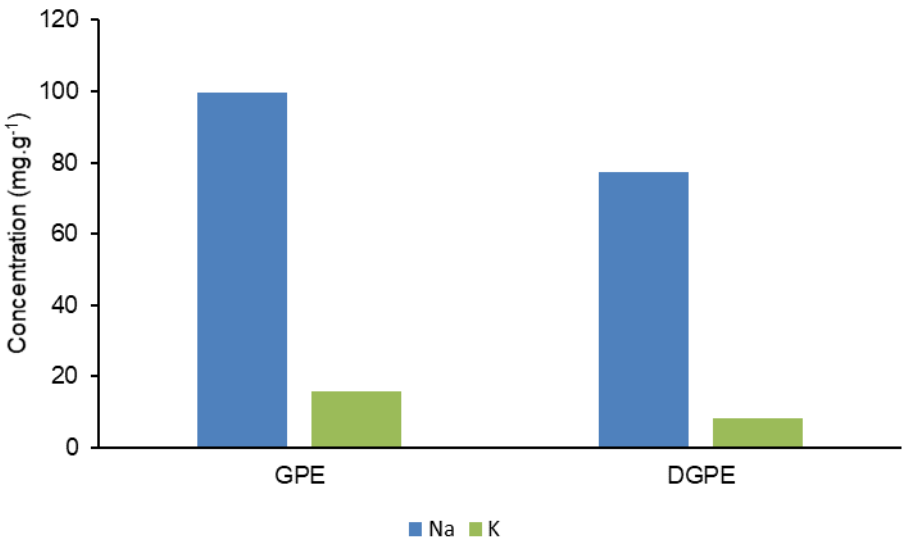


Figure 4.2 – Sodium and potassium concentrations (mg.g^{-1} samples) present in GP extract before and after digestion

During *in vitro* GID, part of Na and K present in GP extract were loss and the concentrations that reached gut were 7.72 ± 0.73 (Na) and 0.82 ± 0.072 (K) g.100g^{-1} sample, significantly lower than the concentrations present in the initial extract ($p < 0.05$). Part of Mg was also absorbed, as the concentration that reached the gut was only 0.13 mg.g^{-1} sample.

The colon contributes to electrolyte homeostasis by conserving sodium, chloride, and potassium. While sodium and chloride can be absorbed against large concentration gradients, potassium absorption requires a favorable concentration gradient (Phillips and Giller, 1973).

4.3.4. Phenolic Compounds

TPC were present in the GPE at concentration of 239.01 ± 4.44 mg GAE.g^{-1} of GPE. The concentration of phenolic compounds in GPE is similar to the reported by Ky and Teissedre, who recovered ca. 195.66 to 215.84 mg GAE.g^{-1} of grape seed (Ky and Teissedre, 2015). Higher yields of phenolic recovery are usually achieved with the use of organic solvents, such as ethanol or acetone, which were not applied in our extracts.

After GID, the concentration of TPC was significantly reduced to 13.59 ± 0.23 mg GAE.g^{-1} sample ($p < 0.05$). A drastically reduction of polyphenols was observed after *in vitro* GID, with only 5.69% of initial TPC reaching the gut. These results are in accordance with the literature that estimates that 90% of polyphenols are digested before reaching the intestine (Tarko *et al*., 2013; Van de Velde *et al*., 2018). This high reduction of TPC can be attributed to degradation of anthocyanins, the major polyphenols present in the GPE, by the acidic gastric conditions.

These results were confirmed by HPLC-DAD analysis (Table 4.2) of both extracts, where it is possible to observe the total anthocyanins degradation, as well as most of polyphenols identified in GPE, including (+) catechin, rutin, neochlorogenic acid and luteolin. These results are in accordance with other authors that concluded that intestinal digestion induce substancial losses in polyphenol families. Lingua and co-workers observed that only 6% of TPC were available in the dialysable fraction of red grapes extract, representing the potential colon available compounds; Sanz- Buenhombre and colleagues recovered part of the vanillic acid after the GID but verified that (+)catechin was completely degraded (Lingua *et al*., 2018; Sanz-Buenhombre *et al*., 2016). It is also possible to observe that syringic and vanillic acids were not detected in GPE but were the most abundant compounds in the DGPE, suggesting that these compounds were initially bound to other molecules, such as fibres that link with the phenolics through hydrogen bonds, hydrophobic interactions or covalent methyl-esterification, protecting them during the digestion.

The poor biaccessibility of phenols are in accordance with the results obtained by Van de Velde, who simulated the digestion of polyphenols present in blackberries and concluded that less than 98% of total anthocyanins were not available for absorption in the gut (Van de Velde *et al*., 2018). These losses are still not completely understood but are probably due to pH changes along gastrointestinal tract interaction with the digestive enzymes and other

chemical reactions that occur during GID such as interaction with other dietary compounds, or changes in solubility (Lingua *et al*., 2018; Giusti *et al*., 2019).

Table 4.2 – Major phenolic compounds found in GPE and DGPE (μg.g^{-1} sample).

RT (min)	λ_{max} (nm)	Phenolic Compound	GPE	DGPE
Non-anthocyanin phenolic compounds				
11.97	324.8	Neochlorogenic acid	302 ± 0.10	-
16.84	279.5	(+) catechin	190 ± 0.00	-
17.23	292.6	Vanillic acid	-	4.54 ± 0.02
25.55	354.7	Luteolin-7-*O*-glucoside	548 ± 0.10	-
26.29	353.5	Rutin	233 ± 0.01	-
26.37	308.9	*p*-coumaric acid	642 ± 0.14	1.47 ± 0.01
Anthocyanin phenolic compounds				
18.01	526.7	Delphinidin-3-*O*-glucoside	165 ± 0.08	-
20.17	518	Peonidin-3-*O*-glucoside	228 ± 0.10	-
20.95	528	Petunidin-3-*O*-glucoside	586 ± 0.12	-
23.77	529	Malvidin-3-*O*-glucoside	721 ± 0.16	-

4.3.5. Antioxidant capacity

Antioxidant capacity is the most studied bioactivity described for GP extracts, and it is related to the presence of polyphenols as well as the high content of antioxidant dietary fiber (Beres *et al*., 2016). However, the different methods, the different antioxidant standards used and the different references to the basic-unit of sample make it difficult to establish comparisons (Llobera and Cañellas, 2007). Hereupon, antioxidant capacity of GPE and DGPE was assessed through ABTS and ORAC methods.

The ABTS radical scavenging activity of GPE was 33.98 ± 0.464 mg AAeq.g^{-1} of GPE Although it is more common to find ABTS activity expressed as Trolox Equivalent (TE),

ascorbic acid (AA) is more hydrophilic and therefore, a more suitable reference to compare our water-soluble extract. Our GP extract radical scavenging activity was 7-fold higher than the results obtained by Oliveira *et al*. (2015a) for strawberry preparations, and within the range activity reported by Kwak *et al.* for different Rosa multiflora extracts, which ranged from 9.8 ± 3.2 to 84.8 ± 8.6 µg AAeq.mL^{-1} (Kwak *et al*., 2016). As expected, antioxidant capacity was lower ($p < 0.05$) for the digested extract, 16.77 ± 0.15 mg AAeq.g^{-1} extract, due to the high loss of polyphenols during GID, including all the anthocyanins, as discussed above.

Antioxidant activity determined by ORAC assay was 3500 ± 0.058 µmol TE.g^{-1} GPE and it is in agreement with the results obtained by Dudonné *et al*. for different aqueous plant extracts, such as *Cistus ladaniferus* (leaf) – 1410 ± 53 µmol TE.g^{-1}, *Eucalyptus globulus* (leaf) – 2846 ± 134 µmol TE.g^{-1}, *Jasminium grandiflorum* (flower) – 2330 ± 64 µmol TE.g^{-1} or Vanilla planifolia – 1593 ± 12 µmol TE.g^{-1} (Dudonné *et al*., 2009). After GID, the oxygen radical absorbance capacity of GPE was reduced to 385.80 ± 0.150 µmol TE.g^{-1} ($p < 0.05$), confirming the results obtained by ABTS method that GID reduces the antioxidant capacity.

Overall, this loss of antioxidant activity confirms the poor bioaccessibility of polyphenols described before, as these compounds largely contribute to the antioxidant bioactivity.

4.3.6. Antimicrobial activity

MICs of GPE were 14 mg.mL^{-1} for Gram-negative bacteria (*E. coli* and *P. aeruginosa*) and 16 mg. mL^{-1} for Gram-positive (MSSA and MRSA), enlightening that all microorganisms were susceptible to GPE.

Growth inhibition curves were performed upon *MSSA, MRSA, Escherichia coli* and *Pseudomonas aeruginosa.* These microorganisms were selected as representative of Gram-

positive and Gram-negative bacteria and using a marker of multiresistant bacteria such as MRSA. Growth inhibition curves for *selected microorganisms*, in the presence of GP and DGP extracts at concentration of 2% (w/v), as measured by turbidity at 660 nm, are presented in Figure 4.3. Corroborating the MIC determined (1.4 - 1.6 %) before, from Figure 4.3, the 2% GP inhibited completely the growth of all strains tested.

It is also possible to observe that the antimicrobial potential of the GPE was affected by the digestion process. DGPE did not affect the growth of Gram-negative bacteria (losing the antimicrobial activity) and although it still exhibits some reduction of the Gram-positive bacteria growth, is the reduction observed is much lower than with the GPE before digestion. The antimicrobial activity of the GPE may be attributed to the presence of several components, including minerals such as sodium and potassium, anthocyanins and some phenolic acids, organic acids such as acetic, citric and tartaric acids; besides the presence of XOS with a well-known antimicrobial activity (Cisowska *et al.*, 2011; Scalbert, 1991; Kallel *et al*., 2015). In fact, it is well-known that natural extracts display higher antimicrobial activity than selected compounds. Bassole *et al*. (2003) attributed antibacterial activity of fruit extracts to an overall synergy between bioactive compounds with antimicrobial properties and other minor components. In fact, the loss of antimicrobial activity after digestion of GPE could be explained by this hypothesis, as the reduction on the antimicrobial potential could be related to the loss of part of these components, including the total degradation of anthocyanins (Table 4.2) and other phenolics, the loss of ca. 30% of sodium and 50% of potassium, and the absorption of part of the organic acids during the digestion.

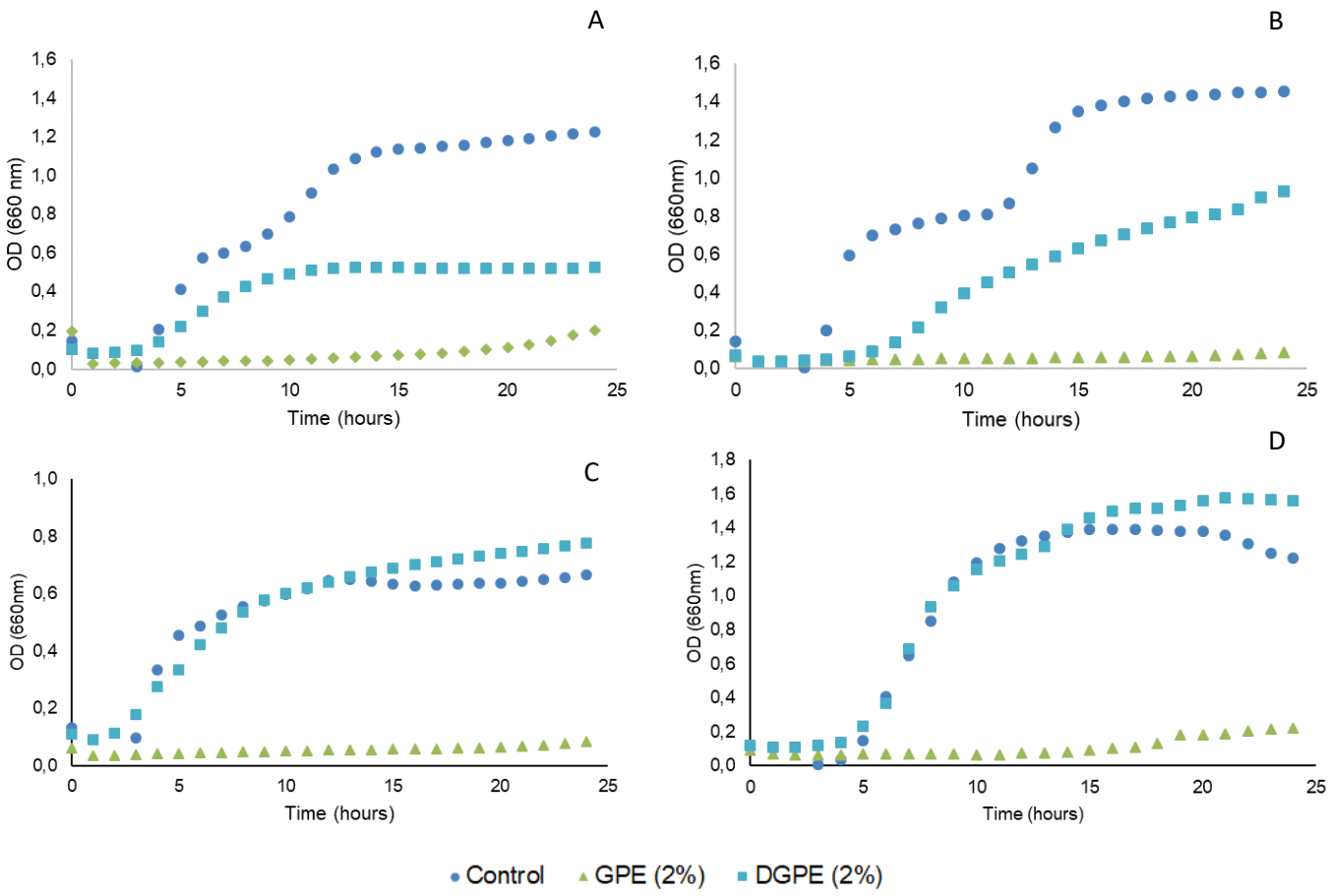


Figure 4.3 – Growth inhibition curves of (A) MSSA, (B) MRSA, (C) *E. coli* and (D) *P. aeruginosa* with a concentration of 2% GP and GID.

The results observed in the present work are in agreement with the literature: Xu *et al*. (2015) evaluated the antimicrobial activity of four different GP extracts on *S. aureus* and *E. coli*, and verified an inhibition of *S. aureus* (MICs ranging 40.6 to 250 $mg.mL^{-1}$); Jayaprakasha and co-workers (2003) obtained a grape seed extract with antimicrobial activity against MSSA, *E. coli* and *P. aeruginosa* and also found to be the most effective antibacterial fraction against Gram-positive bacteria when compared to Gram-negative bacteria; Oliveira and co-workers (2013) obtained Merlot CO_2 supercritical extracts with high activity on *S. aureus*, *Bacillus cereus*, *E. coli* and *P. aeruginosa*.

Once again, it was confirmed the high susceptibility of the bioactive compounds present in GPE to GIT and their loss of bioaccessibility, suggesting the need of an encapsulation system able to protect these compounds along the gastrointestinal tract.

4.3.7. Prebiotic effect

The prebiotic activity of GPE was studied on five strains in basal MRS medium without glucose, at concentrations of 1 and 2% (w/v). Figure 4.4 present the growth of evaluated *Bifidobacteria* and *Lactobacillus* strains, respectively, as measured by turbidity at 660 nm. Frutooligosaccharides (FOS) at the same concentrations were also used as control.

All the probiotic microorganisms grew in the presence of GPE, increasing their growth (OD at 660 nm) along the first 10 h of fermentation for Bifidobacteria and 18 h for Lactobacilli. The use of 2% (w/v) of GPE promoted higher growth up of assayed microorganisms than 1% (w/v) of GPE, as expected. FOS proved to be the best carbon source, at 2% (w/v) for Bifidobacteria and at 1% for Lactobacilli (Figure 4.4). There were not significant differences on the maximum growth of all probiotic strains used when the MRS was supplemented with 1% (w/v) of FOS or with 2% (w/v) of GP extract, except for *L. rhamnosus* that presented a lower growth. On the other hand, GPE lowed *Bifidobacterium* spp. to achieve the maximum OD faster, between 9 and 11 h compared to the 16 – 20 h in the presence of FOS.

The results obtained for the *L. casei* 01 are somehow different from the results obtained by Gullón *et al.* (2014b), who achieved a faster growth of this microorganism in the presence of FOS than with XOS derived from *Eucalyptus globulus* wood, rice husks, wheat bran and from barley wastes.

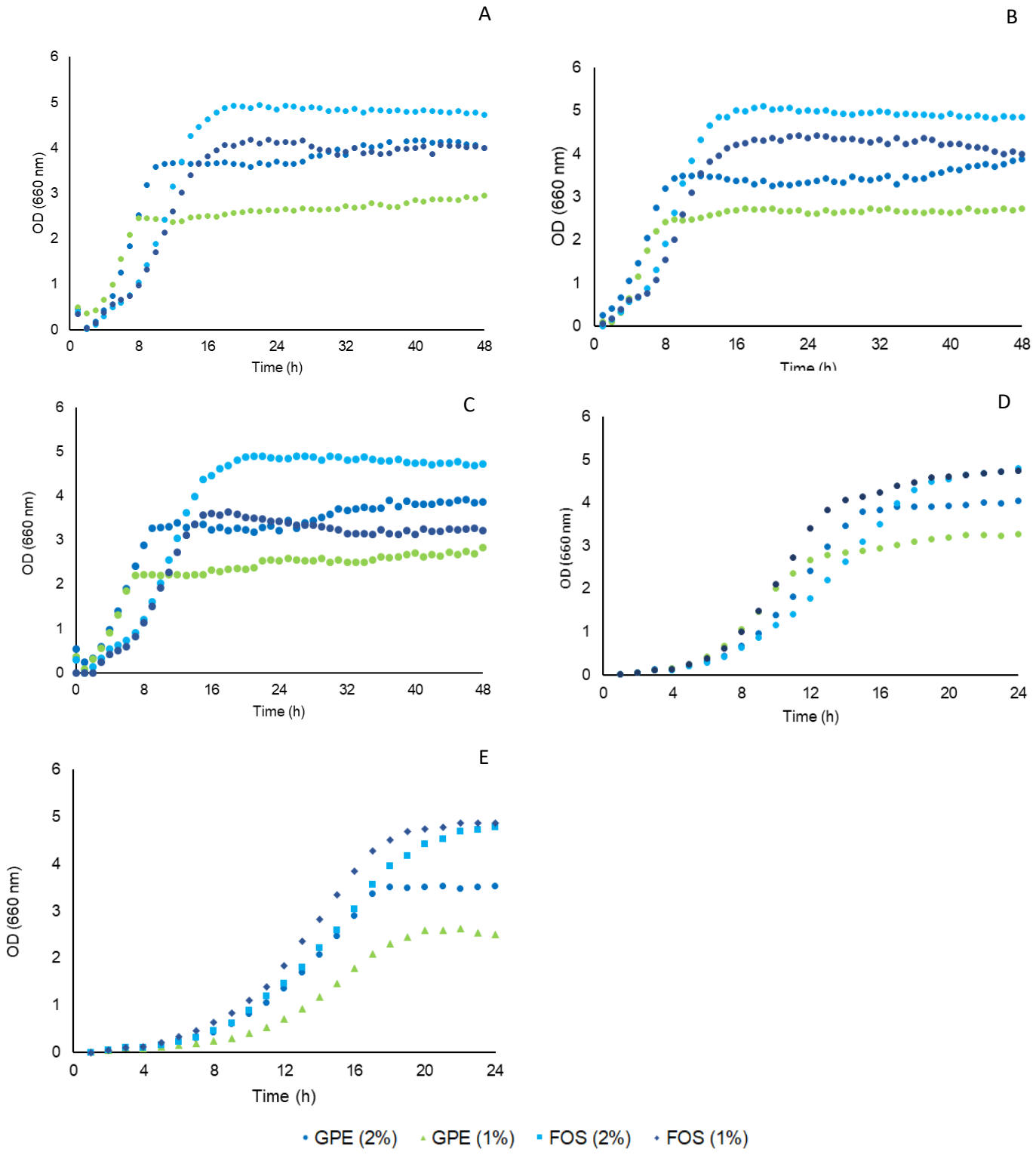


Figure 4.4 – Growth curves of (A) Bifidobacterium *animalis* Bo, (B) *Bifidobacterium longum* BG3, (C) *Bifidobacterium animalis* spp. *lactis* Bb12, (D) *Lactobacillus casei 01,* and (E) *Lactobacillus rhamnosus R11*

Results indicate that GPE can stimulate a fast growth of different probiotic strains, possibly due to the presence of glucose and XOS. These results are in accordance with the well-established prebiotic activity described for XOS including the selective stimulation of gut microbiota growth as the GPE also presented antimicrobial activity against some pathogens, as described above (Imaizumi *et al*., 1991; Macfarlane *et al*., 2006). These beneficial properties of XOS are related to the improvement of gastrointestinal functions as well as increase or change in the composition of short chain fatty acids, increased faecal weight and mineral absorption, immune stimulation, and decreased colonic pH values (Imaizumi *et al*., 1991).

4.3.9. *In vitro* fermentation of DGPE

In order to evaluate the effectiveness of DGPE as a fermentable carbohydrate source for probiotic bacteria, *in vitro* fermentation assays were carried out using a *Lactobacilli* (*L. casei* 01) and a *Bifidobacteria* strain (*B. animalis* Bb12). Table 4.3 shows the number of viable cells (expressed as log CFU.mL^{-1}) for both strains, as well as the pH value evolution in the culture media. FOS were used as positive control once they are already a well-known prebiotic; a negative control (without carbon source) was also used.

The number of viable cells of *Bifidobacterium* Bb12 reached 7.93 log CFU.mL^{-1} with FOS, and 8.14 log CFU.mL^{-1} with the DGPE, after 48 h of fermentation, allowing to conclude that both carbon sources stimulated the growth of this probiotic strain. As expected, without carbon source there was not any cell growth, confirming that the Bb12 growth was stimulated by the fermentable carbon sources, and significantly improved by the use of DGPE ($p < 0.05$).

Table 4.3 – Viable cell count (log CFU.mL^{-1}) of *Bifidobacterium animalis* Bb12 and *Lactobacillus casei* 01 and pH values throughout incubation time (48 h).

Carbon source	Incubation time (h)	Viable cell count (log CFU.mL^{-1})		pH values	
		Bb12	**La01**	**Bb12**	**La01**
FOS	0	7.25 ± 0.03	7.29 ± 0.01	7.09 ± 0.01	6.79 ± 0.02
	4	7.21 ± 0.04 ^{Aa}	7.22 ± 0.06 ^{Aa}	6.37 ± 0.01 ^{Aa}	6.43 ± 0.01 ^{Aa}
	8	7.31 ± 0.02 ^{Aa}	7.38 ± 0.16 ^{Bab}	5.95 ± 0.02 ^{Aa}	6.12 ± 0.02 ^{Aa}
	24	7.50 ± 0.07 ^{ABa}	7.54 ± 0.03 ^{Bab}	5.34 ± 0.01 ^{Aa}	6.09 ± 0.00 ^{Bb}
	48	7.93 ± 0.05 ^{Aa}	7.54 ± 0.03 ^{Bb}	4.93 ± 0.00 ^{Aa}	5.85 ± 0.00 ^{Bb}
DGP	4	7.32 ± 0.04 ^{Bb}	7.41 ± 0.03 ^{Cc}	6.99 ± 0.03 ^{Bb}	6.85 ± 0.06 ^{Cb}
	8	7.45 ± 0.02 ^{BCb}	7.59 ± 0.05 ^{Dc}	6.60 ± 0.02 ^{Bb}	5.64 ± 0.03 ^{Cb}
	24	7.59 ± 0.09 ^{Bb}	7.95 ± 0.03 ^{Cc}	5.46 ± 0.01 ^{Ca}	5.34 ± 0.01 ^{Aa}
	48	8.14 ± 0.01 ^{Ca}	7.93 ± 0.04 ^{Ac}	5.21 ± 0.00 ^{Cc}	5.30 ± 0.03 ^{Cc}
Negative control	4	7.05 ± 0.01^{E}	7.20 ± 0.00^{A}	7.01 ± 0.03^{D}	6.85 ± 0.03^{C}
	8	7.04 ± 0.01^{E}	7.19 ± 0.01^{A}	6.95 ± 0.00^{E}	6.79 ± 0.01^{E}
	24	7.04 ± 0.01^{E}	7.16 ± 0.01^{F}	6.89 ± 0.00^{E}	6.76 ± 0.01^{E}
	48	7.02 ± 0.01^{E}	7.13 ± 0.01^{F}	6.88 ± 0.02^{E}	6.63 ± 0.01^{B}

Values are expressed as mean ± standard deviation (SD) of three replicates.

