Highlights

- Mushroom by-products (*A.bisporus)* were used to stabilize O/W emulsions
- Increasing mushroom by-products proportion led to less creaming and flocculation
- Mushroom by-products (5 and 7.5%) led to higher stability to droplet size variation
- Emulsions with 5 and 7.5% of mushroom by-products showed shear-thinning behaviour
- Mushroom by-products affected emulsions pH and colour but not the Z-potential

Stabilization of oil-in-water emulsions with a mushroom (*Agaricus bisporus*) by-product

Mónica UMAÑA[a], Christelle TURCHIULI[b,c], Valeria EIM[a], Carmen ROSSELLÓ[a], Susana SIMAL[a]*

[a] Department of Chemistry, University of the Balearic Islands, Ctra. Valldemossa, km. 7.5, 07122 Palma de Mallorca, Spain.

[b] Université Paris-Saclay, INRAE, AgroParisTech, UMR SayFood, 91300, Massy, France.

[c]Université Paris-Saclay, IUT d'Orsay, 91400 Orsay, France

*Corresponding author: Tel.: +34 971 172 757. E-mail address: susana.simal@uib.es

fax: +34 971 173 426 Permanent address: Ctra Valldemossa km 7.5, 07122. University of the Balearic Islands, Palma de Mallorca, Spain.

Declarations of interest: none

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[1] a*: CIELab coordinate (redness/greenness); ABTS: 2,2'-azino-bis(3-ethylbenzo- thiazoline-6-sulphonic acid; b*: CIELab coordinate (yellowness/blueness; BS: Backscattering; C: Control emulsion (without MC and with Tween®20); CI: Creaming index; CUPRAC: CUPric Reducing Antioxidant Capacity; d_{50} : Median droplet diameter; dm: Dry matter; FRAP: Ferric Reducing Antioxidant Power; GAE: Gallic acid equivalent; HLB: Hydrophilic-Lipophilic Balance; L*: CIELab coordinate (Luminosity); MC: Mushroom concentrate; MC1.5: Emulsion containing 1.5% w/w of MC; MC3: Emulsion containing 3% w/w of MC; MC5: Emulsion containing 5% w/w of MC; MC7.5: Emulsion containing 7.5% w/w of MC; N_h : Number of height positions of the scan; SH: Layer formed at the bottom of the test tube; TE: Trolox equivalent; TH: Total height; t_{max} : Measurement point corresponding to time t; Z_{min}: Lower height limit of the cell; Z_{max} : Upper height limits of the cell; ΔBS: backscattered light referred to the initial state (t=0 h); ΔE: Total colour change; γ: Shear rate.

Abstract

A concentrate of mushroom (*Agaricus bisporus*) by-products (MC), mainly composed of polysaccharides and proteins, was used as an emulsifier (1.5-7.5% w/w) in oil-in-water emulsions. An emulsion stabilized with Tween®20 was used as control. MC (5 and 7.5% w/w) increased the emulsion viscosity and promoted shear-thinning behaviour. Median droplet diameters of 5 and 7.5% w/w MC emulsions (d_{50} ~2.8 and ~2.1 µm) were similar to that of control but larger ($p<0.05$) in 1.5 and 3% w/w MC emulsions (d_{50} ~5.2 and ~3.4 µm) which also presented flocculation. Emulsions with 5 and 7.5% w/w MC were more stable against droplet size variation than the control. The backscattering profiles showed less droplet migration with MC addition (5 and 7.5% w/w). Stability promoted by MC was due to viscosity increase, steric hindrance, and probably Pickering effect and not to electrostatic forces according to the Z-potential. MC addition promoted perceptible colour changes in the emulsions.

Keywords: Mushroom by-products, natural emulsifier, droplet size, backscattering profile, zeta potential

1 Introduction

In the food industry, many products partly or wholly consist of emulsions. Oil-in-water emulsions, systems of oil droplets dispersed in an aqueous phase, are the most common (Shao et al., 2020). However, emulsions can undercome several instability mechanisms such as gravitational separation (creaming and sedimentation), droplets flocculation and coalescence, Ostwald ripening, and phase inversion (Shao et al., 2020). For all of this, emulsification is probably one of the most challenging processes in the food industry, also being an important step for other operations as microencapsulation of lipophilic compounds by spray drying. An emulsion produced for subsequent spray drying should present some characteristics such as being stable until and during the spray drying process, have a suitable oil droplet size distribution (median oil droplet ~2 μm when using a pilot-scale spray dryer), content sufficient amount of wall material (35-60% w/w of the liquid emulsion), and viscosity compatible with the pumping system (Hernández Sanchez et al., 2015; Janiszewska et al., 2015).

In most cases, it is necessary to add emulsifiers to retard destabilization processes. Among the different types of emulsifiers, biopolymers have received great attention in the last decade due to the increasing interest in the use of sustainable and natural food ingredients (Berton-Carabin and Schroën, 2019; Maravić et al., 2019). Several natural macromolecules have been investigated as emulsifiers: β-glucans, plant proteins, and pectins are some examples (Can Karaca et al., 2015; Maravić et al., 2019; Mota da Silva et al., 2021; Santipanichwong and Suphantharika, 2009).

In the industrial production of mushrooms, high amounts of by-products are generated (from 5 to 20% of production volume), mainly consisting of mushrooms with irregular shape or dimensions and mushroom stalks (Wu et al., 2004). Mushrooms are considered an important source of several bioactive compounds such as polyphenols, polysaccharides (i.e. β-glucans), and sterols (i.e. ergosterol) (Papoutsis et al., 2020). Ergosterol is a sterol that can be found in the cell membranes of fungi where it is critical to maintaining cell membrane integrity, fluidity, and permeability (Abe and Hiraki, 2009). Ergosterol is a high-value molecule since it is a precursor of vitamin D_2 and it has also been related to immunoactivity and antioxidant capacity (Papoutsis et al., 2020; Shao et al., 2010). The chemical synthesis of ergosterol is complex and involves many steps, high energy consumption, and low yield (Blaga et al., 2018). Therefore, ergosterol is usually obtained through yeast fermentation (Feldman et al., 2011). However, recently, there has been a growing interest among scientific researchers in extracting ergosterol from mushrooms to take advantage of the by-products generated by this industry (Gil-Ramírez et al., 2013; Heleno et al., 2016a, 2016b; Morales et al., 2017; Patil et al., 2018; Taofiq et al., 2020). Nevertheless, according to our previous study (Umaña et al., 2020), the residues obtained after extracting ergosterol from mushrooms are still rich in other important bioactive compounds with technological properties such as β-glucans and proteins. Natural

heterogeneous materials which are also rich in proteins and polysaccharides have been evaluated to stabilize oil-in-water emulsions, such as eggplant pulp (Zhu et al., 2020), oat bran (Ralla et al., 2018), and apple, orange, and oat by-products (Huc-Mathis et al., 2021). Moreover, Ruan et al. (2019) were able to obtain mayonnaise type emulsions stabilized with citrus fibre. These authors have observed generally favourable results, reporting that these materials presented thickening properties, surface-active amphiphilic molecules, and/or insoluble particles capable of stabilizing the emulsions (Huc-Mathis et al., 2021; Ralla et al., 2018).

In this context, this work aimed to evaluate the usefulness of mushroom residues obtained after the ergosterol extraction in the stabilization of oil-in-water emulsions for their further processing by spray drying.

2 Materials and methods

2.1 Chemical Reagents

Ergosterol (≥95%, HPLC) and cholecalciferol (≥98%, HPLC) were obtained from Sigma Aldrich (Spain). Polyoxyethylene (20) sorbitan monolaurate (Tween®20) was purchased from Sigma-Aldrich (Gillingham, UK). Glucidex® maltodextrin DE 12 (Roquette, France) was used. Hexane (extra pure), petroleum ether (boiling point 40-60°C, reagent grade), methanol (HPLC grade), and ethanol (96% and absolute extra pure) were obtained from Scharlab (Spain).

2.2 Materials

Commercial sunflower oil was used as the lipid phase of the emulsions, it was acquired in a local store (Cora, France). The mushrooms (*Agaricus bisporu*s.) used in this study were purchased from Xampinyons Mallorquins SA (Palma de Mallorca, Spain). The mushroom cap was separated, freeze-dried, ground, and sieved to a particle size <0.5 mm. Mushrooms are a common source of ergosterol which is a molecule with high commercial value (Guan et al., 2016). Thus, ergosterol was extracted from this material as described by Umaña et al. (2020), in ethanol 96% (ratio solid:solvent 3:100 w/v) and applying acoustic energy (320 ± 6 W/L) (ultrasonic generator UP400S, Hielscher Ultrasonics GmbH, Germany) for 20 min. The solid residue obtained from that extraction was dried in a stove under vacuum at 30°C for 48 h, before grounding and sieving (particle size <0.5 mm). This concentrate made of mushroom by-products obtained after ergosterol extraction will be called mushroom concentrate (MC).

