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ASSESSMENT OF BIOLOGICAL PROPERTIES AND APPLICATIONS OF EXTRACTS OBTAINED FROM AGROINDUSTRIAL SUBPRODUCTS

by

Diana Margarida Andrade Moreira

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ESTUDO DE PROPRIEDADES BIOLÓGICAS E APLICAÇÕES DE EXTRATOS OBTIDOS A PARTIR DE SUBPRODUTOS AGRO-INDUSTRIAIS

Thesis presented to *Escola Superior de Biotecnologia* of the *Universidade Católica Portuguesa* to fulfill the requirements of Master of Science degree in Applied Microbiology

by

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Porque nem todos os anjos moram no céu, esta tese é dedicada a Francisco Andrade.
Obrigada Padrinho, por todo o apoio e amor.

"Science is simply common sense at its best"
Thomas Huxley

"An experiment is a question which science poses to Nature,
and a measurement is the recording of Nature's answer"
Max Planck

Resumo

A oxidação lipídica e o crescimento microbiano, dois dos processos melhor estudados e associados à deterioração dos alimentos, têm ganho grande atenção quando a solução passa pela adição de compostos naturais. A presença de antioxidantes sintéticos em matrizes alimentares detém uma conotação negativa, pelos efeitos indesejáveis associados ao consumo durante longos períodos de tempo. Não obstante, a adição de antioxidantes naturais, menos nocivos e de fontes renováveis, tem vindo a captar uma maior atenção quando conseguem obstruir ambos os processos. Neste trabalho, foram utilizados quatro extratos obtidos a partir de subprodutos/ resíduos agroindustriais, nos quais se procurou avaliar: as atividades antioxidante e antimicrobiana, a capacidade para potenciar o crescimento de bactérias probióticas e uma aplicação associada à indústria alimentar.

A maior capacidade antioxidante foi encontrada no extrato de bagaço uva (GP *extract*), sendo por sua vez o extrato de cascas de amêndoa (AS *extract*) aquele que demonstrou menor capacidade. A identificação dos compostos presentes mostrou que todos os extratos apresentavam perfis diferentes, ainda que existam compostos comuns a todos os extratos, como é o caso do ácido vanílico, 3,4-dihidroxibenzaldeído, vanilina, quercetina, hidroximetilfurfural e 2-furfuraldeído. No que diz respeito à atividade antimicrobiana, concluiu-se que todos os extratos inibiram o crescimento das bactérias patogénicas testadas (*Escherichia coli, Pseudomonas aeruginosa, Listeria monocytogenes, Staphylococcus aureus* e *Salmonella* spp.) No entanto, os extratos não se mostraram mais ativos para bactérias Gram-positivas ou Gram-negativas. A partir da análise das concentrações mínimas bactericidas (CMBs), pode observar-se que o extrato de madeira de eucalipto (EW *extract*) foi o mais ativo, contrariamente ao extrato de carolos de milho (CC extract) e ao de amêndoa. As curvas de sobrevivência/ morte evidenciaram que o efeito antimicrobiano não é imediato, na medida em que, para a maior parte dos casos, acontece após aproximadamente 24h. Os ensaios com bactérias probióticas mostraram que as concentrações de extrato testadas (0.025% e 0.1%) não provocam alterações significativas no crescimento destes microrganismos.

Por fim e como aplicação, recorreu-se à indústria alimentar, pela formulação de um filme que permitiu concluir que, ainda que estes sejam produzidos com diferentes extratos, não apresentam diferenças nas suas propriedades físicas e mecânicas, exceto na cor e no teor de humidade, onde o extrato de cascas de amêndoa e o extrato de carolos de milho apresentaram, respetivamente, valores inferiores aos restantes filmes avaliados. Além disso,

todos os filmes evidenciaram atividade antimicrobiana sobre as bactérias patogénicas testadas, sugerindo o possível uso destes extratos num filme comestível.

Abstract

Lipid oxidation and microbial growth, two of the most well studied processes associated with food spoilage, become the focus of attention when natural compounds are used to circumvent the problem. Synthetic antioxidants associated with food matrices always had a negative connotation due to the undesirable effects related with their consumption over long periods of time. Nevertheless, the addition of natural compounds with antioxidant activity has gained a great importance, particularly when these compounds, less harmful and from renewable resources, can block both processes. In this work, four extracts obtained from by-products / industrial residues were utilized to evaluate: the antioxidant and the antimicrobial activities, the ability to enhance the growth of probiotic bacteria and the applicability associated with the food industry.

Grape pomace (GP) extract was the one with the highest antioxidant activity whereas the extract from almond shells (AS) was the one with the lowest capacity. The identification of the compounds present in the extracts showed different profiles for all extracts although there were common compounds, such as: vanillic acid, 3,4-dihydroxybenzaldehyde, vanillin, quercetin, hydroxymethyfurfural and 2-furfuraldehyde. Concerning the antimicrobial activity, all the extracts inhibited the growth of pathogenic bacteria, namely *Escherichia coli, Pseudomonas aeruginosa, Listeria monocytogenes, Staphylococcus aureus* and *Salmonella* spp. However, no trend was observed, i.e., the extracts did not show a selective action upon either Gram-positive or Gram-negative bacteria. From the analysis of the minimum bactericidal concentrations (MBCs), the eucalypt wood (EW) extract was the most active, whereas the AS and CC (corn cobs) extracts were less active. The time-kill analysis/ survival curves have highlighted the antimicrobial effect of the extracts; however, this was not immediate, in that for the majority of cases, this effect only occurred at approximately 24h. The assays with probiotic bacteria showed that the tested extract concentrations (0.025% and 0.1%) did not significantly affect the growth of these microorganisms.

Finally, for the application associated with the food industry, films with the extracts were produced, and their characterization did not evidence strong differences; statistically significant differences were only observed for color and moisture content, with the AS and CC extracts showing smaller values comparatively to the other films. In addition, all films showed antimicrobial activity against the pathogenic bacteria tested, suggesting the possible use of these extracts in an edible film.

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Contents

List of Figures

List of Tables

Abbreviations

ABTS ..... 2,2'-azinobis-(3-ethybenzothiazoline-6-sulfonate

$ABTS^{\bullet+}$ ..... 2.2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) radical cation

APPH.....2,2'-Azobis (2-methylproprionamidine dihydrochloride)

AS ..... Almond Shells

ATCC ..... American Type Culture Collection

BHA ..... Butylated HydroxyAnisole

BHT ..... Butylated HydroxyToluene

BSA .....Bovine Serum Albumin

CC..... Corn Cobs

CFU ..... Colony Forming Units

CINATE ..... *Centro de Inovação e Apoio Empresarial*

DAD ..... Diode-Array Detection

DPPH.....2,2-Diphenyl-1-Picryhydrazyl Radical

EW.....Eucalypt Wood

FL ..... Fluorescein

FRAP .....Ferric Reducing Antioxidant Power

GP..... Grape Pomace

GRAS .....Generally-Recognized-As-Safe

HAT .....Hydrogen Atom Transfer

HHPP..... High Hydrostatic Pressure Processing

HPLC .....High Performance Liquid Chromatography

IP .....Inhibition Percentage

LCMs .....Lignocellulosic Materials

MAE..... Microwave-Assisted Extraction

MBC .....Minimum Bactericidal Concentration

MBCs .....Minimum Bactericidal Concentrations

MH ..... Müeller Hinton

MIC ..... Minimum Inhibitory Concentration

MICs .....Mininum Inhibitory Concentrations

MRPs .....Maillard Reaction Products

OD ..... Optical Density

ORAC .....Oxygen Radical Absorbance Capacity

PCA ..... Plate Count Agar
PEG .....PolyEthylene Glycol
RNOS .....Related Nitrogen Oxide Species
ROS ..... Reactive Oxygen Species
SCFAs ..... Short Chain Fatty Acids
SCWE ..... Subcritical Water Extraction
SET..... Single Electron Transfer
SFE..... Supercritical Fluid Extraction
TBHQ ..... tert-Butylhydroquinone
TEAC .....Trolox Equivalent Antioxidant Capacity
TRAP .....Total Radical-trapping Antioxidant Parameter
UAE ..... Ultrasound Assisted Extraction
UMAE ..... Ultrasound-Microwave-Assisted Extraction
UV radiation ……..……...Ultraviolet Radiation

1. Introduction

Lipid oxidation and microbial growth are two main degradation pathways for food, and therefore, a major concern for the food industry. Usually, additives are used to slow down or inhibit these processes. The most commonly used antioxidant additives are butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT); however, it has been shown, that these additives can cause DNA damage and carcinogenesis (Alves-Silva *et al.*, 2013).

Due to the undesirable effects of synthetic compounds, actually, there is a recent worldwide tendency to avoid or at least decrease the use of these synthetic additives, creating a need for alternative cheap, renewable, natural and possibly safer sources of natural compounds with antioxidant and antimicrobial activities to stabilize foods against oxidative rancidity and microbial spoilage (Barbosa-Pereira *et al.,* 2014). It is a request that these compounds do not have any negative effects on human health and can thus, be safely used in the food industry (Alves-Silva *et al.,* 2013).

The obtention/ extraction of such compounds from waste materials, e.g. agro-industrial wastes generated by the food processing industry, has been considered in some studies (Moure *et al.,* 2001). The use of antioxidants from natural sources as preservatives in food has great potential because consumers request additive-free, fresher and more natural-tasting food (Almajano *et al.,* 2008). In the following sections, the state of the art related to several aspects of natural sources of antioxidants, extraction methods, chemical composition, biological activities and applications are described.

1.1. Antioxidants

Oxidative stress has been one of the concerns of the modern society in relation to food safety. Various external factors, such as environmental pollutants, smoking, UV radiation, drugs, pesticides, industrial solvents, contribute to the production of free radicals that promote these oxidative reactions. In addition, a variety of diseases are usually associated with these, such as cancer, cardiovascular and degenerative diseases and aging (Nićiforović *et al.,* 2010; Carocho and Ferreira, 2013).

In 1995, Halliwell and Gutteridge defined antioxidants as “any substance that, when present at low concentrations compared with that of an oxidizable substrate, significantly delays or inhibits oxidation of that substrate”, but 12 years later re-defined as “any substance that delays, prevents or removes oxidative damage to a target molecule” (Halliwell, 2007) and

as “any substance that directly scavenges ROS (reactive oxygen species) or indirectly acts to up-regulate antioxidant defences or inhibit ROS production” (Khlebnikov *et al.,* 2007).

In summary, antioxidants seem to be important solution for the lipid oxidation problem, and can be obtained by chemical synthesis or by extraction from natural sources.

1.2. Natural antioxidants

In recent years a large effort to identify natural substances with antioxidant activity has been made, in order to minimize the consumption of synthetic antioxidants. In fact, many studies with animals have reported noxious effects due to the consumption of synthetic antioxidants after a large period of ingestion (Thompson and Moldeus, 1988). Definitely, natural compounds with antioxidant activity seem to be an alternative to synthetic compounds, but a full toxicological analysis is necessary in order to understand the implications associated with the addition of these pure natural compounds in foods (Pokorny and Parkányiová, 2004).

1.2.1. Sources

Vegetable materials are persistently the generous source of many compounds with antioxidant activity. In fact, these materials have been studied as sources of natural antioxidants for application in the food industry.

Natural antioxidants are naturally associated with the human diet and have been found in different vegetable materials such as: *fruits, vegetables and legumes* such as berries, citrus, mango, grapes, cherry, apple, potato, green pepper and bean (Balasundram *et al.,* 2006; Dimitrios, 2006); *herbs and spices* like paprika, oregano, marjoram and rosemary (Moure *et al.,* 2001; Chun *et al.,* 2005; Brewer, 2011), *cereals* such as corn, millets and rye (Moure *et al.,* 2001; Brewer, 2011), *medicinal plants* like sage, thyme and basil (Ollanketo *et al.,* 2002; Miura *et al.,* 2002; Javanmardi *et al.,* 2003; Krishnaiah *et al.,* 2011; Rafiq *et al.,* 2012), *seeds* such as sesame and sunflower (Kimb *et al.,* 2014; Pająk *et al.,* 2014), and *agricultural and industrial residues* **(see Table 1.1.)** (Moure *et al.,* 2001; Balasundram *et al.,* 2006; Ignat *et al.,* 2011). Moreover, different parts of plant also mentioned as potent antioxidants, include the flowers, leaves, bark, seeds, catkins and stems (Moure *et al.,* 2001; Al-Jaber *et al.,* 2011; Shah *et al.,* 2014).

However, natural antioxidants are present in other sources such as beverages (teas, wines and fruit juices), which is a major source of phenolics in human diet. Fruit juices are the source with the highest phenolic content, and they can be offered in commercial samples

as commercial juices (apple, pineapple, orange and grapefruit) or as fresh juices (grape and orange) (Balasundram *et al.,* 2006; Ignat *et al.,* 2011). Conversely, reductions or losses of these phenolic compounds have been reported and they have been attributed to commercial processing procedures (Balasundram *et al.,* 2006). Over the recent few decades, green tea has been cited as a potent antioxidant due to evidences that suggest that regular consumption may lower the chances of developing heart disease and certain types of cancer (Lambert and Elias, 2010; Deka and Vita, 2011; Yang *et al.,* 2011). Coffee also supplies a good source and in fact there is evidence that correlates coffee consumption to the reduction of risk of acquiring Alzheimer's, Parkinson's and heart diseases. (Ascherio *et al.,* 2004; Ignat *et al.,* 2011; Di Castelnuovo *et al.,* 2012).

In addition to the typical sources described above, agro-industrial by-products have gained much importance in the later years. Agricultural, forest and industrial residues are sources with attractive raw materials, especially the wastes or by-products generated by the food processing industries. Food industry is one of the major industries producing a large amount of waste. Many food wastes are employed for antioxidant extraction, for example: grape and wine residues (Makris *et al.,* 2007; Rockenbach *et al.,* 2011; Ky *et al.,* 2014), apple pomace and peels (Schieber *et al.,* 2001; Makris *et al.,* 2007; Suárez *et al.,* 2010), olive (Schieber *et al.,* 2001; Lafka *et al.,* 2011) and citrus peel and seeds (Schieber *et al.,* 2001; Kim and Shin, 2013).

Furthermore, agro-industrial activities have required considerable attention by the expansion of these activities as large quantities of lignocellulosic materials (LCMs) have been accumulated; these LCMs come from a variety of sources such as herbaceous materials, agricultural crops, forestry and wood processing wastes, municipal solid wastes and various industrial wastes. LCMs are mainly made up of cellulose, hemicelluloses and lignin, and they are the most abundant biomass, comprising nearly 70% of the total plant biomass produced by photosynthesis. Therefore, they represent the most abundant renewable organic resource in soil (Sánchez, 2009). Almond shells and corn cobs are two examples of vegetal biomass that have potential to act as natural antioxidants (Conde *et al.,* 2011a).

By-products have the advantage of being renewable, widely distributed, largely available and inexpensive. Some antioxidant activity is also observed in different materials that are not so conventional, where some phenolic compounds have been identified (**Table 1.1.**). Actually, it is a very interesting topic due to the environmental and economic impacts, so it can be a good conscientious challenge for modern society. Therefore, the circular

economy can take some return and can promote industrial symbiosis. But first it is necessary to identify, quantify and characterize these raw materials (Mirabella *et al.*, 2014).

Table 1.1. Agro-industrial by-products that contain phenolic compounds.

Sources	References
Almond shells	Moure *et al.*, 2007; Sfahlan *et al.*, 2009; Conde *et al.*, 2011a
Almond hulls	Takeoka and Dao, 2003; Sfahlan *et al.*, 2009
Chestnut burs	Conde *et al.*, 2011a; Vázquez *et al.*, 2012
Corn cobs	Garrote *et al.*, 2007; Conde *et al.*, 2011a
Eucalypt wood	Garrote *et al.*, 2007; Conde *et al.*, 2011a
Grape pomace	Ruberto *et al.*, 2007; Conde *et al.*, 2011a
Olive tree leaves	Benavente-García *et al.*, 2000; El and Karakaya, 2009

1.2.2. Chemical composition

The most important groups of natural antioxidants include terpenes, vitamins, small proteins and peptides, Maillard reaction products (MRPs), carbohydrates and phenolics.

Terpenes

Terpenes are made from isoprenes that are combinations of five-carbon-base (C_5) units. These molecules are classified in hemi-, mono- (C_{10}), sesqui- (C_{15}), di- (C_{20}), sester- (C_{25}), tri- (C_{30}) and tetraterpenes (C_{40}). When these compounds are accompanied by oxygen they are called terpenoids, which the most common are tetraterpenoids, also called carotenoids (Bakkali *et al.*, 2008). The latter are synthesized by plants and microorganisms (Al-Jaber *et al.*, 2011; Carocho and Ferreira, 2013). Many of these molecules are components of essential oils (Burt, 2004; Bakkali *et al.*, 2008; Brewer, 2011; Sanches-Silva *et al.*, 2014)) and have been reported as having antioxidant activity (Bakkali *et al.*, 2008; Sikora *et al.*, 2008; Brewer, 2011; Xu *et al.*, 2013).

Vitamins

Vitamins also have antioxidant activities, mainly vitamin A, K, C and E. The most frequently found in natural sources are vitamins C and E that are generically called ascorbic acid and tocopherol, respectively (Carocho and Ferreira, 2013) In fact, ascorbic acid is one of the most popular antioxidants (Arrigoni and De Tullio, 2002) and has been reported as the

antioxidant in different products such as, commercial fruit juices (Kabasakalis *et al.*, 2000), mushrooms (Barros *et al.*, 2007), fresh fruits and vegetables such lemon and orange (Szeto *et al.*, 2002; Asami *et al.*, 2003).

Small proteins and peptides

Small proteins and peptides also show antioxidant activity (Chen *et al.*, 1996; Kitt, 2005; Chen *et al.*, 2006; Torruco-Uco *et al.*, 2009; Liu and Zhao, 2010). Antioxidant peptides can be isolated from various protein hydrolyzates and have several advantages including low molecular weight, simple structure, easy absorption, stability under different conditions and lack of immunoreactions. Chen *et al.*(1996) and Elias *et al.*(2008) mentioned different sources of small proteins or peptides with antioxidant activity, such as soybean, egg yolks, potatoes and gelatin.

