DOI 10.1002/mnfr.200800385 Mol. Nutr. Food Res. 2009, *53*, 1036 – 1043

Research Article

Immunostimulatory properties of coffee mannans

Joana Simões[1], Pedro Madureira[2], Fernando M. Nunes[3], Maria do Rosário Domingues[1], Manuel Vilanova[2] and Manuel A. Coimbra[1]

[1] Departamento de Química, Universidade de Aveiro, Aveiro, Portugal
[2] Instituto de Ciências Biomédicas Abel Salazar, Largo Prof. Abel Salazar, Porto, Portugal
[3] Departamento de Química, Universidade de Trás-os-Montes e Alto Douro, Vila Real, Portugal

Coffee infusion mannans are acetylated polysaccharides containing single Gal*p* and Ara*f* residues as side chains of a β-(1→4)-Man*p* backbone. These mannans are structurally similar to the bioactive acetylated mannans from *Aloe vera* (AV). In this study, acetylated mannans were obtained from two coffee infusions prepared from light and dark roasted beans. These samples were tested for their immunostimulatory activity and compared with an extract of AV mannan and with locust bean gum (LBG) galactomannans. The coffee samples, as well as the AV extract, stimulated murine B- and T-lymphocytes, as evaluated by the *in vitro* expression of the surface lymphocyte activation marker CD69, more marked on B- than on T-lymphocytes. In coffee samples, contrarily to the AV, no proliferative effect was noticed. LBG sample did not show any immunostimulatory activity. Because the material that remains in the residue of the hot water extraction was still very rich in mannans, a sequential extraction was performed and a main fraction was recovered with a 4 M NaOH solution. Because this material was insoluble in water, a partial acetylation was performed. These polysaccharides also showed immunostimulatory activity, opening the possibility of exploitation of coffee infusion and coffee residue as sources of bioactive polysaccharides.

Keywords: *Aloe vera* / Coffee / Coffee residue / Galactomannan / Immunostimulatory activity

Received: August 21, 2008; revised: December 8, 2008; accepted: January 19, 2009

1 Introduction

Polysaccharides account for ~29% of roasted coffee infusions high molecular weight material. Depending on the origin of the coffee, the amount of polysaccharides in coffee infusions can vary, *e.g.*, Arabica coffee infusions contain higher amount of polysaccharides than Robusta [1, 2]. Coffee infusion polysaccharides are composed mainly by galactomannans and type II arabinogalactans. Galactomannans account, on average, for 68% of coffee infusion polysaccharides. However, different amounts of galactomannans can be extracted with hot water, depending of the origin of the coffee as well as their degree of roast. Arabica coffee infusions contain higher amount of galactomannans (62–80%) than Robusta (44–67%) and the amount of galactomannans in solution increases with the roasting of the coffee [1–3]. Besides the important functional properties that the polysaccharides confer to the coffee infusions, they are involved in the retention of coffee volatile substances and contribute to the brew viscosity and, thus, to the 'body' of the drink, *i.e.*, the creamy sensation perceived in the mouth [4]. They are also involved in the foam stability of espresso coffee, an important quality attribute of this coffee brew [5, 6]. Recently, they have been shown to have beneficial biological activities, namely, undergoing rapid fermentation in the human colon and thereby contributing to the physiological effects generally associated with fibre fermentation [7–9].

In a recent study, it was possible to isolate and purify from roasted coffee infusions a fraction that can be classified as minimally degraded or transformed galactomannans. Structural characterisation of these galactomannans allowed to assign new structural features [10, 11]. The galactomannans present in coffee infusions are acetylated and contain single arabinose residues as side chains attached at *O*-6 to the β-(1→4)-linked mannose residues in the backbone [10, 11]. These acetylated arabinogalactomannans are structurally similar to the bioactive acetylated mannans of *Aloe vera* (AV)[12]. The acetylated mannans extracted from AV have been shown to have several benefi-

Correspondence: Professor Manuel A. Coimbra, Departamento de Química, Universidade de Aveiro, 3810- 193 Aveiro, Portugal
E-mail: mac@ua.pt
Fax: +351-234-370084

Abbreviations: LBG, locust bean gum; **CR,** coffee residue

cial biological activities such as the decrease of cholesterol levels in mammals, macrophage activation and stimulation of T lymphocyte cells, and action against infectious and tumour diseases [13 – 16]. This structural similarity between coffee infusion mannans and AV bioactive mannans prompt us to evaluate the possible immunostimulatory activity of these polysaccharides.