2.3 Mushroom concentrate characteristics

The moisture content of MC was obtained by AOAC method no. 934.06 (AOAC, 2000). Protein, lipids, and ash content were determined as described by Umaña et al. (2020). The total carbohydrate content was obtained by subtracting the sum of the protein,

lipid, and ash content from 100. Alcohol insoluble residues were obtained to estimate the fibre content of MC (Eim et al., 2008).

Carbohydrate composition was obtained by submitting MC to Seaman hydrolysis and the free monosaccharides were transformed into their alditol acetates. Thereafter the monosaccharides were isothermally separated by gas-liquid chromatography at 220 °C with a 3% OV225 ChromosorbWHP100/120 mesh column (Hewlett Packard 5890A, Waldbronn, Germany) using Ar as the carrier gas flowing at 20 mL/min. Temperatures of injector and FID detector were 230 °C and 240 °C (Dalmau et al., 2017). Uronic acids were determined spectrophotometrically (520 nm) (UV-2401PC, Shidmazu, Japan) (Coimbra et al., 1996). The content of β-glucans was determined with the Mushroom and Yeast β-glucan Assay Procedure kit K-YBGL 10/2005 (Megazyme, Ireland). This process consisted of transforming total glucans and α-1,4-glucans into free D-glucose through total acidic hydrolysis and specific enzymatic hydrolysis, respectively. The free D-glucose was measured spectrophotometrically (510 nm) and the content of β-glucans was calculated as the difference between total and α-glucans (Umaña et al., 2020).

The ergosterol content was measured on mushroom caps before the extraction described in section 2.1 and on the residues obtained from that extraction (MC). To measure the ergosterol content, it was directly extracted in hexane using the method proposed by Shao et al. (2010). Thereafter, it was analyzed by HPLC using a Nova-Pak 4 µm C18 column (3.9 × 150 mm) (Waters, Massachusetts, USA) at 25 °C. The mobile phase was methanol at a flow rate of 1.0 mL/min. Ergosterol was detected with a photodiode array detector (DAD) 2996 (Waters, Massachusetts, USA) at an absorbance of 280 nm (Umaña et al., 2020).

Total polyphenol content was measured according to the Folin Ciocalteau method (González-Centeno et al., 2014), and the antioxidant activity was determined by following three different methods, 2,2'-azino-bis(3-ethylbenzo- thiazoline-6-sulphonic acid) (ABTS), CUPric Reducing Antioxidant Capacity (CUPRAC) and Ferric Reducing Antioxidant Power (FRAP) (González-Centeno et al., 2014, 2015). Results were expressed as g gallic acid equivalent (GAE)/ 100 g dm (dry matter) and g Trolox equivalent (TE)/ 100 g dm for total polyphenol content and antioxidant activity, respectively. Every determination previously described was carried out at least in triplicate.

The solubility in water of MC was determined as described by Pieczykolan and Kurek (2019). Briefly, 0.5 g of powder was added to 50 mL of water and kept under agitation for 5 min. The solution was centrifuged (4000 rpm 15 min), then 25 mL of the supernatant was dried at 105°C until constant weight. Solubility was expressed as a percentage (%). The Hydrophilic-Lipophilic Balance (HLB) of MC was determined with the Griffin equation (Eq 1) (Matsaridou et al., 2012).

$$HLB = 20(1 - Mass\ of\ hydrophobic\ part/Total\ mass) \quad [1]$$

The hydrophobic mass of MC was estimated from the value of the solubility in water % experimentally obtained (100-solubility in water %).

2.4 Preparation of emulsions

Five oil-in-water emulsions were prepared (300 g). One of the emulsions was prepared as a control, with a conventional composition of an emulsion for spray drying containing maltodextrin as wall material and Tween®20 as an emulsifier (HLB of 16.7) (Lian et al., 2019). The other four emulsions were prepared by substituting part of the maltodextrin with MC in different concentrations (1.5, 3.0, 5.0, and 7.5% w/w) and without Tween®20. The formulations of the emulsions are shown in Table 1. The control emulsion (C) was prepared by following the two steps protocol described by Hernandez Sanchez, Cuvelier, & Turchiuli (2015). A pre-emulsion containing Tween®20 as a surfactant was prepared first by rotor-stator homogenization (Polytron 3100D, Canada) for 5 min at 16000 rpm. This emulsion was then diluted with an aqueous solution of maltodextrin and homogenized (5 min at 16000 rpm).

In the case of emulsions containing MC, it was added to the maltodextrin solution and passed through a rotor-stator homogenization (Polytron 3100D, Canada) for 10 min at 16000 rpm. Lastly, the emulsion was prepared by adding the oil and homogenizing (10 min at 16000 rpm) and no commercial emulsifier was added.

2.5 Emulsions characteristics

2.5.1 Apparent viscosity

Apparent viscosity was measured with viscometer RM100 Touch (Lamy Rheology Instruments, France) with DIN 1.1 measuring system for C emulsion (low viscosity) and DIN 1.2 for emulsions containing MC (more viscous). The temperature was maintained at 22 ± 1°C and the shear rate γ (s^{-1}) varied from 50 to 531 s^{-1}.

2.5.2 Emulsion stability

The droplet size distribution of each emulsion was determined by laser light diffraction (Mastersizer 2000, France) immediately after its preparation, and at several times till 48 h. Meanwhile, the emulsions were stored at ambient temperature in hermetic containers (55x70 mm). Before each sampling, emulsions were manually homogenized by gently rotating the containers. The analyses were carried out in wet mode (Hydro 2000) by dispersing a few droplets of each sample in water. The refractive index values used for the dispersing water and the oil droplets were 1.330 and 1.475 respectively (Hernández Sánchez et al., 2016). The particle size distribution of rehydrated MC after homogenization was also determined.

Micrographs of emulsions were obtained with an optical microscope (Olympus BX60 microscope, Japan) equipped with a digital camera (Moticam 3, Motic, Spain) after

diluting the emulsions in distilled water (3:20 v/v). Image analysis was used to estimate the amount of oil that was flocculated. At least 15 micrographs (~30000 droplets) were obtained for each sample and analyzed with ImageJ 1.52a software (Rasband, 2020). First, the total oil was calculated as follows: the photos were transformed into 8-bit, and the "make binary", "fill holes" and "watershed" functions were applied to construct a boundary for each droplet. The area of each droplet was obtained, and the volume was calculated from this value assuming that the particles were spherical. Each droplet volume was summed to obtain the total oil volume. Secondly, the flocculated oil was measured by submitting the micrographs to the same analysis but previously discarding the droplets which were not into floccules by applying the "minimum filter" (radius 1 pixel) and "find maxima" functions (noise tolerance: 10 and output: "segmented particles"). This analysis was carried out on freshly prepared emulsions and after 48 h of their preparation.

The creaming index was determined with the method described by Edris et al. (2016). About 10 mL of emulsions were poured into a 10 mL test tube and left undisturbed (room temperature, ~22°C). The total height (TH) and the layer formed at the bottom of the test tube (SH) were measured with a Vernier caliper at different times (from 0 to 48 h). Then the creaming index (CI) was calculated by using Eq 2:

$$CI = SH/TH \,.\, 100\% \qquad [2]$$

The closer the CI is to zero, the more stable the emulsion against creaming.

The stability of the emulsions was also evaluated by determining the backscattered light (BS) with a Turbiscan (AGS, Formulaction, France) for 24 h. The obtained longitudinal profiles of backscattered light were referred to the initial state (the scan took at time 0) (ΔBS). Turbiscan Stability Index (TSI) was calculated automatically with the TurbiSoft Lab 2.3.1.125 according to Eq 3:

$$TSI(t) = \frac{1}{N_h} \sum_{t_i=1}^{t_{max}} \sum_{Z_i=Z_{min}}^{Z_{max}} |BS(t_i, Z_i) - BS(t_{i-1}, Z_i)| \qquad [3]$$

where: t_{max} is the measurement point corresponding to the time t at which TSI is calculated, Z_{min} and Z_{max} the lower and upper height limits of the cell respectively, and N_h the number of height positions of the scan. Thus, TSI corresponds to a cumulative sum of all the BS variation of the sample. The higher the TSI, the more unstable the system is (Wiśniewska et al., 2014). Destabilization kinetics graphs were constructed by representing the TSI evolution over 24 h.

The droplets' migration was detected using the ΔBS profile on the bottom of the cell. Then the function "peak thickness" was applied from 0 to 9 mm of the cell at 50% of the peak height. The slope of the linear part of a plot of peak thickness against time provided information about the migration rate (Huck-Iriart et al., 2011).

