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Study of different antifungal systems to preserve strawberry jam against fungi spoilage

DOCTORAL THESIS

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Hacen constar que:

La memoria titulada “Study of different antifungal systems to preserve strawberry jam against fungi spoilage” que presenta Dª Susana Ribes Llop para optar al grado de Doctor por la Universitat Politècnica de València, ha sido realizada en el Departamento de Tecnología de Alimentos de la Universitat Politècnica de València bajo su dirección y que reúne las condiciones para ser defendida por su autora.

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Un viaje de 10.000 km empieza con un solo paso

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ABSTRACT

New tendencies in the development of innovative food packages could affect product microbial stability during storage. In this sense, antifungal systems such as emulsions, nanoemulsions and mesoporous silica supports, may be promising alternatives to preserve the product quality without having to apply further thermal treatments.

The formulation of stable antifungal systems and the optimisation of the methodology to be employed to prepare these systems play an important role in: i) their antifungal effectiveness; ii) bioactive compounds losses during their preparation; and iii) their impact on the food product's sensory profile.

Nanoemulsions prepared with essential oils generally presented higher antifungal effect than free essential oils applied at the same concentrations. Furthermore, the emulsifier played a key role in the resulting antifungal activity. Whey protein isolated-based nanoemulsions were more effective in inhibiting mycelial mould growth and spore germination than Tween 80-based ones.

The great *in vitro* effectiveness of nanoemulsions to control mould development allowed to check their application to food products, and strawberry jam was selected as the food matrix. To this end, clove and cinnamon leaf emulsions were incorporated into strawberry jams to control fungal decay. Although these emulsions were able to reduce jam spoilage, their incorporation negatively affected the aroma, taste and the overall acceptance of the jam.

Regarding the methodology employed to prepare oil-in-water emulsions, the magnetic stirring and high pressure homogenisation combination was the most effective to reduce essential oil losses. Moreover, stable cinnamon bark-xanthan gum emulsions with good *in vitro* antifungal properties against the target fungi were obtained. Despite the promising use of cinnamon bark emulsions to control fungi decay in strawberry jam, their incorporation negatively affected the product's aroma, taste and overall acceptance.

Given the strong impact of essential oils on the food product's sensory profile, two new approaches were investigated: i) combinations of different antifungal

compounds in emulsions; and ii) immobilisation of bioactive compounds on mesoporous silica surfaces. The cinnamon bark essential oil, zinc gluconate and *trans*-ferulic acid combination allowed the obtention of a product considered microbiologically acceptable and with good organoleptic characteristics. However, total mould inhibition was not achieved. Consequently, the antifungal and sensory properties of the bioactive agents (eugenol and thymol) immobilised on mesoporous silica surfaces were studied. The preparation of jams with eugenol immobilised on MCM-41 microparticles induced better control of the fungal inhibition compared with the samples functionalised with thymol. The sensory analysis of the jams after incorporating the solids revealed that eugenol and thymol immobilisation cushioned the impact of these compounds on the jam flavour profile.

RESUMEN

Las nuevas tendencias en el desarrollo de envases alimentarios innovadores podrían afectar a la estabilidad microbiana del producto durante el almacenamiento. En este sentido, sistemas antifúngicos tales como emulsiones, nanoemulsiones y soportes mesoporosos de sílice pueden ser alternativas prometedoras para preservar la calidad del producto sin tener que aplicar otros tratamientos térmicos.

La formulación de sistemas antifúngicos estables y la optimización de la metodología a emplear para preparar estos sistemas, tienen un papel importante en: i) su eficacia antifúngica; ii) pérdidas de los compuestos bioactivos producidas durante su preparación; y iii) su impacto en el perfil sensorial del producto alimenticio.

Las nanoemulsiones preparadas con aceites esenciales presentaron por norma general un mayor efecto antifúngico que los aceites libres aplicados a las mismas concentraciones. Asimismo, el tipo de emulsionante utilizado desempeñó un papel clave en la actividad antifúngica de las nanoemulsiones. Las nanoemulsiones preparadas con proteína de suero de leche fueron más eficaces en la inhibición del crecimiento micelial y en la germinación de las esporas que las formuladas con Tween 80.

La gran eficacia *in vitro* de las nanoemulsiones en el control del desarrollo de moho permitió evaluar su aplicación en productos alimenticios, siendo seleccionada la confitura de fresa como matriz alimenticia. Con este fin, las emulsiones de los aceites esenciales de clavo y de hoja de canela fueron incorporadas a las confituras de fresa con el fin de controlar el deterioro fúngico. A pesar de que estas emulsiones fueron capaces de reducir el deterioro fúngico de las confituras de fresa, su incorporación afectó negativamente al aroma, el sabor y la aceptación global de la confitura.

En cuanto a la metodología empleada para preparar emulsiones aceite-agua, la combinación de agitación magnética y homogeneización por altas presiones fue la más eficaz para reducir las pérdidas de aceite esencial. Asimismo, se obtuvieron

emulsiones estables con una alta capacidad antifúngica, determinada *in vitro*, frente a los hongos objeto de estudio. A pesar del prometedor uso de las emulsiones de aceite esencial de la corteza de canela en el control del deterioro fúngico de las confituras de fresa, su incorporación al alimento afectó negativamente al aroma, sabor y aceptación global del producto.

Debido al gran impacto de los aceites esenciales en el perfil sensorial del producto alimenticio, se investigaron dos nuevos enfoques: i) combinaciones de diferentes compuestos antifúngicos en emulsiones; y ii) inmovilización de compuestos bioactivos sobre superficies de sílice mesoporosas. La combinación de aceite esencial de corteza de canela, el gluconato de zinc y el ácido *trans*-ferúlico permitió la obtención de un producto considerado microbiológicamente aceptable con buenas características organolépticas pero no se consiguió la inhibición total del crecimiento de moho. Por ello, se estudiaron las propiedades antifúngicas y sensoriales de agentes bioactivos (eugenol y timol) inmovilizados sobre superficies de sílice mesoporosas. La preparación de las confituras con eugenol inmovilizado en las micropartículas de MCM-41, mostró un mejor control de la inhibición fúngica en comparación con las muestras preparadas con timol funcionalizado. El análisis sensorial de las muestras tras la incorporación de los sólidos a la confitura de fresa, reveló que la inmovilización de eugenol y timol reduce el impacto sensorial de estos compuestos en confituras de fresa.

RESUM

Les noves tendències en el desenvolupament d'envasos alimentaris innovadors podrien afectar l'estabilitat microbiana del producte durant l'emmagatzematge. En aquest sentit, sistemes antifúngics com les emulsions, nanoemulsions i suports mesoporosos de sílice poden ser alternatives prometedores per preservar la qualitat del producte sense haver d'aplicar altres tractaments tèrmics.

La formulació de sistemes antifúngics estables, així com l'optimització de la metodologia a emprar per preparar aquests sistemes, tenen un paper important en: i) la seva eficàcia antifúngica; ii) pèrdues dels compostos bioactius produïdes durant la seva preparació; i iii) el seu impacte en el perfil sensorial del producte alimentari.

Les nanoemulsions preparades amb olis essencials van presentar per norma general, un major efecte antifúngic que els olis lliures aplicats a les mateixes concentracions. Així mateix, el tipus d'emulsionant utilitzat va tenir un paper clau en l'activitat antifúngica de les nanoemulsions. Les nanoemulsions preparades amb proteïna de sèrum de llet van ser més efectives en la inhibició del creixement micelial i en la germinació de les espores que les formulades amb Tween 80.

La gran eficàcia *in vitro* de les nanoemulsions en el control del desenvolupament de fongs va permetre avaluar la seva aplicació a productes alimentaris, sent seleccionada la confitura de maduixa com a matriu alimentària. Amb aquesta finalitat, les emulsions dels olis essencials de clau i de fulla de canyella van ser incorporades a les confitures de maduixa per tal de controlar el deteriorament fúngic. Tot i que aquestes emulsions reduïren el deteriorament fungic de les confitures de maduixa, la seva incorporació va afectar negativament a l'aroma, el sabor i l'acceptació global de la confitura.

Pel que fa a la metodologia emprada per preparar emulsions oli-aigua, la combinació d'agitació magnètica i homogeneïtzació per altes pressions va ser la més eficaç per reduir les pèrdues d'oli essencial. A més, es van obtenir emulsions estables amb una alta capacitat antifúngica, determinada *in vitro*, davant dels

fongs objecte d'estudi. Malgrat el prometedor ús de les emulsions d'oli essencial de l'escorça de canyella en el control del deteriori fúngic de les confitures de maduixa, la seva incorporació a l'aliment va afectar negativament a l'aroma, sabor i acceptació global del producte.

A causa del gran impacte dels olis essencials en el perfil sensorial del producte alimentari, es van investigar dos nous enfocaments: i) combinacions de diferents compostos antifúngics en les emulsions; i ii) immobilització de compostos bioactius sobre superfícies de sílice mesoporoses. La combinació d'oli essencial d'escorça de canyella, el gluconat de zinc i l'àcid *trans*-ferúlic va permetre l'obtenció d'un producte considerat microbiològicament acceptable amb bones característiques organolèptiques però no es va aconseguir la inhibició total del creixement del fong. Per això, es van estudiar les propietats antifúngiques i sensorials d'agents bioactius (eugenol i timol) immobilitzats sobre superfícies de sílice mesoporoses. La preparació de les confitures amb eugenol immobilitzat en les micropartícules de MCM-41, va mostrar un millor control de la inhibició fúngica en comparació amb les mostres preparades amb timol funcionalitzat. L'anàlisi sensorial de les mostres després de la incorporació dels sòlids a la confitura de maduixa, va revelar que la immobilització de eugenol i timol redueix l'impacte sensorial d'aquests compostos en confitures de maduixa.

PREFACE

DISSERTATION OUTLINE

This Thesis studies the use of antifungal systems formulated with different bioactive substances, generally recognised as safe (GRAS), to preserve and control fungi development in strawberry jam.

Jam has been one of the commonest methods employed to preserve fruits for decades (Rababah et al., 2015). They are described as mixtures, with an appropriate gelled consistency, of sugars, pulp and/or purée of one or more fruits and water. Jams must have a soluble dry matter content ≥ 60%, except when sugars have been partially or totally substituted for sweeteners (RD 863/2003; REGLAMENTO (UE) 1129/2011). Traditionally after preparation, the jams are poured into glass jars at 85 °C, sealed with metal covers, inverted for 5 minutes, sterilised, and then, returned to a straight position (Rababah et al., 2015). This preparation type, together with the high sugar content and a low pH, ensures product stability during storage at room temperature. However, novel processing techniques, that focus on maintaining fruit properties, and new packaging formats could result in a final product that offers different characteristics and stability during storage. High hydrostatic pressure (HHP) has been used as an alternative to pasteurisation and sterilisation. HHP is a good non-thermal preservation treatment that can achieve microbial inactivation and product stabilisation without amending sensory qualities (Dervisi et al., 2001). HHP offers new opportunities for the food industry to respond to consumer demands. However, this technology is currently more expensive than traditional processing technologies (high-temperature sterilisation) (Yaldagard et al., 2008).

Conversely, new tendencies in the development of more attractive products focus on the visual aspects of the complete package, including the container, cap and label, and not only on ingredients or preparation (Weissmann et al., 2017). This occurs with PET bottles, which are an alternative to glass bottles. This container type offers several advantages. Its lighter weight reduces the

environmental impact during transport and the losses of foodstuff are decreased since fewer bottles are broken during filling and storage (Dombre et al., 2015). Moreover, in the present study, the most relevant advantages of using PET bottles compared with glass containers are the obtention of a product with an easy application and anti-dip system. However, in order to avoid PET bottles from deforming, jam must be packaged at 45 °C, which implies a possible source of contamination. This is why the product's microbial stability is required during the packaging procedure and after opening the container.

Fungi are the most relevant organisms to contaminate fruit and berry concentrates like jam, with low water activity (a_w). The raw material used during jam manufacturing can contain fungal spores, which are inactivated during the cooking procedure. However, the jam containers used in the food industry may be re-contaminated by indoor fungi spores during container depletion (Nieminen et al., 2008).

Aspergillus and *Penicillium* species are frequently found in postharvest products like fruit and vegetables. The principal changes produced in food products due to spoilage are pigmentation, discolouration, rotting, off-flavours and off-odours (Varga et al., 2008). *Aspergillus* and *Penicillium* species can colonise berries and cause them to decay (Jensen et al., 2013). *Zygosaccharomyces* species are the major spoilage microorganisms of fruit juices, carbonated drinks, ketchup, candied fruits, syrups and nougat, among others (Martorell et al., 2005). Specifically, *Zygosaccharomyces rouxii* and *Z. bailii* are characterised by their ability to tolerate low a_w and pH environments. They are able to grow in products with high sugar contents (>60 %), and exhibit strong resistance to weak-acid preservatives, extreme osmotolerance, and have ability to ferment hexose sugars (James & Stratford, 2003).

Essential oils (EOs) have been extensively used in the last few years for their antimicrobial and antifungal properties (Perdones et al., 2012; Abbaszadeh et al., 2014; Salvia-Trujillo et al., 2014; Monu et al., 2016). EOs have been generally recognised as safe (GRAS) by the FDA in Code 21 of the Federal Regulation, part 182.20, and their natural character makes their use in food products acceptable

by consumers (Burt, 2004). They are the most employed antifungal agents in the present Thesis. However, use of EOs is limited due to their: i) high volatility, which leads to bioactive compounds losses; ii) low solubility in water, which makes their antifungal action in food commodities with high moisture content; and iii) intensive aroma. Therefore, different approaches have been proposed to overcome these drawbacks: i) optimisation of the methodology used to prepare oil-in-water (O/W) emulsions to reduce losses of EOs; ii) the combination of different antifungal compounds to cushion the food product's impact without decreasing antifungal activity; iii) immobilisation of the bioactive agents on the surface of mesoporous silica supports as promising antifungal agents against moulds and yeasts without producing changes in the sensory profile of foods after their incorporation. The obtained results are disposed as follows:

Chapter 1, entitled "**Use of oil-in-water emulsions as potential antifungal delivery systems**", is divided into 3 sections. The first section studies the effect of physically stable nanoemulsion-based delivery systems to control their antifungal action against *A. niger*. Three EOs (cinnamon leaf, lemon and bergamot oils) and two emulsifiers (Tween 80 and whey protein isolate) were employed to prepare O/W nanoemulsions. The *in vitro* antifungal activity of the different systems was evaluated in terms of fungal and mycelial growth inhibition (%), spore germination inhibition (%) and the morphological damages produced to fungal hyphae and hyphal tips.

The positive results obtained in the *in vitro* antifungal tests led to further studies to investigate the antifungal activity of O/W emulsions in food commodities. For this purpose, strawberry jam was selected as the food matrix and the results are included in the second section of **Chapter 1**. This section focuses on employing the O/W emulsions prepared with clove and cinnamon leaf EOs as natural agents. Both EOs contain eugenol as the main compound, which is responsible for their good antifungal properties, along with the other compounds present in these oils. The effect of polymer content was also evaluated. Moreover, the *in vitro* and *in vivo* antifungal tests were carried out against three mould strains: *A. flavus*, *A. niger* and *Penicillium expansum*. The preparation of

O/W emulsions revealed losses of EOs which were affected by the amount of xanthan gum (XG) employed in the formulation of emulsions. Taking into account these results and those obtained in the physicochemical characterisation of the O/W emulsions, the suitable amount of polymer and EO to be used in strawberry jam was established. Furthermore, the influence of storage temperature and the effectiveness of the O/W emulsions on mould growth in strawberry jam were also investigated. To this end, samples were maintained at 4 °C to simulate product cold storage after opening the jam container, and at 25 °C, the optimum growth temperature of fungi. The incorporation of O/W emulsions into strawberry jam allowed to control fungi spoilage of the product but negatively affected the aroma, taste and the overall acceptance of the product, which confirmed that further studies in this area are required.

Optimisation of the methodology used to prepare O/W emulsions in order to diminish losses of EOs was studied in the third section. Furthermore, the evaluation of the antimicrobial activity of O/W emulsions *in vitro,* and also in strawberry jam to control mould and yeasts, was carried out. Different methodologies were followed to prepare emulsions: i) rotor-star device (1 min 10,000 rpm, 3 min 20,000 rpm), followed by high pressure homogenisation (HPH) at 40 or 80 MPa; and ii) magnetic stirring for 15 min, followed by HPH at 40 or 80 MPa. The results indicated that the optimum conditions were the use of magnetic stirring for 15 min followed by HPH at 40 or 80 MPa in order to reduce losses of EOs. This methodology is a good alternative to obtain stable emulsions with high *in vitro* antifungal properties against all the studied fungi. In fact the jam prepared with the emulsions that contained 0.08 mg/g of cinnamon bark EOs exhibited the total inhibition of *A. flavus*, *P. expansum*, *Z. rouxii* and *Z. bailii*. Nevertheless, the incorporation of cinnamon bark-xanthan gum emulsions negatively affected the jam's aroma, taste and overall acceptance, which occurred in the previous chapter.

Taking into account the impact of EOs and their main compounds on the sensory profile of the studied food product, the combination of different antifungal agents and the development of new antifungal systems were

investigated. **Chapter 2** entitled **"Combination of different antifungal agents in oil-in-water emulsions to control jam spoilage",** studies the use of cinnamon bark EO (CBEO), ZG and FA in the formulation of O/W emulsions to control strawberry jam spoilage by fungi with no changes to their sensory characteristics. The *in vitro* and *in vivo* assays of O/W emulsions were carried out against *A. niger*. This mould was selected due to its relevance in the decay of strawberry products and for its resistance against different preservatives from the *in vitro* results, which is a problem for both scientific and industrial areas. The physicochemical characteristics of the different O/W emulsions were also evaluated. Great antifungal properties in the *in vitro* assays were observed by using CBEO, ZG and FA in the formulation of O/W emulsion. The addition of emulsions to strawberry jam did no alter the organoleptic profile of the final product, but total mould inhibition was not accomplished. Therefore, the antifungal activity of the bioactive agents immobilised on mesoporous silica surfaces was investigated in **Chapter 3**, entitled **"Eugenol and thymol immobilised on mesoporous silica-based material as an innovative antifungal system: application in strawberry jam"**. The evaluation of the antifungal effectiveness of the eugenol and thymol bioactive principles, both alone and when immobilised on the surface of MCM-41 particles, and their impact on strawberry jam' final aroma and fungal decay, was studied. The characterisation of the mesoporous silica material was evaluated by standard techniques, such as particle size, ζ-potential values, FESEM images, thermogravimetric and elemental analyses. The jams prepared with the eugenol immobilised on the MCM-41 microparticles exhibited neither mould nor yeast development during the complete evaluation period. The sensory evaluation of jam confirmed that the immobilisation of eugenol and thymol on MCM-41 cushioned the impact of these compounds on the food product's flavour profile.

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PREDOCTORAL STAGE AT A FOREIGN INSTITUTION

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LIST OF ACRONYMS

AB: Apex bifurcations

AN: Anomalies

ANOVA: Analysis of variance

BEO: Bergamot essential oil

CBEO: Cinnamon bark essential oil

CC: Decrease in the cytoplasmic content

CEO: Cinnamon leaf essential oil

CFU: Colony forming units

CR: Cytoplasmic retraction

d_H: Droplet size

***$d_{3,2}$*:** Surface weighted mean diameter

***$d_{4,3}$*:** Volume weighted mean diameter

EO: Essential oil

FA: *trans*-ferulic acid

FDA: Food and Drug Administration

FESEM: Field Emission Scanning Electron Microscopy

GRAS: Generally Recognised as Safe

HPH: High Pressure Homogenisation

HSH: High Shear Homogenisation

IUPAC: International Union of Pure and Applied Chemistry

k: Consistency index

LAB: Lactic acid bacteria

LEO: Lemon essential oil

LSD: Least Significant Differences

MIC: Minimum Inhibitory Concentration/Minimal Inhibitory Concentration

MFC: Minimum Fungicidal Concentration/Minimal Inhibitory Concentration

MSPs: Mesoporous Silica Particles

M_w: molecular weight

n: Flow behaviour index

List of acronyms

n_{ap}: Apparent viscosity

O/W: Oil-in-water

PDA: Potato dextrose agar

PDB: Potato dextrose broth

PdI: Polydispersity index

PET: Polyethylene terephtalate

SC: Separation of the cytoplasm

τ: Shear stress

T80: Tween 80

a_w: water activity

WPI: Whey protein isolate

XG: Xanthan gum

YPDB: Yeast extract peptone dextrose broth

$\dot{\gamma}$: Shear rate

ZG: Zinc gluconate

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1. INTRODUCTION

REVIEW

PREVENTION OF FUNGAL SPOILAGE IN FOOD PRODUCTS USING NATURAL COMPOUNDS: A REVIEW

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Abstract

The kingdom Fungi is the most important group of microorganism contaminating food commodities, and chemical additives are commonly used in the food industry to prevent fungal spoilage. However, the increasing consumer concern about synthetic additives has led to their substitution by natural compounds in foods. The current review provides an overview of using natural agents isolated from different sources (plants, animals and microorganisms) as promising antifungal compounds, including information about their mechanism of action and their use in foods to preserve and prolong shelf life. Compounds derived from plants, chitosan, lactoferrin, and biocontrol agents (lactic acid bacteria, antagonistic yeast and their metabolites) are able to control the decay caused by fungi in a wide variety of foods. Several strategies are employed to reduce the drawbacks of some antifungal agents, like their incorporation into oil-in-water emulsions and nanoemulsions, edible films and active packaging, and their combination with other natural preservatives. These strategies facilitate the addition of volatile agents into food products and, improve their antifungal effectiveness. Moreover, biological agents have been investigated as one of the most promising options in the control of postharvest decay. Numerous mechanisms of action have been elucidated and different approaches have been studied to enhance their antifungal effectiveness.

Keywords: Plant secondary metabolite, essential oil, chitosan, lactic acid bacteria, antagonistic yeast, antifungal protection

1. INTRODUCTION

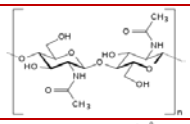
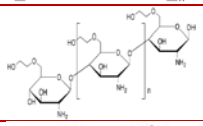
The kingdom Fungi is a group of eukaryotic organisms that includes unicellular microorganisms such as yeasts and moulds. They are the most important group of organisms to contaminate fruits and vegetables, and other food commodities like wine, juice, fruit puree, jams, meat and cheddar cheese, among other food products (Nieminen et al., 2008; Gammariello et al., 2014). Furthermore, postharvest spoilage of fruits, vegetables, and cereals by phytopathogens, particularly fungal pathogens, produces significant economic losses (Liu et al., 2017).

The food industry has resorted to several techniques to prevent fungi growth and spoilage (Davidson and Taylor, 2007). Even though the most effective methods to control food spoilage are achieved by chemical additives, their negative consumer perception and the more severe regulations on the use of fungicides (Wisniewski et al., 2016; Calvo et al., 2017) have increased an interest in new alternatives to protect food products by replacing synthetic agents with natural compounds (Parafati et al., 2015; Russo et al., 2017).

Natural antifungals can be obtained from different sources, including plants, animals and microorganisms (**Table 1**). Plant secondary metabolites are an important source of antifungal bioactive substances, and include essential oils, phenolic compounds, flavonoids and alkaloids among others (Ciocan and Băra, 2007). Among the natural antifungals of animal origin, chitin, chitosan and lactoferrin are reported to possess antifungal activity against a wide range of fungi (Perdones et al., 2012; Wang et al., 2013). Furthermore, lactic acid bacteria (LAB) produces a wide variety of products with antifungal activity, among them, proteinaceous compounds called bacteriocins have shown to inhibit the growth and development of fungi (Hondrodimou et al., 2011). Recently, the use of antagonistic yeasts has attracted more interest since their inhibitory activity is not related with the production of toxic metabolites, which occurs with antibiotics that derive from bacteria, fungi, plants and animals (Vardanyan and Hruby, 2016).

The purpose of this review is to highlight the different sources of natural compounds of plant, animal and microbiological origins that can be used to control food spoilage caused by moulds and yeasts, and to explain their biological mode of action. Hence, this paper focuses on analysing the potential application of these natural compounds in food products to improve their shelf life.

Table 1. Depiction of representative natural antifungals of different sources: plant, animal and microorganisms.

NATURAL ANTIFUNGALS		
PLANT ORIGIN	Essential oils (EOs)	
	Phenolic compounds	Phenolic acids
		Flavonoids
	Glucosinolates	
	Other compounds	
ANIMAL ORIGIN	Chitin	
	Chitosan	
	Lactoferrin	
MICROBIOLOGICAL ORIGIN	Lactic acid bacteria (LAB)	
	Antagonistic yeasts	

2. ANTIFUNGALS OF PLANT ORIGIN

Plant antifungals are usually compounds that belong to their secondary metabolisms. Essential oils (EOs), phenolic compounds, glucosinolates, hexanal and hexanol, among others, are antifungals that derive from plants.

2.1 Essential oils

Essential oils (EOs) are highly complex mixtures of often hundreds of individual aroma compounds that are poorly soluble in water and have a pleasant odour and taste. Moreover, EOs have been recognised as GRAS (Generally Recognised as Safe) by the U.S. Food and Drug Administration (CFR, 2014).

Plant EOs have been used for many years in food and pharmaceutical products for their antifungal, antimycotic and pest control properties (Bajpai et al., 2012). In fact, their antifungal effectiveness has attracted the growing interest of researchers for being used as food preservatives (Vitoratos et al., 2013). The most widely employed EOs as natural food preservatives are cinnamon, clove, lemongrass, oregano, thyme, nutmeg and basil. **Figure 1** shows the main compounds of these EOs.

2.2.1 Mode of action

Several hypotheses have been put forward to explain EOs' antifungal activity: i) direct effects on enzymes and intracellular functions modification due to the presence of OH groups that can form hydrogen bonds (Soylu et al., 2006; da Cruz-Cabral et al., 2013); ii) changes in the morphology of different species of moulds and yeasts as a result of the interactions with membrane enzymes, which diminish cell wall firmness and integrity (Soylu et al., 2006; da Cruz-Cabral et al., 2013); iii) accumulation of EO compounds in the cell membrane because of their molecular structure and the position of functional groups, which leads to cell membrane destabilisation and damage (Rao et al., 2010); and iv) variations in membrane

permeability, granulation of the cytoplasm and cytoplasmic membrane disruption (Bennis et al., 2004; Tao et al., 2014).

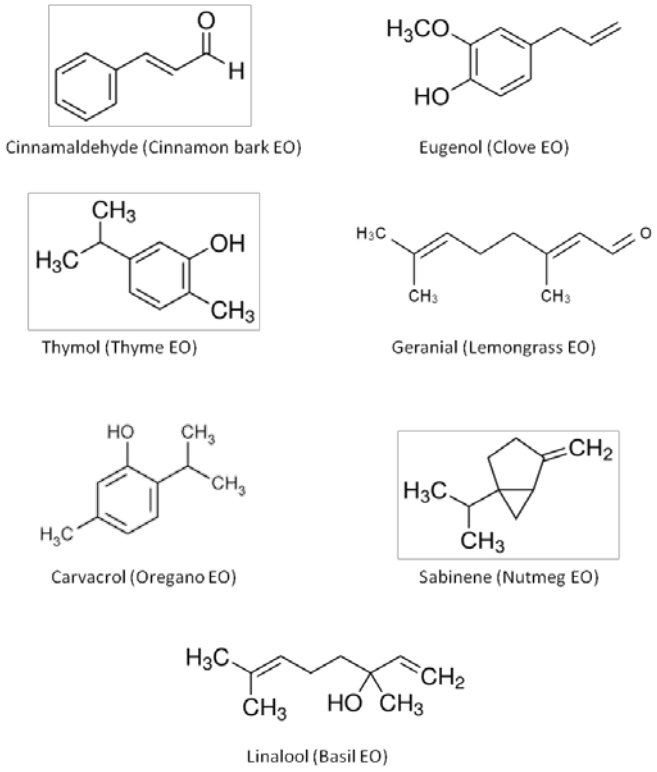


Figure 1. Main compounds of cinnamon, clove, lemon grass, oregano, thyme, nutmeg and basil EOs.

2.1.2 Incorporation in food systems

The strong flavour of EOs makes their incorporation into food products at high doses difficult given the changes in their sensory profile (Perdones et al., 2012). Emulsions, nanoemulsions and edible coatings have exhibited several advantages, such as the encapsulation of functional lipophilic substances, which allow the preservation and lower the concentrations of antifungal agents, diminishing the impact on the sensory profile of food commodities while maintaining their effectiveness (Salvia-Trujillo et al., 2015). In these sense, emulsions incorporating EOs have been successfully applied in jam preservation and tomato plant. Clove and cinnamon leaf emulsions containing a final EO concentration of 0.34 and 0.39 mg/g, respectively, were incorporated into strawberry jams to prevent fungal spoilage (Ribes et al., 2016). The jam samples with the emulsions were inoculated with two moulds of the *Aspergillus* genus and one mould of the *Penicillium* genus and were stored for 63 days at 4 °C and 25 °C. No mould development was noticed at day 49 and 28 for samples stored at cold and ambient temperature, respectively. Soylu et al. (2010) sprayed tomato plants with oil-in-water (O/W) emulsions prepared with different EOs (oregano, lavender and rosemary) to control fungal development. The obtained results showed that oregano EO proved the most effective against *Botrytis cinerea* (77% of protection by using 75 mg/L of oregano EO; and 0% of protection in control samples) and no signs of phytotoxicity were found on the plants treated at the maximum concentration used.

Several studies have dealt with the use of coatings to extend the shelf life of fruit products that are susceptible to fungi contamination due to their composition (high water and fructose content). Fuji apples were coated by dipping them in solutions prepared by mixing apple puree and an alginate solution with EOs (Rojas-Graü et al., 2007). An alginate-apple puree coating that contained 1% and 1.5% of lemongrass, and 0.5% of oregano, inhibited fungi growth during a 21-day period. In the same context, fresh-cut melon was dipped into an alginate edible coating with EOs (Raybaudi-Massilia et al., 2008) in order to prevent fungi

development. The incorporation of EOs into edible coatings reduced the natural fungi population of fresh-cut melon for 21 days, and cinnamon EO and eugenol displayed the most marked antifungal action (<3 log CFU/g). In another study, cinnamon leaf coatings with pectin have also been employed to prevent grape spoilage by *B. cinerea* (Melgarejo-Flores et al., 2013) revealing that samples treated with coatings, which contained 5 g/L of cinnamon leaf EO, presented 100% fungal decay. Recently, Arancibia et al. (2014) formulated biodegradable bilayer films with soya protein isolate, lignin and formaldehyde that contained citronellal and geraniol to control fungi growth in banana samples during 6 days at 15 °C. The incorporation of the films coatings with EO reduced fungi counts; especially at the end of the storage period, when mould and yeast counts were below 0.1 log CFU/g in treated bananas.

In addition to emulsions, nanoemulsions and edible coatings, active packaging by incorporating EOs is another possibility to reduce the impact of EOs on the sensory properties of foods and to extend the shelf life of food products. The use of active packaging systems with EOs has been employed in sliced bread, cheese and apples (Balaguer et al., 2013). In this context, cinnamon EO incorporated into paraffin as a bioactive coating has been tested against *Rhizopus stolonifer* in sliced bread, where 80% and 90% of inhibition was achieved with 4% and 6% of cinnamon leaf on the coating (Rodriguez et al., 2008). Similarly, Balaguer et al. (2013) evaluated gliadin films that contained 5% of cinnamaldehyde as active packaging of bread and cheese spread. The results revealed that the active packaging of bread slices was effective for delaying fungal growth. The antifungal assays with cheese spread were carried out at 4 °C and the results showed no fungal growth for 30 days. Recently, apples inoculated with a mixture of twelve *Penicillium* spp. strains were treated in a chamber with oregano, cinnamon and clove EO. Apparently, 14% of the control apples did not present signs of infection compared with 39-42% of the samples treated with EOs, where oregano EO was still the most effective after 21 storage days, followed by clove and cinnamon EOs (Frankova et al., 2016).

2.2 Phenolic compounds

Phenolic compounds constitute the main class of plant secondary metabolites with more than 8,000 identified phenolic structures. They are present in fruits, legumes, vegetables and whole grains (Pulido et al., 2000). **Figure 2** shows a simplified classification of phenolic compounds that possess biological activity.

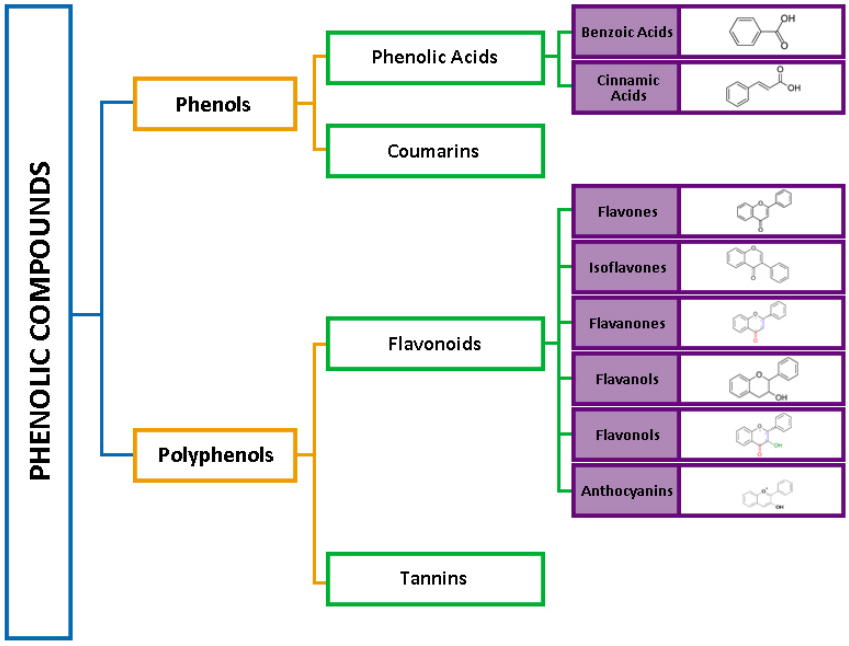


Figure 2. Simplified classification of phenolic compounds with biological activity (Adapted from Hurtado-Fernández et al., 2010).

2.2.1 Mode of action

The mode of action of phenolic compounds is related to: i) membrane dysfunction and disruption, which leads to the dissipation of the pH gradient and electrical potential, interference with the ATP-system in the cell, inhibition of enzymes, inhibition of germination, suppression of mycelia development and germ tub elongation (El-Mogy and Alsanius, 2012; da Cruz-Cabral et al., 2013); and ii) the interaction with membrane proteins, whose conformation and functionality change, increasing concentrations of reactive oxygen species (da Rocha Neto et al., 2015).

2.2.2 Incorporation into food systems

Some researchers have reported the effectiveness of different phenolic compounds as protective natural preservatives for inhibiting spoilage fungi in multiple food systems. To this extend, phenolic compounds from edible herbaceous species have been used to prevent growth of *Monilinia laxa* on nectarines and apricots; *Penicillium digitatum* on oranges; and *B. cinerea* on table grapes. Brown rot due to *M. laxa* growth in apricots and nectarines was inhibited by using phenolic compounds after 6 days. Moreover, on post-treatment day 25, a reduction of 92% in *P. digitatum* growth was observed in oranges by the authors, and a reduction of 53% in *B. cinerea* growth was obtained after 6 days of treating table grapes (Gatto et al., 2011).

Effectiveness of esculetin, ferulic acid, quercetin, resveratrol, scopoletin, scoparone and umbelliferone to control *Penicillium expansum* on Golden Delicious and Granny Smith apples was evaluated by Sanzani et al. (2009). Quercetin and umbelliferone proved to be the most effective compounds, and independently of the application methodology (wound or dipping). These phenolic compounds, either alone or combined, prevented Golden Delicious apples from decaying by 86-92% compared to the control samples by wound

application. Differently, the combination of quercetin and umbelliferone by dipping, highlighted that only 33% of apples showed fungal decay after 8 days of treatment compared with 63% of the control samples (Sanzani et al., 2009). Furthermore, Quaglia et al. (2016) reported the effect of spraying pomegranates with phenolic compounds from olive-mill wastewater after inoculation with *Penicillium* sp. The treatment of pomegranate fruits with phenolic compounds at 4 mg/mL and 8 mg/mL lowered the percentage disease index of *Penicillium adametzioides*, and the disease index was 30% and 15%, respectively.

2.3 Glucosinolates

Glucosinolates are secondary metabolites present in plants in the *Brassicaceae, Capparaceae* and *Caricaceae* families. They are found in grains, roots, peduncles and leaves of plants, and their amounts depend on the vital part of plants and maturation stage (Brown et al., 2003). When plants are wounded, glucosinolates are released from vacuoles and hydrolysed by the enzyme myrosinase to produce isothiocyanates (Grubb and Abel, 2006), which are characterised by their high volatility. Numerous reports focused on the antifungal action of glucosinolates, and isothiocyanates have been found (Troncoso et al., 2005).

2.3.1 Mode of action

The hypotheses formulated to elucidate the mechanism of action of glucosinolates include: i) inhibition of oxygen uptake by fungi through the uncouple action of oxidative phosphorylation in the mitochondria of fungi by inhibiting coupling between electron transport and phosphorylation reactions, and thus hindering ATP synthesis (Kojima and Oawa, 1971); ii) formation of reactive oxygen species which leads to an intolerable level of oxidative stress in fungal cells, and irreparable damage (Wang et al., 2010); iii) the non-specific and

irreversible interaction of isothiocyanates with the sulfhydryl groups, disulphide bonds and amino groups of protein and amino acid residues (Kojima and Oawa, 1971; Banks et al., 1986); and iv) the reaction of some glucosinolates, such as butenyl-isothiocyanate, with some enzymes present at the plasma membrane level to produce fungal growth inhibition or cell death (Sikkema et al., 1995).

2.3.2 Incorporation into food systems

Treatments based on atmospheres enriched with glucosinolates have been demonstrated as a good alternative to control fungi spoilage in fruits and vegetables. Troncoso-Rojas et al. (2005) investigated the use of benzyl-isothiocyanate to control *Alternaria alternata* growth on tomatoes. The results indicated that this compound reduced fruit disease compared with the control samples.

Other works have suggested the use of different glucosinolates to control fungi growth on peppers, blueberries, apples and strawberries. Green bell peppers were exposed to isothiocyanates to investigate their antifungal potential against *A. alternata*. To this end, a sterile filter paper was soaked with the glucosinolates solution and placed inside the bag with the samples. The results showed that the effect of 0.56 mg/mL of isothiocyanate was determinant in reducing fungi rot (Troncoso et al., 2005). Furthermore, vaporisation with allyl-isothiocyanates on blueberries has proven effective against fungi by showing a 3% fungal decay after 21 days of treatment (Wang et al., 2010). Wu et al. (2011) treated apples with isothiocyanate vapours to control *B. cinerea* and *P. expansum* spoilage. The combination of different isothiocyanates reduced fungal growth incidence by up to 85%. Likewise, Ugolini et al. (2014) treated strawberries infected naturally with *B. cinerea* by using an atmosphere enriched with pure allyl-isothiocyanate or one derived from defatted seed meals of *Brassica carinata*. This treatment significantly reduced the decay produced by *B. cinerea* by over 47% and up to 91% for 2 strawberry varieties.

2.4 Other compounds

Some natural constituents, such as hexanal, hexanol, 2-(E)-hexenal, trans-2-hexenal, and 2-nonanone, which are responsible for the aroma of some vegetables and fruits, protect damaged areas from fungi proliferation (Gardini et al., 2002). Their use also provides changes in the flavour and quality of food products owing to the presence of volatiles (Utama et al., 2002).

2.4.1 Mode of action

Even though the mechanism of action of some volatile compounds like antifungals is not clear, most authors agree that: i) the interaction of these compounds with protein sulfhydryl and amino groups causes severe damage to fungal membranes and cell walls, which results in the collapse and deterioration of hyphae (Andersen et al., 1994; Fallik et al., 1998); and ii) membrane disruption, which in turn produces leakage of electrolytes, reduces sugar and, amino acids from cells (Song et al., 2007).

2.4.2 Incorporation into food systems

The application of natural extracts, such as hexanal, trans-2-hexenal, and 2-nonanone, into active packages improves the release of bioactive compounds during storage and diminishes the development of undesirable flavours in foodstuffs (Soares et al., 2009).

Numerous works have reported the use of volatile compounds to prevent fungi development in fruits. Pears, apples, tomatoes, peaches, raspberries and strawberries are some fruits that have been studied by different authors to test the effect of volatile compounds to control fungal growth. Neri et al. (2006a) evaluated trans-2-hexenal antifungal activity on pears and apples. According to the authors, efficacy ranged from 53.6% to 97.8% in pears and apples,

respectively. In another study, Neri et al. (2006b) investigated the effectiveness of trans-2-hexanal against *P. expansum* in pears wounded with a conidial suspension. In this case, the most marked reduction in infection compared with the control samples took place in pears which were treated 24 h after pathogen inoculation (Effectiveness Index: 96.2%).

