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# Structural polymeric features that contribute to *in vitro* immunostimulatory activity of instant coffee

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ABSTRACT

An instant coffee fraction, rich in arabinogalactans, obtained by ultrafiltration, using 1 and 5 kDa membranes, has previously shown *in vitro* stimulatory activity on BALB/c mice spleen B lymphocytes. The compounds inducing lymphocytic activation were shown to elute, mainly near the void volume by size-exclusion chromatography, using Bio-Gel P6 (1–6 kDa). Treatment of the compounds with chymotrypsin, a digestive protease, did not affect the induced B lymphocyte activation. On the contrary, hydrolysis with an α-L-arabinofuranosidase, removing single terminally-linked arabinose residues, decreased the extent of B cell activation. The immunostimulatory activity of these compounds was also observed by *in vitro* experiments, using bone marrow-derived macrophages and dendritic cells as responders. Altogether, these results show the relevance of single arabinose residues, present at the non-reducing end of polymeric compounds, to the coffee stimulatory activity in cells mediating innate and acquired immunity.

## 1. Introduction

During the preparation of roasted coffee beverages, galactomannans and arabinogalactans are the most abundant polysaccharides among the several compounds extracted from coffee (Bradbury & Halliday, 1990). These polysaccharides have been reported to present *in vitro* immunostimulatory activity (Capek et al., 2014; Nosáľová et al., 2011; Passos et al., 2014; Simões et al., 2009). However, the several structural modifications of polysaccharides during the roasting of coffee beans (Moreira et al., 2016; Nunes & Coimbra, 2001), together with their incorporation in high molecular weight brown compounds also known as melanoidins (Moreira, Nunes, Domingues, & Coimbra, 2012; Moreira et al., 2017), make difficult the establishment of structure-function relationships.

Immunostimulatory polysaccharides interact with cellular and humoral components of the immune system, modulating innate and acquired immune responses, either by exhibiting a direct effect themselves or by inducing effects via complex reaction cascades (Ferreira, Passos, Madureira, Vilanova, & Coimbra, 2015; Tzianabos, 2000). Among the different leukocyte populations, macrophages have been the preferred *in vitro* cellular model to study potential immunostimulatory effects of polysaccharides (Schepetkin & Quinn, 2006). Nevertheless, other cellular or molecular immune targets have also been studied, such as monocytes, dendritic cells, neutrophils, lymphocytes or complement proteins. Altogether, these models have provided insights about the structural features associated with polysaccharide immunostimulatory activity, such as, monosaccharide and glycosidic-linkage composition, but also other structural features, including molecular weight, conformation, presence of acetyl and sulphate groups, and branching characteristics (Ferreira et al., 2015).

The immunostimulatory activity of coffee polysaccharides, both galactomannans (Simões et al., 2009) and arabinogalactans (Capek et al., 2014; Nosáľová et al., 2011; Passos et al., 2014), has been assessed using spleen cells, an *in vitro* model that includes several immune cell populations, mediating both innate and acquired immune responses, namely, lymphocytes (B and T cells), macrophages, dendritic cells, and vascular cells (Paul, 2012, chap. 2). Nevertheless, assays reporting the individual stimulation of each type of immune cell should

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provide more precise information about how polysaccharides interact with them (Schepetkin & Quinn, 2006). Although, for galactomannans from espresso coffee beverage and chemically acetylated spent coffee grounds, the immunostimulatory properties have been related to the presence of acetyl groups (Simões, Nunes, Domingues, & Coimbra, 2010, 2011; Simões et al., 2012), for coffee arabinogalactans no structural features have been related to their reported immunostimulatory activity. As no acetyl groups have been reported to occur in coffee arabinogalactans, acetylation should not be a relevant parameter contributing to their immunostimulatory activity. Moreover, as coffee arabinogalactans are polysaccharide-protein complexes (Nunes, Reis, Silva, Domingues, & Coimbra, 2008; Redgwell, Curti, Fischer, Nicolas, & Fay, 2002), the relevance of the protein content for their immunostimulatory effects needs to be assessed. Although arabinogalactan molecular weight may be a property that could be associated with their immunostimulatory activity (Passos et al., 2014), the different assays conducted with instant coffee arabinogalactan-rich fractions, with molecular weights of 1–5 kDa (Passos et al., 2014); 5–6 kDa (Nosáľová et al., 2011); and > 12 kDa (Kwak & Shin, 2016) show that structural features other than molecular weight should be responsible for such activity.

The arabinogalactans from coffee have a main backbone of (β1→3)-D-Gal residues, with some substitutions at the O-6 position, with short chains of (β1→6)-D-Gal residues (Bradbury & Halliday, 1990). The Gal residues of these (β1→6)-D-Gal side chains can be substituted at the O-3 position with single α-Ara residues, (1→5)-linked Ara disaccharides (Redgwell et al., 2002), rhamnoarabinose disaccharides, or rhamnoarabinoarabinose trisaccharides (Moreira et al., 2012; Nunes et al., 2008). Because roasting also promotes non-enzymatic transglycosylation reactions (also known as reversion reactions), hybrid polysaccharide structures, composed of galactomannans and arabinogalactans, also occur in coffee, together with melanoidins (Moreira et al., 2014, 2016, 2017), which can contribute to a large diversity of structural features displayed by the polysaccharides present in coffee beverages.