The coffee residue resultant from coffee beverage preparation is composed by the materials that were not able to be extracted by the hot water. Considering the huge amount of coffee residue produced all over the world, the reutilisation of this by-product is a relevant subject. Some attempts of reutilisation of coffee residue have been made, using it as fertiliser, fuel [17] or an antioxidant material source [18], but, to our knowledge, none of these strategies have yet been routinely implemented. Polysaccharides comprise 50% of green coffee beans dry weight, and galactomannans account for 50% of these polysaccharides [19]. Although up to 40% of the total polysaccharides are degraded during roasting [20], mannans are the least degraded polysaccharides, accounting for nearly 50% of total polysaccharides, even for the darkest roasted coffees [20, 21]. As, depending on the degree of roast, only 6 – 12% of the polysaccharides are extracted during the beverage preparation [3, 20], this residue could be a good source of valuable polysaccharides, especially mannans. Nevertheless, the use of coffee residue as a source of polysaccharides has been hampered due to their insolubility. The acetylation of coffee residue polysaccharides presents a real possibility to render them soluble in water. Additionally, they became with a composition that is similar to the coffee infusion acetylated arabinogalactomannans and AV bioactive mannans, which would confer an added value to this coffee residue mannans. The utilisation of coffee residue as a source of polysaccharide with immunostimulatory activity and the consequent valorisation of this by-product is a possibility keen to be exploited.

In this work, the immunostimulatory activity of coffee infusion mannans isolated and purified from two coffee infusions prepared from roasted coffee beans, and mannans prepared from coffee residue, was studied. The results were compared with the activity of the mannan from AV and the galactomannans of locust bean gum (LBG).

2 Materials and methods

2.1 Coffee infusion samples

Coffee infusions were prepared from Brazilian Arabica coffee beans from two degrees of roast: a light roast, with 5% matter loss on a dry weight basis (DR 5%), and a dark roast, with 10% dry matter loss (DR 10%), as described by Nunes and Coimbra [1, 3]. Briefly, each ground and defatted coffee was extracted with water (50 g/L) at 80°C, 20 min, filtered, concentrated, dialysed (12 – 14 kDa cut-off), frozen and freeze-dried, giving the High Molecular Weight Material (HMWM). The mannan rich fractions were obtained from the HMWM as described by Nunes *et al*. [10, 11]. Briefly, the HMWM was gradually precipitated in ethanol and the fraction insoluble in 50% ethanol (Et50) was recovered and further purified by anion exchange chromatography on Q-Sepharose. The material recovered on the nonretained fraction was further purified by phenylboronic acid (PBA) affinity chromatography, giving origin to fractions CI5 and CI10, for the light and dark roasted coffee infusion, respectively.

2.2 Coffee residue samples

Coffee residue mannans were prepared from espresso coffee residue obtained from a commercial batch of Buondi coffee in a local cafeteria. The coffee residue (1 kg, 360 g dry weight) was sequentially extracted with: (i) 2 L distilled water at 90°C during 1 h, then (ii) with 2 L 0.5 M imidazole pH 7.0 at 70°C during 1 h, (iii) 1 L 0.05 M NaOH, (iv) 1 L 1 M NaOH and (v) 1 L 4 M NaOH. All NaOH extractions were performed during 2 h at room temperature. To prevent peeling reactions and alkaline oxidation of the polysaccharides, the NaOH extraction was carried out under an inert atmosphere (N_2) with O_2 free solutions containing 0.02 M $NaBH_4$. The NaOH solutions were prepared using distilled water previously boiled for 20 min and cooled under a nitrogen atmosphere. After each extraction step the mixture was filtered, concentrated under reduced pressure and dialysed (12 – 14 kDa cut-off) for 3 days, with several changes of distilled water. The alkali extracts were previously acidified to pH 5.0 with glacial acetic acid. After dialysis, the extracts were centrifuged and the precipitates, if present, were recovered separately from the supernatants. All samples were frozen and freeze-dried.

The mannans present in the 4 M NaOH precipitate were solubilised by acetylation according to the methodology proposed by Biswas *et al*. [22] for starch and cellulose. In a glass vial equipped with a magnetic stirrer, 1 g of sample was suspended in 2 mL of acetic anhydride containing 50 mg of iodine, sealed and heated at 100°C for 10 min. The reaction mixture was then cooled to room temperature and treated with a saturated solution of sodium thiosulphate (2 mL) with stirring. The mixture colour changed from dark brown to colourless, indicating the transformation of iodine to iodide. The mixture was poured into 50 mL of ethanol and stirred for 30 min. The dispersion was centrifuged (15 000 rpm, 10 min, 4°C) and the precipitate was recovered, suspended in water and stirred overnight in order to solubilise the acetylated polysaccharides coffee residue (CR)1. The material present in the residue was separated from CR1 and submitted to a new acetylation procedure with acetic anhydride and iodine following the same procedure. The acetylated material recovered from the second acetylation was named CR2.

2.3 *Aloe vera* and locust bean gum samples

The high molecular weight material from AV was obtained by dialysis (12 – 14 kDa cut-off) from a commercial capsule containing 405.5 mg of powder (Molo-Cure, USA).

The galactomannans from LBG had been purified from a commercial sample (HG M200- INDAL) by solubilisation in water (5 g/L) at room temperature during 1 h and then at 90°C during 30 min, and recovered by precipitation in 80% ethanol (12 h at 4°C) followed by filtration [23].