In the same way, hexanal fumigations have been used to prevent mould growth in apples, tomatoes, raspberries and peaches. Apple treatments with hexanal vapours to control fungi development and lesions were evaluated by Fan et al. (2006). Apples were exposed to different concentrations of hexanal vapours at several temperatures. At 4 °C and with 5-7 µmol/L, only 52% of the apples developed lesions, whereas 98% of those treated with 8-12 µmol/L at 22 °C exhibited lesions. Similarly, the incidence obtained from natural raspberry decay caused by *B. cinerea*, after exposure at different concentrations of hexanal vapours, was evaluated (Song et al., 2007). The results highlighted that the incidence of decay lowered by 30% for all the raspberry varieties. Furthermore, Utto et al. (2008) treated tomatoes with hexanal vapours to study their antifungal activity. The tomatoes treated with 200-270 µL/L exhibited 40% of fungal growth. Finally, the use of 2- nonanone to prevent *B. cinerea* growth on strawberries has been investigated by Almenar et al. (2007). Treated fruit presented neither visible injuries nor fungi development, whereas 10% of the control strawberries showed *B. cinerea* development.

3. ANTIFUNGALS OF ANIMAL ORIGIN

Antifungals from animal sources involve compounds that are isolated from animals or are animal-derived. Chitin, chitosan and lactoferrin are the most widely used antifungals that derive from animals.

3.1 Chitin and chitosan

Chitin is an abundant biopolymer found in the exoskeleton of arthropods and crustaceans, fungal cell walls, and other biological materials. Crustacean shells, like those from carbs and shrimps, are the most widely used chitin sources for commercial applications given the availability of waste from seafood processing industries (Hamed et al., 2016).

Chitosan is derived from chitin by deacetylation in alkaline media. It is a copolymer which consists in β-(1–4)-2-acetamido-D-glucose and β-(1–4)-2-amino-D-glucose units. Chitosan exhibits different biological properties, including antifungal, antibacterial and antiviral activities (Chirkov, 2002). Because of its wide biological spectrum, an increasing interest for applications of coatings for perishable foods has been observed in the last few years (Raafat et al., 2008).

3.1.1 Mode of action

Different mechanisms of action of chitosan have been reported in the literature: i) interferences with uptake of minerals, such as Ca^{2+} or other nutrients, that delay the spore germination process (Plascencia-Jatomea et al., 2003); ii) interaction on the spore wall that inhibits spore germination (Plascencia-Jatomea et al., 2003); iii) induction of cell leakage by stress (Zakrzewska et al., 2005); iv) membrane permeabilisation through specific interactions with high-affinity binding sites on the fungal surface (Zakrzewska et al., 2005; Park et al., 2008; Galván Márquez et al., 2013); and v) interactions with DNA and/or RNA which, in turn, inhibit protein synthesis (Galván Márquez et al., 2013).

3.1.2 Incorporation into food systems

Chitosan has demonstrated its great antifungal activity in various food commodities. Chitosan has been approved by the FDA as a GRAS food additive (USFDA, 2013), and its application in the industry is safe for both consumers and the environment (Romanazzi et al., 2017). **Table 2** summarises the relevant antifungal effect of chitosan applications on different plants, seeds and food commodities.

Chitosan has been used to prevent grey mould provoked by *B. cinerea* in cucumber plants (Ben-Shalom et al., 2003) by spraying solutions of chitosan or a chitin oligomers mixture. Treatment proves effective as it lowers the disease index compared with the control plants. Moreover, the antifungal effectiveness of chitosan on the development of *Fusarium oxysporum* f. sp. *albedinis (Foa)*, the agent responsible for Bayoud disorder, in date palm roots was elucidated (El Hassni et al., 2004). In this case, when seedling roots of date palm were treated with the chitosan solution and inoculated with the mould, the seedling mortality lowered.

Rodríguez et al. (2007) investigated the efficacy of chitosan and hydrolysed chitosan to induce enzymatic activities against *Pyricularia grisea* when applied to rice seeds (*Oryza sativa L.*), and also its results on leaf blast intensity in seedlings. For both chitosan types, the greatest disease defence in rice seedlings was obtained using 1,000 mg/L after 14 days of inoculation. Nevertheless, when applying 500 mg/L of chitosan and hydrolysed chitosan, severity was evidenced 14 days after treatment.

Several authors have highlight that grey mould on grapes is one of the most economically important disorders of grapevine and table grapes worldwide (Reglinski et al., 2010; Vasconcelos de Oliveira et al., 2014). The antifungal activity of chitosan against *B. cinera* on Chardonnay grape leaves was studied by Reglinski et al. (2010). Chardonnay leaves treated with chitosan proved more resistant to infection. Moreover, the effectiveness of chitosan on postharvest fungus infection by *B. cinerea* and *P. expansum* on grapes was elucidated (Vasconcelos de Oliveira

et al., 2014). Chitosan treatment reduced the number of infected plants compared with untreated samples.

Some studies have employed chitosan in combination with other biopolymers or antifungal substances used by the food industry as edible coatings or packaging materials to control fungal spoilage in different fruits. Vu et al. (2011) coated berries with modified-chitosan films that contained limonene and peppermint. Strawberries were sprayed, dried and stored for 14 days at 4 °C. On day 8, 45% of the fruits displayed some decay when chitosan-limonene films were applied, whereas the percentage of decay lowered by up to 60% when chitosan alone was applied or chitosan-peppermint was employed.

Some authors have also coated strawberry fruits by dipping them into chitosan solutions. Different combinations of chitosan and calcium gluconate solutions were prepared by Hernández-Muñoz et al. (2008) to coat strawberries. The combination of chitosan and calcium gluconate at a ratio of 1:0.5 has inhibited fungal fruit decay during storage. Conversely, fungal decay was observed in samples coated with chitosan solution. The results showed that only 35% of strawberries were infected after 6 days of treatment when 1% of chitosan was employed. Similarly, strawberries were treated with film-forming dispersions of the chitosan-lemon EO. The results showed that pure chitosan coatings reduced the percentage of samples that displayed visual mould growth compared with the control strawberries. These results confirmed that chitosan antifungal action improved when the lemon EO was incorporated (Perdones et al., 2012).

Sánchez-González et al. (2011) dipped grapes in film-forming dispersions prepared with chitosan, hydroxypropylmethylcellulose and the bergamot EO to prevent their spoilage. The development of moulds and yeast after 18 days were 0.25 log CFU/g and 0.1 log CFU/g for the control and coated samples with the chitosan and bergamot EO, respectively.

The effect of alginate, chitosan, and their combinations, on *Colletotrichum musae* growth has been studied in bananas (Maqbool et al., 2010) by simulating marketing conditions (5 days/25 °C/60% RH). The authors suggested that the bananas coated with alginate would show no fungicidal activity. In fact, the fruits with alginate and chitosan delayed anthracnose for 28 days and food freshness was maintained. Chitosan has been also employed on dry fruit. Walnut kernels were immersed in a coating solution that contained chitosan and green tee extracts (Sabaghi et al., 2015). As a result, chitosan coatings reduced yeast and mould growth on samples. In some samples with green tea and chitosan (10 g/L), no yeast and mould growth was detected throughout the storage period.

3.2 Lactoferrin

Lactoferrin is an iron-binding glycoprotein present in milk and often employed as an antimicrobial agent in human medicine and food preservation. The protein is folded into two homologous globular lobes connected by a short α-helix peptide (Berlutti et al., 2011). Among its protective effects, several authors have reported antifungal activity (Wei et al., 2008; González-Chávez et al., 2009).

3.2.1 Mode of action

The mechanism of action of lactoferrin appears to be complex. Some researchers have suggested that the antifungal mode of action of lactoferrin might be related to: i) membrane rupture and leakage of intracellular proteins and sugars, which inhibit fungal growth (Wang et al., 2013; González-Chávez et al., 2009); ii) reduced ATP production as a result of inhibited mitochondrial respiration (Wang et al., 2013); iii) oxidative injuries (Wang et al., 2013); and iv) Fe^{3+} chelation (Zarember et al., 2007).

3.2.2 Incorporation into food systems

Very few studies are available in the literature that have reported antifungal effects of lactoferrin on food products. In one, Wang et al. (2013) sprayed tomato plants with a solution prepared with different lactoferrin concentrations and Tween 80. Treatment lasted 24 h before *B. cinerea* inoculation took place. The index of the samples that displayed visual mould growth lowered as the lactoferrin dose increased. Therefore, it can be stated that lactoferrin solution was able to protect more than 50% of samples when 100 mg/L of lactoferrin solution was used.

4. ANTIFUNGALS OF MICROBIOLOGICAL ORIGIN

Biopreservation or use of microorganisms and/or their metabolites to prevent fungi spoilage and to extend the shelf life of foodstuffs has attracted growing interest in the scientific and industry areas owing to changes in consumer opinions and demand (Le Lay et al., 2016). For these reasons, alternative methodologies to control postharvest loss caused by fungi have been investigated. Among natural biological agents, attention has been paid to lactic acid bacteria (LAB) and antagonistic yeasts as a result of their excellent effectiveness as antifungal agents (Leroy and De Vuyst, 2004; Gerez et al., 2013) (**Figure 3**).

Table 2. Relevant chitosan applications on plants, seeds and food products (Adapted from Kashyap et al., 2015).

Plant/harvest	Organisms	Effect of chitosan application	References
Cucumber plant	*Botrytis cinerea*	Control the grey mould caused by *Botrytis cinerea*.	(Ben-Shalom et al., 2003)
Roots and seedlings of date palm	*Fusarium oxysporum* f. sp. *albedinis (Foa)*	Reduction of the growth of *Foa* and production of morphological changes in *Foa* mycelium.	(El Hassni et al., 2004)
Seeds of rice	*Pyricularia grisea*	Defense response induction associated with the concentration and type of chitosan. Symptoms of resistance were observed.	(Rodríguez et al., 2007)
Strawberries	Fungi	The combination with calcium gluconate inhibited strawberries decay.	(Hernández-Muñoz et al., 2008)
Banana	*Colletotrichum musae*	Chitosan and alginate combinations inhibited *C. musae* growth.	(Maqbool et al., 2010)
Grapevine leaves	*Botrytis cinerea*	Suppression of mould development on detached grapevine leaves.	(Reglinski et al., 2010)

Table 2. *(Continued)*

Plant/harvest	Organisms	Effect of chitosan application	References
Grapes	Moulds and yeasts	The combination with hydroxypropylmethylcellulose and bergamot oil reduced mould and yeasts development on grapes.	(Sánchez-González et al., 2011)
Strawberries	Moulds	Chitosan films alone or combined with limonene and peppermint reduced fruit decay.	(Vu et al., 2011)
Strawberries	*Botrytis cinerea*	Reduction of the percentage of infected fruits and chitosan coatings with lemon oil presented great anti-*Botrytis* action.	(Perdones et al., 2012)
Grape	*Botrytis cinerea and Penicillium expansum*	Fungal growth was delayed when chitosan was applied as a coating on table grapes artificially contaminated with fungi spores.	(Vasconcelos de Oliveira et al., 2014)
Walnut kernels	Moulds and yeasts	Reduction of mould and yeasts growth in chitosan coatings and inhibition of fungal growth in chitosan-green tea coatings.	(Sabaghi et al., 2015)

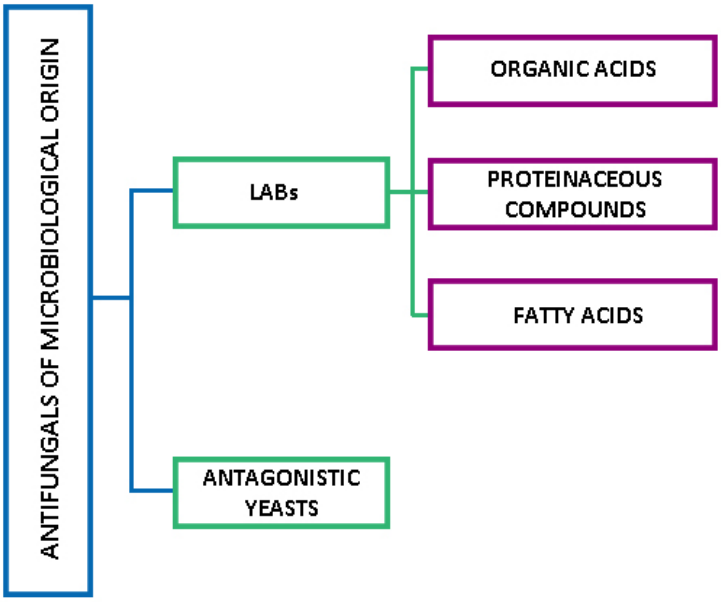


Figure 3. Classification of the antifungals of microbiological origin.

4.1 LABs

LABs are Gram (+), non-sporulating, catalase-negative, acid-resistant and anaerobic aerotolerant microorganisms. Eleven genera have been associated with food products: *Carnobacterium, Enterococcus, Lactococcus, Lactobacillus*, *Lactosphaera, Leuconostoc, Oenococcus, Pediococcus, Streptococcus*, *Vagococcus* and *Weissella* (Vries et al., 2006).

LABs have obtained the GRAS and Qualified Presumption of Safety (QPS) status by the FDA and EU, respectively. They are used in the food industry for their capacity to control fungi spoilage and/or pathogen microorganisms by producing different antimicrobial compounds (Martinez et al., 2013). The main

identified antifungal metabolites include organic acids, proteinaceous compounds and fatty acids: i) the organic acids present in food commodities are additives or carbohydrate end-products produced by LABs, which include acetic, lactic and propionic acids as the main compounds originated by carbohydrate LABs fermentation (Ross et al., 2002); ii) bacteriocins, are a kind of ribosomal synthesised antifungal peptides or proteins (Nes et al., 1996). Various studies describe the production of these compounds originated by *Lactococcus, Streptococcus, Lactobacillus* and *Pediococcus* (Crowley et al., 2013); and iii) fatty acids perform an important function in antifungal activity and require at least one hydroxyl group and one double bond along the carbon backbone (Crowley et al., 2013).

4.1.1 Mode of action

The mode of action of LABs could be related to their stationary phase. There is a possibility that cell lyses could contribute to fungal toxicity. Likewise, other mechanisms that could explain the antifungal effect of LABs include their competition for nutrients, space and exclusion of pathogens from entry sites in the matrix, and alteration of spore membrane, viscosity and permeability (Pawlowska et al., 2012).

Regarding the mechanism of action of LAB products, some authors have put forward the following hypothesis: i) organic acids defuse through the membrane and cause its dissociation by releasing hydrogen ions which, in turn, cause pH to drop. Organic acids also increase membrane permeability and neutralise the electrochemical proton gradient (Oliveira et al., 2014); ii) bacteriocins provoke fungal cell membrane disruption, changes in their permeability to small monovalent cations (K^+) and a large macromolecule like ATP (Sharma and Srivastava, 2014); and iii) fatty acids contribute to high membrane fluidity due to a low sterol content and a high degree of phospholipid fatty acid instauration (Benyagoub et al., 1996).

4.1.2 Incorporation into food systems

Utilisation of LABs to prevent fungi spoilage has been studied in different foodstuffs, which include fruits and vegetables, bakery and dairy products (**Table 3**).

4.1.2.1 Fruits and vegetables

To prevent fungal spoilage on fruits and vegetables, LABs have been employed as promising antifungal agents. Sathe et al. (2007) evaluated the antifungal activity of *Lactobacillus plantarum* CUK501 to prevent fungal spoilage of cucumber provoked by the inoculation of *Aspergillus flavus*, *Fusarium graminearum*, *R. stolonifer* and *B. cinerea*. This treatment effectively avoided the lesions provoked by all the evaluated moulds. In another study, apples were used as models to investigate the antifungal action of *Pediococcus pentosaceus* R47 against *P. expansum* (Rouse et al., 2008). The authors pointed out that no mould growth was detected during the 14-day study. Similarly, Crowley et al. (2012) studied the antifungal effect of *Lb. plantarum* 16 and 62 in orange juice against *Rhodotorula mucilaginosa* and *P. expansum*. The authors highlighted that *R. mucilaginosa* counts were under 10^1 CFU/mL from days 8 to 25 and 8 to 14 for *Lb. plantarum* 16 and 62, respectively.

Grapes have been also employed as a model to determine the antifungal potential of LABs (*Weissella cibaria* 861006 and *Weisella paramesenteroides* 860509) against *Penicillium oxalicum* (Lan et al., 2012). After 2 days, hyphal development was perceived on the surface of the control samples, but no fungal hyphae were detected on the surface of the grapes treated with *W. cibaria* 861006 until day 6 of treatment.

Table 3. LABs in fruit and vegetables, bakery products and dairy products (Adapted from Crowley et al., 2013).

Food products	Antifungal LAB	Organism	Reference
Fruits and vegetables			
Cucumber	*Lactobacillus plantarum* CUK501	*Aspergillus flavus, Fusarium graminearum*, *Rhizopus stolonifer, Botrytis cinerea* and *Sclerotinia minor*	(Sathe et al., 2007)
Apples	*Pediococcus pentosaceus* R47	*Penicillium expansum*	(Rouse et al., 2008)
Orange juice	*Lactobacillus plantarum* 16 and 62	*Rhodotorula mucilaginosa* *Penicillium expansum*	(Crowley et al., 2012)
Grapes	*Weissella cibaria* 860106 and *Weissella paramesenteroides* 860509	*Penicillium oxalicum*	(Lan et al., 2012)
Kumquats	*Lactobacillus plantarum* IMAU10014 and a shuffled mutant strain (F3A3)	*Penicillium digitatum*	(Wang et al., 2013)
Jackfruit	*Lactococcus lactis* subsp. *lactis*	*Rhizopus stolonifer*	(Ghosh et al., 2015)

Table 3. *(Continued)*

Food products	Antifungal LAB	Organism	Reference
Bakery products			
Bread	*Lactobacillus plantarum* CRL 778, *Lactobacillus reuteri* CRL 1100, *Lactobacillus brevis* CRL 772 and CRL 796	*Aspergillus, Fusarium,* and *Penicillium* species	(Gerez et al., 2009)
Bread	*Lactobacillus amylovorus* DSM 19280	*Fusarium culmorum* FST 4.05, *Aspergillus niger* FST4.21, *Penicillium expansum* FST 4.22, and *Penicillium roqueforti* FST 4.11	(Ryan et al., 2011)
Bread	*Lactobacillus plantarum* LB1 *Lactobacillus rossiae* LB5	*Penicillium roqueforti* DPPMAF1	(Rizzello et al., 2011)
Bread Panettone	*Lactobacillus rossiae* LD108, and *Lactobacillus paralimentarius* PB12	*Aspergillus japonicus, Eurotium repens* and *Penicillium roseopurpureum*	(Garofalo et al., 2012)
Bread	*Lactobacillus sakei* KTU05-6, *Pediococcus acidilactici* KTU05-7, *Pediococcus pentosaceus* KTU05-8, *Pediococcus pentosaceus* KTU05-9 and, *Pediococcus pentosaceus* KTU05-10	Moulds	(Cizeikiene et al., 2013)
Bread	*Lactobacillus amylovorus* DSM19280	Moulds	(Axel et al., 2015)

Table 3. *(Continued)*

Food products	Antifungal LAB	Organism	Reference
Dairy products			
Yoghurt and Cheese surface	*Propionibacterium jensenii* SM11, *Lactobacillus paracasei* subsp. *paracasei* SM20, *Lactobacillus paracasei* subsp. *paracasei* SM29, and *Lactobacillus paracasei* subsp. *paracasei* SM63	*Candida pulcherrima, Candida magnoliae, Candida parapsilosis* and *Zygosaccharomyces bailii*	(Schwenninger & Meile, 2004)
Yoghurt	*Lactobacillus rhamnosus* K.C8.3.1l, *Lactobacillus paracasei* K.C8.3.1Hc1, *Lactobacillus zeae* K.V9.3.1Ng and *Lactobacillus harbinensis* K.V9.3.1Np	*Debaryomyces hansenii*, *Kluyveromyces lactis*, *Kluyveromyces marxianus*, *Penicillium brevicompactum*, *Rhodotorula mucilaginosa* and *Yarrowia lipolytica*	(Delavenne et al., 2013)
Yoghurt	*Lactobacillus casei* AST18	*Penicillium* sp.	(Li et al., 2013)
Cottage cheese	*Lactobacillus plantarum* isolates	*Penicillium commune*	(Cheong et al., 2014)
Cheddar cheese	*Lactobacillus amylovorus* DSM 19280	*Penicillium expansum* FST 4.22	(Lynch et al., 2014)
Yoghurt	*Lactobacillus paracasei* DGCC 2132	*Penicillium* sp. nov. DCS 1541 and, *Penicillium solitum* DCS 302	(Aunsbjerg et al., 2015)

In order to investigate the antifungal bio-preservation potential of *Lb. plantarum* IMAU10014 and a shuffled mutant strain F3A3, kumquats were infected with *P. digitatum* by Wang et al. (2013). The results revealed mycelia and spores on the control samples 2 treatment days. However, no mould growth was observed on the kumquats sprayed with the F3A3 strain, which is a promising bio-preservative agent. Gosh et al. (2015) employed jackfruit to study the antifungal activity of LAB against *R. stolonifer*. To this end, harvested jackfruits were treated with *Lactococcus lactis* subsp. *lactis* followed by an application of fungal spores. Only 4-27% rot was observed on jackfruits after 15 days of treatment with the LAB.

4.1.2.2 Bakery products

Bakery products are a problem for the food industry since fungal development provokes high economic loss and health costs (Garofalo et al., 2012; Crowley et al., 2013). For this reason, Gerez et al. (2009) evaluated the ability of LABs to inhibit *Aspergillus, Fusarium*, and *Penicillium* in bread. In this case, LABs were used in the formulation of a mixed starter culture with *Saccharomyces cerevisiae* in bread making. Loaves of bread were surface-sprayed with a conidial suspension of *Aspergillus niger*. When the LAB was used, fungal growth was delayed for 5 days compared to the control samples. A similar methodology was employed with slices of bread to determine the antifungal activity of *Lactobacillus amylovorus* strains. The capacity of both *Lb. amylovorus* strains to inhibit the environmental mould outgrowth in an industrial bakery was studied (Ryan et al., 2011). Therefore, *Lb. amylovorus* sourdough bread delayed the outgrowth of the environmental fungi. Certainly, the maximum shelf life was obtained for the *Lb. amylovorus* sourdough bread tested against the mould flora obtained in the bakery (**Table 3**).

Furthermore, bread slices of sourdough fermented wheat germ bread with *Lb. plantarum* LB1 and *Lactobacillus rossiae* LB5 were nebulised with *Penicillium roqueforti* DPPMAF1 to study their antifungal activity (Rizello et al., 2011). After

21 days of inoculation, mycelial growth was visible in slices of sourdough-fermented wheat germ bread. The contamination score proposed by the authors suggested 10% contamination.

Garofalo et al. (2012) investigated the antifungal effectiveness of *Lb. rossiae* LD108 and *Lactobacillus paralimentarius* PB127 against *Aspergillus japonicus* M1 on biologically acidified dough prepared with sourdoughs to make bread and panettone. The sourdoughs inoculated with *Lb. rossiae* LD 108 were able to prolong the shelf life of this product by around 30 days compared to baker's yeast bread (11 days). On the contrary, the sourdoughs inoculated with *Lb. paralimentarius* PB127 prolonged shelf life by only 19 days compared with baker's yeast bread which prolonged the shelf life of the sample 39 days. The results revealed that the antifungal activity of *Lb. rossiae* LD108 sourdough allowed fungal growth to be inhibited for over 4 months in panettone cakes contaminated with *A. japonicus* M1 spores.

The antifungal activity of *Pediococcus pentosaceus* KTU05-9 used as a starter on wheat bread samples that contained sourdough, and the antifungal activity of *Pediococcus acidilactici* KTU05-7, *P. pentosaceus* KTU05-8 and KTU05-10, sprayed on the bread surface against moulds, was evaluated by Cizeikiene et al. (2013). Their results highlighted that the addition of sourdough prepared with *P. acidilactici* KTU05-7, *P. pentosaceus* KTU05-8 and KTU05-10 reduced fungal spoilage of bread better than the control samples. The *P. acidilactici* KTU05-7, *P. pentosaceus* KTUO5-8 and KTU05-10 single cell suspensions, sprayed on bread surfaces, inhibited the growth of fungi over an 8-day storage period, whereas the control bread exhibited visual fungi colonies. Recently, Axel et al. (2015) tested *Lb. amylovorus* DSM19280 antifungal activity as a starter culture in sourdough. Therefore, slices of bread were exposed to the bakery environment for 5 min on each side and were then packed in sterile bags. The addition of *Lb. amylovorus* DSM19280 prolonged the bread shelf life by 4 days compared to the control samples, where moulds were visible after 2 days.

4.1.2.3 Dairy products

Dairy products are also susceptible to fungal attack and LABs could be used to prevent fungi contamination in cheese and yoghurt products (Table 3). On this view, Schwenninger and Meile (2004) tested the antifungal activity of *Propionibacterium jensenii* SM11 and *Lactobacillus paracasei* subsp. *paracasei* strain SM20, SM29 or SM63 against yeasts, on yoghurt and cheese surfaces. Yoghurts prepared with *P. jensenii* SM11 and *Lb. paracasei* subsp. *paracasei* strains SM20, SM29 or SM63 were contaminated with *Candida pulcherrima*, *Candida magnoliae*, *Candida parapsilosis* and *Zygosaccharomyces bailii.* According to the authors, the samples treated with lactobacilli and propionibacteria at the 10^8 CFU/ mL concentration showed a constant number of yeasts during the evaluation period at levels of 10^2 CFU/mL. Moreover, cheese samples, were permeated in a protective culture (*P. jensenii* SM11 and *Lb. paracasei* subsp. *paracasei* SM20, SM29 or SM63). Afterwards, samples were contaminated with *C. pulcherrima*, *C. magnoliae*, *C. parapsilosis* and *Z. bailii*. The cheeses formulated with *P. jensenii* SM11 and *Lb. paracasei* subsp. *paracasei* SM20 presented yeast development (4.11 log CFU/g on treatment day 21). Likewise, Delavenne et al. (2013), employed yoghurt formulated with *Lactobacillus harbinensis* K.V9.3.1Np, *Lactobacillus rhamnosus* K.C8.3.1I and *Lb. paracasei* K.C8.3.1Hc1 and *Lactobacillus zeae* K.V9.3.1Ng to evaluate their antifungal activity against *Debaryomyces hansenii, Rhodotorula mucilaginosa, Yarrowia lipolytica, Penicillium brevicompactum, Kluyveromyces lactis* and *Kluyveromyces marxianus.* Hence, the surface of yoghurts was contaminated by inoculating the target fungi. Only the *Lb. rhamnosus* K.C8.3.1I strain was able to inhibit *R. mucilaginosa* growth (<2 log CFU/g). Conversely, fungi strains were completely inhibited (<2 log CFU/g) in the samples formulated with *Lb. harbinensis* K.V9.3.1Np. Similarly, Li et al. (2013) studied the antifungal potential of *Lactobacillus casei* AST18 to control spoilage of yoghurts from *Penicillium* sp. In this case, no fungal development was noted in the samples maintained at 4 °C, whereas the

samples stored at 30 °C exhibited mould development after 10 treatment days for the 2% and 4% AST18-added yoghurt samples. The samples that contain 6% and 8% of *Lb. casei* AST18 presented mould growth after 14 days. Indeed, 18 days after beginning treatment, all the samples presented mould development.

Cheong et al. (2014) tested the antifungal effect of *Lb. plantarum* isolates on cottage cheese against *Penicillium commune*. To this end, cheese samples were inoculated with LAB and incubated for 2 days at 24 °C. Cottage cheese samples treated with *Lb. plantarum* isolates started to show mould development 18 days after treatment, while the control samples displayed deterioration after 4 days. Another work focused on preventing the cheese spoilage produced by *P. expansum* FST 4.22, which was carried out by Lynch et al. (2014). Cheese was formulated using a starter mixed with *Lb. amylovorus* DSM 19280 as an adjunct culture. *Lb. amylovorus* DSM 19280 delayed fungi development compared to the control samples (from 8 to 12 days).

Finally, Aunsbjerg et al. (2015) investigated the antifungal action of *Lb. paracasei* DGCC 2132 against *Penicillium* sp. nov. DCS 1541 and *Penicillium solitum* DCS 302. For this purpose, yoghurt samples were inoculated with the target fungi. The results revealed the antifungal properties of LAB against *Penicillium* sp. nov. DCS 1541 and *P. solitum* DCS 302 compared with the control samples after 4 days of treatment at 25 °C.

4.2 Antagonistic yeasts

The use of antagonistic yeasts as biopreservative microorganisms has been studied in depth because they possess some important features that increase their suitability as biocontrol agents. Many have simple nutritional requirements and are able to colonise dry surfaces and to grow on inexpensive substrates in bioreactors (Chanchaichaovivat et al., 2007). Yeast present on fruit surfaces represents the main yeast group used to manage postharvest diseases (Liu et al., 2013).

4.2.1 Mode of action

Numerous authors have suggested different modes of action of antagonistic yeasts against fungi, which seem to be related to: i) antifungal hydrolases (El Ghaouth et al., 2003); ii) production of pigments, which causes iron depletion in the cell environment (Sipiczki et al., 2006); iii) induction of some defence-related proteins attributed to the metabolism of proteins, defence response, transcription, energy metabolism and cell structure (Chan et al., 2007); iv) presence of enzymes associated with sugar metabolism (Chan et al., 2007); v) production of volatile organic compounds (Parafati et al., 2015); vi) tolerance to reactive oxygen species (ROS) (Liu et al., 2012); vii) induction of ROS production in host (Marcarisin et al., 2010); and viii) biofilm formation (Parafati et al., 2015).

4.2.2 Incorporation into food systems

Utilisation of antagonistic yeasts as biocontrol agents has been studied by different authors in apples, grapes, peaches, pears, and strawberries. **Table 4** presents the *in vivo* applications of antagonistic yeasts.

El Ghaouth et al. (2003) determined the prevention of *B. cinerea* disease on apples treated with *Candida saitoana*. No effect on lesion development was noted in the samples treated with the antagonistic yeast 1 day before *B. cinerea* contamination. Conversely, lesion development effectively reduced on the samples to which *C. saitoana* was applied 2 and 3 days prior to mould contamination. In addition, the efficacy of *Candida guillermondii* and *S. cerevisiae* M25 on preserving apples from *P. expansum* was evaluated by Scherm et al. (2003). The lesion diameter was lower than 55% for the *C. guillermondii*-treated samples, but was under 30% for the *S. cerevisiae* M25-treated apples after 7 days of storage. However, all the control samples showed a lesion diameter of 100%.

Li et al. (2011) studied the antagonistic activity of *R. mucilaginosa* against *P. expansum* and *B. cinerea*, which cause grey and blue mould in apples. For this

purpose, apples were wounded and pipetted with different yeast suspensions. The authors revealed that *R. mucilaginosa* inhibited *P. expansum* growth completely, while the disease incidence of the control samples was 97.2%. Indeed, *B. cinerea* decay and lesion diameter decreased by 97.1% and 56.1% in comparison with control samples. Likewise, the efficacy of *Pichia guilliermondii* strain M8 against *B. cinerea* using apples as a model was investigated by Zhang et al. (2011). These authors determined that *P. guilliermondii* M8 was able to lower *B. cinerea* incidence from 45% to 20% compared to the control samples.

Grapes have also been employed as models to describe the antifungal potential of different antagonistic yeasts. *S. cerevisiae* and *Schizosaccharomyces pombe* strains were employed to prevent grey mould on grapes after harvesting (Nally et al., 2012). *S. cerevisiae* and *Sch. pombe* showed a reduction over 70%, whereas 100% of the control samples presented mould development. Calvo-Garrido et al. (2013a) investigated the effectiveness of *Candida sake* CPA-1 combined with Fungicover® against botrytis bunch decay in organic vineyards. The control samples had an incidence and severity of 90% and 22%, respectively. Similarly, the sour rot of grapes to be controlled by biological agents was described by Calvo-Garrido et al. (2013b). The combination of *C. sake* CPA-1 with Fungicover®, *Ulocladium oudemansii,* and chitosan was applied to organic vineyards, which reduced the severity of sour rot from 47% to 70%, compared with the control samples.

Lutz et al. (2013) tested *Cryptoccocus albidus* NPCC 1248, *Pichia membranifaciens* NPCC 1250, *Cryptoccocus victoriae* NPCC 1263 and NPCC 1259 to prevent *P. expansum* and *B. cinerea* growth on pears. The results showed that *C. albidus* NPCC 1248, *P. membranifaciens* NPCC 1250 and *C. victoriae* NPCC 1263 were able to reduce *P. expansum* disease and lesion diameter was ≥ 50%, but was ≥ 30% for *B. cinerea*. The control samples had a disease incidence of 100% for both mould strains, while lesion diameter was 35% and 80% for *P. expansum* and *B. cinerea*, respectively.

Peaches are easily contaminated by *R. stolonifer* in some countries like China, where it is one of the most relevant postharvest problems. For this reason, Xu et

al. (2013) studied the effectiveness of *Pichia caribbica* (JSU-1) against *R. stolonifer* on peaches. The highest concentration of the antagonistic yeast employed in tests, showed the lower incidence (4%) and lesion diameter (26 mm) compared with the control samples (100% and 58 mm, respectively).

Recently, the influence of strawberry preharvest spraying with *Cryptococcus laurentii* on the postharvest decay of fruits was tested against *B. cinerea* by Wei et al. (2014). The disease incidence of grey mould decay was higher than 70% at 4 °C and 20 °C in the control samples. On the contrary, the application on *C. laurentii* at 6, 3 and 0 days prior to harvesting gave the best results, and disease incidence was lower than 22% at both temperatures.

Even though a wide variety of yeasts has been reported to be good postharvest biocontrol agents, very few yeasts that are considered to act as biocontrol products are available on the market (Shemer™, Candifruit™ and Boni-Protect™). Shemer™ (AgroGreen, Asgdod) is a fungicide based on *Metschnikovia fructicola.* It is registered for postharvest use in Israel, but not in Europe. Candifruit™ (SIPCAM INAGRA, S.A., Valencia, Spain) is based on *C. sake,* which has been commercially available only in Spain from 2008 for pome fruits against postharvest pathogens (Mari et al., 2010). Boni-Protect™ (Bio-ferm, Germany) contains two strains of *Aureobasidium pullulans*, isolated from untreated apple trees. Both strains act competitively against fungal pathogens, which is why the development of resistances is not possible. Since 2002, Boni-Protect™ has been used in field trials and can be incorporated without any pre-harvest interval before harvest, or even between picking dates.

Table 4. *In vivo* applications of antagonistic yeasts.

Food products	Antagonistic yeast	Organism	Reference
Apple	*Candida saitoana*	*Botrytis cinerea*	(El Ghaouth et al., 2003)
Apple	*Candida guillermondii* *Saccharomyces cerevisiae* M25	*Penicillium expansum*	(Scherm et al., 2003)
Apple	*Rhodotorula mucilaginosa*	*Penicillium expansum* and *Botrytis cinerea*	(Li et al., 2011)
Apple	*Pichia guilliermondii* M8	*Botrytis cinerea*	(Zhang et al., 2011)
Grapes	*Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*	*Botrytis cinerea*	(Nally et al., 2012)
Grapes	*Candida sake* CPA-1	*Botrytis cinerea*	(Calvo-Garrido et al., 2013a)
Grapes	*Candida sake* CPA-1 and *Ulocladium oudemansii*	*Botrytis cinerea*	(Calvo-Garrido et al., 2013b)
Pear	*Cryptoccocus albidus* NPCC 1248, *Pichia membranifaciens* NPCC 1250, *Cryptoccocus victoriae* NPCC 1263 and *Cryptoccocus victoriae* NPCC 1259	*Penicillium expansum* and *Botrytis cinerea*	(Lutz et al., 2013)
Peaches	*Pichia caribbica* JSU-1	*Rhizopus stolonifer*	(Xu et al., 2013)
Strawberries	*Cryptococcus laurentii* 2.3803	*Botrytis cinerea*	(Wei et al., 2014)

5. FUTURE TRENDS

Despite the promising potential of preventing fungal contamination of natural compounds such as EOs, phenolic compounds and glucosinolates among others, they all have present some important limitations when applied to food products given their impact on the final product's sensory profile (Perdones et al., 2012; Aloui and Khwaldia, 2016). The incorporation of these compounds into O/W emulsions, nanoemulsions, edible coatings and active packaging could overcome these drawbacks, but these techniques do not allow to completely mask the flavour of these antifungal agents. New studies now focus on studying the immobilisation of EOs on the surface of different materials. Immobilisation guarantees microbial action, while the natural agent's volatility is suppressed (Chen et al., 2009; Gharbi et al., 2015; Higueras et al., 2015). The immobilisation process has being used to develop antimicrobial materials that contain peptides and enzymes (Yala et al., 2011; Hanušová et al., 2013), but further research should centre on the practical applications of these innovative systems, particularly on testing the fungicidal effect on packaged foodstuffs during the completely storage period. Toxicity studies should also be carried out to confirm the safety of these materials before being employed in food commodities.

Recently, filters impregnated with silver nanoparticles have been used as antimicrobial agents, and have suggested that this technique can be further used by the food industry in a vast variety of liquid products thanks to its low cost and high efficiency (van Halem et al., 2009; van Erven Cabala et al., 2015; Fernández et al., 2016). These promising results offer new uses of antifungals such as EOs or glucosinolates immobilised on different materials and impregnated on biodegradable filters to preserve liquid food products.

The persistence of antifungal activity during storage periods is another problem to solve. The efficacy of emulsions, edible coatings, and active packaging materials that contain active compounds diminishes with time when applied to food systems (Sung et al., 2013; Nguyen Van Long et al., 2016). To this end,

further studies that focus on developing new antifungal systems which can be restored against losses of active compounds are required.

Regarding the use of biocontrol agents, only some antagonistic yeasts are commercially available. Many present poor performance and inconsistency in commercial circumstances as knowledge about their mode of action is scarce (Spadaro and Droby, 2016; Romanazzi et al., 2016). Future trends should focus on understanding the interactions among antagonistic yeasts, fungi, fruits and the microenvironment in order to develop economical and great formulations and operation processes. Antagonistic yeasts should be incorporated before pathogenic fungi establish on fruits to thus avoid their infection as a result of preventing propagules of pathogenic fungi on the host superficies (Romanazzi et al., 2016). Epiphytic microflora studies should be also considered since microbial communities present on fruit surfaces could affect disease control through their interaction with host fruits, the pathogenic fungi, and biocontrol agents (Massart et al., 2015).

Interactions between plant or animal antifungals with the macromolecules present in food products and external factors should also be studied. This information will provide reliable data to producers and consumers about their advantages compared with chemical additives and fungicides.

In addition, despite the number of works conducted on the laboratory, very few have been done on a large-scale. Hence, further studies conducted in industrial trials and field or greenhouse trials to test different antifungal emulsions, nanoemulsions, coatings and biocontrol agents are needed.

6. CONCLUSIONS

This review shows the main advantages of using different natural antifungals to preserve food products from fungi spoilage. In general, plant EOs, chitosan and biocontrol agents have attracted growing interest of researchers for being used as food preservatives given their antifungal, antimycotic and pest control properties. New strategies are being studied to overcome the drawbacks of using some natural antifungals, such as sensory modifications, which essential oils, phenolic compounds, glucosinolates or other volatile compounds cause in food products. The protection or encapsulation of these natural agents into oil-in-water emulsions and nanoemulsions, their incorporation into edible films and active packaging systems, and their combination with other natural compounds, such as chitosan or phenolic extracts, improve their antifungal properties while reducing their doses. In the same way, use of microorganisms and/or their metabolites to control fungi spoilage is a good alternative to chemical additives for a wide variety of food products.

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2. OBJECTIVES

The general objective of this Doctoral Thesis iss to study the use of different antifungal systems as O/W emulsions and nanoemulsions and mesoporous silica particles, to control deterioration produced by moulds and yeasts in strawberry jam.

In order to achieve the above-mentioned objective, the following specific objectives are established:

- To evaluate the influence of the formulation of nanoemulsion on the *in vitro* antifungal activity of the cinnamon leaf, lemon and bergamot oil nanoemulsions against *A. niger*.
- To elucidate the effect of the clove and cinnamon leaf emulsion formulations on the physico-chemical and antifungal activity against genera *Aspergillus* and *Penicillium*; and to evaluate the impact of these emulsions on the sensory profile of strawberry jam.
- To optimise the methodology used to prepare stable O/W emulsions in order to reduce losses of EO during preparation.
- To analyse the feasibility of using cinnamon bark-xanthan gum emulsions to control the deterioration of strawberry jam caused by *A. flavus*, *A. niger, P. expansum, Z. bailii* and *Z. rouxii*, and the product's sensory profile after incorporating emulsions.
- To study the *in vitro* and *in vivo* antifungal properties of the stable O/W emulsions prepared with different antifungal agents (cinnamon bark essential oil, zinc gluconate and *trans*-ferulic acid) against *A. niger,* and their sensory impact on strawberry jam.
- To evaluate the immobilisation of eugenol and thymol on the surface of mesoporous silica particles to control the strawberry jam spoilage caused by *A. niger* and *Z. bailii,* and to reduce the impact on the jam's sensory profile.