Two instant coffee fraction samples were used in this work to *in vitro* stimulate bone marrow-derived macrophages (BMDM) and dendritic cells (BM-DCs): 1E, a sample with reported *in vitro* immunostimulatory activity by inducing the activation of B-lymphocytes (Passos et al., 2014), and 2E, a sample prepared from a different instant coffee brand. To reveal the structural features that could be contributing to the observed activity, sample 1E was submitted to size-exclusion chromatography, α-chymotrypsin proteolytic digestion, and α-L-arabinofuranosidase hydrolysis.

## 2. Materials and methods

### 2.1. Instant coffee fractions

The instant coffee fractions were prepared as described by Passos et al. (2014) from two instant coffee powders, obtained in a local supermarket from commercial batches from Auchan, Portugal (sample 1) and Nestlé, Portugal (sample 2). All reagents used were of analytical grade or higher available purity. Briefly, each instant coffee powder was dissolved in water (2.5% w/v) with stirring during 10 min at 80 °C. The solution was then cooled to room temperature and, after a minimum period of 48 h at 4 °C, it was decanted and centrifuged at 15,000 rpm for 10 min at 4 °C. The supernatant recovered was frozen and freeze-dried. The supernatants from each instant coffee were named as “Sn1” and “Sn2”, respectively. Afterwards, a solution of each supernatant was subjected to a sequential ultrafiltration, using a stirred ultrafiltration unit (Stirred Cell Model 8200, 200 mL) with regenerated cellulose membranes having molecular weight cut-offs of 100, 30, 10, 5 and 1 kDa (Millipore, Darmstadt, Germany). Each Sn sample was dissolved in 200 mL of water. When the volume of retentate reached 40 mL after passing through the membrane, refilling with water to a volume of 200 mL was done and this procedure was repeated several times. On average, this procedure was repeated 10 times for Sn1 (exhaustive procedure) and twice for Sn2 for each membrane pore size. Because Sn2 was not exhaustively purified, the correspondent fraction of 1–5 kDa was further purified by size exclusion chromatography, using Bio-gel P2 (*SEC*-P2, range 100–1800 Da) and the exclusion volume recovered gave sample 2E. All fractions obtained were freeze-dried and stored under an anhydrous atmosphere. Previously, Passos et al. (2014) showed that the material recovered in the fraction of 1–5 kDa from Sn1 (sample 1E) was the only fraction to induce B-lymphocyte activation. In this study both sample 1E and sample 2E were studied.

### 2.2. Size exclusion chromatography of instant coffee fractions

Size exclusion chromatography, using Bio-gel P6 (range 1–6 kDa), was performed on a XK 2.6/70 column with a flow rate of 0.3 mL/min. The samples were dissolved in 1 mL of distilled water, centrifuged and loaded onto the column previously equilibrated with water. Exclusion ($V_0$) and inclusion volumes ($V_T$) were estimated with Blue Dextran (2000 kDa) and glucose (180 Da), respectively. Fractions of 2 mL were collected up to a total volume of 160 mL, and the remaining volume was collected in a single container (160–350 mL). The collected fractions were monitored by evaporative light scattering detection (ELSD). ELSD was performed in a SEDEX Model 55 by setting the temperature to 57 °C, the pressure to 1.9 bar, and introducing 70 μL of each fraction interspersed with water, using a flow of 4 mL/min. The absorbances at 280, 325, and 405 nm of 1:20 dilutions of each fraction were measured, using a quartz cuvette in a double beam ultraviolet–visible (UV/Vis) spectrophotometer (Lambda 35, Perkin-Elmer, USA). The collected fractions were also assayed for sugars by the phenol-sulfuric acid method (absorption at 490 nm) (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). The appropriate fractions were pooled and freeze-dried.

Size exclusion chromatography, using Bio-gel P2 (SEC-P2, range 100–1800 Da), was performed on a XK 1.6/40 column, using the conditions described for SEC-P6. Fractions of 1 mL were collected and monitored by ELSD and at 280, 325, and 405 nm, as described.

### 2.3. Enzymatic treatments

#### 2.3.1. α-Chymotrypsin treatment

Samples (50 mg) were treated with 20 U of α-chymotrypsin from bovine pancreas (EC 3.4.21.1, Sigma, St. Louis, USA) during 24 h at 25 °C with continuous stirring in 100 mL of 100 mM Tris-HCl buffer, pH 7.8, and 10 mM $CaCl_2$. Enzymatic digestion was terminated by adjusting the pH to 2.0 by the addition of 2 M HCl and neutralization with 2 M NaOH after 15 min. Samples were dialysed, using a membrane cut-off of 1000 Da (Spectrum, Breda, The Netherlands) until reaching distilled water conductivity.

#### 2.3.2. α-L-Arabinofuranosidase treatment

Sample 1E (15 mg) was hydrolysed with 1 U of *Clostridium thermocellum* arabinofuranosidase 51 A (EC 3.2.1.55, Nzytech), purified from a recombinant *Escherichia coli* strain, during 48 h at 37 °C with continuous gentle stirring in 5 mL of 100 mM Na-acetate buffer, pH 5.5, containing 0.02% sodium azide. It was freeze-dried (1EA) and purified through *SEC*-P2 (1EA-P2) as described in Section 2.2.

### 2.4. Fraction chemical characterization

#### 2.4.1. Sugar and glycosidic-linkage analysis

The individual neutral sugars were determined after acid hydrolysis, derivatization to alditol acetates, and analysis by GC-FID, as described by Passos and Coimbra (2013). Sugars were determined in duplicate, only performing a third analysis when major sugars had higher than 5% variability.