Maillard reaction products (MRPs)

The Maillard reaction is a non-enzymatic reaction between carbonyl groups of reducing sugars and amino groups from amino acids, peptides or proteins. MRPs are naturally produced in food during thermal processing and storage (Yilmaz and Toledo, 2005; Vhangani and Van Wyk, 2013). The most studied MRPs for their antioxidant properties are melanoidins, which show scavenging hydroxyl radical, superoxide and hydrogen peroxide antioxidant capacities, as well as metal chelation activity. Some foods with antioxidant activity are affected by chemical interactions that can modify the antioxidant compounds, especially tomato derivatives and roasted coffee (Nicoli *et al.*, 1997; Rufián-Henares and Morales, 2007; Vhangani and Van Wyk, 2013).

Carbohydrates

Several carbohydrates have been studied for their antioxidant properties, especially polysaccharides like dextran, pullulan, mannan and lipopolysaccharides (Tsiapali *et al.*, 2001). The antioxidant activity of these compounds has been reported *in vitro* assays, such as reported for arabinoglucogalactan from *Panax noto* ginseng roots (Wu and Wang, 2008), and *in vivo* studies, such as those with porphyran from *Porphyra* (Zhang *et al*., 2003).

Phenolic compounds

The terms “phenolic”, “polyphenol” and “phenols” can be established in relation to the basic chemical structure of the compounds (Boudet, 2007). In fact, phenolic is a molecule composed with an aromatic ring bearing one or more hydroxyl substituents and functional

derivatives, such as esters, methyl ethers and glycosides. Despite the structural diversity, this group of compounds is often referred to as "polyphenols" (Balasundram *et al.*, 2006; Ignat *et al.*, 2011).

Phenolic acids, flavonoids and tannins are the three main groups of polyphenols that are most recognized for their antioxidant properties.

- *Phenolic acids* are very abundant in nature and they are derivatives of benzoic acid (gallic *p*-hydroxybenzoic, protocatechuic, vanillic and syringic acid) and cinnamic acids (caffeic, ferrulic, *p*-coumaric and sinapic acid) (Ignat *et al.*, 2011).
- *Tannins* are polyphenolic compounds subdivided into two groups: hydrolysable and condensed tannins (Ignat *et al.*, 2011). The most studied condensed tannins are based on the flavan-3-ols (-)-epicatechin and (+)-catechin, also called proanthocyanidins; and hydrolysable tannins are derivatives of gallic acid (Balasundram *et al.*, 2006; Ignat *et al.*, 2011).
- *Flavonoids* constitute the largest group of polyphenols. Because of this, many authors divide the polyphenols in 2 categories: flavonoids and non-flavonoids (Balasundram *et al.*, 2006). These in turn are subdivided into subgroups such as flavonols, flavones, flavanols (or catechins), isoflavons, flavanols and anthocyanidins (Ignat *et al.*, 2011).

Phenolic compounds are secondary metabolites that are derivatives of various metabolic pathways in plants. Generally, these molecules are involved in the protection of plants against pathogens and predators (Balasundram *et al.*, 2006; Ignat *et al.*, 2011). Moreover, they contribute towards the colour and sensory characteristics of fruits and vegetables (Balasundram *et al.*, 2006). In fact, they seem to be a promissory group of natural antioxidants. Various studies have been reported in the last few years, where different sources of these compounds have been identified. Sikora *et al.* (2008) cited different plant materials, such as fruits, vegetables, cereals, legumes and spices, in which phenols are the prevalent antioxidant compounds.

Many properties, beyond the antioxidant activity have also been attributed to these compounds, such as anti-allergenic, anti-atherogenic, anti-inflammatory, antimicrobial, anti-thrombotic, cardioprotective and vasodilating activities (Balasundram *et al.*, 2006).

1.2.3. Obtention

The extraction process is a very important step in the obtention of these antioxidant extracts. Habitually, phenolic compounds, as in the case of those from the food industrial wastes, have been extracted by milling, drying or lyophilizing plant materials. However, fresh materials can also be used by soaking with subsequent solvent extraction (Ignat *et al.*, 2011).

Conventional extraction techniques

Conventional extraction techniques are based on the extracting power of different solvents. Some of these techniques are: Soxhlet extraction (1), maceration (2) and hydrodistillation (3) (Kumar *et al.*, 2011; Azmir *et al.*, 2012; Khoddami *et al.*, 2013).

Soxhlet extraction (1) was first proposed by *Franz Soxhlet* in 1879 and initially developed only for lipid extraction. Now, it is used as a model for comparison to other techniques and it is the most used conventional technique.

Maceration (2) was used in times to obtain essential oils and it is a good way to increase the surface area with the solvent.

Hydrodistillation (3) does not involve organic solvents and it can be performed before dehydration of vegetal materials. This technique can be carried out in three "ways": water distillation, water and steam distillation and direct steam distillation (Azmir et al. 2013; Khoddami et al. 2013).

Solvent extraction, by water, alcohols, acetone or their mixtures, is one of the most common used techniques. The yield of extraction and the compounds extracted (**Table 1.2.**) are directly affected by the solvent. Temperature and time of extraction can also affect the yield of extraction and usually when these parameters increase, the solubility of the analyte is promoted. However, extracted antioxidant compounds are generally degradable by extended extraction times and high temperatures (Ignat *et al.*, 2011; Khoddami *et al.*, 2013). Nevertheless, these techniques with solvents imply a co-extraction of non-phenolic compounds such as proteins and sugars requiring a subsequent purification process.

Table 1.2. Extracted bioactive compounds by different solvents (adapted from Ignat *et al.* (2011) and Azmir *et al.*(2013)).

Water	Ethanol	Methanol	Acetone	Chloroform	Ether	Ethyl acetate
Proanthocyanidins Phenolic acids Tannins Saponins Terpenoids	Tannins Polyphenols Flavonols Terpenoids Alkaloids Free phenolic acid	Anthocyanins Terpenoids Saponins Tannins Flavones Polyphenols Catechins Phenolic acids Flavanones	Flavonoids Some polyphenols (eg. olive leaves)	Terpenoids Flavonoids Flavonols Free phenolic acids	Alkaloids Terpenoids Phenolic acids Phenolic compound Flavonols	Phenolic acids Flavonols Antocyanins

However, conventional extraction techniques have long extraction times, elevated costs of high purity solvents, low evaporations of the huge amount of solvents, low extraction selectivity and possible thermal decomposition of thermo labile compounds (Azmir *et al.,* 2013; Khoddami *et al.,* 2013). To overcome these disadvantages, new promising non-conventional extraction techniques are used.

Non-Conventional extraction techniques

These techniques offer two advantages: short extraction times and decrease of organic solvent consumption, so they are considered "green techniques". Non- conventional extraction techniques include ultrasound assisted extraction (UAE), microwave-assisted extraction (MAE), ultrasound-microwave-assisted extraction (UMAE), high hydrostatic pressure processing (HHPP); subcritical water extraction (SCWE) and supercritical fluid extraction (SFE) (Azmir *et al.,* 2013; Khoddami *et al.,* 2013).

Supercritical fluid extraction has been much discussed as a very promising technique in the food industry. This technique is based on the fact that at the critical point, the properties of the solvent change. In other words, the solvent is exposed to a given temperature and pressure in which there is no gas or liquid (Grigonis *et al.,* 2005; Azmir *et al.,* 2013). The use of CO_2 as the supercritical fluid makes this technique still acquire additional advantages, since it is environmentally safe, non-toxic and readily separated or removable. In fact, this technique is ideal for plant materials, which are composed of thermolabile compounds. However, when CO_2 is used as supercritical fluid the extraction of polar phenolic compound is rather limited (Grigonis *et al.,* 2005; Ignat *et al.,* 2011).

Subcritical water extraction, also known as "high pressure extraction with water", "superheated water extraction" and "hot water extraction", is performed with water at 100-374 ºC applied under sufficient pressure (10-60 bar) to preserve water in the liquid state (Herrero *et al.,* 2006; Rodríguez-Meizoso *et al.,* 2010). It is called subcritical water due to the subcritical conditions used, which leads to breakdown of intermolecular hydrogen bonds. In this procedure, water in subcritical state is also environmentally safe, non-toxic, presents unique solvent and transport properties and surface tension, viscosity and dielectric constant, with values similar to the ones found for methanol. In fact, SCWE is a good solvent technique for ionic species at ambient temperature and pressure (Kim *et al.,* 2009; Khoddami *et al.,* 2013).

In fact, hydrothermal techniques, based on the application of hot, pressurized water or steam, have deserved increasing interest, as they can also be considered as environmentally friendly. Actually, LCMs are commonly treated with water or steam at 160-240 ºC (autohydrolysis processing) that results in both depolymerization of hemicelluloses and breakage of lignin-carbohydrate bonds. The reactions involved in hydrothermal processing are autocatalytic: they start with hydronium ions from water autoionization and their progress is favored by the *in situ* generation of organic acids like phenolic acids. During the process the pH decreases (Garrote *et al.,* 2004; Moure *et al.,* 2005; Conde *et al.,* 2011a).

These techniques improve selectivity, extraction times (shorter) and the big advantage is the non use of toxic organic solvents. Nevertheless, a big disadvantage is that expensive equipment is required (Herrero *et al.,* 2006; Khoddami *et al.,* 2013).

1.2.4. Natural *versus* synthetic antioxidants

Synthetic antioxidants are used in food industry to increase the product shelf-life (Carocho and Ferreira, 2013). Generally, these antioxidants are composed of phenolic structures with different degrees of alkyl substitution (Velioglu *et al.,* 1998).

Some of the most popular synthetic antioxidants used in the food industry are: tert-butyl hydroquinone (TBHQ), butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT). TBHQ is found in foods, especially in fats and fish products. BHA, more prevalent than TBHQ, is found in vegetables, fish and milk products; sometimes also found in plastics. BHT is very similar to BHA and it is present in beverages and cereals. Ascorbic acid, the most used and known synthetic antioxidant, is noted by its presence not only in food matrices but also in cosmetic and pharmaceutical industries. However, the use of synthetic compounds in food matrices has to be further studied, because they may be the cause of loss of nutritional

value and, can even produce toxic substances that may compromise human health (André *et al.*, 2010). Advantages and disadvantages of synthetic and natural antioxidants have been resumed in **Table 1.3.**

Nevertheless, the application of natural antioxidants in the food industry is of interest for at least four reasons: may provide protection to food components against lipid oxidative damage during processing, storage and cooking (Paiva-Martins *et al.*,2007; Bouaziz *et al.*,2008; Pereira de Abreu *et al.*, 2011); may be absorbed into the human body and might exert beneficial effects (Serafini *et al.*, 2000; Mukoda *et al.*, 2001; Madhujith and Shahidi, 2007); could exert beneficial effects, without being absorbed, in the gastrointestinal tract itself (Yin *et al.*, 2008) and in the colon (Lee *et al.*, 2007); and finally could be suitable for therapeutic use due to their biological effects such as anti-inflammatory, anti-ischemic and antithrombotic agents (Morris, 2002; Tanaka and Sugie *et al.*, 2008; Ullah and Khan, 2008).

Table 1.3. Advantages and disadvantages of synthetic and natural antioxidants (Valenzuela and Nieto, 1996).

Synthetic antioxidants	**Natural antioxidants**
Economic	More expensive
Many applications	Restricted use of certain products
Mean and high antioxidant activity	Wide range of antioxidant activity
Some problems about toxicity and security	Known as innocuous substances
Some of them are prohibited	Increasing use and growing applications
Low solubility in water	Wide range of solubility
Decreasing interest	Increasing interest

Nowadays, some natural sources have been tested to be used in the food industry and synthetic antioxidants are eventually used as standards for antioxidant activity measurements and used to compare with natural antioxidants (Carocho and Ferreira, 2013).

1.3. Biological properties

1.3.1. Antioxidant activity

Antioxidant activity and antioxidant capacity are often used interchangeably, but they have different meanings. Activity refers to the rate constant of the reaction between the antioxidant and oxidant species, whereas the capacity refers to the amount (in moles) of a given free radical scavenging by a sample (Apak *et al.*, 2013).

1.3.1.1. Mechanism of action

Antioxidants can act during oxidative stress in different ways: (1) as preventive antioxidants, which hinder ROS formation or scavenge species responsible for oxidation inhibition and prevent decomposition of hydroperoxides into free radicals; (2) by breaking the chain reaction, which convert reactive free radicals into stable molecules and consequently interrupt the propagation of the autoxidation chain reaction; (3) as singlet oxygen quenchers, which transform singlet into triplet oxygen; (4) through synergism with other antioxidants or compounds increasing the activity of chain-breaking antioxidants in a mixture; (5) as reducing agents which turns hydroperoxides into stable molecules; (6) as metal chelators that stabilize metal pro-oxidants like iron and copper cations; and finally as (7) inhibitors of specific oxidative enzymes, especially lipoxygenases (Brewer, 2011; Carocho and Ferreira, 2013). However, the most effective antioxidants are those that interrupt the free radical chain reaction, normally composed by aromatic or phenolic rings that transfer hydrogen to free radicals (Brewer, 2011).

In fact, the free radical chain process of autoxidation can be retarded by two categories of antioxidants: *primary* antioxidants and *secondary* antioxidants (Rajalakshmi and Narasimhan, 1996). *Primary* antioxidants, also known as "chain-breaking" antioxidants by scavenging free radicals, are substances capable of accepting free radicals, which can delay the inhibition step or interrupt the propagation of autoxidation. These antioxidants have a higher affinity for peroxyl radicals than lipids and they are effective at very low concentrations. *Secondary* antioxidants or preventive, act through a mechanism that does not involve direct scavenging of free radicals, and can act in different ways, such as binding of metal ions, absorbing UV radiation, deactivating singlet oxygen, scavenging oxygen and converting hydroperoxides to non-radical species. Usually, they act as antioxidants only if a second minor component is present, such as citric acid, which is an effective reducing agent in the presence of metal ions (Rajalakshmi and Narasimhan, 1996; Reische *et al.*, 1998).

Antioxidants can also act by different mixed and co-operative mechanisms. In this way an antioxidant can behave as a pro-oxidant (depending on the structure, chemical environment and operational conditions), for example ascorbic acid, flavonoids and α-tocopherol in presence of transition metal ions (Carocho and Ferreira, 2013). However, some *primary* antioxidants, when present in high concentrations or certain conditions, can turn to pro-oxidants (Yordi *et al.*, 2012).

1.3.1.2. Measurement of antioxidant activity

The antioxidant activity depends on a number of factors that includes:

(i) Type of substrate, i.e. type of source and ripeness

Evidence shows that different plants have different contents of phenols and consequently, different antioxidant activities. However, in the same matrix (e.g. berry fruits) and the same plant (e.g. blackberry) differences in antioxidant activity are also found depending on ripeness state (Kähkönen *et al.,* 2001) and on the parts of plant that are being analyzed (e.g. leaves, seeds, fruits) (Moure *et al.*, 2001; Al-Jaber *et al.*, 2011).

(ii) Concentration of extract

Antioxidant activity is directly influenced by concentration of the extract and in general, high concentrations reflect higher activity. However, the maximum activity does not always correspond to the highest concentration tested, because it is affected by the source of the extract and by method used to measure this activity (Yen and Wu, 1999).

(iii) Conditions of storage

Compounds with antioxidant activity are affected by external factors such as light and temperature. Storage time and packaging also affect this activity (Poiana *et al.,* 2011). Patthamakanokporn *et al.* (2008) reported various fruits whose antioxidant activity was affected by the conditions and times of storage.

(iv) Partitioning properties of the antioxidant between lipid and aqueous phases

The antioxidant activity of non-polar antioxidants is decreased in the presence of water. In fact, there seems to be a correlation between antioxidant activity and colloidal properties of emulsions. In some cases, surfactants are added (like Tween 20) to improve the solubility of the emulsions (Schwarz *et al.,* 2000). Proteins such as bovine serum albumin (BSA) are also added to hydrophobic solutions as emulsifiers (Rangsansarid and Fukada, 2007).

Therefore these factors and many more, affect the measurement of antioxidant activity. This should therefore be evaluated by different methods, because there is no perfect method that can give an unequivocal result (Litescu *et al.,* 2010). Besides, it is obvious that the specificity and sensitivity of one method may not lead to differences on the recovery of phenolic subclasses (Kähkönen *et al.,* 2001). Various methods have been developed although the results have to be treated with caution. So, the best solution is using various methods to obtain a more reliable result (Litescu *et al.,* 2010; Carocho and Ferreira, 2013).

Various methods use the free radicals or synthetic antioxidants to compare with the antioxidant activity (Carocho and Ferreira, 2013). Generally, methods to measure the antioxidant activity are classified, depending on the type of assessment carried out, into two categories (Litescu *et al.,* 2010): assessment of antioxidant efficacy in relation to free radical species (1) and assessment of antioxidant efficacy using biological significant markers and significant substrates (2).

Assessment of antioxidant efficacy in relation to free radical species (1)

The first category is subdivided into two categories depending on the reaction involved i.e. ability to quench free radicals by hydrogen atom transfer (HAT) or ability to single electron transfer (SET). However, in some cases it is possible to have a combination of both (Prior *et al.,* 2005; Litescu *et al.,* 2010).

HAT-based assays measure the classical ability of an antioxidant to quench free radicals by hydrogen donating, in which antioxidant and subtract compete for thermally generated peroxyl radicals through the decomposition of azo-compounds. These reactions are solvent and pH independent and are usually quite rapid. These include: Oxygen Radical Absorbance Capacity Assay (ORAC), Total Radical-Trapping Antioxidant Parameter Assay (TRAP) and Crocin Bleaching Assay (Prior *et al.,* 2005; Litescu *et al.,* 2010).

SET-based assays measure the capacity of an antioxidant to reduce the oxidant, which changes colour in this process. The degree of colour reduction is correlated with the sample's antioxidant concentration and it involves a redox reaction with the antioxidant as an indicator of the reaction endpoint and measures the antioxidant's reducing capacity. These assays are pH dependent and include: Trolox Equivalent Antioxidant Capacity Assay (TEAC) or ABTS assay, 2,2-Diphenyl-1-Picryhydrazyl Radical Assay (DPPH) and Ferric Reducing Antioxidant Power Assay (FRAP) (Prior *et al.,* 2005; Litescu *et al.,* 2010).

Some authors including Prior *et al.* (2005) classify TEAC and DPPH assays as a combination of HAT- and SET- based assays since both indicator radicals may be neutralized either by direct reduction via electron transfer or by radical quenching via hydrogen atom transfer. Besides these methods, the method is also known as Folin-Ciocalteu or Total Phenolics Assay.