3. CHAPTERS

CHAPTER 1

USE OF OIL-IN-WATER EMULSIONS AS POTENTIAL ANTIFUNGAL DELIVERY SYSTEMS

SECTION 1.1 INFLUENCE OF EMULSIFIER TYPE ON THE ANTIFUNGAL ACTIVITY OF CINNAMON LEAF, LEMON AND BERGAMOT OIL NANOEMULSIONS AGAINST Aspergillus niger

SECTION 1.2 USE OF OIL-IN-WATER EMULSIONS TO CONTROL FUNGAL DETERIORATION OF STRAWBERRY JAMS

SECTION 1.3 APPLICATION OF CINNAMON BARK EMULSIONS TO PROTECT STRAWBERRY JAM FROM FUNGI

SECTION 1.1

INFLUENCE OF EMULSIFIER TYPE ON THE ANTIFUNGAL ACTIVITY OF CINNAMON LEAF, LEMON AND BERGAMOT OIL NANOEMULSIONS AGAINST *Aspergillus niger*

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Abstract

Only exiguous data are currently available on the antifungal properties of essential oil (EO) nanoemulsions against spore-forming microorganisms. The aim of this work is to develop physically stable nanoemulsion-based delivery systems for different EOs (cinnamon leaf, lemon, and bergamot), to exploit their antifungal properties against *Aspergillus niger*. The inhibition of mycelial radial growth and spore germination were used as indicators of antifungal activity of the nanoemulsions, which were prepared at 3 wt% EO, using non-ionic Tween 80 (T80) or anionic whey protein isolate (WPI) (1 wt%) as emulsifiers, and sunflower oil (1 wt%) as ripening inhibitor. The nanoemulsions were physically stable over seven days of accelerated aging at 35 °C. The minimal inhibitory concentrations of free cinnamon leaf and of both citrus EOs were 0.35 and 5.50 μg/g, respectively. The encapsulation of cinnamon leaf EO in nanoemulsions significantly enhanced the inhibiting effect against *A. niger* mycelial growth and spore germination, with respect to the free EO. In contrast, for citrus EOs, the encapsulation in nanoemulsions generally decreased the antifungal activity, likely because of the nanoemulsion acting as a hydrophobic sink for the main constituents of citrus EOs. The emulsifier played a fundamental role in the resulting antifungal activity, with WPI-based nanoemulsions being more effective in inhibiting the mycelial growth and the spore germination of *A. niger* than T80-based ones. The antifungal action was correlated to the morphological alterations observed in *A. niger*, such as the loss of cytoplasm in fungal hyphae and hyphal tip. The results of this study show the importance of nanoemulsions design in the development of efficient and stable natural antifungal agents for food applications.

***Keywords**:* Nanoemulsions; Essential oils; Tween 80; Whey protein isolate; Antifungal activity; *Aspergillus niger*

1. INTRODUCTION

Food product deterioration during storage, caused by fungi, and especially by moulds, is responsible for significant economic losses to the food industry. The most common mould genus is *Aspergillus*, which is a plant, animal, and human pathogen. It can contaminate agricultural products at different stages such as pre-harvesting, harvesting, processing and handling. The changes associated with spoilage by *Aspergillus* species encompass the sensorial, nutritional and qualitative product properties. However, the most notable consequence of their presence is the contamination of food and feeds with mycotoxins (Perrone et al., 2007).

The use of chemical preservatives as antifungal agents to control fungal spoilage had become a common practice in the last decades. However, following the recent increasing consumers' trends towards more natural and healthy food products, food processors have started to search for safer alternatives to replace synthetic additives. Plant products have been recognized and employed for food protection since many years (Rodriguez-Lafuente, Nerin de la Puerta, & Batlle, 2009). Essential oils (EOs) belong to one of the most promising classes of natural antifungal preservatives (Varma & Dubey, 2001; Tian et al., 2011). However, despite their enormous potential of application, the use of EOs as food preservatives is strongly limited by their high volatility, low water-solubility and strong susceptibility to environmental conditions. The encapsulation of EOs in oil-in-water (O/W) nanoemulsions significantly improves their water dispersibility, and, by providing large surface areas of contact with the microorganisms, also contributes to enhancing the antimicrobial effectiveness of EOs (Donsì & Ferrari, 2016).

The main purpose of this study is the investigation of the effects on the antifungal activity against *Aspergillus niger* of three different EOs (cinnamon leaf, lemon, and bergamot), when encapsulated in O/W nanoemulsions, formulated with two different emulsifiers of approved use in foods, such as T80 and WPI. In particular, the antifungal activity of the developed nanoemulsions is evaluated in

terms of the induced inhibition of fungal growth, of mycelial growth, and of spore germination, as well as of the morphological damages caused to fungal hyphae and hyphal tips. The T80, a non-ionic low-mass surfactant, is characterized by high surface activity, which translates in the quick absorption at O/W interfaces, efficiently preventing the coalescence of the droplets (Li et al., 2015). Negatively charged whey proteins are instead able to form a protective membrane around the oil droplets, which prevents droplet aggregation by steric hindrance and electrostatic repulsion (Hebishy, Buffa, Guamis, Blasco-Moreno, & Trujillo, 2015; Teo et al., 2016).

2. MATERIALS AND METHODS

2.1 Microorganism, culture media, and reagents

The strain of *Aspergillus niger* (CECT 20156) was supplied by the Spanish Type Culture Collection (CECT, Burjassot, Spain). For culture media, Potato Dextrose Broth and Potato Dextrose Agar were used, all provided by Scharlab (Barcelona, Spain).

The EOs used in this work were cinnamon leaf (CEO), purchased from Sigma-Aldrich (Milan, Italy), lemon (LEO) and bergamot (BEO), which were both supplied by CAPUA s.r.l. (Reggio Calabria, Italy). In some emulsion formulations, the EOs were mixed with sunflower oil purchased from Sagra (Lucca, Italy). T80 (Sigma-Aldrich, Milan, Italy) and WPI (Volactive UltraWhey 90, Volac Socoor S.r.l., Italy) were employed as emulsifying agents. According to manufacturer specifications, T80 has a molecular weight of 1.31 kDa, whereas WPI, consisting of β-lactoglobulin (50-60% w/w), glycomacropeptide (15-20% w/w), α-lactalbumin (15-20% w/w), bovine serum albumin (1.0-2.0% w/w), immunoglobulin G (1.0-2.0% w/w), immunoglobulin A (0.1-1.0% w/w), and lactoferrin (0.1-0.5% w/w), had an average molecular weight of 18.2 kDa.

A. niger mycelial material and spores were fixed with a lactophenol-cotton blue solution, purchased from Sigma-Aldrich (Milan, Italy).

2.2 Characterization of free EOs

2.2 1 Gas chromatography-mass spectrometry analysis

Gas chromatography-mass spectrometry (GC-MS) analysis of CEO, LEO, and BEO was performed on a 6890/5975 inert GC-MS (Agilent Technologies, USA, equipped with a HP-5 fused silica capillary column (30 m x 0.25 mm x 0.25 µm) to determine the EOs composition. The oven temperature was held at 60 °C for 3 min, then raised to 100 °C at 10 °C/min, to 140 °C at 5 °C/min, and finally to 240 °C at 20 °C/min. Helium gas was used as the carrier gas at a constant flow rate of 1 mL/min. Injector and MS transfer line temperatures were set at 250 °C and 230 °C, respectively. Parameters for MS analysis were EI Ion source, electron energy 70 eV, solvent delay 3 min and m/z 40-550 *amu*. The identification of the EO components was performed by matching mass spectra with the standard mass spectra from the NIST MS Search 2.0 library. The results were expressed as the percentage of relative area (%) of two runs for each EO.

2.2.2 Antifungal activity of free EOs

2.2.2.1 Mycelial growth and Minimal Inhibitory Concentration assay

The CEO, LEO, and BEO were individually examined against *A. niger* as described by Ribes, Fuentes, Talens, and Barat (2016). The mould was inoculated on Potato Dextrose Agar and incubated at 25 °C for 7 days. Subsequently, the spores were counted in a hemocytometer to achieve an inoculum density of 10^6 CFU/mL.

Different EO concentrations were examined on the basis of previous studies (Sharma & Tripathi, 2008; Gemeda, Woldeamanuel, Asrat, & Debella, 2014): 0.10, 0.25, 0.35, 0.50, 1.00, 1.50 and 2.00 µg/g for CEO; and 0.25, 0.50, 1.00, 1.50, 2.00, 2.50, 3.00, 3.50, 4.00, 4.50, 5.00, 5.50 and 6.00 µg/g for LEO and BEO. The selected maximum concentrations are all below the solubilities in water of the main components, which are of 1.9 mol/mol for eugenol and of 1.0 mol/mol for D-limonene (Miller & Hawthorne, 2000), corresponding to 17.3 µg/g and 7.6 µg/g, respectively. The EOs were added to aliquots of 15 g of Potato Dextrose Agar containing 0.1 wt% T80, to ensure the even dispersion of the EOs, according to a procedure previously described (Tao, Jia, & Zhou, 2014; Ribes et al., 2016), into Petri dishes. The EOs were added to the culture medium at 50 °C. Control Petri dishes, without EOs, were prepared following the same procedure. The centre of each plate tested was inoculated with a Potato Dextrose Agar disc (7 mm diameter) taken from the edge of zero-day-old fungi culture, previously spread with 100 µL of the spore solution (10^6 CFU/mL). Each plate was sealed with Parafilm® and incubated for 7 days at 25 °C.

At the end of the incubation period, the edge of the fungi culture was observed using a light microscope at 100×, 200× and 400× magnification. The mycelial material was fixed by using lactophenol-cotton blue solution. Growth inhibition of treatment against control was calculated using equation 1:

$$Mycelial\ growth\ inhibition\ (\%) = [\mathrm{C} - \mathrm{T}/\mathrm{C}]\mathrm{x}\ 100 \quad (1)$$

where *C* and *T* represented the mycelial growth (mm) in the control and treated plates, respectively.

Furthermore, the Minimal Inhibitory Concentration (MIC) of the CEO, LEO, and BEO against *A. niger* was determined. The MIC was defined as the lowest concentration in the serial dilution of the antifungal agents, which resulted in the lack of visible growth after 7 days of incubation at 25 °C.

All tests were performed in duplicate, for each treatment, which was repeated twice.

2.2.2.2 Spore germination assay

Spores from seven-day-old *A. niger* culture were collected by adding 1 mL of sterile water containing 0.1 wt% T80 to each Petri plate and rubbing the surface with a sterile L-shaped spreader. The spores were transferred to a tube containing 5 mL of sterile water and 0.1 wt% T80 and counted in a hemocytometer to achieve an inoculum density of 10^6 CFU/mL.

The same EO concentrations used in the mycelial growth assay were examined. In this case, they were added to 5 mL of Potato Dextrose Broth with 0.1 wt% of T80, to ensure the even dispersion of the EOs, and 100 µL of the inoculum density were added to each tube. Potato Dextrose Broth tubes with no EOs were used as controls. The tubes were incubated 24 h at 25 °C. At the end of the incubation period, germinated spores were observed using a light microscope at 400× magnification. Each slide was fixed in lactophenol-cotton blue solution. The assay was run in duplicate and the efficacy of the EO treatments was evaluated by looking for the presence of germ tubes. Each treatment was repeated twice. Results were expressed in terms of the percentage of spore germination inhibition by comparing control and treated plates according to equation 2:

$$Spore\ germination\ inhibition\ (\%) = (sc - st/sc)x\ 100 \quad (2)$$

where *sc* and *st* are the average numbers of spores germinated in control plates and treated plates, respectively.

2.3 O/W emulsions and nanoemulsions

2.3.1 Preparation and characterization

O/W nanoemulsions with a total oil phase of 4 wt% and an aqueous phase of 96 wt% were prepared using the High Pressure Homogenization (HPH) technique (Donsì, Sessa, & Ferrari, 2012b). The nanoemulsions were prepared with the oil phase consisting exclusively of the EO (4 wt% of total formulation), or of the EO mixed with sunflower oil (ripening inhibitor) at a 3:1 wt ratio, and hence with 3 wt% of EO and 1 wt% of sunflower oil of total formulation. The oil phase consisted of EO, to which sunflower oil was eventually added as Ostwald ripening inhibitor. The aqueous phase contained 1 wt% of T80 or WPI. Primary emulsions were obtained by High Shear Homogenization (HSH) using an Ultra Turrax T25 (IKA Labortechnik, Germany) at 24,000 rpm for 4 min, maintaining the samples in an ice bath. The primary emulsions were then processed 3 times at 200 MPa by using an HPH system equipped with a 100 µm diameter orifice valve (model WS1973, Maximator JET GmbH, Schweinfurt, Germany). The inlet temperature was set at 5 °C and the outlet temperature was reduced to 5 °C by using a heat exchanger immediately downstream of the homogenization valve.

The mean droplet size and the ζ-potential of the samples were determined at 25 °C using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK), equipped with dynamic light scattering and ζ-potential analyser. The samples were loaded in the thermostated cell of the system, with an equilibration time of 5 min. Upon the analysis of the dynamic light scattering data and electrophoretic mobility, the software calculated the mean droplet size (d_H) and the polydispersity index (PdI), using the Stokes-Einstein equation, and the ζ-potential using the Smoluchowsky model. The mean droplet size was measured on undiluted samples, whereas ζ-potential was measured on samples diluted 10-fold with bidistilled water. Accelerated ageing tests were carried out on samples stored at 35 °C in a thermostated orbital shaker, with rotational speed set at 150 rpm.

All the primary emulsions and nanoemulsions prepared were subjected to pH measurement with a Mettler Toledo SevenEasy S20 pH meter (US).
Each measurement was performed in triplicate.

2.3.2 In vitro antifungal activity

The study of the *in vitro* antifungal activity of the O/W emulsions and nanoemulsions was conducted on the basis of the results of the measurements of the antifungal activity of free EOs.

2.3.2.1 Mycelial growth assay

The methodology followed in the mycelial growth assay of the emulsions and nanoemulsions was the same previously described for the free EOs (Section 2.2.2.1). The mycelial growth assay of the O/W emulsions and nanoemulsions was carried out using a final concentration of 0.25 μg/g of CEO and 4.0 μg/g of the citrus oils (LEO and BEO), which were proved to be sufficient to obtain a mycelial growth inhibition higher than 50% than control. Control samples were prepared with T80 or WPI, replacing the EOs with sunflower oil.

The edge of the fungi culture was observed using a light microscope at 100×, 200× and 400× magnification. The mycelial material was fixed by using lactophenol-cotton blue solution.
Each treatment was repeated twice. Each assay was conducted in duplicate.

2.3.2.2 Spore germination assay

In order to evaluate the spore germination of *A. niger*, the same methodology followed in the spore germination test of free EOs was employed. In this case, the final concentrations of encapsulated EOs were 0.1 μg/g of CEO and 1 μg/g of the citrus oils (LEO and BEO) in 5 mL of Potato Dextrose Broth. Control tubes were

prepared with T80 or WPI, replacing the EOs with sunflower oil. The tubes were incubated 24 h at 25 °C. At the end of the incubation period, germinated spores were observed using a light microscope at 400× magnification.
Each treatment was repeated twice. Each count of germinated spores was conducted in duplicate.

2.4 Statistical analysis

The results obtained in the physicochemical characterisation of the O/W emulsions and nanoemulsions and of their antifungal activity were analysed by a multifactor analysis of variance (multifactor ANOVA). The effect of EOs on the radial growth and spore germination inhibition (%) was evaluated by one-way ANOVA. The least significance procedure (LSD) was used for the means comparison at the 5% level of significance. Data were statistically processed by Statgraphics Centurion XVI.

3. RESULTS AND DISCUSSION

3.1 Composition of the EOs

The components of the different EOs (CEO, LEO, and BEO) were identified by GC-MS analysis (**Table 1**). The main compounds of the CEO were eugenol (83.04%), benzyl benzoate (3.84%), caryophyllene (3.04%) and aceteugenol (2.42%). Similar compositions have been identified by Singh, Maurya, de Lampasona and Catalan (2007). Eugenol has been reported to be an excellent fungicide against a wide range of fungi (Bakkali, Averbeck, Averberck, & Idaomar, 2008).

The main components of the LEO were D-limonene (68.56%), β-pinene (13.80%) and τ-terpinene (10.92%). The BEO exhibited a very similar composition,

with the main components being also D-limonene (49.69%), β-pinene (13.19%) and τ-terpinene (9.13%), together with β-linalool (14.56%). These compositions are in agreement with those reported by Franceschi, Grings, Frizzo, Oliveira, and Dariva (2004). Citrus EOs are typically complex mixtures of > 400 compounds, depending on the citrus cultivar, extraction and separation processes (Nannapaneni et al., 2009). Several authors have attributed the antifungal properties of citrus oils to components such as D-limonene, linalool, and citral (Sonboli, Babakhani, & Mehrabian, 2006; Tepe et al., 2006).

3.2 Antifungal activity of free EOs

3.2.1 Inhibition of mycelial growth

The radial growth of *A. niger* after 7 days of incubation was 45 ± 3 mm. The addition of EOs in the culture media inhibited the mycelial growth in a concentration-dependent manner. The results of the mycelial growth inhibition (%) after 7 days of incubation at different EO concentrations have been summarized in **Table 2**.

The CEO showed the highest antifungal activity, with the complete inhibition of the fungal mycelial growth being observed at concentrations >0.25 μg/g. The LEO and BEO exhibited a weaker activity, with the fungal development being completely inhibited at concentrations of LEO or BEO >5.00 μg/g.

More specifically, the CEO caused a mycelial growth reduction of 76% at 0.25 μg/g, and the complete growth inhibition at 0.35 μg/g, whereas both citrus EOs induced an inhibition of only 13% at 0.25 μg/g. The mycelial growth inhibition increased up to 50% for LEO and BEO concentration of 4.00 μg/g and to 100% for 5.50 μg/g.

The antimicrobial activity of a determined EO results from the combination of composition and concentration of each volatile compound, which in turn are

governed by the plant variety, growing conditions and method of extraction of the EO (Salvia-Trujillo, Rojas-Graü, Soliva-Fortuny, & Martín-Belloso, 2015).

Moreover, according to literature data, eugenol, the main compound in CEO, exhibits a stronger antifungal activity than D-limonene, which is attributed to the disturbance of the cytoplasmic membrane, causing the disruption of the proton motive force, electron flow, active transport and coagulation of cell content (Davidson, 1997). In contrast, D-limonene, the major component of both LEO and BEO, is a hydrophobic agent with a high susceptibility to oxidative degradation, which causes a loss of antifungal activity (Sun, 2007). Furthermore, according to previous studies on lemon, orange, and eucalyptus EOs, d-limonene is one of the weakest inhibitors of fungal growth among monoterpene compounds (Combrinck, Regnier, & Kamatou, 2011).

In addition, the MIC value (no visible fungal growth after 7 days of plate incubation) of CEO resulted in being 0.35 μg/g, while the MIC value of both citrus EOs (LEO and BEO) resulted in being 5.50 μg/g.

Table 1. Chemical composition of the cinnamon leaf EO (CEO), lemon EO (LEO), and bergamot EO (BEO). Percentages of the relative areas (%) are the mean of two runs and were obtained from the integration of the peaks identified in the spectra by a selective mass detector.

Compound	CEO (% relative area)	LEO (% relative area)	BEO (% relative area)
β-Phellandrene	-	0.85 ± 0.00	4.74 ± 1.41
Ocimene	-	-	0.32 ± 0.00
β-Pinene	-	13.80 ± 0.04	13.19 ± 0.01
α-Phellandrene	0.89 ± 0.09	-	-
o-Cymene	0.47 ± 0. 19	0.29 ± 0.03	0.22 ± 0.02
D-Limonene	-	**68.56 ± 0.20**	**49.69 ± 2.41**
β-trans-Ocimene	-	-	2.56 ± 0.07
τ-Terpinene	-	10.92 ± 0.02	9.13 ± 0 02
Terpinolene	0.23 ± 0.06	0.39 ± 0.09	-
2-Carene	-	-	0.36 ± 0.24
β-Linalool	1.91 ± 0.06	-	14.56 ± 0.24
α-Terpineol	0.22 ± 0.03	0.14 ± 0.00	0.15 ± 0.01
β-Citral	-	0.78 ± 0.02	-
Citral	-	-	0.50 ± 0.05
α-Citral	-	1.67 ± 0.05	-
Cinnamaldehyde	0.92 ± 0.03	-	-
α-Terpineolacetate	-	-	0.26 ± 0 02
Eugenol	**83.04 ± 0.45**	-	-
Geraniolacetate	-	0.45 ± 0.04	2.24 ± 0.09
Copaene	0.88 ± 0.02	-	-
Caryophyllene	3.04 ± 0.52	0.40 ± 0.02	0.39 ± 0.01
α-Bergamotene	-	0.93 ± 0.18	1.20 ± 0.71
Cinnamylalcohol, acetate	1.09 ± 0.05	-	-
α-Caryophyllene	0.61 ± 0.01	-	-
β-Bisabolene	-	0.83 ± 0.05	0.51 ± 0.01
β-Guaiene	0.09 ± 0.04	-	-
Aceteugenol	2.42 ± 0.00	-	-
Caryophyllene oxide	0.34 ± 0.06	-	-
Benzyl Benzoate	3.84 ± 0.06	-	-

3.2.2 Inhibition of spore germination

The results of the spore germination assay of the CEO, LEO and BEO are also reported in **Table 2**. A remarkable sporicidal activity against *A. niger* was observed for the EOs employed in this study. In general, there was a positive correlation between EO concentrations and the inhibition of spore germination, with similar trends to what was observed for mycelial growth inhibition. At a CEO concentration of 0.10 μg/g, 46% of the spore germination was inhibited, whereas complete inhibition was observed at 0.35 μg/g. The effect of the CEO can be explained in terms of the interference with the process of spore germination, either through the denaturation of the enzymes or the obstruction of the aminoacids involved in germination (Tian et al., 2012). In the case of the LEO and BEO, higher concentrations than CEO were needed to inhibit spore germination. About 50% inhibition was observed at 1.00 μg/g, whereas complete inhibition was reached at concentrations ≥5.5 μg/g of both LEO and BEO. However, for concentrations comprised between 3.5 μg/g and 5.0 μg/g, LEO exhibited a slightly but significantly higher inhibition of spore germination than BEO. Therefore, oil composition appears to affect significantly the resistance of *A. niger* spores to EO treatments.

3.2.3 Morphological characterization of A. niger upon exposure to free EOs

The effect of the three EOs on *A. niger* was examined by light microscopy. The micrographs of the control and treated samples under conditions of significant but not complete inhibition (0.25 μg/g CEO, 3.5 μg/g BEO, and 3.5 μg/g LEO) are shown in **Fig. 1**. Microscope examination of the control samples (**Fig. 1A**) exhibited regular and homogenous mycelial hyphae with cylindrical principal axes, and the cytoplasmic content clearly distinguishable. Samples treated with 3.5 μg/g

of the BEO (**Fig. 1D**) presented a clear decrease in cytoplasmic content, with a visible separation of cytoplasm from the cell wall in the hyphae.

Furthermore, the hyphae of treated samples were thinner than for control samples, due to the cytoplasmic coagulation. Budding of the hyphal tip and anomalous apex bifurcations were also observed after the CEO and LEO exposure (**Fig. 1B** and **C**, respectively). The observed effects on the *A. niger* morphology were similar to those previously reported by Sharma and Tripathi (2008) and by Tian et al. (2011).

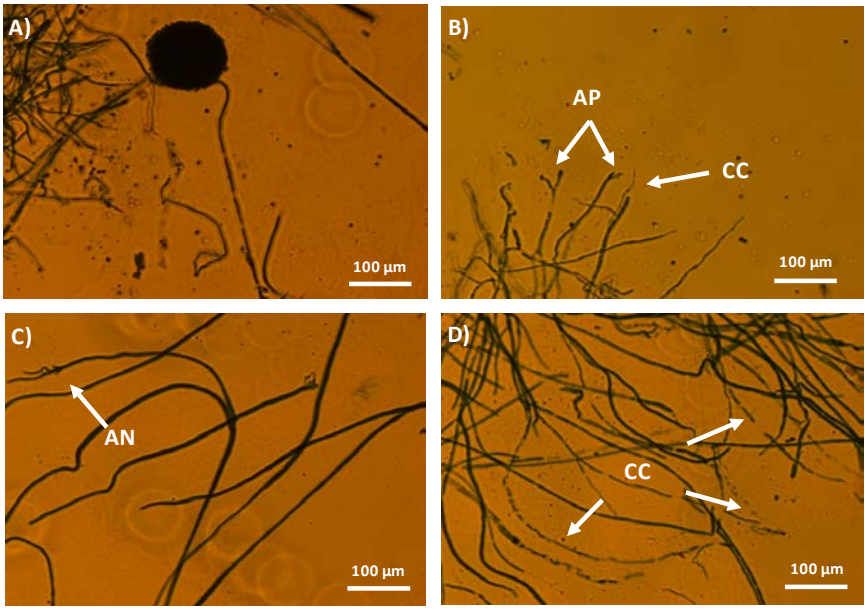

Fig. 1. Bright field micrographs of *Aspergillus niger* treated by free EOs and EO emulsions and nanoemulsions. A) Control samples showing the normal growth of *A. niger*. B, C, D) Anomalies (AN), apex bifurcations (AP) and the decrease in cytoplasmic content (CC) are visible after exposure to free cinnamon leaf EO (CEO), lemon EO (LEO), and bergamot EO (BEO), respectively.

Table 2. Effect of the concentration and type of EOs on the inhibition of the radial fungal growth and the spore germination of *Aspergillus niger* after 7 days of incubation at 25 °C. Mean values (n=2) ± SD.

Concentration of EO (µg/g)	Radial growth inhibition (%) of CEO	Spore germination inhibition (%) of CEO	Radial growth inhibition (%) of LEO	Spore germination inhibition (%) of LEO	Radial growth inhibition (%) of BEO	Spore germination inhibition (%) of BEO
Control	-	-	-	-	-	-
0.10	26.7 ± 9.4^{a}	45.9 ± 8.0^{a}	-	-	-	-
0.25	75.6 ± 3.1^{b}	84.7 ± 2.6^{b}	13.3 ± 3.1^{a}	38.0 ± 1.8^{a}	13.3 ± 3.1^{a}	11.2 ± 14.3^{a}
0.35	100.0 ± 0.0^{c}	100.0 ± 0.0^{c}	-	-	-	-
0.50	100.0 ± 0.0^{c}	100.0 ± 0.0^{c}	18.9 ± 1.6^{ab}	42.3 ± 3.5^{ab}	15.6 ± 6.3^{a}	44.7 ± 3.0^{a}
1.00	100.0 ± 0.0^{c}	100.0 ± 0.0^{c}	27.8 ± 7.9^{bc}	55.5 ± 1.6^{bc}	33.3 ± 6.3^{b}	45.9 ± 11.3^{b}
1.50	100.0 ± 0.0^{c}	100.0 ± 0.0^{c}	26.7 ± 9.4^{bc}	54.8 ± 2.6^{bc}	40.0 ± 9.4^{bcd}	56.5 ± 3.1^{bcd}
2.00	100.0 ± 0.0^{c}	100.0 ± 0.0^{c}	34.4 ± 11.0^{c}	83.9 ± 2.6^{c}	37.8 ± 6.3^{bc}	73.2 ± 6.3^{bc}
2.50	-		35.6 ± 3.1^{cd}	90.0 ± 3.4^{cd}	48.9 ± 6.3^{cd}	90.3 ± 2.6^{cd}
3.00	-		46.7 ± 3.1^{de}	98.7 ± 0.6^{de}	46.7 ± 3.1^{bcd}	92.0 ± 0.0^{bcd}
3.50	-		47.8 ± 4.7^{e}	98.3 ± 1.0^{e}	47.8 ± 4.7^{cd}	95.2 ± 1.0^{cd}
4.00	-		53.3 ± 3.1^{e}	99.1 ± 0.0^{e}	52.2 ± 14.1^{d}	96.7 ± 1.4^{d}
4.50	-		72.2 ± 7.9^{f}	99.1 ± 0.5^{f}	72.2 ± 1.6^{e}	98.0 ± 0.5^{e}
5.00	-		82.2 ± 3.1^{f}	99.4 ± 0.4^{f}	82.2 ± 3.1^{e}	99.2 ± 0.4^{e}
5.50	-		100.0 ± 0.0^{g}	100.0 ± 0.0^{g}	100.0 ± 0.0^{f}	100.0 ± 0.0^{f}
6.00	-		100.0 ± 0.0^{g}	100.0 ± 0.0^{g}	100.0 ± 0.0^{f}	100.0 ± 0.0^{f}

$^{a, b, c, d, e, f, g}$ *Different superscripts indicate significant ($p < 0.05$) differences among EOs concentration (µg/g)*

3.3 Antifungal activity of EO nanoemulsions

3.3.1 Nanoemulsion formulation and physical stability

The composition of the disperse oil phase of nanoemulsions significantly influences both the efficiency of the emulsification process (especially through disperse phase viscosity and interfacial tension) and the physical stability of the nanoemulsions. In particular, the appreciable solubility in water of EOs induces peculiar coalescence phenomena, known as Ostwald ripening, which consists in the molecular diffusion of EOs through the continuous phase, driven by the higher local oil solubility around smaller droplets than larger ones. Ostwald ripening hence results in the growth of larger droplets at the expense of the smaller ones, reducing the physical stability of EO nanoemulsions (Donsì, Annunziata, Sessa, & Ferrari, 2011). The addition of ripening inhibitors, compounds with negligible water solubility such as long chain triglycerides oils, generating an entropy of mixing effect that counteracts the imbalance of droplet size effect (Chang, McLandsborough, & McClements, 2015), is reported to efficiently contrast the occurrence of Ostwald ripening (Donsì & Ferrari, 2016).

In this work, on the basis of previously investigated formulations (Donsì, Cuomo, Marchese, & Ferrari, 2014), the nanoemulsions were prepared with the oil phase consisting only of EO, or of EO mixed with sunflower oil (ripening inhibitor) at a 3:1 wt ratio.

The results are reported in **Fig. 2** in terms of mean droplet size d_H of the nanoemulsions, measured immediately after preparation and after 7 days of accelerated ageing in an orbital shaker at 35 °C, using either T80 (**Fig. 2A**) or WPI (**Fig. 2B**) as emulsifiers. Remarkably, the addition of sunflower oil has two significant advantages: (a) it improves the emulsification process by HPH, contributing to reduce the attainable d_H value, and (b) reduces the physical instability phenomena. In particular, the nanoemulsions containing sunflower oil exhibited always smaller d_H values, which remained constant during the entire

observation period, without showing any sign of incipient coalescence. In contrast, when the nanoemulsions were prepared with pure EOs, the d_H values exhibited measurable variations already after one week of storage. For example, in the case of LEO, the nanoemulsions with EO alone exhibited d_H values of 160 and 430 nm for T80 and WPI, respectively, which increased to 180 and 670 nm after one week, whereas, when sunflower oil was added, the nanoemulsions exhibited d_H values of 140 and 300 nm for T80 and WPI, respectively, which did not significantly vary after one week.

It can hence be concluded that the nanoemulsions formulated with the blend of EOs and sunflower oil (3:1 wt ratio) resulted stable under the accelerated ageing tests conducted at 35 °C, and were therefore used in the subsequent fungitoxic experiments.

3.3.2 Physicochemical characterization

The mean droplet size (d_H) and polydispersity index (PdI) of the nanoemulsions, containing the EOs blended with sunflower oil (3:1 wt ratio), and obtained by HPH are reported in **Table 3**, in comparison with the primary emulsions obtained by HSH. The results show that the type of emulsifier and the emulsification process significantly affected both d_H and PdI.

The use of WPI as emulsifier led to significantly ($p < 0.05$) higher values of both d_H and PdI than the use of T80, in agreement with a previous study (Tastan, Ferrari, Baysal, & Donsì, 2016), due to the different O/W interfacial tensions and dynamics of absorption and reorganization at O/W interfaces, which makes T80 more surface active (Donsì, Sessa et al., 2012). Coherently, the effect of HPH processing was more evident for WPI than for T80.

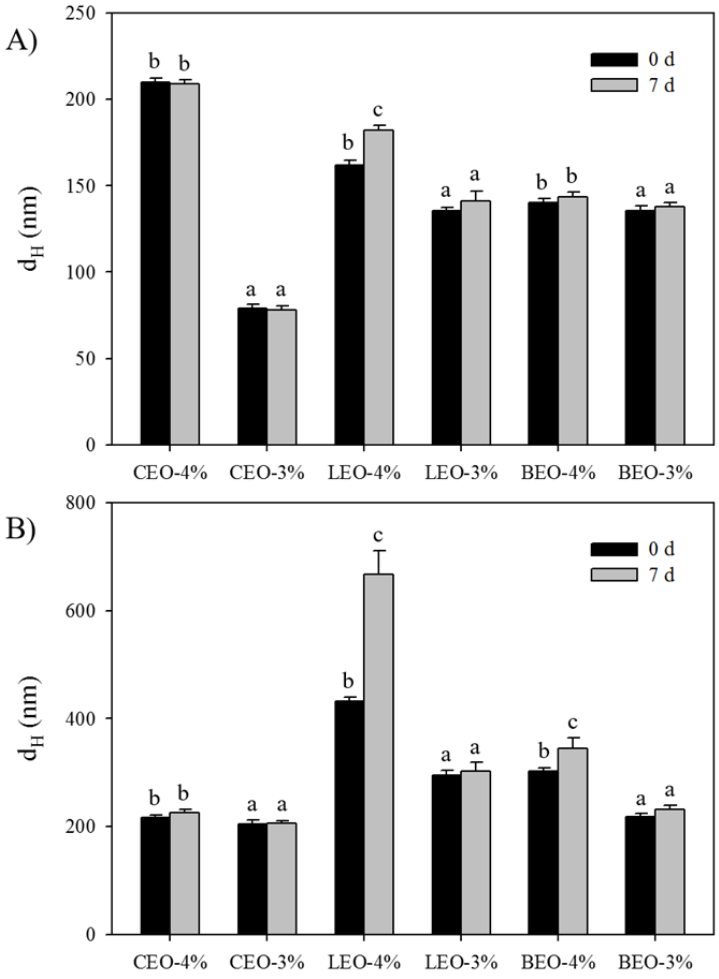

Fig. 2. Mean droplet size d_H of nanoemulsions prepared using T80 (A) or WPI (B) as emulsifiers, and measured immediately after preparation (0 d) or after seven days of storage at 4 °C (7 d). The oil phase was maintained constant at 4 wt%, and consisted either of cinnamon leaf EO (CEO), lemon EO (LEO), and bergamot EO (BEO) alone (4%) or of the EO mixed with sunflower oil at 3:1 wt ratio (3%). Asterisks denote significant differences ($p < 0.05$) within the fresh and aged nanoemulsions containing the same EO.

In the case of T80, the d_H values of primary emulsions obtained by HSH emulsification, which were 84, 311 and 240 nm for CEO, LEO, and BEO, respectively, were significantly ($p < 0.05$) reduced to 79, 143 and 133 nm respectively by HPH processing.

Instead, in the case of WPI, the d_H values of the primary emulsions were in the micrometric range (1.6, 2.5 and 1.3 µm for emulsions containing the CEO, LEO, or BEO, respectively), and were significantly ($p < 0.05$) reduced to submicrometric sizes (212, 296 and 266 nm for the CEO, LEO, and BEO) by HPH emulsification (**Table 3**). Several factors relating to the emulsion formulation affect the final mean droplet size achievable by HPH emulsification, and in particular (a) those influencing the break-up phenomena, such as the viscosity of the disperse and continuous phase and the interfacial tension, and (b) those controlling the recoalescence phenomena, such as emulsifier affinity for and interaction with newly formed interfaces (Donsì et al., 2011, Donsì et al., 2012a and Donsì et al., 2012b). In the present case, the EO represents 75 wt% of the oil phase, with an expected significant impact on the properties of the disperse phase, including the viscosity, the interfacial tension, as well as and the affinity for the emulsifier and the formation of molecular interactions with it.

Emulsification process and surfactant type significantly affected also the PdI values, which measure the spread of the particle size distribution, being 0 the smallest possible value and 1 the largest, and with smaller values indicating narrower size distributions (Jo & Kwon, 2014). The samples prepared with T80 exhibited, after HPH treatment, PdI values ranging from 0.11 to 0.15. In the case of WPI, higher PdI values were observed after HPH, ranging from 0.32 to 0.45, likely because of the macroscopic changes in protein agglomeration induced by HPH processing (Donsì, Senatore, Huang, & Ferrari, 2010).

Fig. 3 and **Fig. 4** show the micrographs of the different primary emulsions and nanoemulsions prepared with T80 and WPI, respectively, highlighting the contribution of HPH processing in obtaining finer and more homogeneous size distributions, especially in the case of WPI. Concerning the T80 formulations, a

large fraction of the emulsion droplets falls below the observability limits of the optical microscope and can not be detected in the micrographs.

Despite the formulation and processing conditions of the nanoemulsions were identical, the T80 nanoemulsions always exhibited significantly smaller mean droplet sizes than the WPI nanoemulsions. Previous studies have shown that low-molecular-mass surfactants (i.e. Tween 20, Tween 80, SDS) lead to the formation of smaller droplets than proteins (i.e. WPI, β-lactoglobulin, caseinate, pea proteins), because of their ability to quickly absorb onto the new droplets surfaces and rearrange in a protective layer, reducing the extent of recoalescence during HPH emulsification (Donsì et al., 2012b and Qian and McClements, 2011). The results of **Table 1** confirm that, independently on the formulation, the use of T80 (M_w of 1.31 kDa) led to emulsions significantly finer in size than the use of WPI (M_w of 18.4 kDa).

However, it must be remarked that also other factors might affect the efficiency of the HPH emulsification process. For example, the differences between low-molecular-mass surfactants and macromolecules become smaller at higher emulsifier concentrations, where larger driving forces increase the mass transfer also of macromolecules to the oil-water interface. At concentrations of 6 wt% for both oil phase and emulsifier, the use of WPI led to the formation of nanoemulsions with a comparable mean droplet size of emulsions formed by the combined use of Tween 20 and monoolein (Tastan et al., 2016). Moreover, also the charge of the emulsifier and its interaction with the oil phase appear to play a significant role in the final emulsion droplet size (Silva, Cerqueira, & Vicente, 2015).

Table 3. Droplet size (d_H) (nm), polydispersity index (PdI), ζ-potential, and pH of the different emulsions formulated with CEO, LEO, BEO and T80 or WPI and produced by high shear homogenization (HSH) or high pressure homogenization (HPH) (The EOs:sunflower oil ratio used in emulsions and nanoemulsions formulation was 3:1). Values are expressed as mean (n=3) ± SD.

		System					
	Emulsifier	**CEO-HSH**	**CEO-HPH**	**LEO-HSH**	**LEO-HPH**	**BEO-HSH**	**BEO-HPH**
d_H (nm)	T80	83.5 ± 0.3 ^{ax}	79.0 ± 1.0 ^{ax}	311.0 ± 7.6 ^{cx}	143.4 ± 2.3 ^{ax}	240.1 ± 0.6 ^{bx}	133.4 ± 1.7 ^{ax}
	WPI	1601.7 ± 17.0 ^{dy}	212.7 ± 2.7 ^{ay}	2490.0 ± 174.9 ^{ey}	296.4 ± 4.5 ^{cy}	1264.3 ± 12.7 ^{dy}	266.1 ± 0.9 ^{by}
PdI (-)	T80	0.089 ± 0.016 ^{ax}	0.106 ± 0.004 ^{ax}	0.456 ± 0.033 ^{cx}	0.150 ± 0.006 ^{ax}	0.273 ± 0.010 ^{bx}	0.146 ± 0.018 ^{ax}
	WPI	1.000 ± 0.000 ^{dy}	0.316 ± 0.020 ^{by}	0.969 ± 0.054 ^{dy}	0.432 ± 0.026 ^{cy}	0.895 ± 0.091 ^{dy}	0.454 ± 0.021 ^{cy}
ζ-potential (mV)	T80	-7.2 ± 0.6 ^{ax}	-8.9 ± 0.4 ^{bx}	-16.0 ± 0.6 ^{cx}	-16.8 ± 0.7 ^{cx}	-6.7 ± 0.2 ^{ax}	-9.2 ± 0.4 ^{bx}
	WPI	-40.1 ± 0.5 ^{dy}	-32.3 ± 0.2 ^{by}	-42.6 ± 0.4 ^{ey}	-40.8 ± 0.4 ^{dy}	-30.8 ± 0.4 ^{ay}	-37.3 ± 0.4 ^{cy}
pH	T80	6.7 ± 0.1 ^{ax}	6.6 ± 0.1 ^{abx}	6.4 ± 0.2 ^{bx}	6.3 ± 0.1 ^{bx}	6.4 ± 0.2 ^{bx}	6.4 ± 0.1 ^{bx}
	WPI	6.7 ± 0.3 ^{ax}	6.6 ± 0.3 ^{ax}	6.4 ± 0.2 ^{ax}	6.5 ± 0.4 ^{ax}	6.5 ± 0.2 ^{ax}	6.3 ± 0.1 ^{bx}

$^{a, b, c, d, e}$ *Different superscripts indicate significant differences among systems (p<0.05)*
$^{x, y}$ *Different superscripts indicate significant differences between emulsifiers (p<0.05)*

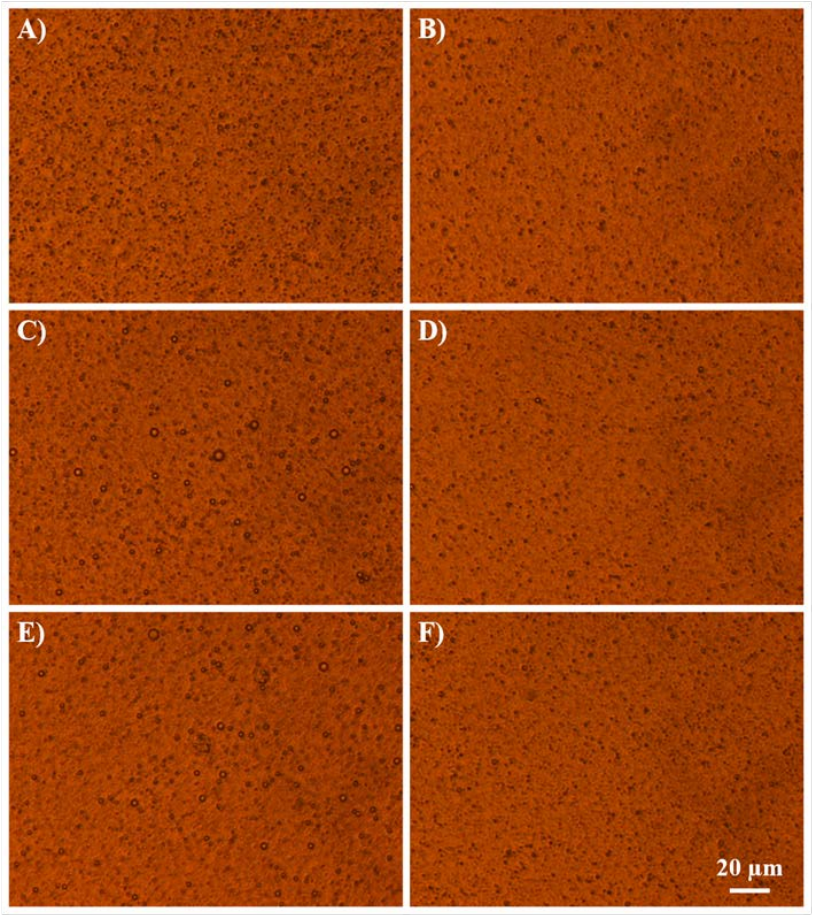

Fig. 3. Bright field micrographs of primary emulsions (A, C, E) and nanoemulsions (B, D, F) prepared with T80 and cinnamon leaf EO (A, B), lemon EO (C, D), and bergamot EO (E, F).