2.4 Sugars analysis

Neutral sugars were released from polysaccharides by treatment with 11 M H_2SO_4 (10 mg/mL) during 3 h at room temperature with occasional stirring followed by hydrolysis for 2.5 h with 1 M H_2SO_4 at 100°C. The sugars were then derivatised to their alditol acetates and analysed by GC flame ionisation detector (GC-FID), as previously described [1, 3].

2.5 Determination of the degree of acetylation of coffee residue mannans

The determination of the degree of acetylation was performed by saponification with NaOH and analysis of the released acetic acid by solid phase micro extraction and GC (SPME-GC) according to the method developed by Nunes *et al*. [24]. The samples (2 – 3 mg) were dispersed in water (2.4 mL) in vials with 1 mL capacity and sonicated for 10 min in a water bath at room temperature. The saponification of the polysaccharides occurred by the addition of 0.8 mL of 2 M NaOH, with a reaction time of 1 h at 25°C. The reaction was finished by the addition of 0.8 mL of 2 M HCl, and the pH was adjusted to 2.0. The vials (10 mL) containing 4.0 mL of sample suspension (sample dispersed in 2.4 mL of water, 0.8 mL of 2 M NaOH and 0.8 mL of 2 M HCl) or standard solutions were thermostatised at 40°C in a water bath, with continuous stirring. After 15 min, the SPME fibre coated with 50/30 μm divinylbenzene/Carboxen on polydimethylsiloxane (DVB/Carboxen/PDMS) was manually inserted through the Teflon septum into the headspace of the vial and exposed at 40°C during 30 min. The SPME coating fibre containing the headspace volatile compounds was introduced into the GC injection port at 250°C and kept for 10 min for the desorption. A Hewlett-Packard 5890 series II gas chromatograph (Hewlett-Packard, Wilmington, USA), equipped with a split/splitless injector and a FID was used.

2.6 Immunostimulatory activity assays

2.6.1 Preparation of polysaccharide extracts

The polysaccharide solutions (1 mg/mL) were prepared as described by Dourado *et al*. [25] by dispersing the polysaccharides in endotoxin-free PBS. The samples were then sterilised by filtering through a 0.22 μm filter (Whatman) and aliquots were collected and assayed for total carbohydrates by the phenol-H_2SO_4 method [26]. For removal of any contaminant bacterial LPS the filtered solutions were passed under sterile conditions through a 5 mL Detoxigel column (Pierce), previously washed several times with endotoxin-free water (25 mL), 1% w/v deoxycolic acid solution (25 mL), and then equilibrated with PBS. The solutions were finally concentrated and total carbohydrates were also determined before and following column passage by the phenol-H_2SO_4 method [26]. No losses were detected following column passage.

2.6.2 Mice

Male C57BL/6 mice (6 – 8 wks old) were purchased from Charles River (Barcelona, Spain), and were kept at the animal facilities of the Institute Abel Salazar, under specific pathogen-free conditions until used. Sterile food and water were supplied *ad libitum*. Nesting and hiding material were provided. Procedures involving mice were performed according to the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS 123) and 86/609/EEC Directive and Portuguese rules (DL 129/92).

2.6.3 *In vitro* mononuclear cell cultures of murine mononuclear spleen cells

Spleen cells were obtained by gently teasing the organ in RPMI-1640 medium (Sigma, St. Louis, USA) supplemented with penicillin (100 IU/mL), streptomycin (50 μg/mL), 2-mercaptoethanol (0.05 M) and 10% of fetal bovine serum (Sigma, St. Louis, USA) (RPMI). Mononuclear cell suspensions were distributed on 96-well plates (10^6cells/well) and cultured for 6 h at 37°C, in 95% humidified atmosphere containing 5% CO_2. Plated cells were stimulated with RPMI medium alone (negative control), 5 μg/mL of LPS from *Salmonella abortus equi* (Sigma, St. Louis) (positive control) or with 12.5 – 100 μg/mL of coffee beverage samples (CI5 and CI10), coffee residue samples (CR1 and CR2), *Aloe vera* extract (AV) and galactomannan from LBG.

2.6.4 Evaluation of the *in vitro* lymphocyte stimulating effect by flow cytometry analysis

For cytometry analysis, C57BL/6 mice spleen cells were resuspended in balanced salt solution (BSS) supplemented with 10 mM of sodium azide and 1% BSA. The following monoclonal antibodies were used for immunofluorescence cytometric analysis in a FACScan (Becton Dickinson, San Jose, CA, USA): FITC-conjugated rat anti-mouse B220 (Pharmingen, San Diego, CA), FITC-conjugated rat anti-mouse CD8 (Pharmingen), FITC-conjugated rat anti-mouse CD4 (Pharmingen) and phycoeritrin-conjugated hamster anti-mouse early activation marker (CD69; Pharmingen). CELLQUEST software (Becton Dickinson) was used to

Table 1. Sugars composition and acetylation of mannan-rich samples used.