Assessment of antioxidant efficacy using biologically significant markers and substrates (2)

This category includes methods for the determination of antioxidant efficacy through the evaluation of the damaging effects on a biological substrate produced by the ROS or RNOS (Related Nitrogen Oxide Species). In other words, the measurement is performed using biological markers as DNA strands, RNA strands and lipids (Litescu *et al.,* 2010). These methods are essentially based on lipid peroxidation, which induces disturbance and alteration of biological membranes. The secondary products of this oxidation could modify very fundamental molecules in biological point of view (like DNA), which can result in various disorders and diseases (Niki, 2010).

This oxidative stress can be initiated in three ways: via a thermal process (when lipid solutions are heated in presence of oxygen to promote the formation of lipoperoxide radicals); by utilization of an azo-initiator which also generates peroxyl radicals (when in presence of lipid or low density lipoproteins produces lipoperoxide) and finally using a hydroxyl radical generating systems that can be created by UV radiation, titanium dioxide or Fenton reaction (Fe (II) - H2O2) (Litescu *et al.,* 2010).

It should be noted that a good radical scavenging activity does not necessarily translate into a good antioxidant activity, and thus not all compounds showing high radical scavenger ability show good antioxidant properties (Litescu *et al.,* 2010). Similarly, uric acid is suggested as a major antioxidant in plasma using TRAP and ORAC methods. However, it is not as efficient as an antioxidant against lipid peroxidation as ascorbic acid is. In summary, the capacity of free radical scavenging activity does not necessarily correlate with antioxidant activity and with the capacity of inhibiting lipid peroxidation (Litescu *et al.,* 2010; Niki, 2010).

1.3.2. Microbial activities

1.3.2.1. Antimicrobial activity

Several studies suggest that phenolic compounds show some activity against pathogenic microorganisms. In fact, various sources of natural antioxidants have been reported for their antimicrobial properties, associated with presence of polyphenols (see **Table 1.4.** and **Table 1.5.**). The evidence that increasing numbers of microorganisms are now resistant to the available antibiotics is an emergent problem (Daglia, 2012; Alves *et al.*, 2013). So, it is necessary more than ever to find new alternatives. In fact, plants have chemical compounds (*e.g.* essential oils and organic acids) that provide them with protection against microbial infection (Bell *et al.*, 2005). Antimicrobial activity of natural compounds is an interesting way to develop new healthy foods, as well as to be used in medical and pharmaceutical applications (Nohynek *et al.*, 2006).

For many of these sources and by-products, a very good correlation between phenolic content and antimicrobial activity has been found (Estevinho *et al.*, 2008; Pérez *et al.*, 2009; Delgado Adámez *et al.*, 2012; Silván *et al.*, 2013). Moreover, many studies have reported antifungal and antiviral activities of these compounds (El and Karakaya, 2009; Chanda *et al.*, 2010; Alves-Silva *et al.*, 2013).

Phenolic compounds like tannins and alkaloids are the most important antimicrobial agents, however for antiviral activity the presence of hydroxyl and ester groups is required (Atri *et al.*, 2012; Ifesan *et al.*, 2013). Antimicrobial properties of alkyl esters, such as *p*-hydroxybenzoic acid (also called parabens), are well known due to their large utilization in pharmaceutical, food and beverage industries (Merkl *et al.*, 2010).

Table 1.4. Examples of natural sources of antimicrobial agents.

Sources	**Phenolic compounds**	**Microorganisms**	**References**
Ginja berry plant	Terpenes	*Bacillus subtilis* *Escherichia coli* *Pseudomonas aeruginosa* *Staphylococcus aureus*	Piccirillo *et al.*, 2013
Berry extracts	Antocyanins Proantocyanins Monomeric phenols Flavonoids	*E.coli* *Enterococcus faecalis* *Listeria monocytogenes* *Salmonella typhimurium* *S. aureus* *Streptococcus mutans* *Yersinia enterocolitica*	Puupponen-Pimiä *et al.*, 2001; Yoo *et al.*, 2011; Lacombe *et al.*, 2012
Essential oils (herbs eg. Bush-basil)	Terpenes Eugenol	*Achromobacter denitrificans* *Aeromonas hydrophila* *L. innocua* *Serratia marcescens*	Alves-Silva *et al.*, 2013
Oregano extracts	Phenolic acids	*Helicobacter pylori*	Chun *et al.*, 2005
Grape seeds extracts	Proantocyanidins Catechins Epicatechins	*Brochothrix thermosphact* *Campylobacter spp.* *L. innocua* *S. aureus* *Salmonella* spp.	Delgado Adámez *et al.*, 2012; Silván *et al.*, 2013
Honey extracts	Phenolic acids	*B. subtillis* *E. coli* *Klebsiella pneumonia* *P. aeruginosa* *S. aureus* *Staphylococcus lentus*	Estevinho *et al.*, 2008
Herbal teas extracts (eg. green tea, black tea, rosemary)	Catechins Caffeic acid	*Brochothrix thermosphacta* *E.coli* *L. monocytogenes* *Pseudomonas putida* *Salmonella* spp. *Shigella flexneri* *Streptococcus mutans* *Streptococcus sobrinus*	Oh *et al.*, 2013; Rozoy *et al.*, 2013

Table 1.5. Examples of antimicrobial agents in by-products.

By-products	Phenolic compounds	Microorganisms	References
Brewery waste	Procatechins Caffeic acid *p*-coumaric acid Ferrulic acid Catechins	*S. aureus* *L. monocytogenes* *Salmonella* spp. *E. coli* *E. coli 0157:H7*	Barbosa-Pereira *et al.,* 2014
Fruits and vegetable peels	Tannins Phenolic acids	*K. pneumoniae.* *S. subflata* *Corynebacterium rubrum* *Salmonella typhimurium* *Enterobacter aerogenes* *Proteus mirabilis* *B.cereus* *L. monocytogenes* *S.aureus* *E. coli* *Vibrio cholerea*	Chanda *et al.,* 2010; Wonghirundecha and Sumpavapol, 2012

Mechanisms of action

The antimicrobial activity of phenolic compounds is determined by their chemical structure, in particular by the number and position of substitution of the benzene ring (Alves *et al.,* 2013).

Many studies report that membrane damage is the principal mechanism of action of phenolic compounds (Vaquero *et al.,* 2007; Campos *et al.,* 2009; Nohynek *et al.,* 2006; Lou *et al.,* 2012). In fact, phenolic compounds are known for their membrane-active properties, which are related to diffusion through the cytoplasmic membrane (Campos *et al.,* 2009). However, differences between Gram-positive and Gram-negative bacteria are argued to be a consequence of the dissimilar protection granted by the differing cell walls. Actually, it is known that the external membrane of Gram negative bacteria (lipidic membrane) works like a barrier to external agents such as digestive enzymes and some hydrophobic antibiotics (Nohynek *et al.,* 2006). In opposition, some hydrophobic compounds, such as ellargic acid, are active against these bacteria. Taking this into account, other mechanisms may be involved when the activity of some phenolics upon this particular group of bacteria is considered (Vattem *et al*., 2004; Souza *et al.,* 2010).

Another mechanism that has been published is the phenolic-protein interaction: for example, if these compounds interact with extracellular enzymes they can promote a deprivation of nutrients required for microbial growth and metabolism. The antioxidant

activity associated to phenolic compounds is usually related with their capacity to chelate metallic ions that can be enzyme cofactors, for example (Vattem *et al*., 2004; Vermerris and Nicholson, 2008). Another mechanism of action is "cited" by Puupponen-Pimiä *et al.* (2001) related to DNA alterations, when they report that a strain of *E. coli*, which has a DNA repair mechanism, is less sensitive to anthocyanidins than a strain that does not have such mechanism. On the other hand, phenolic acids are capable to cross the cytoplasmic membrane, promoting the acidification of the intracellular medium. Consequently, the cells start to spend energy regulating the pH and prevent hyper acidification by removing the protons. However, at higher concentrations this protective mechanism does not work and the bacteria eventually die (Vattem *et al.*, 2004; Adams and Moss, 2008). Additionally, others mechanisms were reported by Hyldgaard *et al.* (2012) such as changes in membrane fatty acid composition, might interactions with intracellular compounds, leaked of H+ and K+ ions and ATP and inhibition of enzymes involved in ATP synthesis.

In conformity, sources of phenolic compounds are constituted by various molecules whereby multiple mechanisms might be involved and synergic effects can be observed (Puuponen-Pimia et al., 2001; Nohynek *et al.*, 2006; Hyldgaard *et al.*, 2012).

1.3.2.2. Growth enhancement of probiotic bacteria

Probiotic bacteria are usually defined as "viable microorganisms (bacteria or yeasts) that exhibit a beneficial effect on the health of the host when ingested" (Salminen et al., 1998)). These bacteria influence, in special, gut health by: energy extraction homeostasis, immunity stimulation and/or modulation, regulation of intestinal motility and permeability, elimination of pathogenic microorganisms through competition for nutrients or direct agglutination; competition for receptor sites by blocking the adhesion of other cells; production of antimicrobial substances such as bacteriocins, organic acids and hydrogen peroxide; binding and metabolization of toxic compounds, mucus production and pH reduction through stimulation of lactic acid-producing microorganisms (Aluko, 2012).

Probiotics are generally-recognized-as-safe (GRAS) and the best known genera including such strains are *Lactobacillus* and *Bifidobacterium* (Salminen *et al.*, 1998; Kottol *et al.*, 2014).

Effects of plant extracts such as grape pomace and olive leaves extracts, rich in phenolic compounds, upon the growth of probiotic bacteria have been reported extensively (Hervert-Hernández et al. 2009; Haddadin, 2010; Tabasco et al. 2011; Sánchez-Patán et al. 2012; Vodnar and Socaciu, 2012). Catechins have been reported as the polyphenols that more

positively influence the growth of *Lactobacillus* and *Bifidobacterium* strains (Haddadin, 2010; Kottol *et al.*, 2014). This positive effect can be explained by the ability of these probiotic bacteria to use phenolic compounds as substrates during growth and polyphenols as an energy source. Besides positively affecting bacteria metabolism, phenolic compounds can enhance consumption of nutrients such as sugars. It is well known that promoting growth of probiotic bacteria with phenolic compounds is dependent on: microbial strains, polyphenol structure and dosages assayed (Hervert-Hernández *et al.*, 2009; Aluko, 2012). The metabolism of polyphenols such as catechins by probiotic bacteria generates short-chain fatty acids (SCFAs) that are associated with beneficial effects both at the cellular and systemic levels. Actually, the production of SCFAs in the colon is important to decrease the pH and consequently inhibit the ability of pathogenic bacteria to grow and colonize the gut epithelium. Moreover, this decrease in pH also increases the absorption of minerals such as calcium and magnesium (Aluko, 2012).

1.3.3. Other Properties

The balance between oxidation and antioxidation is critical to maintain the health of biological systems. Therefore, several biological properties have been attributed to natural antioxidants that provide insight on the potential effects on health and in disease conditions.

Actually, the incorporation of phenolic compounds in the human diet has been correlated with the prevention of vascular pathologies, cancer, atherosclerosis, type II diabetes, Alzheimer's disease, hypertension, obesity, osteoporosis, chronic liver disease, nephritis or chronic renal and gastrointestinal diseases (Moure *et al.*, 2001; Nićiforović *et al.*, 2010; Yordi *et al.*, 2012, Carocho and Ferreira, 2013).

Various berry fruits have been associated with gastrointestinal disorders, treatment of urinary infections and prevention of certain cancers (Nohynek *et al.*, 2006). Grape and wines have shown protection against atherothrombotic episodes such as myocardial ischemia (Ky *et al., 2014)*. Generally, fruits and vegetables have been related to cardiovascular and cerebrovascular disease prevention, associated with the presence of flavonoids. *In vitro* studies demonstrated that some polyphenols act by blocking cell proliferation, which can be connected with the proliferation of cancerous cells (Yordi *et al.*, 2012). Coffee consumption has been correlated with the risk reduction of neurodegenerative diseases as Alzheimer's and

Parkinson's (Ignat *et al.*, 2011; Di Castelnuovo *et al.*, 2012), which could be justified by the presence of vitamins C and E and flavonoids (Yordi *et al.*, 2012).

1.4. Potential food, cosmetic and pharmaceutical applications

The wide biodiversity of phenolic compounds justifies their wide range of applications in diverse industries. Natural compounds with antioxidant activity have been frequently reported as having applicability in the food and cosmetic industries (Moure *et al.*, 2001; Davis and Perez, 2009; Ayala-Zavala *et al.*, 2011; Selani *et al.*, 2011). Furthermore, acquired antibiotic resistances make the pharmaceutical industry one of the most promising stakeholders due to the demand to formulate new and more effective drugs (Daglia, 2012).

In the pharmaceutical industry, these are promising compounds because they are easily obtained, which is a great advantage, especially at the economic level. Moreover, the ecological consciousness suggests that "natural" products are safe, which also gives an additional interest (Rates, 2001). Honey is one of many sources that have been mentioned to be used in this industry due to the antimicrobial, anti-inflammatory and tissue repair properties (Estevinho *et al.*, 2008).

In the cosmetic industry, the benefits of polyphenols focus in tissue healing, also favored by the antimicrobial activity. One example is *Aloe vera*, which is already currently found in some gels and cream formulations for a range of skin disorders. *Aloe vera* is also found in the cosmetic industry applied to hair products (Davis and Perez, 2009).

However, the major application of polyphenols still focuses in the food industry. Similarly with synthetic antioxidants, natural compounds have been used not only to increase the shelf-life but also to prevent lipid oxidation of foods (Moure *et al.*, 2001). Nevertheless, some organoleptic characteristics of the extracts might be a disadvantage and hinder their incorporation in food matrices, as already occurs with rosemary that, despite having an excellent antioxidant activity, has a very intense aroma (Moure *et al.*, 2001). In fact, these compounds can improve the overall quality of some foods and therefore, a new generation of food packaging ought to be developed including phenolic compounds. These packages should be able to increase the shelf-life and, simultaneously, decrease the risk of contamination by pathogenic microorganisms. Moreover, the possibility of conjugating various properties to obtain an improved food packaging might increase the range of applications to diverse food matrices (Appendini and Hotchkiss, 2002).

1.5. Work objectives

Several residual sources are rich in certain compounds, particularly phenolics, known for their antioxidant and antimicrobial activities, among other properties. Taking this information into account, the general objective of this work was to use antioxidant extracts from agro-industrial wastes, such as corn cobs, almond shells, eucalypt wood and grape pomace, to assess their suitability as either antimicrobial agents against foodborne pathogens or as growth stimulators-support of beneficial bacteria. To achieve this general objective, we proposed the following specific objectives:

1- Measurement of the antioxidant activity and chemical characterization of the extracts. For this, three assays for the antioxidant activity were done and the extracts were characterized by HPLC analysis.

2- Evaluation of the antimicrobial activity of these extracts against selected foodborne pathogens. This was done through the determination of the minimum inhibitory and bactericidal concentrations (MICs and MBCs, respectively), complemented by time-kill analysis/ survival curves.

3- Assessment of the ability of the antioxidant extracts to enhance the growth of selected probiotic bacteria. This was done by comparing growth curves with and without the selected extracts.

4- Assessment of the applicability of these antioxidant extracts in the production of edible films. An antimicrobial and antioxidant edible film, using an alginate-sodium matrix was formulated. Film characterization included color measurement, water solubility and swelling ratio, thickness measurement, moisture and extract content.

2. Materials and methods

2.1. Antioxidant extracts: sources and obtention

In this work, we tested four antioxidant extracts obtained from the following agro-industrial sources: corn cobs (CC), eucalypt wood (EW), grape pomace (GP) and almond shells (AS). These extracts were kindly supplied by the laboratory of Chemical Engineering of the University of Vigo (Ourense, Spain). The extracts were obtained as described by Conde *et al.* (2011a), briefly, the selected lignocellulosic wastes were subjected to autohydrolysis processing in media containing hot, compressed water, at temperatures in the 200-240 ºC range. The aqueous phases from treatments were extracted with ethyl acetate and the soluble solids were re-dissolved in 80% ethanol (to obtain a more hydrophilic product) and then lyophilized and kept at room temperature until further use.

2.2. Chemical characterization

The antioxidant activity of extracts was measured using three different methods: DPPH radical scavenging assay, ABTS radical scavenging capacity assay and ORAC-FL assay. For chemical analysis, extracts were prepared dissolving the antioxidants at 5 mg/mL in methanol (80% (v/v)) (Panreac Química S.A.U, Spain), except for the ORAC assay (here they were prepared as for the microbial assays).

2.2.1. 2,2-Diphenyl-1-picryhydrazyl (DPPH) radical scavenging assay

The antioxidant activity of extracts was measured in terms of hydrogen donating or radical scavenging ability, using DPPH radical scavenging method adapted from Gadow *et al.* (1997). This radical is one of few stable organic nitrogen radicals, which bears a deep purple colour. So, when free radical DPPH reacts with hydrogen donors, it is reduced to the corresponding hydrazine (colourless), as illustrated below.

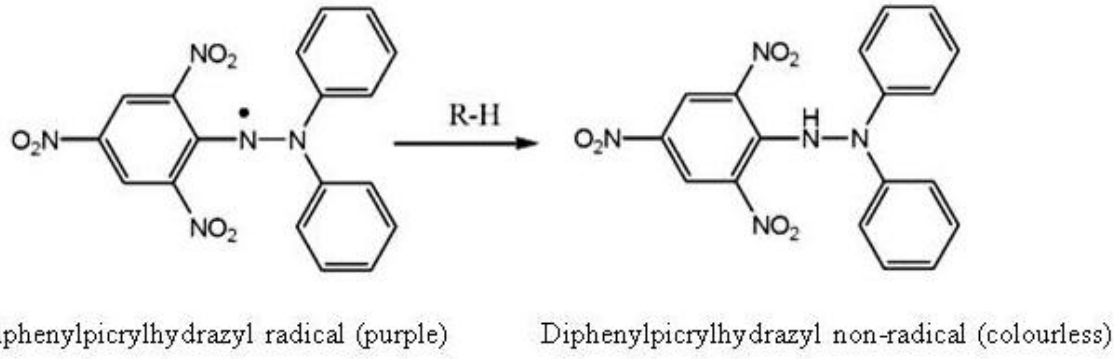

Figure 2.1. DPPH assay mechanism.

From an experimental point, 3 mL of $6x10^{-5}$ M methanolic solution of DPPH (Sigma Aldrich, EUA) were added to 75 µL of a methanolic solution of each antioxidant extract (5 mg/mL). The decrease in absorbance at 515 nm was measured for 16 minutes after DPPH addition with an UVmini 1240 UV-Vis spectrophotometer (Shimadzu, Japan), until the absorbance was stable. Methanol was used to zero the spectrophotometer. Control was done without antioxidant extract, in order to know the initial absorbance of the DPPH radical.

