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In vitro uptake and immune functionality of digested Rosemary extract delivered through food grade vehicles

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ABSTRACT

The digestion, absorption, uptake and bioavailability of a rosemary supercritical fluid extract encapsulated in oil in water emulsion were studied. Two emulsions with opposite surface charge were prepared, containing 7% canola oil, and either 2% lactoferrin or whey protein isolate. When absorption and uptake of carnosic acid and carnosol were followed on Caco-2 cell monolayers, there were no differences with protein type. However, when co-cultures of HT-29 MTX were employed, the presence of mucus caused a higher retention of carnosic acid in the apical layer for lactoferrin emulsions. The immune activity of the bioavailable fractions collected from cell absorption experiments was tested *ex vivo* on murine splenocytes. Although transport through the intestinal barrier models was low, the bioavailable fractions showed a significant effect on splenocytes proliferation. These results demonstrated the potential of using rosemary supercritical extract through protein stabilized oil in water emulsions, as a food with immunomodulatory functionality.

1. Introduction

The functional role of herbs and spices and their constituents is a hot topic in food related plant research. Rosemary (*Rosmarinus officinalis* L.) is a common used food flavouring herb that has extensively been studied due to its prominent immunomodulatory, antioxidant, antitumor, antiviral and antimicrobial activities (Arranz et al., 2015a; Aruoma et al., 1996; Moreno, Scheyer, Romano, & Vojnov, 2006; Vicente et al., 2013). Chemical analysis of extracts obtained from rosemary leaves demonstrated high content of phenolic compounds, among them carnosic acid and carnosol (Bai et al., 2010), which are mainly responsible for the beneficial health effects attributed to rosemary extract (Anderson, 1990; Arranz et al., 2015a).

The application of supercritical fluid extraction (SFE) on rosemary leaves provides advantages as compared to conventional methods such as solvent extraction or hydrodistillation (Carvalho, Moura, Rosa, & Meireles, 2005; Díaz-Maroto, Díaz-Maroto Hidalgo, Sánchez-Palomo, & Pérez-Coello, 2005). Rosemary supercritical extracts (RSE) showed a higher recovery of carnosic acid and carnosol and consequently an improvement on its antioxidant activity (Tena, Valcárcel, Hidalgo, & Ubera, 1997). Extraction method optimization has been performed on various processing parameters, including CO_2, pressure or temperature, as well as addition of co-solvents, for example, ethanol, to increase the extraction yield of diterpenoid polyphenols (Tena et al., 1997; Vicente et al., 2013).

SFE has been adopted not only by the chemical industry, but also by the food and pharmaceutical industry, due to the relatively fast and high recovery of extracts, the use of CO_2 instead of organic solvents and the elimination of concentration steps (Herrero, Cifuentes, & Ibañez, 2006). In the case of extracts obtained from rosemary by SFE, their incorporation in functional foods represents a challenge, as they possess a very low solubility in water. Therefore, the use of delivery systems to incorporate a rosemary supercritical extract (RSE) and preserve its functionality is necessary. Emulsions of the oil in water kind have been described as excellent carriers for lipophilic compounds, as conjugated linoleic acid or curcumin, helping to delay degradation during upper digestive tract transit and increase bioavailability of hydrophobic compounds tested in *in vitro* models of the intestinal barrier (Fernandez-Avila, Arranz, Guri, Trujillo, & Corredig, 2016; Gülseren, Guri, & Corredig, 2014). However, only few studies have addressed the encapsulation of RSE in emulsions, while there is a good deal of research referring the biological activities of the extract.

The encapsulation and bioavailability of RSE in an emulsion system or protein based nanostructures (casein micelles) was recently studied, and the research highlighted the opportunities of such systems for successful incorporation of RSE in food products (Arranz et al., 2015c). When tested *in vitro* on a Caco-2 cell model, both emulsion droplets

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and casein micelles encapsulating RSE significantly protected the extract from degradation during digestion and showed an increased bioavailability for carnosic acid and carnosol. In addition to the application of RSE as a biologically functional ingredient, the extract has also been suggested as a stabilizer of water in oil in water emulsions (Cizauskaite et al., 2015).

Other approaches of encapsulation of rosemary essential oil in emulsions using maltodextrin as coating material and subsequent freeze drying or modified starch and maltodextrin dispersions using spray drying have been recently reported (Fernandes, Borges, & Botrel, 2014; Turasan, Sahin, & Sumnu, 2015). Application of microencapsulated aqueous rosemary extract as powder formulation in cottage cheese have been described by Ribeiro et al. (2016), where rosemary was employed as an antioxidant agent.

When designing functional foods, the preservation of the beneficial health properties of the bioactives is crucial. Structural changes during digestion, aligned with the release of bioactive molecules and the interactions with the intestinal mucus, and their functionality after uptake should be taken under consideration. The assessment of bioactivity after absorption could be approached using different *in vitro*, *ex vivo* or *in vivo* models, depending on the research question (Arranz, Corredig, & Guri, 2016).

The consumption of molecules showing immunomodulatory activity is often correlated to a decrease in risk of chronic diseases such as diabetes or atherosclerosis, or some types of cancer. The functionality of the immune system is maintained due to the activity of organs and tissues, among them the spleen is the largest single lymphoid organ in the body (Anderson, 1990). Lymphocytes are densely packed in the white pulp of the spleen, containing T and B cell zones that complete their maturation and activation due to the presence of antigens (Abbas, Lichtman, & Pillai, 2014).

