# 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.

***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). 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, 2017a). 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 et al., 2017a).

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, 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 (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 ºC, 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 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 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 minutes 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 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).

*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 ºC 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 and, 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

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.

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 into the 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 was 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 different O/W emulsions, 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 always of 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 have reported the antifungal effectiveness of CBEO against the *Zygosaccharomyces* 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.

The antifungal action of ZG and FA is shown in Figure 1B and 1C, respectively. 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 of ZG was determined to be 6 mg/g (Figure 1 B). The

MIC of ZG was also 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.

In general, a higher antifungal activity of the phenolic compound, compared with 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. Mohapatra, 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 above 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, when FA was added.

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 the aforementioned 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 a 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 treatment during the preparation procedure, which would cause 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 was tested, against *A. niger* at 25 °C for 7 days (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 in this section with those achieved while evaluating the antifungal properties of free CBEO. 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. In the case of the CBEO emulsion, the reduction of the antifungal activity observed could be attributed to the CBEO losses originated during the emulsion preparation because of the mechanical stress applied to the samples during the homogenisation procedure. Indeed, Liang, Xu, Shoemaker, Li, Zhong, and Huang (2012) and, Shah, Davidson, and Zhong (2013) reported that peppermint EO nanoemulsions prepared with modified starch and, eugenol nanodispersed by whey protein-

maltodextrin conjugates showed lower antimicrobial activity than non-encapsulated agents, respectively. This fact suggests that the use of macromolecules as emulsifiers, with which the EO interacts, could also reduce the antifungal effectiveness of emulsions (Donsì & Ferrari, 2016).

*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.

*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).

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

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

Different factors concerning the formulation of the emulsions have an important role in the final mean droplet size attainable by emulsification, and specially: i) those affecting the break-up phenomena, like the viscosity of the disperse and continuous phases and the interfacial tension, and ii) those regulating the recoalescence phenomena, as the emulsifier affinity for an interaction with newly formed interfaces (Donsì et al., 2011; Donsì, Annunziata, Sessa, & Ferrari, 2012).

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 1). 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 1. 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 as a consequence of the electrostatic repulsion among the droplets. 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). The polymer was used as an emulsion stabiliser as it can absorb into the interfacial layer (Dickinson, 2009). In addition, the stabilisation effect of hydrocolloids was because of the viscosity modification in the continuous phase by lowering the rate of creaming and coalescence (Dickinson, 2009).

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 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. Ribes et al. (2017a) evaluated the antifungal effect of emulsions containing 0.08 and 0.10 mg/g of CBEO and, the results noticed that those emulsions were not sufficient to inhibit *A. niger* growth, resulting the main microorganism originating the strawberry jam spoilage.

On the contrary, a 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 synergism activity between non-encapsulated phenolic and EOs compounds has been elucidated by Miyague, Macedo, Meca, Holley, and Luciano (2015), highlighting the potential application for the food industry due to the strong taste of EOs. Moreover, combination of FA with metal salts has been also studied (Kalinowska et al., 2014). These authors reported a higher antimicrobial activity of ferulates compared with FA. This biological activity was attributed to the fact that combination of both agents increases the compound lipophilicity what makes them easier to penetrate the lipid layers of the cell membrane and interact with components of the bacterial or fungal cell. Further studies are needed to clarify the possible synergistic effect of combining the three compounds in O/W emulsions.

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 interfere 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 (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.

*3.4.2 Sensory evaluation*

The sensory analysis was performed in order to evaluate the sensory acceptance of the strawberry jam that contained different O/W emulsions (Figure 5). Three different samples were tested by assessors: i) control strawberry jam; ii) strawberry jam prepared with O/W emulsions containing 0.08 mg/g of CBEO (MFC value); and iii) strawberry jam that contained the O/W emulsion with 0.06 mg/g CBEO-1 mg/g ZG- 1 mg/g FA, which showed no fungal development in the *in vitro* tests. Incorporation of the O/W emulsion containing CBEO-ZG – FA into strawberry jam did not alter samples' aspect, aroma, taste, unctuousness and overall acceptance compared with the control jam. In this case, 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 with CBEO-ZG –FA into strawberry jam did not modify its sensory acceptance. Remarkably, the strawberry jams that contained the emulsion formulated with 0.08 mg/g of CBEO scored lower for the aroma, taste and overall acceptance attributes compared with the control samples. These outcomes are in agreement with those obtained in a previous work where O/W emulsions formulated exclusively with

0.08 and 0.10 mg/g of CBEO were incorporated to strawberry jam samples and the acceptability of the final product was evaluated (Ribes et al., 2017a).