The percent inhibition of the DPPH radical by the antioxidant extracts was calculated according to the formula (2.2.1.) by *Yen and Duh* (1994):

$$\% \text{ inhibition} = \frac{\text{Abs t=0} - \text{Abs t=16}}{\text{Abs t=0}} \times 100 \qquad (2.2.1.)$$

where $Abs_{t=0}$ is the absorbance of the control at t = 0 min and $Abs_{t=16}$ is the absorbance of the antioxidant extract at t = 16 min.

A standard curve was constructed (as shown in Appendix I, **Table 6.1.**) by plotting absorbance against known concentrations of ascorbic acid (Sigma Aldrich, USA) on methanol solution (0-1000 µM). The results are expressed in g/L of ascorbic acid equivalents, which was the synthetic antioxidant used to construct the calibration curve. All assays were performed in triplicate.

2.2.2. 2,2'-Azinobis-(3-ethybenzothiazoline-6-sulfonate) (ABTS) radical scavenging assay

The antioxidant activity of extracts was also measured using the ABTS method previously described by Miller *et al.* (1993). It is reported as a decolouration assay, like the DPPH method, applicable to both lipophilic and hydrophilic antioxidants. This method is performed as described by Re *et al.* (1999), it is based on the scavenging of 2,2'-azinobis-(3-ethybenzothiazoline-6-sulfonate), also called ABTS. Free radical ($ABTS^{\bullet+}$) is generated by oxidation with potassium persulfate, that after this, reacts with hydrogen donors undergoing a reduction, as illustrated below (**Figure 2.2.**)

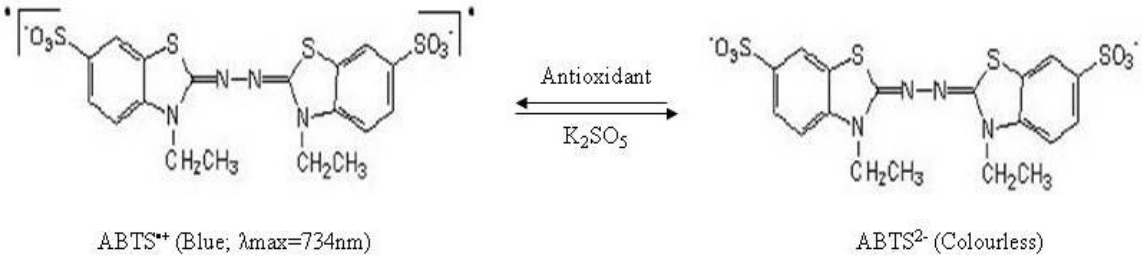

Figure 2.2. ABTS assay mechanism: $ABTS^{\bullet+}$ in presence of antioxidant.

The $ABTS^{\bullet+}$ solution was prepared through the addition, at a 1:1 (v/v) proportion, of a 7 mmol/L solution of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (Sigma, USA) to a 2.45 mmol/L potassium persulfate solution (Merck, Darmstadt, Germany). The mix was left in the dark for 16 h to let the reaction occur. After, this concentrated solution was diluted using deionized water, to obtain an initial optical density (OD) of 0.700 ± 0.020, measured at 734 nm with an UVmini 1240 UV-Vis spectrophotometer (Shimadzu, Japan).

For the analysis, 10 µL aliquots of the extract solutions were added to 1 mL of $ABTS^{\bullet+}$ solution and left to react for 6 min. Since the inhibition percentage (IP) must be between 20 and 80%, the sample was diluted when needed. The IP was calculated using the equation bellow (2.2.2.), in which the $OD_{ABTS\bullet+}$ represents the initial $ABTS^{\bullet+}$ OD and OD_{Sample} represents the OD read after the 6 min reaction.

$$IP = \frac{OD_{ABTS^{\bullet+}} \quad OD_{Sample}}{OD_{ABTS^{\bullet+}}} \times 100$$

(2.2.2.)

A standard curve was constructed (as shown in Appendix I, **Table 6.2.**) by plotting absorbance against known concentrations of ascorbic acid in methanol solution (0-0.25 g/L). The results are expressed in g/L of ascorbic acid equivalents, which was the synthetic antioxidant used for the calibration curve. All assays were performed in triplicate, considering three different replicates of each analysed triplicate.

2.2.3. Oxygen radical absorbance capacity (ORAC-FL) assay

Besides the antioxidant assays cited above, the antioxidant capacity of extracts was also measured using the ORAC assay. Initially described by Glazer (1990) and developed further to include antioxidants samples by Cao *et al.* (1993), the ORAC assay was performed with fluorescein (FL) as described by Dávalos *et al.* (2004). This method is based on the scavenging capacity of antioxidants against the peroxyl radical, which reflects classical radical chain breaking antioxidant capacity by hydrogen atom transfer. In summary, the peroxyl radical reacts with a fluorescent probe to form a non-fluorescent product, which can be quantitated easily by fluorescence, so the antioxidant capacity is determined by a decreased rate and amount of product formed over time.

A stock solution of fluorescein disodium (Sigma Aldrich, USA) at 1166.1 µM concentration in phosphate buffer (pH 7.4; 0.075M) was prepared and stored in the dark at -18 ºC. Prior to the analysis, an aliquot of this stock solution was diluted with phosphate buffer (pH 7.4; 0.075M) to reach a final concentration of 116.66 nM. The reaction was carried out in black polystyrene 96-well microplates (96 x 320 µL, Thermo Fisher Scientific, USA) with 120 µL of the diluted fluorescein solution (116.66 nM) added to 20 µL of antioxidant solution and the mixture was incubated at 40 ºC for 15 min. After incubation, 60 µL of APPH (2,2'-Azobis (2-methylproprionamidine dihydrochloride)) solution (Sigma Aldrich, USA) at 14 mM freshly prepared was added rapidly. A control sample was performed by the same procedure, except that the test material was replaced by 20 µL of phosphate buffer. Fluorescence was recorded during 104 cycles (≈140 min), using a microplate reader with 485 nm excitation and 520 nm emission filters.

Antioxidant curves (fluorescence versus time) were first normalized to the curve of the blank corresponding to the same assay by multiplying original data by the factor $fluorescence_{blank,t=0}$/ $fluorescence_{sample,t=0}$. From the normalized curves, the area under the fluorescence decay curve (AUC) was calculated as according to the formula (2.2.3.) by Dávalos *et al.* (2004):

$$AUC = 1 + \sum_{i=1}^{i=80} f_i / f_0 \qquad (2.2.3.)$$

where f_0 is the initial fluorescence reading at t=0 min and f_i is the fluorescence reading at time I (t≈140 min). The net AUC corresponding to a sample was calculated by subtracting the AUC corresponding to the blank. Regression equations between net AUC and antioxidant concentration were calculated for all the samples.

The results are expressed as µM Trolox (Sigma Aldrich, USA) equivalents that are a water-soluble vitamin E analogue, using a standard curve (0.2-1.6 ŋmol) as shown in Appendix I, **Figure 6.3**. All reaction mixtures were prepared in duplicate and at least three independent runs were performed for each sample.

2.2.4. HPLC analysis

The samples from antimicrobial experiments were analyzed by High Performance Liquid Chromatography with Diode-Array Detection (HPLC –DAD) (Agilent 1100 Series, Agilent Technologies, USA) to identify the phenolic compounds present. The HPLC pumps, autosampler, column oven and diode-array system were monitored and controlled using the HP Chem Station computer program (Agilent Technologies) and equipped with a Synergi 4u Hydro-RP 80A from Phenomenex (250 mm x 4.6 mm) with precolumn operating at 50ºC. The wavelength used for the identification of phenolic compounds was 280 nm. The volume injection was 5 µL, and with a flow rate of 1 mL/ min. A non-linear gradient of solvent A: 89 % H_2O, 10 % CH_3OH (Panreac Química S.A.U., Spain), 1% CH_3COOH (Fisher Scientific and solvent B: 89 % CH_3OH, 10 % H2O, 1% CH_3COOH (Fisher Chemical, UK), was used as follows: 0 min, 100% A; 30 min, 60% A, 40% B; 40 min, 100% A.

The different peaks of the spectra obtained at 280 nm (phenolic acids) were analysed by comparison of retention times and spectra with that of several pure standards from Sigma-Aldrich (gallic acid, 3,4-dihydroxybenzaldehyd, vanillic acid, 4-hydroxybenzaldehyd, vanillin, syringaldehyde, p-coumaric acid, acetovanillone, ferulic acid, quercetin,

hydroxymethylfurfural, 2-furfuraldehyde). Three independent analyses were performed for each of the triplicate samples from each antimicrobial experiment.

2.3. Microbiological assays

2.3.1. Antimicrobial activity assessment

Pathogenic bacteria: source, maintenance and culture conditions

The antibacterial properties of the antioxidant extracts from agro-industrial wastes were tested against five bacterial indicator strains, three Gram-positive and two Gram-negative bacteria. All microorganisms were isolated from contaminated food (*Centro de Inovação e Apoio Empresarial (CINATE), Escola Superior de Biotecnologia, Universidade Católica Portuguesa, Portugal)* (see **Table 2.1**.), except for *Escherichia coli* ATCC 25922 (American Type Culture Collection, USA). *Escherichia coli* ATCC 25922 was, therefore, used as reference microorganism to carry out the antimicrobial susceptibility assays.

Table 2.1. Food sources from which the indicator microorganisms were isolated

Strain	Gram	Food source	Identification Code
Listeria monocytogenes	+	Goat cheese	3375
Staphylococcus aureus	+	Unspecified	SA18N
Pseudomonas aeruginosa	-	Unspecified	PA
Salmonella spp.	-	Meat product	463

The microorganisms were supplied by CINATE in Plate Count Agar (PCA). With the objective to make a stock of each provided bacterial strain, an isolated colony from each plate was transferred to a tube containing Müeller Hinton (MH) broth (Biokar diagnostics, France) and incubated at 37ºC overnight. Then, 500 µL of each bacterial suspension were transferred into cryovial tubes (VWR International, USA) containing 500 µL of 60% (v/v) glycerol (Fisher Chemical, UK) and maintained at -80ºC.

Before use, the frozen stock cultures were reactivated as follows: the volume of each cryovial tube was transferred into 9 mL of MH broth and incubated for 8-10 hours at 37ºC; then, 1 mL of this initial reactivation tube was transferred into a tube containing 9 mL of fresh MH broth and incubated at 37ºC overnight under aerobic conditions.

2.3.1.1. Diffusion method: preliminary screening assays

The initial antimicrobial screening was performed using the well diffusion assay. Müeller Hinton Agar (Biokar diagnostics, France) was used for all foodborne bacteria. Plates containing 20 mL of culture media were inoculated with a bacterial suspension (0.5 turbidity in the McFarland scale). Four-millimeter wells were punctured into the agar and filled with 40 µL of each tested extract solution. Sterile water was used as negative control (Adebolu and Oladimeji, 2005; Burdulis *et al*., 2009; Puupponen-Pimiä et al. 2001). After incubation (24 h at 37 ºC), confluent bacterial growth was observed surrounding the wells. Inhibition of the bacterial growth (clear zones around the wells) was measured in millimeters. All assays were performed in triplicate.

2.3.1.2. Microdilution method: determination of minimum inhibitory and minimum bactericidal concentrations

The minimum inhibitory concentration (MIC) (in mg of extract/mL) and minimum bactericidal concentration (MBC) (in mg of extract/mL) were determined for all extracts. The MIC values were considered to be the lowest concentration of extract required to inhibit bacterial growth (assessed by lack of turbidity) after 24h of incubation. The MBC values were considered to be the lowest concentration of extract where growth was prevented and, in addition, the initial viability was reduced by at least 99.9% after 24 h (Fernandes *et al.,* 2008; Tavaria *et al.,* 2013).

MIC and MBC values were determined using 96-well microplates (96 x 320 µL, Thermo Fisher Scientific, USA), one for each microorganism. These values were determined for all combinations of pathogenic bacteria with all antioxidant extracts.

Test solutions were prepared by dissolving the antioxidant extracts in MH broth at different concentrations; to facilitate the dissolution of extracts Bovine Serum Albumin at 2% (w/v) (BSA, Nzytech, USA) was used, afterwards the mixture was placed in an ultra-sonicator (Bandelin Sonorex, Bandelin Electronic GmbH & Co, Germany) for 3-10 minutes. Several concentrations of extracts were tested: 1%, 2% and 3% (v/v) solutions were prepared from a stock solution at 4% (w/v), to a final volume of 1 mL.

Each of the above test solutions were inoculated at 2% (v/v) with each tested inoculum of ca. 10^7-10^8 CFU (colony forming units)/mL, after overnight incubation at 37ºC. For example, 2% concentration of CC extract an eppendorf was prepared as follows: 500 µL stock solution extract (4% (w/v)), 480 µL MH broth (with 2% (w/v) BSA added) and 20 µL of one

of the inocula. After this, 300 µL of this mixture were put in three wells of the 96-well microplates, and incubated at 37ºC for 24h.

A positive control was performed to ensure bacterial viability using fresh MH broth supplemented with BSA at 2% w/v (the same preparation used to dissolve the antioxidant extracts). Two negative controls, sterile MH broth and stock solution of each extract without inoculum were put in the same plate, to ensure the absence of contamination during the manipulation process.

Bacterial growth reductions were analyzed by comparing viable cell counts between the positive control and treatments with extracts at 24h and at 0h. For this, aliquots of 100 µL obtained from each well of the microplate were diluted using peptone water through serial decimal dilutions and 20 µL of each dilution were plated on MH agar. Viable counts were determined using the drop count method described by Miles and Misra (1938). Plates were incubated at 37ºC for approximately 24h. All these assays were carried out in triplicate.

2.3.1.3. Time-kill analysis/ Survival curves

The antimicrobial activity of extracts was evaluated also by survival or inhibition curves. All extracts were tested on *E. coli* ATCC 25922 and the CC extract was chosen to be used with all other bacterial strains. To corroborate the previous results, the concentrations used to determinate MIC and MBC values were repeated, namely 1%, 2%, 3% (v/v) and 4% (w/v), in order to also understand how microorganisms react to the action of the extracts during the incubation period. Test solutions were prepared by dissolving the antioxidant extracts with MH broth at different concentrations; to facilitate the dissolution of the extracts, BSA at 2% (w/v) was used, and afterwards the mixture was placed in an ultra-sonicator for 3-10 minutes. After obtaining the inhibitory curve with these concentrations, a new range of concentrations was tested.

These different assays were prepared in eppendorf tubes at a final volume of 1mL with MH broth, inoculated at 2% (v/v) and incubated at 37ºC for 24h. A positive control (MH broth + 2% inocula) and two negative controls (stock solution of the extract and MH broth) were performed to ensure the viability of bacteria and the sterility of the solutions used, respectively. Each sample was analysed in duplicate.

Viable cell counts were determined on four sampling times: 3, 6, 12 and 24h. For that, at each time, 100 µL were taken and plated on MH agar, after decimal dilutions with peptone water, using the drop count method described above. Plates were incubated at 37ºC for

approximately 24h. The initial viable counts were determined with the positive control at 0h. All these assays were plated in quadruplicate.

Results were given by plotting the log CFU *versus* time. Whenever a result was below the quantification limit, the method's detection limit ($\log_{10} 500$) was assumed.

2.3.2. Effect of the antioxidant extracts upon growth of probiotic bacteria

Probiotic bacteria: source, maintenance and culture conditions

The effect of agro-industrial extracts on the growth of probiotic bacteria was evaluated using two bacterial strains: *Lactobacillus casei* (code: *L. casei* 01 - nu-trish® L. caseo-01 CHR-HANSEN) and *Lactobacillus acidophilus* (code: *L. acidophilus* 10- DELVO PRO LAFTI-L10 DSL produced by DSM Food Specialities), provided as pure-lyophilized cultures.

Stock cultures of both bacterial strains were maintained in cryovial tubes containing 500 µL of MRS broth (Biokar diagnostics, France) and 500 µL of glycerol 60% (v/v). Frozen stock cultures were activated by transferring the volume of cryovial tube into 9 mL of MRS broth and incubating for 6-8 hours at 37ºC: then, 1 mL of the initial reactivation tube was transferred to a tube containing 9 mL of fresh MRS growth broth medium.

2.3.2.1. Microdilution method: assessment of the effect by optical density measurement

Test solutions were prepared by dissolving the antioxidant extracts with MRS broth at different concentrations, namely, 0.025 and 0.1% (w/v) to facilitate the dissolution of extracts BSA at 2% (w/v) was used and, after the mixture, placed in an ultra-sonicator for 3-10 minutes. Three-hundred µL of this mixture were put on each well (in triplicate) on a 96-well microplate. Test solutions, prepared with MRS broth, were inoculated at 2% (v/v) with probiotic inocula (*ca.* 10^7-10^8 CFU /mL), following overnight incubation at 37ºC. For example, to 0.025 % (w/v) of CC extract was placed into eppendorf: 250 µL stock solution extract (0.1 % (w/v)), 730 µL MRS broth (with 2% (w/v) BSA) and 20 µL inoculum.

A positive control, of MRS broth and the probiotic culture, was performed to confirm the strain viability. Two negative controls, sterile MRS broth and stock solution of each extract were performed in the same microplate to ensure the absence of contamination of the dissolutions.

Bacterial growth was followed by optical density at 620nm, using a microplate reader (FLUOstar OPTIMA – BMG LabTech, Germany) that measured this value during 48h. All these assays were carried out in triplicate.

2.4. Agro-industrial extracts incorporation in food packaging: edible film

Films were prepared by dissolving the antioxidant extracts (containing Tween 80 (Sigma Aldrich, EUA), functioning as a dispersant) with deionised H_2O. .Afterwards, the mixture was ultra-sonicated for 3-10 minutes.

2.4.1. Film formulation

With the objective to find the better edible film several formulations were tested. In the next paragraphs we described the combinations that were made.

Using as reference the matrix described by Campos *et al.* (2013), initially, methylcellulose was tested and small changes (see Appendix II, **Table 6.1.**) were applied to observe how the concentrations and the compounds affected the different films, i.e., films would be modified according only to the visual aspect.

Another matrix (see Appendix II, **Table 6.2.**, F-SA1) was tested with sodium alginate (FMC BioPolymer, Norway) cross-linked to calcium chloride ($CaCl_{2:}$ VWR International, USA), which $CaCl_2$ was incorporated only to remove the film from the plate.