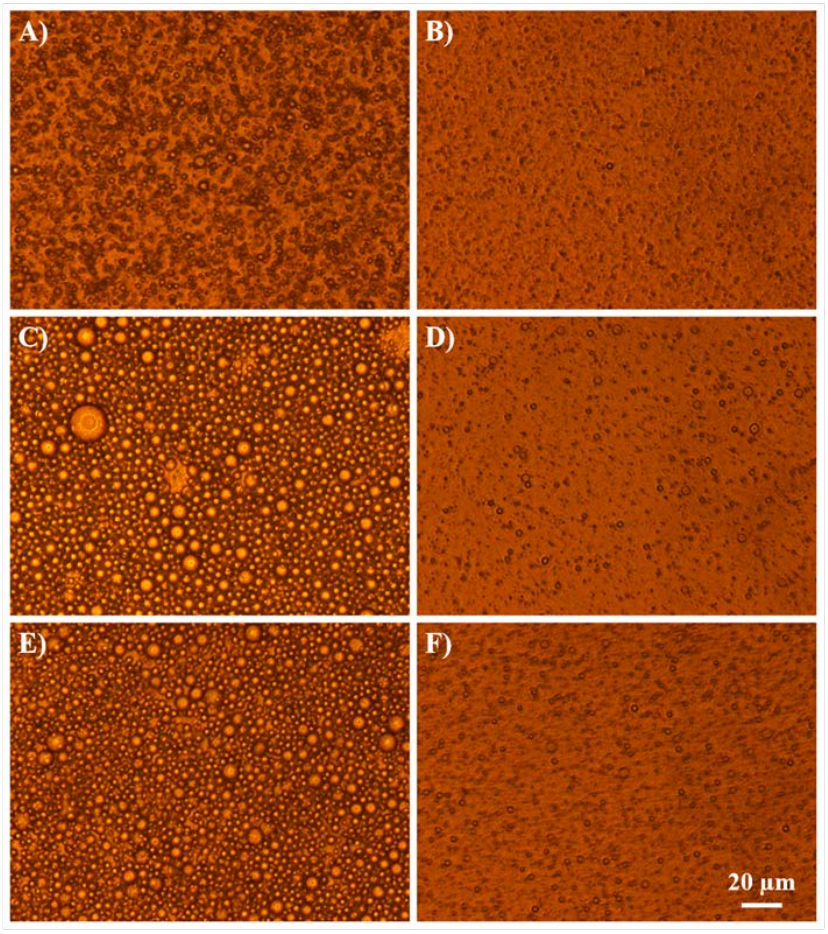

Fig. 4. Bright field micrographs of primary emulsions (A, C, E) and nanoemulsions (B, D, F) prepared with WPI and cinnamon leaf EO (A, B), lemon EO (C, D), and bergamot EO (E, F).

The average ζ-potential values of all the formulations are also reported in **Table 3**. The ζ-potential is an indirect measurement of the electrical charge of the colloidal particles, which gives indications on their stability during storage. Absolute ζ-potential value > 30 mV indicates that electrostatic repulsion among droplets likely contributes to preventing their aggregation. The average ζ-potential values of emulsions prepared with the non-ionic T80 were slightly negative (around −8.0 mV for the CEO and BEO samples, and around −16.4 mV for the LEO emulsion), suggesting a preferential stabilization mechanism by steric hindrance. It is possible that the anionic species from the free fatty acids used in the emulsions formulation (in sunflower oil and in EOs) are preferentially located near the droplet surfaces, affecting the surface charge.

In contrast, the ζ-potential values observed in the case of WPI-based EO nanoemulsions ranged from −30 to −43 mV, suggesting that electrostatic repulsive forces might significantly contribute to the droplet stabilization, similarly to what previously reported for submicron peanut O/W emulsions (Benzaria et al., 2013).

The differences observed in the ζ-potential of the emulsions prepared with different EOs and the same surfactant could be attributed to the differences in the dissociation degree and the number of ionizable compounds of the EOs (Salvia-Trujillo et al., 2015).

Furthermore, HPH processing also induced some minor changes in the surface charge of the particles, which, in the case of WPI, have been explained in terms of the interactions between the lipid phase and proteins in the emulsions (Lee, Lefèvre, Subidare, & Paquin, 2009).

The pH of the primary emulsions and nanoemulsions were always comprised between 6.3 and 6.7.

3.3.3 Inhibition of mycelial growth

The radial growth inhibition of *A. niger* after 7 days of incubation in the presence of EO nanoemulsions is shown in **Fig. 5**, in comparison with the free EOs and the corresponding primary emulsions, prepared by HSH. The CEO nanoemulsions exhibited significantly higher antifungal properties than free CEO. In particular, at a CEO final concentration of 0.25 μg/g, the radial mycelial growth was completely inhibited by using the nanoemulsions, whereas free CEO at the same concentration reached only 75% inhibition. The nanoemulsions likely contributed to improving CEO dispersibility and stability in water, as well as ameliorating the contact with the fungi and extending over time the EO antifungal properties.

Interestingly, LEO and BEO nanoemulsions, at an EO final concentration of 4.0 μg/g, exhibited a reduction of antifungal activity with respect to free EOs: the inhibition of the radial mycelial growth induced by the nanoemulsions was always lower than by free EOs. The sustained release over time of the EOs from the nanoemulsion droplets, driven by EO partition between the oil droplets and the aqueous phase, is likely to control the antimicrobial activity of EOs (Donsì, Annunziata et al., 2012). Eugenol is reported to have an octanol-water partition coefficient log *P* comprised between 2.5 (Fujisawa & Masuhara, 1981) and 2.6 (Li, Fabiano-Tixier, Ginies, & Chemat, 2014), whereas the log *P* value of d-limonene is reported between 4.4 (Li et al., 2014) and 4.6 (El-Kattan, Asbill, Kim, & Michniak, 2001).

Therefore, based on the log *P* values of their main components, it can be hypothesized that CEO tends to dissolve in the aqueous phase at significantly higher (>2 orders of magnitude) concentrations than LEO and BEO, supporting the observation that in the case of citrus oil nanoemulsions, the limited driving forces towards the oil release to the aqueous phase reduces the resulting antimicrobial activity. However, despite an initial lower inactivation rate of encapsulated EOs than free EOs, some authors reported that, over prolonged periods of time, emulsions and nanoemulsions, by ensuring a sustained release, can significantly prolong the antimicrobial activity (Majeed et al., 2016).

Therefore, it can be concluded that, among the different mechanisms intervening to limit the fungitoxic action of d-limonene, the presence of the ripening inhibitor (sunflower oil in the present case) acting as a hydrophobic sink (Chang, McLandsborough, & McClements, 2013) is likely to play a fundamental role.

The data of **Fig. 5** also show that WPI-based nanoemulsions exhibit a higher inhibition of mycelial growth than T80 based ones. Several mechanisms appear to be involved in the antimicrobial action of EO nanoemulsions, including (a) the internalization of the oil droplets in the microbial cells, owing to the passive transport through the cell membrane, and hence depending primarily on the mean droplet size, (b) the fusion of the emulsion droplets with the cell membrane, which promotes the targeted release of the EOs at the desired sites, and depends strongly on the type of emulsifier used, (c) the sustained release over time of the EOs from the nanoemulsion, driven by EO partition between the oil and the aqueous phase, and hence depending on oil phase composition, and (d) the electrostatic interaction of positively charged nanoemulsion droplets with negatively charged microbial cell walls, which increases the concentration of EOs in the vicinity of microbial cells, depending on emulsion formulation and emulsifier charge (Donsì & Ferrari, 2016). The larger mean droplet size of WPI nanoemulsions is expected to limit their extent of internalization with respect to T80 nanoemulsions, and, similarly, their prevalent negative charge is expected to cause a higher electrostatic repulsion from the highly anionic surface of fungi cells

(due to their membrane composition, based on glucan, chitin, and glycoproteins) than nanoemulsions based on non-ionic T80. Furthermore, a slower release of EO would be expected from WPI nanoemulsions with respect to T80 nanoemulsions, for which the formation of T80 surfactant micelles might promote the EO solubilisation. Therefore, the observation that the inhibition of the mycelial growth was higher for WPI nanoemulsions suggests that the fusion of the emulsion droplets with the cell membrane might be the dominating factor in the present case. This hypothesis is supported by previous studies, which show that the complex surface composition of fungal cells might offer a large number of suitable interaction sites with the emulsifier, hence driving the targeted release of the EOs (Ziani, Chang, McLandsborough, & McClements, 2011).

The effect of mean droplet size on emulsion fungitoxic activity was also studied, by comparing the mycelial growth inhibition of nanoemulsions with that of primary emulsions, showing that finer and more homogeneous droplet size distributions, in addition to improving the physical stability of the nanoemulsions, also significantly increased ($p < 0.05$) the antifungal activity of the EOs against *A. niger*. This behavior is clearly visible in the case of the LEO samples. The LEO emulsions formulated with T80 and prepared by HSH exhibited a mycelial growth inhibition of 18%, whereas the nanoemulsions exhibited an inhibition of 24%. Similarly, the mycelial growth inhibition for the LEO samples formulated with WPI showed a significant increase ($p < 0.05$) in the spore germination inhibition from 28% to 40%. Owing to their sub-micrometric droplet size, O/W nanoemulsions can penetrate more easily through the fungi membrane than micrometric emulsion droplets, leading to a marked increase in antifungal activity. In

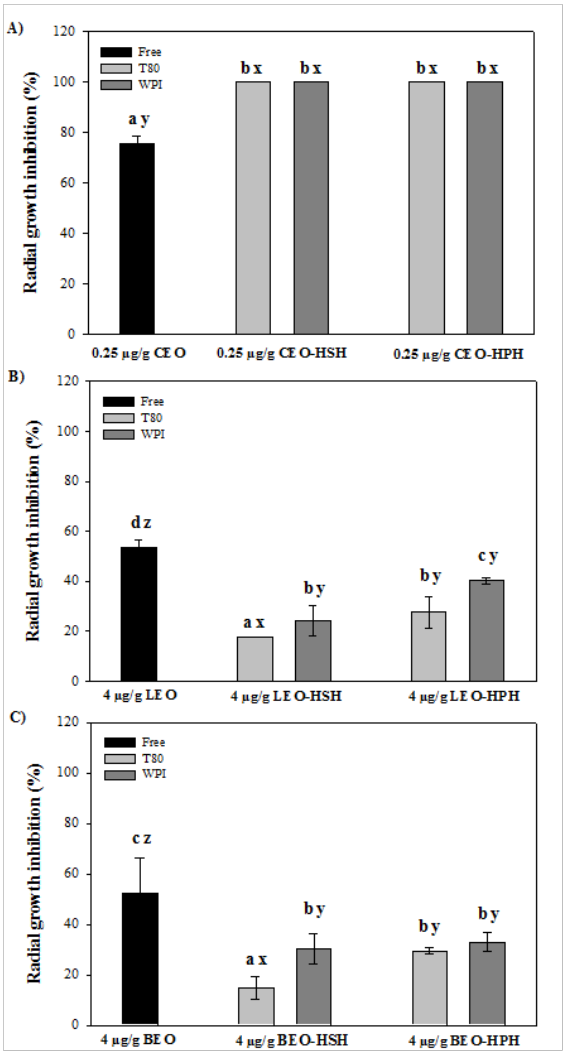

Fig. 5. Radial growth inhibition of *Aspergillus niger* after 7 days of direct exposure to EO nanoemulsions in comparison with free EO and EO emulsions prepared by high shear homogenization (HSH): A) cinnamon leaf EO (CEO) formulations, B) lemon EO (LEO) formulations, and C) bergamot EO (BEO) formulations. Mean value (n = 2) ± SD. Different letters (a, b, c, d) indicate significant differences among systems; and (x, y, z) indicate significant differences among samples with and without emulsifiers ($p < 0.05$).

agreement with these results, several previous research papers report the significant improvement of the antimicrobial activity, when encapsulated in submicrometric emulsions, of different EOs, such as limonene (Donsì et al., 2011), thyme (Chang, McLandsborough, & McClements, 2012) and peppermint EOs (Liang et al., 2012) among others.

3.3.4 Inhibition of spore germination

The inhibition of spore germination of *A. niger* by exposure to the EO nanoemulsions is shown in **Fig. 6**. In comparison with the inhibition of mycelial growth, the role played by nanoemulsions on the inhibition of spore germination exhibits some differences.

The inhibition of spore germination showed a statistically significant ($p < 0.05$) enhancement upon emulsification for CEO (both T80 and WPI) and BEO (only WPI), whereas in the case of LEO (both T80 and WPI) and BEO (only T80) a decrease was observed. The differences with respect to the effect on mycelial growth likely depend on the different mechanisms involved in the inhibition of spore germination, which is based on the denaturation of the enzymes or in the obstruction of the aminoacids implicated in the germination process, interfering with their normal activity (Tian et al., 2012).

Similarly, to the inhibition of mycelial growth, the WPI-based nanoemulsions were more effective in inhibiting the spore germination than T80-based ones, which can also be explained in terms of the larger number of sites suitable for interaction with WPI than with T80 also on the spore surface.

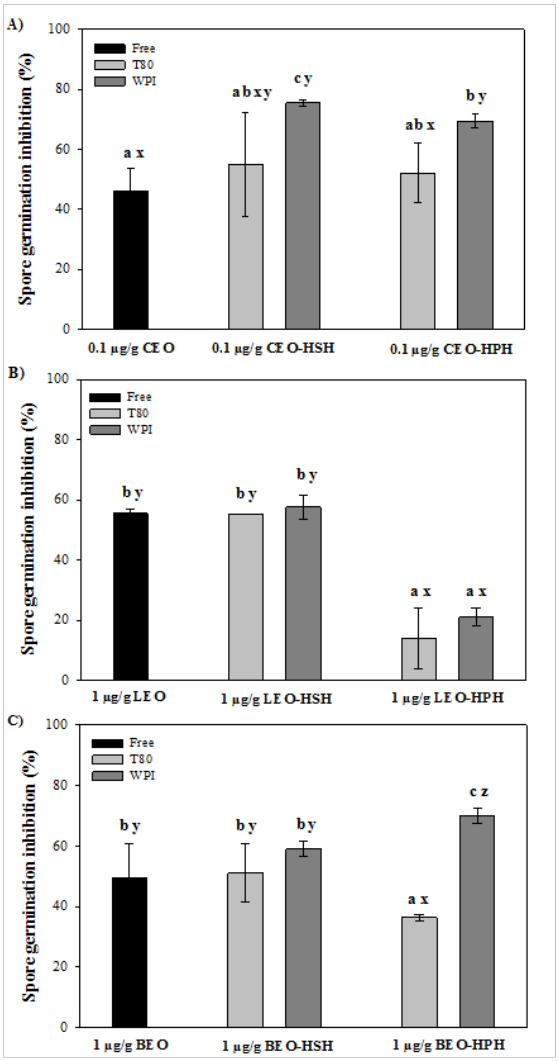

Fig. 6. Spore germination inhibition of *Aspergillus niger* after 7 days of direct exposure to EO nanoemulsions in comparison with free EO and EO emulsions prepared by high shear homogenization (HSH): A) cinnamon leaf EO (CEO) formulations, B) lemon EO (LEO) formulations, and C) bergamot EO (BEO) formulations. Mean value (n = 2) ± SD. Different letters (a, b, c) indicate significant differences among systems; and (x, y, z) indicate significant differences among samples with and without emulsifiers ($p < 0.05$).

No clear dependence of spore germination inhibition on mean droplet size could be identified, with no significant difference between primary emulsions and nanoemulsions in the case of CEO and BEO, and significantly ($p < 0.05$) lower inhibition for nanoemulsion in the case of LEO. It can be speculated that the mechanisms of action of the different EOs, which result in different levels of spore inhibition at the examined concentrations because of the different constituents, with the main component of CEO, eugenol, being more active than D-limonene, the main component of both LEO and BEO (Combrinck et al., 2011 and Davidson, 1997), are affected at different extent by the delivery system. However, further studies are needed to clarify this aspect.

3.3.5 Morphological characterization of A. niger upon exposure to encapsulated EOs

The effect of the EO encapsulation systems was also examined by light microscopy. The results of the control and treated samples (LEO and BEO formulations) are presented in **Fig. 7**. Control samples, comprising fungi treated with blank emulsions (**Fig. 7A** and **B**) and with blank nanoemulsions (**Fig. 7C** and **D**) exhibited large and globular conidial heads, with the conidiophore being clearly visible. Mycelial hyphae showed a tubular shape and a regular structure, where the cytoplasmic content could be unequivocally distinguished. The EO emulsions (**Fig. 7E–H**) and nanoemulsions (**Fig. 7I–L**) caused a decrease in the cytoplasmic content, with a separation of the cytoplasm from the cell wall in the hyphae, independently on the surfactant used. According to Sharma and Tripathi (2008), the O/W emulsions containing citrus EOs could interfere with enzymatic reactions of wall synthesis, affecting fungal morphogenesis and growth.

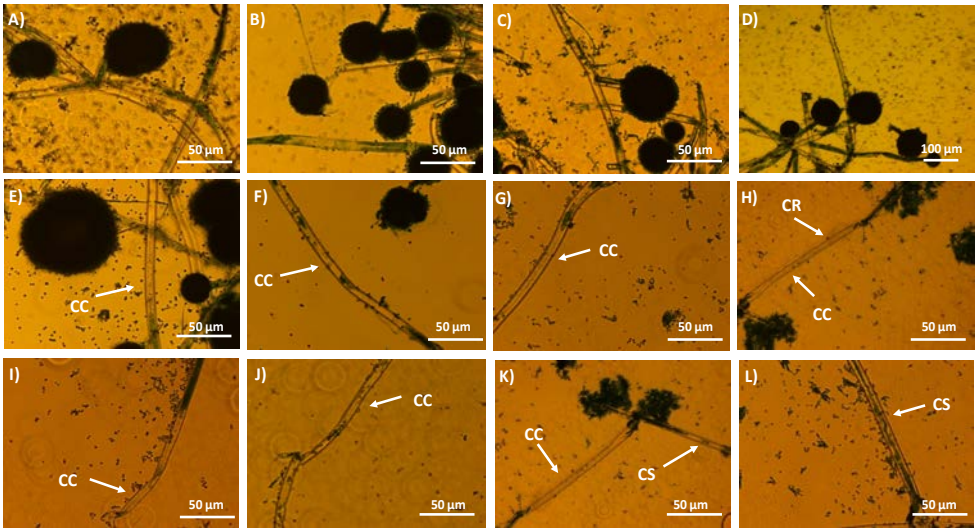

Fig. 7. Bright field micrographs of *Aspergillus niger* treated by EO emulsions and nanoemulsions. A, B) Normal growth of *A. niger* after the treatment with blank emulsions prepared with T80 and WPI, respectively. C, D). Normal growth of *A. niger* in samples treated with blank nanoemulsions prepared with T80 and WPI, respectively. E, F, G, H) Clear decrease in the cytoplasmic content (CC) and cytoplasmic retraction (CR) after the treatment with high shear homogenization (HSH) emulsions formulated with lemon EO (LEO) and T80 and WPI, and with bergamot EO (BEO) and T80 and WPI, respectively. I, J, K, L) Decrease in the cytoplasmic content (CC) with a separation of the cytoplasm (CS) from cell wall in hyphae after the treatment with high pressure homogenization (HPH) nanoemulsions formulated with LEO and T80 and WPI, and with BEO and T80 and WPI, respectively.

4. CONCLUSIONS

The cinnamon leaf, lemon and bergamot oils possess strong fungitoxic activities, but their use is limited by their scarce water solubility. The EOs were encapsulated in nanoemulsions formulated with sunflower oil as ripening inhibitor (at a 1:3 wt ratio with the EO) and T80 or WPI as emulsifiers, to obtain physically stable nanoemulsions at a final EO concentration of 3 wt%, which have been investigated to inhibit the mycelial growth and the spore germination of *A. niger*, in comparison with free EOs.

In the case of cinnamon leaf oil, the nanoemulsions always showed significantly better antifungal properties than the free EO. On the contrary, in the case of citrus EO, the encapsulation in nanoemulsions, in comparison with the free EOs, did not induce any significant improvement of the antifungal activity, for bergamot EO, or had an antagonistic effect, in the case of lemon EO. The different composition of the EOs, with eugenol being the main component of cinnamon leaf oil and D-limonene being the main component of citrus oils, and their different interaction with the nanoemulsion ingredients, likely explain the different contribution of the nanoemulsions.

Remarkably, the nanoemulsions based on WPI always exhibited better antifungal activity than those based on T80, likely due to the larger numbers of sites of interaction with the WPI than with T80 available on the surface of fungal cells and spores.

The results, therefore, demonstrate the promising advantages of using nanoemulsions as physically stable antifungal agents to control *A. niger* growth and spore germination. However, further studies are needed to better elucidate some key aspects in the design of EO nanoemulsion, such as the role played by formulation and morphology on the specific mechanisms of action of the EO components, on the interaction with the microorganism target sites, and the *in product* behavior.

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SECTION 1.2

USE OF OIL-IN-WATER EMULSIONS TO CONTROL FUNGAL DETERIORATION OF STRAWBERRY JAMS

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Food Chemistry, 211 (2016), 92-99

Abstract

This work aimed to control the fungal deterioration of strawberry jams. The antifungal activity of the clove, cinnamon leaf, lemon and mandarin essential oils and their effectiveness in oil-in-water emulsions were evaluated. According to the results obtained, only clove and cinnamon leaf oils were selected to prepare emulsions. All the tested emulsions were stable, independently the amount of polymer and essential oil used. Essential oil loss was affected by the amount of polymer employed to prepare the emulsions. The oil-in-water emulsions with 5.0 mg/g xanthan gum, and with 0.55 mg/g clove or 0.65 mg/g cinnamon leaf essential oil, were used for the *in vivo* tests. The jams prepared with the oil-in-water emulsions showed a lower fungal decay compared with jams without emulsion. The present work demonstrated that emulsions can be employed to prevent strawberry jam mould spoilage.

Keywords: Essential oils; Oil-in-water emulsion; Strawberry jam; *Aspergillus flavus*; *Aspergillus niger*; *Penicillium expansum*

1. INTRODUCTION

Fungal contamination is a serious problem in the food industry because it has negative impacts on final products. Fungi are the most important microorganisms to contaminate fruit and berry concentrates, like jams with low water activity. Fungal spores present in the raw materials of jams are inactivated while jams are cooked. However, the large jam containers used in the food industry may be re-contaminated by spores of indoor fungi; e.g. during partial container depletion (Nieminen et al., 2008).

There are thousands of known species of moulds, and the commonest genera are *Aspergillus* and *Penicillium*. Moulds are difficult to inhibit because of their complex structure, and using chemical agents is one of the main techniques resorted for controlling their growth. However, consumer concern about human health has forced the food industry to search for new strategies as alternatives to chemical additives in order to control food spoilage caused by moulds.

Essential oils (EOs) extracted from many plants and fruits are used as antimicrobial agents against bacteria, moulds and yeasts (Perdones et al., 2012 and Salvia-Trujillo et al., 2014). Their natural character renders them desirable for use in food products. Many EOs have been recognised as safe (GRAS) by the FDA in 21 Code of Federal Regulation part 182.20 (CFR, 2014), and they are widely accepted by consumers (Burt, 2004).

The antimicrobial activity of EOs is attributed mainly to their content in volatile compounds. Eugenol (4-allyl-2-methoxyphenol) is the main compound in cinnamon leaf EO (75–95% (w/w)) (Vangalapati, Satya, Prakash, & Avanigadda, 2012). This EO has demonstrated potent antioxidant and antibacterial activity (Bakkali, Averbeck, Averbeck, & Idaomar, 2008). Eugenol is a naturally-occurring phenol extracted from cloves. Different studies have demonstrated the bactericidal and antifungal activity of clove EO and eugenol (Hua et al., 2014, Jayashree and Subramanyamm, 1999, Liang et al., 2015 and Velluti et al., 2003). The antimicrobial activity of EO from citrus fruits has been widely demonstrated

against mould, yeast and bacteria (Belletti et al., 2010, Espina et al., 2011 and Viuda-Martos et al., 2008).

The volatile compounds present in EOs are highly insoluble in water because of their lipophilic nature, whose contact with microorganisms in high moisture content foods may be limited (Kalemba & Kunicka, 2003). One way to avoid this problem and to enhance their aqueous solubility and stability is to incorporate essential oils into oil-in water (O/W) emulsions. In this case, their sensory impact on food products could reduce and their water solubility could increase for the contact with microorganisms to suffice and to improve antimicrobial effectiveness (Hill, Gomes, & Taylor, 2013).

The objective of this study was to evaluate the mould decay of strawberry jams by using natural preservatives. For this purpose, the *in vitro* antifungal activity of different EOs and O/W emulsions, and the effectiveness of these emulsions when incorporated into strawberry jams were evaluated.

2. MATERIALS AND METHODS

2.1 Screening the antifungal activity of EOs

Clove, cinnamon leaf, lemon and mandarin EOs (Sigma-Aldrich, St. Louis, USA) were individually tested against *Aspergillus flavus* (CECT 2685), *Aspergillus niger* (CECT 20156) and *Penicillium expansum* (CECT 20140). The method described by Viuda-Martos et al. (2008) was employed with minor modifications. The stock cultures of fungi were supplied by the Spanish Type Culture Collection (CECT, Burjassot, Spain).

The fungi were inoculated on Potato Dextrose Agar (PDA, Scharlab, Barcelona, Spain) and incubated at 25 °C for 7 days. Afterwards, spores were counted in a hemocytometer to achieve an inoculum density of 10^6 CFU/mL. Different EO concentrations were tested after taking into account previous studies (Omidbeygi et al., 2007, Perdones et al., 2012 and Viuda-Martos et al., 2008). The concentrations of tested EOs were: 0.40, 0.45, 0.50 and 0.55 mg/g for clove oil; 0.50, 0.55, 0.60 and 0.65 mg/g for cinnamon leaf oil; 10, 12.50, 15 and 17.50 mg/g for lemon oil; 27.50, 30, 32.50 and 35 mg/g for mandarin oil. Aliquots of 15 g of PDA with the EOs and 0.1% (w/w) Tween 80 (Scharlab, Barcelona, Spain) were poured into Petri dishes. EOs were added to the culture medium at 50 °C and Tween 80 was added to the medium to ensure good EO distribution. The petri dishes without EO were used as control samples. The centre of each plate was inoculated with a PDA disc (7 mm diameter) taken from the edge of 0-day-old fungi culture, previously spread with 100 µL of the spore solution (10^6 CFU/mL). Each plate was sealed with Parafilm® and incubated for 7 days at 25 °C. Radial mycelial growth was evaluated daily for 7 days by measuring the diameter of each fungus. Values were expressed in mm diameter/day. All tests were run in duplicate.

2.2 Study of O/W emulsions

2.2.1 Emulsion preparation

Xanthan gum (XG, Satiaxane™ CX 911, Cargill, Barcelona, Spain) was dispersed in distilled water at 2.5, 5.0 and 7.5 mg/g, and stirred overnight at room temperature. After biopolymer dissolution, the clove and cinnamon leaf EOs were added to reach the final concentrations of 0.55, 0.65, 0.75 mg/g and 0.65, 0.75, 0.85 mg/g, respectively. The mixture was emulsified in a rotor-stator homogeniser (Ultraturrax, IKA®, Germany) at 10,000 rpm for 1 min and 20,000 rpm for 3 min. These emulsions were degasified at room temperature with a vacuum pump.

2.2.2 Physico-chemical characterisation of O/W emulsions

The particle size was determined with a laser diffractometer (Mastersizer 2000, Malvern Instruments, Worcestershire, UK). Emulsions were diluted in deionised water at 2,000 rpm until an obscuration rate of 10% was obtained. The Mie theory was applied by considering a refractive index of 1.50 and absorption of 0.01.

The ζ-potential was carried out according to Sánchez-González, Cháfer, Chiralt, and González-Martínez (2010), using a Zetasizer nano-Z (Malvern Instruments, Worcestershire, UK).

The rheological behaviour of emulsions was analysed by a rotational rheometer (Haake Rheostress 1, Thermo Electric Corporation, Karlsruhe, Germany) with a type Z34DIN Ti sensor system of coaxial cylinders, assessed as described by Sánchez-González et al. (2010). Shear stress (τ) was measured as according to shear rate ($\dot{\gamma}$) from 0 to 512 s^{-1}. Apparent viscosity values were calculated at 100 s^{-1}.

2.2.3 GC-MS analysis

The clove and cinnamon leaf EOs and O/W emulsions composition were analysed by GC-MS. Two grams of the EO or O/W emulsions were suspended in a tube that contained 15 mL of *n*-hexane. The mixture was shaken gently and filtered through filter paper. *n*-Hexane was evaporated at 40 °C in a rota-vapour, and the obtained extracts were added to 2 mL of n-hexane and analysed by GC–MS.

The GC/MS analysis of the EOs was performed in a 6890/5975 inert GC–MS (Agilent Technologies, Santa Clara, CA, US), equipped with a HP-5 fused silica capillary column (30 m × 0.25 mm × 0.25 µm). The oven temperature was held at 60 °C for 3 min, and then raised to 100 °C at 10 °C/min, to 140 °C at 5 °C/min, and finally to 240 °C at 20 °C/min. Helium gas was used as the carrier gas at a constant

flow rate of 1 mL/min. The injector and MS transfer line temperatures were set at 250 °C and 230 °C, respectively. The parameters for the MS analysis were EI Ion source, electron energy 70 eV, solvent delay 3 min and *m/z* 40–550 amu. EO components were identified by matching mass spectra with the standard mass spectra from the NIST MS Search 2.0 library.

2.2.4 Antifungal activity of O/W emulsions

The antifungal activity of the clove and cinnamon leaf emulsions against *A. flavus*, *A. niger* and *P. expansum* was determined by the methodology described in Section 2.1. In this case, 0.5 g of each O/W emulsion was added to 49.5 g of PDA at 50 °C. Next, aliquots of 15 g of PDA with the emulsions were poured into Petri dishes. The PDA with a dispersion prepared with distilled water and XG was used as a control. A disc of mycelial material was placed in the center of each plate and then incubated. Radial mycelial growth was evaluated daily for 7 days. The results were expressed in mm diameter/day. All tests were run in duplicate.

2.3 Study of O/W emulsions in strawberry jam

2.3.1 Emulsion preparation

Jam preparation was adapted from Igual, Contreras, and Martínez-Navarrete (2010). Strawberry jam was obtained by mixing fruit and sugar in a ratio of 65:35 and cooked at 100 °C for 30 min to reach a 60 °Brix in the product as described in the Spanish quality regulation for fruit jam (BOE, 2003). This process was carried out in an electrical food processor (Thermomix TM 31, Vorwerk M.S.L, Spain). The amount of emulsions added to strawberry jam was established to achieve a concentration of 1 g of the O/W emulsion in 100 g of jam in the final product.

2.3.2 Evaluation the antifungal activity of O/W emulsions in strawberry jams

Fifteen grams of strawberry jam that contained the O/W emulsions were inoculated with 100 µL of the spore solution (10^6 CFU/mL). Jams were poured into Petri dishes and incubated for 63 days at two different temperatures: 4 °C to simulate product cold storage after opening the jam container, and at 25 °C, the optimum growth temperature of fungi. Three Petri dishes were prepared per temperature condition, microorganism and day of analysis (n=54). Mould counts were done in PDA plates after a 72-hour incubation at 25 °C (Pascual & Calderón, 2000). All assays were performed in duplicate.

2.3.3. Sensory analysis

A sensory analysis was carried out by 30 non-expert untrained assessors. The group of assessors was composed of 12 men and 18 women, and panellists' ages ranged from 21 to 50 years. Tests were done on a structured 9-point hedonic scale (9=very much like and 1=very much dislike) (UNE-ISO 4123), by which the colour, aroma, taste, consistency and overall acceptance attributes were evaluated. All the samples were presented to the panellists at room temperature in a transparent plastic glass coded with three random numbers.

2.4 Statistical analysis

The results obtained in the physico-chemical characterisation of the O/W emulsions, and the antifungal evaluation of the EO and O/W emulsions, were analysed by a multifactor analysis of variance (multifactor ANOVA). The effect of incorporating the O/W emulsion into the sensory attributes of strawberry jam was evaluated by a one-way ANOVA. The least significance procedure (LSD) was used to test for any differences between averages at the 5% level of significance. Data were statistically processed by Statgraphics Centurion XVI.

3. RESULTS AND DISCUSSION

3.1 Screening the antifungal activity of EOs

The clove, cinnamon leaf, lemon and mandarin EOs all at the tested concentrations had the capacity to reduce or inhibit the growth of *A. flavus*, A. *niger* and *P. expansum* (**Fig. 1**) since fungi showed slightly retarded growth compared with the control plates, even for the lowest EO concentrations. This behaviour suggests that the active compounds of the EOs could affect initial mould development, and could cause a delay in mould growth, which would confirm their fungistatic effect (Manso, Cacho-Nerin, Becerril, & Nerín, 2013).

The clove, cinnamon leaf and lemon EOs at all the tested concentrations increased the *Lag phase* of *A. flavus*, *A. niger* and *P. expansum*, with a diminution of the germination rate. The clove EO provoked a higher delay in mould growth compared to the other EOs tested. The clove EO at the lowest concentration managed to reduce mycelial growth more than 78% for all the studied moulds.

The clove, cinnamon leaf and lemon EOs inhibited growth of moulds at the assayed highest concentrations (0.55 and 17.5 mg/g, respectively) during the whole study period, except for the cinnamon leaf EO tested against *P. expansum*, for which mycelial growth reduced, but was not inhibited. The high lemon EO

concentration needed to inhibit fungi development could be due to D-limonene's susceptibility to oxidative degradation, which could cause loss of activity (Sun, 2007).

The mandarin EO caused the lowest percentage of mycelial reduction in all the studied moulds (**Fig.1**). The highest mandarin EO concentration tested achieved only percentage reductions of 62%, 61% and 73% for *A. flavus*, *A. niger* and *P. expansum*, respectively.

3.2 Study of O/W emulsions

3.2.1 Physico-chemical characterisation of O/W emulsions

Table 1 shows the $d_{3,2}$ and $d_{4,3}$ values for the particle size analysis, the ζ-potential and the rheological parameters for the various emulsions.

Oil content and XG concentration had a significant impact on $d_{3,2}$ and $d_{4,3}$. Regarding oil content, the clove emulsions at the lowest assayed EO concentration (0.55 mg/g) exhibited a $d_{3,2}$ of 5.19±0.05 μm when the XG concentration was 2.5 mg/g, whereas an increased droplet mean diameter was observed (7.32±0.21 μm) at the highest EO concentration (0.75 mg/g) (**Table 1**).

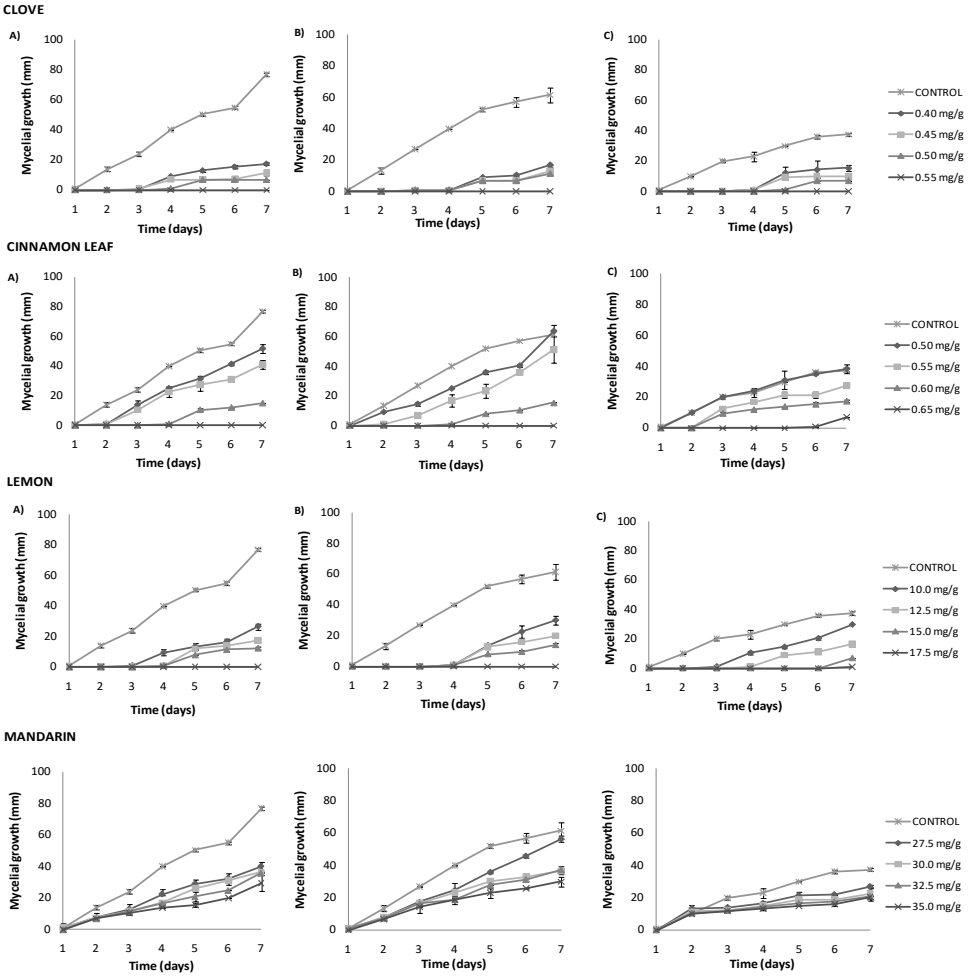

Fig. 1. Antifungal activity of the clove, cinnamon leaf, lemon and mandarin EOs against (A) *Aspergillus flavus*, (B) *Aspergillus niger* and (C) *Penicillium expansum* at 25 °Cfor 7 days. Diameter of mycelial growth (mean value and standard deviation (n=2)).

The same behaviour was observed for $d_{4,3}$ when 2.5 mg/g of XG was used. The mean size values increased from 10.4±2.6 to 17.5±0.7 µm when larger amounts of the clove EO were employed. Generally, the cinnamon leaf O/W emulsions exhibited the same tendency for the $d_{3,2}$ and $d_{4,3}$ values observed in the emulsions formulated with the clove EO.

As observed, the higher the oil content in the emulsions, the bigger particle size became. This could be due to the increase in the dispersed phase concentration, which could facilitate the droplet flocculation rate and therefore the reduction in the ratio between the interfacial stabilising material and the dispersed phase (McClements, 2005). Similar results have been reported by Sánchez-González et al. (2010) in emulsions of bergamot EO and chitosan aqueous systems.

Regarding polymer concentration, the increase in the XG concentration led to a reduction in the droplet mean diameter of emulsions with significant differences ($p<0.05$) (Krstonošić, Dokić, Dokić, & Dapčević, 2009). The cinnamon leaf emulsions prepared with the lowest EO concentration gave $d_{3,2}$ values of around 8.36±0.04 µm when the polymer concentration was 2.5 mg/g. However, a smaller droplet mean diameter was observed when 7.5 mg/g of XG was used at the same EO concentration (6.33±0.29 µm) (**Table 1**). This could be due to the ability of particles to cover the surface of droplets and to produce a thick interfacial layer around them (Frelichoswska, Bolzinger, & Chevalier, 2009). In contrast, the clove emulsions presented similar $d_{3,2}$ values despite the increase in the XG concentration (**Table 1**). This suggested that the clove EO amphiphilic components could have greater surfactant activity, and could thus contribute to reduce the droplet particle size under equal homogenisation conditions. This trend has also been observed by Bonilla, Atarés, Vargas, and Chiralt (2012) when they used thyme oil in chitosan-based films.