Sample	Sugars composition (% mol)				Total sugars (%)	Man/Gal	No. of acetyl groups per sugar residues (mol acetic acid/mol sugar residue)
	Ara	Man	Gal	Glc			
CI5	2	89	7	1	76	13	0.08
CI10	1	92	5	1	63	18	0.08
CR1	6	69	23	2	55	3	0.84
CR2	1	90	6	2	44	15	0.94
AV	4	41	5	41	34	8	n.d.
LBG	1	77	20	3	86	4	n.d.

CI5 and CI10, Coffee infusion fractions isolated and purified from coffee infusions, as described in section 2.1; CR1 and CR2, acetylated fractions from coffee residue, as described in section 2.2; AV, *Aloe vera* sample; LBG, locust bean gum sample.
n.d., not determined.

process the gathered data. Dead cells were excluded by propidium iodide incorporation.

2.6.5 Statistical analysis

The results obtained were analysed by one-way ANOVA in order to detect significantly ($p < 0.05$ or $p < 0.01$) difference between the means. Significantly different means relative to control were assigned after *post hoc* analysis using the Dunnet test.

3 Results and discussion

3.1 Chemical characterisation and immunostimulatory properties of coffee infusion mannans

The coffee infusions mannans were purified from two coffee infusions with two different degrees of roast using a multistep purification procedure that included ethanol precipitation, anion exchange chromatography and PBA affinity chromatography, a methodology that was shown to be specific for the isolation of relatively high substituted galactomannans [10]. The purified coffee infusion galactomannans accounted for 12 and 8.3% of its high molecular weight material (HMWM) for the light and dark roasted coffee, corresponding to 1.0 and 0.66%, respectively, of the whole roasted coffee. This means that for 150 mL of a typical coffee infusion prepared with a proportion of 50 g of ground roasted coffee *per* 1 L of water, the amount of acetylated galactomannans is ~77 mg for the light roasted coffee and ~50 mg for the dark. The sugars composition of these purified galactomannan-rich fractions is presented in Table 1. The mannose/galactose (Man/Gal) ratio for the light roasted coffee (CI5) was 13 and 18 for the dark roasted coffee (CI10). This allows to infer that the galactomannans from CI5 had a higher amount of galactose side chains than those of CI10. The relative content of arabinose residues, also known to occur as mannan side chains, was also higher in CI5. Anyway, both galactomannans presented an equal abundance of acetyl groups that accounted for 8% on a molar basis in relation to the sugar residues, indicating that, on average, 2 in 25 sugar residues were acetylated. It has been shown [10, 11] that this acetylation occurs preferentially on the mannan backbone in the *O*-2 position of the mannose residues. These structural features of the coffee infusion galactomannans show resemblance with the immunostimulatory polysaccharide acemannan of AV, although this has been classified as an acetylated glucomannan, having a higher percentage of acetyl groups (0.91 acetyl groups *per* sugar residue [27]).

The *in vitro* immunostimulatory properties of coffee infusions acetylated mannans were tested and compared with a commercial extract of AV bioactive mannans (sugars composition shown in Table 1). Additionally, a LBG galactomannans sample was used as a different source of mannans, working as a control sample. The Man/Gal ratio of LBG galactomannan was 4 (Table 1), due to the higher relative content of galactose, having a higher degree of branching than CI5 and CI10.

Figure 1 shows the *in vitro* lymphocyte stimulatory effect of CI5 and CI10 samples evaluated by flow cytometry analysis of the expression of the early activation marker CD69 on the surface of C57BL/6 mice spleen B- and T-cells. B-lymphocytes were activated by both coffee infusion mannans, as well as by AV, but not LBG. The stimulatory effect observed of 89% for CI5 and 74% for CI10 was similar to that obtained for AV (74%) and significantly different from that observed for the LBG galactomannan (5%). LBG showed a value not significantly different from the PBS blank test (4%). The amount of material to reach the reported maximum activation percentages was 20 μg/mL for CI5 and 100 μg/mL for CI10, whereas for AV was an intermediate concentration (50 μg/mL).

The flow cytometry analysis of CD69 expression on the surface of C57/BL6 mice $CD4^+$ and $CD8^+$ T- cells in spleen mononuclear cell cultures (Fig. 1) also shows a stimulatory effect on these lymphocyte populations by coffee infusion mannans. On $CD4^+$ cells, the maximum observed activation was 39 – 40% for both coffee infusions samples, values comparable to that obtained for AV (59%). The amount of mate-

