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Combination of different antifungal agents in oil-in-water emulsions to control strawberry jam spoilage

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ABSTRACT

The combination of antifungal agents (cinnamon bark oil, zinc gluconate and *trans*-ferulic acid) in oil-in-water emulsions to control the fungal spoilage of strawberry jams, minimising essential oil's sensory impact, was evaluated in this work. The *in vitro* assays of free antifungal agents were performed against five fungal strains; meanwhile, the emulsions assays were conducted against *Aspergillus niger* given its strong resistance and its relevance in strawberry products. The emulsion formulated with 0.08 mg/g of essential oil was able to inhibit mould growth after the incubation period. The incorporation of zinc gluconate or *trans*-ferulic acid, independently of the concentration used, allowed to reduce a 25% the amount of essential oil needed to inhibit the microbial growth. The combination of antifungal agents in the emulsions has demonstrated to be an effective alternative to reduce the amount of essential oil employed, maintaining the hygienic quality and sensory profile of the 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 & Taylor, 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). 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). Combining EOs with other antifungal agents 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 against moulds and yeasts (Manso et al., 2013). The main constituent of this EO is *trans*-cinnamaldehyde (Ribes, Fuentes, Talens, & Barat, 2017). Indeed, cinnamon is broadly employed as a natural preservative and flavouring substance by the food industry to extend the shelf life of foods. Recently, cinnamon bark emulsions have been used to control mould growth in strawberry jams, being *Aspergillus niger* the most resistant microorganism after 28 days of analysis (Ribes, Fuentes, Talens, Barat, 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). 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). Recently, zinc salts have been used as antifungals in table olives to reduce yeast growth (Bautista-Gallego et al., 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 (Kanski, Aksenova, Stoyanova, & Butterfield, 2002). It also acts as an inhibi-

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tor of fungal enzymes (Daglia, 2012), and many authors have reported its *in vivo* and *in vitro* antifungal activity (Daglia, 2012; Ferrochio, Cendoya, Farnochi, Massad, & Ramirez, 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 could 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* due to its frequent isolation in strawberry product; 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). In emulsion preparation, cinnamon bark essential oil (>60%) (CBEO) (Ernesto Ventós S.A., Barcelona, Spain), xanthan gum (XG) (Cargill, Barcelona, Spain), zinc gluconate (ZG) (Solubility in water at 20 °C, 8 g/100 mL) (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 °C 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 Eq. (1) (Ribes, Fuentes, Talens, Barat, Ferrari, et al., 2017):

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

where *C* and *T* represent diameter of the 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, and all the tests were run in triplicate.

2.3. O/W emulsions

2.3.1. Preparation

The O/W emulsions were prepared mixing the natural agents, Tween 80 and XG during 15 min by using a magnetic stirrer, followed by one single pass at 40 MPa by a high pressure homogenisation (HPH) system (Panda Plus 2000, Gea Niro Soavi S.p.A., Parma, Italy). The concentrations of each antifungal agent tested in 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. 10 mg/g of Tween 80 and 5 mg/g of XG were used in all the emulsions. These concentrations were defined taking into consideration previous works (Ribes et al., 2016; Salvia-Trujillo, Rojas-Graü, Soliva-Fortuny, & Martín-Belloso, 2013). Small molecule surfactants, e.g. Tween 80, are used in food grade emulsions as they can stabilise the emulsion by reducing the O/W interfacial tension. Indeed, the XG is used as stabiliser to enlarge the long-term stability of emulsions by viscosity modification.

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 conducted by GC–MS. These losses are referred to as *trans*-cinnamaldehyde, which is the main CBEO compound (Ribes, Fuentes, Talens, Barat, 2017). 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-min 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 × 0.25 mm × 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).