The effect produced by incorporating larger amounts of polymer was less marked for the $d_{4,3}$ values, for both types of O/W emulsions.

The surface charge of oil droplets in the emulsions prepared with the EOs is shown in **Table 1**. According to McClements (2005), if the electrical charge of

droplets is high enough, the emulsion may be stable against aggregation due to repulsive forces between droplets. Generally, particles with a ζ-potential that is more positive than +30 mV, or more negative than −30 mV, are considered stable. The electrical charge of the lipid droplets of the emulsions were between −67.0±0.6 and −72.0±1.9 for the clove EO emulsions, and between −64.5±1.1 and 70.7±0.9 for the cinnamon leaf EO emulsions. Therefore, it can be stated that both types of O/W emulsions are stable. In the present work, the strong negative ζ-potential observed in the emulsions was due to the presence of XG, which is an anionic hydrocolloid. The polymer was used as an emulsion stabiliser as these stabilisers can absorb into the interfacial layer (Dickinson, 2009). In addition, the stabilisation action of hydrocolloids was due to the viscosity modification in the continuous phase by lowering the rate of creaming and coalescence (Dickinson, 2009 and Garti and Leser, 2001).

Regarding rheological characteristics, all the emulsions showed a shear thickening behaviour with flow behaviour index (*n*) values at around 0.47. No thixotropic effects were observed from the comparison made of the up and down curves. The curves were predicted by the Ostwald de Waele model. **Table 1** shows the consistency index (*k*), the flow behaviour index (*n*) and the apparent viscosity values calculated at 100 s^{-1}, which is the typical shear rate of different unit operations, such as mastication (McClements, 2005).

Table 1. Polymer (mg/g) and EO concentration (mg/g), particle size ($d_{3,2}$ and $d_{4,3}$), ζ-potential, Ostwald de Waele model parameters (n, k) and apparent viscosity (η_{ap} at 100 s^{-1}) of the O/W emulsions prepared with the clove and cinnamon leaf EOs. Mean values (n=3) and standard deviation.

Polymer concentration (mg/g)	EO concentration (mg/g)	$d_{3,2}$ (μm)	$d_{4,3}$ (μm)	ζ-potential (mV)	$0 \leq \dot{\gamma} \leq 512\ s^{-1}$		
					k (Pa·s)^n	n	η_{ap} (Pa·s)
CLOVE EO							
2.5	0.55	5.19 ± 0.05 ^{aA}	10.4 ± 2.6 ^{aA}	- 67.0 ± 0.6 ^{bC}	0.130 ± 0.009 ^{aA}	0.595 ± 0.007 ^{cC}	0.020 ± 0.001 ^{aA}
2.5	0.65	6.28 ± 0.02 ^{bC}	15.0 ± 1.1 ^{bB}	- 67.2 ± 4.5 ^{bC}	0.131 ± 0.006 ^{aA}	0.595 ± 0.005 ^{cC}	0.020 ± 0.001 ^{aA}
2.5	0.75	7.32 ± 0.21 ^{cC}	17.5 ± 0.7 ^{bcC}	- 67.0 ± 0.3 ^{bC}	0.120 ± 0.006 ^{aA}	0.605 ± 0.005 ^{cC}	0.019 ± 0.001 ^{aA}
5.0	0.55	5.21 ± 0.05 ^{aA}	10.8 ± 2.3 ^{aA}	- 72.0 ± 1.9 ^{aA}	0.523 ± 0.028 ^{bB}	0.472 ± 0.004 ^{bB}	0.047 ± 0.002 ^{bB}
5.0	0.65	6.48 ± 0.36 ^{bB}	15.4 ± 1.2 ^{bB}	- 70.4 ± 0.9 ^{aAB}	0.519 ± 0.025 ^{bB}	0.471 ± 0.004 ^{bB}	0.046 ± 0.002 ^{bB}
5.0	0.75	7.12 ± 0.22 ^{cB}	17.9 ± 0.7 ^{bcC}	- 70.4 ± 0.3 ^{aAB}	0.515 ± 0.041 ^{bB}	0.475 ± 0.007 ^{bB}	0.045 ± 0.002 ^{bB}
7.5	0.55	5.13 ± 0.05 ^{aA}	11.6 ± 2.2 ^{aA}	- 69.8 ± 0.1 ^{aB}	1.312 ± 0.096 ^{cC}	0.401 ± 0.008 ^{aA}	0.081 ± 0.006 ^{cC}
7.5	0.65	5.19 ± 0.03 ^{aA}	10.9 ± 0.8 ^{aA}	- 70.8 ± 0.3 ^{aAB}	1.359 ± 0.081 ^{cC}	0.395 ± 0.007 ^{aA}	0.086 ± 0.005 ^{cC}
7.5	0.75	5.14 ± 0.25 ^{aA}	10.8 ± 0.4 ^{aA}	- 70.2 ± 0.7 ^{aAB}	1.312 ± 0.064 ^{cC}	0.399 ± 0.004 ^{aA}	0.082 ± 0.003 ^{cC}
CINNAMON LEAF EO							
2.5	0.65	8.36 ± 0.04 ^{bB}	17.6 ± 0.3 ^{bB}	- 64.9 ± 0.8 ^{cC}	0.55 ± 0.04 ^{aA}	0.472 ± 0.041 ^{cC}	0.047 ± 0.002 ^{aA}
2.5	0.75	8.59 ± 0.48 ^{bcB}	19.6 ± 3.9 ^{bcB}	- 64.5 ± 1.1 ^{cC}	0.57 ± 0.05 ^{aA}	0.460 ± 0.007 ^{cC}	0.047 ± 0.002 ^{aA}
2.5	0.85	8.92 ± 0.37 ^{cB}	22.8 ± 1.7 ^{cC}	- 64.7 ± 0.9 ^{cC}	0.55 ± 0.04 ^{aA}	0.468 ± 0.006 ^{cC}	0.047 ± 0.002 ^{aA}
5.0	0.65	6.46 ± 0.02 ^{aA}	13.6 ± 1.5 ^{aA}	- 65.1 ± 1.2 ^{cC}	0.55 ± 0.08 ^{aA}	0.467 ± 0.004 ^{cC}	0.047 ± 0.004 ^{aA}
5.0	0.75	6.21 ± 0.23 ^{aA}	13.9 ± 5.1 ^{aA}	- 65.4 ± 1.0 ^{cC}	0.51 ± 0.02 ^{aA}	0.470 ± 0.002 ^{cC}	0.044 ± 0.001 ^{aA}
5.0	0.85	8.82 ± 0.21 ^{cB}	18.9 ± 0.8 ^{bcB}	- 67.3 ± 1.3 ^{bB}	0.59 ± 0.03 ^{aA}	0.453 ± 0.005 ^{cC}	0.048 ± 0.002 ^{aA}
7.5	0.65	6.33 ± 0.29 ^{aC}	21.0 ± 3.3 ^{bcBC}	- 70.4 ± 1.4 ^{aA}	1.40 ± 0.12 ^{bB}	0.294 ± 0.095 ^{aA}	0.070 ± 0.002 ^{bB}
7.5	0.75	6.48 ± 0.17 ^{aA}	19.3 ± 0.3 ^{bcB}	- 70.7 ± 0.9 ^{aA}	1.69 ± 0.05 ^{cA}	0.381 ± 0.002 ^{bB}	0.098 ± 0.002 ^{cC}
7.5	0.85	6.71 ± 0.18 ^{aA}	21.7 ± 4.8 ^{cC}	- 70.5 ± 0.2 ^{aA}	1.73 ± 0.01 ^{cA}	0.378 ± 0.007 ^{bB}	0.098 ± 0.002 ^{cC}

Different superscripts in the same column indicate significant differences (p<0.05) due to polymer concentration (a, b, c, d, e, f) or due to EOs concentration (A, B, C, D, E, F, G, H, I)

The incorporation of EOs into the XG dispersions did not produce significant changes in the rheological characteristics of the emulsions. Notwithstanding, an increase in the XG concentration in the emulsions resulted in significant increases ($p<0.05$) in the consistency index (k), which led to more consistent fluids and is related to the apparent viscosity (η_{ap}) of the emulsions. The clove emulsions formulated with 2.5 mg/g of XG obtained k values of 0.127±0.007 Pa s. The same emulsions prepared with 7.5 mg/g of polymer gave k values of 1.327±0.080 Pa s (**Table 1**). The same tendency was observed for the emulsions formulated with cinnamon leaf EOs.

As shown in **Table 1**, the apparent viscosity values increased significantly ($p<0.05$) when larger amounts of XG were incorporated. These values oscillated between 0.02 and 0.08 Pa s for the clove emulsions, and from 0.05 to 0.10 Pa s for the cinnamon leaf emulsions. It is well-known that even at low polymer concentrations, XG dispersions exhibit high viscosities (Laneuville, Turgeon, & Paquin, 2013). Nevertheless, in the samples with the same amount of XG, the nap values remained constant in spite of using higher EO concentrations. This could be explained by the promotion of the EO-polymer interactions and the complex structure of the network formed by XG, which could cushion the impact of the EOs concentration on η_{ap}. This agrees with previously reported results (Martínez-Padilla, García-Rivera, Romero-Arreola, & Casas-Alencáster, 2015) on foams with whey protein concentrate and XG.

3.2.2 GC–MS analysis

Screening the antifungal activity of EOs indicated that the clove and cinnamon leaf oils were more interesting for controlling mould growth in food products. Components of these EOs were identified by the GC–MS analysis. In both EOs, eugenol (ca. 86%) was the main compound (**Table 2**), and similar results have been reported by different authors (Espina et al., 2011 and Singh et al., 2007). The main compound of cinnamon EO from leaves is usually eugenol and, in some cases, there is a small amount of cinnamaldehyde (Tzortzakis, 2009 and Vangalapati et al., 2012). The clove and cinnamon leaf extracts and their main compound, eugenol, have been reported as one of the most effective natural antimicrobial agents (Amiri et al., 2008, Hill et al., 2013, Omidbeygi et al., 2007 and Singh et al., 2007).

Despite the similar amount of eugenol in both EOs, significant differences in terms of their antifungal activity have been previously observed. The clove EO has marked antifungal activity at the highest tested concentration (0.55 mg/g), whereas the cinnamon leaf EO inhibited the growth of *A. flavus* and *A. niger*, and reduced the growth of *P. expansum* at 0.65 mg/g. These results could suggest synergistic interactions between eugenol and sesquiterpene hydrocarbons (caryophyllene, β-caryophyllene and δ-cadinene) against the evaluated fungi strains. Some studies have reported the antifungal activity of sesquiterpens constituents against several damping-off, root pathogens, etc. (Chang et al., 2008 and Kumar et al., 2014). The sum of the relative areas of eugenol and the sesquiterpene hydrocarbons were 94.67% and 87.76% for the clove and cinnamon leaf EOs, respectively.

Emulsions were also analysed and the EOs losses during emulsion preparation were determined. Estimated EOs losses were referred to eugenol, the main component of both EOs. Eugenol losses after emulsions preparation came close to 40% in all cases. EOs losses could be attributed to stress applied to samples and to the heating achieved during the homogenisation process. Depending on the type of EO employed for emulsion preparation, non-significant differences were

observed. However, EO losses were affected by the amount of XG employed to prepare the O/W emulsions; for instance, 2.5 mg/g of XG led to the greatest eugenol loss compared to 5.0 and 7.5 mg/g XG, while no significant differences were observed between these samples. The lower viscosity of the samples formulated with 2.5 mg/g of XG, compared with the emulsions that contained 5.0 and 7.5 mg/g of XG, could cause the diffusion of EOs to the surface of the emulsions to facilitate evaporation and its subsequent loss (Perdones, Escriche, Chiralt, & Vargas, 2016). The greater the viscosity of the samples, the greater the immobilisation of oil droplets.

3.2.3 Antifungal activity of O/W emulsions

As previously mentioned, only the clove and cinnamon leaf EOs were selected and their concentrations in emulsions were established by considering the results of the *in vitro* evaluation. As a result, the assayed EO concentrations were 0.55, 0.65 and 0.75 mg/g for clove and 0.65, 0.75 and 0.85 mg/g for cinnamon leaf. No mycelial growth was observed for any tested condition (data not shown), which indicated that the O/W emulsions with 0.55 mg/g of clove and 0.65 mg/g of cinnamon leaf sufficed to inhibit *A. flavus*, *A. niger* and *P. expansum* growth over 7 days.

Table 2. Chemical composition of the cinnamon leaf and clove EOs. Percentages of relative area (%) are the mean of two runs and were obtained from electronic integration measurements using selective mass detector.

Compound	Cinnamon leaf EO (% relative area)	Clove EO (% relative area)
R-α-Pinene	0.71 ± 0.40	-
Camphene	0.23 ± 0.22	-
β-Pinene	0.18 ± 0.25	-
α-Phellandrene	0.77 ± 0.60	-
o-cymene	0.48 ± 0.26	-
D-Limonene	0.90 ± 0.50	-
Eucalyptol	0.04 ± 0.40	-
β-Linalool	1.10 ± 0.32	-
Borneol	0.10 ± 0.94	-
1-Terpinen-4-ol	0.30 ± 0.12	-
Thymol	0.10 ± 0.55	-
α-Terpineol	0.10 ± 0.00	-
Cinnamaldehyde	0.50 ± 0.00	-
Safrene	1.29 ± 0.22	-
Eugenol	**84.51 ± 4.16**	**84.48** ± 1.09
Caryophyllene	3.09 ± 0.19	9.90 ± 0.21
Cinnamylacetate	0.70 ± 0.92	-
β-Caryophyllene	0.57 ± 0.18	2.83 ± 0.18
δ-Cadinene	0.16 ± 0.14	0.39 ± 0.00
Aceteugenol	1.66 ± 0.36	2.40 ± 0.34
Caryophyllene oxide	0.10 ± 0.41	-
Benzyl Benzoate	3.41 ± 0.00	-

According to the EO losses which occurred during emulsion preparation, the final clove and cinnamon leaf EO content in their O/W emulsions was 0.34 mg/g and 0.39 mg/g, respectively. When comparing the results with those obtained in the *in vitro* tests, an increased antifungal activity was noted. This could be attributed to improved water solubility of the encapsulated compounds by enhancing the EOs diffusion rate and, therefore, antifungal activity at the tested concentrations against *A. flavus*, *A. niger* and *P. expansum*.

According to these results, and to those obtained in the physico-chemical characterisation of the O/W emulsions, the suitable amount of polymer and EO to be employed in strawberry jams was established. The emulsions prepared with 0.55 and 0.65 mg/g of the clove and cinnamon leaf EO, respectively, and with 5.0 mg/g of XG, were added to strawberry jam. Considering this formulation, the final concentration of clove and cinnamon leaf EO in the strawberry jams was 0.34 mg/g and 0.39 mg/g, respectively.

3.3 Study O/W emulsions in strawberry jam

3.3.1 Evaluation of the antifungal activity of O/W emulsions in strawberry jam

In order to evaluate the influence of temperature and the effectiveness of the O/W emulsion on mould growth, samples were stored at 4 °C and 25 °C for 63 days (**Fig. 2**). In the control samples, the mycelial growth rate was affected by storage temperature for all the tested moulds.

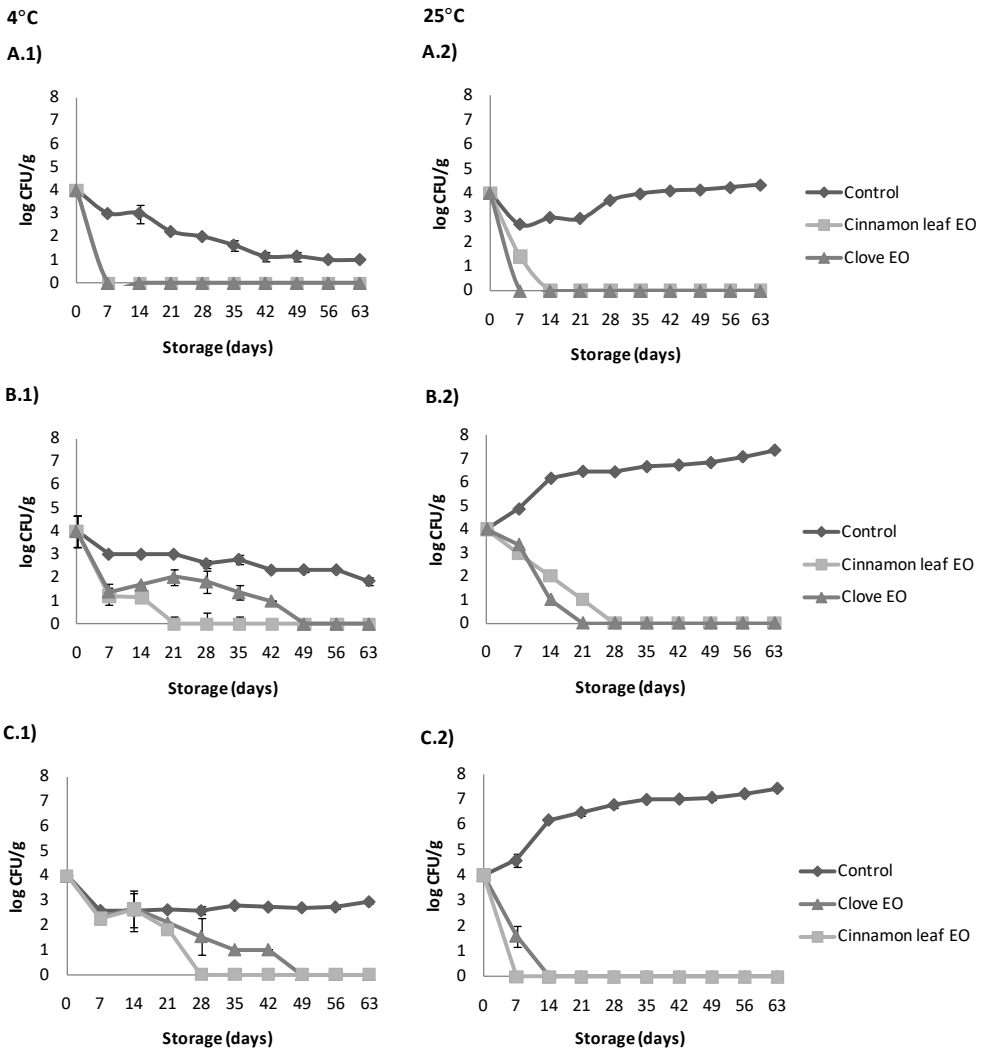

Fig. 2. Effect of the oil-in-water emulsions on the growth of (A) *Aspergillus flavus*, (B) *Aspergillus niger*, (C) *Penicillium expansum* on strawberry jams stored at 4 °C and 25 °C for 63 days. Mean value and standard deviation (n = 2).

The jams prepared with the O/W emulsions inoculated with *A. flavus* and stored at 4 °C showed no fungal growth during the evaluation period. EO type significantly affected ($p<0.05$) the antifungal activity of the O/W emulsions. At refrigeration temperature, the clove and cinnamon O/W emulsions took longer to inhibit *A. niger* and *P. expansum* growth compared with *A. flavus*. The inhibitory effect was stronger when cinnamon leaf O/W emulsion was added to the strawberry jams, compared to clove emulsions. In this regard, no fungal growth was inhibited at days 21 and 28 for *A. niger* and *P. expansum*, respectively when cinnamon leaf was used; however, the clove emulsions inhibited fungal growth at day 49.

Inhibition of *A. flavus* on the samples that contained clove emulsions took place during the first 7 storage days at 25 °C, whereas inhibited fungi growth was observed on day 14 when the cinnamon leaf EO was used. Unexpectedly, the opposite behaviour was observed for *P. expansum*, whose inhibition at 25 °C was faster than at 4 °C independently of the EO type employed.

Despite the oil content in jam samples being the same as that tested in agar media, the time needed to observe the effectiveness of EOs against moulds in jams was longer. This could be related to the different diffusions of the active compounds, which could be easier in agar media than in jam, or could be due to the fact that the antifungals lost through evaporation throughout the storage period were limited since the matrix structure differed and the mass transfer process occurred differently (Perdones et al., 2012). This behaviour could also be explained by the low water content in food compared to agar media, which could hinder the transfer of EO to the active site in the microbial cell (Omidbeygi et al., 2007). Other crucial factors must be taken into account, such as antagonistic interactions with other ingredients (e.g. proteins or carbohydrates) (Pitt & Hocking, 2009).

3.3.2 Sensory analysis

A sensory analysis was run to check the acceptability of the strawberry jams that contained the O/W emulsions. The samples tested by panellists consisted in jams with the clove and cinnamon leaf O/W emulsions at the established concentrations, and a jam sample with no EO added, which was used as a control. The average scores marked by the assessors for all the evaluated attributes are shown in **Fig. 3**.

The strawberry jams with the added O/W emulsions obtained lower scores for the aroma, taste and overall acceptance attributes compared with the control samples. The consistency and colour attributes did not significantly differ ($p>0.05$) from the control samples. The lowest scores were found in the jams with the cinnamon leaf O/W emulsions because of the higher EO content used and the strong impact of this EO on the typical strawberry jam flavour. However, the incorporation of the O/W emulsions into jams did not affect their texture and colour evaluations.

Further studies should be carried out to obtain a good relation between the antimicrobial effectiveness of the active compounds and their sensory impact on the final product.

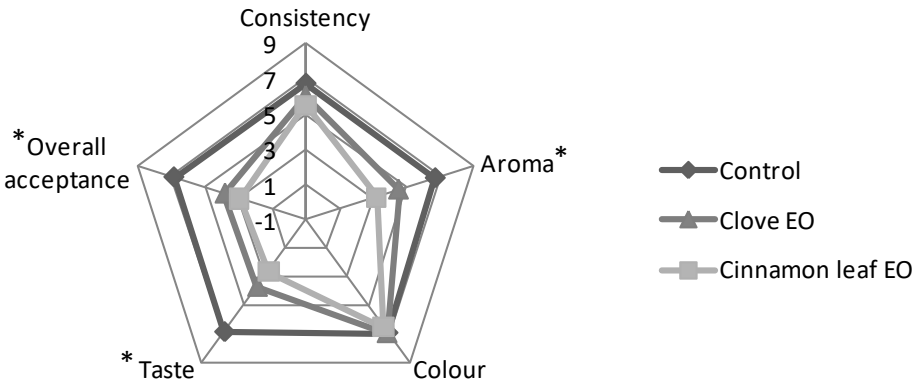

Fig. 3. Sensory profile of strawberry jams. *Indicates 95% significant differences according to the ANOVA test (n = 30).

4. CONCLUSION

The clove and cinnamon leaf EOs showed the highest antifungal properties. The physic-chemical characterisation of the different O/W emulsions prepared with these EOs revealed that the EO concentration in the emulsion brings about changes in particle size. XG contributes to the stability of emulsions by adsorption on the oil droplet surface. Indeed, the higher the polymer content, the shorter the droplet mean diameter. The main compound of the clove and cinnamon leaf EOs was eugenol, which is responsible, together with sesquiterpene hydrocarbons, for their antifungal activity. The O/W emulsions preparation led to EO losses of about 40%, and such losses were affected by the amount of XG employed, and was not due to the EO type. The antifungal activity of the clove and cinnamon leaf O/W emulsions against several strains, such as *A. flavus*, *A. niger* and *Penicillium expansum*, was evidenced in the *in vitro* and *in vivo* tests.

The incorporation of the O/W emulsions into strawberry jam did not modify the texture or colour of the product, but negatively affected aroma, taste and the overall acceptance of jam.

The combined results demonstrate the promising advantages of using emulsions as natural additives to preserve and/or extend the shelf life of strawberry jams. Nevertheless, further studies are needed to reduce the sensory impact on final products, such as high pressure homogenisers to reduce the oil droplet's particle size, increasing the interfacial area exposed to microbial cells, or combining different natural agents in order to improve synergistic effects in foodstuff.

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SECTION 1.3

APPLICATION OF CINNAMON BARK EMULSIONS TO PROTECT STRAWBERRY JAM FROM FUNGI

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Abstract

The objective of the work was to evaluate the use of cinnamon bark-xanthan gum emulsions to preserve strawberry jam. The optimisation of the methodology used to prepare the emulsions and, the evaluation of their antimicrobial activity in culture media and in the strawberry jam were investigated. Emulsions were prepared in either a rotor-stator homogeniser or a magnetic stirrer combined with a high pressure homogeniser. Microorganism suspensions (10^3 and 10^6 CFU/mL), essential oil concentration and microbial sensitivity were decisive in the emulsions' antimicrobial activity. The high stress applied to samples and their heating during homogenisation caused essential oil content losses. The jams prepared with the oil-in-water emulsions inoculated with *Aspergillus flavus, Penicillium expansum, Zygosaccharomyces rouxii* and *Zygosaccharomyces bailii* exhibited no growth during the 28 days of analysis. The obtained results indicated the suitability of cinnamon bark oil-xanthan gum emulsions for preserving strawberry jam.

Keywords: Natural agents; cinnamaldehyde; oil-in-water emulsions; preservation; strawberry jam

1. INTRODUCTION

Jams are defined as mixtures, with a suitable gelled consistency, of sugars, pulp and/or purée of one or more fruits and water. Despite jam is a stable product due to its high sugar level (69%, USDA, 2016), there are particular microorganisms, such as moulds and yeasts, which are able to grow in products with an elevated amount of sugar.

The use of chemical additives is very effective to prevent food spoilage owing to moulds and yeasts proliferation. Nevertheless, consumers have become more concerned about the adverse impact of synthetic additives on human health (Stević, Berić, Šavikin, Soković, Godevac, Dimkić & Stanković, 2014). In this sense, natural preservatives such as essential oils (EOs) had been extensively used during the last years due to its antioxidant and antimicrobial properties (Perdones, Sánchez-González, Chiralt, & Vargas, 2012).

EOs are categorised as flavourings in Europe (Official Journal of the European Communities, Commission Decision 2002/113/EC, notified under document number C (2002) 88) and their constituents are categorised as GRAS (Generally Recognized as Safe) by the U.S Food and Drug Administration. Cinnamon EO has demonstrated a strong antimicrobial activity but few reports show the behaviour versus moulds and yeasts (Manso, Becerril, Nerín, & Gómez-Lus, 2015). EOs contain volatile compounds and they are highly insoluble in water because of their lipophilic nature, and may have limited contact with microorganisms in high moisture content foods (Kalemba & Kunicka, 2003). This problem can be successfully overcome by using oil-in-water (O/W) emulsions, improving the water solubility of EOs, ensuring sufficient contact with microorganisms and enhancing their antimicrobial effectiveness (Hill, Gomes, & Taylor, 2013). O/W emulsions can be obtained by a two-step process (McClements, 2005). A coarse emulsion, or premix, is firstly obtained by employing a rotor-stator type device. Then the premix is processed in a high pressure homogeniser. High pressure homogenisation (HPH) reduces particle droplet size and is used to produce

emulsions with uniform composition and greater stability (Lee, Lefèvre, Subirade, & Paquin, 2009).

The main objective of this work was to study the use of cinnamon bark oil-in-water emulsions to preserve strawberry jams from fungi contamination. The optimisation of the methodology employed to prepare the emulsions by reducing active compounds losses, and their antimicrobial potential against moulds and spoilage yeasts in strawberry jam were investigated.

2. MATERIALS AND METHODS

2.1 Microorganism, culture media and reagents

Strains of *Aspergillus flavus* (CECT 20156), *Aspergillus niger* (CECT 20156), *Penicillium expansum* (CECT 20140), *Zygosaccharomyces rouxii* (CECT 1229) and *Zygosaccharomyces bailii* (CECT 12001) were supplied by the Spanish Type Culture Collection (CECT, Burjassot, Spain). For culture media, Potato Dextrose Agar (PDA), Yeast Peptone Dextrose broth (YPDB) and agar were used, all provided by Scharlab (Barcelona, Spain).

In the emulsions formulation, the cinnamon bark EO (CBEO) was supplied by Ernesto Ventós S.A. (Barcelona, Spain) and the xanthan gum (XG, SatiaxaneTM CX 911) by Cargill (Barcelona, Spain). *Trans-cinnamaldehyde* 99% was supplied by Sigma-Aldrich (St. Louis, USA) and *n*-Hexane by Scharlau (Barcelona, Spain).

2.2 Screening the antimicrobial activity of the CBEO

The CBEO was individually tested against *A. flavus, A. niger* and *P. expansum* following the methodology proposed by Ribes, Fuentes, Talens, and Barat (2016). Moulds were inoculated on PDA and incubated at 25 °C for 7 days. The spore solutions (10^3 and 10^6 CFU/mL) harvested from a 7-day-old PDA were prepared in NaCl 0.7% with a haemocytometer. Next 100 µL of each fungal suspension were

spread on the surface of a PDA Petri dish and an agar plug of this dish (7 mm diameter) was transferred to the centre of 15 g PDA's Petri dishes with different EO concentrations, which were established by considering previous studies (Kocevski, Du, Kan, Jing, & Pavlović, 2013; Manso et al., 2015). The tested EO concentrations were: 0.03, 0.04, and 0.05 mg/g. To secure EO distribution, 0.1% of Tween 80 was added to the medium. The controls with the same amount of Tween 80 were added to the test. Each dish was sealed with Parafilm® and incubated for 7 days at 25 °C.

Radial mycelial growth was determined after 1, 3, 5 and 7 days of incubation by measuring the diameter of the fungal colony. Values were expressed as mm diameter/day.

The Minimal Inhibitory Concentration (MIC) and the Minimal Fungicidal Concentration (MFC) of the CBEO were evaluated by observing the revival or growth of the inhibited mycelial disc transferred to the untreated PDA for 7 days. The dishes that showed no growth were taken as the MFC value, whereas those with mycelial growth indicated the MIC value.

The antimicrobial activity of the CBEO against *Z. rouxii* and *Z. bailii* was also evaluated by the methodology adapted from Tyagi, Gottardi, Malik, and Guerzoni (2014). Yeast strains were grown in YPD broth medium at 25 °C for 48 h in an orbital shaking incubator at 120 rpm. Cells were counted in a haemocytometer to obtain an inoculum density of 10^3 and10^6 CFU/mL.

The tested CBEO concentrations were the same as those previously described, and they were established by considering previous works (Tzortzakis, 2009; Kocevski et al., 2013). Aliquots of 15 g of YPD agar with the EO and 0.1% Tween 80 were poured into Petri dishes. Next 100 µL of the cell solution were spread on the surface of the YPD agar media dishes. As controls, the YPD agar dishes were supplemented with the same amount of Tween 80. The inoculated plates were incubated at 25 °C for 48 h. The MIC values were determined at the lowest EO concentration with non-visible growth. All the tests were run in triplicate.

2.3 Study of O/W emulsions

2.3.1 Emulsions preparation

The CBEO (0.06, 0.08, 0.10, 0.12 mg/g) was used as a lipid phase. To prepare the aqueous phase, 5 mg/g of XG were dispersed in distilled water and stirred overnight at room temperature. Primary emulsions were obtained following different steps: i) using a rotor-stator homogeniser (Ultraturrax, IKA®, Germany) at 10,000 rpm for 1 min and 20,000 rpm for 3 min; or ii) using a magnetic stirrer for 15 min. In both cases, primary emulsions were subjected to HPH in a Panda Plus 2000 (Gea Niro Soavi S. p. A., Parma, Italy) at 40 or 80 MPa.

2.3.2 Gas chromatography-mass spectrometry analysis

The final EO content in the CBEO emulsions was quantified according to the methodology employed for emulsion preparation: rotor-stator device and/or a high pressure homogenisation at 40 and 80 MPa. For this purpose, 5 mg/g of the XG were dispersed in distilled water and stirred overnight at room temperature. After biopolymer dissolution, the CBEO was added to reach a final concentration of 0.50 mg/g.

After preparing the O/W emulsions, and independently of the process used, the EO was extracted by adding 15 mL of *n*-hexane to 2 g of the O/W emulsion, followed by 2-minute vortex agitations. The mixture was shaken gently and filtered through filter paper. The *n*-hexane was evaporated at 40 °C in a rota-vapour. The obtained extracts were added to 2 mL of *n*-hexane and analysed in the 6890/5975 inert GC-MS (Agilent Technologies, USA), equipped with a HP-5 fused silica capillary column (30 m x 0.25 mm x 0.25 µm). The oven temperature was held at 60 °C for 3 min, and then raised to 100 °C at 10 °C/min, to 140 °C at 5 °C/min, and finally to 240 °C at 20 °C/min. Helium gas was used as the carrier gas at a constant flow rate of 1 mL/min. The injector and MS transfer line

temperatures were set at 250 °C and 230 °C, respectively. The parameters for the MS analysis were EI Ion source, electron energy 70 eV, solvent delay 3 min and *m/z* 40–550 amu. EO components were identified by matching mass spectra with the standard mass spectra from the NIST MS Search 2.0 library (Ribes et al., 2016). The analysis was repeated three times for each sample.

According to the results obtained in this part of the study, and those obtained while evaluating the antimicrobial activity of the CBEO, the concentration of the EOs in the emulsions (0.06, 0.08, 0.10, 0.12 mg/g) and the methodology for preparing emulsions (use of magnetic stirrer for 15 min and HPH process) were established.

2.3.3 Physico-chemical characterisation of the O/W emulsions

The pH of the emulsions was measured by a Crison Basic 20+ pH meter (Crison S.A. Barcelona, Spain), and density was determined in a pycnometer.

Particle size was determined in a laser diffractometer (Mastersizer 2000, Malvern Instruments, Worcestershire, UK) following the methodology described by Ribes et al. (2016).

The ζ-potential was determined according to Ribes et al. (2016) with a Zetasizer nano-Z (Malvern Instruments, Worcestershire, UK). All the analyses were run in triplicate.

2.3.4 Antimicrobial activity of the O/W emulsions

The antifungal activity of the CBEO emulsions against *A. flavus, A. niger* and *P. expansum* was determined by the methodology described in Section 2.2. In this case, 0.50 g of each emulsion (0.06, 0.08, 0.10, 0.12 mg/g of the CBEO and 5 mg/g of XG) was added to 49.50 g of PDA at 50 °C. The controls with a dispersion prepared with distilled water and XG were added to the test. Each Petri dish was sealed with Parafilm® and incubated for 7 days at 25 °C. Radial mycelial growth was determined after 1, 3, 5 and 7 days. Values were expressed as mm diameter/day. The MIC or MFC values of the O/W emulsions were studied.

The antimicrobial action of the CBEO emulsions against *Z. rouxii* and *Z. bailii* was also assessed by the previously described methodology. 100 µL of the cell solution (10^3 or 10^6 CFU/mL) was spread on the surface of each dish that contained YPD agar with emulsion. The YPD agar with the dispersion prepared with distilled water and XG was used as a control. The inoculated plates were stored at 25 °C for 48 h. The MIC values were determined. All the tests were run in triplicate.

2.4 Study of the O/W emulsions in strawberry jam

2.4.1 Jam preparation

Strawberry jam was prepared according to Ribes et al. (2016). The O/W emulsions were added to jam after cooling the product at ambient temperature and then homogenising. The amount of emulsions added to strawberry jam was established in order to achieve a concentration of 1 g of the O/W emulsion in 100 g of jam in the final product.

2.4.2 Sensory analysis

A sensory analysis was carried out by a semi-trained panel. The group of assessors was formed by 11 men and 19 women, whose ages ranged from 21 to 50 years. They were recruited due to their interest and availability, following the general guidelines UNE-ISO 8586:2012. Training sessions were carried out in order to introduce the panellists to the sensory analysis and to identify and score the quality attributes which describes the samples. Tests were run on a structured 9-point hedonic scale (9=like very much and 1=dislike very much) (UNE-ISO 4121), by which colour, aroma, taste, consistency and overall acceptance attributes were evaluated. All the samples were presented to panellists at room temperature under normal lighting conditions in a transparent plastic cup coded with random, three-digit numbers. Bread pieces and spoons were provided to the panellists; drinking water was also provided for oral rinsing.

2.4.3 Shelf-life of inoculated strawberry jam

Fifteen grams of strawberry jam that contained the O/W emulsions (0.08 and 0.10 mg/g of EO and 5 mg/g of XG, homogenised at 40 MPa) were inoculated with 100 µL of the spore and cell solution (10^3 CFU/ mL). Plates were incubated at 25 °C for 28 days. Three Petri dishes were prepared per EO concentration, microorganism and analysis day (n=150). Moulds and yeast counts were taken in PDA plates after 72 h of incubation at 25 °C (Pascual & Calderón, 2000). All the assays were performed in triplicate.

2.5 Statistical analysis

The results obtained in the physico-chemical characterisation of the O/W emulsions and the antifungal evaluation of the EO and O/W emulsions were

analysed by a multifactor analysis of variance (multifactor ANOVA). The effect of incorporating the O/W emulsion on the sensory attributes of strawberry jam was evaluated by a one-way ANOVA. The least significance procedure (LSD) was used to test for any differences between averages at the 5% level of significance. Data were statistically processed by Statgraphics Centurion XVI.

3. RESULTS AND DISCUSSION

3.1 Antimicrobial activity of the CBEO

The results obtained while screening the antifungal activity of the CBEO are found in **Fig. 1**. The CBEO increased the *Lag phase* of all the moulds evaluated, with a diminution on the germination rate for both fungal suspensions (10^3 CFU/mL and 10^6 CFU/mL). At the highest EO concentration (0.05 mg/g), mycelial growth was totally inhibited in all the studied moulds, irrespectively of the fungal concentration employed. The use of 0.03 and 0.04 mg/g of the CBEO reduced the growth of *A. flavus*, *A. niger* and *P. expansum*, regardless of the evaluated fungal suspension.

The inoculum concentration affects the degree of inhibition. In the most diluted suspension (10^3 CFU/mL), the CBEO caused the total inhibition of *P. expansum*, independently of the EO concentration employed. Furthermore, *A. flavus* and *A. niger* were totally inhibited when 0.04 and 0.05 mg/g of the CBEO was used, respectively. This behaviour reflects the greater resistance of *A. niger* and the highest sensitivity of *P. expansum* to CBEO exposure. The highest assessed fungal concentration showed 100% mycelial growth inhibition when 0.05 mg/g of the CBEO was incorporated into the media.

Antifungal activity could be the result of different activity sites on microbial cells, such as damage of the enzymatic cell systems that correlate with the energy production or structural compounds of EOs, or even the denaturation of the enzymes involved in spore germination (Gutiérrez, Batlle, Sánchez, & Nerín,

2010). The efficacy of cinnamaldehyde, the main CBEO compound, to inhibit growth of the fungi of genera *Penicillium* and *Aspergillus* has been demonstrated by López, Sánchez, Batlle, and Nerín (2007). They found that *P. islandicum* and *A. flavus* were completely inhibited by 4.36 µL/L and 34.9 µL/L, respectively, of a cinnamaldehyde-fortified cinnamon EO in the vapour phase, and reported the MIC of cinnamaldehyde against *A. flavus* to be 21.8 µL/L.

The MFC values for *P. expansum*, *A. flavus* and *A. niger* were 0.03, 0.04 and 0.05 mg/g, respectively, at the most diluted spore suspension. However, 0.05 mg/g of the CBEO was the MIC at 10^6 CFU/mL for the three strains. These results indicate the relation between the EO concentration and spore solution, and confirm that the concentration of fungal suspensions plays an important role in fungal development (Manso, Cacho-Nerin, Becerril, & Nerín, 2013).

The MIC of the CBEO was determined against different yeast strains (*Z. rouxii* and *Z. bailii*) at the 10^3 and 10^6 CFU/mL cell suspensions. The EO exhibited concentration-dependent inhibition of growth, and the MIC of the CBEO varied from 0.04 to 0.05 mg/g. The results indicated greater antimicrobial activity of the CBEO against *Z. rouxii* than against *Z. bailii*, with a MIC value of 0.04 mg/g. The highest MIC value (0.05 mg/g) at 10^3 cells/mL was shown against *Z. bailii* (data not shown). The same trend was observed for the MIC value when the highest cell suspension was used (10^6 CFU/mL). The obtained data indicated that the yeast suspension concentration plays a key role in reducing yeast spoilage. Similar results were obtained by Monu, Techathuvanan, Wallis, Critzer, and Davidson (2016) when determining the MIC of cinnamon bark and *trans-cinnamaldehyde* against *Z. bailii*.

3.2 Study of the O/W emulsions

3.2.1 Gas chromatography-mass spectrometry analysis

The CBEO components were identified by a GC-MS analysis (**Table 1**). The main EO compounds were *trans-cinnamaldehyde* (74.56%), caryophyllene (6.5%), eugenol (5.14%), cinnamylacetate (2.83%) and β-linalool (2.62%). Similar results have been reported by different authors (Fei, Yi-cheng, Xing-qian, & Yu-ting, 2011; Mazzarrino et al., 2015). The antifungal properties of the CBEO and their main component, *trans-cinnamaldehyde*, have been demonstrated by several authors (Manso et al., 2013). Some research works have attributed the antifungal properties of cinnamaldehyde to the high electrophilic properties of the carbonyl group adjacent to the double bound, which render it reactive with the nucleophiles present in microorganisms (Gill & Holley, 2004).

Given the volatility of EOs, it is important to quantify the EO retained by O/W emulsions, and to, therefore, adjust the EO content to be used in emulsion formulations. These results are useful for optimising the methodology to prepare O/W emulsions. Emulsions were analysed by a GC-MS analysis, and losses of EOs while being prepared using different treatments (rotor-stator homogenisation and/or HPH process) were determined. EO losses were referred to *trans-cinnamaldehyde*.