However, it was necessary readjust the method and so two different contacts with calcium chloride were performed, i.e., $CaCl_2$ was not incorporated only to cross-linking but also in alginato sodium mixture (see Appendix II, **Table 6.2.**). The final formulation was carried out as following (**Table 2.2.**): film preparation by casting in a two-stage contact with calcium ion: sodium alginate and calcium chloride were dissolved into distilled water. The solution was heated with stirring until it was completely uniform and all solids were dissolved. Then, the plasticizers, glycerol (Fisher Chemical, UK) and PEG 400 (Sigma Aldrich, EUA), were added in the same proportion. Afterwards, the oligosaccharides from glucuronoxylooligosaccharides from *Eucalyptus globules* wood, obtained as described by *Gullón et al.* (2011), were added to the above mixture. Finally, antioxidant extracts at 2% (w/v) were incorporated into the alginate film solution. The film-forming solution was cast on a Pirex-coated plate followed at controlled temperature in the lab during about 24 h. After drying, an undetermined volume of 2% $CaCl_2$ solution was poured onto the dried alginate film for some seconds and re-dried at room temperature until the film formed could be easily removed from the cast. The films were stored in a desiccator for two days before analysis (Norajit *et al.*, 2010). Each type of film was prepared in duplicate.

Table 2.2. Final film formulations tested.

Polymeric matrix	Plasticizer	Others compounds	Drying conditions	Cross-Linking	References
Sodium Alginate (1% (w/v)) + $CaCl_2$ (0.5% (w/v))	Glycerol + PEG 400 (1.5 g/ g alginate of each)	Tween 80 (0.8% (v/v)) + Oligosaccharide (1% (w/v))	24h, room temperature	$CaCl_2$ (2% (w/v))	Modified from Norajit *et al.* (2010)

In the preliminary assays a final volume of 15 mL was used, but when the first good visual films were obtained, the ideal final volume was determined. Five final volumes (10, 12, 13, 15 and 17 mL) were tested in glass plates with approximately 9.5 cm of diameter. The ideal volume was defined empirically as the one giving best malleability, a film with a desired thickness, neither too thin nor too thick.

2.4.2. Properties of alginate films: characterization

Extract content

To determine the extract content in each film, all discs were weighed (each film was prepared in duplicated). The extract concentration was calculated from the difference in weight between the films containing the extract and the control film with no extract (Campos *et al.*, 2013).

Moisture content

Film samples (0.2 g) were weighed into aluminum crucibles and dried at 105 ºC in an oven for 24 h. Moisture content was determined as a percentage of the initial film weight loss during drying and was reported on a wet basis (Norajit *et al.*, 2010). The analysis was performed by triplicate for each film.

Film thickness

Film thickness was measured using a micrometer (MI20, Adamel Lhomargy, Rossie en Brie, France). Six measurements were performed on different sections of the films; the average value was calculated for each film, with the corresponding standard deviation (Norajit *et al.*, 2010).

Water solubility

Film samples (2 x 2 cm) were first dried at 105ºC for 24 h to determine the initial dry matter (three portions of each film were used to carry out this analysis). A portion of the film was then immersed in 50 ml of distilled water and then placed in a shaker water bath at 25 and 80 ºC. After 24 h, the samples were filtered through Whatman No. 1 filter paper. The papers containing any unsolubilized film were dried at 105 ºC for 24 h. The water solubility (WS) of the film was calculated using the following equation (2.4.2.1.) by Norajit *et al.* (2010):

$$WS\ (\%) = [(W_o - W_f)/W_o] \times 100 \qquad (2.4.2.1.)$$

where Wo and Wf are initial and unsolubilized dry matter, respectively.

Film color

Film color was evaluated with a portable CR-400 Chroma Meter (Minolta, Osaka, Japan). The CIELab color scale was used to determine the lightness (L), redness (+a*)/greenness (-a*) and yellowness (+b*)/blueness (-b*) of the films. Film samples were measured on the surface of a white standard plate with color coordinates L=97.59, a= -0.07 and b=1.89 (Campos *et al.*, 2013). Film color was expressed as the total difference in color, ΔE, calculated with the formula (2.4.2.2.) by Campos *et al*. (2013):

$$\Delta E = [(L_{film} - L_{control})^2 + (a_{film} - a_{control})^2 + (b_{film} - bc_{control})^2]^{1/2} \qquad (2.4.2.2.)$$

For each film, two pieces were measured; from each piece of film, three readings were taken.

Antimicrobial activity testing: disc diffusion assay

Antibacterial activity testing of the edible films was carried out using the agar diffusion method according to Chen, Yeh, and Chiang (1996). The edible films were cut into 10 mm diameter discs and then placed on MH agar plates, which had been previously seeded with 0.1 mL of inoculum containing approximately 10^5–10^6 CFU/mL of tested bacteria. The plates were then incubated at 37 ºC for 24 h. Observations on the diameter of the inhibitory zone surrounding film discs and contact area of edible film with agar surface were made (Pranoto *et al.*, 2005). Each experiment was performed in duplicate and all films were tested.

2.5. Statistical analysis

The statistical differences were evaluated using GraphPad Prism 5.00 (California, USA). The normality of the distributions was evaluated through the Kolmogorov Smirnov's Test and the differences were evaluated using One-way ANOVA associated with Tukey's Multiple Comparison Test. Differences were considered significant when $p \leq 0.05$.

3. Results and Discussion

3.1. Chemical characterization

3.1.1. Antioxidant measurement assays

Several authors have reported antioxidant activity in compounds or similar compounds obtained from different lignocellulosic materials that were used to produce the tested extracts in this work (Cruz *et al.*, 2001; Mandalari *et al.*, 2010; Conde *et al.*, 2011a; Delgado Adámez *et al.*, 2012; Silván *et al.*, 2013).

Since antioxidants often act by mixed and cooperative mechanisms, the results from a single *in vitro* assay may be scarcely representative of the complex conditions that characterize many food systems. Due to the multiple components of extracts and to the variety of possible substrates, the separation of single products and their further individual assessment may be costly and inefficient, since the possible synergistic interactions among the various antioxidant compounds may be lost. On the other hand, when the antioxidant activity is measured by an individual assay, the corresponding results may reflect only the chemical reactivity under the specific conditions of the test. Alternatively, the conclusions drawn from different tests may be contradictory (for example, due to mixed effects involving many factors). This problem has been addressed assuming that the antioxidant capacity of a given concentrate may be better assessed on the basis of the results achieved in several antioxidant activity tests. In this way, the effects of factors such as the behavior of the biological substrate employed, the operational conditions or the concentration of active compounds can be better evaluated (Conde *et al.*, 2011a).

Based on the above information, **Table 3.1.** lists for the four tested extracts the data obtained by several antioxidant assays and their statistical analysis. Results show a great diversity in their antioxidant activity; we can observe that for both ET-based assays (DPPH and ABTS assays) the extracts with higher antioxidant capacity in descending order were: GP > EW > CC > AS. For the HAT-based assay i.e ORAC assay, no statistically significant differences were observed between all methanolic extracts. However, when performed in the presence of BSA this assay showed statistically significant differences (p<0.05) and overall higher antioxidant capacities were obtained when compared to the assays carried out without BSA; this value in descending order of activity was: CC = GP > EW > AS. These differences should be analyzed with due caution to the extent that these are different assays, so sometimes it is difficult to compare methods based on different mechanisms to measure the antioxidant

activity. Actually, ABTS and DPPH assays could easily provide comparable results (Litescu *et al.,* 2010) as is the case of the results obtained from these two methods, where no statistically significant differences were observed. However, antioxidant activity may be dependent on the oxidizing target and conditions used in the test system (Frankel and Meyer, 2000). So, the addition of BSA could be the reason for such dissimilar results between extracts in methanol and with BSA in the ORAC assay. A control with BSA was performed to ensure that this compound would not interfere with the method. Actually, the influence of proteins like BSA in the antioxidant activity of phenolic compounds is not well understood; it is known that interactions may occur mainly at low pH (pH≈5) (Frankel and Meyer, 2000; Ferrer-Gallego *et al.,* 2012). Through these interactions, some studies have shown that this protein can in some cases enhance the antioxidant capacities (Frankel and Meyer, 2000; Bonoli-Carbognin *et al.,* 2008) Conde *et al.,* 2011b). However, no statistically significant differences were observed between both groups, so even if there are interactions, these do not significantly affect the antioxidant capacity.

Table 3.1. Antioxidant capacities of the tested extracts by the different assays. Averages with the same superscript letter in the same column indicate no significant difference by the Tukey's Multiple Comparison test ($p<0.05$).

Sample	ABTS (g/L ascorbic acid equivalent)	DPPH (g/L ascorbic acid equivalent)	ORAC (µM trolox equivalent)	
			Methanol	BSA
Corn Cobs	1.12 ± 0.01^{a}	0.63 ± 0.02^{a}	43.04 ± 4.04^{a}	51.17 ± 9.41^{a}
Eucalypt Wood	1.44 ± 0.04^{b}	0.73 ± 0.01^{b}	41.38 ± 6.64^{a}	29.02 ± 6.54^{b}
Grape Pomace	1.77 ± 0.04^{c}	1.09 ± 0.01^{c}	48.72 ± 5.55^{a}	34.49 ± 5.05^{a}
Almond Shells	0.97 ± 0.03^{d}	0.55 ± 0.01^{d}	40.23 ± 6.66^{a}	31.06 ± 4.86^{c}

3.1.2. Identification of phenolic compounds by HPLC analysis

Table 3.2. shows the compounds identified by HPLC qualitative analysis. Compounds identified by HPLC have been classified in four groups: phenolic acids, aldehydes, flavonoids (all of them of phenolic nature), and sugar derived compounds (with non-phenolic nature). This is in agreement with the information reported by Conde *et al.* (2011a).

Table 3.2. Compounds identified in the tested extracts by HPLC analysis (+: presence; -: absence).

Group	CC	EW	AS	GP
Phenolic acids				
Gallic acid	-	+	+	+
Protocatechuic acid	-	-	-	+
4-hydroxybenzoic acid	+	-	-	+
Vanillic acid	+	+	+	+
Syringic acid	-	+	+	+
***p*-coumaric acid**	+	-	-	-
Ferulic acid	+	-	-	-
Ellagic acid	-	+	-	+
Aldehydes				
3,4-Dihydrixybenzaldehyde	+	+	+	+
4-hydroxybenzaldehyde	+	-	+	-
Vanillin	+	+	+	+
Flavonoids				
Quercetin	+	+	+	+
Sugar derived compounds				
Hydroxymethylfurfural	+	+	+	+
2-Furfuraldehyde	+	+	+	+

Through hydrothermolysis of lignocellulosic materials, lignin depolymerization leads to the solubilization of polymeric and oligomeric fractions made up of guaiacyl, syringyl and p-hydroxyphenyl units. HPLC qualitative analysis of antioxidant extracts confirmed the presence of gallic, protocatechuic, 4-hydroxybenzoic, vanillic, syringic, *p*-coumaric, ferulic and ellagic acids; 3,4-dihydroxybenzaldehyde, 4-hydroxybenzaldehyde and vanillin were the identified aldehydes, and quercetin was the identified flavonoid. In reported studies dealing with the autohydrolysis or steam explosion of LCMs (Conde *et al.*, 2009) lignin-derived products such as p-hydroxybenzoic acid, other hydroxyphenyl acids (including ferulic, vanillic, syringic and coumaric acids) and aldehydes (such as syringaldehyde, p-

hydroxybenzaldehyde and vanillin) have been identified in the ethyl acetate soluble fraction of liquors.

During hydrothermal treatment of LCMs, hemicelluloses are partially hydrolyzed and decomposed. Compounds generated from sugar degradation were found in the autohydrolysis medium, including furfural and hydroxymethylfurfural (coming from dehydration of pentoses and hexoses, respectively). These compounds were present in all the extracts used in this work. Additionally, other compounds common to composition of all studied extracts were: vanillic acid, 3,4-dihydroxybenzaldehyde, vanillin, quercetin, and 2-furfuraldehyde. Gallic acid and syringic acid were also identified in all studied extracts, except for the CC extract. Futhermore, ellagic acid was identified only in EW and GP extracts; *p*-coumaric and ferulic acid were also only identified in the CC extract.

In fact, these results are in agreement with those reported by other authors. Moure *et al.* (2007) reported the presence of syringic and vannilic acids in almond shells. Ruberto *et al.* (2007) also reported quercetin in grape pomace. Garrote *et al.* (2007) reported the presence of vanillin in eucalypt wood liquors; this compound was also identified in corn cobs liquors in addition to hydroxymethylfurfural and 3,4-dihydroxybenzaldeyde. Others studies that used the same sources have also reported some of the compounds identified in these extracts. For example, quercetin and gallic acid are commonly identified in grape matrices (Iacopini *et al.*, 2008; Tabasco *et al.*, 2011; Silván *et al.*, 2013) and vanillin and syringic acid are usually associated to eucalypt matrices (Amakura *et al.*, 2002; Moure *et al.*, 2007).

These small differences may be the basis of possible differences in the remaining assays. According to the above compositional data, the fractions studied in this work are complex mixtures of various phenolic and non phenolic compounds and their activity may be the result of synergistic effects between these compounds.

3.2. Microbiological assays

The antimicrobial properties of several antioxidants have been proposed either to develop new food preservatives, due to the increasing consumer pressure on the food industry to avoid synthetic preservatives, or to develop innovative therapies for the treatment of various microbial infections, considering the increase in microbial resistance against conventional antibiotic therapies (Daglia, 2012).

In this work, we evaluated the antimicrobial properties of antioxidant extracts from LCMs, which are rich in phenolic acids, aldehydes, flavonoids and sugar derived compounds (Conde *et al.,* 2011a).

3.2.1. Diffusion method: preliminary screening assays

Several methodologies can be used to determine the antimicrobial activity of antioxidants from several sources. One of the most common approaches used to screen for this property are the agar diffusion methods.

So, an agar diffusion assay was employed in this study in order to screen which extracts exhibited some effect upon target microorganisms and select the best ones to be used in the subsequent antimicrobial assays (Clark *et al.,* 1990; Nascimento *et al.,* 2000; Burdulis *et al.,* 2009).

This method was chose because despite being a qualitative assay, it gives an idea of the presence or absence of substances with antimicrobial activity and normally, they are used when large numbers of samples and/or large numbers of bacterial strains are screened (Puupponen-Pimiä *et al.,* 2001; Burt, 2004).

However, although this method is indicated to evaluate the antimicrobial activity of antioxidants, no antimicrobial activity was observed with the antioxidant extracts tested here. Although of practical nature this method may be hampered by numerous factors that influence the diffusion process, such as size and shape of particles (Valgas *et al.,* 2007), which may have been responsible for the non-detection observed. In addition, non-polar samples may face difficulties to diffuse in the media and cationic antioxidant extracts may adsorb to the surface of the plate and not diffuse into the medium. In fact, when antioxidant extracts were tentatively hydrated with water, it was observed that they were not soluble. Consequently, these extracts may display a good antibacterial activity, but which is not noticeable by this method (Griffin *et al.,* 2000; Valgas *et al.,* 2007).

Optimization of the dissolution method of the antioxidant extracts

The first difficulty found when work with the extracts began was their inadequate water solubility which could not allow to ensure that all potentially active compounds were available in the medium.

Based in bibliographic references, the extract concentrates were subsequently resuspended in bovine serum albumin (BSA) and a good dissolution of extracts was observed (this fact was confirmed by HPLC analyses as described above); Bonoli-Carbognin *et al.* (2008) have reported that BSA enhances the antioxidant effect of water-soluble phenolic compounds in oil-in-water emulsions. Almajano *et al.* (2008), concluded that BSA exerts its synergistic effect with antioxidants because of the formation of a protein-antioxidant adduct in model food emulsions that depends on the antioxidant structure. In fact, Conde *et al.* (2011b) reported the use of this protein-like emulsifier in crude extracts obtained from the same sources. Therefore, if one of the applications of these extracts is their incorporation into food matrices, it is to expect that the use of this protein would not have negative effects upon their antioxidant activity neither on their antimicrobial potential.

Furthermore, the addition of BSA to MH medium did not inhibit or enhance the growth of the tested bacteria in this work in comparison with the MH without BSA, as was reflected by the plate counts throughout time. This behavior is in agreement with the information reported by Tian *et al.* (2010) who observed no inhibitory effects on the growth of the pathogenic bacterium *E. coli* O157:H7 using BSA as protein control at 2% (w/v). Additionally, certain authors used BSA as a negative protein control, such as reported by Ochiai *et al.* (2013) when studying the inhibitory effect of α-amylase against periodontal pathogenic bacterium *Porphyromonas gingivalis*; that the authors compared the growth of the bacterium in the presence of α-amylase and other protein (BSA) and concluded that no inhibitory effect was observed in the presence of BSA.

In fact, in this work the time curve presented for the positive control (with culture medium) and control with BSA didn't show significant differences. Therefore, based on the above information, the inhibitory effect observed in the antimicrobial activity experiments was attributed to action of compounds present in the antioxidant extracts.

3.2.2. Microdilution method: determination of minimum inhibitory and minimum bactericidal concentrations

In view of the negative results obtained with the agar well diffusion method, the microdilution method was also tested, and considered as a quantitative assay allowing the determination of the MICs and MCBs by mixing the bacteria directly with the antioxidant extracts. However, when the extract solutions were prepared it was observed that they were quite turbid, strongly colored and dark. This fact made it impossible to observe visually the presence or absence of growing bacteria. Furthermore, the microdilution method is based on

the estimation of the MIC through a sharp decline in the absorbance value. The MBC, on the other hand, is determined by subculturing the dilutions that would have shown no evidence of growth in the MIC determination assay (Ncube *et al.*, 2008). For this reason, it was not feasible to determine MICs values, but it was only possible to find MBCs values (see **Table 3.3.**).

Table 3.3. Minimum bactericidal concentration (MBCs) for each pathogenic bacteria tested with the different extracts.

	MBC % (w/v)			
	CC	**EW**	**GP**	**AS**
***Escherichia coli* ATCC 25922**	4	3	3	4
***Salmonella* spp. 463**	3	3	3	2
Pseudomonas aeruginosa	3	2	3	4
***Listeria monocytogenes* 3375**	2	2	2	3
***Staphylococcus aureus* 18 N**	4	2	3	3

Table 3.3. shows the MBCs for all the tested strains against the different extracts; so, it is apparent that *E. coli* ATCC 25922, was one of the most resistant strains, requiring higher concentrations of CC and AS extracts for a bactericidal effect (4% (w/v)). Oppositely, *Listeria monocytogenes* 3375 was the more susceptible bacterium needing the lowest concentrations for all tested extracts, namely 2% (w/v) for CC, EW and GP extracts.