Different capital letters mean significant differences between microorganisms and different lower letters means significant differences between carbon sources ($p < 0.05$).

Regarding *Lactobacillus casei* 01, the number of viable cells grew until 24 hours (7.54 and 7.95 log CFU.mL^{-1} for FOS and DGPE, respectively) and stabilized between 24 and 48 hours (7.54 and 7.93 log CFU.mL^{-1} for FOS and DGPE, respectively). These results for La01 are somehow expected, as it is generally known that *Lactobacilli spp*. usually reach the maximum growth after ca. 24 h.

With the pH values evolution along the fermentation process, a similar behavior was observed i.e., media with carbon source in its composition suffered a decrease of pH values, while the negative control was slightly affected by changes in pH values. For the media

fermented by Bb12, pH reduction was more pronounced in the presence of FOS than with DGPE (4.93 and 5.21, respectively), while for the media fermented by La01, a reverse effect was observed: the pH value after 48 h was lower with DGPE (5.30) than with FOS (5.85).

HPLC-DAD analysis of short-chain fatty acids (SCFA) in cell-free supernatants confirmed the production of acetic, butyric, formic, and propionic acids during fermentation. Table 4.4 shows the concentrations of these organic acids along the 48 h of fermentation. An overview of this table allows to observe that acetic acid was the SCFA produced in the highest quantity, followed by propionic acid, while butyric acid presented the lowest concentrations in all the assays. It is known that the production of acetic acid by *Bifidobacteria* spp. is higher than the production by *Lactobacilli* spp., which is observed in the fermentation with FOS; when using DGPE, this difference was only observed after 24 hours (Gullón *et al*., 2014b). Furthermore, the presence of acetic acid in higher quantity could be due to the fact that this organic acid was used for the production of the extract (Costa *et al*., 2018).

Regarding the *Bifidobacterium* strain fermentation, the production of acetic and formic acids was significantly higher ($p < 0.05$) when the DGPE was used as carbon source, while the propionic acid was higher when using FOS, after 24 h. The production of butyric acid during fermentation with Bb12 was not affected by the carbon sources, as it was not found significant differences in the concentration of this SCFA.

Regarding the fermentation with the *Lactobacillus* strain, the same behavior in the production of acetic and propionic acids was found, i.e, the use of DGPE improved the production of these SCFA comparing to FOS. On the other hand, the production of butyric acid was improved by the use of DGPE ($p < 0.05$) and the production of formic acid did not present significant differences ($p > 0.05$) after 4 h of incubation.

Table 4.4 – Organic acids concentration (mg.mL^{-1}) along the fermentation using FOS and DGP extract as carbon sources

Incubation time	FOS		DGP extract	
	Bb12	La01	Bb12	La01
	Acetic acid	**Acetic acid**	**Acetic acid**	**Acetic acid**
4h	1.221 ± 0.014^{Aa}	1.369 ± 0.013^{Bb}	2.770 ± 0.021^{Cc}	2.471 ± 0.024^{Bd}
8h	1.052 ± 0.023^{Aa}	1.051 ± 0.008^{Aa}	2.388 ± 0.012^{Bb}	2.406 ± 0.032^{Cb}
24h	1.509 ± 0.009^{Aa}	1.209 ± 0.017^{Bb}	2.583 ± 0.024^{Ca}	2.845 ± 0.027^{Dd}
48h	1.697 ± 0.012^{Aa}	1.137 ± 0.014^{Bb}	2.683 ± 0.023^{Cc}	2.699 ± 0.021^{cD}
	Butyric acid	**Butyric acid**	**Butyric acid**	**Butyric acid**
4h	0.114 ± 0.002^{Aa}	0.111 ± 0.001^{Aa}	0.104 ± 0.005^{Aa}	0.122 ± 0.008^{Aa}
8h	0.092 ± 0.002^{Aa}	0.142 ± 0.002^{Bb}	0.092 ± 0.001^{Aa}	0.186 ± 0.009^{Cb}
24h	0.092 ± 0.001^{Aa}	0.157 ± 0.002^{Bb}	0.106 ± 0.002^{Aa}	0.211 ± 0.012^{Cc}
48h	0.129 ± 0.005^{Aa}	0.147 ± 0.002^{Bb}	0.134 ± 0.002^{Ac}	0.199 ± 0.011^{Cd}
	Formic acid	**Formic acid**	**Formic acid**	**Formic acid**
4h	0.140 ± 0.003^{Aa}	0.184 ± 0.004^{Bb}	0.294 ± 0.007^{Cc}	0.367 ± 0.009^{Dd}
8h	0.131 ± 0.003^{Aa}	0.183 ± 0.004^{Bb}	0.168 ± 0.006^{Cc}	0.181 ± 0.001^{Bd}
24h	0.309 ± 0.007^{Aa}	0.323 ± 0.003^{Ba}	0.417 ± 0.005^{Cb}	0.300 ± 0.002^{Bc}
48h	0.425 ± 0.004^{Aa}	0.291 ± 0.007^{Bb}	0.683 ± 0.007^{Cc}	0.271 ± 0.007^{Bc}
	Propionic acid	**Propionic acid**	**Propionic acid**	**Propionic acid**
4h	0.670 ± 0.002^{Aa}	0.514 ± 0.006^{Bb}	0.751 ± 0.009^{Cc}	0.616 ± 0.011^{Dd}
8h	0.473 ± 0.006^{Aa}	0.455 ± 0.003^{Ba}	0.511 ± 0.005^{Cb}	0.693 ± 0.012^{Dd}
24h	0.735 ± 0.001^{Aa}	0.558 ± 0.006^{Bb}	0.515 ± 0.002^{Cc}	0.689 ± 0.007^{Dd}
48h	0.791 ± 0.001^{Aa}	0.487 ± 0.005^{Bb}	0.601 ± 0.006^{Cc}	0.788 ± 0.009^{Dd}

Different capital letters mean significant differences between microorganisms and different lower letters means significant differences between carbon sources ($p < 0.05$).

Selective fermentations of prebiotic carbohydrates by *Bifidobacteria* and *Lactobacilli* depend on different factors, including the strain and substrate (Gullón *et al*., 2015). GP and its extract contain significant amounts of carbohydrates (approximately 50 g.100g^{-1}) and showed that it can act as a fermentable source of polysaccharides, as they can promote the growth of both Bb12 and La01 and produce organic acids.

4.3.10. Cytotoxicity

Toxicity of GPE before and after digestion was evaluated through XTT cell proliferation assay upon Caco-2 intestinal cells, using extracts concentrations of 1 and 2% (w/v), as these were the concentrations that showed potential bioactivities. Figure 4.5 presents the metabolism inhibition of Caco-2 cells in the presence of GPE and DGPE.

The XTT cell proliferation assay is an effective method to measure cell growth and drug sensitivity in tumor cell lines (ATCC, 2011). GP extract at concentration of 2% promoted Caco-2 cells metabolism by 37.05 ± 2.56% and by 20.91 ± 2.27%, respectively. After the digestion of GPE, it is possible to observe a slightly, but not significant ($p > 0.05$), inhibition of metabolism: 5.75 ± 2.81% and 9.43 ± 1.90% for DGPE at concentrations of 2 and 1%, respectively.

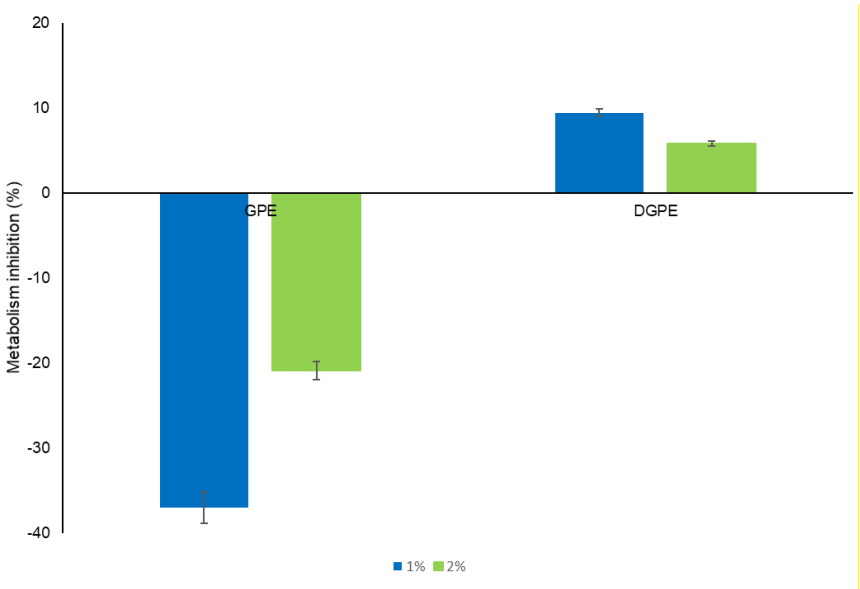


Figure 4.5 – Metabolism of Caco-2 intestinal cells upon the presence of GP extract at concentrations of 1 and 2% (w/v).

It is possible to conclude that GPE is safe to be used as food ingredient, at concentrations up to 2% (w/v). It is also important to correlate the Caco-2 cells viability with the antimicrobial

and prebiotic activities, once 2% (w/v) of GPE was enough to proliferate probiotic strains and inhibit some pathogenic bacteria growth.

4.3. Conclusions

This work described the potential bioactive properties of a grape pomace extract produced through a novel eco-friendly method. Freeze-dried GPE presented high content of total dietary fiber, other carbohydrates such as xylobiose, minerals and phenolic compounds. *In vitro* GID simulation of GPE allowed to conclude that xylobiose was resistant to GI conditions, and that this extract has a prebiotic potential, at concentration of 2% (w/v), establishing a potential carbon source that can be fermented by *Lactobacillus* and *Bifidbacterium* spp. GPE proved to have antimicrobial activity against pathogenic bacteria, with large spectrum being active against Gram-negative and Gram-positive strains, although this effect was lost after the GID. Similar impact of gastrointestinal digestion occurred to polyphenols and consequently to the antioxidant capacity, which strongly decreased after digestion.

The reduction on the antimicrobial and antioxidant capacities of the GPE after the GID process suggests the need for an alternative system to protect it from the harsh GID conditions, such as an encapsulation system, to guarantee its ability to act on the intestinal environment. These results allow to conclude that a multifunctional extract with different bioactive properties could be obtained from GP presenting relevant potential for food applications as ingredient in the development of new functional foods.

Part III

Improvement of Bioavailibility

CHAPTER 5

Polymeric microparticles as oral delivery systems for grape pomace extract towards the improvement of biological activities

Abstract

GP is known for its bioactive compounds and their impact upon gastrointestinal (GI) health. However, bioaccessibility of bioactive compounds from grape pomace is often poor due to their degradation during digestion. This work aimed to encapsulate bioactive GP extract (GPE) into chitosan (CS) and alginate (Alg) microparticles (MPs) to mitigate degradation in the GI tract. The synthesis of Alg and CS MPs was optimized using a rotatable central composite design and MPs were characterized for their size, polydispersity, zeta potential and total phenolics (TPC) association efficiency. The best Alg and CS formulations showed sizes ranging 523 - 853 nm, polydispersity indexes of 0.11 - 0.36, zeta potential of -15.0 - +14.9 mV and TPC association efficiencies of 68 and 65%, respectively. Analysis by FTIR confirmed that no additional chemical groups were formed after association of the polymers with GPE. Both formulations improved the bioaccessibility of different phenolic compounds following *in vitro* simulation of digestion through the GI tract, leading to increased antioxidant and antimicrobial activities. In addition, the permeability of phenolics and xylooligosaccharides through a Caco-2/HT29-MTX co-culture was reduced, suggesting a

higher residence time in the intestine. Cy5.5 was used for tracking of the CS MPs, which did not affect the metabolic activity of Caco-2 and HT29-MTX cells up to a concentration of 0.5 $mg.mL^{-1}$. Confocal microscopy images confirmed the adsorption of MPs to the cellular layer and suggested a reduction of the tight junction protein occludin when cells were incubated with Cy5.5-CS in solution. Therefore, encapsulation of GPE can offer protection against degradation at the GI tract and thus improve its biological activity with significant impact for oral delivery applications, including functional foods and beverages.

5.1. Introduction

In the last decades, the pharmaceutical, cosmetic and food industries have been looking into alternatives to the existing preservative compounds that could bypass the increasing issues with microorganism resistance and side effects to the human health as well as to find natural solutions to replace synthetic ones (Rodrigues *et al*., 2013). GP is mainly composed by grape skins, seeds and stalks, is one of the major by-product generated worldwide by the winery industries, and is rich in polyphenols and antioxidant fibres, which are known to benefit human health (Beres *et al*., 2017; Oliveira *et al*., 2013). Grapes and GP are rich in phenolic compounds, including anthocyanins, procyanidins, gallic acid and resveratrol, which are associated with i) the inhibition of low-density lipoprotein oxidation, ii) the protection against free radical-mediated tissue injury, and iii) the modulation of vascular cell function (Wang *et al*., 2017). These compounds are also known for their antimicrobial activity, as they are capable to cross the semipermeable membrane of microorganisms and interact with the cytoplasm or cellular proteins, inhibiting the growth of antibiotic resistant pathogens and toxin-producing fungi (Yavad *et al*., 2015). It has been previously developed a bioactive grape pomace extract (GPE), with potential antioxidant and antimicrobial activities

promoted by the rich composition in polyphenols, soluble dietary fibres, including XOS, and minerals (Costa *et al*., 2019).

Despite the antioxidant and antimicrobial capacity of grapes and GP demonstrated *in vitro*, their biological activity *in vivo* depends on their bioavailability – *i.e.* the fraction that remains available after absorption in the gut and further metabolism (Wang *et al*., 2017; Chedea *et al*., 2018). The degradation of phenolic compounds during digestion reduces their antioxidant effect and their capacity to inhibit the growth of common colon pathogenic bacteria such as *Listeria monocytogenes* and *Escherichia coli* (Caccetta *et al*., 2000; Costa *et al*., 2019). Most polyphenols are digested by pancreatin-bile salt digestion, and anthocyanins, which are more sensitive to acidic pH, are mostly degraded by pepsin in the stomach (Pérez-Vicente *et al*., 2002; Wang *et al*., 2017; Chedea *et al*., 2018). Thus, the oral administration of these bioactive compounds calls for the formulation of a system that offers protection from gastric and intestinal digestion in order to maintain their structural integrity before reaching the gut (Munin and Edwards-Lévy, 2011).

The encapsulation of polyphenols has been explored as a method to improve their bioavailability and overcome biological barriers. Natural hydrophilic polymers such as chitosan (CS) and alginate (Alg) have generated significant interest (Liang *et al*., 2011; Madureira *et al*., 2015b). (Diebold and Calonge, 2010; Costa *et al*., 2015b). Alginate is a linear and anionic polysaccharide consisting of α-L-guluronate and β-D-mannuronate monomers, randomly distributed in the polymer chain (Motwani *et al*., 2007; Costa *et al.*, 2015b). CS is a cationic polysaccharide produced by the deacetylation of chitin, with demonstrated antimicrobial activity against many bacteria, filamentous fungi and yeast (De la Fuente *et al*., 2010; Kong *et al*., 2010). Both polymers have been widely used by the pharmaceutical and food industries for the development of oral and topical delivery systems, as they are biodegradable, non-toxic, biocompatible, and mucoadhesive capacity (Motwani

et al., 2007; Costa *et al.*, 2015b). In fact, the mucoadhesive capacity of polymers is of great interest for the formulation of oral delivery systems, as it allows to increase the residence time of the encapsulated compounds at the site of activity (George and Abraham, 2006). The encapsulation of natural extracts using Alg and/ or CS carriers has been commonly used to improve different properties: encapsulation of yerba mate extracts rich in polyphenols into Alg and CS systems to be incorporated as food ingredient, microcapsules of Alg to improve the storage stability of polyphenols from grape, and encapsulation of polyphenols from Asian pigeonwings into alginate microcapsules to improve their resistance to GI digestion (Anbinder *et al*., 2011; Aizpurua-Olaizola *et al*., 2016; Pasukamonset *et al*., 2016). Nonetheless, encapsulation of natural extracts from grape by-products into alginate or chitosan delivery systems to improve their bioaccessibility is not described.

This work aimed the encapsulation of GPE into chitosan and alginate microparticles for oral delivery applications. The overarching objective was to offer protection against digestion in the GI tract and improve the bioaccessibility of XOS and phenolic compounds, thus maximizing their antioxidant and antimicrobial activities, without absorption by the intestinal mucosa.

5.2. Material and Methods

5.2.1. Materials

GPE was obtained by enzymatic extraction followed by freeze-drying, as previously described (Costa *et al*., 2018). Low molecular weight (LMW) chitosan, alginic acid sodium salt from brown algae, sodium tripolyphosphate (TPP), gallic acid, caffeic acid, (+) cathechin, p-coumaric acid, rutin, syringic acid, vanillic acid, cyanidin–3-glucoside, peonidin-3-o-glucoside, delphinidin-3-glucoside standards, digestion enzymes, paraformaldehyde (PFA), Triton™ X-100, phalloidin-tetramethylrhodamine B

isothiocyanate (phalloidin-TRITC), bovine serum albumin (BSA), fluoroshield™ and resazurin sodium salt were purchased from Sigma Aldrich (St. Louis, Missouri, USA). Luteolin-7-o-glucoside and neochlorogenic acid standards were purchased from Extrasynthese (France), and XOS standards from Megazyme (Bray, Ireland). 4', 6-diamidino-2-phenylindole, dilactate (DAPI, Invitrogen™) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). 8-well chamber µ-slides were acquired from Ibidi GmbH (Gräfelfing, Germany). Bile salts were obtained from Oxoid™ (Hampshire, UK), and Cyanine5.5 NHS dye probe from Lumiprobe GmbH (Hannover, Germany). DMEM and MEM media were purchased from Gibco (Massachusetts, USA). Fetal Bovine Serum (FBS) was obtained from BioWest (Nuaillé, France), and Penicillin-Streptomycin-Fungizone from Lonza (Basel, Switzerland).

5.2.2. MPs production

GPE-loaded chitosan NPs (CS-GPE MPs) were prepared following the methods reported in Zahoor *et al*. (2005) with slight modifications. Briefly, TPP (1 $mg.mL^{-1}$) was added dropwise to a solution of GPE and CS under stirring. Alginate MPs (Alg-GPE MPs) were prepared following the methods of Zahoor *et al*. (2005) and Sarmento *et al*. (2006 and 2007) with slight modifications. GPE was dissolved into a sodium alginate solution followed by the dropwise addition of $CaCl_2$ (2 $mg.mL^{-1}$) under stirring. Both MPs formulations were further stirred for 30 seconds at 1,200 rpm after preparation. As a control, empty CS and Alg MPs were produced following the same methods.

The parameters for the production of CS-GPE and Alg-GPE MPs were optimized by response surface methodology, according to a 2^2 central composite design, using the particle size, zeta potential and total phenolics (TPC) compounds association efficiency as responses. The CS/Alg (X_1) and GPE (X_2) concentrations were used as independent variables, evaluated

in five levels, according to Table 5.1. The following polynomial equation was fitted to the data, where βn are constant regression coefficients, y is the response (size, zeta potential or TPC association efficiency), and x_1 and x_2 are the coded independent variables (concentrations of CS/Alg and GPE).

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{12} x_1 x_2 \quad (5.1)$$

Table 5.1 – Coded variables studied in the production of Alg-GPE and CS-GPE MPs.

Coded variables	-1.41	-1.0	0	+1.0	+1.41
GPE concentration (mg.mL^{-1})	12.93	15.00	20.00	25.00	27.07
Alg concentration (mg.mL^{-1})	1.76	3.00	6.00	9.00	10.24
CS concentration (mg.mL^{-1})	0.88	1.00	3.00	4.00	5.12

5.2.3. MPs characterization

5.2.3.1. Particle size, polydispersity index and zeta potential

The particle size, polydispersity index and zeta potential were determined by dynamic and phase analysis light scattering using a Zetasizer Nano ZSP (Malvern Instruments Ltd, Worcestershire, UK). The MPs formulations were loaded into polystyrene and folded capillary cells and measured at 25 °C using a 10 mW He-Ne laser (633 nm) and a detector angle of 173°. The samples were submitted to 6 runs with 20 cycles each.

5.2.3.2. Association efficiency of polyphenols

MPs were pelleted by centrifugation (3857 *x g*, 120 min) and the supernatants were collected for determination of TPC association efficiency (AE). The AE was calculated as the difference between the amount of TPC present in the supernatant and the initial amount of

TPC present in the GPE used in the preparation of the MPs. TPC were determined by the Folin-Ciocalteu method (Singleton and Rossi, 1965).

A qualitative profile of polyphenols was obtained by HPLC-DAD (wavelengths ranging from 190 to 600 nm), according to the method described by Oliveira *et al.* (2015). The analysis was conducted on a Waters Liquid Chromatograph (Waters Alliance, Mildford MA, USA), using a C18 guard column (Symmetry® C18) and an Alltech adsorbosil C18 reversed-phase packing column (250 x 4.6 mm ID, 5-µm particle size, and 125 Å pore size). Mobile phase: Solvent A: acetonitrile 100% with 0.2% TFA; Solvent B: acetonitrile / water 5:95 (v/v) with 0.2% TFA; flow rate of 1 $mg.mL^{-1}$. All compounds were identified and quantified by comparison to pure standards and using an external calibration curve. Before the HPLC analysis, all the samples were filtered using 0.45 µm polyester syringe filters (Macherey-Nagel, Germany).

5.2.3.3. Molecular interactions analysis

ATR-FTIR analysis was conducted in the absorbance range of 4000 to 400 cm^{-1} using a Spectrum 100 FTIR spectrometer, (Perkin Elmer, Massachusetts, USA) equipped with an attenuated total reflectance (ATR) accessory (PIKE Technologies, USA) with a diamond/Se crystal plate.

5.2.4. In vitro simulation of GI digestion

The digestive conditions in the GI tract were simulated following the methods described by Madureira *et al.* (2011) with slight modifications. Alg-GPE and CS-GPE MPs were used at 45 $mg.mL^{-1}$ in pure water.

Stomach: The pH was adjusted to 2.0 using HCl 1 M and the gastric juice simulated using pepsin (25 $mg.mL^{-1}$). The solution was added to the samples at a ratio of 0.05:1 and incubated for 120 min (long digestion) at 37 ˚C and under agitation (130 rpm).

Intestine: Simulation of gut conditions was performed by initial adjustment of the pH to 6 using $NaHCO_3$ 1 M. The intestinal juice was simulated by the use of pancreatin (2 $mg.mL^{-1}$) and bile salts (12 $mg.mL^{-1}$). The solution was added to the sample at a ratio of 0.25:1 and incubated for 60 min at 37 ˚C and under agitation (45 rpm).

After *in* vitro simulation of GI digestion, the enzymes were removed by dialysis using 3 kDa membranes and the samples freeze-dried. All assays were performed in triplicate and after each stage, the release profile of the polyphenols was assessed by HPLC, as described above.

5.2.5. Antioxidant capacity

5.2.5.1. ABTS Radical Scavenging Activity

The free radical-scavenging activity was measured using the $ABTS^+$ radical cation de-colorization assay described by Gião *et al.* (2007). Briefly, $ABTS^+$ solution was produced by reacting a 7 mM ABTS solution filtered through a 0.45 μm filter, with potassium persulfate (2.45 mM) in the ratio of 1:1 (v/v). The $ABTS^+$ solution was diluted with ultrapure water to an absorbance of 0.70 ± 0.02 at 734 nm (Shimadzu 1240 UV–visible spectrophotometer). One mL of diluted $ABTS^+$ solution was added to 10 μL of MPs and the absorbance measured exactly 6 min after mixing. Ascorbic acid was used as a standard to prepare the calibration curve in the range of 0.02–0.50 $mg.mL^{-1}$.