2.5.3 pH, Z-potential, and conductivity

The pH of the emulsions was measured using a pH meter (Crison, pH 25, Spain). To determine the Z-potential, the emulsions were diluted 1/500 to prevent multiple scattering effects (Talón et al., 2019). The pH of the diluted emulsions was adjusted to their original pH when needed (only in the control emulsion). The Z-Potential was determined at 25°C using a Nano Zetasizer (Malvern, Nano ZS90, UK). This parameter was calculated from the measured electrophoretic mobility of the droplets using the Smoluchowsky model. The conductivity of the emulsions was measured directly without dilution with a conductivity meter (HI 9033, Hanna Instruments, United States) at 24 ± 1°C.

2.5.4 Colour

The colour of the emulsions and MC was evaluated with a CM-5 colourimeter (Konica Minolta, Japan) (Vallespir et al., 2018) and expressed using the CIELab* coordinates: L* (luminosity), a* (redness/ greenness), and b* (yellowness/blueness). The total colour change (ΔE) was calculated to compare each emulsion containing MC with the C emulsion (Eq 4).

$$\Delta E = \sqrt{\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2}} \quad [4]$$

2.6 Statistical analysis

Every emulsion was prepared at least in duplicate and each determination was carried out at least in duplicate as well. Statistical analyses were performed using R 3.1.0 software (R Core Team, 2017a). The existence of significant statistical ($p<0.05$) differences was evaluated by applying the ANOVA test and means were compared with the Tukey's test (de Mendiburu, 2016; R Core Team, 2017b).

3 Results and discussion

3.1 Mushroom concentrate characteristics

The MC composition is shown in Table 2. As can be seen, MC was mainly composed of carbohydrates and proteins. Ashes were the third major compound, and very low moisture and lipid content was observed. MC showed a relatively high fibre content. The lipids content was lower than those reported in previous studies (Manzi et al., 2001; Reis et al., 2012) (~90% lower) as most of the lipophilic compounds were removed during the ergosterol extraction.

The carbohydrate composition indicated that mannose and glucose are the main monosaccharides of MC, this is because the fungal cell wall consists of mannoproteins, β-D-Glucans, and chitin (Sánchez, 2017). Most of the glucose corresponded to glucans polysaccharides (~88%) being β-glucans the most abundant (~59% of total glucans).

Other authors reported similar (6.23 g/100 g dm; Sari et al. 2017) or higher (10.96 g/100 g dm, Khan et al.2017) β-glucans content in *Agaricus bisporus*.

The ergosterol measured on the mushroom caps before extraction was 0.62 ± 0.01 g/100 g dm, meaning that ~93% of the initial ergosterol was removed during extraction. The small presence of ergosterol and the content of polyphenols make MC an interesting material due to the antioxidant activity of these molecules (González-Centeno et al., 2015; Shao et al., 2010). The MC antioxidant activity, confirmed with three assays (ABTS, FRAP, and CUPRAC), is interesting because it could prevent oil oxidation during emulsification, storage, and further processing such as spray drying. MC was mainly insoluble in water, probably due to the presence of insoluble polysaccharides such as chitin and β-glucans (Cheung, 2013). β-glucans can be soluble or insoluble in water depending on their structure, for instance, protein-bound glucans are insoluble in water (Rop et al., 2009). According to Hess et al. (2018), about 71% of *Agaricus bisporus* fibre is water-insoluble. The value of HLB estimated for MC was 9.0. It is generally accepted that surfactants with high HLB (>7) can form oil-in-water emulsions (Khan et al., 2011; Premlal Ranjith and Wijewardene, 2006). This suggests that MC could be used for this type of emulsions.

3.2 Emulsions characteristics

3.2.1 Apparent viscosity

The apparent viscosity against the shear rate is represented in Figure 1. The C emulsion showed a constant viscosity of ~94 mPa·s, independent of the shear rate variations. Thus, this emulsion followed a Newtonian behaviour. On the other hand, different rheological behaviours were observed in the emulsions containing MC. In the case of the lowest MC concentration (MC1.5), the viscosity increased with the shear rate. Thus, this could be described as a shear-thickening behaviour. Similarly, the viscosity of MC3 remained stable on the first measures but from 300 s^{-1}, it increased with the shear rate, thus MC1.5 and MC3 could be classified as dilatant fluids. McClements (2015) stated that shear-thickening might occur because the shear applied promotes collisions among the droplets. As a result, occurs flocculation, and the emulsion viscosity increases. Interestingly, both the MC5 and MC7.5 emulsions showed the opposite behaviour. Thus, the viscosity decreased when increasing the shear rate which is known as a shear-thinning behaviour meaning that MC5 and MC7.5 could be classified as pseudoplastic fluids. According to Koocheki et al. (2009), as the shear rate is increased, polymer chains and non-soluble particles become aligned in the direction of the flow decreasing the viscosity (Leverrier et al., 2016). This effect has been observed by different authors in oil-in-water emulsions when using emulsifier materials rich in fibre such as eggplant materials (Zhu et al., 2020), or with high protein content as pea flour (Sridharan et al., 2020). The addition of MC in the higher proportions (MC5 and MC7.5) also significantly ($p<0.05$) increased the viscosity of the

emulsions. This agrees with several investigations that evaluated different concentrations of vegetable fibre in oil-in-water emulsions (Maravić et al., 2019; Zhu et al., 2020). MC concentrations of 1.5 and 3% w/w were too low to promote a significant ($p>0.05$) increase in the emulsions' viscosity or a shear-thinning behaviour. Shear-thinning behaviour with higher viscosities are desirable characteristics in emulsions because it prevents droplets from creaming but the emulsion still flows easily when pouring it (McClements, 2015). Moreover, the viscosity of MC5 and MC7.5 emulsions was relatively low and similar to the C emulsion at high shear rates (~124 and ~167 mPa·s at 531 s^{-1} for MC5 and MC7.5, respectively). Then, these emulsions would be expected to be pumped efficiently during processing as spray drying (Koocheki et al., 2009).

3.2.2 Emulsion stability

The droplet size distribution of the five emulsions was determined over time to evaluate their stability. In the case of emulsions containing MC, the particle size distribution of the non-soluble fraction of MC was subtracted from the whole volume size distribution of each emulsion at each time. To illustrate this operation, the droplet size distribution of MC before adding the oil is showed in Supplementary 1. The same figure presents the distribution of the emulsion containing both oil and MC non-soluble particles (5% w/w) and the result of the subtraction of MC from the emulsion (MC5). As it can be seen in Supplementary 1, MC showed a mono-modal particle size distribution (d_{50} ~20 µm). A micrograph of MC particles can be seen in Supplementary 2. On the other hand, MC5 showed a shoulder on the left (~3 µm) corresponding to the oil droplets. After subtracting MC, the oil droplet size distribution could be obtained.

The initial droplet size distribution of the five emulsions is shown in Figure 2. The d_{10} percentile, median diameter (d_{50}), d_{90} percentile, and the span of emulsions immediately after their preparation and after 48 h, are presented in Figure 3. The C, MC3, MC5, and MC7.5 emulsions showed a mono-modal initial oil droplet size distribution, while MC1.5 showed a shoulder on the right ~10 µm (Figure 2). The C and MC7.5 distributions were shifted to the left compared to the rest of the distributions (Figure 2). According to d_{50}, MC1.5 contained the largest droplets (d_{50} 5.2 µm) followed by MC3 (d_{50} 3.4 µm) (Figure 3). Increasing the MC concentration resulted in smaller droplets (d_{50} was 2.8 and 2.1 µm for MC5 and MC7.5, respectively), similar to C emulsion (d_{50} 2.2 µm). Ralla et al. (2018) also observed a droplet size decrease in oil-in-water emulsions when increasing oat concentrations from 0.1 to 5.0% w/w. Apparently, in the case of MC, 1.5 and 3% w/w is insufficient to properly cover the oil droplets during homogenization.

Figure 2 shows the emulsions droplet size variation over time of C, MC1.5, MC3, MC5, and MC7.5. In the case of C emulsion, after 5 h, larger oil droplets (~30 µm) were observed. This trend of coalescence continued even more over time. This can also be

observed in the variation of d_{10}, $d_{50,}$ and d_{90} (Figure 3), since these three percentiles along with span, significantly ($p<0.05$) increased over time; after 48 h d_{50} was ~12 µm.