Although it is clear that the encapsulation of RSE in emulsion droplets will increase the stability and bioavailability of the bioactives, the effect of interfacial composition, and in particular charge, on the uptake is not known. The aim of this study was to evaluate the functionality of digested RSE incorporated in different milk protein stabilized oil in water emulsions. Canola oil in water emulsions were stabilized with two oppositely charged milk proteins at neutral pH; lactoferrin (LF) or whey protein isolate (WPI), to define how the matrix design could influence the bioaccessibility of the main constituents of RSE, carnosic acid and carnosol. The bioavailability of digested samples was tested on Caco-2 cells and furthermore the effect the interactions between the mucus layer and the two emulsion systems were assessed using mixed cultures of Caco-2 and MTX-HT29 (Guri, Gülseren, & Corredig, 2013). The recovery of the main phenolic compounds in the bioavailable fraction was determined by ultra-performance liquid chromatography-tandem mass spectrometry (UPLC/MS/MS). More importantly, the basolateral bioavailable fractions were tested *in vitro* by measuring the immune response on splenocytes. The results will then confirm the hypothesis that food emulsions containing RSE may be used as functional ingredients with immunomodulatory functionality.

2. Materials and methods

2.1. Materials

Lactoferrin powder (93% lactoferrin, 4.7% other milk proteins, 1% moisture, 1.3% ash) was obtained from BioPole S.A. (Epalinges, Lausanne, Switzerland) and whey protein isolate (98% protein, 4.5% moisture and 0.2% fat) was obtained from Land O'Lakes (St. Paul, MN, USA). Canola oil (100% pure) was purchased from a local supermarket. Ethanol (100%) and phosphoric acid (85%) (HPLC grade) were purchased from Panreac (Barcelona, Spain), and acetonitrile was obtained from Lab Scan (Dublin, Ireland). CO_2 (N38) was supplied from Carburos Metálicos (Madrid, Spain). Sulforhodamine B (SRB), pepsin (P7000), pancreatin (P1750), phospholipase A2 (P6534), bile salts (B8631), Dulbecco's modified Eagle medium (DMEM), RPMI 1640 no phenol red medium, 2-mercaptoethanol, HEPES buffer and carnosol (>95%) were purchased from Sigma-Aldrich Corporation (Oakville, ON, Canada). Fetal bovine serum (FBS) heat inactivated, nonessential amino acids (NEAA), trypsin 1 mM EDTA, HEPES buffer, L-glutamine, and penicillin–streptomycin (10,000 units of penicillin and 10,000 mg of streptomycin per mL) were purchased from Invitrogen (Canada Inc., Burlington, ON, Canada). Carnosic acid (≥97%) was supplied from Fluka (Madrid, Spain). Dried rosemary leaves (*R. officinalis* L.) were bought from an herbal shop (Murcia, Spain). Cells (Caco-2 and HT29-MTX) between passages 25 to 45 were used during this study.

2.2. Extraction and encapsulation of rosemary

Supercritical rosemary extract was obtained as previously described (Arranz et al., 2015c). Briefly, a pilot-plant supercritical fluid extractor, SF2000, Thar Technology (Pittsburgh, PA, USA) was used, the extraction was performed at 15 MPa and 313 K using 60 g/min CO_2 flow rate and ethanol (5% w/w) as co-solvent.

Oil in water emulsions containing 7% canola oil, with or without rosemary extract (5 mg/mL) and stabilized with 2% of WPI or LF were prepared. First, WPI and LF were dissolved in ultrapure water and stored overnight at 4 °C to allow complete hydration. Oil was premixed in protein solutions using a hand-held homogenizer (Polytron PT 1200, Kinematica, Fisher Scientific, Mississagua, ON, Canada) at 30000 rpm for 1 min and emulsions were prepared by high-pressure homogenization at 475 KPa for four passes using a microfluidizer (model M-110Y, Microfluidics Corporation, Newton, MA, USA). Emulsions were stored at 4 °C for 30 days.

2.3. Particle size distribution and ζ-potential measurements

The particle size distribution of the fresh, stored and digested emulsions was measured using static light scattering (Mastersizer 2000, Malvern Instruments, Worcestershire, UK). The volume weighted mean diameter ($d_{4,3}$, μm) was determined on the fresh and stored emulsions. Water was used as dispersant and refractive indices used for the measurements were 1.33 and 1.46, for water and canola oil, respectively. ζ-Potential of the fresh and stored emulsions was measured by dynamic light scattering (Zetasizer Nano, Malvern Instruments) in samples diluted in ultrapure grade water (1:100).

2.4. In vitro digestion

Emulsion samples and a control aqueous rosemary solution containing 2% ethanol to ensure dissolution of the extract (referred therefore after as aqueous rosemary suspension) were subjected to a simulated gastric and duodenal *in vitro* digestion process using Infogest protocol with some modifications suited to the food matrix being analyzed (Malaki Nik, Corredig, & Wright, 2010; Minekus et al., 2014). Emulsion and control samples (10 mL each) were mixed with 10 mL of gastric fluids containing 25,000 U/mL of pepsin and pH 3, for 30 min incubation in a shaking water bath (220 strokes/min). Then duodenal phase was initiated by the addition of 20 mL of juices containing a lower pancreatin concentration (5 mg/mL) to decrease rate of lipolysis (Malaki Nik et al., 2010), 5 μL phospholipase A_2 (stock solution 6.7 mg/mL) added to mimic enzymatic physiological profile, 0.4 mM bile salts and 1 mM phospholipids for 2 h at 37 °C. Samples were immediately transferred to ice for further analysis and in addition digestion was stopped by dilution (1:6) in culture medium containing 10% of FBS prior to cell culture analysis.

2.5. Free fatty acid release determination

The concentration of free fatty acid (FFA) release in the digestated samples was quantified using an enzymatic kit (NEFA, Wako