Trans-cinnamaldehyde losses in the O/W emulsions prepared with the rotor-stator device were around 40%, and became higher in combination with HPH (**Fig. 2**). In contrast, the % of *trans-cinnamaldehyde* losses in the emulsions obtained by magnetic stirring, and subjected to 40 and 80 MPa of pressure, were 6.80±1.29 and 15.27±2.21, respectively. The emulsion subjected to high pressure showed a significant ($p<0.05$) reduction in the % of *trans-cinnamaldehyde* losses compared with the emulsion obtained in the rotor-stator type device. This could be caused by the high stress applied to samples and their heating during the homogenisation process, which would promote the degradation of constituents. Indeed, the higher the pressure applied during the homogenisation process, the greater the

degradation of the EO compounds. These results agree with those reported by Donsì, Annunziata, Sessa and Ferrari (2011) for a terpenes mixture, who observed the degradation of different active compounds, due to the stress that samples had to withstand during high shear homogenisation and HPH.

3.2.2 Physico-chemical characterisation of stable O/W emulsions

Different formulations and pressures were used to obtain stable emulsions. The pH, density, $d_{3,2}$, $d_{4,3}$ and ζ-potential values for the different emulsions are summarised in **Table 2**.

The pH values of the emulsions prepared at 40 MPa varied between 6.56±0.02 and 7.30±0.05 at ambient temperature, and the values obtained from the emulsions prepared at 80 MPa varied between 6.80±0.02 and 7.37±0.02. The pH decrease may be related with the acid nature and dissociation in the aqueous solution of some CBEO compounds. Similar results were reported by Sánchez-González, Vargas, González-Martínez, Chiralt, and Cháfer (2009) and Sánchez-González, Chiralt, González-Martínez, and Cháfer (2011) when incorporating different EOs into hydroxypropylmethylcellulose film-forming dispersions.

No changes were observed for density when EO content increased.

As can be observed in **Table 2**, the higher the oil content in emulsions, the bigger particle size becomes. This could be due to an increase in the dispersed phase concentration, which facilitates the droplet flocculation rate, as well as the reduction in the ratio between the interfacial stabilising material and the dispersed phase (McClements, 2005).

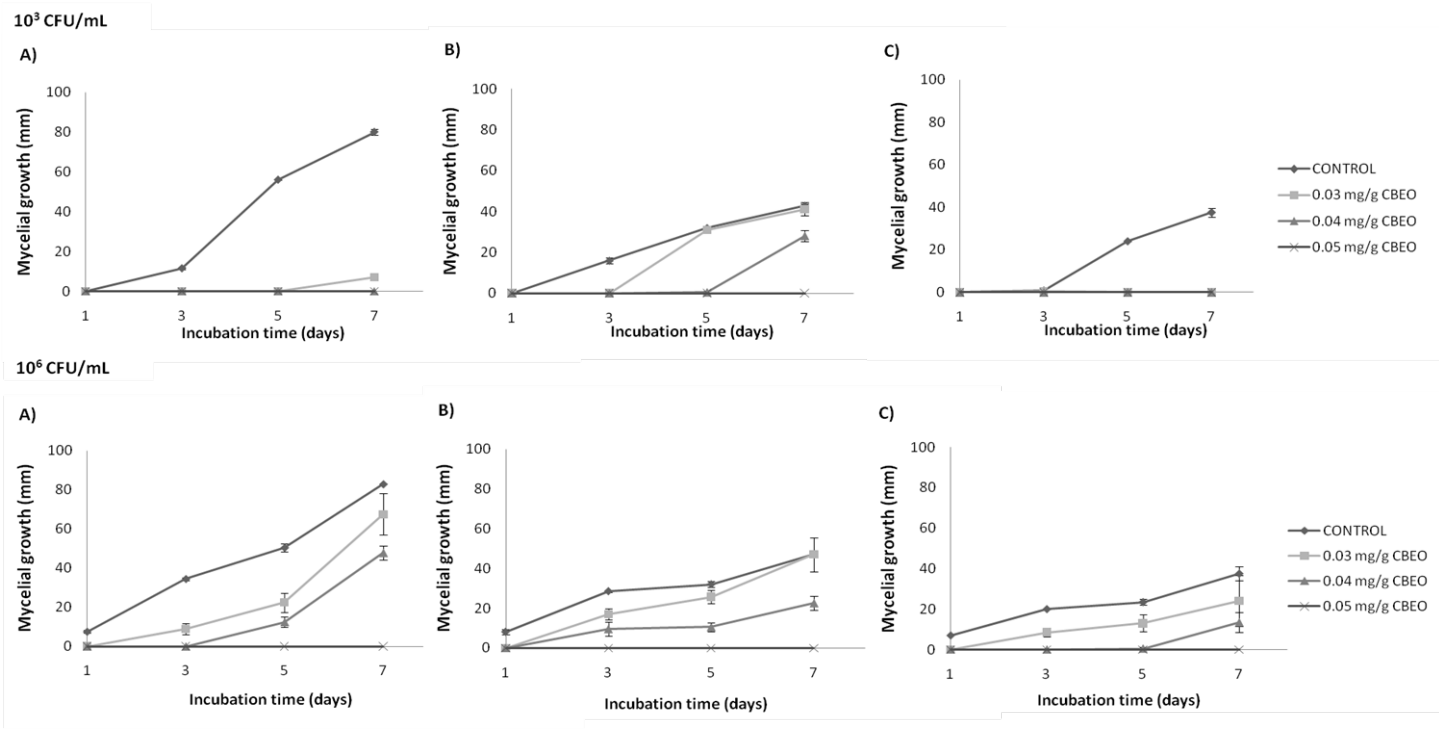

Fig. 1. Antimicrobial activity of the CBEO against (A) *Aspergillus flavus*, (B) *Aspergillus niger* and (C) *Penicillium expansum* after 7 days of incubation at 25 °C (fungal suspensions: 10^3 and 10^6 CFU/mL). Media values (n=3) ± SD.

Similar results have been reported by Sánchez-González, Cháfer, Chiralt, and González-Martínez (2010) in emulsions of bergamot EO and chitosan aqueous systems. Only for the emulsions prepared with 0.10 mg/g and 0.12 mg/g of the CBEO a significant ($p<0.05$) impact on $d_{3,2}$ was observed. The mean size values lowered from 3.397±0.127 to 3.112±0.228 µm in the emulsions prepared using 0.10 mg/g of the CBEO at 40 and 80 MPa, respectively. The reduction in the mean size values for the emulsions formulated

Table 1. Chemical composition of CBEO. Percentages of relative area (%) are the mean of three runs and were obtained from electronic integration measurements using selective mass detector.

Compound	CBEO (% relative area)
α-Phellandrene	0.91 ± 0.03
2-Carene	0.45 ± 0.01
o-cymene	1.21 ± 0.03
D-Limonene	0.44 ± 0.00
β-Phellandrene	1.77 ± 0.05
β-Linalool	2.62 ± 0.05
1-Terpinen-4-ol	0.21 ± 0.01
α-Terpineol	0.59 ± 0.00
Trans-cinnamaldehyde	74.56 ± 0.09
Eugenol	5.14 ± 0.13
Copaene	0.86 ± 0.04
Caryophyllene	6.54 ± 0.07
Cinnamylacetate	2.83 ± 0.01
α-caryophyllene	1.17 ± 0.02
Caryophyllene oxide	0.69 ± 0.03

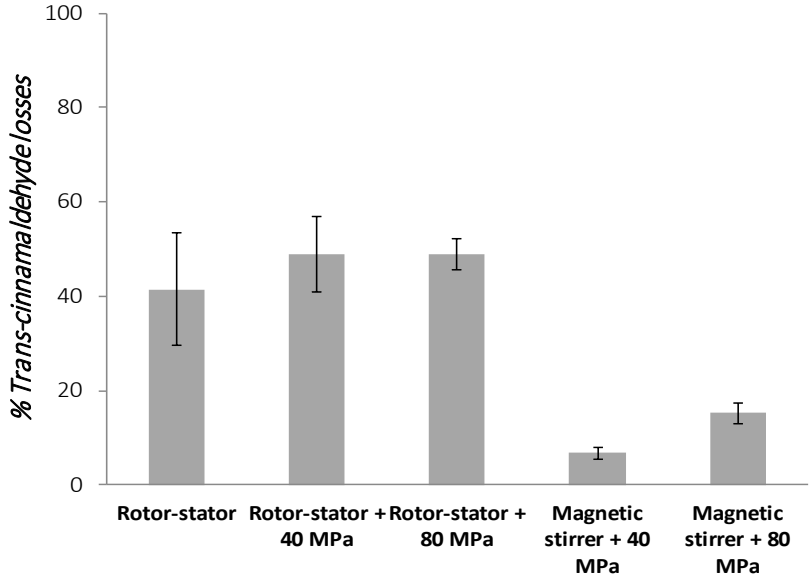

Fig. 2. Percentage (%) of *trans-cinnamaldehyde* loss from the CBEO emulsions with different treatments (rotor-stator and/or HPH at 40 or 80 MPa). Mean values (n=3) ± SD.

with 0.12 mg/g of the EO was more marked, and the mean size values lowered from 3.397±0.127 to 2.949±0.073 µm in the emulsions subjected to 40 and 80 MPa, respectively. In contrast, the primary emulsions formulated with 0.06, 0.10 and 0.12 mg/g of the EO and subjected to high pressure had a significant ($p<0.05$) impact on $d_{4,3}$, and showed a reduction around 1.5 µm. Only in case of the emulsion with 0.08 mg/g of the CBEO the impact of HPH on $d_{4,3}$ did not affect significantly (**Table 2**).

According to McClements (2005), if the electrical charge of droplets was sufficiently high, the emulsion could become stable against aggregation due to repulsive forces between droplets.

Generally, particles with a more positive ζ-potential than +30 mV, or a more negative one than -30 mV, are considered stable (Heurtault, Saulnier, Pech,

Proust, & Benoit, 2003). The electrical charge of oil droplets in emulsion is shown in **Table 2**. A strong negative ζ-potential was observed in emulsions. The increase in pressure applied during the homogenisation procedure of emulsions diminished the surface charge of particles with significant differences ($p<0.05$). The decrease in their ζ-potential values was more negative than -45.0 mV. The mechanical stress during HPH can break up the XG, and thus increase the number of molecules to be potentially adsorbed on the O/W interface. This would explain the observed ζ-potential strengthening (Salvia-Trujillo, Rojas Graü, Soliva Fortuny, & Martín Belloso, 2015).

The obtained O/W emulsions were stable regardless of the effect caused in the electrical charge of droplets by HPH.

3.2.3 Antimicrobial activity of the O/W emulsions

According to the results obtained in the Section 3.1 and the % of *trans-cinnamaldehyde* losses, the concentrations of the tested EO were 0.06, 0.08, 0.10, 0.012 mg/g.

The antifungal activity of the CBEO emulsions obtained at 40 and 80 MPa against *A. flavus*, *A. niger* and *P. expansum* for 7 days by using 10^3 and 10^6 CFU/mL is shown in **Fig. 3**. The O/W emulsions under the tested conditions increased the *Lag phase* of all the tested moulds, and the germination rate lowered. The O/W emulsions prepared with 0.08 mg/g of the CBEO at 40 MPa for the lowest assayed spore solution (10^3 CFU/mL) had a significant antifungal effect ($p<0.05$) on all the studied moulds. These emulsions inhibited the growth of *A. flavus*, *A. niger* and *P. expansum* for 7 days.

Table 2. Mean values (n=3) ± SD of pH, density (g/cm^3), particle size ($d_{3,2}$ and $d_{4,3}$), and ζ-potential of cinnamon bark-xanthan gum O/W emulsion.

	Pressure (MPa)	0.06 mg/g EO	0.08 mg/g EO	0.10 mg/g EO	0.12 mg/g EO
pH	40	7.30 ± 0.05 ^{cx}	7.28 ± 0.02 ^{cx}	6.79 ± 0.02 ^{bx}	6.56 ± 0.05 ^{ax}
	80	7.37 ± 0.02 ^{cx}	7.34 ± 0.02 ^{cy}	6.95 ± 0.01 ^{bx}	6.80 ± 0.02 ^{ay}
ρ (g/cm^3)	40	1.001 ± 0.001 ^{ax}	1.001 ± 0.001 ^{ax}	1.001 ± 0.000 ^{ax}	1.001 ± 0.001 ^{ax}
	80	1.002 ± 0.001 ^{ax}	1.001 ± 0.001 ^{ax}	1.001 ± 0.000 ^{ax}	1.001 ± 0.002 ^{ax}
$d_{3,2}$ (μm)	40	2.520 ± 0.018 ^{ax}	3.215 ± 0.144 ^{bx}	3.397 ± 0.127 ^{bcy}	3.532 ± 0.101 ^{cy}
	80	2.501 ± 0.063 ^{ax}	3.112 ± 0.228 ^{bx}	2.949 ± 0.073 ^{bcx}	3.226 ± 0.138 ^{cx}
$d_{4,3}$ (μm)	40	6.921 ± 0.426 ^{ay}	7.547 ± 0.114 ^{ax}	8.666 ± 0.255 ^{by}	9.712 ± 0.597 ^{cy}
	80	5.571 ± 0.071 ^{bx}	7.490 ± 0.543 ^{bx}	7.438 ± 0.198 ^{bx}	7.008 ± 0.541 ^{bx}
ζ-potential (mV)	40	- 51.9 ± 1.7 ^{ax}	- 47.5 ± 0.5 ^{bx}	- 47.0 ± 1.5 ^{bcx}	- 45.1 ± 0.6 ^{cx}
	80	- 54.9 ± 1.1 ^{ay}	- 53.4 ± 1.4 ^{ay}	- 53.8 ± 0.6 ^{ay}	- 51.0 ± 0.4 ^{by}

$^{a, b, c, d,}$ *Different superscripts indicate significant differences among EO concentrations (p<0.05).*

$^{x, y}$ *Different superscripts indicate significant differences among different pressure (p<0.05).*

The assays in which the fungal suspension was 10^6 CFU/mL obtained an increased mycelia growth rate for the three evaluated fungi. These results once again confirmed the relevance of the initial fungal concentrations on fungal development (Manso et al., 2013). Under these conditions, the O/W emulsions prepared with 0.08 mg/g of the CBEO at 40 MPa were able to inhibit the total growth of *A. flavus*, *A. niger* and *P. expansum*. On the contrary, when the EO concentration rose above 0.08 mg/g and the pressure applied to the primary emulsions was 80 MPa, *A. niger* development on culture media occurred.

A similar trend in the growth rate was observed for both fungal suspensions when the pressures applied to the primary emulsions increased. The O/W emulsions formulated with 0.06 mg/g of the CBEO at 80 MPa showed higher mycelial growth than the same emulsions prepared at 40 MPa. This finding could be related with the % of *trans-cinnamaldehyde* losses, which could diminish the antifungal effectiveness of the O/W emulsions.

The MIC and MFC values of the O/W emulsions formulated with 0.08 mg/g of the CBEO at 40MPa and 0.10 mg/g of CBEO homogenised at 40 and 80 MPa were also evaluated. The MFC of the O/W emulsions process at 40 MPa was 0.08 mg/g.

The MIC of the O/W emulsions was also determined against *Z. rouxii* and *Z. bailii* at different cell suspensions (10^3 and 10^6 CFU/mL). The lowest MIC (0.06 mg/g) value was obtained at 10^3 CFU/mL for both strains by subjecting the primary emulsions to 40 MPa. In contrast at 10^6 CFU/mL, a remarkable antimicrobial effect was observed for *Z. rouxii*. At this cell suspension, the emulsion's MIC values for *Z. rouxii* and for *Z. bailii* were 0.06 and 0.08 mg/g of the CBEO, respectively, when applying 40 MPa of pressure (data not shown). As previously mentioned, the higher the pressures applied in homogenisation, the bigger the % t*rans-cinnamaldehyde* losses. This fact affected yeast growth inhibition, which became less effective due to loss of active compounds.

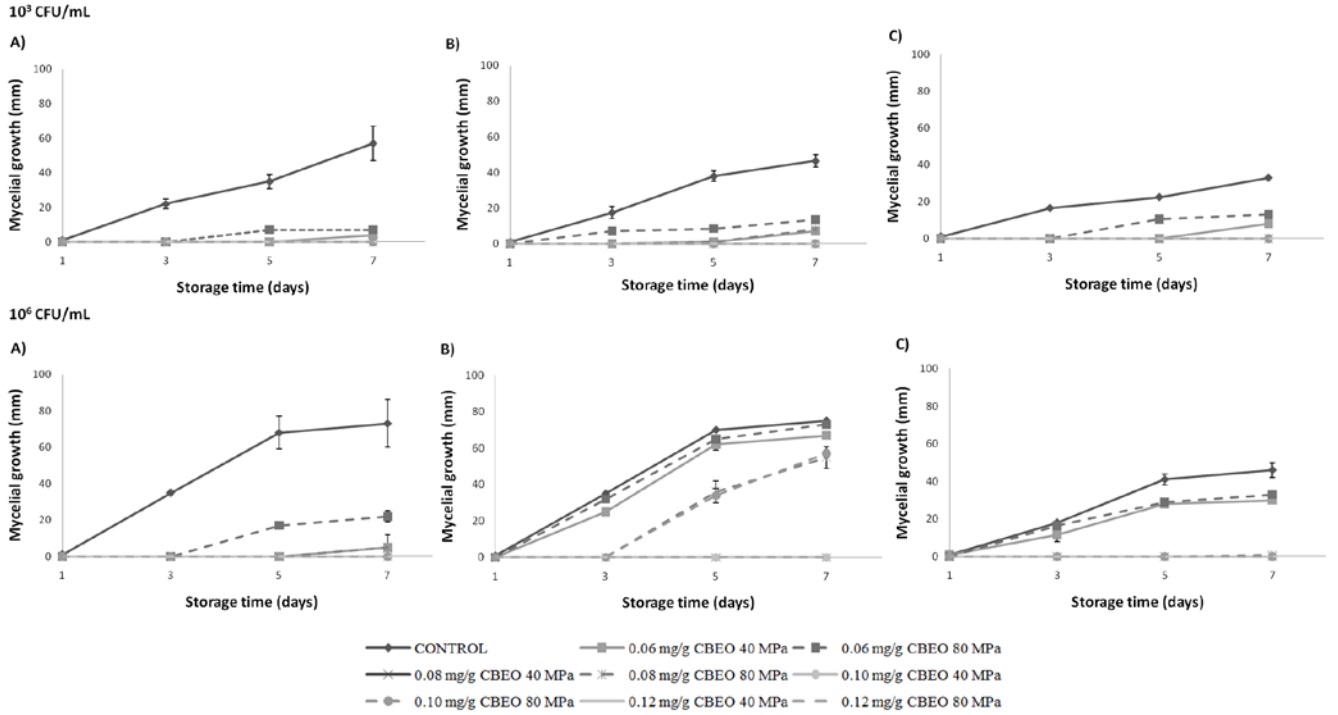

Fig. 3. Antimicrobial activity of the O/W emulsions obtained at 40 and 80 MPa against (A) *Aspergillus flavus*, (B) *Aspergillus niger* and (C) *Penicillium expansum*, after 7 days of incubation at 25 °C. Fungal suspensions: 10^3 and 10^6 CFU/mL. Media values (n=3) ± SD.

3.3 Study of the O/W emulsions on strawberry jam

3.3.1 Sensory analysis

A sensory analysis was carried out to check the acceptability of the strawberry jam that contained the O/W emulsions. The samples tested by panellists consisted of the jam with the CBEO emulsions at the established concentrations. One jam sample with no EO was used as a control. The strawberry jam with the O/W emulsions scored lower for the aroma, taste and overall acceptance attributes compared with the control samples. No significant differences were observed between EO concentrations. Consistency and colour attributes did not significantly differ ($p>0.05$) from the control samples (**Fig. 4**).

3.3.2 Study of the O/W emulsions on strawberry jam

The emulsions prepared with 0.08 and 0.10 mg/g of the CBEO and homogenised at 40 MPa, were added to strawberry jam. Jams were inoculated to simulate a possible product contamination and samples with no inoculation were used as controls.

Microbial development on the strawberry samples that contained the O/W emulsions for 28 days at 25 °C was studied (**Fig. 5**). The jams prepared with the O/W emulsions inoculated with *A. flavus*, *P. expansum*, *Z. rouxii* and *Z. bailii* showed no growth throughout the study. For *A. niger*, a reduction of around 1 log was observed between the control plates and the samples. These results agree with those reported above. *A. niger* showed the greatest resistance against the CBEO treatment and the O/W emulsions added to strawberry jam.

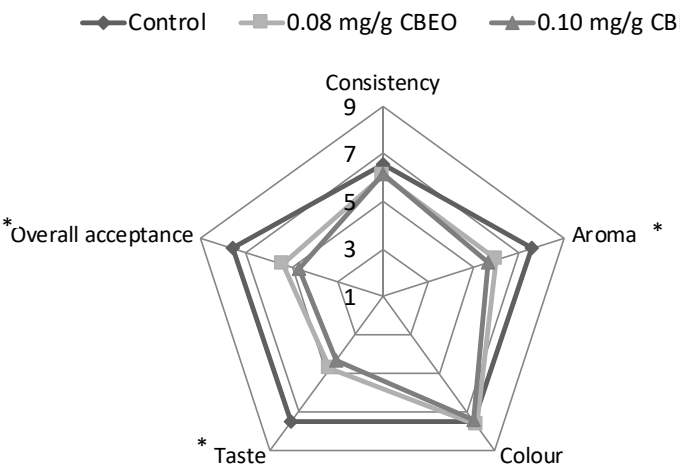

Fig. 4. Sensory profile of strawberry jam. *Indicates 95% significant differences according to the ANOVA test (n=30).

Strawberry is sensitive to pathogens, and fungal contamination is common in this product. Major threatening fungi that reduce the post-harvest storage life of strawberries include *Botrytis*, *Aspergillus*, *Rhizopus* and *Penicillium* (Lazar, Jobling, & Benkeblia, 2010; Sharma, 2014). Various reports have demonstrated that *A. niger* species members are responsible for the post-harvest decay of fresh fruits like apples, peaches, citrus, grapes, strawberries and tomatoes, among others (Perrone et al., 2007). This opportunistic effect could suggest the greater resistance of *A. niger* to the O/W emulsions incorporated into strawberry jam.

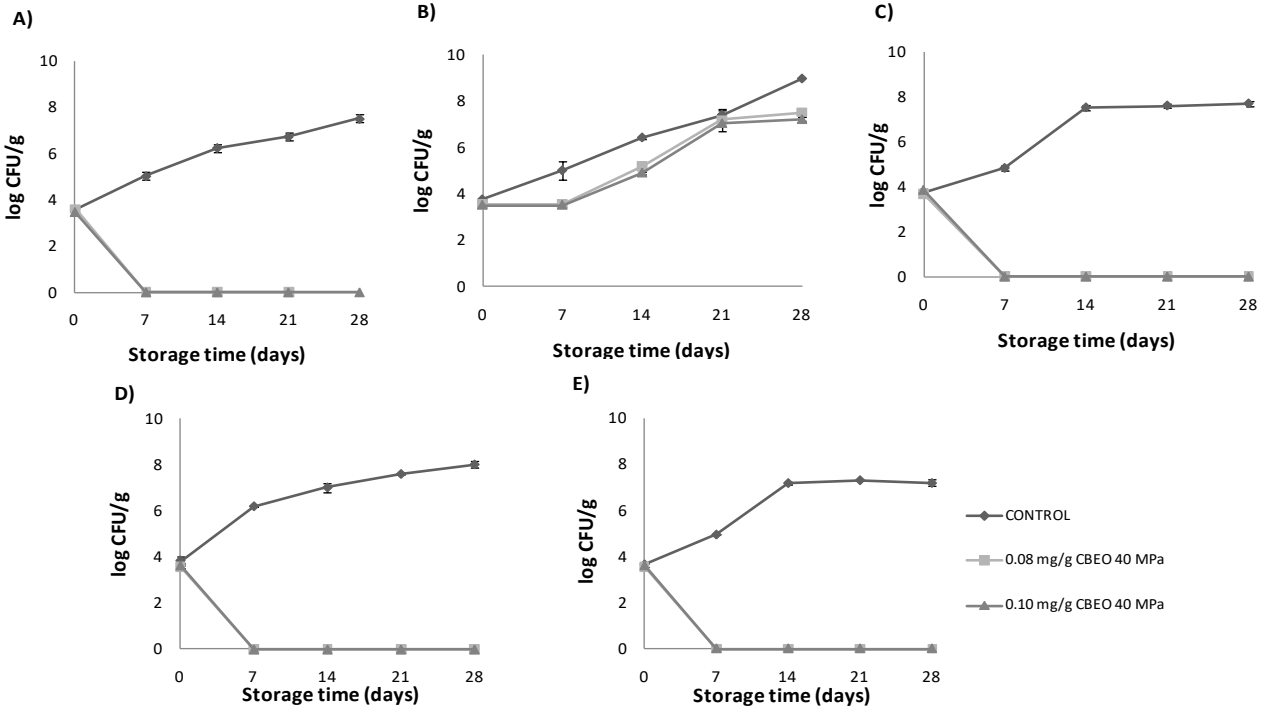

Fig. 5. Effect of the O/W emulsions on growth of A) *Aspergillus flavus*, B) *Aspergillus niger*, C) *Penicillium expansum*, D) *Zygosaccharomyces rouxii* and E) *Zygosaccharomyces bailii* on the strawberry jam stored at 25 °C. Inoculum density: 10^3 CFU/mL. Mean values (n=3) ± SD.

4. CONCLUSIONS

The optimisation of the methodology to prepare cinnamon bark-xanthan gum emulsions achieves a *trans-cinnamaldehyde* losses around 40%, which are higher in combination with high pressure homogenisation. Nevertheless, the losses of the emulsions obtained by magnetic stirring, and subjected to 40 and 80 MPa of pressure, are below 16%. Moreover, the antimicrobial activity of the emulsions was determined by fungal suspension, essential oils concentration and microbial sensitivity to essential oils.

The incorporation of emulsions containing 0.08 mg/g of cinnamon bark oil into strawberry jam allows their preservation against *Aspergillus flavus*, *Penicillium expansum*, *Zygosaccharomyces rouxii* and *Zygosaccharomyces bailii* during the whole evaluation period. Furthermore, this incorporation does no modify product texture or colour, but negatively affects the aroma, taste and overall acceptance of jam.

Although, the obtained results suggest some advantages in the use of the cinnamon bark emulsions as natural preservatives in strawberry jam, more studies are needed to reduce the sensory impact of essential oils. The combination of different natural antifungal agents such as phenolic compounds or zinc salts could be a promising alternative to reduce or suppress the changes produced in foods due to the strong flavour of essential oils.

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CHAPTER 2

COMBINATION OF DIFFERENT ANTIFUNGAL AGENTS IN OIL-IN-WATER EMULSIONS TO CONTROL STRAWBERRY JAM SPOILAGE

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Submitted to Food Control

Abstract

The aim of this work is to evaluate the combination of several antifungal agents (cinnamon bark oil, zinc gluconate and *trans*-ferulic acid) in oil-in-water emulsions to control the fungal spoilage of strawberry jams Furthermore, the effect of these oil-in-water emulsions on the sensory profile of samples was investigated. The *in vitro* assays of the free antifungal agents were carried out against five fungal strains. Nevertheless, the effectiveness of the oil-in-water emulsion was tested against *Aspergillus niger* given its strong *in vitro* resistance and its relevance in strawberry products. The emulsions formulated with 0.08 mg/g of cinnamon bark essential oil were able to inhibit mould growth after the incubation period. Indeed, the emulsions formulated with 0.06 mg/g of cinnamon bark essential oil and with different zinc gluconate concentrations (1, 2, 4 and 6 mg/g) inhibited the mycelial growth for 7 days. The mycelial growth inhibition was also achieved when 0.06 mg/g of cinnamon bark essential oil and 1 mg/g of *trans*-ferulic acid were employed in the emulsion formulation. Furthermore, the emulsion formulated with the three active agents was used for an *in vivo* assay, and exhibited less fungal spoilage compared with the control jam (without emulsion). Incorporation of emulsion into strawberry jam did not modify its sensory characteristics. The present work demonstrates that the cinnamon bark essential oil, zinc gluconate and *trans*-ferulic acid combination in oil-in-water emulsions can be used to preserve strawberry jam from fungal spoilage.

Keywords: Essential oil; zinc gluconate; *trans*-ferulic acid; antifungal agent; strawberry jam

1. INTRODUCTION

Numerous techniques, including heat treatment, acidification, drying, incorporation of additives, or their combinations, have been used by the food industry to prevent fungal growth and spoilage (Davidson and Taylor, 2007; Farkas, 2007). Using synthetic additives to control fungi is the most effective method, but negative consumer perception has forced the food industry to find other natural alternatives (Ribes, Fuentes, Talens, & Barat, 2016).

In the last few years, plant essential oils (EOs) have attracted interest in both academia and food industry fields thanks to their antifungal properties (Manso, Cacho-Nerin, Becerril, & Nerín, 2013; Perdones, Sánchez-González, Chiralt, & Vargas, 2012). However, the use of plant EOs for preserving food commodities has some limitations due to their intensive aroma, difficult dispersion in the food matrix and possible interactions with other ingredients. Some authors have proposed the use of oil-in-water (O/W) emulsions to overcome these problems (Chang, McLandsborough, & McClements, 2012; Perdones et al., 2012; Weiss, Gaysinksy, Davidson, & McClements, 2009). Combining EOs with other antifungal agents, such as zinc salts and phenolic compounds, could help to reduce the amount of EOs needed to prevent fungi from growing.

Cinnamon bark EO has demonstrated a strong antimicrobial activity against foodborne pathogens but few reports show the behaviour versus moulds and yeasts (Manso, Becerril, Nerín, & Gómez-Lus, 2015; Manso et al., 2013). The main constituent of this EO is *trans*-cinnamaldehyde (Ribes, Fuentes, Talens, & Barat, 2017a). Indeed, cinnamon is broadly employed as a natural preservative and flavouring substance by the food industry to extend the shelf life of foods (Ribeiro-Santos et al., 2017).

Zinc (Zn) is an important essential mineral for humans given its activity in the metabolism of nutrients that form part of enzyme systems (Hess & Brown, 2009). Zinc deficiency affects children's growth and increases the risk and severity of different infections (Bautista-Gallego, Moreno-Baquero, Garrido-Fernández, & López-López, 2013). This mineral is also used in the food industry given its ability

to form green colour complexes with chlorophyll derivates, especially at high temperature (Ngo & Zhao, 2007). Zinc exhibits cytotoxic activity when used above a threshold concentration (Romero-Gil, Rejano-Zapata, Garrido-Fernández, & Arroyo-López, 2016). Recently, zinc salts have been used as antifungals in table olives to reduce yeast growth (Bautista-Gallego, Arroyo-López, Garrido-Fernández, García-García, López-López, & Rodríguez-Gómez, 2010), and also in cracked table olives where presence of zinc salts, e.g., $ZnCl_2$, more markedly reduced the yeast population during shelf life than other traditional preservatives (Bautista-Gallego, Arroyo-López, Romero-Gil, Rodríguez-Gómez, & Garrido-Fernández, 2011). Among the different zinc salts available, the use of zinc gluconate (ZG) is authorised in the EU to fortify food products (Directive 2002/46/CE), and the Food and Drug Administration (FDA) has recognised zinc gluconate as being safe (GRAS) in Code 21 of Federal Regulations, part 182.8988 (CFR, 2015).

Ferulic acid (FA) is a phenolic compound present in fruits and vegetables. FA exhibits strong antioxidant activity, and acts as a scavenger against hydroxyl and peroxyl radicals (Kansi, Aksenova, Stoyanova, & Butterfield, 2002). It also acts as an inhibitor of fungal enzymes (Daglia, 2012), and many authors have reported its *in vivo* and *in vitro* antifungal activity (Ferrochio, Cendoya, Farnochi, Massad, & Ramirez, 2013; Zabka & Pavela, 2013). Other FA effects on human metabolism have been explored, e.g., anti-inflammatory, anti-thrombosis, UV-protector and anticancer properties (Lima, Flores, Santana-Cruz, Leyva-Gómez, & Krötzsch, 2013). As a result of its antioxidant and antimicrobial activity, and also of its health benefits and low toxicity, FA is used as a food additive in food commodities, beverages and cosmetics in Japan (Lima et al., 2013). Nevertheless, its solubility in aqueous solutions is low (Mota, Queimada, Pinho, & Macedo, 2008), and it is susceptible to light exposure. Nonetheless, all these drawbacks can be solved by incorporating it into O/W emulsions.

The main objectives of this work were to: i) evaluate the *in vitro* antifungal activity of cinnamon bark essential oil, zinc gluconate and *trans*-ferulic acid against *Aspergillus flavus*, *Aspergillus niger*, *Penicillium expansum*,

Zygosaccharomyces rouxii and *Zygosaccharomyces bailii*; ii) investigate the combination of these compounds in O/W emulsions to control the spoilage of strawberry jams against *Aspergillus niger*; iii) evaluate the effect of emulsion incorporation on the sensory acceptance of strawberry jam.

2. MATERIAL AND METHODS

2.1 Strains, media and chemicals

Strains *Aspergillus flavus* (CECT 20156), *Aspergillus niger* (CECT 20156), *Penicillium expansum* (CECT 20140), *Zygosaccharomyces rouxii* (CECT 1229) and *Zygosaccharomyces bailii* (CECT 12001) were supplied by the Spanish Type Culture Collection (CECT, Burjassot, Spain). Potato Dextrose Agar (PDA), Yeast Peptone Dextrose broth (YPDB), agar and n-hexane were purchased from Scharlab (Barcelona, Spain). While preparing emulsions cinnamon bark essential oil (>60%) (CBEO) (Ernesto Ventós S.A., Barcelona, Spain), xanthan gum (XG) (Cargill, Barcelona, Spain), zinc gluconate (ZG) (Guinama, Valencia, Spain) and *trans*-ferulic acid (FA), and Tween 80 (Sigma-Aldrich, Madrid, Spain) were used. *Trans*-cinnamaldehyde (99%) was supplied by Sigma-Aldrich (Madrid, Spain).

2.2 Antifungal properties of CBEO, ZG and FA: *in vitro* conditions

CBEO, ZG and FA activity against *A. flavus*, *A. niger* and *P. expansum* was examined according to Ribes et al. (2016). Moulds were inoculated on PDA and incubated at 25 ºC for 7 days, and the spores were counted in a haemocytometer to achieve an inoculum density of 10^6 CFU/mL. Next 100 µL of the fungal suspension were spread on the surface of a PDA plates. An agar plug of this dish (7 mm diameter) was transferred to the centre of 15 g PDA's Petri dish with

different antifungal concentrations: 0, 0.02, 0.04 and 0.06 mg/g for CBEO, 0, 1, 2, 3, 4, 5, 6 and 7 mg/g for ZG, and 0, 1, 2, 3, and 4 mg/g for FA. The antifungal agents were added to the culture medium, containing 10 mg/g of Tween 80 to ensure their dispersion, at 50 °C. The control sets, with no natural agents, were prepared by the same procedure. Each plate was incubated at 25 °C for 7 days. Growth inhibition of treatment against the control samples was calculated with Equation 1 (Ribes, Fuentes, Talens, Barat, Ferrari, & Donsì, 2017b):

$$\textit{Mycelial growth inhibition (\%)} = (C\text{-}T/C) \times 100 \quad (1)$$

where *C* and *T* represent mycelial growth (mm) in the control and treated plates, respectively.

The minimal inhibitory concentration (MIC) and the minimal fungicidal concentration (MFC) of CBEO, ZG, and FA were evaluated by observing the revival or growth of the inhibited mycelial disc transferred to PDA for 7 days. The dishes that showed no visual growth were taken as the MFC value, whereas those with mycelial growth indicated the MIC value.

The antifungal effectiveness of natural preservatives (CBEO, ZG, and FA) against *Z. rouxii* and *Z. bailii* was evaluated by the methodology adapted from Ribes et al. (2016). The tested CBEO, ZG, and FA concentrations were the same as those previously described. A suspension of yeast strains, 100 µL of 10^6 CFU/mL counted by a haemocytometer, grown in 50 mL of YPD broth at 25 °C for 48 h, was spread on 15 g of YPD agar that contained the natural preservatives and Tween 80 (10 mg/g). The control Petri dishes, with no antifungal agents, were prepared following the same procedure. Plates were incubated at 25 °C for 48 h.

The lowest CBEO, ZG or FA concentration that achieved the visual inhibition of yeast growth was the MIC.

All the tests were run in triplicate.

2.3 O/W emulsions

2.3.1 Preparation

The O/W emulsions that contained the different natural agents (CBEO, ZG and FA), 10 mg/g of Tween 80 and 5 mg/g of XG were prepared by using a magnetic stirrer, which operated for 15 min. The concentrations of each antifungal compound tested during emulsion preparation were: 0.02, 0.04, 0.06 and 0.08 mg/g of CBEO; 1, 2, 4 and 6 mg/g of ZG and; 1, 2.5 and 4 mg/g of FA. Furthermore, the concentrations of the Tween 80 and the XG were defined taking into consideration previous works (Ribes et al., 2016; Salvia-Trujillo, Rojas-Graü, Soliva-Fortuny, & Martín-Belloso, 2013). Afterwards, the O/W emulsions were processed one time at 40 MPa by a high pressure homogenisation (HPH) system (Panda Plus 2000, Gea Niro Soavi S.p.A., Parma, Italy).

2.3.2 Determination of CBEO losses by gas chromatography-mass spectrometry analysis

Determination of CBEO losses after preparing emulsions, which were subjected to HPH fluid dynamic stresses, was analysed by a GC-MS analysis. These losses are referred to as *trans*-cinnamaldehyde, which is the main CBEO compound (Ribes et al., 2017a). To this end, 5 mg/g of XG were dispersed in distilled water and stirred overnight at room temperature.

Next CBEO was incorporated to achieve a final concentration of 0.50 mg/g. CBEO was extracted by incorporating 15 mL of n-hexane into 2 g of the emulsion, followed by 2-minute vortex agitations. The mixture was filtered through filter paper and n-hexane was evaporated at 40 °C in a rota-vapour. The resulting extracts were incorporated into 2 mL of n-hexane and analysed in a 6890/5975 inert GC/MS (Agilent Technologies, USA), equipped with an HP-5 fused silica capillary column (30 m x 0.25 mm x 0.25 µm). The methodology followed was that described by Ribes et al. (2016). The analysis was repeated 3 times for each sample.

2.3.3 Antifungal properties of the O/W emulsions against Aspergillus niger: in vitro conditions

The study of the *in vitro* antifungal activity of the CBEO, ZG, and FA emulsions was conducted by considering the results obtained above. *A. niger* was selected as the target microorganism for both its resistance *in vitro* and its prevalence in the post-harvest storage life of strawberry products (Farzaneh, Kiani, Sharifi, Reisi, & Hadian, 2015; Jensen et al., 2013; Nieminen, Neubauer, Sivelä, Vatamo, Silfverberg, & Salkinoja-Salonen, 2008).

2.3.3.1 Antifungal properties of the CBEO emulsions

The antifungal properties of the O/W emulsions formulated with CBEO were evaluated according to Ribes et al. (2016), with minor modifications. Moulds were inoculated and incubated on PDA at 25 °C for 7 days. Next the spores were counted in a haemocytometer to obtain an inoculum density of 10^6 CFU/mL. The CBEO content in the emulsions formulation was 0.06 and 0.08 mg/g. Each emulsion (0.50 g) was added to media (49.5 g of PDA) at 50 °C. Then a PDA disc, spread previously with 100 µL of the spore solution (10^6 CFU/mL), was placed in

the centre of each plate. Positive controls were prepared with a dispersion of distilled water, Tween 80 and XG. Plates were incubated at 25 °C for 7 days. Growth inhibition was calculated as described in Section 2.2.

The MIC and MFC of the emulsions were evaluated as previously described. Each assay was conducted in triplicate.

2.3.3.2 The O/W emulsions formulated by combining CBEO, ZG, and FA

Emulsions were formulated using the CBEO combined with ZG and/or FA. The method followed to test their antifungal activity is defined in Section 2.3.3.1. For the combination with ZG, the used amounts of CBEO were 0.02, 0.04, 0.06 and 0.08 mg/g, and the employed ZG concentrations were 1, 2, 4 and 6 mg/g. For the combination with FA, the employed CBEO concentrations were 0.02, 0.04 and 0.06 mg/g, and the concentrations of tested FA were 1, 2.5 and 4 mg/g. These concentrations were established by considering the results of the *in vitro* antifungal effect of: i) free FA and ii) the O/W emulsions formulated with CBEO and ZG. For the triple combination, 0.06 mg/g of CBEO, 1 mg/g of ZG and 1 mg/g of FA were used. Each assay was conducted in triplicate.

2.3.4 Characterisation of the O/W emulsions

According to the *in vitro* results of the antifungal properties of the different O/W emulsions prepared with various agents, the final characterised formulations are described in **Table 1**.

The pH of the emulsions was measured by a Crison Basic 20+ pH meter (Crison S.A. Barcelona, Spain). Particle size was determined by a laser diffractometer (Mastersizer 2000, Malvern Instruments, Worcestershire, UK), as described by Ribes et al. (2016), by applying the Mie theory (refractive index of 1.50, absorption index of 0.01). The ζ-potential was carried out according to Ribes et al. (2016) using a Zetasizer Nano-Z (Malvern Instruments, Worcestershire, UK), and

the Smoluchowsky mathematical model was employed to transform the electrophoretic mobility measures into ζ-potential values. Each measurement was taken in triplicate.