5.2.5.2. Oxygen Radical Absorbance Capacity (ORAC)

The ORAC assay was performed according to the methods described by Contreras *et al*. (2001). The reaction was carried out at 40 ˚C by adding 120 μL fluorescein (116.6 nM) to 20 μL of sample (diluted 1:500 to 1:3200 in 75 mM PBS, pH 7.4), followed by the addition of 60 μL of free radical-generating AAPH (14 mM) after 10 min. The antioxidant Trolox (6.25 – 200 μM) was used as control. The fluorescence (λex = 485 nm, λem = 520 nm) was measured for 137 min (104 cycles), in black polystyrene 96-well microplates using a

FLUOstar OPTIMA plate reader (BMG Labtech, Offenburg, Germany). AAPH and Trolox solutions were prepared daily, and fluorescein was diluted from a stock solution (1.17 mM) in 75 mM PBS (pH 7.4). All reaction mixtures were prepared in duplicate and three independent runs were performed for each sample. The final ORAC values were expressed as μmol of Trolox equivalent per gram of sample (μmol TE.g^{-1} of MPs).

5.2.6. Antimicrobial activity of Alg-GPE and CS-GPE MPs

The antimicrobial activity of Alg-GPE and CS-GPE MPs was determined for methicillin-susceptible *Staphylococcus aureus* (MSSA) ATCC 6538, *Escherichia coli* O157:H7, *Pseudomonas aeruginosa* ATCC 10145, *Salmonella enteritidis* ATCC 13076, *Listeria monocytogenes* (food isolate from ESB collection) and *Candida albicans DSM 1386*. Digested Alg-GPE MPs and CS-GPE MPs at a concentration of 2.5% (w/v) were inoculated in Mueller-Hinton broth 1% (v/v) using an inoculum of 10^6 CFU.mL^{-1}, and incubated at 37 °C for 24 h. Viable counts were determined at 0, 3, 6, 12 and 24 h, using decimal dilutions and plating in Plate Count Agar (PCA) using the drop method (MSSA, *E. coli*, *P. aeruginosa* and *L. monocytogenes*) or spread plate technique (*S. enteritidis* and *C. albicans*). Inoculated MHB without extract was used as positive control. The PCA plates used for the viable counts were then incubated for 24 h at 37 °C. All assays were done in duplicate and results are presented as log CFU versus time.

5.2.7. *In vitro* intestinal permeability

An *in vitro* Caco-2/HT29-MTX co-culture model was used to determine the intestinal permeability of the encapsulated bioactive compounds, after digestion. Caco-2 and HT29-MTX were used as enterocytes and mucin-secreting mature goblet cells respectively, representing the two major cell populations found in the intestinal epithelium. The human

Caco-2 cell line is of colonic origin, but upon confluence, differentiates to form a well-differentiated polarized monolayer of columnar absorptive cells with a brush border and expressing typical metabolic enzymes and transporters (Lea, 2015).

5.2.7.1. Cell culture

Caco-2 (passage 63) and HT29-MTX (passage 55) cells were grown in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS), 100 $U.mL^{-1}$ penicillin and 100 $\mu g.mL^{-1}$ streptomycin, and non-essential amino acids in a humidified chamber at 37 ˚C and 5% CO_2. The culture media was replaced every 2-3 days until cell seeding.

The cells were seeded on collagen-coated membrane inserts (0.4 μm pore size - Corning, New York, USA) placed in 12-well culture plates and 0.5 and 1.5 mL of media were added to the apical and basolateral compartments, respectively. The media was replaced every 2 days for 21 days and the TEER was measured to validate the barrier integrity.

5.2.7.2. Trans-epithelial electrical resistance (TEER) measurement

The TEER was measured to assess barrier integrity, following a protocol for static epithelial models (Gaillard and De Boer, 2000) The cell-culture inserts were allowed to equilibrate to RT for 10 min and the TEER was measured using a Millicell® ERS-2 volt-ohm-meter (Merck Millipore, Billerica, MA, USA) with STX chopstick electrodes. TEER values in $\Omega \cdot cm^2$ were calculated by subtracting the TEER of cell-free transwells® from the cell-cultured transwell inserts, and multiplying by the surface area of the well.

5.2.7.3. Permeability assay

On the day of the study, the culture media was removed from both compartments and replaced with 1.5 mL PBS (pH 7) in the basolateral compartment and with free GPE and Alg-GPE MPs and CS-GPE MPs suspensions in the apical compartment. The permeability was determined at 0, 15, 30, 45, 60, 120, 180, and 240 min, by collecting 500 μL from the basolateral compartment for quantification of polyphenols, as described above, and XOS. At

each time point, the basolateral compartment was replenished with 500 µL of fresh PBS. The experiments were performed in triplicate.

5.2.7.4. Quantification of XOS

Permeability of XOS was determined by quantification using refractive-index high performance liquid chromatography (RI-HPLC) (Beckman Coulter System Gold HPLC system, California, USA), using a Waters Ultrahydrogel 120 (7.8 x 300 mm) column, with ultrapure water as mobile phase, and an isocratic flow rate of 0.5 $mL.min^{-1}$. XOS were identified by comparison to the retention times of pure XOS standards (Megazymes, Ireland), and quantified by external standard calibration. Before the analysis, all the samples were filtered using 0.45 µm polyester syringe filters (Macherey-Nagel, Germany).

5.2.8. Cytotoxicity, cellular uptake and permeability of soluble or nanoparticulate CS

To study the cellular uptake of the encapsulated GPE, CS was chosen as a model to evaluate interaction between the polymer, in soluble or nanoparticulate form, and the intestinal barrier. The performance of the CS-GPE MPs in the previous assays showed promisor results as potential oral delivery system, as well as the CS positive charge that has been described to adhere to the negatively charged cell surfaces, thus increasing the cellular uptake, validate this selection (Woitiski *et al*., 2011; Je *et al*., 2017).

To evaluate the cellular uptake of the CS-MPs, a Cy5.5 dye was used for tracking. The expression of the tight-junction protein occludin was assessed by immunocytochemistry to evaluate the capacity of CS (in solution or in the form of MPs) to modulate the opening of cellular tight junctions.

5.2.8.1. Synthesis of Cy5.5 labelled CS (Cy5.5-CS)

LMW chitosan (5 mg.mL^{-1}) was dissolved in 1% (v/v) glacial acetic acid and the pH adjusted to 6 using NaOH 5 M. Cy5.5 Mono NHS ester (1 mg.mL^{-1} in DMSO) was added at a 0.005 molar ratio of reactive carboxylic groups to chitosan free amine groups. The reaction occurred overnight in the dark at 50 ˚C at a stirring rate of 500 rpm. Unconjugated Cy5.5 molecules were removed by ultrafiltration using a stirred UF cell module (Millipore, CA) under pressurized nitrogen (40 psi) at RT and an Ultracel 10kDa MW cut-off (MWCO) membrane until their presence could not be detected by quantification of the filtrates fluorescence using an Horiba FluoroMax-4 (Kyoto, Japan; λ_{em} = 675 - 800 nm; λ_{ex} = 650 nm). The Cy5.5-CS conjugate was recovered by adjusting the pH to 9 followed by centrifugation (17920 x *g*, 20 min). The pellet was washed with ultrapure H_2O at pH 9 twice and freeze dried until further use. CS and CS-GPE MPs were produced using the Cy5.5-CS (Cy5.5-CS MPs and Cy5.5-CS-GPE MPs) and characterized for size and polydispersity as above.

5.2.8.2. Cytotoxicity assay

The cytotoxicity of Cy5.5-CS and Cy5.5-CS-GPE MPs was determined indirectly by the resazurin reduction assay. Caco-2 and HT29–MTX cells were plated separately in 24-well plates, at 2.5x10^4 cells/cm^2. After 24 h, the media was changed and Cy5.5-CS, Cy5.5-CS MPs and Cy5.5-CS-GPE MPs dispersed in PBS, were added (1:10) to yield the final concentrations of 0.5, 0.05 and 0.005 mg.mL^{-1}. PBS and DMSO were used as negative and positive controls, respectively. The cells were incubated for 24 h, after which the media was replenished with fresh media supplemented with 10 µg.mL^{-1} resazurin sodium salt. The cells were further incubated for 3 h at 37 °C and the cell metabolic activity was determined by measuring the fluorescence of resofurin (λ_{ex}=530 nm, λ_{em}=590 nm) using a BioTeK®

Synergy H1 (Winnoski, VT, USA) multi-plate reader. The results are expressed as a percentage in relation to the PBS control.

5.2.8.3. Cellular uptake

The Caco-2/HT29-MTX co-cultures described above were cultured in Ibidi 8-well µ-slides and grown for 21 days after which dispersions of the Cy5.5-CS, Cy5.5-CS MPs and Cy5.5-CS-GPE MPs in serum-free culture media were added and incubated for 18 h. Following incubation, the cells were extensively washed, fixed in 4% (w/v) paraformaldehyde for 20 min and permeabilised by incubation with a 0.2% (v/v) Triton X-100 solution in PBS for 10 min. The fixed cultures were then incubated in a 2% (w/v) BSA solution in PBS to minimise non-specific antibody bonding. After blocking, cells were incubated with 5 $\mu g.mL^{-1}$. Alexa Fluor® 488-conjugated occludin monoclonal antibody (OC-3F10) for 1h followed by incubation with phalloidin-TRITC for 1 h. Finally, cells were washed extensively, counter-stained with DAPI (0.2 $\mu g.mL^{-1}$) for 10 min and preserved in Fluoroshield mounting medium. The stained cell layers were imaged using a Zeiss LSM780 confocal laser scanning microscope (Oberkochen, Germany) and analysed using Zen 2010 software and ImageJ.

5.2.9. Statistical analyses

For the central composite design, the analysis of variance (ANOVA), test for the lack of fit, determination of the regression coefficients and the generation of surface responses were carried out using the Statistica 7.0 software (StatSoft, Tulsa, USA). All other statistical analyses were done using the IBM SPSS statistics program v23.0 (Illinois, USA), using the Student T-test for independent samples and analysis of variance (ANOVA) with Tukey post-hoc test. Differences were considered significant at a level of $p < 0.05$.

5.3. Results and Discussion

5.3.1. MPs characterization

Formulations of Alg-GPE NPs and CS-GPE MPs for intestinal delivery of a bioactive GPE were optimized and studied to understand how the polymer and extract concentrations influence the physical properties of the MPs. Tables 5.2 and 5.3 summarize for the Alg-GPE and CS-GPE MPs respectively the results of the complete experimental design regarding particle size, zeta potential and. The surface responses for size and TPC association efficiency were selected for having the largest impact on the optimization of the MPs and are outlined in Figure 5.1a and 5.1b, respectively.

Table 5.2 – Factors and responses of experimental design for Alg-GPE MPs

Trial	Alginate conc. ($mg.mL^{-1}$)	GPE conc. ($mg.mL^{-1}$)	Size (nm)	Zeta Potential (mV)	Polydispersity	Phenolics AE (%)
1	3.00 (-1)	15.00 (-1)	523.5 ± 7.5	-15.0 ± 0.8	0.112	68.0 ± 0.5
2	3.00 (-1)	25.00 (+1)	932.0 ± 5.9	-18.9 ± 0.9	0.361	65.4 ± 0.3
3	9.00 (+1)	15.00 (-1)	531.3 ± 22.7	-32.7 ± 1.9	0.151	52.6 ± 1.6
4	9.00 (+1)	25.00 (+1)	953.0 ± 7.3	-24.0 ± 2.2	0.319	50.1 ± 0.4
5	1.76 (-1.41)	20.00 (0)	558.1 ± 8.2	-26.2 ± 2.1	0.146	63.0 ± 2.6
6	10.24 (+1.41)	20.00 (0)	627.0 ± 12.3	-30.8 ± 2.7	0.212	43.5 ± 0.7
7	6.00 (0)	12.93 (-1.41)	270.9 ± 5.8	-25.8 ± 0.3	0.319	62.0 ± 0.7
8	6.00 (0)	27.07 (+1.41)	1051.0 ± 25.3	-23.5 ± 0.9	0.332	50.1 ± 0.4
9	6.00 (0)	20.00 (0)	580.7 ± 3.4	-25.7 ± 1.2	0.263	55.1 ± 2.1
10	6.00 (0)	20.00 (0)	583.2 ± 4.7	-24.8 ± 0.6	0.222	56.0 ± 0.4
11	6.00 (0)	20.00 (0)	577.3 ± 2.2	-25.2 ± 0.9	0.262	57.0 ± 0.6

Table 5.3 – Factors and responses of experimental design for CS-GPE MPs

Trial	Chitosan conc. ($mg.mL^{-1}$)	GPE conc. ($mg.mL^{-1}$)	Size (nm)	Zeta Potential (mV)	Polydispersity	Phenolics AE (%)
1	1.00 (-1)	15.00 (-1)	536.9 ± 8.8	13.2 ± 0.9	0.257	37.9 ± 0.4
2	1.00 (-1)	25.00 (+1)	707.3 ± 5.0	13.5 ± 0.4	0.502	45.3 ± 0.4
3	4.00 (+1)	15.00 (-1)	619.0 ± 6.0	14.7 ± 0.8	0.093	52.5 ± 0.7
4	4.00 (+1)	25.00 (+1)	780.6 ± 4.6	14.8 ± 0.6	0.744	60.2 ± 0.6
5	0.88 (-1.41)	20.00 (0)	571.4 ± 4.3	14.8 ± 0.5	0.874	38.8 ± 1.4
6	5.12 (+1.41)	20.00 (0)	853.0 ± 5.9	14.9 ± 0.7	0.358	64.7 ± 3.1
7	3.00 (0)	12.9 (-1.41)	418.7 ± 4.3	7.42 ± 0.5	0.372	50.9 ± 0.25
8	3.00 (0)	27.1 (+1.41)	791.2 ± 5.3	12.6 ± 0.1	0.932	54.2 ± 0.1
9	3.00 (0)	20.00 (0)	639.7 ± 13.6	13.9 ± 0.4	0.797	57.8 ± 0.3
10	3.00 (0)	20.00 (0)	634.3 ± 8.0	14.1 ± 0.5	0.552	55.2 ± 0.2
11	3.00 (0)	20.00 (0)	637.5 ± 8.9	13.2 ± 0.4	0.681	56.3 ± 0.6

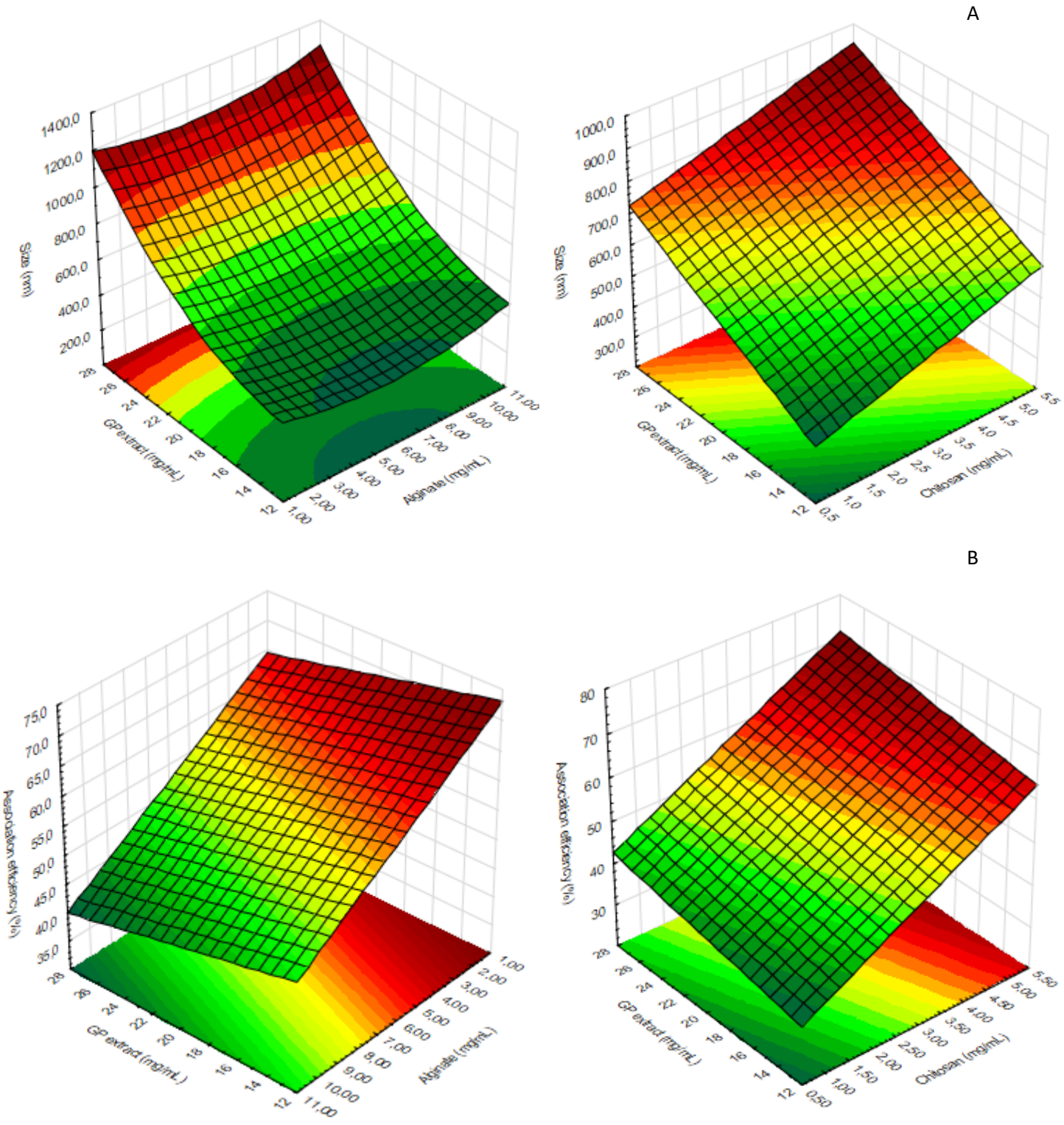


Figure 5.1 – Surface response for (A) microparticle size of Alg-GPE and CS-GPE MPs, and (B) TPC association efficiency of Alg-GPE and CS-GPE MPs.

The different formulations of Alg-GPE MPs yielded particles with sizes ranging from 270 to 1051 nm and AE ranging from 38.8 to 64.7%. The supernatants obtained from centrifugation were analyzed by HPLC-DAD to assess the qualitative profile of non-encapsulated polyphenols, indicating the presence of rutin and malvidin-3-*O*-glucoside. The zeta potential varied between -15 and -32 mV, indicating that the Alg MPs should resist aggregation.

CS-GPE MPs were obtained with sizes ranging from 418 to 853 nm, AE of TPC comprised between 38 and 60%, and zeta potential varying between 7 and 15 mV. Non-encapsulated polyphenols in CS-GPE MPs showed the same profile as Alg-GPE MPs, as only rutin and malvidin-3-*O*-glucoside were not encapsulated.

Table 5.4 shows the regression coefficients for the coded polynomial equations, with *F* values and the determination coefficients (R^2). The resulting equations were tested for adequacy and fitting by ANOVA. The fitted models were suitable according to the R^2 values. The size and association efficiency of TPC of the Alg-GPE MPs followed a second-order and a first-order equation respectively, and in both cases, the concentration of GPE was more determinant than the concentration of alginate suggesting that an increase in GPE could improve the AE but would also significantly increase the particle size. For CS-GPE MPs, the size and AE followed a linear behaviour and both were mainly affected by the concentration of the polymer rather than the GPE suggesting that a higher concentration of TPC would not necessarily increase the AE.

By setting the desirability of dependent variables to maximum, it was possible to obtain the best formulations for both polymers. The optimized formulation for Alg-GPE MPs was set at 3 $mg.mL^{-1}$ of alginate and 15 $mg.mL^{-1}$ of GPE and for CS-GPE MPs at 5.12 $mg.mL^{-1}$ of polymer and 20 $mg.mL^{-1}$ of GPE.

Table 5.4 – Coded second-order regression coefficients for MPs size or association efficiency.

Coefficient*	MPs size		Association Efficiency	
	Alginate	**Chitosan**	**Alginate**	**Chitosan**
$ß_0$	818.79	100.77	82.16	24.45
$ß_1$	43.42	43.78	0.55	5.84
$ß_2$	60.74	21.47	2.43	0.54
$ß_{11}$	3.69	-	-	-
$ß_{22}$	2.70	-	-	-
$ß_{12}$	0.22	-	-	-
R^2	0.94	0.91	0.94	0.95
F	31.24	18.65	28.61	41.25

* Coefficients of polynomial equation $y = B_0 + B_1x_1 + b_2x_2 + b_{11}x_1^2 + b_{22}x_2^2 + b_{12}x_1x_2$, where x_1 is chitosan or alginate concentration and x_2 is GPE concentration.

5.3.2. Molecular interactions analysis

Analysis by FTIR was done to identify potential physicochemical interactions between GPE and the Alg or CS MPs that could lead to the formation of new covalent bonds. Figure 5.2 shows the FTIR spectra of GPE, empty MPs and GPE-loaded MPs, of Alg and CS, respectively.

The most sensitive absorption region of the GPE major components is comprised between 1600 and 800 cm^{-1}. The GPE spectra showed four major peaks, at 1540, 1380, 1040 and 995 cm^{-1} that correspond to the stretching of N-O bonds of nitro compounds, bending of C-H bonds, stretching of CO-O-CO bonds of anhydride compounds and to the bending of C=C bonds, respectively. Infrared spectrum also presented two medium peaks at 1215 and 1080 cm^{-1}, characteristic of the C-O stretch in the C-OH group as well as the C-C stretch in the carbohydrate structure (Anjos *et al*., 2015).

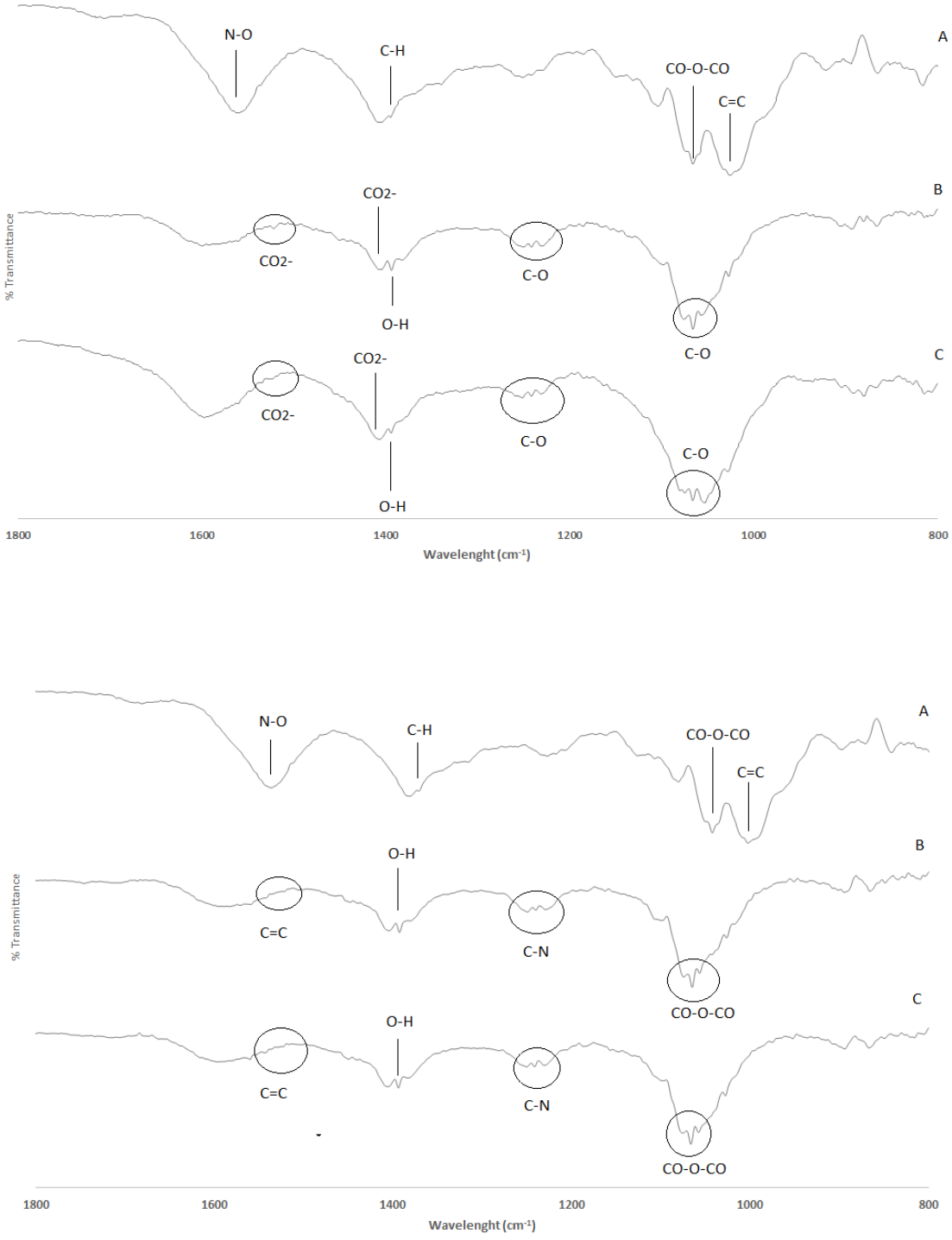


Figure 5.2 (A) - FTIR spectra of (A) GPE, (B) GPE-Alg MPs and (C) Alg-MPs and (B) – FTIR spectra of (A) GPE, (B) GPE-CS MPs and (C) CS-MPs.