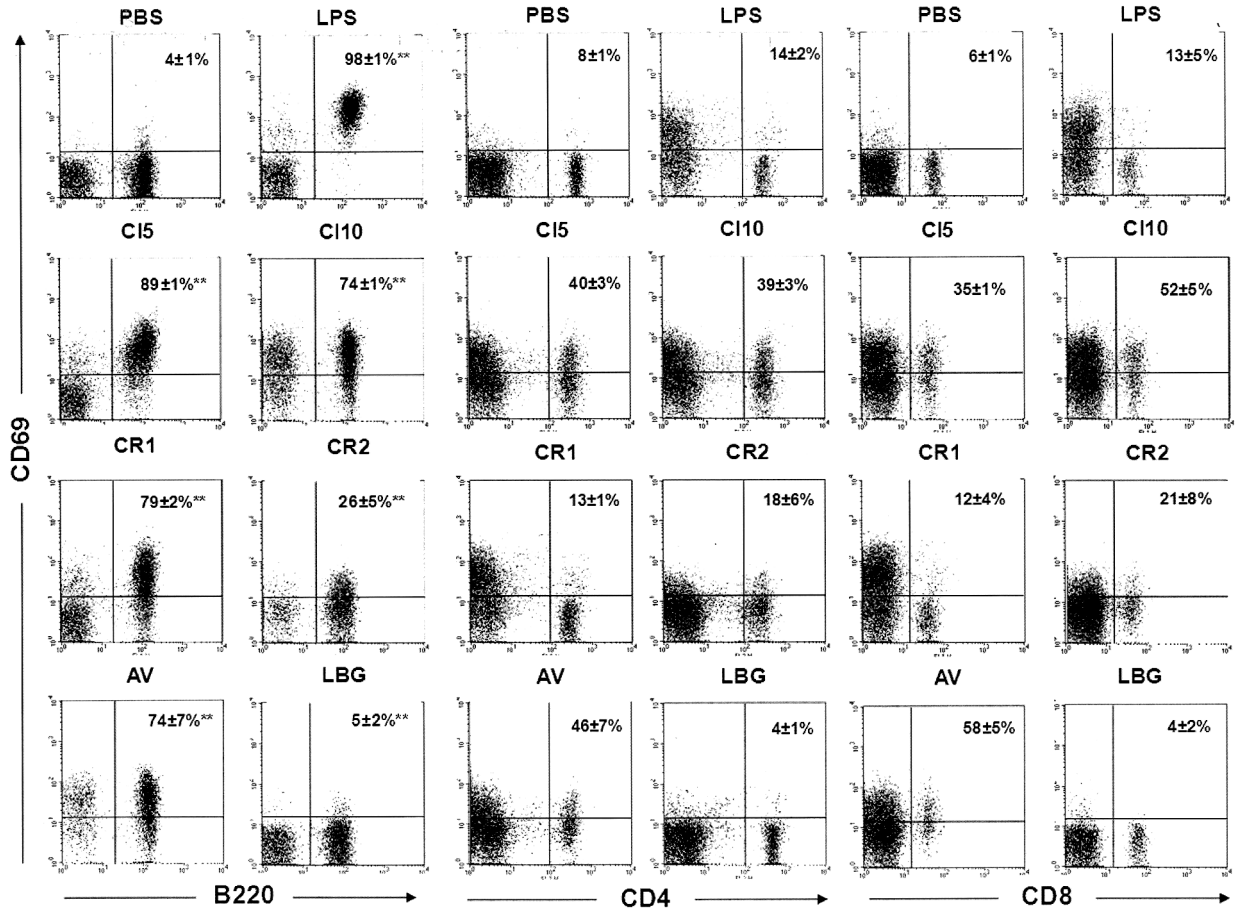

Figure 1. *In vitro* lymphocyte stimulatory effect of galactomannans of coffee infusion (CI5 and CI10), coffee residue (CR1 and CR2), *Aloe vera* (AV) and LBG. Typical example of dot plots showing CD69 expression on the surface of B cells (B220), $CD4^+$ T cells or $CD8^+$ T cells in C57BL/6 mice spleen mononuclear cell cultures stimulated with medium alone (RPMI), 5 μg/mL of LPS, or with previously titrated optimal doses of CR1, CR2, CI5, CI10 or AV on each lymphocyte population. For B-cells, these were of 50 μg/mL (CR1), 50 μg/mL (CR2), 20 μg/mL (CI5), 100 μg/mL (CI10) and 50 μg/mL (AV). For $CD4^+$ cells these were of 100 μg/mL (CR1), 25 μg/mL (CR2), 40 μg/mL (CI5), 100 μg/mL (CI10) and 25 μg/mL (AV). For $CD8^+$ cells these were of 100 μg/mL (CR1), 25 μg/mL (CR2), 40 μg/mL (CI5), 100 μg/mL (CI10) and 25 μg/mL (AV). Numbers inside dot plots indicate the mean values (±SD) of the proportion of $CD69^+$ cells within the gated population ($B220^+$, $CD4^+$ or $CD8^+$) determined on three wells *per* experimental conditions. In this and in the following pictures the significance of the results, as compared with control, is indicated (*, $p < 0.05$; **, $p < 0.01$). This is a representative example of four independent experiments.

rial required to reach the reported maximum activation percentages was 40 μg/mL for CI5 and 100 μg/mL for CI10, but it was lower (25 μg/mL) for AV. For $CD8^+$ cells, a maximum activation of 35 and 52% was obtained for CI5 and CI10 samples, respectively (Fig. 1), which are values comparable to that obtained for AV (47%). The amount of material to reach the reported maximum activation percentages was higher (40 μg/mL for CI5 and 100 μg/mL for CI10 samples) than that required for AV (25 μg/mL). LBG mannan extract did not show stimulatory activity for $CD4^+$ or $CD8^+$ T-cells in spleen mononuclear cell cultures (Fig. 1).