Emulsion MC1.5 also showed some variation of oil droplet size over time. Thus, after 48 h the droplet size distribution became bimodal. This could be due to a heterogeneous mechanism of coalescence or flocculation, meaning that a fraction of the droplets became affected by this process and the rest remained stable (McClements, 2015). Furthermore, a significant ($p<0.05$) increase was observed on d_{90} after 48 h (Figure 3). In the case of MC3 emulsion, relatively good stability was observed, however, after 48 h, the curve was slightly shifted to the right (Figure 2). This resulted in a significant ($p<0.05$) increase of d_{10} (Figure 3), indicating the disappearance of the smallest droplets. Regarding the emulsions containing the highest percentages of MC (MC5 and MC7.5), they both showed high stability against droplet size variation over time. Thus, the curves corresponding to the droplet size distribution at different times were coincident among them (Figures 2). Moreover, no significant ($p>0.05$) differences were observed in the percentiles and span of the distribution after 48 h (Figure 3) for both MC5 and MC7.5. Hence, these MC proportions prevented the coalescence and flocculation of the oil droplets.

Micrographs of the emulsions can be seen in Figure 4. Initially, C emulsion presented small and uniform droplets with very few floccules (~3% oil volume). Emulsion MC1.5 and MC3, on the other hand, were already flocculated. The image analysis results indicated that ~23% volume of the oil was flocculated in emulsion MC1.5 and ~16% in emulsion MC3 from the very beginning. This agrees with the observation previously described concerning the viscosity variation with the shear rates for these emulsions (section 3.2.1). The floccules of MC1.5 might correspond to the peak observed on the right of the droplet size distribution of this emulsion (Figure 2). Emulsions stabilized with a limited concentration of biopolymers can present flocculation by bridging when a single polymer molecule adsorbs onto two or more droplets creating an almost permanent link (Berton-Carabin and Schroën, 2015; Pons, 2000). As can be seen in Figure 4, MC5 and MC7.5 presented small droplets with fewer floccules than MC1.5 and MC3. According to the image analysis, there was ~9% volume oil flocculated in MC5 and this value was ~6% for MC7.5. Similarly, Castel et al. (2017) observed bridging flocculation in oil-in-water emulsions stabilized with Brea gum (5% w/w), and this phenomenon was not observed when increasing the Brea gum concentration (10-20% w/w). Figure 4 also shows micrographs of the emulsions after 48 h. The C emulsion presented larger droplets after 48 h and a very slight increase in the flocculation (up to 4%). MC1.5 and MC3 showed slight increases in the droplet size and, according to image analysis, MC1.5 presented ~26% of the oil volume in floccules and this value was ~19% for MC3, this means increases of ~13% and ~19% of the flocculated oil after 48 h for MC1.5 and MC3, respectively. As can be seen in Figure 4, MC5 and MC7.5

remained stable with small droplets even after 48 h. Moreover, the flocculated oil did not increase in these emulsions.

The creaming index variation over time is shown in Figure 5. The C emulsion exhibited high stability according to this parameter since only small creaming was observed after 24 h (~2%) and 48 h (~4%). These results differ from the findings previously described of the droplet size variation over time. Increases in droplet size usually lead to higher instability against creaming (McClements, 2007). However, it should be considered that the visual detection of creaming is limited when boundaries are diffuse.

The MC1.5 emulsion showed the lowest stability against creaming. Thus, after only 5 h, CI was ~57% and continued increasing until reaching a maximum of ~60% after 24 h. Increasing MC proportion resulted in better stability against creaming. Thus, the MC7.5 emulsion showed the highest stability among the four emulsions containing MC (no creaming observed even after 24 h), followed by MC5 and MC3.

The stability of the emulsions was also evaluated by backscattering light using a Turbiscan instrument (Figure 6). A decline in ΔBS of C emulsion was observed in the bottom zone (from ~2 to 10 mm of the cell height). This suggests a decrease in the concentration of oil particles in the bottom. On the other hand, a pronounced increase of ΔBS was observed in the top zone (from ~30 to 40 mm) representing the growth of oil droplets concentration. Therefore, the migration of oil droplets from the bottom to the top was detected, which indicates that creaming occurred in the C emulsion even when it was not detected through the creaming index. On the other hand, in the middle zone (10-30 mm) a decrease in ΔBS was observed, probably due to particle size variation. MC1.5 emulsion also exhibited a ΔBS decrease in the bottom zone and an increase in the top zone. A similar profile was observed in MC3 emulsion but the ΔBS increase in the top zone was less pronounced for this sample. The droplet migration rate calculated from the ΔBS profiles of these emulsions was 0.39, 0.40, and 0.41 mm/h for C, MC1.5, and MC3, respectively. Huck-Iriart et al. (2011), prepared sunflower oil-in-water emulsions stabilized with sodium caseinate and observed that creaming took place as a result of either floccules migration or small droplet migration. Thus, in the case of MC1.5 and MC3 emulsions, creaming could occur due to the floccules migration, while for C emulsion, this phenomenon resulted from small droplets migration. On the other hand, MC5 and MC7.5 also exhibited ΔBS decreases in the bottom zone. However, unlike the rest of the emulsions, MC5 and MC7.5 did not show a peak of ΔBS increase in the top zone. This indicates that some migration of the droplets begun at the bottom of the cell, but they did not arrive at the top. This agrees with the creaming index results since MC5 and MC7.5 emulsions showed significantly ($p<0.05$) lower creaming percentages than MC1.5 and MC3.

The Turbiscan Stability Index (TSI) of the emulsions is illustrated in Figure 7. On the first hours of the measurement, the TSI practically did not vary among the samples. After 6

h, the TSI average value was 1.7 ± 0.1 for all the emulsions. However, as expected, the TSI increased faster for C, MC1.5, and MC3 emulsions comparing with MC5 and MC7.5. Thus, after 20 h, TSI of C emulsion was 3.9 and 4.7, and 4.8 for MC1.5 and MC3 emulsions. On the other hand, the MC5 and MC7.5 emulsions showed the highest stability according to this parameter with a TSI value of ~2.9 and 2.3 after 20 h, respectively.

Overall, 5 and 7.5% w/w of MC addition improved the stability of the emulsions against droplet size variation and creaming. Lower concentrations of MC (1.5 and 3% w/w) resulted in larger droplets, with floccules and faster creaming. Santipanichwong and Suphantharika (2009) studied the influence of β-glucans from different sources (curdlan, coming from barley, oat, and yeast) in oil-in-water emulsions and observed that this polysaccharide improved the creaming stability of the emulsions possibly due to the viscosity increase. This observation coincides with the results obtained in our study since MC promoted a significant ($p<0.05$) increase of the emulsion viscosity when it represented 5 and 7.5% w/w of the emulsion, these emulsions exhibiting high stability. Higher viscosity hinders droplets mobility according to Stoke's law. Other authors who have stabilized emulsions with heterogeneous natural materials containing polysaccharides have also reported similar results. For instance, Zhu et al. (2020) and Maravić et al., (2019), also explain the better stability of emulsions containing higher concentrations of eggplant pulp and sugar beet fibre respectively, by an increase in the viscosity promoted by polysaccharides. The formation of a network of polysaccharides in which droplets are trapped has also been stated by these authors (Maravić et al., 2019; Santipanichwong and Suphantharika, 2009; Zhu et al., 2020). Thus, the soluble part of MC (~45%, Table 2) containing soluble β-glucans and other polysaccharides was able to increase the water viscosity, as it was reported in the apparent viscosity results for emulsions MC5 and MC7.5, and probably form a network of fibre. On the other hand, the non-soluble material of MC was in form of solid particles (a micrograph of these particles is shown in Supplementary 2) which may have also an important effect on the stability of the emulsions. Huc-Mathis et al. (2021) stabilized oil-in-water emulsions with apple, beet sugar and oat by-products, mainly composed of insoluble material (~90-94%) and reported that the main stabilizing mechanism was a Pickering one. Pickering emulsions are emulsions physically stabilized by solid colloidal particles, that are wetted by oil and water and act as emulsifiers, being remarkable stable against droplet size variations (Berton-Carabin and Schroën, 2015).

3.2.3 pH, Z-potential, and conductivity

The emulsions' pHs are shown in Table 3. As it can be seen, emulsions containing MC showed significantly ($p<0.05$) higher pH than emulsion C. No significant ($p>0.05$) differences in the pH among the emulsions containing MC were observed. Previous studies have reported similar pH of different types of emulsions containing mushroom

materials. For instance, Kurt and Gençcelep (2018) prepared model meat emulsions with a mushroom powder (*Agaricus bisporus*) (0.5-3% w/w) and reported a pH of 6.29 ± 0.04. Choe et al. (2018), evaluated the use of winter mushroom powder (*Flammulina velutipes*) (0-2% w/w) as an alternative to phosphates in emulsion-type sausages, and observed significant ($p<0.05$) higher pH with mushroom powder (6.08 and 6.33 without and with 2% w/w of mushroom powder respectively). The increases in the pH of the emulsions when adding MC (comparing with the C emulsion) might be caused by the presence of basic amino acids in mushrooms such as lysine, histidine, and arginine (Choe et al., 2018; Ito et al., 2017). Some authors have stated that mushroom proteins perform a buffering effect (Ko and Kim, 2007). This buffering effect of the mushroom proteins is evidenced by the fact that the pH of the emulsions was maintained when adding different concentrations of MC.