The spectra of both Alg MPs and Alg-GPE MPs presented a similar profile, suggesting that GPE was mostly entrapped within these MPs. Representative bands include a broad band

between 1660 and 1530 cm^{-1} that could be attributed to a C=C or to an antisymmetric CO_2- stretch; a double band at 1405 and 1395 cm^{-1} attributed to a symmetric CO_2- stretch and a O-H bond deformation; a wide band between 1270 and 1200 cm^{-1} due to C-O stretching of aromatic esters; and a 1110 – 1010 cm^{-1} wide broad band that occurs in GPE, and Alg MPs and Alg-GPE MPs that are characteristic of a C-O bond stretching vibration of pyranosyl ring and to C-O stretching from C-C-H and C-O-H deformation (Lawrie *et al*., 2007; Daemi and Barikani, 2012).

CS MPs and CS-GPE MPs were similar. Representative bands include a broad band at 1090 – 1000 cm^{-1} that is attributed to CO-O-CO stretching; peaks at 1250, 1240 and 1230 cm^{-1} corresponding to C-N stretching of amine and amide groups in CS; a carboxylic acid O-H bond bending at 1395 – 1380 cm^{-1} and; a broad band between 1660 and 1530 cm^{-1} that can be attributed to a C = C or to an antisymmetric CO_2- stretching. The FTIR spectra allowed to conclude that the association between the polymers and GPE did not promote any chemical changes that could alter the properties of the GPE.

5.3.3. Bioaccessibility of polyphenols during gastrointestinal digestion

An *in vitro* gastrointestinal (GI) simulation model was used to mimic the physiological conditions of digestion and evaluate the release profile of phenolic compounds from the MPs along the GI tract and determine their bioaccessibility. It has been previously reported that GPE is rich in different phenolic compounds, including p-coumaric acid, rutin, neochlorogenic acid and different anthocyanins such as malvidin and petunidin. However, the bioactive potential of GPE is partially lost during GID mainly due to the degradation of polyphenols that confer antimicrobial and antioxidant activities (Costa *et al.*, 2019).

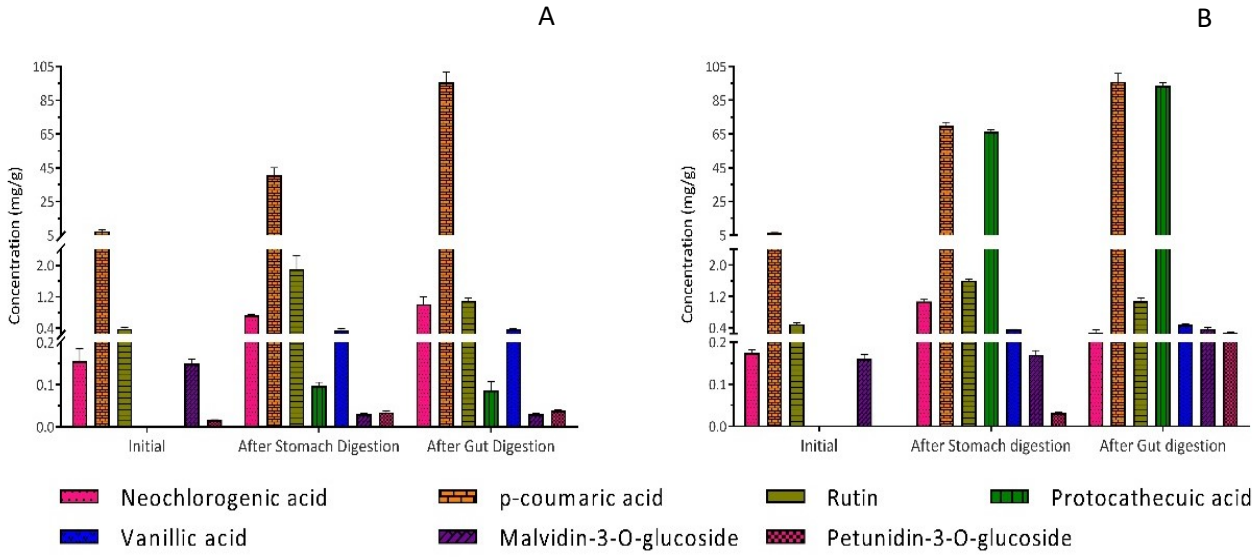


Figure 5.3 – Release of phenolic compounds along the *in vitro* gastrointestinal digestion from encapsulated extract in (A) Alg-GPE MPs and (B) CS-GPE MPs.

Figure 5.3 shows that before digestion only neochlorogenic acid, *p*-coumaric acid and rutin could be detected, suggesting that these compounds were not completely encapsulated with the particles and remained in digestion in the stomach, it was possible to determine the presence protocathecuic and vanillic acids, malvidin-3-*O*-glucoside and petunidin-3-*O*-glucoside. CS-GPE MPs yielded a higher concentration of released compounds given its solubility in acidic media, as found in the stomach. Digestion in the gut led to more polyphenols being released from the polymeric matrix thus increasing their concentration. At acidic pH, the hydroxyl groups of phenolic compounds are deprotonated, while the CS and Alg amine groups are ionized, resulting in moderately swollen MPs that allow the release of polyphenols along the tract (Liang, 2017). Alg-GPE and CS-GPE MPs did not show significant differences in the phenolics release profile, except for *p*-coumaric acid after digestion in the stomach and protocathecuic acid after both digestion phases, which were significantly more released from GPE-CS MPs ($p < 0.05$).

These results are in accordance with those obtained by Liang *et al*., who showed that CS MPs were capable to prevent the degradation of tea polyphenols in the GI tract (Liang *et al*., 2017). Gibis and co-workers also encapsulated a grape seed extract into CS/pectin – coated liposomes and concluded that these carriers were capable to protect polyphenols (Gibis *et al*., 2012). The efficiency of Alg, in the form of beads or hydrogel, has also been reported for the protection of a yerba mate phenolic-rich extract during the simulation of GI digestion (Córdoba *et al*., 2013). Here, Alg-GPE and CS-GPE MPs also prove be able to protect GPE and reduce the degradation of polyphenols along the GI tract leading to a higher bioaccessibility.

5.3.4. Antioxidant capacity

The antioxidant capacity of GPE, Alg-GPE and CS-GPE MPs through ABTS and ORAC methods is presented in Figure 5.4. The antioxidant capacity of GPE was measured by the ABTS assay and estimated at 33.98 ± 0.47 mg AAeq.g^{-1} of extract and decreased to 16.77 ± 0.15 mg AAeq.g^{-1} of extract after digestion, indicating a 50.7% reduction of the antioxidant potential. Alg-GPE MPs displayed a smaller reduction of antioxidant capacity at 31.9% following a reduction from 22.75 ± 1.04 to 15.50 mg ± 1.99 AAeq.g^{-1} of MPs after digestion. CS-GPE MPs, in their turn, presented an antioxidant capacity of 20.75 ± 1.17 mg AAeq.g^{-1} of MPs, which increased to 23.20 mg AAeq.g^{-1} ± 0.70 after digestion – an increase of 11.8%. The ORAC assay showed a similar trend: GPE in free form decreased its antioxidant activity after digestion from 3500 to 386 μmol TE.g AAeq.g^{-1}, corresponding to an 89% reduction. However, when encapsulated in the Alg-GPE MPs the reduction was lowered to 52% (2268 to 1087 μmol TE.g^{-1}) and the antioxidant activity actually increased by 20% when encapsulated in the CS MPs (2058 to 2463 μmol TE.g AAeq.g^{-1}). In both assays, the capacity

of CS MPs to protect the antioxidant activity of GPE was significantly higher than that of the Alg MPs ($p < 0.05$).

Although the encapsulation of GPE decreased its initial antioxidant capacity when compared to the free extract (due to lower availability of free phenolics), both carriers systems proved efficient to protect the antioxidant capacity of the extract along the GI tract. The antioxidant capacity of GPE was higher when encapsulated in the CS-GPE MPs ($p < 0.05$) than in Alg-GPE MPs, which is in line with the results obtained by HPLC-DAD (Figure 5.3) that showed a higher concentration of polyphenols after digestion for the CS-GPE MPs.

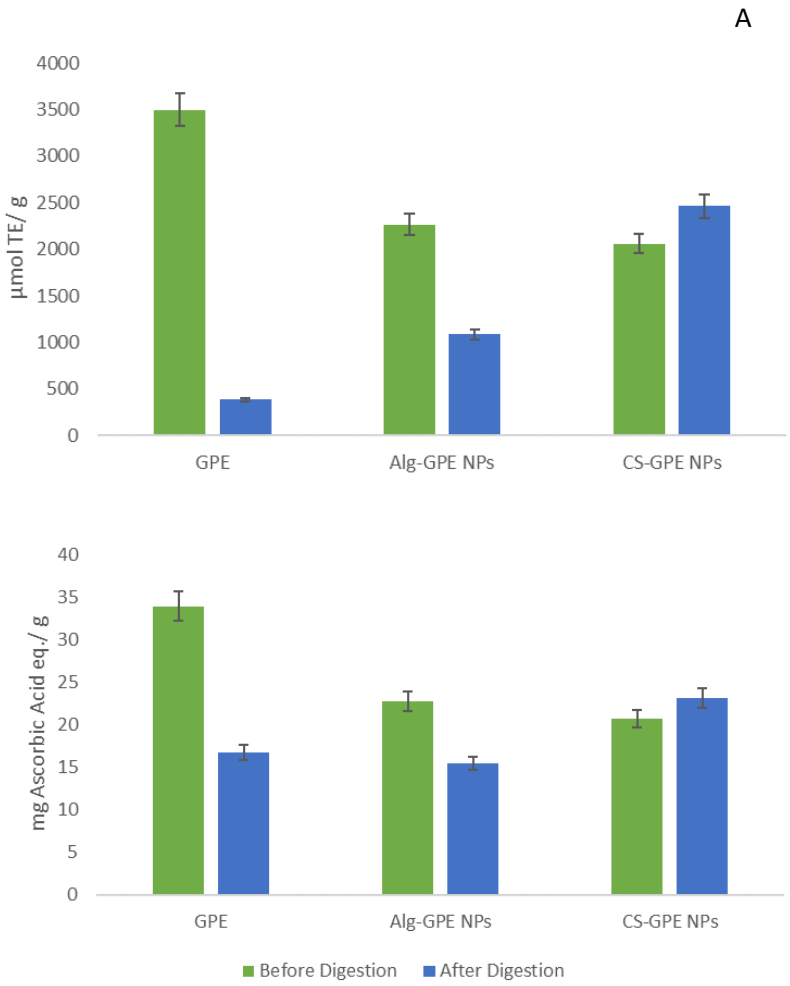


Figure 5.4 – Antioxidant capacity of GPE, Alg-GPE MPs and CS-GPE MPs, before and after GI digestion, determined through a) ABTS and b) ORAC methods.

These results are in accordance with the studies by Pasukamonset and co-workers (2016), who showed that the encapsulation of a polyphenol-rich extract from Asian pigeonwings petal flower into alginate beads allowed to reduce the degradation of polyphenols and increase their biological activity, through the improvement of antioxidant capacity.

This study demonstrated that the loss of antioxidant capacity observed for GPE after digestion through the GI tract could be overcome by encapsulation in Alg or CS MPs, particularly CS MPs that allowed to improve the antioxidant capacity of GPE, further supporting the use of these carriers as solutions to improve the bioaccessibility of polyphenols from GPE to the intestine.

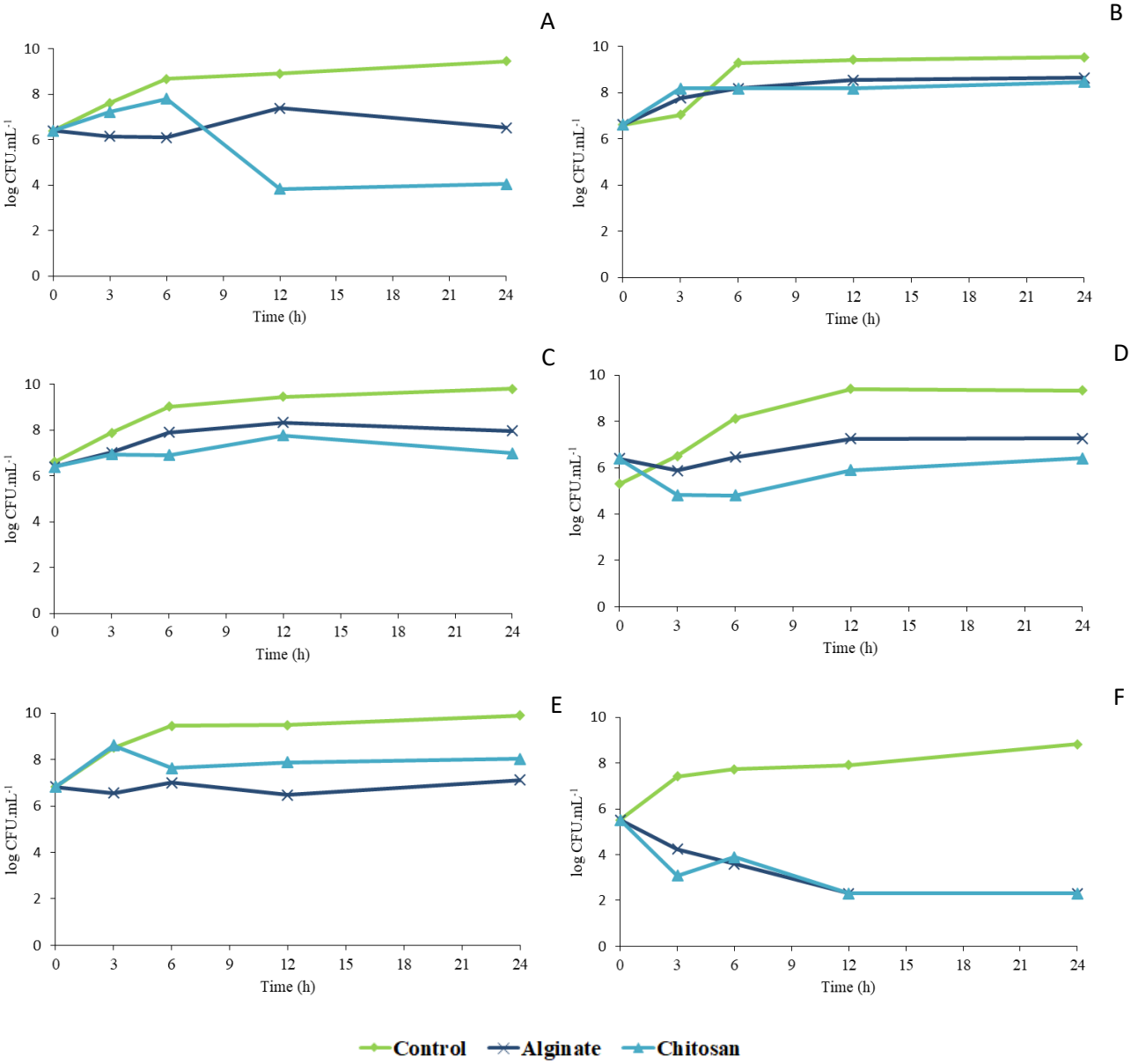
5.3.5. Antimicrobial activity of Alg-GPE and CS-GPE NPs

Owing to its resistance to gastric conditions and attributed antimicrobial properties, CS has been widely used within the context of encapsulation for the oral delivery of antimicrobial agents (Hejazi and Amiji, 2003; Madureira *et al*., 2015a). The use of Alg for the delivery of antibiotics also suggests that it could be used to protect the antimicrobial potential of GPE.

It has been previously reported that GPE presents significant antimicrobial activity against some pathogenic microorganisms, although this activity was loss after the GID (Costa *et al*., 2019). The encapsulation into polymeric particles was performed to improve the resistance of the extract along the GI digestion, improving their antimicrobial activity within the intestine.

The antimicrobial activity of encapsulated GPE after digestion was tested against common pathogenic microorganisms responsible for GI disorders. Figure 5.5 shows that GPE encapsulated in both CS-GPE and Alg-GPE MPs was able to inhibit the growth of all six pathogens studied when compared to the control – inoculated MHB without extract. Specifically, encapsulation in Alg-GPE MPs was able to promote a 3-log reduction of

MSSA, a 2-log reduction of *L. monocytogenes*, *P. aeruginosa* and *S. enteritidis*, and a 1-log reduction of *E. coli*, which was the least sensitive strain. The encapsulation of GPE in CS-GPE MPs promoted a 5-log reduction of MSSA, a 3-log reduction of *L. monocytogenes* and *P. aeruginosa*, and a 1-log reduction against *E. coli* and *S. enteritidis*. *Candida albicans* was



the most sensitive strain with both types of MPs promoting a 6-log reduction of viable cell numbers.

Figure 5.5 – Effect of GPE encapsulated into Alg or CS MPs on total viable counts of (A) MSSA, (B) *E. coli*, (C) *L. monocytogenes*, (D) *P. aeruginosa*, (E) *S. enteritidis* and (F) *C. albicans*.

These results are in accordance with the literature, which has shown that GPE acts in the colon as a powerful antimicrobial agent (Madureira *et al*., 2015b). The bioaccessibility studies showed that CS MPs were more efficient in the protection and consequent release of most anthocyanins and other phenolics such as neochlorogenic acid, which are known for their antimicrobial activity. These compounds act on cell membrane fluidity and fatty acid profile leading to membrane disruption (Silva *et al*., 2015; Costa *et al*., 2019). This phenomenon is more evident in Gram-positive bacteria (MSSA and *L. monocytogenes*), which due to their thicker peptidoglycan layer, present more affinity for the action of polyphenols (Madureira *et al*., 2015) – explaining the higher effect of CS-GPE MPs on these bacteria. The concentration of anthocyanins after digestion in the gut was lower for the Alg-GPE NPs, which exhibited a lower antimicrobial activity against MSSA and *L. monocytogenes* when compared to the CS-GPE MPs. Nevertheless, the Alg-GPE MPs were able to inhibit the growth of the six strains reflecting the antimicrobial activity of the other polyphenols released after digestion.

5.3.6. *In vitro* intestinal permeability

Free GPE, Alg-GPE MPs and CS-GPE MPs were added to the apical compartment of a Caco-2/HT29-MTX co-culture model and the permeability of released xylobiose and phenolics was measured over time. The integrity of the intestinal epithelium was assessed by measuring the TEER (Figure 5.6) along the experiment. The addition of free GPE caused an immediate reduction of the TEER to 20% when compared to the control. This effect was significantly mitigated by the encapsulation in both Alg-GPE and CS-GPE MPs where the reduction in TEER became apparent only after 30 min of incubation and to a lesser extent (80% over the control).

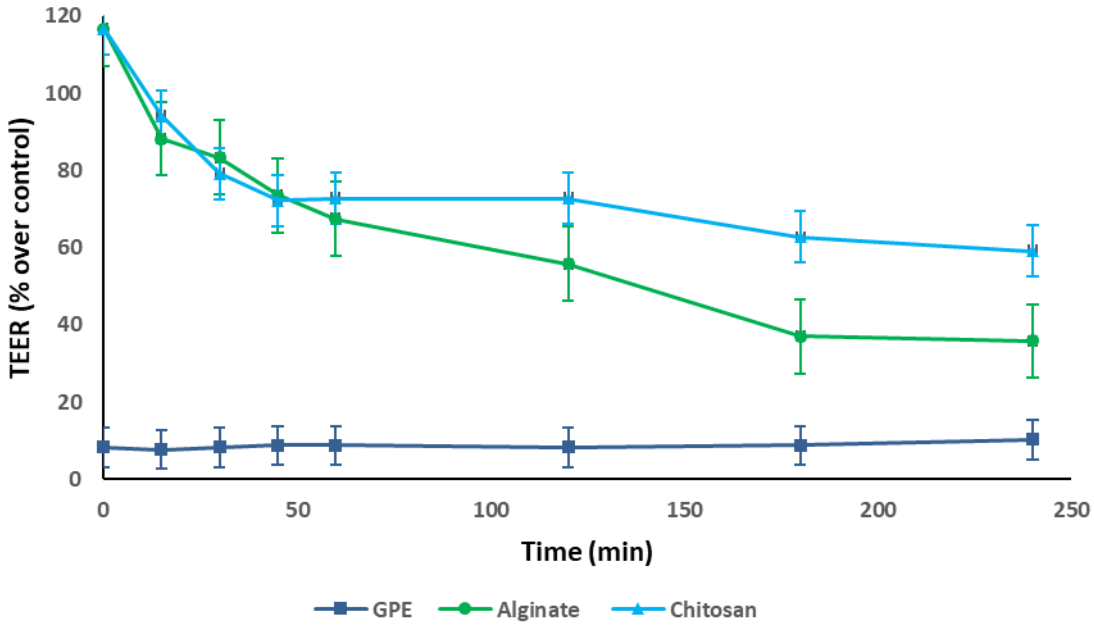


Figure 5.6 –TEER of Caco-2/HT29-MTX cells along the permeability assay.

Figure 5.7 shows that the cumulative intestinal permeability of xylobiose from the free extract was significantly higher when compared to both the Alg or CS delivery systems. The drop in TEER observed with the free GPE suggests the opening of intercellular tight junctions that, together with smaller particle size, could lead to increased intestinal permeability. There were no significant differences in the permeability of xylobiose from the Alg-GPE and CS-GPE MPs during the first 60 min of incubation. However, the permeation was higher for the CS-GPE MPs between the 60-min to 210-min time points ($p < 0.05$). These results are in accordance with the work by Courts, which showed that dietary oligosaccharides can cross tight junctions without needing to hydrolyse into monosaccharides (Courts, 2013). Furthermore, this study suggested that both Alg and CS provide a controlled release of xylobiose, decreasing its permeability across the intestinal epithelium and therefore, allowing higher amounts to remain in the gut and be fermented by the microflora.

Regarding the permeation of polyphenols across the Caco-2/HT29-MTX co-culture only chlorogenic and vanillic acids were capable of crossing the cell layer. The mass ratios of chlorogenic acid from free GPE and Alg-GPE MPs that permeated the intestinal epithelium were of 0.01% and 0.07%, respectively. These results are in accordance with the study conducted by Konishi and Kobayashi, who showed that more than 99% of the loaded chlorogenic acid remained in the apical compartment (2004).