We observed that the same extract showed the same MBC values for the different strains; so, a 4% (w/v) CC extract was required to kill *E.coli* ATCC 25922 and *S. aureus* 18N. Therefore, these two strains (Gram negative and Gram positive, respectively) were the most resistant against this extract. In the case of EW extract, the most susceptible strains were *Pseudomonas aeruginosa*, *L. monocytogenes* 3375 and *S. aureus* 18N only requiring 2% (w/v) extract to be bactericidal. The GP extract was shown to be the most effective against *L. monocytogenes* 3375 (MBC 2% (w/v)) and the AS was the most active against *Salmonella* spp. 463 (MBC 2% (w/v)).

All extracts at the tested concentrations showed antimicrobial activity against all pathogenic bacteria evaluated. These results were in agreement with those obtained by several authors that have reported antimicrobial activities against pathogenic bacteria using antioxidants from LCMs; for example, Cruz *et al.* (2001) reported that the ethyl acetate extracts obtained from acid hydrolyzates of *Eucalyptus globulus* wood were most active for inhibiting bacteria (*Streptococcus bovis* CECT 213, *Escherichia coli* CECT 434,

Staphylococcus aureus CECT 59, *Salmonella* spp. and *Enterococcus faecalis* (isolated from food and from a clinical uroculture, respectively) than the extracts obtained from corn cobs. These authors further stated than the higher inhibitory action on microbial growth corresponded to ethyl acetate hydrolyzates from *Eucalyptus* wood with MIC values ranging from 0.01-0.5% (w/v) in comparison with 0.1-1% (w/v) for ethyl acetate hydrolyzates from corn cobs. The maximum inhibitory activity was observed for *Eucalyptus* wood hydrolyzates against *Staphylococcus aureus*, which was the most sensitive microorganism towards the assayed compounds.

Mandalari *et al.* (2010) have studied the antimicrobial activity of flavonoid-rich fractions from natural almond skin and blanched almond skin, two by-products from the almond industry. They observed that these extracts were active against the Gram-positive foodborne pathogens such as *S. aureus* and *L. monocytogenes*, but were not active against any of the tested Gram-negative bacteria (with the exception of natural almond skin showing activity against enteric *Salmonella*). This difference in the antibacterial potential could be explained by the amount of flavonoids present in the natural almond skin and the activity showed by blanched almond skin may result from the interaction of different polyphenols retained by this by-product.

Delgado Adámez *et al.* (2012) assessed the effects of the grape seed extract juice (GSEJ) and grape seed extract wine (GSEW) on the bacteria implicated in foodborne illnesses. These GSEJ and GSEW extracts exhibited antibacterial action against all bacteria tested, being more effective against Gram-positive bacteria than Gram-negative bacteria. These authors observed that no inhibition was produced in control samples (0 % of inhibition), while for all seed extracts the inhibition was nearly 100 % for 100 and 50 µL/mL seed extracts dilutions. The extent of the inhibitory effects of the extracts could be attributed to their phenolic composition revealed by their total phenol content.

Silván *et al.* (2013), reported the antibacterial activity of grape seed extract (GSE) against different *Campylobacter* spp. strains proving the strong capacity of GSE to inhibit them and showed that phenolic acids, catechins and proanthocyanidins were the main compounds responsible for the observed behavior.

From the obtained results it is not possible to establish a clear mechanism to explain the action of the extracts tested in this work on the tested bacteria. However, based on the existing literature we can hypothesize upon their mechanisms of action. Taking into account the differences between Gram-negative and Gram-positive bacteria, it is possible that the compounds present in the extracts may be causing damage to the cytoplasmic membrane (Campos *et al.,* 2009; Nohynek *et al.,* 2009; Lacombe *et al.,* 2012; Oh *et al.,* 2013), which justifies the fact that the greatest resistance is observed for Gram-negative bacteria. In fact, Gram-positive bacteria are the most susceptible to the action of these extracts but apparently the tested extracts were able to act on both Gram-negative and Gram-positive bacteria, i.e., these antioxidants do not seem to be selective for a particular Gram type bacteria. Our extracts are composed by various phenolic compounds, such as phenolic acids, aldehydes, flavonoids and sugar derived compounds (see **Table 3.2**. in Section 3.1.) In fact, Hyldgaard *et al.* (2012) and Lou *et al.* (2012) reported various antimicrobial mechanisms by phenolic compounds besides membrane damage such as changes in membrane fatty acid composition, possible interactions with intracellular compounds, leakage of H^+ and K^+ ions and ATP, inhibition of enzymes involved in ATP synthesis, binding to DNA to inhibit cellular functions among others. These mechanisms were reported simultaneously for various and for the same compound; additionally, Hyldgaard *et al.* (2012) and also Puuponen-Pimia *et al.* (2001) reported that the inherent activity of compounds may not rely exclusively on the ratio in which the main active constituents are present but also interactions between these and minor constituents that can exert synergistic antimicrobial activities. Therefore, based on the complexity of the compounds found and in the presented results, it is possible that other or simultaneous antimicrobial mechanisms of action might be involved.

The EW extract was the one that showed the greatest antimicrobial activity. On the other hand, the less effective extracts (as antimicrobials) were CC and AS extracts. Although the relationship between antimicrobial and antioxidant activity is not well defined, several publications have reported compounds with antioxidant activity that exert antimicrobial effects and have also tried to correlate them (Delgado Adámez *et al.,* 2012; Gutiérrez-Larraínzar *et al.,* 2012; Shami *et al.,* 2013). Unfortunately, for the results here presented and discussed, it was not possible to correlate both activities because there was a lack of evidence with respect to this. As well as the antioxidant potential of these natural extracts may be due

to the presence of different active phenolic compounds, aldehydes, flavonoids and sugar derived compounds or to the synergistic effects among them, the same is probably happening with respect to the antimicrobial activity (Puuponen-Pimia *et al.,* 2001; Hyldgaard *et al.,* 2012). For this reason, it is very difficult to establish the mechanism or mechanisms that are involved in bacterial damage.

Despite the impossibility to determine the MIC values, in some cases it was possible to observe that the bacterial growth decreased slightly or a bacteriostatic effect occurred comparatively to the positive control, being necessary to take this into account.

Thus, for *E. coli* ATCC 25992, 2% (w/v) of CC and AS extracts and 1% of the EW extract have shown bacteriostatic effect. CC and AS extracts at 3% (w/v) showed reductions of 4-5 logarithmic cycles and EW extract at 2% (w/v) showed reductions of 3 cycles. For GP extract even though it had no bacteriostatic effect at any concentration, 4 log cycle reductions were observed at 2% (w/v).

For *Salmonella* 463 it was not possible to observe concentrations with bacteriostatic effect. In fact, there was only a slight reduction of 2 logarithmic cycles with the CC extract at 2% (w/v).

The GP extract showed bacteriostatic effect at 2% (w/v) for *Pseudomonas aeruginosa.* In addition, reductions of 2and 3 log cycles with CC and AS extracts, respectively, at 2% and 3% (w/v), were observed.

A bacteriostatic effect was observed at 1% (w/v) for CC, AS and GP extracts against *Listeria monocytogenes* 3375. At the same concentration, a slight reduction of 2 log cycles was observed for the EW extract. For *S. aureus* 18 N it was observed a bacteriostatic effect with the EW extract at 1% (w/v). In addition, the AS extract at 2% (w/v) was able to reduce bacterial counts by 3 logs.

Several authors reported on the antimicrobial effect of plant extracts with action upon both Gram-positive and Gram-negative bacteria (Estevinho *et al.,* 2008; Mandalari *et al.,* 2010; Shami *et al.,* 2013)). Nevertheless, it is consensual that Gram-positive bacteria are, in general, more susceptible than Gram-negative counterparts to plant-originated antimicrobials (Nohynek *et al.,* 2006; Burdulis *et al.,* 2009; Oh et al., 2013; Senthilkumar and Venkatesalu, 2013) due to their lipopolysaccharide outer membrane that restricts diffusion of hydrophobic compounds. The antimicrobial activity is, generally ascribed to the high phenolic content (Piccirillo *et al.,* 2013; Senthilkumar and Venkatesalu, 2013), which cause disruption of the

cellular membrane, inhibition of ATPase activity or leakage of cell constituents. However, some authors (Puupponen-Pimia *et al.*, 2001) reported different results when studying eight extracts from Finnish berries; these inhibited the growth of Gram-negative bacteria but not of Gram-positive ones. These contrasting results suggest that there are possible synergistic effects taking place between the major and minor components of these extracts, so this should be further exploited, before the antimicrobial effect can be delegated to a specific set of compounds.

3.2.3. Time-kill analysis/Survival curves

With the aim of obtaining some information about the effect of the antioxidant extracts upon the inhibited bacteria, time-kill analysis was performed. Taking into account the deep dark color and turbidity of the extracts, it was not possible to evaluate their antimicrobial properties using a spectrophotometric method, so the agar dilution method was used instead, as described above (see Section 2), to draw the inactivation curves.

The obtained results are in agreement with the results presented above (**Table 3.3.),** i.e., the concentrations that exhibited reductions in bacterial growth or that showed bactericidal activity also showed the same effect in this experiment. The growth reductions observed above, were also observed here. Some values of CFU obtained for determination of MBCs are below the detection limit of the method, so the new range of concentrations was performed taking into account the MBCs values and the standard deviation.

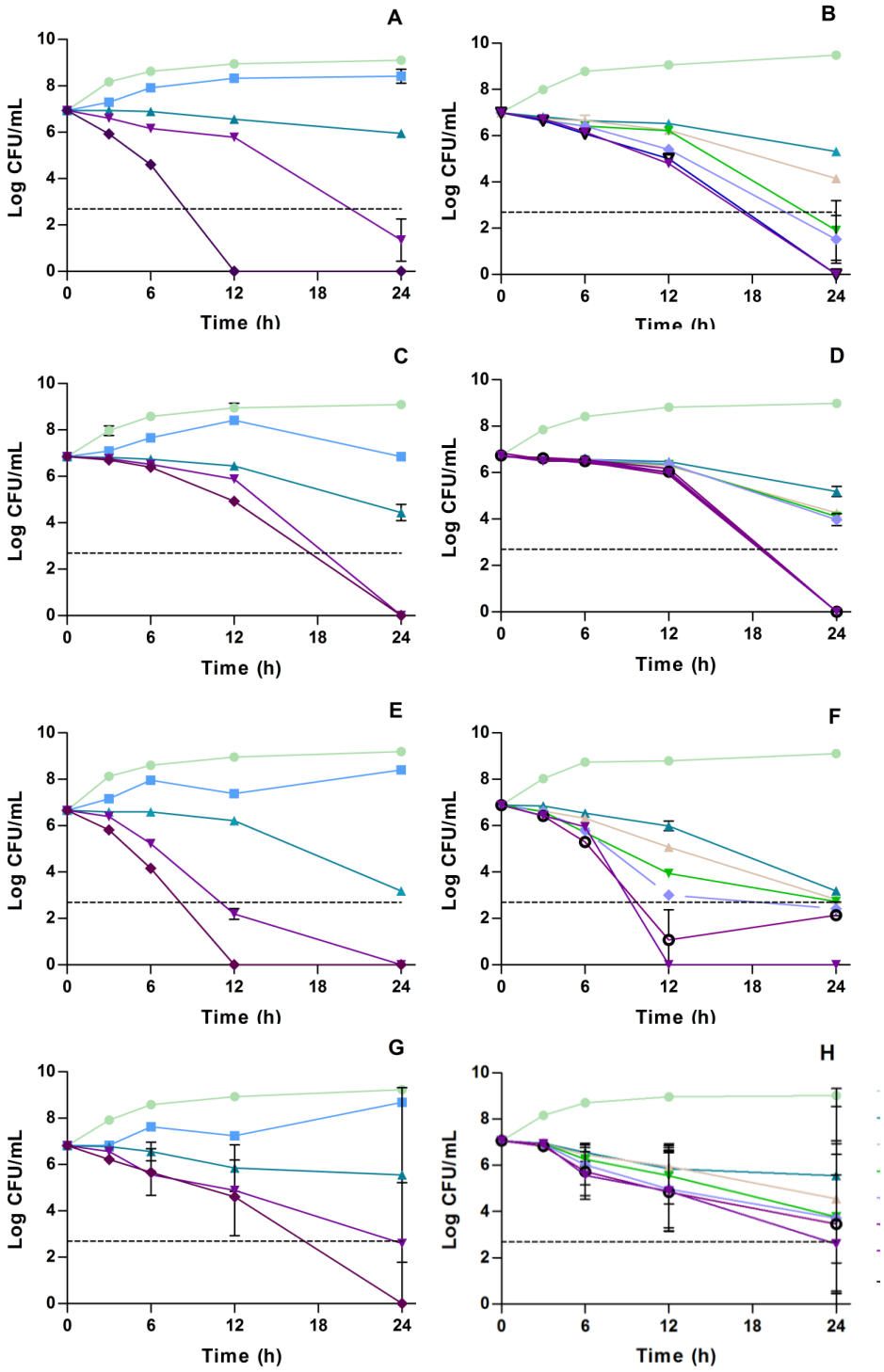

Figure 3.1. Effect of the CC (A, B), EW (C, D), GP (E, F) and AS (G, H) extracts against *E. coli* ATCC 25922. Each point represents the average value of four determinations; vertical bars represent standard deviation for each set of determinations. Legend: Positive Control; 1% (w/v): 2% (w/v); 2.2% (w/v); 2.4% (w/v); 2.6% (w/v); 2.8% (w/v); 3% (w/v); 4% (w/v); ----- Below method's detection limit.

Therefore, for *E. coli* ATCC 25922 a very significant change in MBC could be seen, which decreased from 4% to 2.8% (w/v) (**Figure 3.1.** B); this alteration could reflect an experimental error. With the same logic and also with the same bacterium, for AS extract (**Figure 3.1.** G-H), the new range of concentrations was not established only with the information of the MBC values, but also in accordance with what was observed with the other extracts; they all seemed to change significantly between the concentration 2% and 3%. At the end, and based on the obtained results, we concluded that indeed this range did not allow us to observe any bacteriostatic effect, and we should have tested concentrations between 3% and 4%.

Generally, the bactericidal effect was observed approximately after 24h (see **Figure 3.1.** B, C, D, G and H) of contact with the antioxidant extract, this can be explained hypothetically because the effect of compounds present in the extracts is not instant and takes time for the damage in the cells to become bactericidal. Actually, approximately 24h os mentioned, because the sampling before was at 12h, so in fact the bactericidal effect could be observed between 12-24h. However, some exceptions are evidenced; for the CC extract (**Figure 3.1.** A), the bactericidal effects are observed approximately after 6h at 4% (w/v) and approximately after 12h at 3% (w/v) for *Salmonella* spp. 463 (**Figure 3.2.** D) . In addition, the same effect was observed approximately after 12h at 4% (v/w) for *E. coli* ATCC 25922 (**Figure 3.1.** A), *P. aeruginosa* and *L. monocytogenes* 3375 (**Figure 3.2.** A and B). GP extract at 4% (w/v) (**Figure 3.1.** E) also showed bactericidal effect approximately after 12h for *E. coli* ATCC 25922.

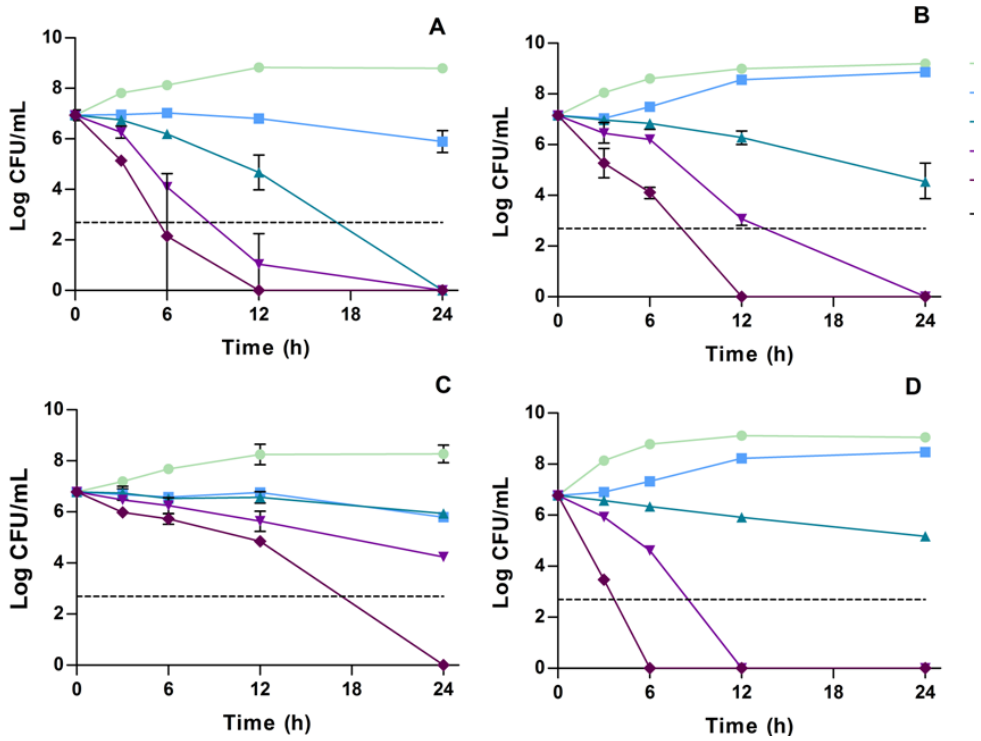

Figure 3.2. Effect of the CC extract against pathogenic bacteria: *L. monocytogenes* 3375 (A), *P. aeruginosa* (B), *S. aureus* 18 N (C) and *Salmonella* spp. 463 (D). Each point represents the average value of four determinations; vertical bars represent standard deviation for each set of determinations. Legend: Positive Control; 1% (w/v): 2% (w/v); 3% (w/v); 4% (w/v); Below method's detection limit.