The results achieved 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, maintaining the hygienic quality of the product.

## 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 (0.06 mg/g-1 mg/g-1 mg/g) increases 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 detailed 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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**Figure captions**

**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 ºC. Mean value (n=3) ± SD. Different letters (a, b, c, d) indicate significant differences among the preservative concentrations ($p<0.05$).

**Figure 2.** Mycelial growth inhibition (%) of cinnamon bark EO (CBEO) mixed with different concentrations of zinc gluconate (1, 2, 4 and 6 mg/g of ZG), against *Aspergillus niger* at 25 ºC 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 ZG concentrations ($p<0.05$).

**Figure 3.** Mycelial growth inhibition (%) of cinnamon bark EO (CBEO) mixed with different concentrations of *trans*-ferulic acid (1, 2.5 and 4 mg/g of FA) 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, B, C) indicate significant differences among the FA concentrations ($p<0.05$).

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

**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).

**Table 1.** Mean values (n=3) ± SD of pH, particle size ($d_{3,2}$ *and* $d_{4,3}$), and the ζ-potential of O/W emulsions (0.06 mg/g of CBEO: cinnamon bark EO; 1 mg/g of ZG: zinc gluconate; 1 mg/g of 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 formulations ($p<0.05$).*

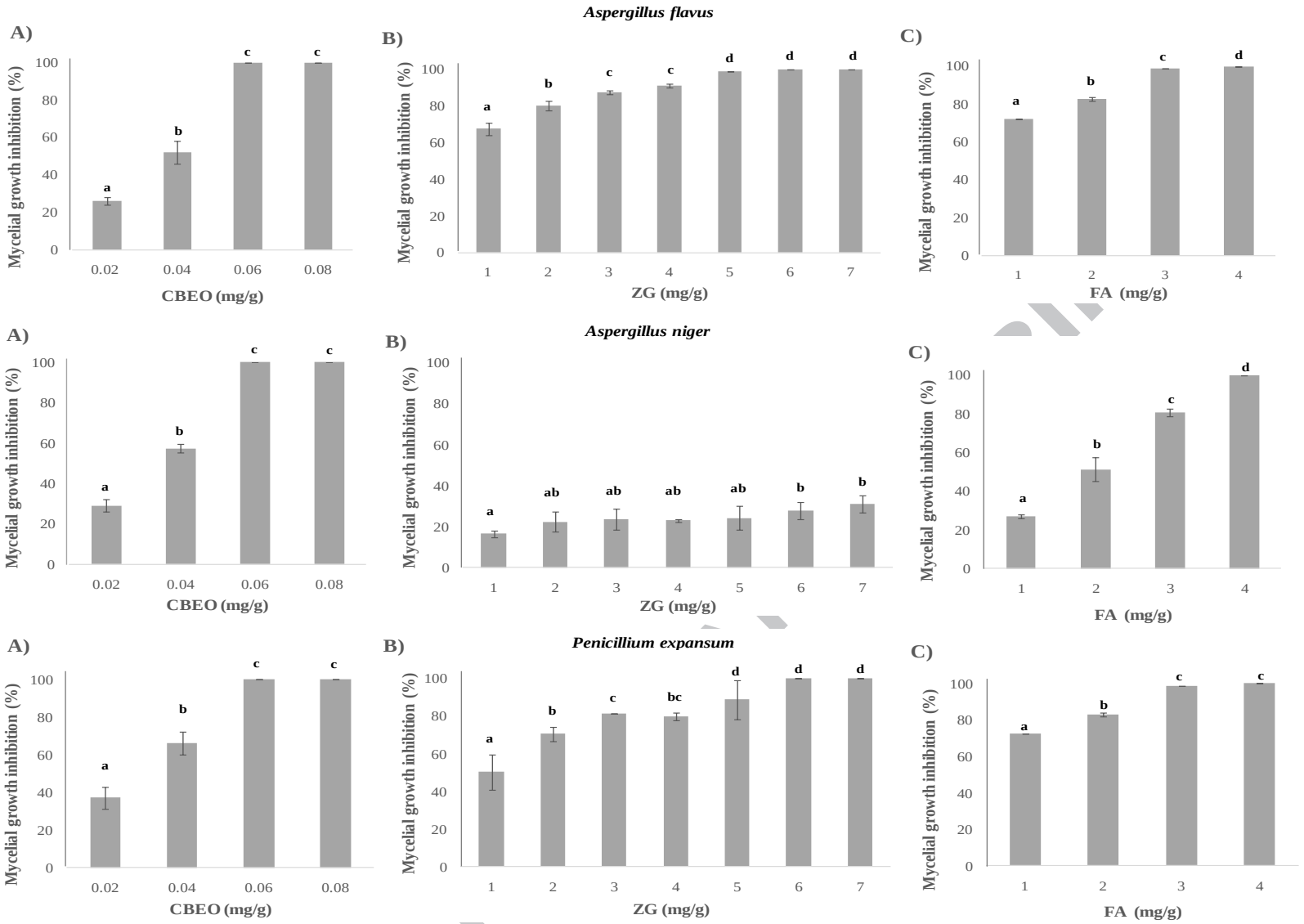