The Z-potential is also shown in Table 3. There were no significant differences ($p>0.05$) among the Z-potential absolute values of the five emulsions. They all showed negative values which indicate that the surfaces of the droplets had negative electric charges which originated from adsorbed excess OH^- ions, and, in the case of emulsions containing MC, the dissociated side polar groups of proteins molecules might have been adsorbed as well (Wiącek and Chibowski, 2005). According to several authors, emulsions with a Z-potential absolute value higher than 30 mV are considered highly stable systems through electrostatic repulsions (Bhattacharjee, 2016; Fioramonti et al., 2019; Masum et al., 2019). However, it is conventionally accepted that the larger the absolute value of Z-potential, the greater the electrostatic repulsion between the droplets, and thus, better stability is expected (Dickinson, 2009). This does not agree with the results observed in our study. Similarly, Nakauma et al. (2008) evaluated the emulsifying properties of sugar beet pectin, soybean polysaccharide, and gum Arabic and observed significantly ($p<0.05$) higher Z-potential absolute magnitude in emulsions with sugar beet pectin than in the rest of the emulsions. Nevertheless, according to the evaluation of the increase of the droplet size upon storage (3 days at 60°C), emulsion with sugar beet pectin showed the poorest stability. The authors explained that the Z-potential itself is not an unequivocal diagnostic parameter when, besides electrostatic repulsions, other factors such as steric repulsions affect the stability of the emulsions.

MC has an important content of proteins (~19%, section 3.1), and stabilization of oil droplets by adding proteins has been mainly attributed to electrostatic repulsion (Ozturk et al., 2015). However, the proteins' capacity to stabilize emulsions through electrostatic repulsion, depends on several factors including the pH, which should be sufficiently above or below the proteins' isoelectric point (McClements and Gumus, 2016; Mota da Silva et al., 2021). The isoelectric point for *Agaricus bisporus* proteins such as tyrosinase is ~4.9 (Ismaya et al., 2017), while other proteins as lectins have been reported to have an isoelectric point from 5.5 to 6.7 (Ismaya et al., 2020). In our study, the natural pH caused by MC (pH ~6.9) was maintained, and probably it was not

different enough from mushrooms proteins isoelectric point. The presence and concentration of salt ions in the continuous phase might also affect the Z-potential (Zhang et al., 2019). When increasing the MC concentration, the soluble part of this material such as soluble minerals was also increased (ashes were ~10% dm of MC, section 3.1). Considering that mushrooms (*Agaricus bisporus*), are relatively rich in minerals as potassium, magnesium, calcium, and sodium (Vetter, 2003), they might have affected the Z-potential. To confirm this, the conductivity of the emulsions was determined (Table 3). As expected, the C emulsion showed the lowest conductivity and this parameter progressively increased when increasing MC concentration, suggesting a higher presence of ions. Ionic impurities have already been reported to change oil droplets' electrical charge (Wen et al., 2014).

Overall, the high stability observed in MC5 and MC7.5 can be attributed to steric repulsion and Pickering effect rather than to electrostatic repulsions under the conditions evaluated in this study. Zhu et al. (2020) prepared emulsions containing eggplant pulp which was mainly composed of polysaccharides (~40% dm), soluble sugar (~35% dm), and proteins (~12% dm), and concluded that adsorption of massive polysaccharides associated with proteins occurred on the droplet surface, leading to a steric effect. They also considered that the adsorption of eggplant components on the droplet surface might have increased the effective density of oil droplets, decreasing the density difference between the droplets and the continuous phase. Even when MC proteins do not seem to promote electrostatic repulsions in this study, their presence might still be important, since polysaccharides themselves are not considered to be surface-active (Santipanichwong and Suphantharika, 2009). According to Setiowati et al. (2020), protein and polysaccharides work synergistically because proteins have an amphiphilic character that allows them to be adsorbed onto the oil-water interface, whilst hydrophilic groups of the polysaccharides are oriented to the aqueous phase. This promotes a steric hindrance effect which hinders coalescence and flocculation.

3.2.4 Colour

Supplementary 3 shows a photograph of each emulsion and its colour CIELab coordinates. MC CIELab coordinates are presented in Table 2, this material had a light brown tone probably as a result of some Maillard browning reaction during the ergosterol extraction. C emulsion presented a whitish colour while the emulsions containing MC presented different tones of brown. Increasing the MC concentration resulted in significant ($p<0.05$) decreases of L^* and significant ($p<0.05$) increases of a^* and b^*. Moreover, according to the ΔE calculated between each emulsion and the control, these colour differences were perceptible by the human eye ($\Delta E>2.3$) (Gaurav, 2003).

4 Conclusions

This work attempted to evaluate the addition of a natural material coming from mushroom (*Agaricus bisporus*) in oil-in-water emulsions suitable for subsequent spray drying. Interesting results are reported that indicate that, with an appropriate concentration, this material is a potential alternative for synthetic emulsifiers. Thus, the mushroom concentrate increased the viscosity of the emulsion and promoted a shear-thinning behaviour when it represented 5 and 7.5% w/w of the emulsion. With these concentrations, mushroom concentrate also promoted high stability of the emulsion in terms of droplet size variation over time, since no change was observed in this parameter even after 48 h; especially compared with the control (stabilized with a commercial emulsifier) which presented larger droplets after only 5 h. These emulsions (5 and 7.5% w/w of mushroom concentrate) also showed better stability against creaming according to the backscattering profiles evolution and the creaming index. The Z-potential results indicate that electrostatic repulsion is not an important force in the stabilizing capacity of the mushroom concentrate, meaning that stability promoted by this material was mainly due to a viscosity increase, a steric hindrance of the droplets mobility, and probably a Pickering effect. Furthermore, this mushroom material might also improve the nutritional quality of the final emulsions since it is a source of bioactive polysaccharides (β-glucans) and proteins. It has been observed that the addition of MC affected the colour of the emulsions, thus, further studies should focus on determining how this material affects other sensorial properties of the emulsions.

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Figure 1 revised

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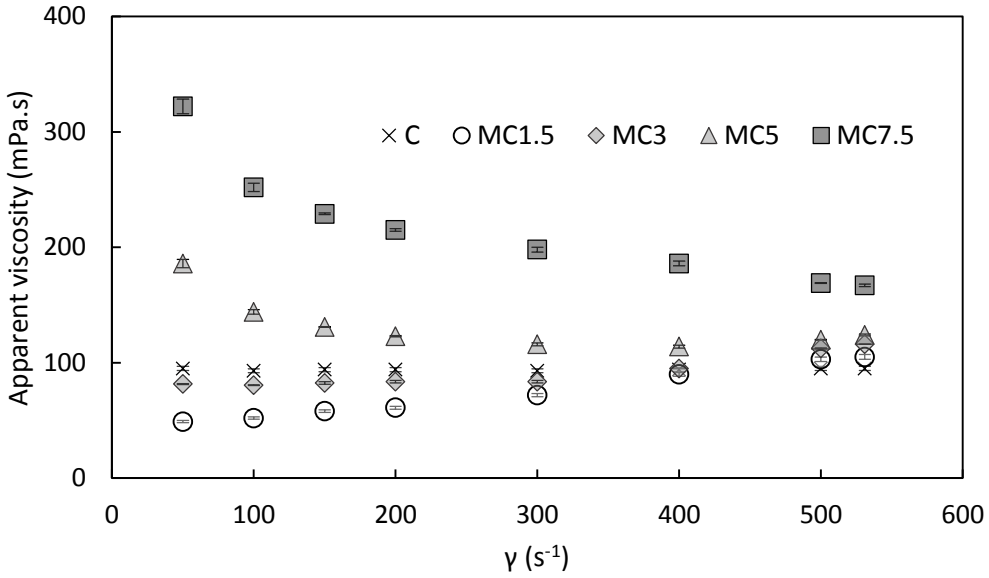