3.2 Chemical characterisation and immunostimulatory properties of coffee residue mannans

The sugars analysis of coffee residue (Table 2) shows that mannose (57%) is the main sugar residue, followed by galactose (26%), glucose (11%) and arabinose (6%). This sugars abundance is similar to that observed for the cell wall material [20] and for the whole roasted coffee [28] with a medium degree of roast. This sugars composition shows that the mannans are the major polysaccharides of coffee residue. In order to extract and purify the mannans, the cof-

Table 2. Sugars composition of coffee residue and fractions from the extraction with 4 M NaOH (NaOH sn, NaOH ppt and cellulosic residue).

Sample	Yield (%)	Sugar composition (% mol)				Total sugars (%)
		Ara	Man	Gal	Glc	
Coffee residue	–	6	57	26	11	35
NaOH sn	1.4	12	14	69	3	40
NaOH ppt	4.6	2	87	8	2	73
Cellulosic residue	49.1	5	60	20	14	54

fee residue was sequentially extracted with several aqueous solvents. Although coffee residue material was obtained in all extracts, the best solvent for extraction of the mannans was 4 M NaOH, allowing to obtain 6.0% of the starting material (Table 2). Upon neutralisation and dialysis, the precipitate (NaOH ppt) was separated from the supernatant (NaOH sn). The sugar composition of NaOH ppt (Table 2) showed to be very similar to that found for the roasted coffee infusion mannans and very different from the one observed for NaOH sn fraction, which was rich in galactose and arabinose (Table 2), characteristic of arabinogalactans. Anyway, a large proportion of mannans remains in the coffee residue (Table 2) needing stronger conditions than those used for their extraction.

Polysaccharides are linked together by many intermolecular hydrogen bonds which, due to their abundance, are responsible for their low solubility in water. The insolubility increases with the increase of the degree of polymerisation and decreases with the increase of the number of branching residues or acetyl groups in the polymers. As the mannans isolated from both, coffee infusion and coffee residue, have a comparable Man/Gal ratio, this is not a factor that could explain their water insolubility. This could be possibly related to a higher degree of polymerisation of coffee residue mannans when compared to those present in coffee infusions and/or to a lower degree of acetylation. Chemical acetylation is a process that can be used to promote the solubilisation of polysaccharides by functionalisation of hydroxyl groups and, consequently, decreasing the extent of polymeric intermolecular hydrogen bonds.

The acetylation procedure performed on the 4 M NaOH ppt allowed to obtain a yield of 8.4% (CR1). The main sugar residue present in fraction CR1 was mannose. However, this fraction contains a large amount of galactose (23%) and arabinose (6%). This sugars composition suggests the presence of mannans but, due to the amount of galactose residues, the presence of arabinogalactans cannot be excluded, possibly due to the co-solubilisation with the mannans. The amount of acetyl groups, on a molar basis in relation to the sugar residues, was 84%, which shows that the acetylation was performed efficiently. With the purpose of increasing the yield of soluble mannans from coffee residue and trying to obtain a pure mannan, another acetylation procedure was performed on the resultant residue, allowing to obtain CR2 with an additional yield of 3.7%. Sugars analysis (Table 2) shows that CR2 fractions had a sugars composition similar to those obtained for coffee infusions mannans. The amount of acetyl groups, on a molar basis in relation to the sugar residues, was 94%, a value comparable to that observed for CR1. However, this value is ten times higher than the one observed for the acetylation of coffee infusions but is close to that reported for AV acemannan.

The *in vitro* lymphocyte stimulatory effect of the chemically acetylated coffee residue mannans is also shown in Fig. 1. The B-lymphocytes were activated by the chemically acetylated coffee residue mannans with a maximum stimulatory effect observed of 79 and 26% for CR1 and CR2, respectively. These values were significantly different from those observed for the negative control (PBS, 4%) and LBG (5%) and the value for CR1 was similar to the one obtained for coffee infusions and AV. The amount of material to reach the reported maximum activation percentages was 50 μg/mL for CR1 and for CR2, which was similar to the concentration used for AV. The flow cytometry analysis of CD69 expression on the surface of C57/BL6 mice $CD4^+$ and $CD8^+$ T-cells in spleen mononuclear cell cultures also shows a stimulatory effect on theses lymphocyte population by coffee residue chemically acetylated mannans. On $CD4^+$ cells, the maximum observed activation was 13 – 18% for chemically acetylated coffee residue mannans, values lower than those obtained for AV (59%) and for coffee infusions (39 – 40%). The amount of material required to reach the reported maximum activation percentages was 100 μg/mL for CR1 and 25 μg/mL for CR2, the later a value similar to the concentration used for AV. For CD8+ cells, a maximum activation of 17 and 21% was obtained for CR1 and CR2, respectively. These values, although significantly different from those observed for LBG (4%), were lower than that obtained for AV (47%) and coffee infusions mannans (35 – 52%). The amount of material to reach the reported maximum activation percentages was 100 μg/mL for CR1 and 25 μg/mL for AV.