**Figure 1**

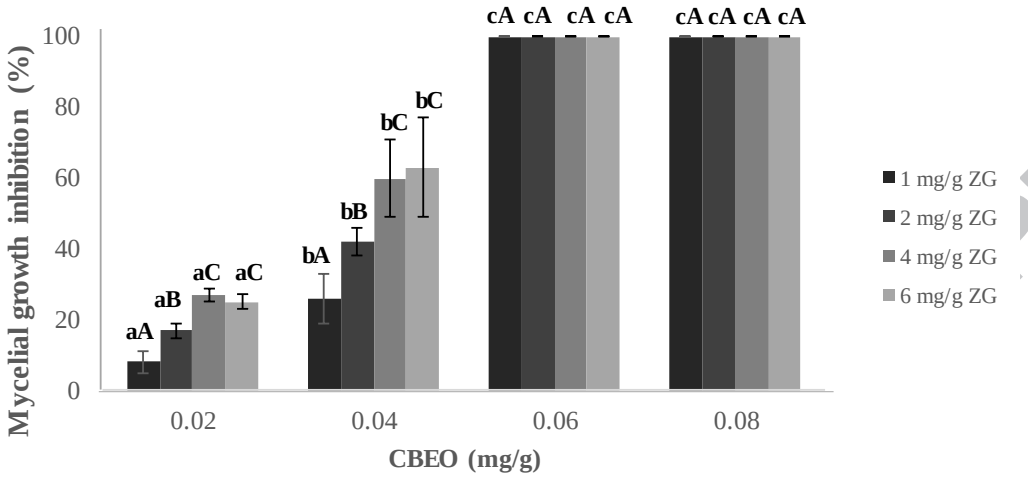


**Figure 2**

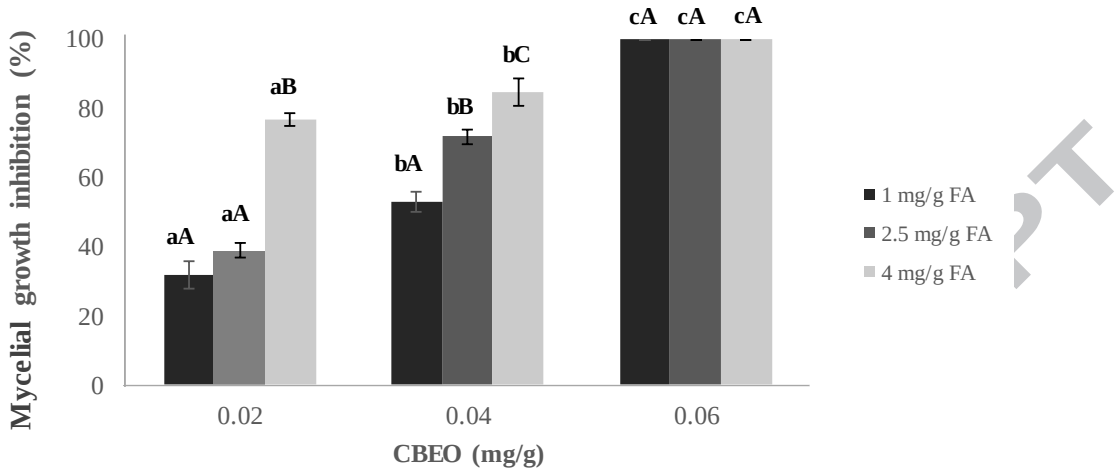


**Figure 3**

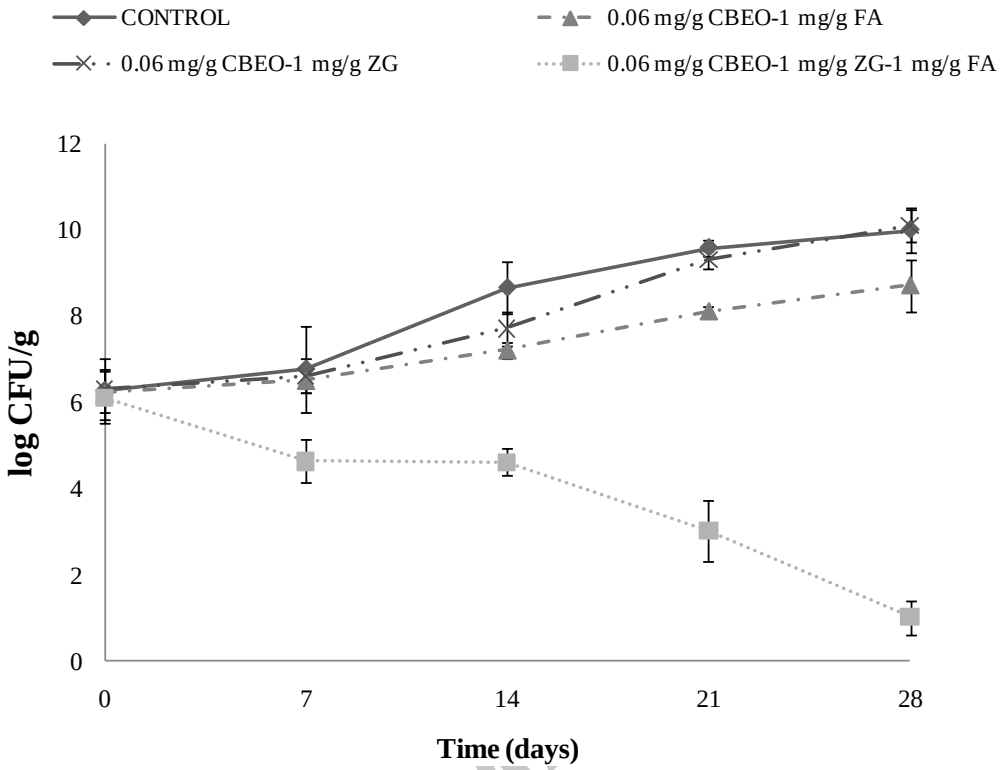


**Figure 4**

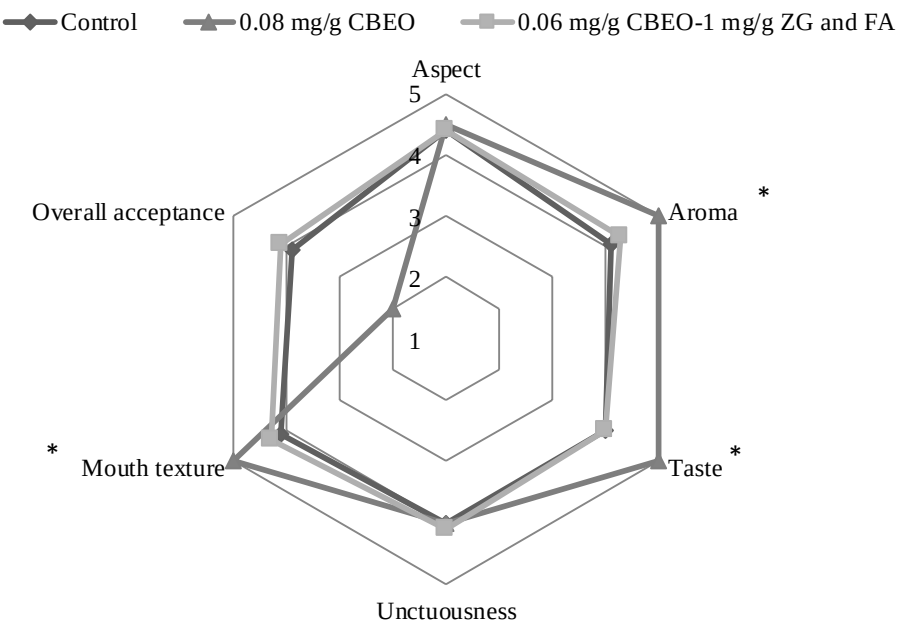


**Figure 5**

Highlights

- Cinnamon bark oil, zinc gluconate and trans-ferulic acid were used as antifungals
- Innovative delivery systems formulated with different antifungal agents were studied
- Emulsions' impact on the sensory profile of strawberry jam was tested
- Hygienic quality of strawberry jam was maintained using novel emulsions
- The profile of the strawberry jam was not affected by emulsions' addition