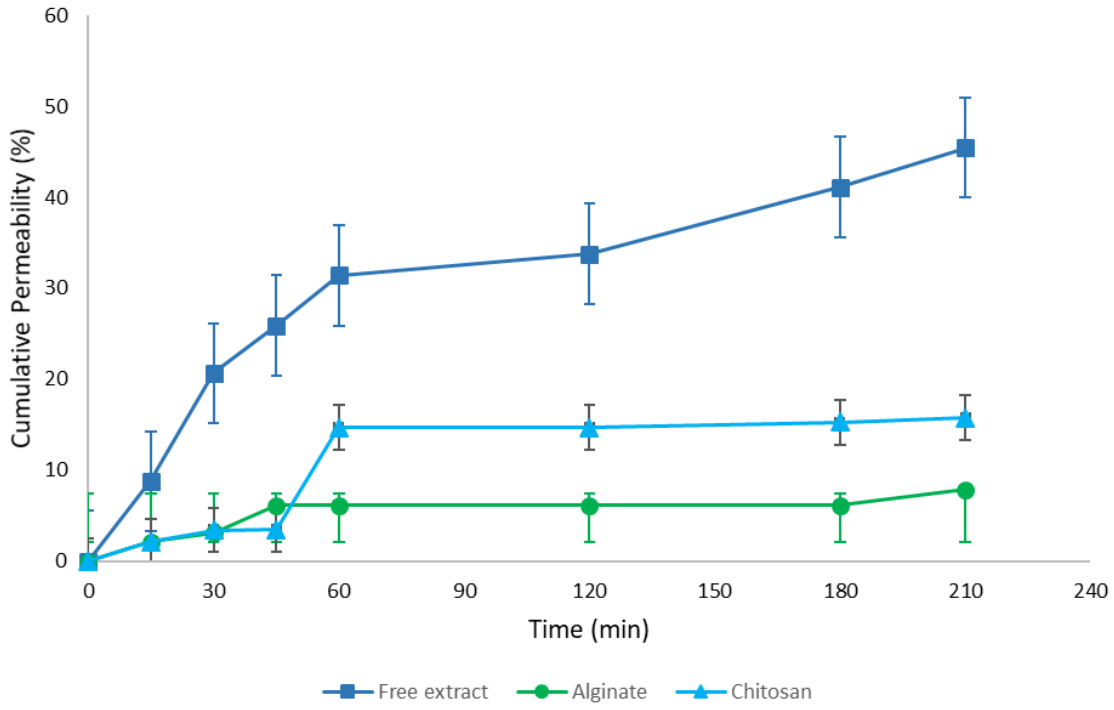


Figure 5.7 – Permeability of free and encapsulated XOS across Caco-2 and HT29-MTX co-culture up to 4 h.

The cumulative permeation of vanillic acid in free GPE was 6.6%, after 60 min. The encapsulation into CS-GPE MPs reduced the permeability to 0.53%, after 60 min, and the encapsulation into Alg-GPE MPs significantly increased the permeation of vanillic acid across the membrane to 75% after 45. The differences observed for the permeability of vanillic acid from Alg MPs and from CS MPs may be explained by the negative charge of vanillic acid at neutral pH, such as the pH of the intestinal epithelium in *in vitro* studies. The

negatively charged vanillic acid and positively charged chitosan may interact in these conditions, explaining the less permeation across the membrane, as compared to negatively charged sodium alginate.

5.3.7. Cellular uptake of chitosan

5.3.7.1. Synthesis of fluorescently-labelled chitosan

Cy5.5 Mono NHS Ester reacted with CS amine to produce Cy5.5-labelled chitosan (Cy5.5-CS). The recovery yield was 41% and the removal of unconjugated dye was confirmed by the absence of fluorescence in the diafiltrates, after extensive washing with ultrapure water (Figure 5.8).

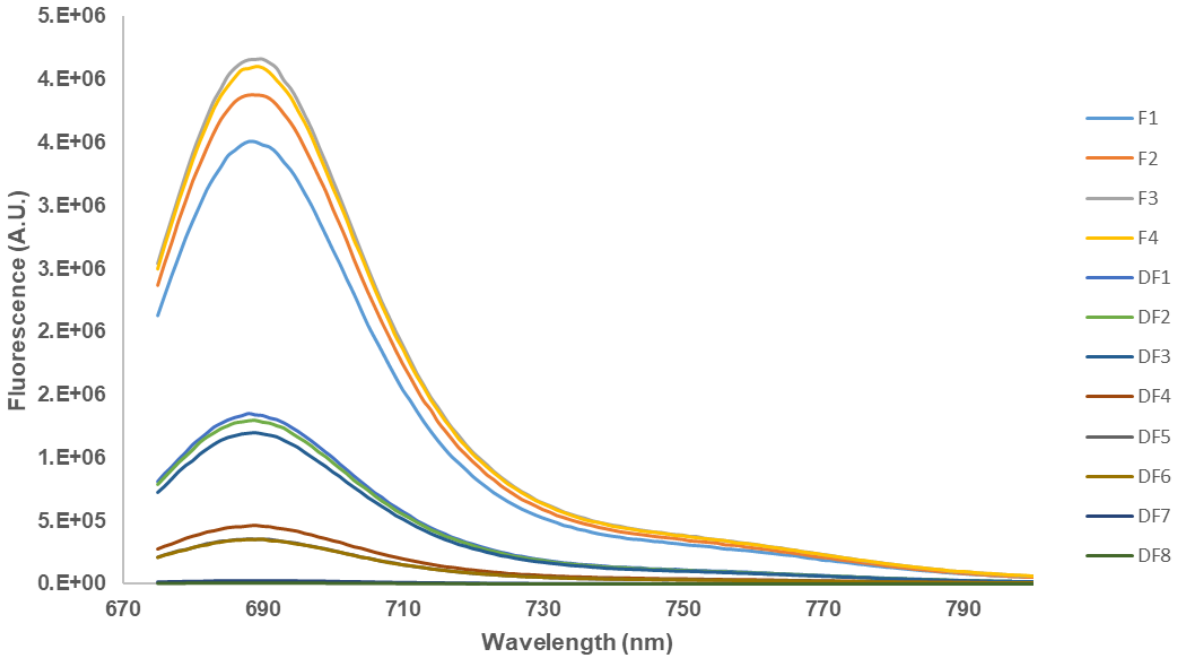


Figure 5.8 – Fluorescence emission spectra (λex=650 nm) of the Cy5.5-CS before and after filtration through 10 kDa MWCO Amicon Ultra-15 centrifugal filters.

Cy5.5-CS was used to produce empty and GPE-CS particles (Cy5.5-CS particles and Cy5.5-CS-GPE particles) using the same conditions as the label-free CS-GPE MPs. The Cy5.5-CS-

GPE particles showed a significantly larger size ($p < 0.05$) at 1323.8 ± 412.9 nm, and were highly polydisperse (PdI = 0.688). In addition, the zeta potential of the Cy5.5-CS-GPE particles decreased to around 0 mV, owing to the negative charge of Cy5.5, which could reduce the stability of the particles (Pedrosa *et al*., 2017). In order to produce more stable MPs, studies of the isoelectric point of GPE and Cy5.5-CS could be performed to adjust the pH value of NPs and thus improve their stability.

5.3.7.2. Biocompatibility of Cy5.5-labelled chitosan

The biocompatibility of Cy5.5-CS and of the Cy5.5-CS and Cy5.5-CS-GPE particles was assessed by the determination of their effect on the metabolic activity of Caco-2 and HT29-MTX cells after 24 h of incubation. Figure 5.9 shows that the viability of both cell lines was significantly reduced ($p < 0.001$) after incubation with DMSO (negative control). In contrast, the Cy5.5-CS and Cy5.5-CS-GPE particles did not have any significant effect on the metabolic activity of both cell lines when compared to the control (PBS) for the range of concentrations tested (5 to 500 $\mu g.mL^{-1}$). The cytotoxicity of GPE, before and after digestion, has been previously assessed and no harmful effects were observed (Costa *et al*., 2019). These results showed that the Cy5.5-CS and the empty and GPE-loaded Cy5.5-CS particles were non-toxic to Caco-2 and HT29-MTX cells at concentrations up to 0.5 $mg.mL^{-1}$, which was the concentration used for the permeability studies of chitosan across the Caco-2/HT29-MTX co-cultures.

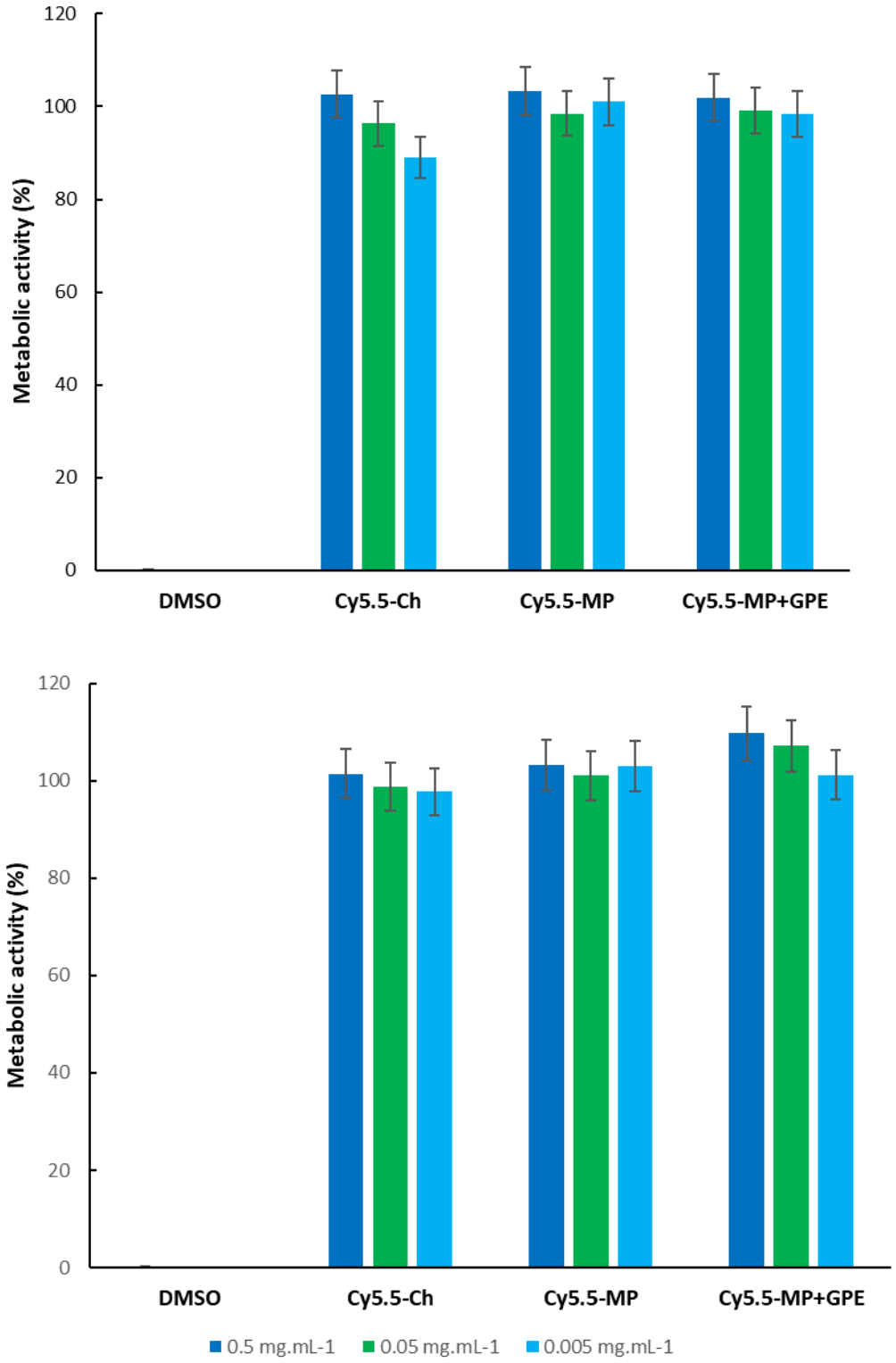


Figure 5.9 – Cell viability expressed as metabolic activity upon contact with Cy5.5-CS, Cy5.5-CS MPs and Cy5.5-CS-GPE MPs for (A) Caco-2 cells and (B) HT29 – MTX.

5.3.7.3. Cellular uptake

Images obtained from laser scanning confocal microscopy of the Caco-2/HT29-MTX on ibidi microslides after incubation with Cy5.5-CS, Cy5.5-CS NPs and Cy5.5-CS-GPE NPs suggest a reduction in the expression of the tight junction protein occludin when the cells were incubated with the Cy5.5-CS solution (Figure 5.10), which is in accordance with the literature that described the capacity of CS to open the cellular tight junctions (Vllasaliu *et al*., 2012; Zhang *et al*., 2014). However, it was much unexpected that Cy5.5-CS-GPE NPs promoted the integrity of the tight junctions, contradicting the literature and the results obtained for Cy5.5-CS and Cy5.5-CS NPs. Confocal microscopy images of the intestinal cell layer after 18 h contact with the Cy5.5-CS, Cy5.5-CS NPs and Cy5.5-CS-GPE NPs, allowed to qualitatively observe that Caco-2/HT29-MTX did not internalize the Cy5.5-CS NPs and Cy5.5-CS-GPE MPs, neither Cy5.5-CS even after the degradation of the tight junctions.

The orthogonal views show that the agglomerates remained on top of the cell layer and did not permeate the cells. We hypothesize that the renowned mucoadhesive properties of CS may be leading to the agglomeration of Cy5.5-CS on the mucus produced by the HT29-MTX cells.

In fact, it was unexpected that Cy5.5-CS was not able to permeate the epithelium, as its absorption-promoting capacity endorsed by the ability to induce opening of the epithelial tight junctions is widely described. Also, the molecular weight of the polymer (50 – 120 kDa), prior to modification with Cy5.5, can also difficult the absorption of CS from the gastrointestinal tract, as its permeability decreases with the molecular weight, and low bioavailability is described for CS with molecular weights higher than 20 kDa (Chae *et al*., 2005).

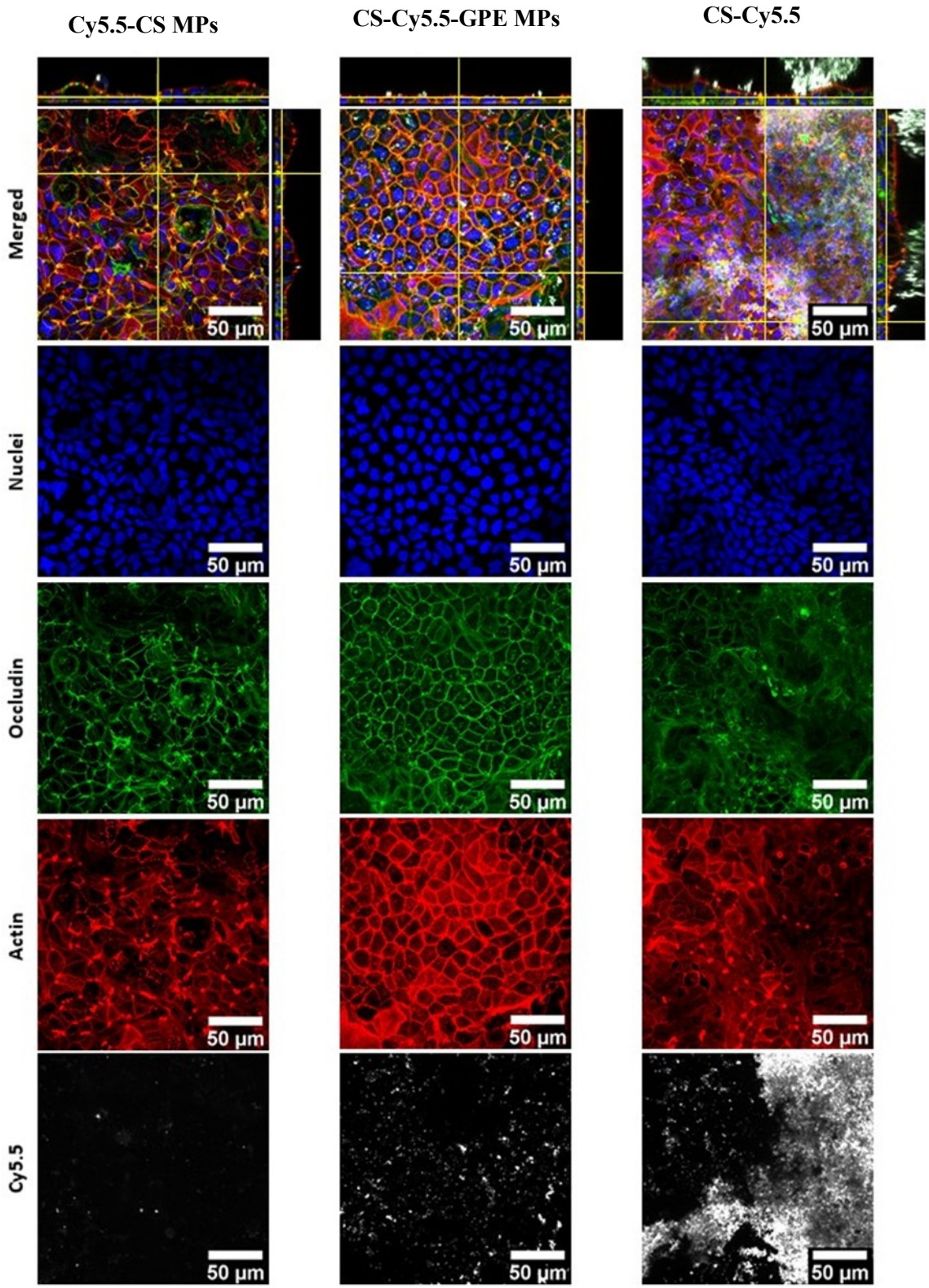


Figure 5.10 – Confocal analysis Caco-2/ HT29-MTX cells exposed to Cy5.5-CS, Cy5.5-CS MPs and Cy5.5-CS-GPE MPs. Cells were stained with DAPI (blue) for the nucleus, Alexa fluor 488 (green) for occludins, and phalloidin-TRITC for actin (red).

These results confirm that CS-GPE MPs are an interesting vehicle to deliver bioactive compounds to the intestine, as they provide protection against gastric digestion, allow the compounds to remain longer in the intestine, reducing their permeability across the epithelium. Moreover, it was proved that the polymer was not internalized by intestinal cells, thereof possible side effects promoted by its intake could be minimized.

5.3. Conclusions

This work studied the potential of Alg and CS MPs to be used as an oral delivery system of a bioactive grape pomace extract. The encapsulation of GPE into alginate and chitosan through ionic gelation allowed the formation of MPs with sizes comprised between 400 and 1000 nm and high association efficiency of phenolic compounds and XOS. Both systems allowed to protect the extract from the gastrointestinal digestion conditions and thus increase the bioaccessibility of different polyphenols, including anthocyanins, which was reflected on an improvement of the antioxidant and antimicrobial activities of GPE, including the inhibition of intestinal pathogenic microorganisms. Permeability studies across a Caco-2/ HT29-MTX co-culture model showed that the encapsulation of GPE decreased the permeability of xylobiose, allowing it to remain longer in the intestine and potentially improving the prebiotic potential of GPE. The modification of the chitosan microparticles with Cy5.5 for tracking did not affect the biocompatibility of GPE-loaded MPs with both Caco-2 and HT29-MTX cell lines and analysis by confocal microscopy confirmed the integrity of cellular tight junctions after incubation with the fluorescently-labelled particles for 18 h.

CHAPTER 6

Development of a functional beverage through incorporation of an encapsulated grape bioactive extract

Abstract

The goal of this study was the development of a novel healthy coconut beverage through the incorporation of 2.5% (w/v) GPE encapsulated into alginate (GPE-Alg) or chitosan (GPE-CS) particles. Physico-chemical stability under two storage conditions (ready-to-reconstitute beverage at room temperature, or liquid beverage at 4 ˚C) was evaluated throughout 60 days, regarding pH value, total soluble solids, color, total anthocyanins and TPC. The antimicrobial and prebiotic potential after the digestion of the beverages using a simulated gastrointestinal system was assessed for different pathogens and probiotic strains, respectively. Sensory analysis was also performed to evaluate the differences promoted by the incorporation of the encapsulated GPE.

Incorporation of GPE-Alg in the coconut water did not affect the pH of beverage but introduced some differences in total soluble solids and color, while GPE-CS affected all these three parameters. Freeze-drying of beverage accelerated the rate of degradation of total phenolics and anthocyanins, comparing to the liquid formulation storage at 4 ˚C. The GPE-Alg presented higher half-life time of degradation for phenolic acids and GPE-CS presented

higher half-life time degradation for anthocyanins. Digested functional beverages with GPE-Alg and GPE-CS decreased the growth of *Staphylococcus aureus*, *Listeria monocytogenes* and *Candida albicans*, while promoting the growth of different bifidobacteria and lactobacilli strains. Sensory analysis allowed to conclude that the incorporation of GPE-Alg and GPE-CS did not promote significant differences in most of evaluated attributes.

Therefore, results suggest that the incorporation of bioactive GPE in coconut water could be used for development of a novel functional beverage, rich in phenolic compounds (mainly anthocyanins) and with potential antimicrobial and prebiotic activities. This study opens the opportunity in the application of food by-products in the development of novel efficient functional foods and beverages.

6.1. Introduction

In the last decade, the increasing consciousness that diet and health are closely related and nutrition can have benefits in the mitigation of some diseases, has led to an increased demand for new healthier foods and beverages (Corbo *et al*., 2014; Nazir *et al*., 2019). The wide diffusion of these functional foods throughout the highly competitive market has thinned the line between nutrition and pharmaceutics, so the concepts of functional food and nutraceutics are often blurred (Corbo *et al*., 2014). Although, there is no a sole consensual definition of functional food, it is accepted that they should have some properties, including the enhance of a biological property or help in the prevention of disease, which means that it should have benefits beyond the nutritional function, and finally should be in the form of a common food or beverage, meaning that it can be consumed as part of the daily diet and routine (Nazir *et al*., 2019).

Nowadays, beverages are the most common category of functional foods as they are convenient to consume within the modern busy lifestyle, highly stable at shelf or refrigerated

storage, and the incorporation of the bioactive ingredient is usually easier (Corbo *et al*., 2014; Nazir *et al*., 2019). The formulation of these products usually is designed considering the addition of one or more bioactive ingredient, leading to a final beverage fortified or enrichment with polyphenols, prebiotics or probiotics, proteins, or minerals that provide the beverage different functional properties, such as antioxidant, prebiotic, immunostimulant, cholesterol regulation, among others (Corbo *et al*., 2014; Nazir *et al*., 2019).

Together with the functional foods trend, consumers are also demanding for more sustainable food products, as it is even more evident the negative impact of the food production systems, including the agriculture and food industries, in the climate changes, mainly caused by the accumulation of by-products (Asioli *et al*., 2017). Currently, by-products applications include their use as fertilizers, compost, fuel or animal feed, and in the worst-case scenario, as wastes are incinerated or landfilled, which constitute a high environmental impact, without generating value and thus, leading to high management costs (Martin *et al*., 2016). These agroindustrial by-products are also a considerable source of bioactive compounds, such as carotenoids, phenolic compounds, dietary fibers and proteins, which can be applied as ingredients in functional foods, increasing their nutritional value and providing beneficial properties to the consumer. The development of new ingredients from agro-food by-products allows us to simultaneously address two imperative issues in food processing, namely to increase the sustainability of processes and supply new raw materials of interest.

Coconut water is largely consumed in tropical countries such as Brazil and Thailand, appreciated for its sweetness and freshness, and it is taken directly from the inner part of the fruit (Walter *et al.*, 2014; Prades *et al*., 2011). It is rich in sugars as fructose, glucose, and sucrose, and in minerals, including potassium, calcium, magnesium and phosphorous, with varying concentrations along the maturation process (Walter *et al.*, 2014). The World Health

Organization highly recommends this drink for rehydration in the cases of cholera and diarrhea due to its high content in potassium, which makes it also a natural isotonic drink for athletes (Walter *et al.*, 2014). Coconut water is also a major by-product in coconut processing plants, such in the processing of ready-to-eat coconut, namely fresh minimally processed or desiccated coconut (Prades *et al*., 2011). Nevertheless, due to its low pH (5.8) and high water activity, in addition to high sugar content, this product allows a fast microbial growth, which requires an extra care when using this product (Prades *et al*., 2011; Walter *et al.*, 2014)

Grape pomace (GP) is also another major industrial by-product that has been described as bioactives source, due to the presence of polyphenols, oligosaccharides, minerals that provide its aqueous extract with antioxidant, antimicrobial and prebiotic potentials (Costa *et al*., 2019). It was also found that the compounds that confer these properties to the extract are not resistant to the gastrointestinal digestion, needing to be encapsulated into polymeric systems that allowed the GPE reach the gut and maintain its biological properties (Costa *et al*., 2019).