When tested in spleen mononuclear cell cultures, LBG did not show any stimulatory activity (Fig. 1).

4 Concluding remarks

The acetylated arabinogalactomannans from light and dark coffee infusions were shown to induce the *in vitro* expression of the surface lymphocyte activation marker CD69 on murine B and T lymphocytes. This effect was more marked on B- than on T-lymphocytes and did not lead to a detectable proliferative response by the stimulated cells (data not shown). In these assays, the acetylated arabinogalactomannans from coffee infusions showed a similar activity to that of AV mannans from a commercial source which was used for comparison. LBG galactomannan did not show any immunostimulatory activity.

These results show that, beyond the prebiotic activity and dietary fibre properties of coffee infusion mannans [7 – 9], these polysaccharides have also another important biological attribute: the immunostimulatory activity. Due to their indigestibility and high molecular weight, mannans are not expected to be absorbed by the gut but can stimulate the mucosal immune system, as has been reported for the non-digestible oligosaccharides [29]. Although no specific studies have been performed for mannans, dietary fibre, in general, has been known to quench minor dietary constituents, such as minerals, reducing their absorption in the small intestine [29]. However, dietary fibre fermentation has been shown to improve mineral absorption, produce several products that allow reducing gut infections, increase the levels of intestinal SCFAs, suppression of colon cancer initiation, alleviation of constipation, among other effects [29]. In the human colon, coffee mannans undergo rapid fermentation producing mannooligosaccharides and SCFAs [7 – 9]. In dog experiments, the colonic fermentation of mannooligosaccharides showed improvement of gut health by altering positively the microbial population and enhancing the immune capacity, as well as decreasing the concentration of putrefactive compounds [30].

This research opens the possibility of exploitation of coffee mannans as a source of bioactive polysaccharides with immunostimulatory activity. These polymers can be included as ingredients in daily intake food products in a large range of segments, namely, dairy industry (yogurt, milk and functional beverages); breakfast cereals, biscuits, bread, and snacks; soft drinks, and mineral and spring water. Also, development of coffee beverages with higher and optimised content in functional polysaccharides can be hypothesised.

A significant amount of water soluble coffee residue mannans can be recovered by 4 M NaOH solutions followed by partial acetylation. These mannans have similar immunostimulatory activity than those present in coffee infusions, although with a somewhat lower potency, but with lower amounts of material required for maximum activation, especially for CR2. These results show that the occurrence of acetyl groups in the polysaccharide may be an important structural characteristic for their immunostimulatory properties. Anyway, a more extensive study on the immunostimulatory and immunomodulatory properties of these coffee-derived polysaccharides will be necessary to ascertain possible applications of these molecules. Studies to establish structure – function relationships are required in order to understand the specific responses to the different polysaccharide structures.

The authors gratefully acknowledge the financial support provided of the project POCI/QUI/59337/2004, and Research Units 62/94 – QOPNA and Centro de Química de Vila Real, provided by FCT (Foundation for Science and Technology). Joana Simões thanks to FCT for the doctoral grant SFRH/BD/28572/2006.

The authors have declared no conflict of interest.

5 References

[1] Nunes, F. M., Coimbra, M. A., Chemical characterization of the high molecular weight material extracted with hot water from green and roasted arabica coffee, *J. Agric. Food Chem.* 2001, *49*, 1773 – 1782.

[2] Nunes, F. M., Coimbra, M. A., Chemical characterization of the high molecular weight material extracted with hot water from green and roasted arabica coffee, *J. Agric. Food Chem.* 2002, *50*, 7046 – 7052.

[3] Nunes, F. M., Coimbra, M. A., Chemical characterization of the high molecular weight material extracted with hot water from green and roasted robusta coffee as affected by the degree of roast, *J. Agric. Food Chem.* 2002, *50*, 1429 – 1434.

[4] Illy, A., Viani, R., *Espresso Coffee. The Chemistry of Quality*, Academic Press, London, 1995.

[5] Nunes, F. M., Coimbra, M. A., Duarte, A. C., Delgadillo, I., Foamability, foam stability, and chemical composition of espresso coffee as affected by the degree of roast, *J. Agric. Food Chem.* 1997, *45*, 3238 – 3243.

[6] Nunes, F. M., Coimbra, M. A., Influence of polysaccharide composition in foam stability of espresso coffee, *Carbohydr. Polym.* 1998, *37*, 283 – 285.

[7] Gniechwitz, D., Reichardt, N., Blaut, M., Steinhart, H., Bunzel, M., Dietary fiber from coffee beverage: degradation by human fecal microbiota, *J. Agric. Food Chem.* 2007, *55*, 6989 – 6996.

[8] Gniechwitz, D., Brueckel, B., Reichardt, N., Blaut, M., *et al*., Coffee dietary fiber contents and structural characteristics as influenced by coffee type and technological and brewing procedures, *J. Agric. Food Chem.* 2007, *55*, 11027 – 11034.