Table 1. Formulations of the different tested O/W emulsions (CBEO: cinnamon bark essential oil; ZG: zinc gluconate; FA: *trans*-ferulic acid).

Code	CBEO (mg/g)	ZG (mg/g)	FA (mg/g)
CBEO	0.06	-	-
CBEO-ZG	0.06	1	-
CBEO-FA	0.06	-	1
CBEO-ZG-FA	0.06	1	1

2.4 Effect of using O/W emulsions on strawberry jam

2.4.1 Jam preparation

Strawberry jam was prepared by mixing fruit and sugar in a ratio of 65:35, and cooked at 100 °C for 30 min to reach a 60 °Brix in the product as described in the Spanish quality regulation for fruit jam (BOE, 2003) (Ribes et al., 2016). The CBEO-ZG, CBEO-FA and CBEO-ZG-FA emulsions were added to jam after cooling at 25 °C, and then homogenised. The amount of emulsions incorporated to strawberry jam was defined to achieve a concentration of 1 g of the O/W emulsion in 100 g of jam in the final product.

2.4.2 Strawberry jam spoilage by A. niger

Fifteen grams of strawberry jam with the O/W emulsions were inoculated with 100 µL of the *A. niger* solution (10^6 CFU/ mL). Plates were incubated at 25 °C for 28 days. Ten grams of each sample were placed in sterile plastic bags containing 90 mL of tryptone phosphate water and homogenised for 1 min in a Stomacher blender (Masticator IUL, S.A. Instruments, Germany). Serial dilutions were prepared and 0.1 mL were spread on the surface of the PDA plates.

Three Petri dishes were prepared per formulation and analysis day, plus the control samples (n=60). Mould counts were made on PDA plates after 72 h of incubation at 25 °C (Pascual & Calderón, 2000). All the assays were conducted in triplicate.

2.4.3 Sensory evaluation

To test the sensory acceptance of the strawberry jam with the emulsion (CBEO-ZG-FA), a semi-trained panel composed of 13 men and 17 women, whose ages ranged between 22 and 50 years, made a sensory evaluation. Tests were run on a 5-point hedonic scale (1=dislike very much, 5=like very much) (UNE-ISO 4121:2003). The following sensory parameters were evaluated: visual aspect, aroma, taste, unctuousness, mouth texture and overall acceptance. Each sample was given to panelists at room temperature in a transparent plastic glass, and was coded with three arbitrary numbers.

2.5 Statistical analysis

The results of the *in vitro* antifungal evaluation of the natural agents and CBEO emulsions, the physico-chemical analysis of the O/W emulsions, and the effect of incorporating the O/W emulsion into strawberry jam on the sensory attributes of the samples were evaluated by a one-way ANOVA. The results obtained in the *in*

vitro antifungal activity of the CBEO-ZG emulsions and the CBEO-FA emulsions and the *in vivo* antifungal activity of the O/W emulsions were analysed by a multifactor analysis of variance (multifactor ANOVA). The least significance procedure (LSD) was used to test for any differences between averages at the 5% level of significance. Data were statistically processed by Statgraphics Centurion XVI.

3. RESULTS AND DISCUSSION

3.1 Antifungal properties of CBEO, ZG and FA: *in vitro* conditions

The CBEO mycelial growth inhibition (%) of *A. flavus, A. niger, and P. expansum,* compared with the control samples, after 7 days of incubation is summarised in **Figure 1A**. Incorporation of the CBEO into the media reduced mycelial growth in a dose-dependent manner. The lowest tested CBEO concentration achieved mycelial growth inhibitions of 26%, 29% and 37% for *A. flavus, A. niger* and *P. expansum*, respectively. Fungal development was inhibited when the CBEO concentration was above 0.04 mg/g. The MFC values of CBEO against the three tested moulds were 0.06 mg/g.

The MIC of the CBEO was 0.04 and 0.06 mg/g for *Z. rouxii* and *Z. bailii,* respectively (data not shown). Previous studies available in the literature have reported the antifungal effectiveness of CBEO against the *Zygosaccharomyce*s genus. Monu, Techathuvanan, Wallis, Critzer, and Davidson (2016) reported the *in vitro* effectiveness of CBEO and its main compound, *trans*-cinnamaldehyde, against *Z. bailii,* which gave a MIC value of 50 mg/L. Indeed, Bang, Lee, Park, and Rhee (2000) tested the antifungal activity of *trans*-cinnamaldehyde against *Saccharomyces cerevisiae* and attributed its activity to the effect on cell membrane integrity.

The antifungal action of ZG and FA is shown in **Figure 1B** and **C**. ZG brought about mycelial growth inhibition of up to 50% at concentrations above 1 mg/g for *A. flavus* and *P. expansum*. Mould growth inhibition using ZG was observed at concentrations above 5 mg/g against *A. flavus* and *P. expansum,* whereas the highest ZG concentration employed only induced 31% inhibition for *A. niger.* These differences could be due to the distinct sensitivity of the moulds, being *A. niger* the most resistant against ZG.

In the case of *A. flavus* and *P. expansum,* the MFC value was established at 6 mg/g (**Figure 1 B**).

Additionally, the MIC of ZG was determined against *Z. rouxii* and *Z. bailii*. These values were 4 and 3 mg/g for *Z. rouxii* and *Z. bailii*, respectively (data not shown). No studies that report the activity or mode of action of this zinc salt against *Z. rouxii* and *Z. bailii* are encountered in the literature. However, Pagani, Casamayor, Serrano, Atrian, and Ariño (2007) investigated the disruption of iron homeostasis in *S. cerevisiae* by zinc chloride. Exposure to high Zn concentrations (6 mM $ZnCl_2$) generated reactive oxygen species, and reduced glutathione and iron content. The authors suggested that an excess Zn concentration could alter the function of iron sulphur-containing proteins.

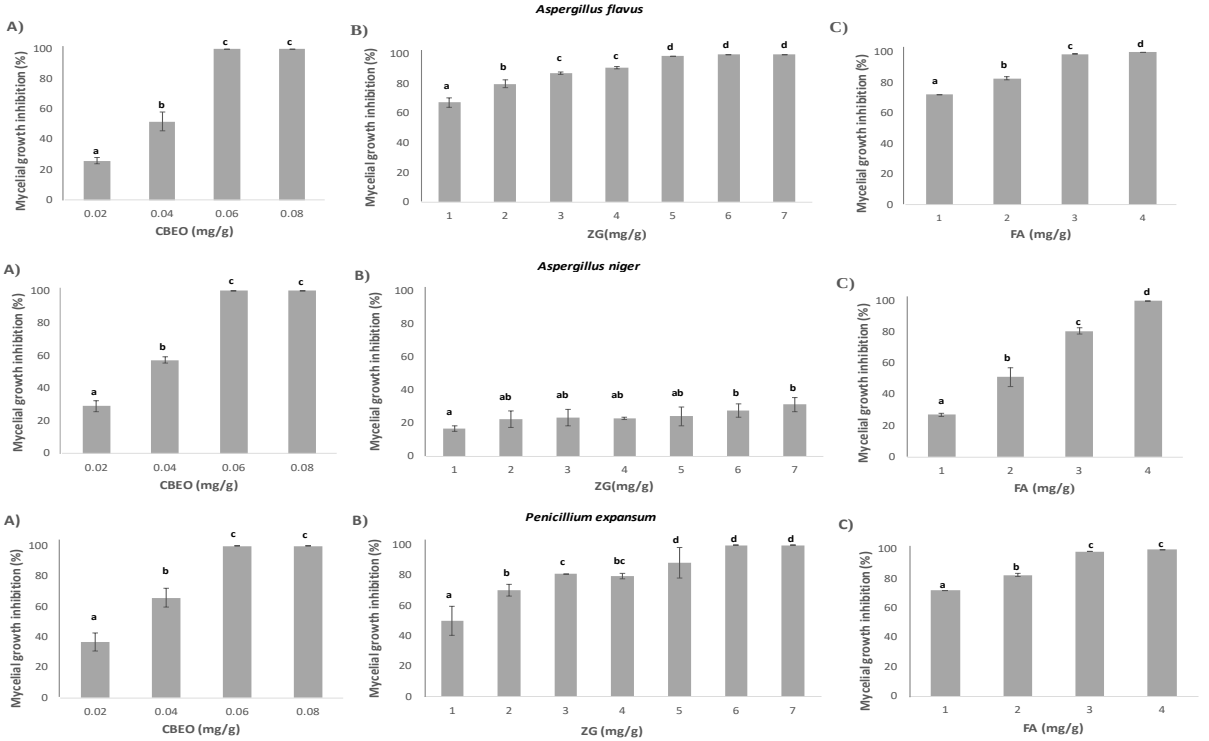

Figure 1. Mycelial growth inhibition (%) of A) cinnamon bark EO (CBEO), B) zinc gluconate (ZG) and C) *trans*-ferulic acid (FA) at different concentrations against *Aspergillus flavus*, *Aspergillus niger* and *Penicillium expansum* after 7 days of incubation at 25 °C. Mean value (n=3) ± SD. Different letters (a, b, c, d) indicate significant differences among the preservative concentrations (*p<0.05*).

In general, greater antifungal activity of the phenolic compound, compared with that obtained when using ZG, was observed in the *in vitro* assays of FA against *A. flavus*, *A. niger* and *P. expansum*, (Figure 1C). Mycelial growth inhibition of up to 60% was exhibited at concentrations higher than 1 mg/g of FA for *A. flavus* and *P. expansum*, whereas 2 mg/g of FA were needed to accomplish inhibition of up to 50% in *A. niger*. Total inhibition was observed at 3 and 4 mg/g of FA for the *Penicillium* genus and the *Aspergillus* genus, respectively. Mohapotrea, Pati, and Ray (2000) suggested that concentrations of phenols that ranged from 3 to 5 µg/mL were required for normal fungi metabolism, but concentrations up to 5 µg/mL were inhibitory. Nesci and Etcheverry (2006) found that *A. flavus* and *A. parasiticus* growth and aflatoxin B1 levels decreased in comparison with the controls, which suggests that FA can be considered an effective fungitoxicant for both *Aspergillus* species.

Studying the effect of FA on *Z. rouxii* and *Z. bailii* growth revealed that high FA concentrations inhibited yeast growth. The MIC values were 2 and 3 mg/g for *Z. bailii* and *Z. rouxii,* respectively (data not shown). Pastorkova, Zakova, Landa, Novakova, Vadlejch, and Kokoska (2013) demonstrated that p-coumaric and FA exhibited selective inhibitory effects on *Z. rouxii* with MICs higher than or equal to 256 µg/mL. Recently, Rojo, Arroyo López, Lerena, Mercado, Torres, and Combina (2015) showed FA to be the most effective phenolic compound to prevent *Z. rouxii* growth in high sugar media at a low pH. In this study, no data about MIC were reported by the authors because total *Z. rouxii* inhibition was not achieved at the maximal concentration of the assayed antimicrobial compound (22 mM).

3.3 O/W emulsions analysis

3.3.1 Determination of CBEO losses by GC-MS analysis

CBEO losses, referred to as *trans*-cinnamaldehyde, after preparing O/W emulsions were 7%. These losses could be due to the high fluid dynamic stress applied to the emulsions by the HPH during the preparation procedure, which would display the degradation of EO constituents. The results obtained in this work agreed with those reported by Donsì Annunziata, Sessa, and Ferrari (2011), who highlighted the degradation of different active agents, such as α-phellandrene, terpinolene, p-cymene and thujene, among others, as a result of the fluid dynamic stress suffered by samples during high shear homogenisation and HPH.

3.3.2 Antifungal properties of O/W emulsions against A. niger: in vitro conditions

3.3.2.1 CBEO emulsions

The effectiveness of CBEO emulsions, prepared by 0.06 and 0.08 mg/g of the EO, against *A. niger* at 25 °C for 7 days was tested (data not shown). Only the samples that contained 0.08 mg/g of the antifungal agent did not show growth, and this value corresponded to its MFC.

Loss of effectiveness was observed when comparing the results obtained and while evaluating the antifungal properties of CBEO and CBEO emulsions. The use of 0.06 mg/g and 0.08 mg/g of CBEO as antifungal agents inhibited *A. niger* growth, whereas the emulsions that contained 0.06 mg/g of CBEO did not inhibit it. The results obtained when evaluating CBEO losses while preparing emulsions could be attributed to the EOs losses brought about by the mechanical stress applied to samples during the homogenisation process which, in turn, could reduce the antifungal activity.

3.3.2.2 The O/W emulsions formulated with CBEO and ZG

O/W emulsions were formulated by combining bioactive agents to lower the employed EO concentration and to improve the antifungal action of emulsions against *A. niger*.

The antifungal activity of the emulsions formulated at different CBEO concentrations (0.02, 0.04, 0.06 and 0.08 mg/g) and combined with ZG (1, 2, 4 and 6 mg/g) against *A. niger* is shown in **Figure 2**. The CBEO and ZG combination enhanced their antifungal action compared to the antifungal properties of free ZG and CBEO. Mycelial growth was inhibited when 0.06 mg/g of CBEO was incorporated into media, even at the lowest ZG concentration (1 mg/g), over 7 days. These results suggest possible synergistic interactions between CBEO and ZG.

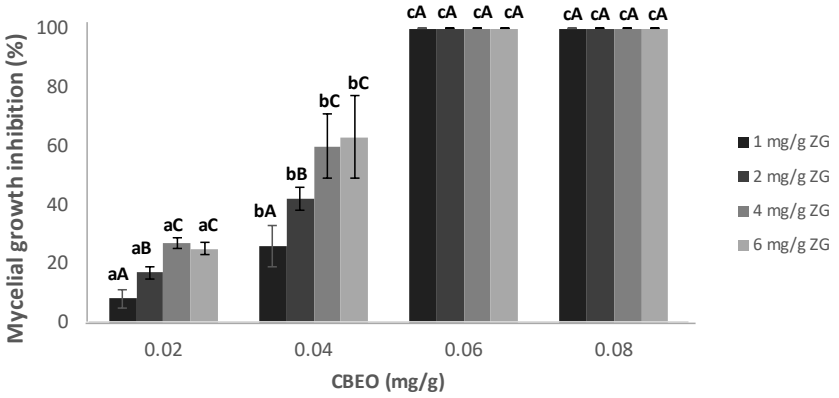

Figure 2. Mycelial growth inhibition (%) of cinnamon bark EO (CBEO) mixed with zinc gluconate (ZG) at different concentrations against *Aspergillus niger* at 25 °C for 7 days. Mean value (n=3) ± SD. Different letters (a, b, c) indicate significant differences among the CBEO concentrations ($p<0.05$), and (A, C, C) indicate significant differences among the ZG concentrations ($p<0.05$).

3.3.2.3 The O/W emulsions formulated with CBEO and FA

Figure 3 shows the antifungal activity of the emulsions prepared with CBEO and FA at different concentrations against *A. niger*. When 0.04 and 2.5 mg/g of CBEO and the FA were, respectively, combined, 72% mycelial growth inhibition was observed. Total mycelial growth inhibition was achieved when 0.06 of CBEO was used, regardless of FA content. However, FA alone achieved only total *A. niger* inhibition when the 4 mg/g concentration was tested (**Figure 1C**).

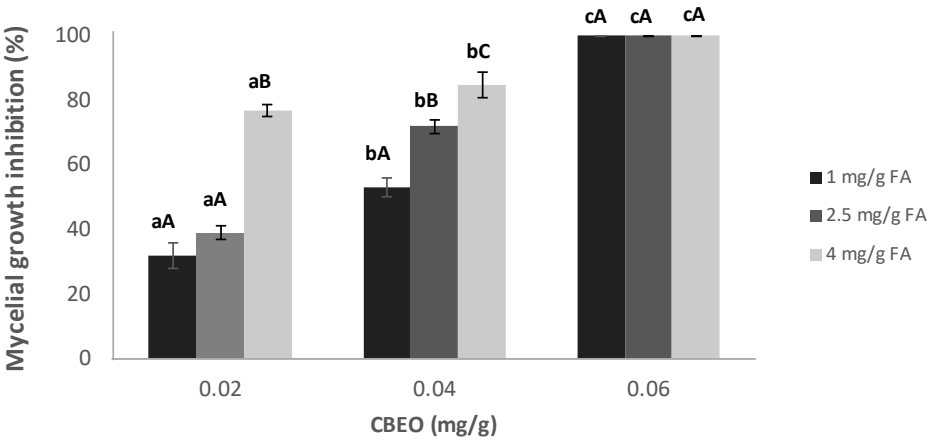

Figure 3. Mycelial growth inhibition (%) of cinnamon bark EO (CBEO) mixed with *trans*-ferulic acid (FA) at different concentrations against *Aspergillus niger* incubated at 25 °C for 7 days. Mean value (n=3) ± SD. Different letters (a, b, c) indicate significant differences among the CBEO concentrations ($p<0.05$) and (A, B, C) indicate significant differences among the FA concentrations ($p<0.05$).

3.3.2.4 The O/W emulsions formulated with CBEO, ZG and FA

The antifungal activity of the O/W emulsions prepared with 0.06 mg/g of CBEO, 1 mg/g of ZG and 1 mg/g of FA was tested against *A. niger*. No mycelial growth was observed for the tested formulation. This result highlighted that this emulsion was sufficient to inhibit *A. niger* growth for 7 days (data not shown).

These results suggested that the synergistic activity among the different natural preservatives incorporated into media allowed *in vitro A. niger* growth inhibition.

3.3.3 Physico-chemical characterisation

Table 2 shows the pH, $d_{3,2}$, $d_{4,3}$ and the ζ-potential values for the O/W emulsions prepared with different antifungal compounds (**Table 1**).

The pH values of the different formulated emulsions were between 6.75 and 7.15. The CBEO and CBEO-FA emulsions obtained the lowest pH values (6.73-6.75). Similar results have been obtained by Harwansh, Mukherjee, Bahadur, and Biswas (2015) in FA-loaded nanoemulsions.

As observed, the higher the total preservative concentration in the emulsion, the bigger particle size becomes. The emulsions that contained only CBEO exhibited a $d_{3,2}$ of 2.149±0.043 µm, whereas an increased droplet mean diameter was noted (2.449±0.038 µm) at the highest final concentration of the preservatives used in the emulsion formulation (CBEO-ZG-FA) (**Table 2**). The same trend occurred with the $d_{4,3}$ values. The mean size values significantly ($p<0.05$) increased from 5.649±0.594 to 6.612±0.683 µm when larger amounts of antifungal agents were employed while preparing emulsions. Interestingly, among the emulsions that contained two antifungal compounds, the larger particle size values ($d_{3,2}$ of 2.409±0.027 µm and $d_{4,3}$ of 6.326±0.161 µm) were observed when CBEO and FA were used for emulsion preparation. This could be due to the characteristics of the dispersed phase, which could facilitate the droplet

flocculation rate, as well as the reduction in the ratio between the interfacial stabilising material and the dispersed phase (McClements, 2005).

The ζ-potential values of all the formulations are also reported in **Table 2**. The ζ-potential is an indirect measure of the electrical charge of colloidal particles, which provides an indication as to their stability during storage. ζ-potential values of > 30 mV or < -30 mV indicated that the electrostatic repulsion among droplets likely contributed to prevent their aggregation (Harwansh et al., 2015). The electrical charge of the lipid droplets of the emulsions was negative, and values were within a range from - 44.3±3.0 to -58.9±1.5 mV. This result indicated the excellent stability of the emulsions. However, it is worth mentioning that the increment in the number of antifungal compounds, in the formulation of the emulsions, increased the mean particle size and decreased the ζ-potential of the samples. This effect could be explained by the differences found between the adsorption of the surface-active compounds at the oil-water interface (Salvia-Trujillo, Rojas-Graü, Soliva-Fortuny, & Martín-Belloso, 2015). Similar results have been obtained by Harwansh et al. (2015) and Salvia-Trujillo et al. (2015) in FA-loaded nanoemulsions-based gel and in nanoemulsions with different incorporated EOs, respectively.

The strong negative charge of the different O/W emulsions noted in the present study was probably influenced by XG, which is an anionic biopolymer (Ribes et al., 2016).

Table 2. Mean values (n=3) ± SD of pH, particle size ($d_{3,2}$ *and* $d_{4,3}$), and the ζ-potential of O/W emulsions (CBEO: cinnamon bark EO; ZG: zinc gluconate; FA: *trans*-ferulic acid).

Samples	pH	$d_{3,2}$ (µm)	$d_{4,3}$ (µm)	ζ-potential (mV)
CBEO	6.73 ± 0.04 [a]	2.149 ± 0.043 [a]	5.649 ± 0.594 [a]	-58.9 ± 1.5 [c]
CBEO- ZG	7.15 ± 0.05 [c]	2.196 ± 0.030 [a]	5.705 ± 0.383 [a]	-52.1 ± 1.6 [b]
CBEO- FA	6.75 ± 0.03 [a]	2.409 ± 0.027 [b]	6.326 ± 0.161 [b]	-51.3 ± 1.5 [b]
CBEO- ZG- FA	6.93 ± 0.04 [b]	2.449 ± 0.038 [b]	6.612 ± 0.683 [c]	-44.3 ± 3.0 [a]

[a, b, c, d,] Different superscripts indicate significant differences among the EO concentrations ($p<0.05$).

3.4 Effect of using O/W emulsions on strawberry jam

3.4.1 Strawberry jam spoilage by A. niger

The *in vivo* antifungal activity of emulsions CBEO-ZG, CBEO-FA and CBEO-ZG-FA (**Table 1**) against *A. niger* at 25 °C for 28 days is shown in **Figure 4**.

Strawberry jams prepared with either the CBEO-ZG or the CBEO-FA emulsion did not show any fungicidal activity compared to the control samples. Marked fungicidal activity was observed in the sample that contained emulsion CBEO- ZG-FA. In this case, a reduction of 2 log-cycles after 7 days of *A. niger* inoculation took place. The fungicidal effect could be the result of the interactions between the main and minor EO compounds, ZG and FA. This synergistic effect allowed mould growth to lower to 1 log CFU/g after 28 days. The limit of microbiological growth employed to determine the shelf life of samples was one of the most restrictive found in food products: the total count of yeast and moulds was 10^2 CFU/g (Pascual & Calderón, 2000). However, total fungi inhibition could interfered with the complex growth environment in food products (Omidbeygi, Barzegar, Hamidi, & Naghdibadi, 2007), which could protect microbial cells from antifungal products. The factors present in complex food commodities, like fat content, proteins, sugar, water, pH and enzymes, could reduce the antifungal effectiveness of EOs (Firouzi, Shekarforoush, Nazer, Borumand, & Jooyandeh, 2007; Friedly, Crandall, Ricke, Roman, O'Bryan, & Chalova, 2009) and interfere with the fungicidal effect of these antifungal compounds.

Finally, the emulsion prepared with 0.06 mg/g of CBEO, 1 mg/g of ZG and 1 mg/g of FA offered the best mould growth reduction results. This formulation was selected to carry out the sensory evaluation in strawberry jam.

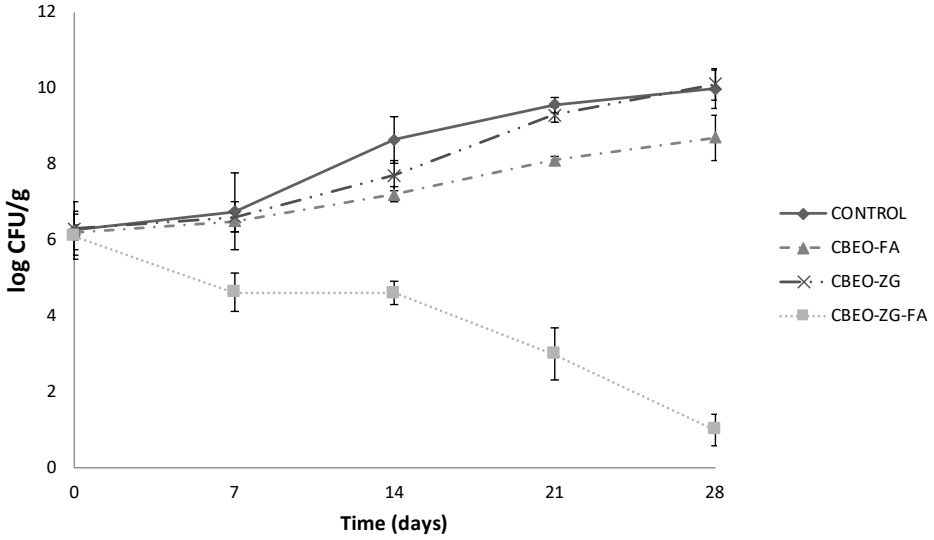

Figure 4. Effect of O/W emulsion on growth against *Aspergillus niger* incubated at 25 °C for 7 days. Mean value (n=3) ± SD (CBEO: cinnamon bark EO; ZG: zinc gluconate; FA: *trans*-ferulic acid).

3.4.2 Sensory evaluation

The sensory evaluation results of the control strawberry jams and the samples treated with antifungal agents (CBEO-ZG-FA) are shown in Figure 5. Incorporation of the O/W emulsion into strawberry jam did not alter samples' aspect, aroma, taste, unctuousness and overall acceptance compared with the control jam. Only mouth texture was the attribute that exhibited a significant difference ($p>0.05$) compared to the control sample. These results indicated that incorporating the O/W emulsion into strawberry jam did not modify its sensory acceptance. These results were especially satisfactory since the main study objective was to develop a new strategy to reduce the impact of EOs on the food sensory profile given their strong aroma and taste.

Acosta-Estrada, Lazo-Vélez, Nava-Valdez, Gutiérrez-Uribe, and Serna-Saldívar (2014) developed a new additive based on nejayote solids from the wastewater of the alkaline-cooking of maize, with large amounts of dietary fibre, calcium and FA. Bread that contained this additive showed no significant colour and texture differences. Nevertheless, increasing amounts of nejayote lowered the bread's scored flavour and odour. This negative effect was not observed in our study.

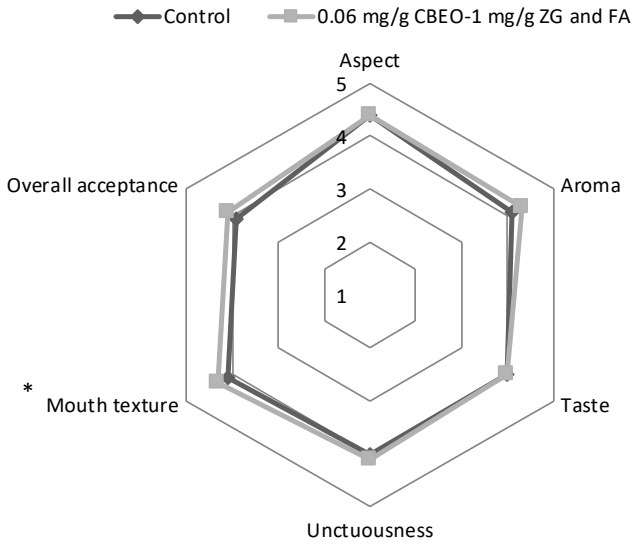

Figure 5. Average score of the different attributes evaluated in the control strawberry jam and the strawberry jam with O/W emulsion samples. 0: very unpleasant and 5: very pleasant. *Indicates significant differences between samples ($p<0.05$) (n=30). (CBEO: cinnamon bark EO; ZG: zinc gluconate; FA: trans-ferulic acid).

4. CONCLUSIONS

Cinnamon bark essential oil, zinc gluconate and *trans*-ferulic acid exhibit antifungal activity against *Aspergillus flavus, Penicillium expansum, Zygosaccharomyces rouxii* and *Zygosaccharomyces bailii.* The physico-chemical characterisation of oil-in-water emulsions reveals changes in particle size and the ζ-potential values associated with the number of natural agents embedded. Higher final preservative content leads to larger particle sizes. The differences in the ζ-potential values among formulations are probably due to differences between the adsorption of surface-active compounds at the oil-water interface. The combination of cinnamon bark essential oil, zinc gluconate and *trans*-ferulic acid allows to increase the effectiveness of O/W emulsion against *Aspergillus niger*.

The combination of cinnamon bark essential oil, zinc gluconate and *trans*-ferulic acid in emulsions is a new approach to control strawberry jam spoilage, and one that does not bring about any changes in its sensory characteristics. Nevertheless, more studies should be conducted to achieve complete fungi growth inhibition, and to investigate antifungal effectiveness against moulds and yeasts in other food commodities.

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CHAPTER 3

EUGENOL AND THYMOL IMMOBILISED ON MESOPOROUS SILICA-BASED MATERIAL AS AN INNOVATIVE ANTIFUNGAL SYSTEM: APPLICATION IN STRAWBERRY JAM

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Abstract

Essential oils and their main compounds have been studied in-depth for their antifungal properties against a wide variety of microorganisms. However, the strong odour emitted by them, even at low concentrations, makes their incorporation into food matrices difficult. Immobilisation of antimicrobial compounds on solid surfaces could be a strategy to reduce their odour impact. The antifungal effectiveness of eugenol and thymol bioactive agents, free and immobilised on mesoporous silica microparticles (MCM-41 family), and their impact on the final aroma and fungal decay of strawberry jam, were evaluated herein. Free eugenol and thymol exhibited good antifungal properties against the fungi strains tested, and thymol proved more effective. The antifungal activity of immobilised eugenol and thymol displayed greater antifungal activity for immobilised eugenol. The jams prepared with immobilised eugenol on MCM-41 microparticles exhibited no mould and yeast development during the studied storage time. The sensory evaluation confirmed that eugenol and thymol immobilisation reduced their typical strong impact on strawberry jam flavour. This work demonstrates the promising use of immobilised eugenol on mesoporous silica microparticles to control strawberry jam decay.

Keywords: Antifungal activity, thymol, eugenol, mesoporous silica support, strawberry jam.

1. INTRODUCTION

Yeasts and moulds can grow on raw and processed foods where the environmental conditions for most bacteria are unfavourable (Krisch et al., 2011). These microorganisms are broadly distributed and able to decay different food commodities, such as wine, cheese, vinegar, juices, fruits, sugar and meat (Gammariello et al., 2014). Chemical preservatives have been extensively used in recent years to control fungi development. Nevertheless, their negative consumer perception and changes in national regulations have forced food manufacturers to produce food commodities free of chemical additives (Ribes et al., 2016).

Essential oils (EOs) are natural volatile compounds from aromatic plants with a strong odour (Burt, 2004). Many EOs have been generally recognised as safe (GRAS) by the Food and Drug Administration (FDA) in 21 Code of Federal Regulations 182.20 (CFR, 2015). Their high content in phenolic derivatives, e.g., eugenol and thymol, etc. (Zabka and Pavela, 2013; Abbaszadeh et al., 2014), make the antifungal properties of EOs a good alternative to synthetic chemical preservatives. Eugenol is a naturally-occurring phenol extracted from buds and leaves of clove (Ribes et al., 2016) that is effective against fungi due to cytoplasmic membrane disturbance (Mihai and Popa, 2015). Thymol is the main monoterpene phenol found in the EOs extracted from *Lamiaceae* family plants, with strong antifungal activity against a wide range of fungal microorganisms, including *Aspergillus* and *Penicillium* species, among others (Klarić et al., 2006). However, the concentration of both compounds required to control fungal decay in foods modifies the food product's sensory profile given their strong flavour. For this reason, research that seeks for alternatives to minimise the sensorial impact of EOs on food products that do no diminish their antimicrobial effectiveness are very important. A potential approach is the immobilisation bioactive compounds from EOs on surfaces.

Among the potential supports to immobilise active molecules, siliceous materials like mesoporous silica particles are very promising thanks to their unique features, such as stability, biocompatibility and large load capacity

(Bernardos and Kourimska, 2013). In this context, MCM-41 (Mobil Composition of Matter) is a member of the mesoporous materials' family according to the IUPAC (International Union of Pure and Applied Chemistry) classification (Dünder-Tekkaya and Yürüm, 2016), known to have a large specific surface and specific volume, and is easy to functionalise and highly stable. MCM-41-based materials have also been reported to resist harsh conditions of the stomach, acid matrices and microbial action (Pérez-Esteve et al., 2015b). The chemical formula for MCM-41 is SiO_2, which is a common additive (E551) in the food industry (Barahona et al., 2016). Given their easy preparation and properties, MCM-41 particles have been used in the present work as promising silica supports where bioactive agents can be immobilised.

The main purpose of this work was to investigate the feasibility of immobilised eugenol and thymol on mesoporous silica particles (MCM-41 family) as an antifungal system, and to study their antifungal effectiveness and sensory impact of the materials on strawberry jam.

2. MATERIALS AND METHODS

2.1 Microbial strains, culture media and chemicals

Strains *Aspergillus flavus* (CECT 20156), *Aspergillus niger* (CECT 20156), *Penicillium expansum* (CECT 20140), *Zygosaccharomyces rouxii* (CECT 1229) and *Zygosaccharomyces bailii* (CECT 12001) were used as test microorganisms, and were supplied by the Spanish Type Culture Collection (CECT, Burjassot, Spain). For mould species, potato dextrose agar (PDA) and potato dextrose broth (PDB) were used, while yeast peptone dextrose broth (YPDB) and agar were employed for the yeast species. All the culture media were purchased from Scharlab (Barcelona, Spain).

Eugenol (99% w/w) and thymol (≥ 99% w/w) were provided by Sigma-Aldrich (Madrid, Spain). For the synthesis of MCM-41 microparticles and the derivatisation and immobilisation of the antifungal compounds, *N*-

cetyltrimethylammonium bromide (CTABr), sodium hydroxide (NaOH), triethanolamine ($TEAH_3$), tetraethylorthosilicate (TEOS), (3-Aminopropyl)triethoxysilane (APTES), trimethylamine, paraformaldehyde, diethyl ether, chloroform, n-butanone, dimethyl sulfoxide (provide all of them by Sigma-Aldrich, Madrid, Spain), acetonitrile, hydrochloric acid (HCl), magnesium sulphate ($MgSO_4$), potassium hydroxide (KOH) and sulfuric acid (H_2SO_4) (Scharlab, Barcelona, Spain) were employed.

2.2 Antifungal activity of free eugenol and thymol

Free bioactive compounds eugenol and thymol were individually examined against *A. flavus, A. niger* and *P. expansum,* as described by Manso et al. (2013) with minor modifications. Spore suspensions of 10^6 CFU/mL were prepared in NaCl (0.7% w/v) and Tween 80 (0.1% w/v), and confirmed using a hematocytometer. MIC (Minimal Inhibitory Concentration) values were obtained by macrodilution in Erlenmeyer flasks that contained 15 mL of PDB and 1% (w/v) of Tween 80 to secure the total active compounds dispersions. A solution of 1,000 mg/kg of thymol was obtained by dissolving the appropriate amount in dimethyl sulfoxide. Different concentrations of bioactive compounds were tested: 0.1, 0.2, 0.3 and 0.4 mg/mL. The control samples, with no antifungal agents, were prepared following the same procedure. Each Erlenmeyer flask that contained free eugenol and thymol were inoculated with 100 µL of the spore suspension and incubated under orbital stirring (180 rpm) at 25 °C for 72 h. The results were expressed as log CFU/mL.

After incubation, the lowest eugenol and thymol non-growth concentration was established as the MIC. To determine the minimal fungicidal concentration (MFC), 100 µL of the non-growth suspensions were seeded onto Petri plates prepared with 15 g of PDA. MFC was defined as the lowest concentration at which no colonies developed after 72 h of incubation at 25 °C.

The antifungal effectiveness of free eugenol and thymol against *Z. rouxii* and *Z. bailii* was also evaluated, for which the methodology followed was similar to that described in the mould assays. Cell suspensions of 10^6 CFU/mL were prepared in Tween 80 (0.1 % w/v), and confirmed using a hematocytometer. The MIC values were obtained by macrodilution in Erlenmeyer flasks that contained 15 mL of YPDB and 1% (w/v) of Tween 80 to secure the total bioactive compounds dispersions. The preparation of the thymol solution and the control samples, and the concentration of the tested bioactive agents, were the same as those previously described. Each Erlenmeyer flask that contained free eugenol and thymol was inoculated with 100 µL of 10^6 CFU/mL, and incubated under orbital stirring (180 rpm) for 48 h at 25 °C. The results were expressed as log CFU/mL.

After incubation, the MIC and the MFC values were determined as described above for moulds, but by employing YPD agar as the culture media. All the tests were conducted in triplicate.

2.3 Study of mesoporous silica particles

2.3.1 Synthesis of MCM-41 microparticles

Synthesis of the mesoporous MCM-41 microparticles was carried out using the so-called “atrane route” described by Pérez-Esteve et al. (2015a). To this end, 52.4 g of $TEAH_3$ and 0.98 g of a NaOH solution were stirred vigorously at 120 °C. After lowering the temperature to 70 °C, 22 mL of TEOS were slowly added to control silica condensation, and stirred to reach 118 °C. Afterwards, 9.36 g of CTABr were added to the solution until completely dissolved, which allowed the incorporation of 180 mL of deionised water, which was vigorously stirred at 70 °C. This step led to the formation of a white suspension, which was aged at 100 °C for 24 h. The obtained solid was washed with deionised water and ethanol until pH 7, and then dried at 70 °C. Finally, the as-synthesised solid was calcined at 550 °C for 5 h to remove the surfactant molecules.

2.3.2 Derivatisation of the bioactive compounds

Eugenol and thymol aldehydes were prepared by preserving the presence of their hydroxyl group given the important role that these hydroxyl moieties play in antifungal activity (Rao et al., 2010; Ahmad et al., 2011). The eugenol aldehyde was obtained by a Reimer-Tiemann reaction. For this purpose, 150 mL of water at 80 °C were used to dissolve 3.39 g of eugenol. Afterwards, the temperature was lowered to 60 °C, and 22.4 g of KOH and 7 mL of chloroform were added. The last reagent was incorporated at a ratio of 1 mL/h for 7 h due to the exothermic character of this reaction. The reaction mixture was kept at 60 °C for 8 h. Finally, 50 mL of H_2SO_4 (10% v/v) were added and the mixture was extracted using n-butanone. The organic phase was rotavapored at reduced pressure to obtain the eugenol aldehyde.

The thymol aldehyde was synthesised by mixing 6 g of thymol, 150 mL of acetonitrile, 20.9 mL of trimethylamine and 3.81 g of $MgSO_4$. This mixture was stirred for 15 min at room temperature in an argon atmosphere. Then 8.1 g of paraformaldehyde were added to the mixture and refluxed for 3.5 h at 83 °C. After cooling the solution, it was acidified using 320 mL of HCl (5% v/v) and stirred for 15 min at room temperature in an argon atmosphere. The organic phase was extracted using diethyl ether, and then removing the volatiles at reduced pressure. The reaction yield was calculated by ^1H NMR in a Bruker AV400 spectrometer (Bruker Daltonik GmbH, Bremen, Germany) which operated at room temperature.

2.3.3 Immobilisation of the bioactive compounds on the surface of MCM-41 microparticles

The immobilisation of the eugenol and thymol aldehydes on the surface of MCM-41 microparticles was carried out through the synthesis of the corresponding alkoxysilane derivatives. The eugenol or thymol aldehyde was mixed with 20 mL of dichloromethane, 3.08 mL of APTES and 0.5 g of $MgSO_4$. The solution was stirred for 1 h at 38 °C in reflux. The mixture was filtered and the organic phase was removed at reduced pressure to obtain the corresponding eugenol or thymol alkoxysilane derivative. Then 2 g of the MCM-41, 60 mL of acetonitrile and 4 mL of the corresponding alkoxysilane derivatives were stirred for 5.5 h at room temperature. Solids were filtered, washed with acetonitrile and dried for 24 h at low pressure.

2.3.4 Characterisation of MCM-41 microparticles

The characterisation of the microparticulated MCM-41 (bare and functionalised with eugenol and thymol) was performed by particle size distribution, ζ-potential, field emission scanning electron microscopy (FESEM), thermogravimetric analyses (TGA) and an elemental analysis.

Particle size distribution was determined in deionised water using a laser diffractometer (Mastersizer 2000, Malvern Instruments, Worcestershire, UK), and applying the Mie theory (refractive index of 1.45, absorption index of 0.1). The ζ-potential analysis was run in a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). Samples were diluted with deionised water (1 mg/mL) and sonicated before being measured. The Smoluchowsky mathematical model was used to convert the electrophoretic mobility measurements into ζ-potential values.

Particle size distribution and the ζ-potential analysis were performed in triplicate. FESEM images were obtained by a Zeiss Ultra 55 (Carl Zeiss NTS GmbH, Oberkochen, Germany) and observed in the secondary electron mode. Thermogravimetric analyses were carried out on a TGA/SDTA 851e balance (Mettler Toledo, Columbus, USA), from 25 to 1,000°C with a heating rate of 10 °C/min in an oxidant atmosphere (air, 80 mL/min). An elemental analysis for C, H, and N was performed by a combustion analysis in a CHNOS model Vario EL III (Elemental Analyses System GMHB, Langenselbold, Germany).

2.3.5 Antifungal activity of eugenol and thymol immobilised on the surface of MCM-41microparticles

The evaluation of the antifungal activity of MCM-41 with the bioactive agents against *A. flavus, A. niger, P. expansum*, *Z. rouxii* and *Z. bailii* was made by the methodology described in Section 2.2. The concentration of the MCM-41 microparticles functionalised with eugenol or thymol was established based on the thermogravimetric and elemental analyses to add equal amounts of eugenol and thymol (0, 0.1, 0.2, 0.3 and 0.4 mg/mL) to the media than in the case of the free bioactive agents. By these means, the comparison of the antifungal activity between the pure and immobilised bioactive agents was made.