The CC extract was the only extract tested that was performed for all four pathogenic microorganisms (**Figure 3.2.**), so some conclusions could be drawn. For this extract, the most susceptible bacterium was *L. monocytogenes* 3375 for which MBC was 2% (w/v), but the extract acted more rapidly as an antimicrobial with *Salmonella* spp. 463; however, this rapid effect could be seen also in *L. monocytogenes* 3375, taking into account the large range of standard deviation. In addition, a certain resistance to the antimicrobial action of the extracts by *S. aureus* 18 N could be seen. These observations suggest that the action of the extracts was not only upon the outer membrane, but rather due to some other mechanism. This resistance of Gram-positive bacteria was also reported earlier (Schieber *et al.*, 2001; Puupponen-Pimiä *et al.*, 2001; Burt, 2004; Chanda *et al.*, 2010; Delgado Adámez *et al., 2012).*

However, it is important to note that some values were below the methods' detection limit and others exhibit high standard deviation values, so certain conclusions should be drawn with care.

In summary, differences in action of the various extracts against various bacteria are evident; and it seems to be bacteria/extract dependent. Therefore, differences in the chemical profile of extracts should, in fact, affect the resistance or susceptibility of bacteria, but the bacterial morphology also plays an important role.

3.2.4. Growth enhancement of probiotic bacteria

Antioxidant compounds with biological effects are susceptible to be metabolized by intestinal bacteria during the gastrointestinal passage, prior to being absorbed. The metabolic activity of the colonic microbiota on bioactive food components can modify the host's exposure to these components affecting their bioavailability and potential bioactivity. However, research on the possible stimulatory role of antioxidant compounds on beneficial intestinal bacteria growth is poorly studied (Vasile *et al.*, 2011).

Furthermore, few studies refer to the effect of antioxidants from natural sources on probiotic bacteria. Accordingly, the knowledge of the interaction between a particular microorganism and extracts from natural sources containing antioxidant compounds is indispensable for appropriate utilization of those extracts. The doubts appear especially in the situation when food containing probiotic bacteria is supplemented with plant raw material rich in antioxidants.

Lactic acid bacteria (LAB) are frequently encountered in the fermentation of plant materials where phenolic compounds are abundant. However, nowadays most of the metabolism of phenolic compounds remains unknown; several authors have reported phenolic compounds that enhance the growth of these bacteria, such as Rodríguez *et al.* (2009) that reported simultaneously the growth of *Lactobacillus hilgardii* in presence of gallic acid and catechins and on the other hand the degradation of these compounds by this bacterium. So, the concentrations that were tested in the assays with probiotic bacteria were lower than those tested for pathogenic bacteria (0.025 and 0.1 % (w/v)) because in fact certain phenolic compounds can enhance the growth of probiotic bacteria but, even though these bacteria seem to be more resistant to phenolic acid than pathogenic bacteria, the extracts are a mixture of phenolic compounds and so antimicrobial activity could be observed. Besides, based on the observed effects, an increase in the extract concentrations could involve a strong inhibition of probiotic bacteria. Small concentrations implied that color and turbidity of extracts were clearer in comparison with the concentrations evaluated for the pathogenic bacteria; so, this assay was performed using a spectrophotometric method.

Taking into account the ideas exposed above, the aim of this task was to evaluate the growth ability and/or the survival capacity of *Lactobacillus acidophilus* 10 and *Lactobacillus casei* 01 in presence of the antioxidant extracts obtained from agro-industrial wastes (Conde *et al*., 2011a).

From **Figure 3.3.** and **Figure 3.4.**, it is possible to observe that the addition of 2% (w/v) BSA to culture medium positively affected the growth of *L. acidophilus* 10 (**Figure 3.3.**), but this effect was not observed for *L. casei* 01 (**Figure 3.4.**). In fact, Farnworth *et al.* (2007) reported on some proteins which enhance the growth of some probiotic bacteria whereas Tian *et al*. (2010) have reported that BSA did not affect the growth of these bacteria.

Even if these antioxidant extracts were negatively affecting the growth of pathogenic bacteria, i.e. triggering antimicrobial mechanisms, the contradictory effect was observed for probiotic bacteria. Actually, some studies report on antimicrobial substances able to enhance the growth of probiotic bacteria; Sánchez-Maldonado *et al.* (2011) reported that *Lactobacillus* spp. are more tolerant to phenolic acids in comparison with *E. coli* and *Bacillus subtilis*. However, in this work, no substantial differences were evidenced upon growth of probiotic bacteria in the presence of the studied antioxidant extracts.

The growth of *L. acidophilus* 10 was not affected by the CC extract (**Figure 3.3. A**). In fact, the same effect (**Figure 3.4. A**) was observed on *L. casei* 01. So, the increase of growth observed at 0.25 and 1 mg/mL of CC extract in the case of *L. acidophilus* 10, when compared with the positive control supplemented with BSA, could be justified by the presence of BSA in the medium, which was used to resuspend the extract.

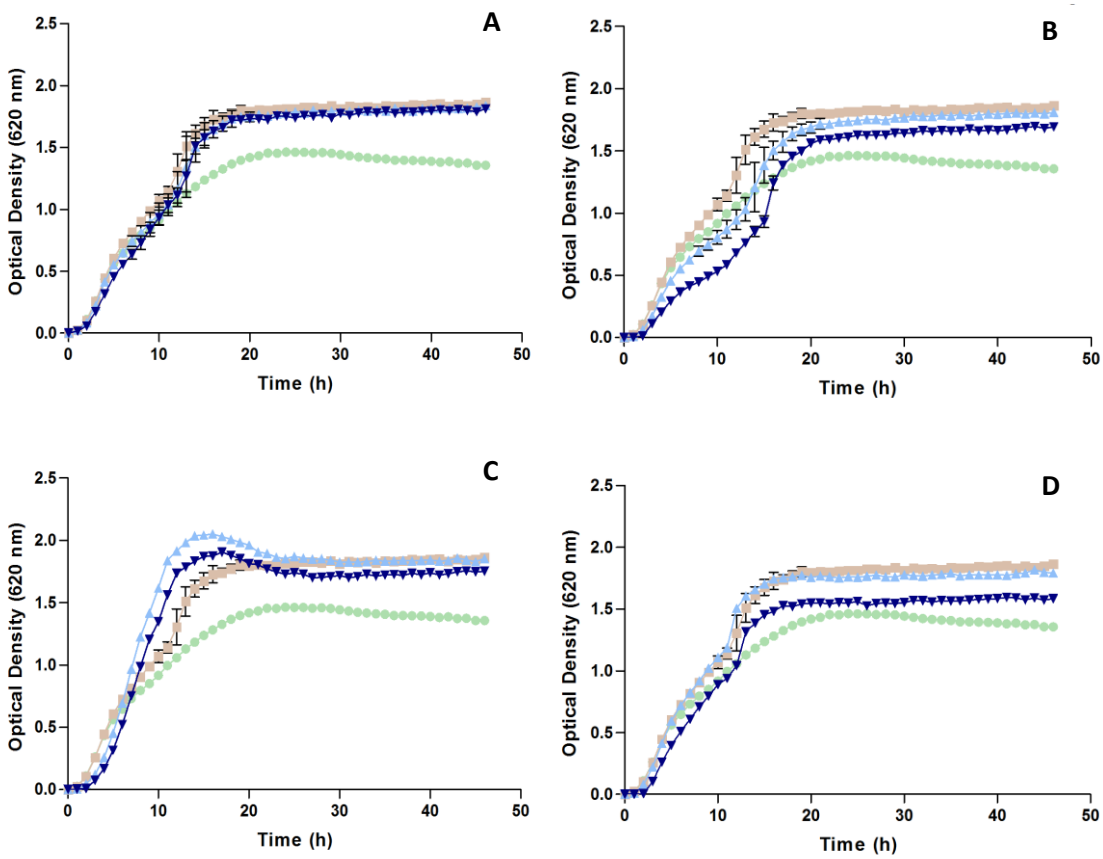

Figure 3.3. Effect of the CC (A), EW (B), GP (C) and AS (D) extracts upon growth of *L. acidophilus* L10. Each point represents the average value of three determinations. Vertical bars represent standard deviation for each set of determinations. Legend: Positive Control (MRS); BSA Control (MRS+BSA (2% (w/v)); 0.025 % (w/v); 0.1 % (w/v).

In the case of the AS extract (**Figure 3.3.** D), a slight inhibition at 0.1% (w/v) was observed for *L. acidophilus* 10 comparatively with its curve in BSA control and AS at 0.025% (w/v); although it showed similar log phases the amount of cells (namely optical density) at 48h was lower than the BSA control . In fact, the same effect was observed for *L. casei* 01 when grown on AS extract at 0.1% (w/v) (**Figure 3.4.** D) and BSA did not affect the growth of this bacterium. So, these inhibitions may reflect that higher concentrations of the AS extract may induce the opposite desired effect and therefore are able to completely inhibit the growth of probiotic bacteria.

Higher concentrations of the EW extract can also eventually inhibit or delay the growth of these two probiotic bacteria, although this decrease in growth was less evident for *L. casei* 01 (**Figure 3.3.** B and **Figure 3.4.** B). This slight inhibition may be due to this extract present a higher amount of sugar derived compounds reported by Conde *et al.* (2011a) (hydroxymethylfurfural and 2-furfuraldehyde), than the other tested extracts. Actually, this inhibitory effect of the EW extract, more evident for *L. acidophilus* 10, affected notoriously the log phase of this bacterium by both concentrations (0.025 and 0.1 % (w/v)), becoming longer thus implying a less exponential log phase, so the growth rate was slightly more slow; however it is important to take into account the closer amount of cells at 48h and the standard deviations observed at log phases.

The growth of *L. casei* 01 was not affected by the GP extract (**Figure 3.4.** C), but the same effect was not verified for *L. acidophilus* 10 (**Figure 3.3.** C). The log phase of the latter was positively affected, i.e. this phase was shorter and sharper than the control with BSA (MRS+BSA). So, the growth of *L. acidophilus* 10 was favored because it reached a higher cell concentration (higher OD) in less time in comparison with its growth in the control media (MRS+BSA), i.e., the final cell biomass is still the same at 48h but the growth ratio at log phases increased. Taking into account the above observations, it is possible to conclude that higher concentrations of the GP extract may eventually enhance the growth of this probiotic bacterium.

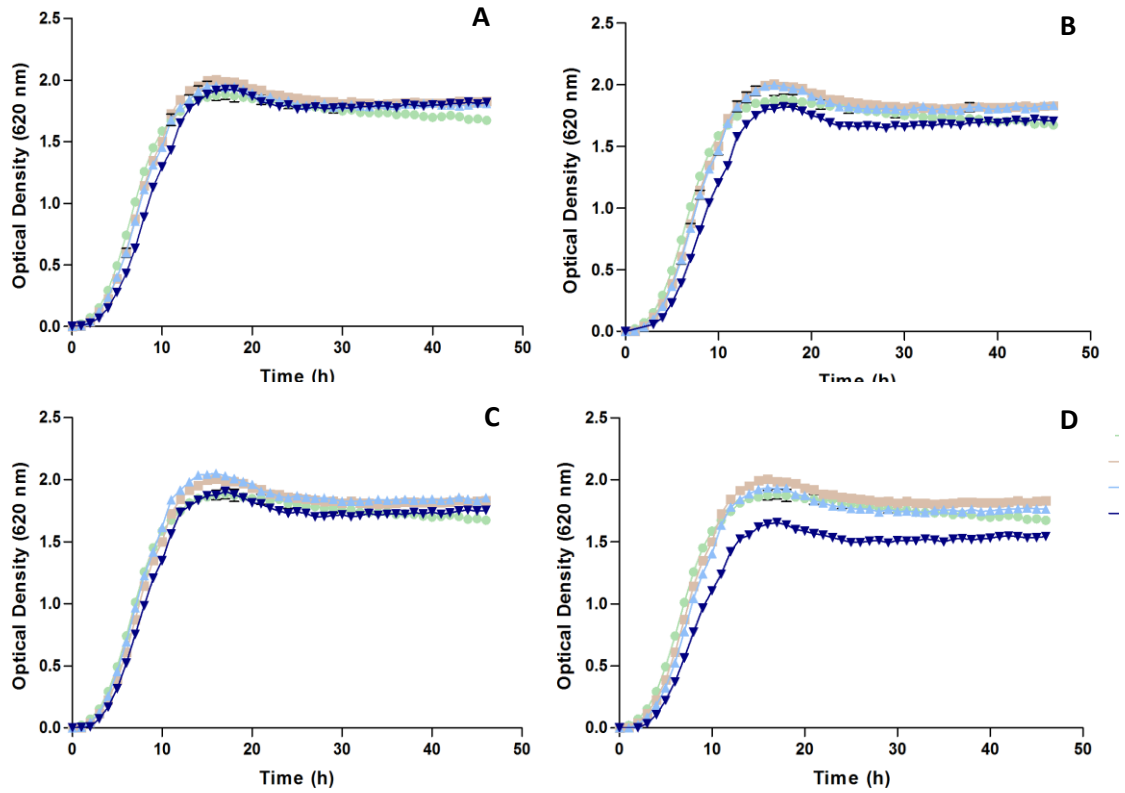

Figure 3.4. Effect of the CC (A), EW (B), GP (C) and AS (D) extracts upon growth of *L. casei* L01. Each point represents the average value of three determinations. Vertical bars represent standard deviation for each set of determinations. Legend: Positive Control (MRS); BSA Control (MRS+BSA (2% (w/v)); 0.025 % (w/v); 0.1 % (w/v).

The differences observed for each probiotic strain with respect to the several antioxidant extracts may be due both to the existing compounds and to the amount thereof. In fact, the GP extract contains fewer amounts of sugar-derived compounds that are generally inhibitors of bacterial growth (Conde *et al.*, 2011a).

3.3. Application as an edible film

Based on the results obtained and taking into account that the extracts have both antimicrobial and antioxidant capacities, an application was sought. Food industries seem to be a good alternative, mainly if talking about food packaging, because in this way, it is possible to take advantage of both extract properties and at the same time carry out different applications, depending on whether the edible film has antimicrobial or antioxidant properties or, in alternative, the conjugation of both properties.

Moreover, antimicrobial edible films and coatings may provide increased inhibitory effects against spoilage and pathogenic bacteria by maintaining effective concentrations of the active compounds on the food surfaces (Cerqueira *et al.,* 2011). On the other hand, the transport and release of various active compounds (antioxidants, flavorings, anti-browning and antimicrobial compounds, vitamins or enzymes) is one of the most important aspects within the features of edible films and coatings (Cerqueira *et al.,* 2011). As a result of use of edible films/coatings, wrapped or coated foods, shelf-life can be extended, the risk of pathogen growth on food surfaces can be reduced and the sensory quality enhanced (Vargas *et al.,* 2008)

In this work, glycerol and PEG400 were udes as plasticizers as well as glucuronoxylooligosaccharides from *Eucalyptus globulus* wood, an agroindustrial byproduct rich in hemicelluloses (obtained as described by Gullón *et al.,* 2011). Hemicellulose is one of the alternative materials that can be used for the production of edible films/coatings based on their edibility and biodegradability. The advantages of using biopolymers to produce such packaging films include their renewability, availability, limited environmental impact and simplified end-of-life disposal issues (Cerqueira *et al.,* 2011). Moreover, the use of renewable sources for packaging materials, such as polysaccharides from biological origin, is one of the main trends of this industry.

Since the incorporation of antimicrobials might influence the physical properties, in this work active films were submitted to physical characterization and the results were compared to the values obtained for the control films (according to methodology by Bierhalz *et al.,* 2012).

3.3.1. Edible film formulation

The several formulations tested in this work in order to obtain an acceptable film from the viewpoint of physical and visual properties are listed in Appendix II. However, only the final formulation (see final formulation in Section 2, **Table 2.2.**) of each extract (including replicates) was tested and used in the characterization assays for all extracts, except for the EW extract which film formulation was not made due to lack of sample.

A final volume of 10 mL of film formulation was prepared, as mentioned in the previous section, and this ideal volume was defined empirically as the one giving the best malleability, neither too thin nor too thick. In **Figure 3.5.** it is possible to observe the visual aspect of the AS extract film formulation.

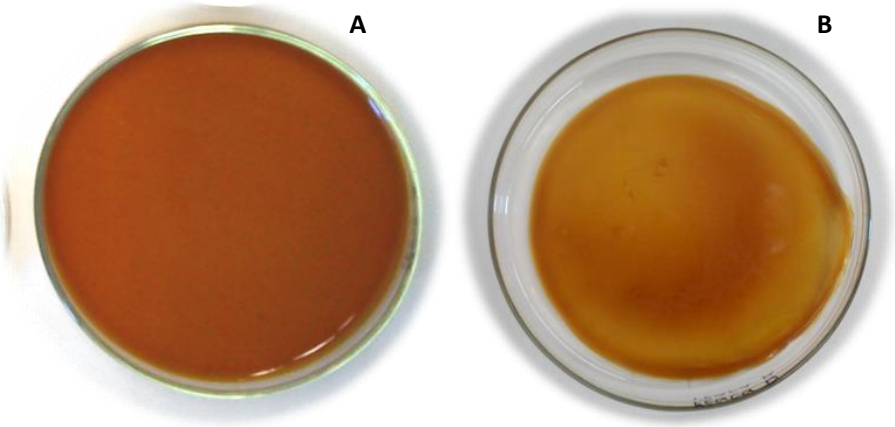

Figure 3.5. Visual aspect of the final film formulated with the AS extract before (A) and after 24h (B) of drying.

3.3.2. Properties of films: characterization

The results obtained for the different properties measured are given in **Table 3.4**.

Extract content

The percentages of extract present in each film were significantly slightly higher than those incorporated initially during the film preparation. This effect of concentration could be due to the loss of water during the drying process. The weight of films containing extracts was higher than the control films, as expected (see **Table 3.4.**).

Moisture content

The moisture content of film samples is shown in **Table 3.4**. Alginate film without glucuronoxylooligosaccharides (XOS) from *Eucalyptus globulus* wood (EW) and antioxidant extracts (Control 1) had a moisture content percentage similar to the alginate film incorporating CC extract and XOS from EW (CC-film); these films showed lower percentage of moisture content when compared with the other tested films. Additionally, these films showed statistically significant differences when compared to AS and GP- films ($p<0.05$), that can be hypothetically associated to the composition of the films. However, the alginate films containing XOS from EW (Control 2) showed the higher moisture content percentage; which was significantly different than the other films assayed in this work.

Film thickness

Thickness is an important parameter for packaging films in terms of mechanical properties and gas transfer rates (Goksu *et al.*, 2007). **Table 3.4**. gives the data for the thickness of all films, including the controls. The results showed that all films containing extracts had a higher film thickness in comparison with the controls. In fact, no statistically significant differences (p>0.05) were observed between films containing extracts and they also appeared visually firmer.