[9] Reichardt, N., Gniechwitz, D., Steinhart, H., Bunzel, M., Blaut, M., Characterization of high molecular weight coffee fractions and their fermentation by human intestinal microbiota, *Mol. Nutr. Food Res.* 2009, *53*, 287 – 299.

[10] Nunes, F. M., Domingues, M. R., Coimbra, M. A., Influence of polysaccharide composition in foam stability of espresso coffee, *Carbohydr. Res.* 2005, *340*, 1689 – 1698.

[11] Nunes, F. M., Reis, A., Domingues, M. R., Coimbra, M. A., Characterization of galactomannan derivatives in roasted coffee beverages, *J. Agric. Food Chem.* 2006, *54*, 3428 – 3439.

[12] Chow, J. T. N., Williamson, D. A., Yates, K. Y., Gous, W. J., Chemical characterization of the immunomodulation polysaccharide of *Aloe vera* L., *Carbohydr. Polym.* 2005, *340*, 1131 – 1142.

[13] Lee, J. K., Lee, M. K., Yun, Y. P., Kim, Y., Acemannan purified from *Aloe vera* induces phenotype and functional maturation of immature dendritic cells, *Int. Immunopharmacol.* 2001, *1*, 1275 – 1284.

[14] Schepetkin, I. A., Quinn, M. T., Botanical polysaccharides: macrophage immunomodulation and therapeutic potential, *Int. Immunopharmacol*. 2006, *6*, 317 – 333.

[15] Choi, S., Chung, M.-H., A review on the relationship between *aloe vera* components and their biologic effects, *Semin. Integr. Med.* 2003, *1*, 53 – 62.

[16] Zhang, L., Tizard, I. R., Activation of a mouse macrophage cell line by acemannan: the major carbohydrate fraction from *Aloe vera* gel, *Immunopharmacology* 1996, *35*, 119 – 128.

[17] Silva, M. A., Nebra, S. A., Silva, M. J. M., Sanchez, C. G., The use of biomass residues in the Brazilian soluble coffee industry, *Biomass Bioenerg.* 1998, *14*, 457 – 467.

[18] Yen, W. J., Wang, B. S., Chang, L. W., Duh, P. D., Antioxidant properties of roasted coffee residues, *J. Agric. Food Chem.* 2005, *53*, 2658 – 2663.

[19] Fischer, M., Reimann, S., Trovato, V., Redgwell, R. J., Polysaccharides of green arabica and robusta coffee beans, *Carbohydr. Res.* 2001, *330*, 93 – 101.

[20] Redgwell, R. J., Trovato, V., Curti, D., Fischer, M., Effect of roasting on degradation and structural features of polysaccharides in Arabica coffee beans, *Carbohydr. Res.* 2002, *337*, 421 – 431.

[21] Oosterveld, A., Voragen, A. G. J., Schols, H. A., Effect of roasting on the carbohydrate composition of *Coffea arabica* beans, *Carbohydr. Polym.* 2003, *54*, 183 – 192.

[22] Biswas, A., Shogren, R. L., Willett, J. L., Solvent-free process to esterify polysaccharides, *Biomacromolecules* 2005, *6*, 1843 – 1845.

[23] Tavares, C., Monteiro, S. R., Moreno, N., Lopes da Silva, J. A., Does the branching degree of galactomannans influence their effect on whey protein gelation?, *Colloid Surf. A Physicochem. Eng. Asp.* 2005, *270*, 213 – 219.

[24] Nunes, C., Rocha, S. M., Coimbra, M. A., Simple and solvent-free methodology for quantification of methanol and acetic acid content of plant polysaccharides based on headspace solid phase microextraction-gas chromatography (HS-SPME-GC-FID), *Carbohydr. Polym.* 2006, *64*, 306 – 311.

[25] Dourado, F., Madureira, P., Carvalho, V., Coelho, R., *et al*., Purification, structure and immunobiological activity of an arabinan-rich pectic polysaccharide from the cell walls of *Prunus dulcis* seeds, *Carbohydr. Res.* 2004, *339*, 2555 – 2566.

[26] Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., Smith, F., Colorimetric method for determination of sugars and related substances, *Anal. Chem.* 1956, *28*, 350 – 356.

[27] McAnalley, B. H. U. S. Patent No. 4,735,935.

[28] Oosterveld, A., Harmsen, J. S., Voragen, A. G. J., Schols, H. A., Extraction and characterization of polysaccharides from green and roasted coffee arabica beans, *Carbohydr. Polym.* 2002, *52*, 285 – 296.

[29] Swennen, K., Courtin, C. M., Delcour, J. A., Nondigestible oligosaccharides with prebiotic properties, *Crit. Rev. Food Sci. Nutr.* 2006, *46*, 459 – 471.

[30] Swanson, K. S., Grieshop, C. M., Flickinger, E. A., Bauer, L. L., *et al*., Supplemental fructooligosaccharides and mannanoligosaccharides influence immune function, ileal and total tract nutrient digestibilities, microbial populations and concentrations of protein catabolites in the large bowel of dogs, *J. Nutr.* 2002, *132*, 980 – 989.