The elemental analysis data revealed that the content of the eugenol and thymol immobilised on the surface of MCM-41 microparticles was 65 mg/g and 76 mg/g, respectively. These values were used to determine that the concentrations of the eugenol and thymol immobilised on the MCM-41 surface incorporated into the media corresponded to 0.1, 0.2, 0.3 and 0.4 mg/mL of the free bioactive compound. Positive controls were prepared with bare MCM-41 and 1% (w/v) of Tween 80. The results were expressed as log CFU/mL. Each assay was performed in triplicate.

2.4 Studying the MCM-41 microparticles with eugenol and thymol in strawberry jam

To evaluate the *in vivo* antifungal effect of the bioactive agents immobilised on the surface of MCM-41 microparticles, strawberry jams were prepared and inoculated with two representative microorganisms. *A. niger* was selected as a mould for its frequent isolation in strawberries, while *Z. bailii* was used as a target yeast for its ability to grow in high sugar products (60% w/w) and at a low pH (Stratford et al., 2013). The concentrations of the free and immobilised eugenol and thymol on MCM-41 were selected according to the MFCs determined in the *in vitro* assays.

2.4.1 Jam preparation

Jam preparation was obtained according to the procedure reported by Ribes et al. (2016). Strawberry jam was obtained by mixing fruit and sugar in a ratio of 65:35 and cooked at 100 °C for 30 min to reach a 60 °Brix in the product as described in the Spanish quality regulation for fruit jam (BOE, 2003). This process was carried out in an electrical food processor (Thermomix TM 31, Vorwerk M.S.L, Spain). The free and immobilised eugenol and thymol, as well as the bare MCM-41 microparticles, were added to 15 g of strawberry jams, once suspended in Tween 80 in order to ensure the complete distribution of the agents, at ambient temperature and then homogenising adequately by using a sterilised spatula.

2.4.2 Antifungal effectiveness in strawberry jam

The *in vivo* antifungal effectiveness of the free and immobilised bioactive agents against *A. niger* and *Z. bailii* was examined by the methodology described by Ribes et al. (2016). Fifteen grams of strawberry jam (control, control with bare MCM-41 microparticles, free bioactive agents and bioactive agents immobilised

on MCM-41) were inoculated with 100 µL of the fungi solution (10^6 CFU/mL) and incubated at 25 °C for 28 days.

At each analysis day, 10 grams of every sample were placed in sterile plastic bags containing 90 mL of tryptone phosphate water and homogenised for 1 min in a Stomacher blender (Masticator IUL, S.A. Instruments, Germany). Serial dilutions were prepared and 0.1 mL were spread on the surface of the agar plates. Three Petri dishes were prepared per formulation, microorganism and analysis day, plus the control samples (n=120). *A. niger* and *Z. bailii* counts were done on PDA and YPD agar plates, respectively, after a 72-hour incubation at 25 °C (Pascual and Calderón, 2000). All the assays were performed in triplicate.

2.4.3 Sensory evaluation

A sensory analysis was carried out to evaluate the feasibility of immobilisation to reduce the impact provoked by eugenol and thymol on strawberry jam (ISO 4121: 2003). For this purpose, a panel of 12 trained judges participated in this study. Panellists were trained during preliminary sessions to identify the typical aromas of eugenol and thymol, calibrated using aqueous solutions of these compounds at different concentrations (0, 0.1, 0.2, 0.3 and 0.5 mg of the bioactive compound per g of solution) (ISO 8586: 2012). During the assessment, a 5-point aroma intensity scale was used: from 0, no descriptor, to 5, extremely intense. Each panellist evaluated the intensity aroma of eugenol or thymol on strawberry jam samples, which contained these compounds that were free and immobilised on the MCM-41 surface. The concentrations of the free and immobilised eugenol and thymol on MCM-41 were selected according to the MFCs determined in the *in vitro* assays.

In order to quantify the effect of immobilisation to reduce the effect of bioactive compounds on strawberry jam aroma, the rates given to the samples with the immobilised compound were compared to those given to strawberry jam with free eugenol and thymol at the same concentration.

2.5 Statistical analysis

The results obtained in the *in vitro* and *in vivo* tests to evaluate the antifungal activity of the free eugenol and thymol, when immobilised on the surface of MCM-41, were analysed by a multifactor analysis of variance (multifactor ANOVA). The characterisation of the mesoporous silica particles the sensory analysis were evaluated by a one-way ANOVA. The least significance procedure (LSD) was employed to test for differences between averages at the 5% significance level. Data were statistically processed by Statgraphics Centurion XVI.

3. RESULTS AND DISCUSSION

3.1 Antifungal activity of free eugenol and thymol

The counts of *A. flavus*, *A. niger*, *P. expansum, Z. bailii* and *Z. rouxii* after free eugenol and thymol treatment are shown in **Figure 1**. Both the bioactive compounds showed significant ($p<0.05$) antifungal activity, which affected fungi growth in a dose-dependent manner. With eugenol, the use of 0.3 mg/mL led to a reduction of between 3 and 5 log-cycles for the *Aspergillus* and *Zygosaccharomyces* genera after 72 h and 48 h, respectively, of its inoculation. The growth inhibition of *A. flavus, A. niger, Z. bailii* and *Z. rouxii* was achieved by employing 0.4 mg/mL of eugenol (MFC). With *P. expansum,* inhibition was attained by using 0.2 mg/mL of eugenol, and this concentration was the MFC.

When 0.2 mg/mL of thymol were added to the media, a reduction of between 3 and 4 log-cycles took place for the *Aspergillus* and *Zygosaccharomyces* genera after 72 h and 48 h, respectively, of its inoculation. Thymol inhibited the growth of all the target microorganisms tested at 0.4 mg/mL, which corresponded to the MFC value.

It is worth mentioning that the discontinuous horizontal line indicates the CFU/mL after inoculation (**Figure 1**). Above this line, no antifungal effect was achieved, while this line indicated the fungistatic activity of free eugenol or

thymol. In addition, below the discontinuous line a fungicidal effect is observed. Taking it into account, a significant ($p<0.05$) fungicidal effect was exhibited when using ≥ 0.3 mg/mL of eugenol and thymol for *A. niger*, *Z. bailii* and *Z. rouxii*, which was stronger for thymol. Indeed, the fungicidal effect of thymol against *A. flavus* was evidenced when 0.2 mg/mL were employed. For *P. expansum*, the treatment with 0.1 and 0.2 mg/mL of eugenol and thymol, respectively, showed clear fungistatic activity.

The differences in the molecular structure of both the antifungal agents most likely determine their antifungal effectiveness. The hydroxyl group present in thymol is responsible for the strong ability to dissolve and accumulate in the cell membrane, and lead to its destabilisation due to marked proton transfer disruption (Rao et al., 2010; Ahmad et al., 2011). Furthermore, the generally weaker antifungal activity of eugenol at low concentrations could be related to its lower hydrophobicity, and also to the presence of a methoxyl group in orthoposition, which diminished its ability to release a proton from the hydroxyl group (Ben Arfa et al., 2006). Similar results have been obtained by Abbaszadeh et al. (2014) when they applied eugenol as an alternative agent to control fungi development. However, the MFC values of thymol against the *Aspergillus, Penicillium* and *Zygosaccharomyces* species were lower than the data obtained in this study. Abbaszadeh et al. (2014) showed the influence of thymol with the MFC values of 150 and 250 µg/mL against *A. flavus* and *A. niger*, respectively. In another study, Monu et al. (2016) found that eugenol and thymol inhibited *Z. bailii* growth at 200 mg/L. The differences between these findings and the results reported herein could be due to the strains selected, the type of assay employed and incubation times used.

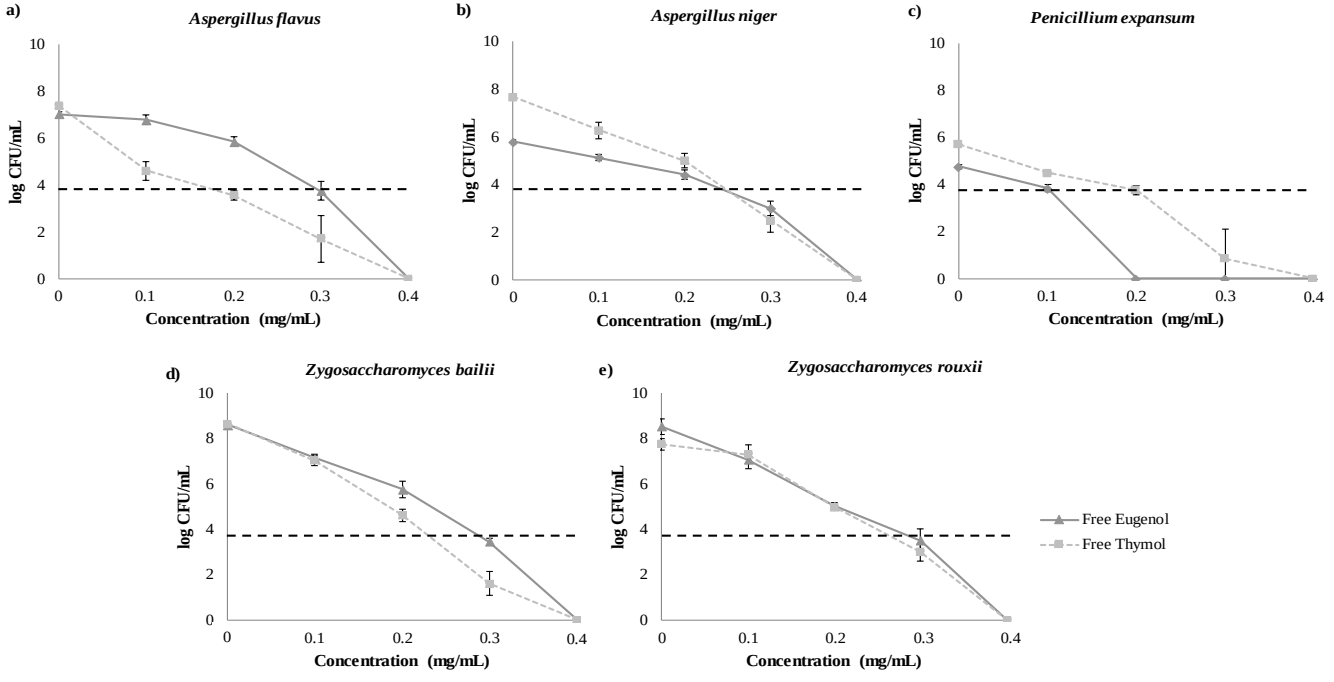

Figure 1. Antifungal activity of free eugenol and thymol against a) *Aspergillus flavus*, b) *Aspergillus niger* and c) *Penicillium expansum* at 25 °C for 72 h; and against d) *Zygosaccharomyces bailii* and e) *Zygosaccharomyces rouxii* at 25 °C for 48 h. Mean value (n=3) ± standard deviation. The discontinuous horizontal line indicates the initial CFU/mL values.

3.2 Characterisation of the bare and functionalised MCM-41 microparticles

Antifungal microparticles were prepared by the immobilisation of eugenol and thymol on the surface of the MCM-41 support. In a first step, both bioactive compounds were reacted with APTES to obtain the corresponding trialcoxysilane derivative. The efficiency of the alkoxysilane derivatisation process was evaluated by the ^{1}H NMR analysis. For the two bioactive agents, the product yield estimated from the ^{1}H NMR spectra was 20-40%. The alkoxysilanes derivatives reacted in a second step with the silanol groups of the MCM-41 microparticles yielded the final functionalised solids.

Bare and functionalised MCM-41 microparticles were characterised by standard techniques. **Table 1** summarises the $d_{0.5}$ and the ζ-potential values for the MCM-41 microparticles (bare and immobilised with eugenol and thymol). The bare MCM-41 microparticles showed a $d_{0.5}$ of 3.13±0.14 μm, whereas an increased particle mean diameter was obtained when the particles were functionalised with eugenol and thymol (4.37±0.12 and 4.1±0.2 μm, respectively).

The ζ-potential values of the samples are provided in **Table 1**. The bare MCM-41 microparticles had negative ζ-potential values (-35.9±1.4). After the immobilisation of eugenol and thymol on the mesoporous material surface, the ζ-potential changed to weak negative or positive values in agreement with the functionalisation of the MCM-41 surface with eugenol and thymol. The change we noted in the ζ-potential values upon functionalisation has also been observed by Pérez-Esteve et al. (2016) in mesoporous silica supports loaded with folic acid and functionalised with amines, and also by Mathew et al. (2016) in succinamic acid functionalised MCM-41 particles.

Figure 2 shows the FESEM images of the bare and functionalised MCM-41. As seen, no changes on the surface of the mesoporous supports were detected when comparing the bare MCM-41 and the functionalised samples, which confirms that the immobilisation of eugenol and thymol on the surface did not affect the integrity of the mesoporous silica particles. As previously mentioned, the content

of the eugenol and thymol immobilised on the surface of the MCM-41 microparticles, obtained from the thermogravimetric and elemental analyses, was 65 mg and 76 mg per gram of solid, respectively. These data were used to calculate the amount of MCM-41 functionalised with eugenol and thymol needed to provide an equivalent dose of bioactive compounds compared to the free molecule (Section 2.3.5).

Table 1. Particle size ($d_{0.5}$) and ζ-potential values of MCM-41 microparticles (bare) and with eugenol and thymol derivates immobilised on its surface (Eugenol-MCM-41 and Thymol-MCM-41). Values are expressed as mean (n=3) ± standard deviation.

Sample	$d_{0.5}$ (μm)	ζ-potential (mV)
Bare MCM-41	3.13 ± 0.14 [a]	-35.9 ± 1.4 [a]
Eugenol-MCM-41	4.37 ± 0.12 [c]	-0.4 ± 0.4 [b]
Thymol-MCM-41	4.1 ± 0.2 [b]	10.8 ± 2.1 [c]

[a, b, c] *Different superscripts indicate differences among mesoporous silica materials*

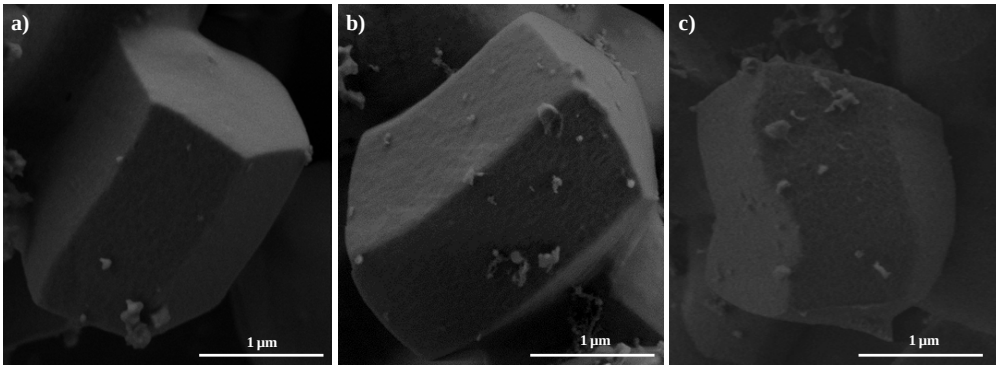

Figure 2. FESEM images of a) bare MCM-41, b) eugenol-MCM-41, c) thymol-MCM-41 microparticles.

3.3 Antifungal activity of eugenol and thymol immobilised on the surface of MCM-41 microparticles

The antifungal activity of eugenol and thymol immobilised on the surface of MCM-41 against *A. flavus, A. niger, P. expansum*, *Z. bailii* and *Z. rouxii* is summarised in **Figure 3**.

No growth inhibition was observed in any mould and yeast strains in the presence of the bare MCM-41. These results agree with those obtained by Wehling et al. (2013), who also evaluated the antimicrobial activity of bare silica particles. In contrast, mould and yeast growth significantly reduced ($p<0.05$) in the presence of increasing amounts of MCM-41 functionalised with eugenol and thymol. The MCM-41 that contained eugenol as an antifungal agent exhibited greater effectiveness than the thymol immobilised on the MCM-41 microparticles against all the evaluated fungi species. Growth of *P. expansum*, *Z. bailii and Z. rouxii* was inhibited by using 0.2 mg/mL of eugenol (MFC) immobilised on the surface of MCM-41, whereas the genus *Aspergillus* presented less sensitivity at this concentration. The total inhibition of *A. flavus* and *A. niger* was attained at 0.3

and 0.4 mg/mL of eugenol immobilised on the MCM-41 microparticles, respectively, which were the MFC concentrations.

Conversely when MCM-41 functinalised with thymol was tested against all the target microorganisms, weak antifungal efficacy was observed at low thymol concentrations. The use of 0.2 mg/mL of thymol immobilised on MCM-41 led to a reduction of between 2 and 4 log-cycles, whereas, the eugenol immobilised on the MCM-41 microparticles at the same concentration attained a 5 log reduction for *A. niger* and inhibited fungi development as in the case of *P. expansum*, *Z. bailii* and *Z. rouxii*. As previously mentioned, this tendency was not observed for *A. flavus* (**Figure 3**). Inhibition of *A. flavus* was observed when using 0.3 mg/mL of thymol immobilised on MCM-41, which corresponds to its MFC value. The MFC values for *A. niger, P. expansum, Z. bailii* and *Z. rouxii* were observed by using 0.4 mg/g of thymol immobilised on silica particles.

In addition, a significant ($p<0.05$) fungicidal effect was observed when using ≥ 0.2 mg/mL of eugenol and thymol immobilised on MCM-41 microparticles for *A. niger*, *P. expansum*, *Z. bailii* and *Z. rouxii*, and was stronger for eugenol. Moreover, the fungicidal effect of eugenol and thymol immobilised on the MCM-41 microparticles against *A. flavus* was exhibited when 0.3 mg/mL were utilised (**Figure 3**).

As far as we know, this is the first time that studies which evaluate the antifungal efficacy of eugenol and thymol derivatives immobilised on the surface of microparticulated MCM-41 against the genera *Aspergillus, Penicillium* and *Zygosaccharomyces* have been reported.

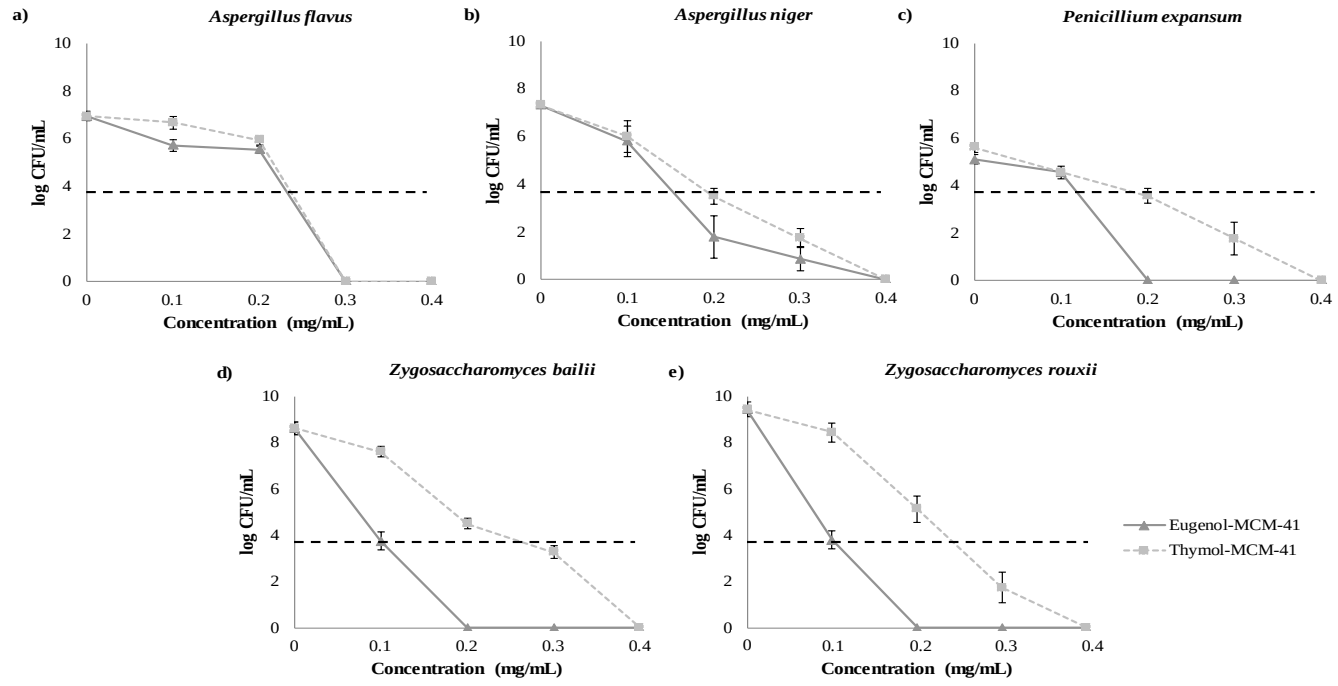

Figure 3. Antifungal activity of eugenol and thymol immobilised on the surface of MCM-41 microparticles against a) *Aspergillus flavus*, b) *Aspergillus niger* and c) *Penicillium expansum* at 25 °C for 72 h; and against d) *Zygosaccharomyces bailii* and e) *Zygosaccharomyces rouxii* at 25 °C for 48 h. Mean value (n=3) ± standard deviation. The discontinuous horizontal line indicates the initial CFU/mL values.

When the results of the free and immobilised eugenol and thymol were compared, it was generally established that greater antifungal effectiveness was observed when these compounds were immobilised on the MCM-41 support. This could be due to: i) the intense antifungal activity of the MCM-41 microparticle after eugenol and thymol immobilisation due to the high density of the antifungal compound on the mesoporous material surface; and/or ii) the volatility reduction of bioactive agents when immobilised on the surface of MCM-41 microparticles.

3.4 Studying the MCM-41 microparticles functionalised with eugenol and thymol in strawberry jam

3.4.1 Antifungal effectiveness in strawberry jam

The development of *A. niger* and *Z. bailii* in non-inoculated and inoculated strawberry jams stored at 25 °C for 28 days is shown in **Figure 4**. As noted, the jams prepared with free and immobilised eugenol exhibited no mould and yeast development throughout the evaluation period. On the contrary, the samples prepared with the free thymol and thymol-MCM-41 did not inhibit the fungal growth of the samples. However, it is noteworthy that the strawberry jams prepared with the free thymol, and inoculated with *A. niger* and *Z. bailii*, exhibited a more fungi development compared to the samples that contained the thymol-MCM-41 microparticles. These results agree with those reported in the *in vitro* antifungal assays (Section 3.3), where the antifungal effectiveness of the MCM-41 that contained the immobilised bioactive agents was enhanced.

Despite the same amount of the free and immobilised thymol being used in the jam samples and culture media against *A. niger* and *Z. bailii*, different antifungal activity was observed. This could be related to the presence of antagonistic interactions with other ingredients, such as carbohydrates (Pitt and Hocking, 2009). Firouzi et al. (2007) reported that despite *in vitro* assays with EOs and their main components, such as oregano and nutmeg, displayed substantial antimicrobial activity, the amounts required when used in food systems increased (approx. 1–3% higher).

3.4.2 Sensory evaluation

In order to test the feasibility of immobilisation to avoid the drawbacks of aromas when incorporating eugenol and thymol into food samples, a sensory evaluation was made. The assessment results indicated that, even though immobilisation could not eliminate the typical thymol and eugenol aroma in the jam samples, this technique was able to significantly reduce the aroma intensity of these compounds in strawberry jam. A comparison of the jam samples with the free and immobilised bioactive compounds established that immobilisation reduced the intensity of eugenol and thymol aromas more than 92% and 96%, respectively. These results confirm the feasibility of immobilisation as a technique to avoid the impact of eugenol and thymol on the sensory profile of food samples. This promising technique could be employed with other substances that are not currently viable given their adverse impact on the sensory perception of applied foods.

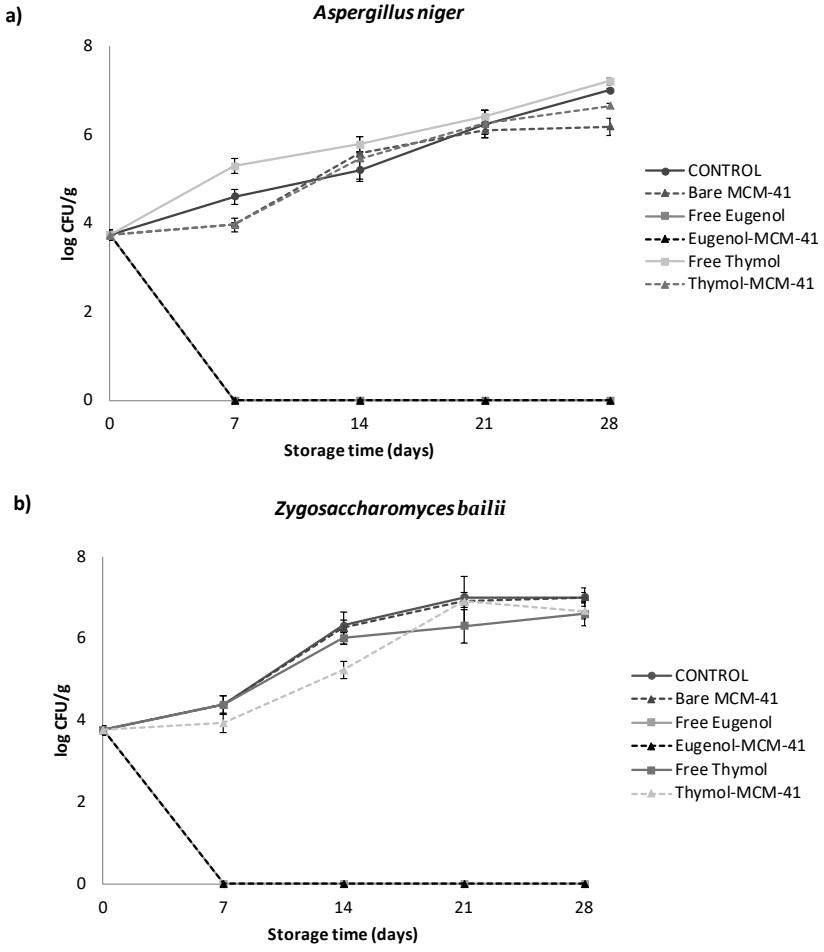

Figure 4. Influence of the free and immobilised eugenol and thymol on the growth of (a) *Aspergillus niger* and (b) *Zygosaccharomyces bailii* in inoculated strawberry jams for 28 days at 25 °C. Mean values (n=3) ± standard deviation.

4. CONCLUSIONS

Free eugenol and thymol exhibit excellent properties as antifungal agents against several mould and yeast strains. When incorporated in their free form, eugenol induces better preservation of strawberry jam in terms of fungal spoilage compared to thymol. However, after immobilisation on MCM-41 microparticles, both bioactive agents have improved the antifungal properties and their impact on jam odour compared to the free compounds are weaker. These results suggest that the use of bioactive agents from plants immobilised on the surface of mesoporous silica materials acts as promising antifungal agents for controlling mould and yeast spoilage, and by diminishing the current industrial limitation due to their strong flavour at the same time.

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4. GENERAL DISCUSSION

In the present Doctoral Thesis, the feasibility of using different antifungal systems to preserve strawberry jam, and the effect of their incorporation on the sensory profile of jams, have been investigated.

Antifungal systems, such as the O/W emulsions and nanoemulsions and, mesoporous silica supports, were used to control strawberry jams spoilage. Different antifungal agents (EOs: bergamot, cinnamon bark, cinnamon leaf, clove, lemon and mandarin; EOs' main compound: eugenol and thymol; phenolic compounds: *trans*-ferulic acid; zinc salt: zinc gluconate) and emulsifiers (Tween 80 and WPI) were used in the formulation of these antifungal systems. XG was also used as a biopolymer in the formulation of some emulsions.

Several strategies were adopted to achieve a product with excellent microbiological and sensory qualities. **Table 1** summarises the different strategies followed, the results achieved and the future studies or perspectives required after following each approach.

The **first approach** was based on using O/W emulsions as stable delivery systems. First of all, nanoemulsions-based delivery systems with different EOs (cinnamon leaf, lemon and bergamot) against *A. niger* were developed.

The cinnamon leaf, lemon and bergamot EOs nanoemulsions were prepared with the oil phase consisting only of EO or of EO mixed with sunflower oil as a ripening inhibitor at the 3:1 wt ratio. T80 and WPI were used as emulsifiers (1 wt%). This formulation proved stable under the accelerating ageing tests conducted at 35 °C, and was employed in the fungitoxic experiments.

The cinnamon leaf nanoemulsions exhibited significantly better *in vitro* antifungal properties than the free EO. However with the citrus EOs (lemon and bergamot), encapsulation in nanoemulsions generally diminished their antifungal activity. The different composition of EOs and their different interactions with the nanoemulsion ingredients likely explain the distinct contribution of nanoemulsions. Probably for citrus EOs, nanoemulsion acts as a hydrophobic sink for their main constituents. In addition, the nanoemulsions based on WPI exhibited better *in vitro* antifungal activity than those based on T80. This is likely

due to the larger numbers of sites of interaction with WPI than with the T80 available on the surface of fungal cells and spores.

By taking into account the relevance of the formulation of emulsions in developing efficient and stable antifungal systems for food applications, the antifungal activity of O/W emulsions to control the fungal deterioration of strawberry jam was investigated. O/W emulsions were formulated with different concentrations of clove (0.55, 0.65 and 0.75 mg/g), cinnamon leaf EO (0.65, 0.75 and 0.85 mg/g) and XG (2.5, 5.0 and 7.5 mg/g).

The *in vitro* antifungal activity of O/W emulsions indicated that those emulsions which contained 0.55 mg/g of clove and 0.65 mg/g of cinnamon leaf were able to inhibit *A. flavus*, *A. niger* and *P. expansum* growth over 7 days. Furthermore, the XG concentration employed in the formulation of emulsions strongly impacted losses of EOs, which took place while preparing the emulsions. The use of 2.5 mg/g of XG led to the most severe eugenol losses, compared to 5.0 and 7.5 mg/g XG, while no significant differences were observed between these samples. The lower viscosity of the samples formulated with 2.5 mg/g of XG, compared to the emulsions that contained 5.0 and 7.5 mg/g of XG, could cause the diffusion of EOs to the surface of the emulsions, which promotes the volatilisation and subsequent loss. Therefore, the emulsions prepared with 0.55 and 0.65 mg/g of the clove and cinnamon leaf EO, respectively, and with 5.0 mg/g of XG, were selected to be added to strawberry jam.

The effectiveness of the O/W emulsion to control mould growth, was studied at 4 °C (by reproducing product cold storage after opening the jam container) and at 25 °C (the optimum growth temperature of fungi) for 63 days. The antifungal activity of the clove and cinnamon leaf O/W emulsions against several strains, such as *A. flavus*, *A. niger* and *P. expansum*, was confirmed in the *in vivo* tests at both temperatures. Finally, the incorporation of the O/W emulsions into strawberry jam did not modify the product's texture and colour, but its affected aroma, taste and the overall acceptance of the jam.

After considering the previous effects of incorporating O/W emulsions into strawberry jams, the use of cinnamon bark-xanthan gum emulsions was also

evaluated. To this end, the optimisation of the methodology followed to prepare the O/W emulsions (to reduce the losses of EO) and the evaluation of their antifungal activity in culture media and in strawberry jam were studied. Emulsions were prepared in a rotor-stator homogeniser or by magnetic stirring combined with a high pressure homogeniser. Losses of EO, after prepararing the emulsions, referred to *trans-cinnamaldehyde,* which is the main compound of CBEO. The emulsion processed with magnetic stirring and subjected to high pressure displayed a significant reduction in the *trans-cinnamaldehyde* losses, compared with the emulsion prepared in the rotor-stator type device. Indeed, the greater the pressure applied during the homogenisation process, the more degraded the EO compounds became. According to the results obtained while optimising of the methodology employed to prepare emulsions, this was carried out by mixing and dispersing ingredients with a magnetic stirrer for 15 min, followed by HPH at 40 or 80 MPa. Furthermore, the concentrations of the CBEO used were established at 0.06, 0.08, 0.10 and 0.12 mg/g. The *in vitro* antifungal activity of the O/W emulsions showed higher mycelial growth when using 80 MPa than 40 MPa at the same CBEO concentrations. This finding could be related to the *trans-cinnamaldehyde* losses, which could diminish the antifungal effectiveness of O/W emulsions. By considering these results, the *in vivo* study was performed using the emulsions composed of 0.08 and 0.10 mg/g of the CBEO, and homogenised at 40 MPa. The incorporation of the CBEO emulsions into strawberry jam induced their preservation against *A. flavus, P. expansum*, *Z. rouxii* and *Z. bailii*. Nevertheless, its incorporation adversely affected the jam's aroma, taste and overall acceptance.

The **second strategy** was defined by bearing all this in mind. The combination of different antifungal compounds, such as CBEO, ZG and FA, in O/W emulsions was investigated. This part of the Thesis aimed to achieve a formulation with excellent antifungal *in vivo* properties, which in turn, did not alter the sensory profile of strawberry jam as a food matrix. *A. niger* was selected as the target fungi for its relevance in strawberry decay and because of its high resistance against different antifungal agents. Different concentrations of the antifungal compounds were used in the *in vitro* and *in vivo* tests, and also in the physico-

chemical characterisation of O/W emulsions. In this sense, the emulsion prepared with 0.06 mg/g of CBEO, 1 mg/g of ZG and 1 mg/g of FA exhibited great *in vitro* antifungal effects against *A. niger*. In addition, strawberry jam was prepared by incorporating into it three different emulsions that contained: i) 0.06 mg/g of CBEO and 1 mg/g of ZG; ii) 0.06 mg/g of CBEO and 1 mg/g of FA and; iii) 0.06 mg/g of CBEO, 1 mg/g of ZG and 1 mg/g of FA. The strawberry jams prepared with the emulsions that contained CBEO and ZG, or CBEO and FA, did no present antifungal activity, compared to the control samples. Conversely, the strong fungicidal effect observed the in emulsions that contained 0.06 mg/g of CBEO, 1 mg/g of ZG and 1 mg/g of FA could be the result of the interactions among the main and minor EO compounds, ZG and FA. This synergistic effect favoured reduced mould growth with a total number of 10^1 CFU/g after 28 days of inoculation, which is considered microbiologically admissible for jam (limits of microbiological growth: yeasts and moulds 10^2 CFU/g). Additionally, the minor modification of the typical organoleptic properties of strawberry jam after incorporating the emulsion indicated the potential use of this delivery system in food products. This strategy allowed the obtention of a food product with good sensory properties and microbiologically acceptable.

In spite of the satisfying results obtained in the aforementioned strategy, a **third approach** was investigated to accomplish the complete inhibition of the moulds and yeasts capable of growing in strawberry jam. Hence the antifungal activity of the bioactive agents immobilised on the surface of mesoporous silica particles was evaluated. Furthermore, the sensory impact of the silica supports with eugenol and thymol after their incorporation into strawberry jam was studied. The jams prepared with the free and immobilised eugenol exhibited no mould and yeast growth throughout the evaluation period. The samples prepared with free thymol exhibited greater fungi development in comparison with the samples prepared with thymol-MCM-41. The antagonistic interactions with other ingredients, such as carbohydrates, present in the food matrix could be the reason for the different antifungal activity noted between eugenol and thymol after immobilising on the MCM-41 surface. Regarding the sensory analysis, the

immobilisation of eugenol and thymol reduced their typically strong impact on strawberry jam flavour.

The results obtained in the present Doctoral Thesis represent a major advance in the drawbacks presented by different systems with potential antifungal properties. Nevertheless, further studies with other bioactive agents, silica substrates and polymers in a wide variety of food matrices should be conducted. Moreover, toxicity studies to reinforce the safety of silica supports, such as MCM-41 particles with bioactive agents to be employed in food commodities, are required.

Table 1. Summary of the present Thesis: strategies, results achieved and the future perspectives of each strategy.

STRATEGIES	RESULTS ACHIEVED	FUTURE PERSPECTIVES
	CEO, LEO and BEO nanoemulsions (EO:sunflower oil at the 3:1 wt ratio) prepared with T80 or WPI (1 wt%) were stable under accelerating ageing test at 35 °C. Good antifungal properties of CEO nanoemulsions against *A. niger*. Nanoemulsions formulated with WPI exhibited great *in vitro* antifungal effects against *A. niger.*	Incorporation of stable delivery systems into food products.
First strategy	Excellent physico-chemical properties of the clove and cinnamon leaf emulsions formulated with 5 mg/g of XG. Great *in vitro* and *in vivo* antifungal activity of the clove and cinnamon leaf emulsions against *A. flavus*, *A. niger* and *P. expansum.* Sensory analysis highlights differences between the control and strawberry jam samples.	A further inquiry on the final product's sensory profile after incorporating the O/W emulsions is required.
	Optimisation of the methodology employed to prepare O/W emulsions. Good *in vitro* antifungal properties of the CBEO emulsions against *A. flavus, A. niger, P. expansum, Z. rouxii* and *Z. bailii.* Excellent *in vivo* antifungal properties of the CBEO emulsions against *A. flavus, P. expansum, Z. rouxii* and *Z. bailii.* The sensory evaluation revealed differences between the control and strawberry jam samples.	More studies concerning the finals product's sensory impact due to the emulsions incorporation are needed.

Table 1. *(Continued)*

STRATEGY	RESULTS ACHIEVED	FUTURE PERSPECTIVES
Second strategy	The emulsions prepared with 0.06 mg/g of CBEO, 1 mg/g of ZG and 1 mg/g of FA exhibited great *in vitro* antifungal effects against *A. niger*. Slight modification of the typical organoleptic properties of strawberry jam as a result of incorporating emulsions. Microbiologically acceptable final product.	Delivery systems achieved the total inhibition of moulds and yeasts without affecting the food sensory characteristics.
Third strategy	Eugenol and thymol immobilisation on the surface of MCM-41 microparticles exhibited good *in vitro* antifungal properties. Free and immobilised eugenol added to strawberry jam showed no mould growth. Eugenol and thymol immobilisation masked their typical strong impact on strawberry jam.	Studies about bioactive agents, silica particles and biopolymers in different food products. Toxicological studies to confirm the security of silica particles that contain bioactive agents, anchored or immobilised.

5. CONCLUSIONS

Based on the results obtained in the present Doctoral Thesis, and given their interpretation, the following statements are concluded:

O/W emulsions and nanoemulsions

- Formulation of nanoemulsions plays an important role in antifungal activity. Despite the formulation and processing conditions of nanoemulsions being identical, the nanoemulsions based on WPI exhibits better *in vitro* antifungal activity than those based on T80, probably due to the larger numbers of sites of interaction with WPI than with the T80 available on the surface of fungal cells and spores.
- The concentration of each component in the formulation of the clove and cinnamon leaf emulsions determines their physico-chemical properties. The higher the oil content in emulsions, the bigger particle sizes become. Conversely, higher xanthan gum concentrations lead to a smaller droplet mean diameter of emulsions, the reduction of the EOs losses and, increase the consistency index of emulsions. Regarding the ζ-potential analysis, both types of emulsions are stable. Additionally, the clove and cinnamon leaf emulsions prepared with 0.55 and 0.65 mg/g, respectively, inhibits the development of *A. flavus*, *A. niger* and *P. expansum* in culture media and in strawberry jam. After incorporating the emulsions into strawberry jams, changes in the product's aroma, taste and overall acceptance are detected.
- Optimisation of the methodology used to prepare oil-in-water emulsions reduces the essential oil losses. The emulsions prepared by magnetic stirring for 15 min and the high pressure homogenisation process at 40 MPa present the lower essential oil losses, which are related to the heating and stress applied in the homogenisation procedure.
- Incorporation of the cinnamon bark-xanthan gum emulsions, containing 0.08 mg/g of EO and prepared by magnetic stirring and high pressure homogenisation at 40 MPa, into strawberry jams induces their preservation against several fungi strains. This incorporation does not alter product texture or colour, but negatively affects the jam's aroma, taste and overall acceptance.

Conclusions

- The combination of different antifungal agents such as cinnamon bark oil (0.06 mg/g), zinc gluconate (1 mg/g) and *trans*-ferulic acid (1 mg/g) determines the *in vitro* antifungal properties of emulsions, lowering the essential oil concentration used. The *in vivo* test exhibits the reduction of the mould growth with a total number of 10^1 CFU/g after 28 days of inoculation, which is considered microbiologically acceptable for jam. Furthermore, the incorporation of emulsions into strawberry jam does not change its organoleptic profile.

Mesoporous silica particles

- Immobilisation of eugenol and thymol on the surface of microparticulated MCM-41 improves their antifungal activity compared to the free bioactive agents. This could be due to the intense antifungal activity of the MCM-41 microparticles after the immobilisation of eugenol and thymol, whit a high density of the antifungal compound on its surface and/or owing to the volatility reduction of bioactive agents when immobilising on MCM-41 microparticles.
- Immobilisation of eugenol and thymol on the surface of MCM-41 microparticles enhances their impact on jam aroma compared to the free bioactive agents. A comparison of jam samples with the free and immobilised bioactive compounds confirmes that immobilisation reduces the intensity of eugenol and thymol aromas more than 92% and 95%, respectively.