Figure 2

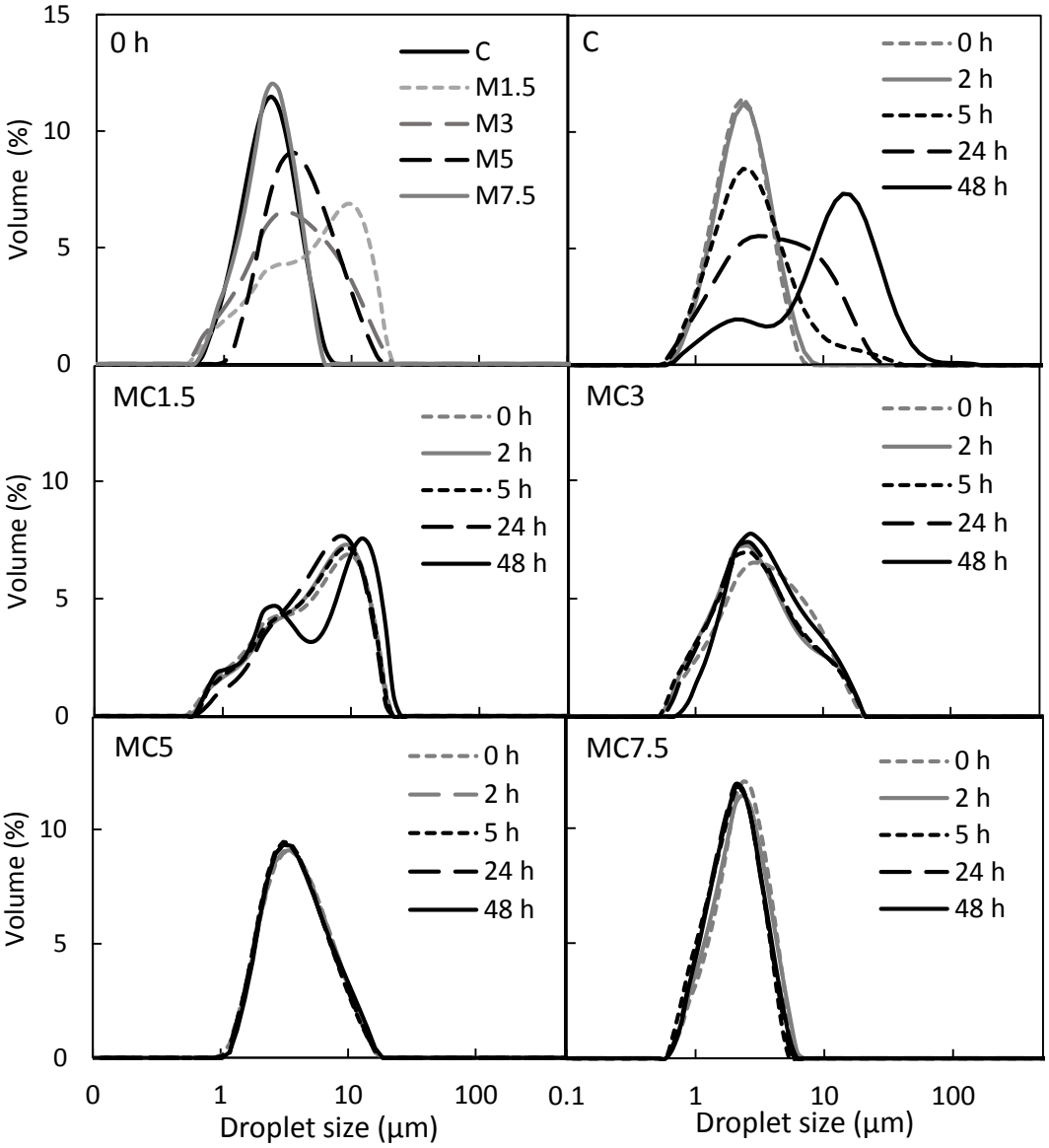
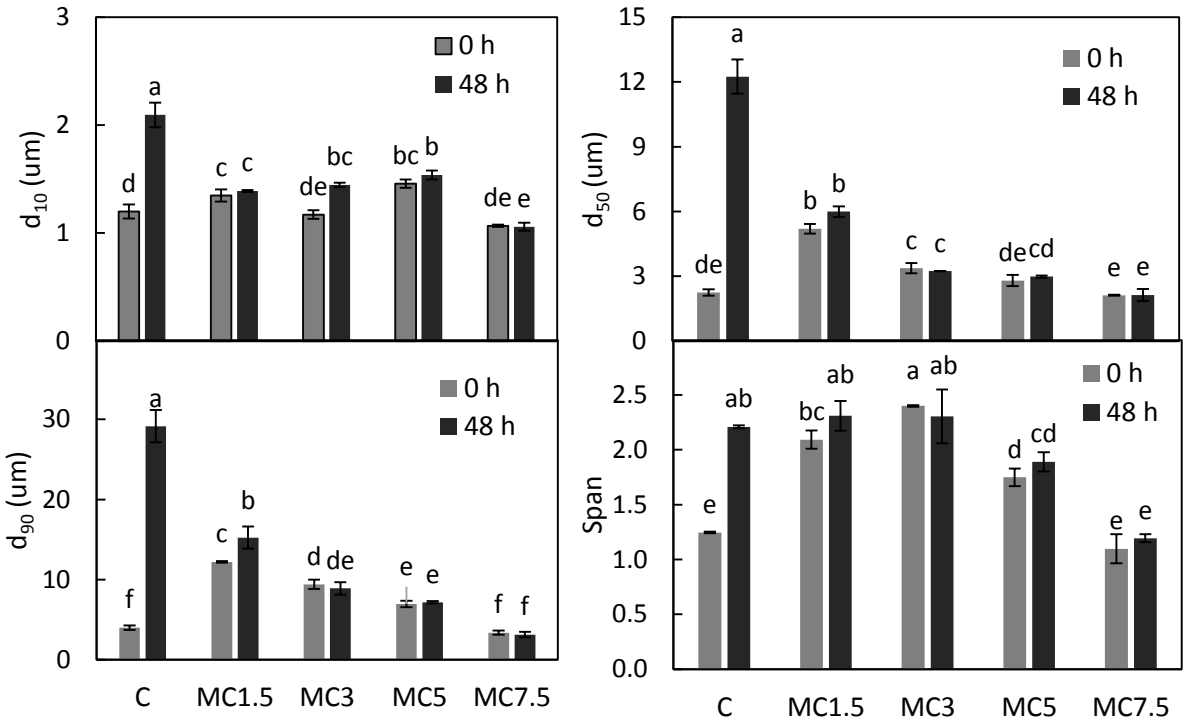


Figure 3



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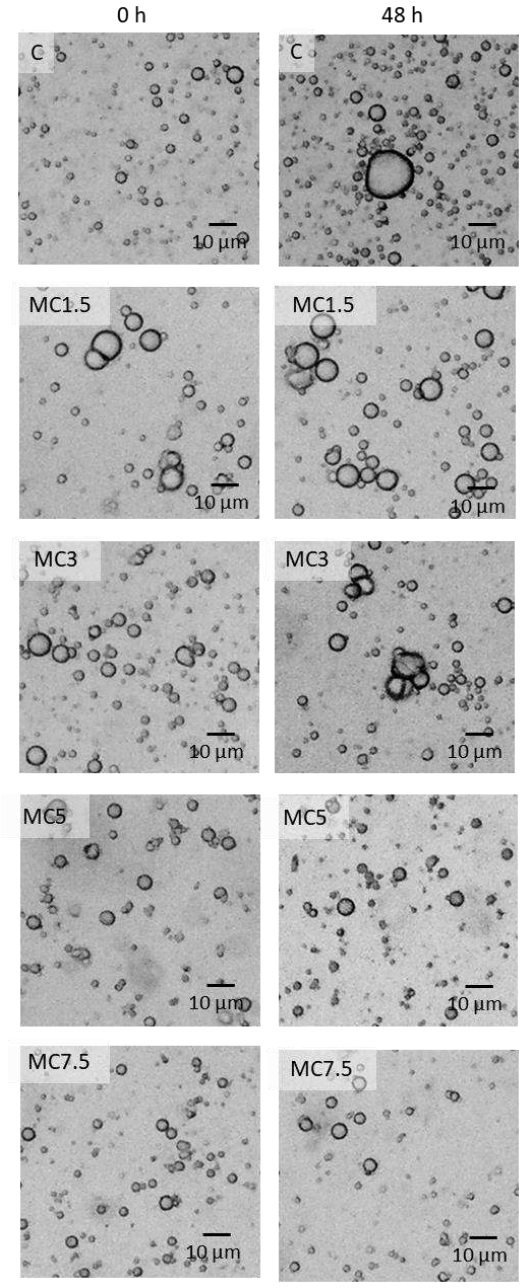