Table 3.4. Extract content, moisture content, weight, film thickness and water solubility of alginate films containing antioxidant extracts from lignocellulosic materials. Averages with the same superscript letter in the same column indicate no significant difference by the Tukey's Multiple Comparison test (p<0.05).

Alginate films	Extract content (mg/g film)	Moisture content (%, dry basis)	Weight (mg)	Thickness (mm)	Water solubility (%)
Control 1	-	25.18 ± 0.86^{a}	673.01 ± 37.02^{a}	121.50 ± 0.71^{a}	71.19 ± 2.78^{a}
Control 2	-	31.24 ± 0.70^{b}	985.37 ± 18.98^{b}	138.67 ± 0.94^{b}	72.06 ± 1.98^{a}
AS-film	264.28 ± 16.50^{a}	27.88 ± 0.77^{c}	1339.67 ± 30.04^{c}	189.50 ± 3.77^{c}	68.16 ± 6.37^{a}
GP-film	283.46 ± 15.37^{a}	26.89 ± 0.76^{c}	1375.50 ± 29.51^{c}	183.83 ± 0.94^{c}	69.71 ± 3.16^{a}
CC-film	238.69 ± 20.25^{a}	25.37 ± 0.80^{a}	1294.77 ± 34.43^{c}	185.08 ± 6.25^{c}	69.86 ± 2.32^{a}

The incorporation of XOS from EW (Control 2) led to films with a significantly higher thickness than the ones without XOS (Control 1); this is in agreement with the results obtained by Goksu *et al.* (2007), that reported that with the increasing xylan concentration from the cotton stalk xylan-lignin films, the number of the xylan layers and the amount of the entrapped water within the films also increased, resulting in an increase in film thickness.

Bierhalz *et al.* (2012), reported that the thickness of the films did not significantly change with the presence of natamycin. However, the results also showed that there was a significant increase in thickness when alginate was present in the formulation. This behavior may be associated to the differences of molecular mass of the biopolymers. According to Sriamornsak and Kennedy (2008), the pectin films tend to be thinner than alginate films because they achieve a more compact molecular packing, fact attributed to the lower molecular mass of the pectin in relation to the alginate. These authors obtained pectin films that were half as thick as their pure alginate counterparts.

Water solubility

One of the most important properties of packaging films is its solubility in aqueous solutions. Potential applications may require low water solubility to enhance product integrity and water resistance. On the other hand, in some cases such as food coating, high film solubility in water, before consumption of the product, might be beneficial (Gosku *et al.,* 2007).

Table 3.4. shows the water solubility of film samples determined at 25 ºC. The films containing antioxidant extracts showed similar solubility to the control films. The solubility in water was about 70% for all film types. So, the addition of the antioxidant extracts to alginate films did not bring about a noticeable significant increase in film solubility in comparison with the control films.

Film color

Packaging film color is important not only from the consumer's view point but also for the packaging of light-sensitive materials. Investigation of the color of packaging films in the literature is, however, limited (Goksu *et al.,* 2007).

Alginate edible films without antioxidant extracts incorporation appeared as clear and transparent films. Addition of the antioxidant extracts affected the color, and therefore the visual appearance of the films. This observation was also described by Pranoto *et al*. (2005).

Table 3.5. shows the values obtained for the three parameters (L, a*, b*) obtained using the CIELab color scale. All three parameters L, a* and b* are affected by the addition of the extract.

L values (lightness) decreased with the incorporation of antioxidant extracts (see **Table 3.5.**), which indicates that the color of the edible film tends to darken. The L value of control films was significantly higher than the value obtained for the films containing antioxidant extracts. Among the films containing extract, the higher L value was observed for the AS-film and in fact this was visually the least colored film.

The parameter a* (redness/ greenness equilibrium) was significantly lower in controls than the value obtained for films with antioxidants. Significant differences were also observed between films containing extract. The color tended to become reddish when a* value increased; the AS-film was the film which showed the greatest tendency, due to the highest a* value presented.

The same trend observed in the controls for the parameter a* was noted for b* (yellowness/ blueness equilibrium), so statistcally significant differences (p<0.05) in b*

values was observed between the controls and films containing extract. The color tended to become yellowish when the b* value increased; the AS-film was also the film that showed more tendency, due to the higher b* value presented. However, it is important to refer that no significant differences were presented for this parameter for the GP and CC-films. In fact, as visually observed, the AS-film was the less colored film containing extract.

In summary, all parameters were affected by addiction of extracts, so these data are in agreement with those reported by Campos et al. (2013) where they observed that the color parameters were influenced by the addition of *Ginja* cherry stem extract to methylcellulose films.

Table 3.5. CIELab coordinates for films prepared with different amounts of extract. ΔE value was calculated using Control 2 since it did not contain extract. Averages with the same superscript letter in the same column indicate no significant difference by Tukey's Multiple Comparison test (p<0.05).

Parameter	L	a*	b*	ΔE
Control 1	89.51 ± 1.23^{a}	1.73 ± 0.04^{a}	-1.56 ± 0.63^{a}	-
Control 2	85.37 ± 1.67^{a}	1.45 ± 0.11^{a}	0.45 ± 0.55^{a}	-
AS-film	42.04 ± 5.04^{b}	26.40 ± 0.11^{b}	22.6 ± 4.80^{b}	55.89 ± 3.75^{a}
GP-film	29.64 ± 3.71^{c}	21.91 ± 1.93^{c}	13.29 ± 2.05^{c}	61.92 ± 3.82^{b}
CC-film	28.60 ± 2.64^{c}	24.21 ± 0.58^{d}	14.82 ± 1.14^{c}	62.86 ± 1.31^{b}

Antimicrobial activity of the edible films: disc diffusion assay

The results of the antibacterial activities of edible films incorporated with antioxidant extracts are presented in **Table 3.6**. This was done using the five selected bacteria, which were the bacteria also used in the previous antimicrobial activity assessment. The disc diffusion assay method for edible films was used. Based on the aforementioned antimicrobial assays, 2% (w/v) of antioxidant extracts were incorporated, this concentration was selected because the drying process may influence this value, as previously observed by Campos *et al.* (2013). In fact, the results listed in **Table 3.4.** showed extract concentration to be slightly higher upon gravimetric analysis (see **Table 3.4.**). As can be seen in **Table 3.6.**, the control films did not inhibit the tested bacteria. Therefore, the effects observed using the films with incorporated antioxidant extracts were attributed only to the active compounds present in the extracts.

The results were consistent with the previous *in vitro* tests in MH broth described previously, and in which all microorganisms showed a similar sensitivity against the agro industrial antioxidant extracts.

These results prove that the active compounds present in the antioxidant extracts from agroindustrial by-products could be immobilized in the alginate film and subsequently released, thereby inhibiting the target microorganisms. This data coincide with that reported by Pranoto *et al.* (2005), who incorporated garlic oil in alginate films and evaluated their antimicrobial activity checking their effectiveness against *E. coli*, *Salmonella typhimurium*, *Staphylococcus aureus* and *Bacillus cereus* and testing the diffusion capacity of films on the media and the subsequently inhibition of those bacteria.

Therefore, an antibacterial alginate edible film incorporating AS, GP or CC extracts containing active compounds, is promising and has good potential in many food applications.

Table 3.6. Diameter (mm) inhibition zone of antioxidant extracts incorporated in an edible film against Gram-negative and Gram-positive bacteria (NO: inhibition not observed).

Strain type	Microorganism tested	Control 1	Control 2	AS-film	GP-film	CC-film
Gram-Negative	*E. coli* ATCC 25922	NO	NO	15.0 ± 0.1	13.6 ± 0.3	14.3 ± 0.3
	P. aeruginosa	NO	NO	14.2 ± 0.1	13.1 ± 0.1	13.7 ± 0.1
	Salmonella spp.463	NO	NO	14.0 ± 0.1	12.8 ± 0.1	13.5 ± 0.1
Gram-Positive	*L. monocytogenes* 3375	NO	NO	15.5 ± 0.1	14.6 ± 0.4	14.9 ± 0.1
	S. aureus 18 N	NO	NO	14.6 ± 0.4	13.9 ± 0.1	14.1 ± 0.1

4. Conclusions

The grape pomace (GP) extract was the extract with the highest antioxidant activity whereas the extract from the almond shells (AS) was the one with the lowest capacity. The chemical identification allowed identification of phenolic and non-phenolic compounds. The compounds present in the extracts showed different profiles in all extracts although there were some common compounds, such as: vannilic acid, 3,4-dihydroxybenzaldehyde, vanillin, quercetin, hydroxymethyfurfural and 2-furfuraldehyde. Differences were observed by the absence of gallic acid and syringic acid in CC extract. Furthermore, ellagic acid was identified only in the EW and GP extracts; *p*-coumaric and ferulic acid were also only identified in CC extract. These differences can hypothetically justify differences in the microbial assays.

All the tested extracts showed antimicrobial activity against *Escherichia coli, Pseudomonas aeruginosa, Listeria monocytogenes, Staphylococcus aureus* and *Salmonella* spp. However, the results did not show a selective action upon either Gram-positive or Gram-negative bacteria. In fact, these extracts are a mixture of phenolic and non-phenolic compounds, so this absence of trend may be expected. From the analysis of the minimum bactericidal concentrations (MBCs), the EW extract was the most active, whereas the AS and CC extracts were less active. The time-kill analysis/ survival curves have highlighted the antimicrobial effect of the extracts; however, this effect was not immediate, in that for the majority of the cases, this effect only occurred at approximately 24h.

The assays with probiotic bacteria showed that the tested extract concentrations (0.025% and 0.1%) did not significantly affect the growth of these microorganisms, although it has been observed that higher concentrations of the AS extract and the GP extract could inhibit or enhance, respectively, the growth of tested probiotic bacteria.

Finally, the edible films produced with the extracts showed similar characteristics; significant differences were observed only for color and moisture content, with the films containing the AS and CC extracts showing smaller values comparatively to the other films. All films displayed antimicrobial activity against the pathogenic bacteria tested, suggesting the possible use of these extracts in an edible film.

5. Future Work

The present work employed an exploratory approach towards the evaluation of some relevant and interesting properties of by-products antioxidant extracts against pathogenic bacteria and beneficial bacteria. Consequently, there is a wide space for further investigation on this topic. Considering this thesis as a base, it would be important to perform a more in-depth study on the antimicrobial, pro-probiotic and antioxidant activities.

The first interesting future work is based on overcoming the difficulty in dissolving these extracts. In fact, it might be interesting to see how the addition of other emulsifiers affect the studied activities. Additionally, it would be interesting to change the polarity of the extracts through the use of resins to facilitate applicability.

A particularly interesting study would be the assessment of the biological antioxidant activity using an analysis of the antioxidant and pro-oxidant effect upon DNA. The evaluation of an extract's capacity to protect a biological molecule or to damage it becomes of particular importance by using, for example, cell cultures to evaluate the cellular uptake of these compounds, their action mechanisms and possible undesirable metabolic interferences.

In addition, possessing a more detailed composition, possibly using a mass spectrophotometer, would be interesting to draw a parallel between the activities of the identified compounds (both individually and in several mixtures). In this way, it would be easier to identify the compounds responsible for inhibitions and establish the synergistic and antagonistic interactions that may occur. Furthermore, it would be interesting to study the mechanism of action of the extracts exerting antimicrobial activity. Membrane damage could be evaluated using SEM (Scanning Electron Microscope) analysis or employing a fluorescence technique based on membrane integrity. In fact, after the compounds are identified, it would be easier to evaluate the mechanism of action behind the antimicrobial effect.

Finally, the metabolism of the extracts by probiotic bacteria is another interesting topic that could contribute in helping understand if these compounds could be used as co-adjuvants in improving gut health.

6. Appendices

6.1. Appendix I – Calibration curves

6.1.1. Ascorbic acid equivalent calibration curve: DPPH assay

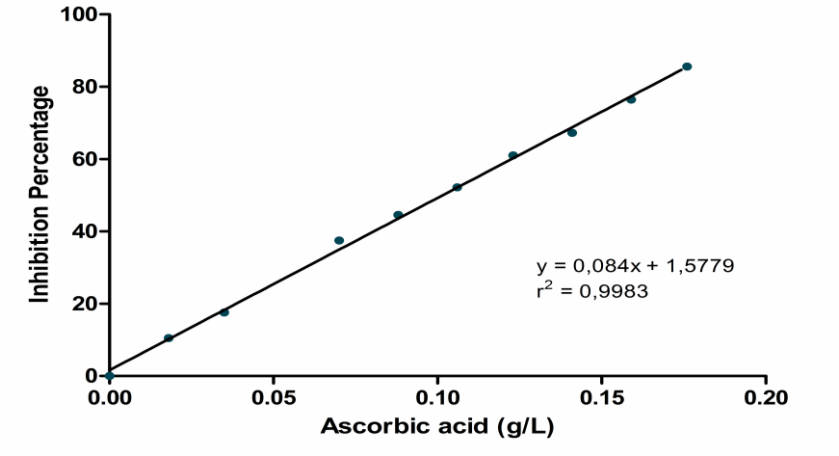

Figure 6.1. Calibration curve using standard ascorbic acid solutions (ABTS assay).

6.1.2. Ascorbic acid equivalent calibration curve:ABTS assay

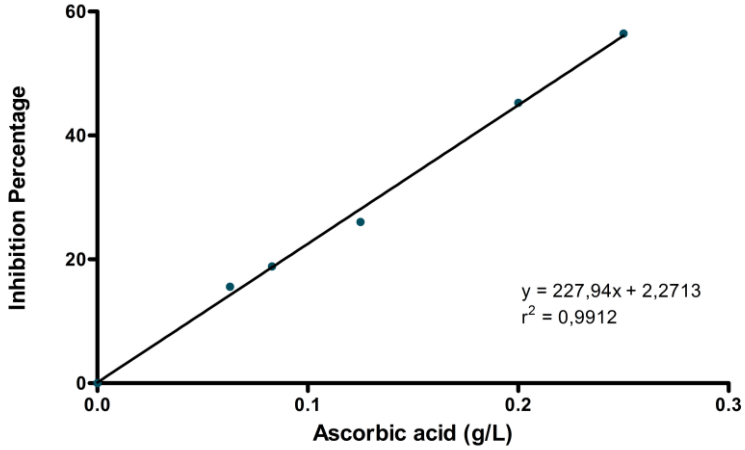

Figure 6.2. Calibration curve using standard ascorbic acid solutions (DPPH assay).

6.1.3. Trolox equivalent calibration curve: ORAC assay

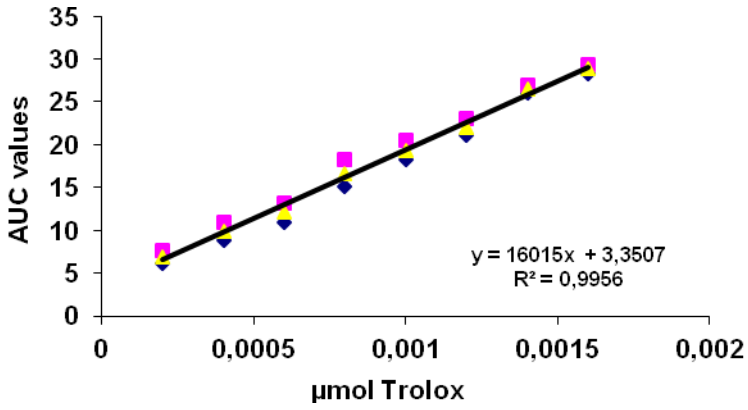

Figure 6.3. Calibration curve using standard trolox solutions (ORAC-FL assay).

6.2. Appendix II - Films formulations

Table 6.1. Different films formulations tested with a metylcellulose matrix and 2% (w/v) of extract.

Polymeric matrix	Films	Plasticizer	Others compounds	Drying conditions	References (Modified from)
Methycellulose (1.2% (w/v))	F-MC1	Glycerol 50% (v/v) (5% (v/v))	Tween 80 (0,5% (v/v))	24h, 37ºC	Campos *et al.*, 2013
	F-MC2	Glycerol 50% (v/v) (10% (v/v))	Tween 80 (0,5% (v/v))	24h, 37ºC	Campos *et al.*, 2013
	F-MC3	Glycerol 50% (v/v) (5% (v/v))	Tween 80 (0,75% (v/v))	24h, 37ºC	Campos *et al.*, 2013
	F-MC4	Glycerol 50% (v/v) (7,5% (v/v))	Tween 80 (0,5% (v/v))	24h, 37ºC	Campos *et al.*, 2013

Table 6.2. Different films formulations tested in a sodium alginate matrix and 2% (v/w) of extract.

Polymeric matrix	Films	Plasticizer	Others compounds	Drying conditions	Cross-Linking	References (Modified from)
Sodium Alginate (1% (w/v))	F-SA1	Glycerol 50% (v/v) (1% (v/v))	Tween 80 (0,8% (v/v))	24h, room temperature	$CaCl_2$ (0,1% (w/v))	Pranoto *et al.*, 2005
Sodium Alginate (1% (w/v)) + $CaCl_2$ (0.5% (w/v))	F-SA2	Glycerol (1.5 g/ g alginate)	Tween 80 (0,8% (v/v))	24h, room temperature	$CaCl_2$ (2% (w/v))	Norajit *et al.*, 2010
	F-SA3	Glycerol (1.5 g/ g alginate)	Tween 80 (0,8% (v/v)) + Oligosaccharide (1% (w/v))	24h, room temperature	$CaCl_2$ (2% (w/v))	Norajit *et al,*. 2010
	F-SA4	Glycerol (1.5 g/ g alginate)	Tween 80 (0,8% (v/v)) + Arabic gum (1% (w/v))	24h, room temperature	$CaCl_2$ (2% (w/v))	Norajit *et al.*, 2010
	F-SA5	Glycerol + PEG 400 (each one:1.5 g/ g alginate)	Tween 80 (0,8% (v/v)) + Oligosaccharide (1% (w/v))	24h, room temperature	$CaCl_2$ (2% (w/v))	Cha *et al.*, 2002; Norajit *et al.*, 2010
	F-SA6	Glycerol + PEG 400 (each one:1.5 g/ g alginate)	Tween 80 (0,8% (v/v)) + Arabic gum (1% (w/v))	24h, room temperature	$CaCl_2$ (2% (w/v))	Cha *et al.*, 2002; Norajit *et al.*, 2010
	F-SA7	Glycerol (1.5 g/ g alginate)+ PEG 400 (3 g/ g alginate)	Tween 80 (0,8% (v/v)) + Oligosaccharide (1% (w/v))	24h, room temperature	$CaCl_2$ (2% (w/v))	Cha *et al.*, 2002; Norajit *et al.*, 2010

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