GP as a source of phenols and fiber has been applied in the formulation of a wide variety of functional foods, including fermented milks, yogurt, ice-creams, salad dressing and cheeses (Dos Santos *et al*., 2017; Karnopp *et al*., 2017; Hwang *et al*., 2009; Lucera *et al*., 2019; Tseng and Zhao, 2013). Nonetheless, its application in the formulation of coconut non-fermented beverages has never been exploit. Considering the nutritional properties and convenience of coconut water, makes this an ideal carrier to incorporate functional ingredients such as a bioactive GPE to develop a new functional beverage, which is able to inhibit the pathogens responsible for diarrhea and modulate the intestinal microflora, at the same time it can restore the body hydration and electrolytes.

Thus, the main objective of this study was to develop a new healthy coconut beverage by the incorporation of a bioactive GPE encapsulated into polymeric particles, alginate (GPE-

Alg) or chitosan (GPE-CS) with proven antioxidant, antimicrobial and prebiotic activities. In addition, evaluation of their bioactive properties, product stability and consumer acceptance were also studied.

6.2. Material and Methods

6.2.1. Raw materials

Water from mature coconuts was provided by Nuvi Fruits S. A. (Torres Vedras, Portugal) as byproduct, vacuum-filtered and pasteurized at 80 ˚C for 5 min. Coconut water was characterized for brix degree, pH, minerals, titratable acidity and sugars profile. GP was provided by Ouro Verde Winery (Bahia, Brazil), and was oven-dried at 45 ˚C for 24 h, milled and sieved in a Bonina 0.25 df depulper (Itametal, Bahia, Brazil).

6.2.2. Production of coconut beverages with encapsulated extract

6.2.2.1. Production of GPE

GPE was obtained through enzymatic extraction of a GP flour, using an enzymatic cocktail with xylanase activity produced by *Aspergillus niger* 3T5B8, as previously described.

6.2.2.2. Encapsulation of GPE

GP was then encapsulated into alginate or chitosan particles, as previously described and illustrated in Figure 6.1. Briefly, GPE was dissolved into an alginate solution (3.0 $mg.mL^{-1}$), at final concentration of 15 $mg.mL^{-1}$, followed by ionic gelation with $CaCl_2$, and chitosan particles were produced by dissolving the GPE at final concentration of 20 $mg.mL^{-1}$ into low molecular weight chitosan acidic solution (5.12 $mg.mL^{-1}$) followed by ionic gelation with sodium tripolyphosphate (TPP). The particle size of alginate particles was 523 nm and of chitosan particles was 853 nm, safe and suitable for gastrointestinal delivery.

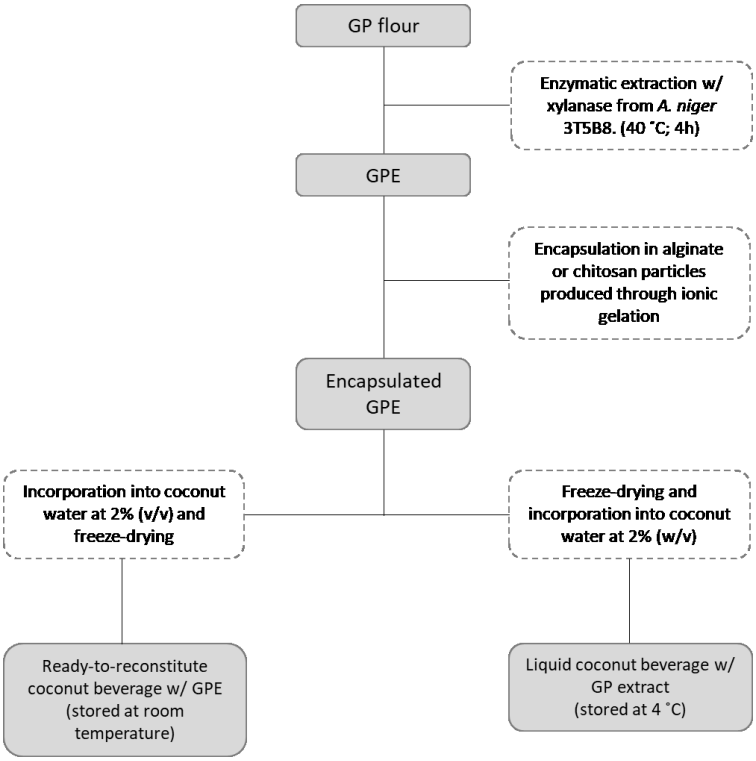


Figure 6.1 – Flow diagram of production of functional coconut beverages with encapsulated GPE.

6.2.2.3. Production of functional coconut beverages

Functional coconut beverages were produced using two different approaches: a) a liquid beverage through the incorporation of freeze-dried GPE-loaded particles in coconut water, at final concentration of 2% (w/v); b) a ready-to-reconstitute (RTR) beverage formulated through the incorporation of liquid GPE-loaded particles in coconut water at final concentration of 2% (v/v), followed by freeze-drying.

6.2.3. Assessment of coconut beverages stability

Stability of coconut water functionalized with GPE-Alg or GPE-CS was assessed using two storage methods: liquid coconut beverages, produced through method A, were stored at 4 ˚C; ready-to-reconstitute coconut beverages, produced through method B, were stored in a

desiccator, protected from light and solubilized in ultra-pure water at final concentration of 10% at the time point of stability measurement. Stability measurements were performed in triplicate at 0, 2, 7, 14, 30 and 60 days. Plain coconut water was used as control.

6.2.3.1. Microbiological control

Microbiological stability was assessed through direct plating into Plate Count Agar (PCA) for quantification of total viable cells, Potato Dextrose Agar (PDA) for quantification of molds and yeasts, De Man, Rogosa and Sharpe Agar (MRS) for quantification of lactic acid bacteria (LAB), MacConkey Agar for quantification Gram-negative enteric bacteria, and Mannitol Salt Agar (MSA) for quantification of *Staphylococcus aureus*. Microorganisms enumeration was performed by decimal dilutions in 0.1% (w/v) peptone water, and plated 100 µL in the different media through spread plate technique. PCA, MacConkey Agar and MSA plates were incubated for 24 h at 37 °C, MRS plates were incubated for 48 h at 37 °C and PDA plates were incubated for 5 days at 30 °C. All assays were performed in duplicate.

6.2.3.2. Determination of pH and total soluble solids

Total soluble solids (˚Bx) were measured at 20 ˚C, using a digital refractometer (Hanna Instruments, Portugal). pH value of coconut beverage was using a potentiometer (Crison Instruments, Barcelona, Spain).

6.2.3.3. Determination of color

The color was evaluated with a portable CR-400 colorimeter (Konica Minolta, New Jersey, USA), through a CIELab color scale that evaluates the degree of lightness (L), redness to greenness (a) and yellowness to blueness (b). Color of coconut beverages was measured against a white standard plate, with standard color coordinates L = 97.7, a = 0.04 and b = 1.47. These values were then used to calculate the overall change in color (ΔE) along the storage period, according to Eq. 6.1 (Chung *et al.* 2016).

$$\Delta E = [(L_{CB} - L_{standard})^2 + (a_{CB} - a_{standard})^2 + (b_{CB} - b_{standard})^2]^{1/2} \quad (5.1)$$

6.2.3.4. Total phenolic compounds and anthocyanins

TPC content in coconut beverages was determined by Folin–Ciocalteu method (Singleton and Rossi, 1965). Quantification was done at 750 nm (UV mini 1240, Shimadzu, Tokyo, Japan) with gallic acid as standard in the range of 0.015–1.00 $mg.mL^{-1}$.

The total anthocyanins content in the coconut beverage was determined by measuring their absorbance at a wavelength of 523 nm, using a UV-vis spectrophotometer (Shimadzu, Japan).

6.2.3.4.1. *Kinetics of total phenolics and anthocyanins degradation*

The degradation of coconut beverages was monitored by phenolic and anthocyanins degradation kinetics using first-order models, as described by Chung *et al.* (2016). The first order reaction was expressed by Eq 6.2, where C_t is the phenolics content at *t* days of storage, C_0 is the initial phenolic content, *k* is the reaction rate constant, and *t* is the days of storage.

$$\ln\left(\frac{C_t}{C_0}\right) = -kt \quad (6.2)$$

The half-life time ($t_{½}$) for a first order reaction can be calculated using eq 6.3.

$$t_{1/2} = \frac{\ln(2)}{k} \quad (6.3)$$

6.2.4. Sensory evaluation

Sensory evaluation was performed only at day zero, by a group of nine semi-trained panelists. Participants were informed about the general aim of the work and test procedure.

Coconut beverages were evaluated using Attribute difference-from-control tests (Meilgaard *et al*., 2007). Each panelist received a portion of plain coconut water labeled as "control sample" and three coded samples: a second portion of plain coconut water (blind control sample) plus the GPE-CS and GPE-Alg test samples. The blind control and test samples were coded with three-digit random numbers and were presented to panelists in a balanced order. Panelist were asked to compare and rate the samples for general appearance, color, aroma, flavor and texture using a continuous line scale (0 = similar to the control sample, 10 = very different from the control sample), apparent viscosity and coconut odor intensity using a continuous bipolar line scale (-5 = much weaker than to the control sample, 0 = similar to the control sample, 5 = much stronger than the control sample).

6.2.5. Assessment of functional properties of the coconut beverage

6.2.5.1. *In vitro* simulation of gastrointestinal digestion

Simulation of the gastrointestinal digestion of the coconut beverages was performed following the method described by Madureira *et al.* (2011) with slight modifications.

Mouth Digestion: The pH value was adjusted to 6.9, using HCl 1 M. Artificial saliva was simulated by using α-amylase at 100 $U.mL^{-1}$ and added at a rate of 0.6 $mg.mL^{-1}$ of digestion. Incubation was made during 2 min at 37 ˚C and 200 rpm.

Stomach Digestion: The pH value was adjusted to 2.0 using HCl 1 M. Gastric juice was simulated by dissolving pepsin 25 $mg.mL^{-1}$, and added at a ratio of 0.05 $mL.mL^{-1}$ of sample. Incubation lasted 120 min (long digestion), at 37 ˚C and 130 rpm.

Gut digestion: Simulation of gut conditions was performed by initial adjustment of pH value to 6.0 using $NaHCO_3$ 1 M. The intestinal juice was simulated by dissolving 2 $g.L^{-1}$ of pancreatin and 12 $g.L^{-1}$ bile salts. This solution was then added at a concentration of 0.25 $mL.mL^{-1}$ of sample. All samples were incubated during 1 h, at 37 ˚C and 45 rpm.

After gut digestion, enzymes were removed using 3 kDa cut-off filters and the sample was freeze-dried. All assays were performed in triplicate.

6.2.5.2. Antimicrobial activity

Antimicrobial activity of coconut beverages after digestion was determined upon *Staphylococcus aureus* (MSSA) ATCC 25923, *Listeria monocytogenes* (food isolate from ESB collection) and *Candida albicans*, as these microorganisms are representative of Gram-positive and Gram-negative pathogens, and yeasts.

For determination of growth inhibition curves, inocula were prepared by suspending each bacterial colony into Mueller-Hinton broth, with a final concentration of ca. 10^8 CFU.mL^{-1}. Two microliters of each inoculum were transferred to a 96-well microplate, every well was fulfilled (to final volume of 200 µL) with the coconut beverages dissolved in MHB, at final concentration of 2% (w/v). Microplate was incubated in a microplate reader (Multiskan GO, Thermo Scientific) at 37 °C for 24 h, with absorbance measurements at 620 nm registered every hour. Three controls were also performed: the first one containing inoculum and MHB (positive control), the second one containing the coconut beverages (negative control) and the third one containing only MH broth.

6.2.5.3. Prebiotic potential

Potential prebiotic effect of coconut beverages after digestion was determined for *Bifidobacterium animalis* Bo (CSK, Ede, Netherlands), *Bifidobacterium longum* BG3 (Cell Biotech, Hellerup, Denmark), *Bifidobacterium animalis* spp. *lactis* Bb12, *Lactobacillus casei 01* (Chr. Hansen, Hørsholm, Denmark), *Lactobacillus rhamnosus R11* (Lallemand, Montreal, Canada), and *Lactobacillus plantarum* 299v (Probi AB, Lund, Sweden). Strains were stored at − 80 °C in de MRS broth (Biokar Diagnostics, Beauvais, France) with 30% (v/v) glycerol.

L. casei, *L. rhamnosus, and L. plantarum* inocula were prepared by suspending each bacterial colony into MRS broth, achieving a concentration of 10^7 CFU.mL^{-1}. The microplate wells were fulfilled with 245 µL syringe-filtered (0.22 µm) coconut beverages diluted in basal MRS broth without glucose, at concentration of 2% (w/v), and 5 µL of the inoculum. Microplate was incubated (Multiskan GO, Thermo Scientific) at 37 °C for 24 h with agitation, and cellular growth was monitored by measuring the Optical Density (OD) at 620 nm.

B. animalis Bo and *B. lactis* BB12 inocula were prepared under anaerobic atmosphere, by suspending each bacterial colony into MRS broth supplemented with 0.05% (v/v) L-cysteine-HCl, achieving a concentration of 10^7 CFU.mL^{-1}). The microplate wells were fulfilled with 245 µL syringe-filtered (0.22 µm) coconut beverages diluted in basal MRS broth without glucose, at concentration of 2% (w/v), 5 µL of the inoculum, and 50 µL of paraffin. Microplate was incubated (Multiskan GO, Thermo Scientific) at 37 °C for 48 h with agitation and cellular growth was monitored by measuring the OD at 620 nm.

For the evaluation of the prebiotic effect of the coconut beverages, growth rates of the bacteria tested were calculated in order to compare with the growth obtained with MRS basal media. This calculation was made by determination of the slope of the trend line of the OD 620 nm over log phase of the growth curves (Sousa *et al.*, 2015).

6.2.6. Statistical analysis

Statistical analysis was performed with IBM SPSS Statistic Program v 23.0 (Illinois, USA), using analysis of variance (ANOVA) with Tukey post-hoc test, and Wilcoxon test for non-parametric data. Differences were considered significant at a level of $p < 0.05$.

6.3. Results

Coconut water obtained from mature coconuts from dried fruits industry presented 9.0 ± 0.1 ˚Bx of total soluble solids, pH value of 5.79 ± 0.13 and titratable acidity of 0.14 ± 0.01 g. 100 mL^{-1}, expressed as citric acid. These values are slightly higher than the values described by Nambiar and co-workers, which were 6.5 ˚Bx, pH 4.5 and titratable acidity of 0.09 g citric acid·100 mL^{-1}, although these values are regarding tender coconuts and the increase of soluble solids, pH and acidity increases with aging (Nambiar *et al*., 2017). Regarding the pH value, it is in accordance with Prades and co-workers, who studied the pH of different coconut waters from mature coconuts and determined pH values ranged between 5.1 and 6.1, and with the results obtained by Halim and colleagues, who determined pH values in the range of 5.3 to 6.3 for mature coconuts (Prades *et al*., 2012; Halim *et al*., 2018). The ratio between total soluble solids and acidity is 64.3 is in accordance with other values reported for coconut water (between 60 and 70), indicating the beverage quality as low pH values and high sugar concentration promote the development of yeasts (Nambiar *et al.*, 2017; Costa *et al*., 2015a).

Coconut water presented high concentration of minerals, 4.78 ± 0.04 g.100 mL^{-1}, and sugars: 1.26 ± 0.07 g.100 mL^{-1} of glucose, 1.20 ± 0.05 g.100 mL^{-1} of fructose, 0.96 ± 0.11 g.100 mL^{-1} of sucrose, 0.15 ± 0.01 g.100 mL^{-1} of xylose and 1.08 ± 0.01 mg.mL^{-1} of cellobiose, which contribute to the high sweetness of the coconut water. The concentration of total sugars, 4.65 g.100 mL^{-1}, is slightly higher than the average of total sugars present in the different coconut varieties analyzed by Prades and co-workers, which ranged from 1.9 to 4.4 g.100 mL^{-1} (Prades *et al*., 2012). Nevertheless, the concentrations of glucose and fructose are in accordance with the values described for these sugars, which ranges 1.5 to 1.7 g.100 mL^{-1} for glucose and around 1.4 g.100 mL^{-1} for fructose (Prades *et al*., 2012; Vigliar *et al*., 2006).

Coconut water presented some microbial contaminations, in the range of 10^2 CFU.mL^{-1}, and specifically, 10^1 CFU.mL^{-1} for Gram-negative bacilli (MacConkey Agar medium) and 10^2 CFU.mL^{-1} for yeasts and molds (PDA). The European Regulation on microbiological quality in foods and drinks (WHO, 2016) demands that the presence of thermotolerant coliforms, determined through MacConkey medium, can not be present, thus the coconut water was pasteurized before further use. After pasteurization, no viable cells were found in the different media.

6.3.1. Stability studies

6.3.1.1. pH and total soluble solids

Functional coconut beverages with GPE-Alg or GPE-CS particles were stored in liquid form at 4 ˚C or in ready-to-reconstitute form at room temperature, along 60 days. Plain coconut water was stored at both conditions as a control. Microbiological control performed during the storage showed that the beverages were stable along the 60 days of storage as no viable cells were detected in all culture media used.

Differences in pH value and total soluble sugars were analyzed between the functional beverages and the control, between the storage conditions and along the storage period, and it ranged between 5.17 and 6.08. Figure 6.2A presents the results of pH measurement of different beverage formulations along the storage period. The pH of with GPE-Alg coconut beverages did not differ from the control ($p > 0.05$), except for the ready-to-reconstitute formulation at day 0, but the addition of chitosan promoted a significant decrease of pH value ($p < 0.05$) in both storage conditions, which should be expected due to the presence of acetic acid in these particles, contributing to a decrease in pH values. The ready-to-reconstitute functional beverages presented differences ($p < 0.05$) after day 7: the pH value of GPE-Alg beverage increased, while the pH value of GPE-CS beverage decreased due to

the particles degradation, releasing acetic acid, decreasing the pH and thus, a general degradation of the beverage.

The storage conditions did not affect pH of control and GPE-Alg beverage, but ready-to-reconstitute beverage with GPE-CS presented significant differences from GPE-CS liquid beverage ($p < 0.05$) after day 7, since the pH value drastically decreased after this period of storage, although promoted by the degradation of particles and release of acetic acid and not by microbiological spoilage. Overall, the pH value of all the formulations was acidic-neutral, very close to the saliva pH (6.2 to 7.6), and definitely within the pH range of commercial non-dairy beverages, which is from 2.1 to 7.4, and in the range of average pH of coconut waters (Reddy *et al*., 2016; Halim *et al.*, 2018)). Despite being in the range of coconut waters pH, this parameter in highly important for determination of quality parameters in coconut waters, and GPE-CS beverages presented a significant decrease along the storage period, independently of the storage condition, suggesting that chitosan particles are not very stable in aqueous media and their solubility in water should be further improved.

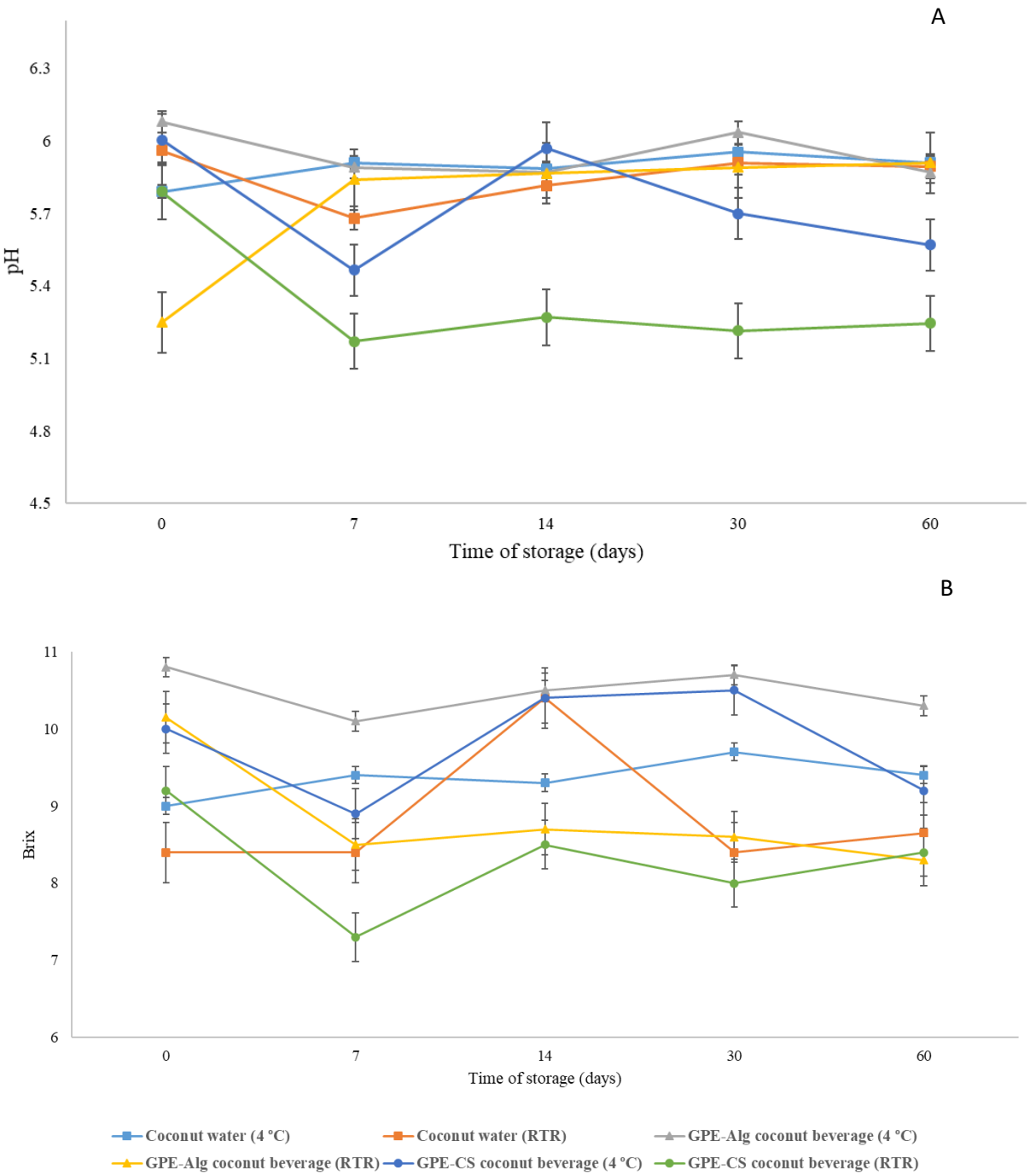


Figure 6.2 – Stability of A) pH and B) Brix degree along 60 days of storage for coconut water and GPE-coconut beverages, at different conditions.

Regarding total soluble solids (Figure 6.2B), some differences were found between the formulations stored at the same condition: GPE-CS coconut beverage stored at 4 ˚C presented higher ($p < 0.05$) ˚Bx when compared to the control coconut water, between days

0 and 30, and coconut beverage with GPE-Alg presented higher ($p < 0.05$) soluble solids than the control and the GPE-CS beverage. It should be expected that the introduction of particles loaded with GPE increase the concentration of total soluble solids, thus the fact that ˚Bx of control is lower than the functional beverages confirms that. Furthermore, the solubility of GPE-Alg particles in aqueous solutions is higher than the solubility of GPE-CS in the same medium, justifying the higher concentration of total soluble solids verified for GPE-Alg beverages. Other similar works report a decrease in total soluble solids measurements in coconut water after 7 days of storage due to a reduction in sugar concentration, which was not verified for the control sample in this work, confirming that the decrease of solids in functional beverages was more related to the degradation of GPE particles than to the degradation of sugars.

For ready-to-reconstitute formulations, some significant differences ($p < 0.05$) were observed between the formulations along the storage: at day 0 the coconut water beverage with GPE-Alg presented significantly higher ˚Bx than others, due to the higher solubility of GPE-Alg particles in aqueous media. The coconut water beverage with GPE-CS presented lower ˚Bx at day 7, and after 14 days the control presented higher ˚Bx, however after the 30 days no significant differences were observed ($p > 0.05$) between beverages. Total soluble solids in liquid formulations of coconut did not change along the storage for the control and for the GPE-Alg beverage, but the beverage with GPE-CS presented some fluctuations throughout time ($p < 0.05$). Major differences were observed for the storage condition, since for all the formulations, the ready-to-reconstitute beverage was different from the sample stored at 4 ˚C ($p < 0.05$), due to the differences in the coconut beverage concentration, as the ready-to-reconstitute beverages had to be dissolved in ultra-pure water for the physicochemical characterization.

5.3.1.2. Color

The coconut beverages were evaluated for color variation between the beverages along the storage time and regarding the control. The color characteristics of beverages are provided in Figure 6.3.

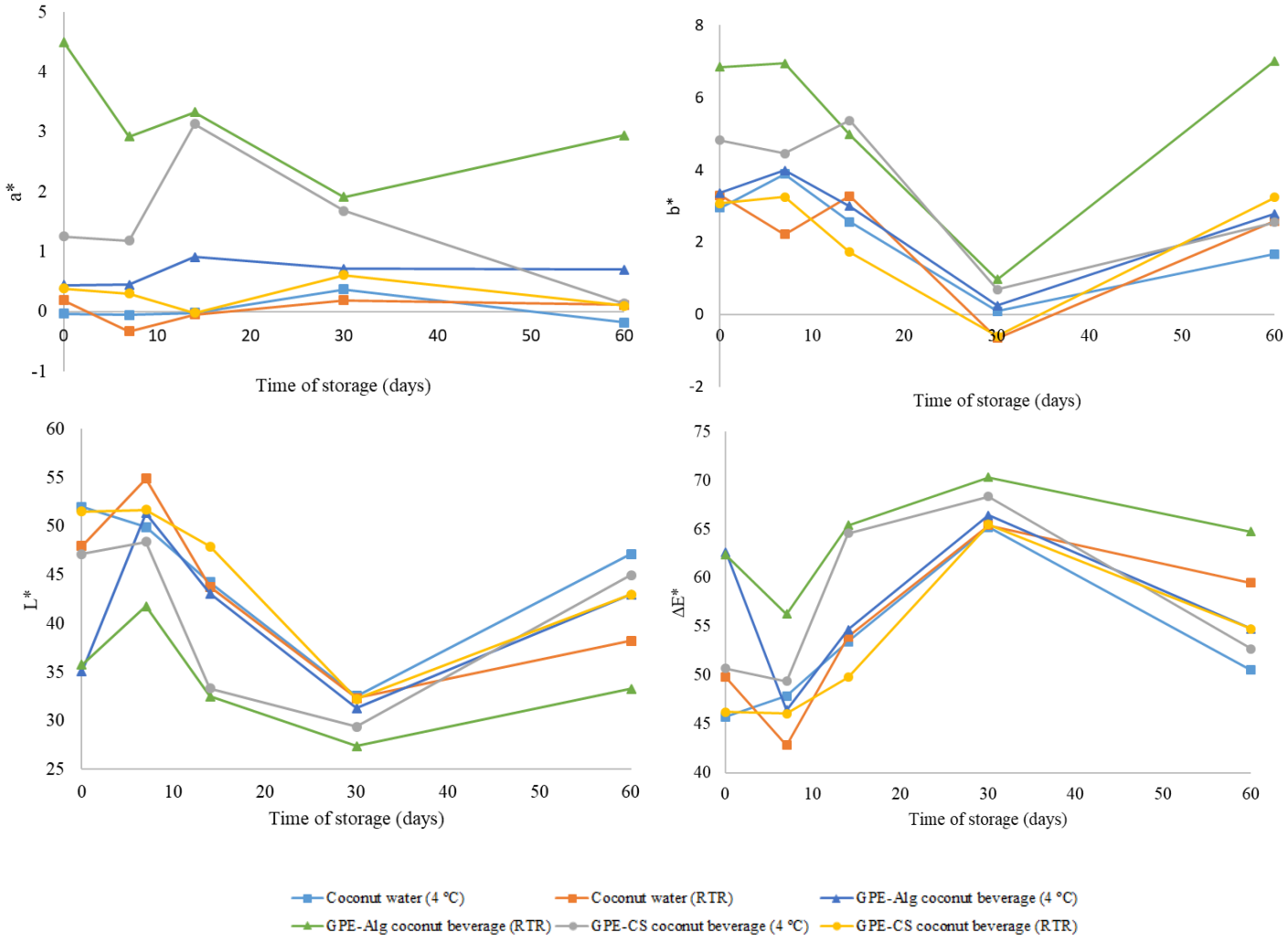


Figure 6.3 – Color parameters of coconut beverages along the 60-day storage period

All the samples presented average lightness values (L*) ranging between 27.39 and 54.90, slightly inferior than the L* values described for coconut water in powder, which range 52.15 to 96.66, due to the incorporation of GPE that promote a darkening in the beverages (Nambiar *et al.*, 2017). The a* values were negative near zero for control, very different

from the a* values obtained by Nambiar and co-workers, who determined a* values for spray-dried coconut water between 0.26 and 11.87 (Nambiar *et al*., 2017). Low positive a* values were found for the coconut beverages with GPE-Alg or GPE-CS, indicating a light red to pink color, provided by the presence of anthocyanins in the GPE, which were able to remain unchanged during the storage period, in accordance with the results obtained by Chung *et al*., who developed a model beverage containing anthocyanins and showed that the color did not changed along the accelerated stability study, for 5 days at 40 ˚C (2016). Low positive b* values indicate a yellowish color, naturally present in the water from mature coconuts, thus b* values presented more differences between the control and the functional beverages, than between the GPE-Alg and the GPE-CS coconut beverages. The control did not show differences in color (ΔE*) between the storage at 4 ˚C or ready-to-reconstitute beverages, but some significant differences were observed within the same samples along the storage, indicating some physicochemical differences along the storage, confirming the results provided by the differences verified in pH values and total soluble solids during the storage period. The GPE-CS coconut beverages did not show differences ($p > 0.05$) comparing to the control, but the GPE-Alg coconut beverages presented some significant differences ($p < 0.05$) from the other formulations, especially when stored at 4 ˚C, which can be explained by the high solubility of GPE particles. The color of the coconut beverages with GPE-CS or GPE-Alg presented some significant differences ($p < 0.05$) along the storage time, in both storage conditions, specifically a significant reduction in ΔE at day 7, which can be related to the decrease in anthocyanins content at day 7 and to the continuous degradation of TPC along the storage period.

6.3.1.3. Phenolic compounds

The degradation profile of TPC is presented in Figure 6.4 and the parameters of the kinetic reaction are provided in Table 6.1. Coconut water and coconut water beverage with GPE-Alg followed a similar profile of TPC degradation, with a slight increase of TPC after 7 days of storage, under both conditions, followed by an accentuate degradation until 14 days and a slight decrease until the end of the storage period. Coconut water beverage with GPE-CS presented a continuous degradation of TPC, presenting the main reduction between the 0 and 7 days of storage. Coconut water beverage with GPE-Alg presented the higher concentration of TPC during the first seven days of storing, but after 14 days the beverages stored at 4 ˚C presented the highest concentration of those compounds. After the 60 days of storage, all the systems presented similar concentrations.

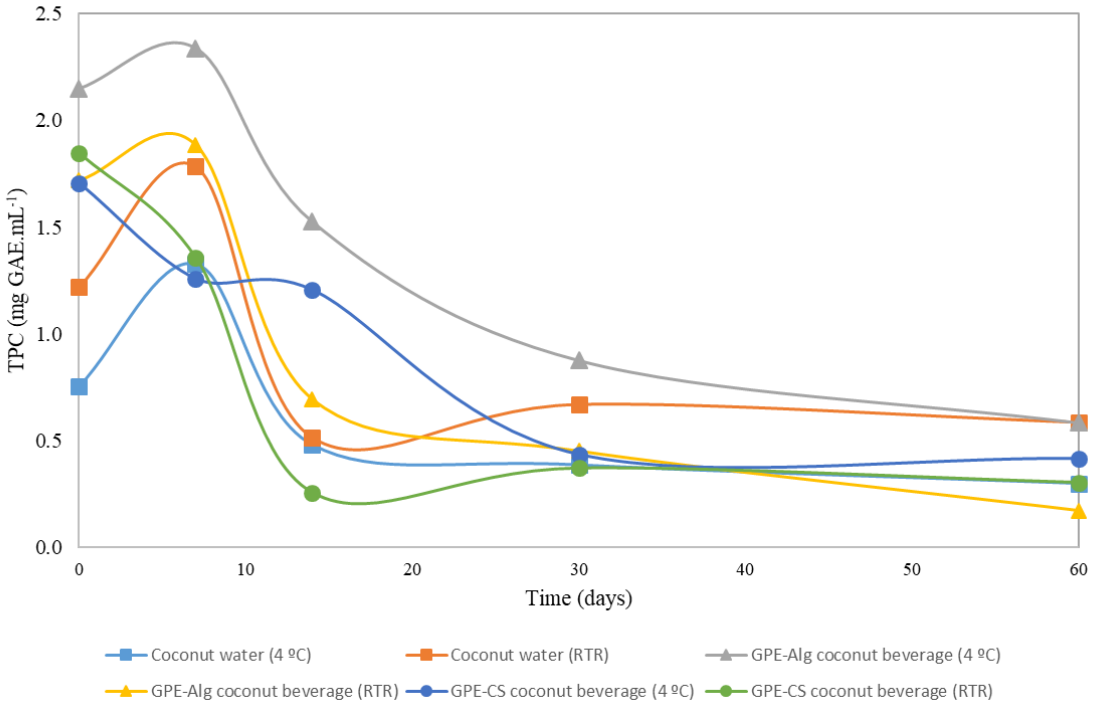


Figure 6.4 – Stability of total phenolics in coconut beverages, along 60 day storage period.

Table 6.1 – Kinetic reaction parameters for phenolic compounds stability in all coconut beverages.

Formulation	Rate constant k	R^2	$t\frac{1}{2}$ (days)
Coconut Water (4˚C)	0.0272	0.72	25.5
Coconut Water (RTR*)	0.0410	0.57	16.9
GPE-Alg coconut beverage (4˚C)	0.0272	0.99	25.5
GPE-Alg coconut beverage (RTR*)	0.0547	0.89	12.7
GPE-CS coconut beverage (4˚C)	0.0379	0.60	20.3
GPE-CS coconut beverage (RTR*)	0.0759	0.46	7.1

*RTR: ready-to-reconstitute

The kinetic reaction order of TPC degradation was calculated using a first-order model ($0.46 < R^2 < 0.99$) for all the formulations, as described by Chung (2016). The half-life time of phenolic compound degradation was higher for the liquid beverage stored at 4 ˚C than for the ready-to-reconstitute beverages, thus indicating that storage conditions influence the stability of polyphenols in beverages. The coconut water beverage with GPE-Alg also presented higher half-life time (25 days at 4 ˚C, and 16 and 12 days, respectively, for ready-to-reconstitute beverages) than the coconut water beverage with GPE-CS (20 days at 4 ˚C and 7 days for ready-to-reconstitute).

The degradation profile of anthocyanins is presented in Figure 6.5 and the parameters of the kinetic reaction are provided in Table 6.2. The kinetic reaction order of anthocyanins degradation was calculated using a first-order model ($R^2 = 0.99$) for all the formulations, as described by Chung (2016). Like as observed for the degradation of TPC, the half-life time of anthocyanins degradation was also higher for the liquid beverages stored at 4 ˚C than for the ready-to-reconstitute beverages stored. However, coconut water beverage with GPE-CS

presented, in both storage systems, higher half-life time of anthocyanins than coconut water beverage with GPE-Alg.

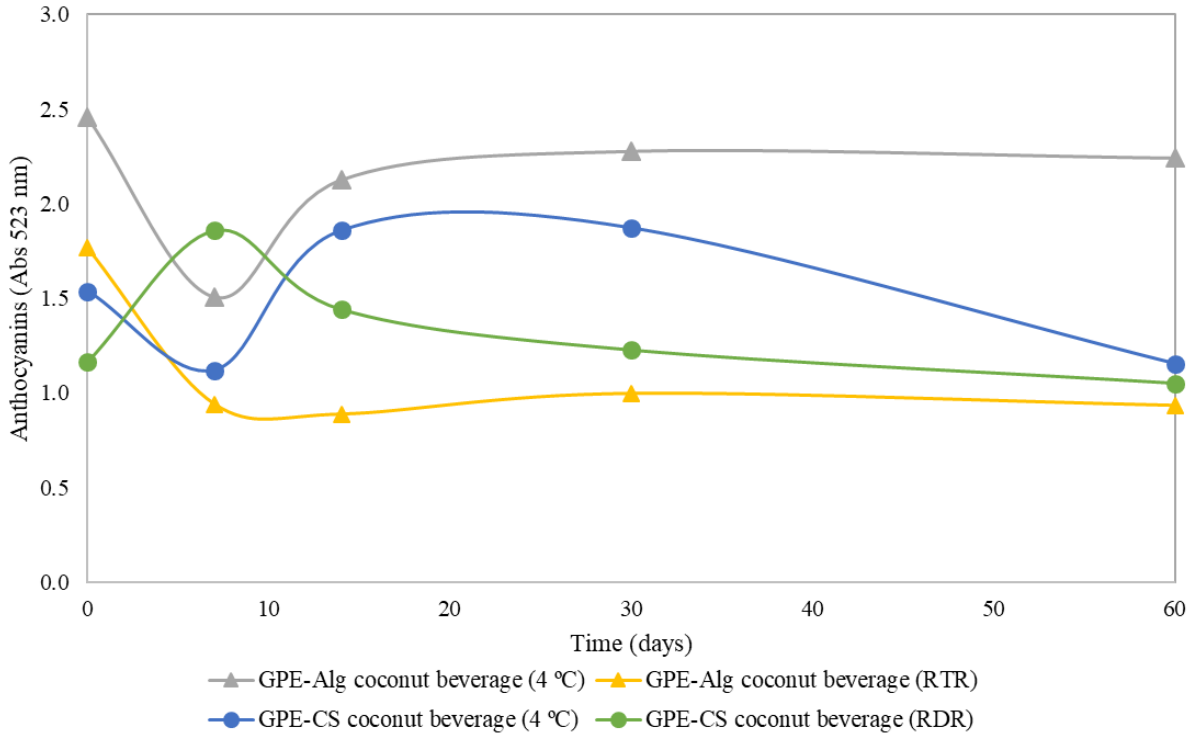


Figure 6.5 – Stability of anthocyanins in GPE-coconut beverages, along 60 day storage period.

When in solution media, like liquid beverages, anthocyanins may undergo different degradative reactions due to the high water activity, gradually reducing with other phenolic compounds into polymeric pigments (Monteiro *et al*., 2017). Furthermore, the origin and type of anthocyanin as well as the matrix where they are included can influence the stability of the anthocyanin, although there are scarce studies on the stability of polyphenols in fruit-derived beverages neither the potential of their encapsulation. Nonetheless, the degradation of anthocyanins occurred at a similar rate than the other polyphenols, when encapsulated into alginate particles, and at a slower rate when encapsulated into chitosan, confirming that,

under the optimal storage conditions, the anthocyanin content in fruits and fruit-extracts remains unchanged or eventually increase during the storage (Beer *et al*., 2012).

Table 6.2 – Kinetic reaction for anthocyanins stability in coconut beverages containing GPE-Alg or GPE-CS

Formulation	Rate constant k	R^2	$t\frac{1}{2}$ (days)
GPE-Alg coconut beverage (4˚C)	0.0286	0.99	24.3
GPE-Alg coconut beverage (RTR*)	0.0900	0.99	7.7
GPE-CS coconut beverage (4˚C)	0.0078	0.99	89.3
GPE-Alg coconut beverage (RTR*)	0.0147	0.99	47.1

**RTR: ready-to-reconstitute*

6.3.2. Sensory evaluation

In order to evaluate the sensorial differences in coconut water beverage triggered by the introduction of GPE-Alg or GPE-CS, a sensory evaluation test was performed by nine semi-trained panelists. Figure 6.6 displays the averages of differences at day 0 between evaluation of coconut water control and coconut water beverage with GPE-Alg or GPE-CS.

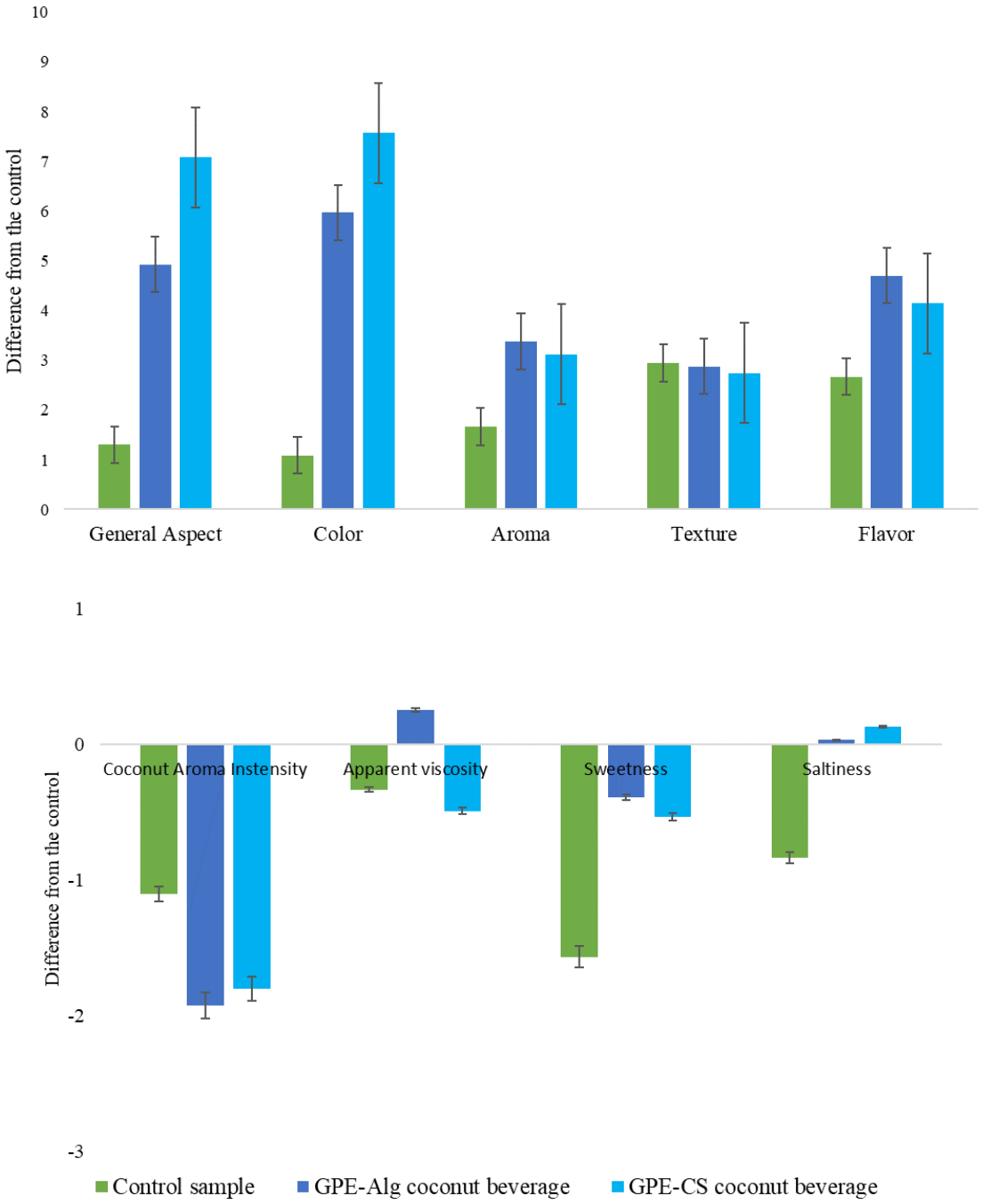


Figure 6.6 - Averages of differences at day 0 between evaluation of coconut water control and coconut water beverages with GPE-Alg or GPE-CS.

No significant differences ($p > 0.05$) in texture, coconut aroma intensity, sweetness and saltiness, were observed between the (blind) the coconut water control and the coconut water beverage with GPE-Alg or GPE-CS. Significant differences ($p < 0.05$) were found for

general appearance and aroma for both the coconut water beverage with GPE-Alg or GPE-CS and for flavor of beverage with GPE-Alg. Differences between the flavor of beverage with GPE-CS and the control were also considered significant ($p < 0.01$), which could be related with the slight bitter flavor of chitosan and with its inability to mask the bitterness of GPE provided by some phytochemicals. The major difference ($p < 0.01$) was regarding the color of the beverage, which was somehow expected due to the GPE content in anthocyanins, which are able to provide a pinkish color and corroborates the results for color evaluation. These results allow to conclude that the incorporation GPE-Alg or GPE-CS into coconut water did not affect most of the coconut water sensory attributes, although some optimization process could be performed to decrease the differences in flavor. A consumer study should also be performed in future to analyze the acceptance of this type of functional beverages.

6.3.3. Bioactive properties

6.3.3.1. Antimicrobial activity

Growth inhibition curves of GPE-Alg and GPE-CS coconut beverages were performed for MSSA, *Listeria monocytogenes*, and *Candida albicans*. Growth inhibition curves for selected microorganisms, in the presence of the digested coconut water control and ready-to-reconstitute beverages with GPE-Alg or GPE-CS, at concentration of 2.5% (w/v), as measured by turbidity at 630 nm, are presented in Figure 6.7.

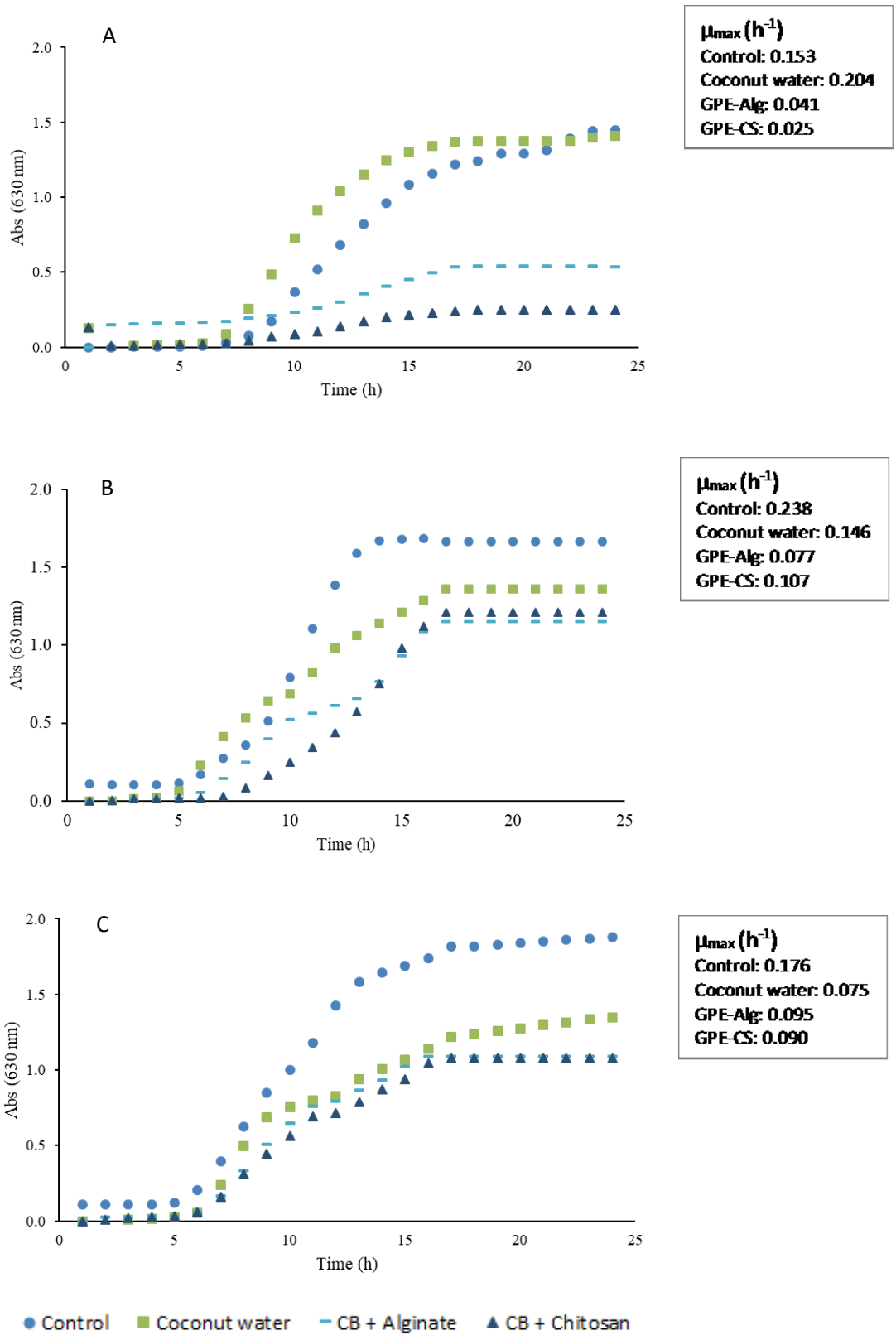


Figure 6.7 – Growth inhibition curves of coconut water, GPE-Alg coconut beverage, and GPE-CS coconut beverage against *Staphylococcus aureus (A)*, *Listeria monocytogenes (B)* and *Candida albicans (C)*.

As previously described, the GPE has inhibitory effect upon different microorganisms, due to the presence of different polyphenols, including anthocyanins, minerals and organic acids (Costa *et al*., 2019). Antimicrobial properties of chitosan are also well described and the encapsulation within this polymer may enhance its antimicrobial activity (Zheng and Zhu, 2003; Qin *et al*., 2006; Du *et al*., 2009). As observed in figure 6.7, digested ready-to-reconstitute beverages with GPE-Alg and GPE-CS had similar antimicrobial behavior upon *C. albicans* and *L. monocytogenes*, but the activity of digested beverage with GPE-CS upon *S. aureus* was higher than GPE-Alg beverage. Using a concentration of only 2.5% (w/v), both functional coconut beverages decreased the growth of *S. aureus* after 24 h of incubation and the growth of *C. albicans* and *L. monocytogenes* after 12 h of incubation. Although GPE-Alg and GPE-CS beverages could not inhibit the growth of *L. monocytogenes* and *C. albicans*, they were able to retard the microbial growth. For L. *monocytogenes*, the growth rate decreased from 0.238 h^{-1} to 0.077 h^{-1} and 0.107 h^{-1} in the presence of GPE-Alg and GPE-CS, respectively. Regarding *C. albicans*, the growth rate decreased from 0.176 h^{-1} to 0.095 and 0.090 with the incorporation of GPE-Alg h^{-1} or GPE-CS h^{-1}, respectively.

Unlike the potential described for other coconut components, such as oil, control coconut water did not have antimicrobial potential, mostly due to the high water content, it is severely prone to contaminations (Oliveira *et al*., 2018). Nevertheless, these results are somehow in accordance with the antimicrobial properties of a fermented coconut beverage developed by Kantachote *et al.* (2017) that successfully inhibited *Staphylococcus aureus, Bacillus cereus and Listeria monocytogenes*, although the characteristic low pH of fermented beverages could additionally increase this effect. Furthermore, Prado and co-workers isolated lactic acid bacteria from coconut water and tested for antimicrobial capacity against different intestinal pathogens, observing high inhibitory capacity against Staphylococcus aureus but no significant effect against *Listeria monocytogenes* (2015).

The potential of functional GPE-Alg and GPE-CS coconut beverages to inhibit the growth of *S. aureus* and hold back the growth of other microorganisms responsible for the proliferation of intestinal infections, possibly reducing its severe symptoms such as diarrhea and consequently, dehydration.

6.3.3.2. Prebiotic potential

The prebiotic potential of the coconut beverages was studied using five strains in basal MRS medium without glucose, at concentrations of 2% (w/v) of each pre-digested ready-to-reconstitute beverage. Figure 6.8 and 6.9 present the growth of evaluated lactobacilli and bifidobacteria strains, respectively, as measured by turbidity at 660 nm. Frutooligosaccharides (FOS) at the same concentration was also used as positive control at 2% (w/v). All the probiotic microorganisms grew in the presence of both coconut beverages, increasing their growth (OD at 660 nm) along the fermentation period. FOS proved to be the most efficient prebiotic component, except for *Lactobacillus plantarum*, in which digested beverages with GPE-Alg or GPE-CS promoted higher OD. In the case of *L. rhamnosus* and *L. casei* growth, coconut water control and incorporating GPE-Alg or GPE-CS differences between themselves neither from FOS. In the case of BB-12, both functional beverages presented a prebiotic potential similar to FOS, while the growth in presence of coconut water control was lower. At last, for *B. animalis* Bo, it was verified a decrease of growth after 20 h, in the presence of FOS, which was not observe in the presence of functional beverages incorporating GPE-Alg or GPE-CS. Although coconut water is rich in sugars that are able to stimulate the growth of probiotic microorganisms, the incorporation of the GPE-Alg or GPE-CS promoted an enhanced prebiotic potential effect, due to the presence of xylooligosaccharides that are able to change the composition of short chain fatty acids, increased faecal weight and mineral absorption, and decreased colonic pH values (Costa *et al*., 2019).

Based on maximum specific growth rates (μ_{max}) achieved, lactobacilli exhibited higher capacity to growth and specifically, Lactobacillus plantarum exhibited the best capacity to growth, achieving the fastest growth in the presence of coconut water supplemented with GPE-Alg (0.408 h^{-1}). GPE-CS presented a slightly lower μ_{max} value of 0.404 h^{-1}, but without statistical differences ($p > 0.05$), and both functional beverages presented significant higher ($p < 0.05$) μ_{max} than FOS. *L. rhamosus* was grew faster in the presence of GPE-CS (μ_{max} 0.383) than with GPE-Alg (μ_{max} 0.336), but both presented better results than FOS, which μ_{max} was 0.324. La01 and Bb12 grew faster with FOS than with the functional beverages. Finally, for Bifidobacterium B0 the growth rates in the presence of GPE-Alg (0.153 h^{-1}) and FOS (0.148 h^{-1}) were similar and significantly higher than the μ_{max} with GPE-CS (0.112 h^{-1}).

Coconut fermented beverages are a novel alternative to fermented dairy beverages, coconut functional beverages incorporating GPE-Alg or GPE-CS can be a potential alternative to these fermented drinks, as they can also play a relevant role in the modulation of intestinal microflora (promoting gut healthy bacteria and inhibiting gut pathogens), avoiding the inherent difficulties associated to the stabilization of probiotic strains on food matrices.

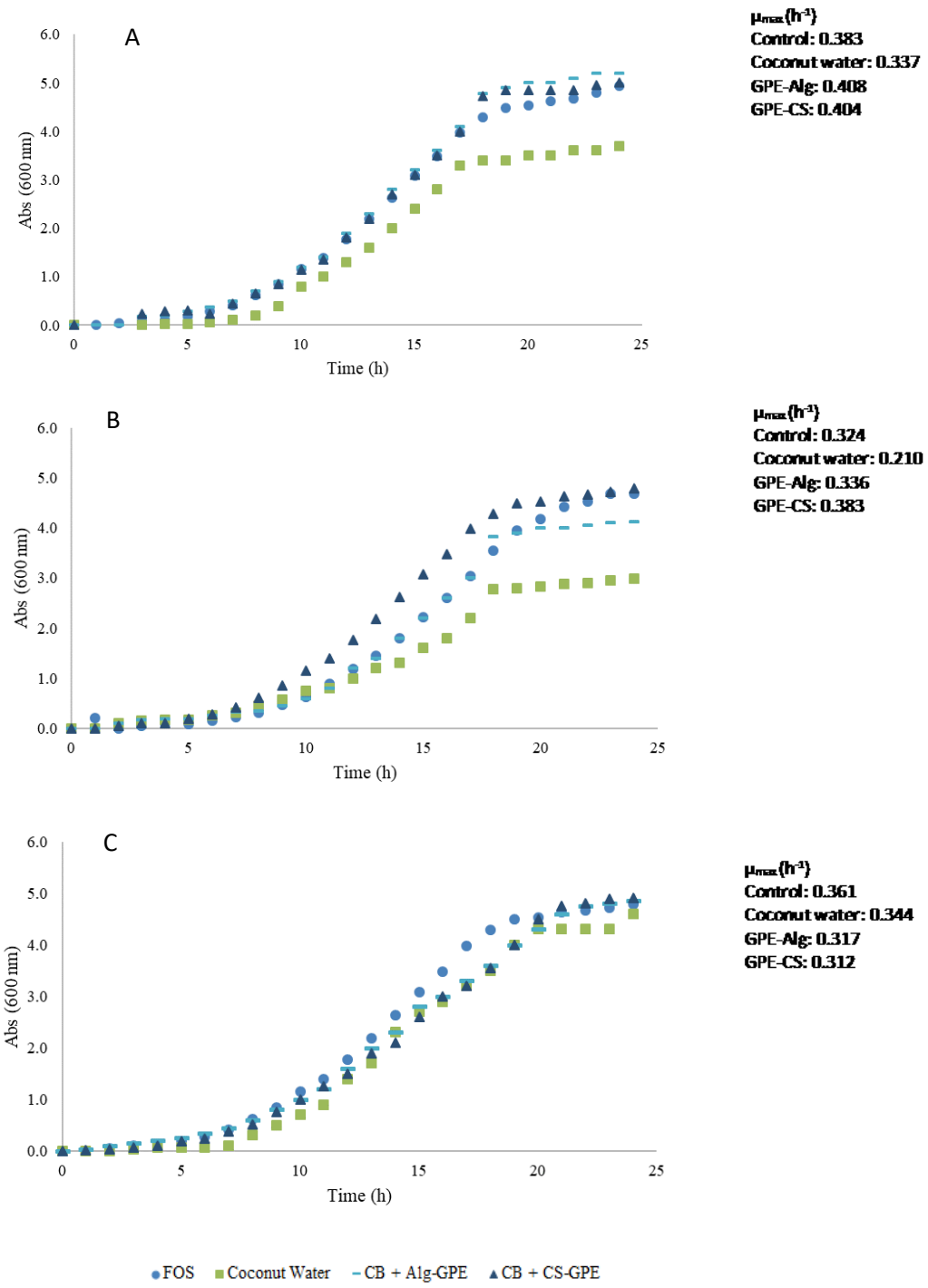


Figure 6.8 – Growth curves of *Lactobacillus plantarum* (A), *Lactobacillus rhamnosus* R11 (B) and *Lactobacillus casei* 01 (C) with FOS, coconut water, GPE-Alg coconut beverage and GPE-CS coconut beverage.

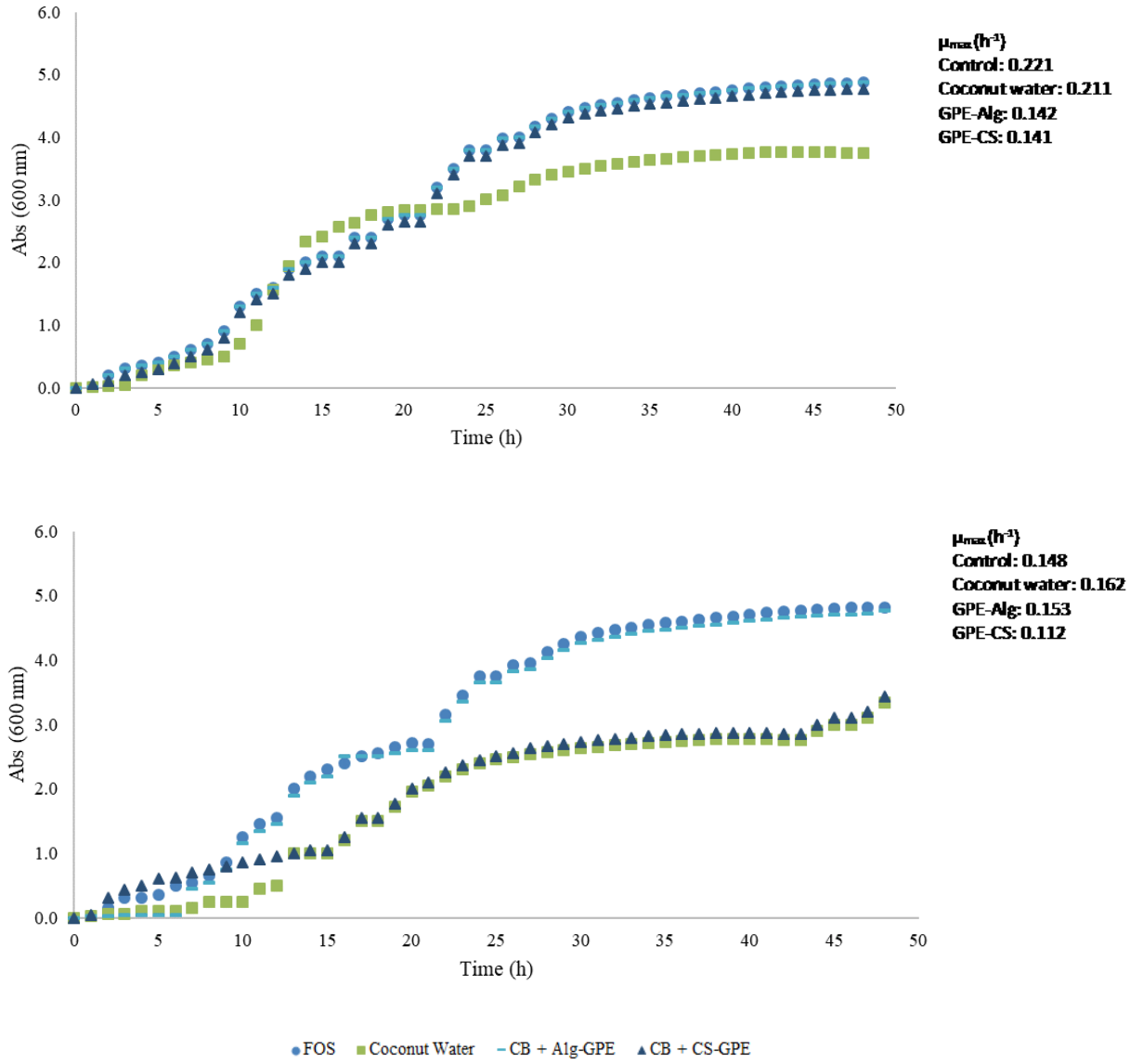


Figure 6.9 – Growth curves of *Bifidobacterium animalis* subsp. *lactis* BB-12 and *Bifidobacterium animalis* Bo with FOS, coconut water, GPE-Alg coconut beverage and GPE-CS coconut beverage.

6.4. Conclusions

Coconut water as a relevant by-product from ready to eat or processed fruit industry, may act as a sustainable beverage matrix for the incorporation of encapsulated bioactive grape pomace extract to produce a functional coconut beverage. The incorporation of GPE encapsulated into alginate or chitosan particles to produce for the first time functional coconut water beverages introduced some differences in physico-chemical parameters,

namely in total soluble solids and color, but most sensory attributes of coconut water were not affected.

In addition, the encapsulation of the bioactive compounds present in the GPE allows them to reach the intestine with minimal losses during the gastrointestinal digestion. The potential antimicrobial activity of digested functional coconut water beverages against intestinal pathogens was validated and related to the high level of total phenolics and total anthocyanins. Also, the digested coconut beverages incorporating GPE-Alg or GPE-CS showed the capacity to enhance the growth of probiotic strains associated to the high content in xylooligosaccharides.

This work allows to demonstrate the potential of a coconut-based functional beverage to modulate the intestinal microflora, which in addition to the hydrating capacity and high mineral content of the coconut water, can be used as a co-adjuvant in the treatment of severe diarrheas, protecting the intestine and restoring hydration. As non-dairy functional beverages, coconut beverages incorporating grape pomace extract encapsulated into alginate or chitosan particles can also be consumed by vegetarians and lactose-intolerant consumers.

Part IV

Conclusions and Future Work

CHAPTER 7

General Conclusions

The main purpose of this thesis was the valorization of the hemicellulose fraction of grape pomace, through the production of xylan-derived oligosaccharides with potential application on the development of novel bioactive food ingredients.

Grape pomace flour composition was evaluated and different methods were used to produce xylooligosaccharides from *Syrah* grape pomace flour. The enzymatic production of XOS was affected by both enzyme type and enzyme loading, and the mixture of xylanases produced by *A. niger* 3T5B8 at 100 UI.g^{-1} was found to be the most favorable condition. The use of enzymatic cocktails demonstrated to be an alternative to conventional methods such as acid and alkaline extraction, as they allowed to obtain similar yields of XOS production but within an eco-friendly and sustainable process.

Main results indicate that it was possible to produce a functional ingredient using a green approach, with high soluble fiber and XOS content, rich in phenolic compounds and exhibiting some relevant bioactive properties, including antioxidant capacity, antimicrobial properties and prebiotic potential. However, simulation of gastrointestinal digestion revealed that the antioxidant and antimicrobial activities were lost before reaching the intestine, blocking the possibility of a potential food application. In detail, the freeze-dried enzymatic grape pomace extract was characterized for its chemical and biological properties. It was

observed that GPE presented high content of total dietary fiber, including xylobiose, other carbohydrates, minerals and phenolic compounds. The *in vitro* simulation of GPE digestion revealed that xylobiose was resistant to gastrointestinal conditions that provide it prebiotic potential, at concentration of 2% (w/v), establishing a potential carbon source that can be fermented by *Lactobacillus* and *Bifidbacterium* spp. It was also proved that GPE has antimicrobial activity against different pathogenic bacteria, although this effect was absent after the GID. Similar impact of GID occurred on polyphenols and anthocyanins, consequently affecting the antioxidant capacity, which strongly decreased. The reduction of the antimicrobial and antioxidant capacities after the GID suggested that, in order to assure the GPE bioavailability, it would be necessary to use an alternative system to protect it from the harsh gastrointestinal conditions, such as an encapsulation system that allow the delivery of GPE into the intestine.

In order to improve its bioavailability in the intestine, the extract was encapsulated into polymeric microparticles, aiming to reduce the loss of bioactive compounds along the gastrointestinal tract. Alginate or chitosan microparticles were used as oral delivery system for GPE. The encapsulation of the GPE into alginate or chitosan particles through ionic gelation allowed the formation of NPs with sizes between 400 and 1000 nm and high association efficiency of phenolic compounds and XOS, characteristics that are suitable for gastrointestinal delivery. Both systems allowed to protect the GPE along the GI tract and deliver it in the intestine, increasing the bioavailability of different phenolic compounds, including anthocyanins, which mirrored in an improvement of the antioxidant and antimicrobial activities, including the inhibition of intestinal pathogenic microorganisms. Permeability studies across Caco-2/ HT29-MTX co-culture cell layer showed that encapsulation of GPE decreased the permeability of xylobiose across the cell membrane, allowing the GPE to remain longer within the intestine, which could also improve the

prebiotic potential of GPE, although further studies are needed to prove this. Modification of chitosan microparticles with Cyanine5.5 for cellular uptake studies did not affect the biocompatibility of CS-GPE-loaded MPs with Caco-2 and HT29-MTX cell lines, and confocal microscopy analysis confirmed the integrity of these cells tight junctions after the contact with chitosan MPs, although these were not internalized within the intestinal cells.

Finally, alginate or chitosan GPE-loaded MPs were applied in the development of a functional food, as a proof of concept of the GPE potential. Coconut water, as a major by-product from food industry, act as a sustainable food matrix for the incorporation of the encapsulated bioactive GPE to produce a functional coconut beverage. The potential antimicrobial activity against intestinal pathogens, in addition to the capacity of enhancing the growth of probiotic strains, allowed the alginate and chitosan - coconut beverages to modulate the intestinal microflora, which in addition to the hydrating capacity and high mineral content of the coconut water, can be used as a co-adjuvant in the treatment of severe diarrheas, protecting the intestine and restoring hydration. As non-dairy functional beverages, alginate and chitosan coconut beverage can also be consumed by vegetarians and lactose-intolerant consumers.

The findings from this work will contribute for the sustainability idea of wine industry in circular economy context, using a new eco-friendly approach to valorize wine by-products into value-added ingredients with proven biological impact. Moreover, the encapsulation of the bioactive grape extract improved its bioaccessibility, a breakthrough that may be translated to other natural extracts and ingredients as an innovative step towards the valorization of agroindustrial by-products.

CHAPTER 8

Future Prospects

This work purposes a novel method for green production of xylan-derived oligosaccharides from grape pomace, adding value to this industrial by-product and allowing to re-introduce it in the food production chain, leading to new research lines in a circular economy context. Nevertheless, further steps could be taken in the valorization process of grape pomace and in this section, we purpose some points that might be interesting and rewarding to explore in the future.

Firstly, as this work lies in the concept of circular economy, an integral valorization approach should have been considered. After the recovery of hemicellulose fraction, there is still a remaining solid residue, mainly composed by cellulose and lignin and it could be fractionated and characterized for further valorization. Lignin is widely known for the functionalization that can provide to materials, such as dispersant and emulsifying capacity, or the production of thermoplastics due to its resistance to high temperatures. Therefore, with a content in lignin about 30 g.100 g^{-1}, grape pomace could be an excellent raw material to exploit.

Although a promisor eco-friendly extract with multiple biological activities has been produced, the primary objective of this thesis was the production of XOS from grape pomace and the final content of XOS in GPE was ca. 5.5 $g \cdot 100g^{-1}$. The purity of GPE in XOS could be improved with further purification of these molecules (ultrafiltration and other

techniques), although due to the relatively low concentration of hemicellulose found in the GPE used for this work (ca.10 $g.100g^{-1}$), an increase in the purity degree would certainly decrease the extraction yield and could also lead to the loss of bioactivities. Also regarding the extraction, other research line that would be interesting to look at is the optimization of the scale-up process. In Chapter 3, a reduced quantity of GP biomass was used for the optimization of the enzymatic method, resulting in the production of XOS with DP 4 or 5. Then, the scale-up process presented in Chapter 3 for the production of higher amount of GPE resulted, majorly, in XOS with DP 2 and residual concentration of XOS with DP 3 or DP 4. These results suggest that the interaction between the substrate and the enzyme need to be optimized to improve the stability and quality of the produced extracts.

Concerning Chapter 4, it would also be relevant to understand the backbone composition of the polysaccharides present in the GPE, and confirm if they are hemicellulose-derived such xylan or arabinan. Moreover, considering the prebiotic potential that GPE demonstrated on different probiotic strains, it could be interesting to simulate an *in vitro* human gut microbiota model to study the impact of GPE on different bacterial groups and their metabolic activity, which are related with human well-being. If the digestion of GPE promoted its prebiotic potential, it also decreased the potential of antioxidant and antimicrobial activities, suggesting that the application of GPE in food ingredients could not be the most advantageous matrix. Instead, this natural ingredient could possibly present other benefits if applied to cosmetic products, acting as a preservative agent due to antimicrobial and antioxidant capacity. Moreover, considering the high concentration of GPE in fiber and carbohydrates, it could be studied some technological properties such as oil absorption and swelling capacity, and use GPE as and additive in the formulation of specific cosmetics, such as gel-based cleansing products.

In Chapter 5, the optimization of MPs formulation could potential benefit with the study of the influence of other variables, including the concentration of TPP. The influence of the extract encapsulation in the prebiotic potential should also be studied to assure this biological activity was not loss and, finally, it would be interesting to perform the cellular uptake assay for alginate MPs and understand how they interact with intestinal cells. Giving the prebiotic potential of GPE, it could be very interesting to co-encapsulate it with different probiotic strains, possibly by means of spray-drying techniques, creating a prebiotic-probiotic gastrointestinal resistant capsule.

Regarding the proof-of-concept, in Chapter 6, it is important to study the solubility of chitosan MPs in order to optimize some sensorial aspects such viscosity and color. Other food matrices could also be exploited, based on the trends of functional foods market. The fermentation of this product with different strains should also be performed to analyze the cell viability and the production of short-chain fatty acids as well as a simulation of *in vitro* human gut microbiota model, in order to obtain more information on the impact of this functional food in the gastrointestinal health.

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