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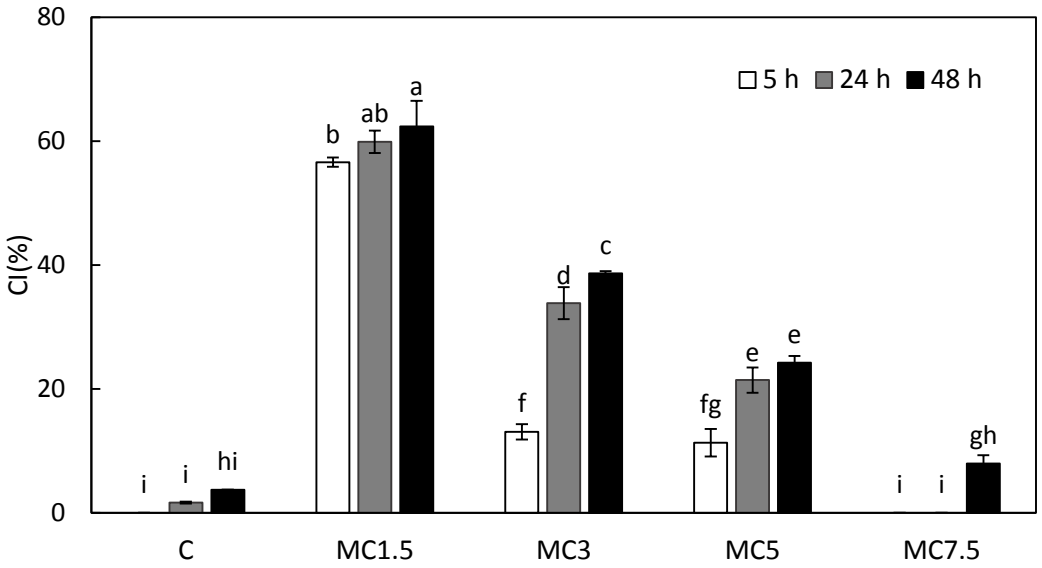

Figure 6

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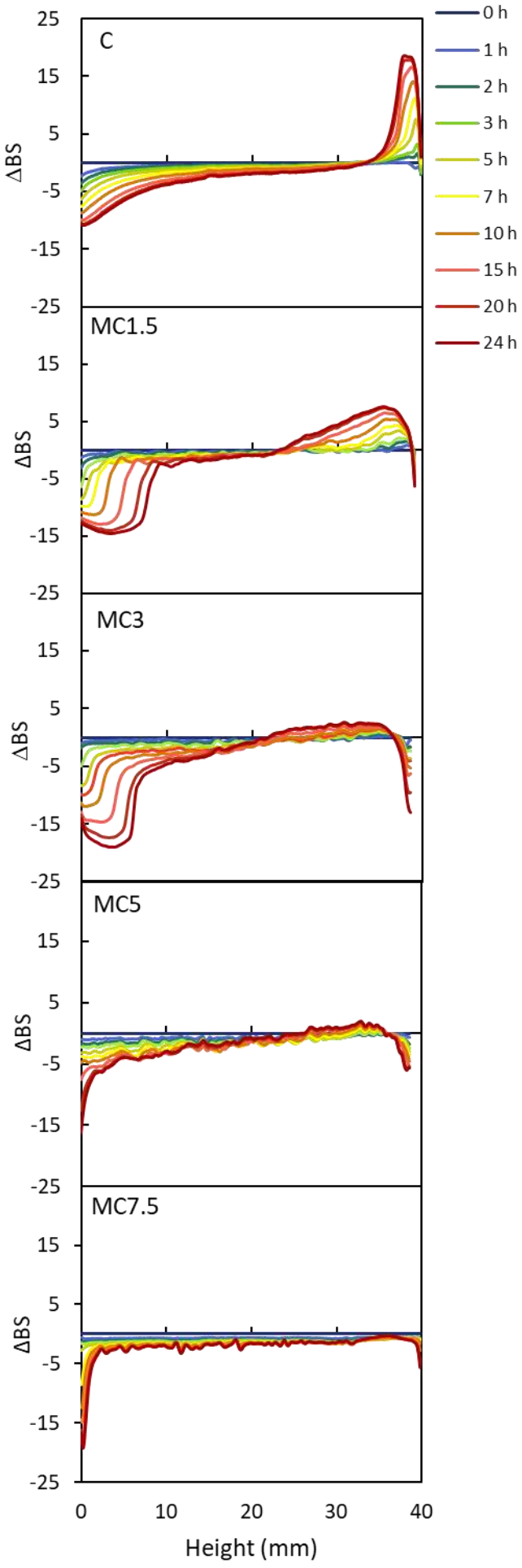

*colour online only

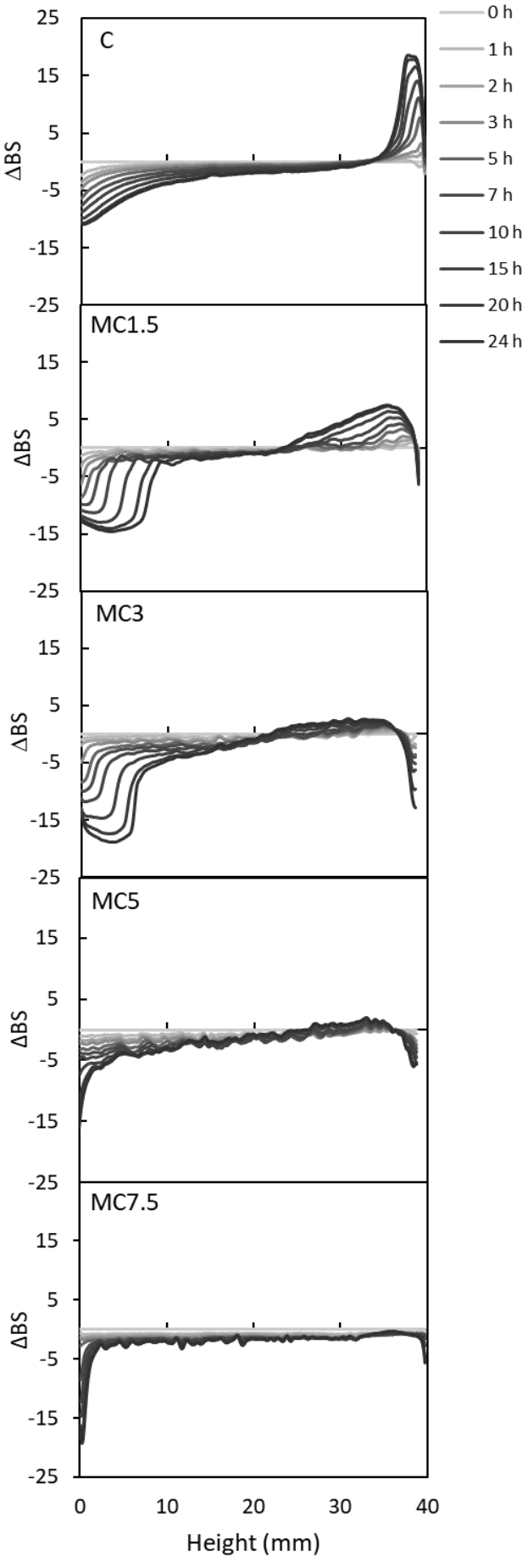

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Figure 7

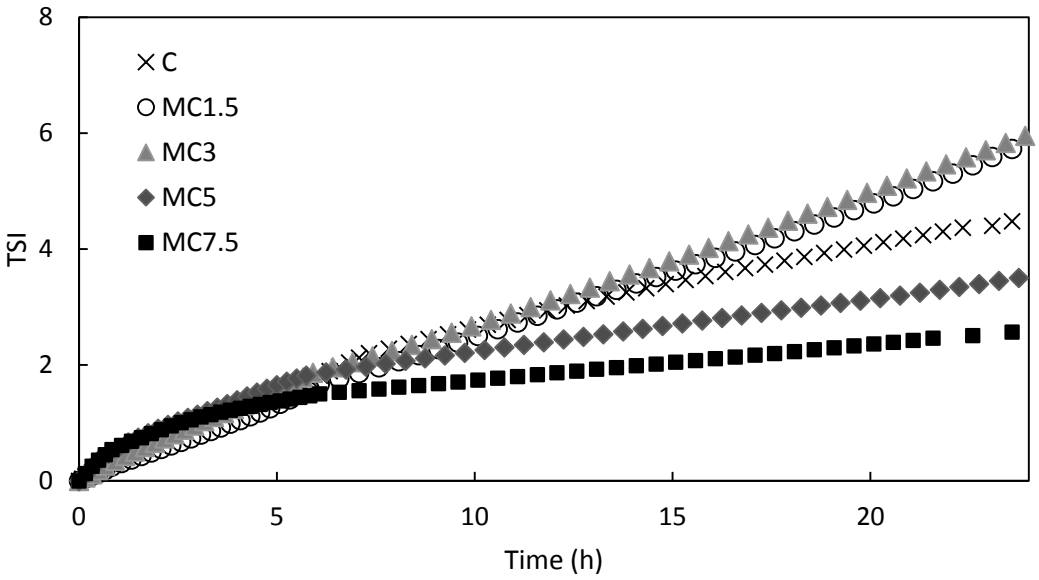


Figure captions

Figure 1. Evolution of the apparent viscosity with the shear rate at 25°C for C, MC1.5, MC3, MC5, and MC7.5 emulsions.

Figure 2. Oil droplet size distribution in emulsions C, MC1.5, MC3, MC5 and MC7.5 at time 0 h and oil droplet size distributions over the time in C, MC1.5, MC3, MC5 and MC7.5 emulsions.

Figure 3. Percentiles d_{10}, $d_{50,}$ and d_{90} and span of the oil droplet size distribution in C, MC1.5, MC3, MC5, and MC7.5 emulsions at time 0 h and 48 h. Different letters on the top of the bars of the same percentile indicate significant differences ($p<0.05$) among the samples.

Figure 4. Micrographs of C, MC1.5, MC3, MC5, and MC7.5 emulsions at time 0 h and 48 h.

Figure 5. Evolution of the creaming index over time of C, MC1.5, MC3, MC5, and MC7.5 emulsions. Different letters on the top of the bars indicate significant differences ($p<0.05$) among the samples.

Figure 6. Evolution of the backscattering profiles (ΔBS) of C, MC1.5. MC3, MC5, and MC7.5 emulsions for 24 h.

Figure 7. Evolution of the Turbiscan Stability Index (TSI) of C, MC1.5, MC3, MC5, and MC7.5 emulsions.

Table 1

Composition of the emulsions prepared with or without mushroom concentrate (MC)

Component (g/100 g emulsion)	C	MC1.5	MC3	MC5	MC7.5
Water	60.00	60.00	60.00	60.00	60.00
Maltodextrin	35.86	34.50	33.00	31.00	28.50
Oil	4.00	4.00	4.00	4.00	4.00
MC	-	1.50	3.00	5.00	7.50
Tween®20	0.14	-	-	-	-

Table 2

Composition and characteristics of the mushroom concentrate (MC)

Compound			
Moisture (g/100 g dm)	2.71	±	0.03
Lipid (g/100 g dm)	0.09	±	0.04
Proteins (g/100 g dm)	19.13	±	0.30
Ashes (g/100 g dm)	9.59	±	0.02
Fibre (g/100 g dm)	42.05	±	0.35
Total carbohydrate (g/100 g dm)	71.16	±	0.38
Carbohydrate composition (g/100 g dm)			
Fucose	0.13	±	0.01
Xylose	0.52	±	0.06
Mannose	16.98	±	0.57
Galactose	1.34	±	0.05
Glucose	11.60	±	0.60
Uronic acids	3.15	±	0.17
α-glucans	4.20	±	0.08
β-glucans	6.01	±	0.23
Ergosterol (mg/g dm)	0.40	±	0.10
Total polyphenols content (mg GAE/g dm)	2.83	±	0.24
Antioxidant activity (mg TE/g dm)			
ABTS	8.14	±	0.87
CUPRAC	5.64	±	0.74
FRAP	3.59	±	0.22
Solubility (%)	44.93	±	0.38
CIELab* colour coordinates			
L^*	76.73	±	0.01
a^*	2.65	±	0.01
b^*	25.01	±	0.01

An average of at least three replicates is reported.

Table 3

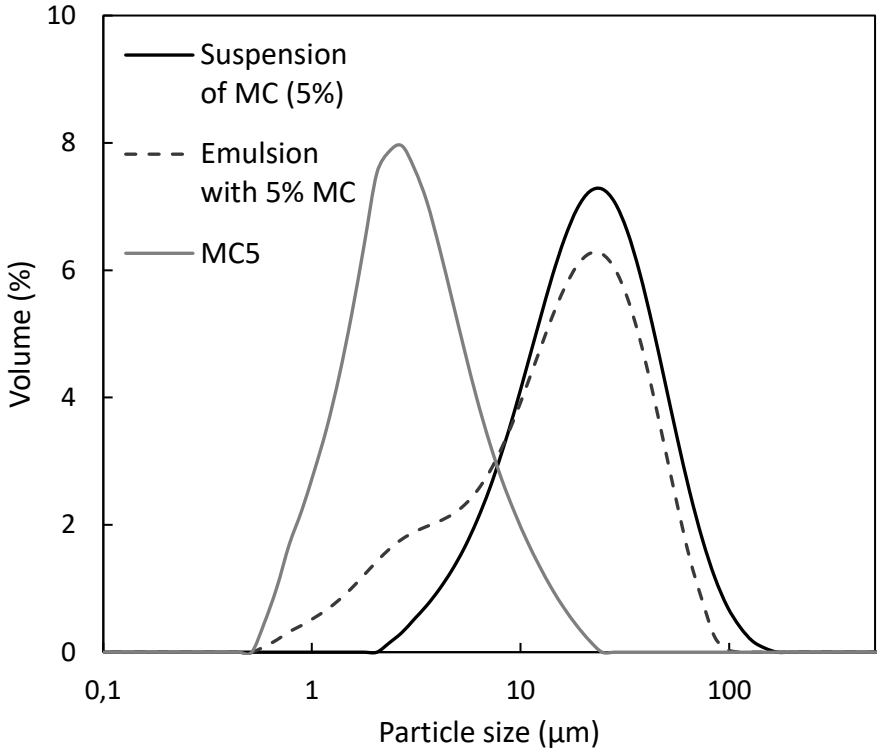
pH, Z-potential, and conductivity of C, MC1.5, MC3, MC5, and MC7.5 emulsions.

Emulsion	pH	Z-potential (mV)	Conductivity (mS/cm)
C	4.44 ± 0.09[b]	-42.15 ± 1.80[a]	0.1 ± 0.0[e]
MC1.5	6.89 ± 0.07[a]	-44.30 ± 1.66[a]	0.8 ± 0.1[d]
MC3	6.87 ± 0.07[a]	-44.36 ± 3.14[a]	1.5 ± 0.1[c]
MC5	6.89 ± 0.08[a]	-43.87 ± 1.02[a]	2.5 ± 0.1[b]
MC7.5	6.83 ± 0.15[a]	-40.88 ± 1.47[a]	3.5 ± 0.1[a]

Different letters for the same variable indicate significant differences ($p<0.05$) among the samples.

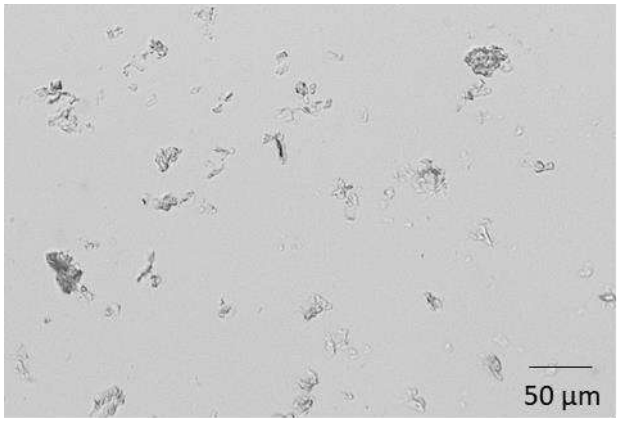
Supplementary 1

Particle size distribution of MC (5%), an emulsion containing MC (5%), and oil droplet size distribution of emulsion containing 5% MC after subtracting MC particle size distribution (MC5).



Supplementary 2

Micrograph of non-soluble particles of MC (5% w/w).



Supplementary 3

Photographs and CIELab coordinates of C, MC1.5, MC3, MC5, and MC7.5 emulsions (obtained immediately after the preparation of the emulsions)

	C	MC1.5	MC3	MC5	MC7.5
					
L^*	75.67 ± 0.16^a	56.93 ± 0.09^b	53.93 ± 0.25^c	50.09 ± 0.28^d	43.09 ± 0.25^e
a^*	-0.60 ± 0.01^e	0.02 ± 0.01^d	1.73 ± 0.02^c	3.24 ± 0.03^b	3.57 ± 0.06^s
b^*	-0.76 ± 0.01^e	15.83 ± 0.01^d	21.01 ± 0.05^c	22.55 ± 0.04^b	23.53 ± 0.02^a
ΔE		25.04 ± 0.05	30.85 ± 0.10	35.48 ± 0.11	40.25 ± 0.08

Different letters indicate significant differences ($p<0.05$) among the samples.

CRediT author statement

Mónica UMAÑA: Methodology, Investigation, Data curation, Validation, Writing-original draft. **Christelle TURCHIULI:** Conceptualization, Methodology, Investigation, Writing-Review & Editing. **Valeria EIM:** Validation, Visualization, Supervision. **Carmen ROSSELLÓ:** Resources, Writing-Review & Editing, Supervision. **Susana SIMAL:** Conceptualization, Formal analysis, Writing - Review & Editing, Supervision, Funding acquisition, Project administration.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: