Polysaccharides from by-products of the Wonderful and Laffan pomegranate varieties: New insight into extraction and

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Corresponding Author: Professor Nadia Mulinacci, ASSOCIATE PROFESSOR

Corresponding Author's Institution: UNIVERSITY OF FLORENCE

First Author: Mohamad Khatib

Order of Authors: Mohamad Khatib; Camilla Giuliani; Federico Rossi; Alessandra Adessi; Amal Al-Tamimi ; Giuseppe Mazzola; Diana Di Gioia; Marzia Innocenti; Nadia Mulinacci, ASSOCIATE PROFESSOR

Abstract: The main crude polysaccharides (CPS), extracted from two widely cultivated pomegranate varieties, Laffan and Wonderful, were studied and characterized. We obtained the highest CPS extraction yield (approximatively 10% w/w on dried matter) by 1 h of decoction (ratio 1/40 w/v). The predominant polymers (75-80%) of the CPS samples shown a hydrodynamic volume close to 2000 kDa by size exclusion chromatography and the exocarp and mesocarp profiles were very similar. The proton spectra (1H-NMR), according to sugar composition and gelling ability, confirmed the main polysaccharide fractions were pectin with different acylation and methylation degree. The CPS from Laffan and Wonderful mesocarp showed prebiotic properties in vitro with Lactobacillus and Bifidobacterium strains. The composition of the decoction (12 % ellagitannins and 10 % of CPS) obtained by a green extraction process of pomegranate by-products, makes it a suitable component of functional food formulations.

Università degli Studi di Firenze

DIPARTIMENTO DI NEUROSCIENZE, PSICOLOGIA, AREA DEL FARMACO E SALUTE DEL BAMBINO
"NEUROFARBA"

Florence, May 5, 2017
Dear Editor,

We intend to submit the revised manuscript to your attention for publication as full article:

Polysaccharides from By-products of the Wonderful and Laffan Pomegranate Varieties: New Insight into extraction and characterization.

By

Mohamad Khatib, Camilla Giuliani, Federico Rossi, Alessandra Adessi, Amal Al-Tamimi, Giuseppe Mazzola, Diana Di Gioia, Marzia Innocenti, Nadia Mulinacci

Explanation of the manuscript's significance

In this fourth review we have tried to improve the quality of the manuscript by modifying the highlights, the abstracts and the conclusions and the text was again controlled for the language. Hoping to properly understand the editor's request, the changes have been made to improve the clarity and effectiveness of the text.

Hoping you can consider the work suitable for publication in Food Chemistry in this form.

Best Regards
Prof. Nadia Mulinacci

Viale G. Pieraccini, 6, 50139 FIRENZE Tel. (055) 4271.287-8 Fax: (055) 4271.280

Editor Comments:

1. Highlights are not meaningful. Revise and see previously published highlights in Food Chemistry.

2. The English usage in certain parts is poor and needs to be improved. For example, The aim of this study therefore was....Change to...The aim of this study was therefore.....

Answer

The highlights are now completely changed and, in our opinion are more meaningful

The text was again controlled for the English language and, some little changes are all over the text. Furthermore, hoping to properly understand the editor's requests we applied substantial modifications to Abstract and Conclusions. The aim was again to improve the clarity and the effectiveness of the text.

All the applied changes are highlighted in red, particularly for the abstract (lines 25-35) and for the conclusions (lines 424-425; 429-434; 438-439; 443-446).

Highlights

The highest extractive yields of polysaccharides were obtained by 1h of decoction

Polysaccharides profiles by SEC were similar for the Laffan and Wonderful varieties

^{1}H-NMR confirmed the presence of pectin at different methylation/acylation degree

Crude polysaccharides from Laffan and Wonderful showed in vitro prebiotic properties

Polysaccharides from By-products of the Wonderful and Laffan Pomegranate Varieties: New Insight into extraction and characterization.

Mohamad Khatib, Camilla Giuliani, [2]Federico Rossi, [2]Alessandra Adessi, [1]Amal Al-Tamimi
[3]Giuseppe Mazzola, [3]Diana Di Gioia, Marzia Innocenti, *Nadia Mulinacci

Department of Neuroscience, Psychology, Drug and Child Health, Pharmaceutical and Nutraceutical Section, University of Florence, Via Ugo Schiff 6, Sesto Fiorentino, (FI) Italy.

1 Biology Department of Ecology, Princess Noura University, Al Imam Abdullah Ibn Saud Ibn Abdul Aziz Road, Riyadh, Saudi Arabia.

2 Department of the Sciences of Agrifood Production and the Environment, University of Florence, Piazzale delle Cascine, 18 - 50144 Firenze, Italy.

3 Department of Agricultural Sciences, University of Bologna, Viale Fanin, 44 - 40127 Bologna,Italy.

*Corresponding author: Nadia Mulinacci
Dipartimento di NEUROFARBA, Università degli Studi di Firenze, Via Ugo Schiff 6, 50019 Sesto Fiorentino, Firenze, Italy.
Tel.: 0039-055-4573773

e-mail: nadia.mulinacci@unifi.it

Abstract

The main crude polysaccharides (CPS), extracted from two widely cultivated pomegranate varieties, Laffan and Wonderful, were studied and characterized. We obtained the highest CPS extraction yield (approximatively 10% w/w on dried matter) by 1 h of decoction (ratio 1/40 w/v). The predominant polymers (75-80%) of the CPS samples shown a hydrodynamic volume close to 2000 kDa by size exclusion chromatography and the exocarp and mesocarp profiles were very similar. The proton spectra (^{1}H-NMR), according to sugar composition and gelling ability, confirmed the main polysaccharide fractions were pectin with different acylation and methylation degree. The CPS from Laffan and Wonderful mesocarp showed prebiotic properties *in vitro* with *Lactobacillus* and *Bifidobacterium* strains. The composition of the decoction (12 % ellagitannins and 10 % of CPS) obtained by a green extraction process of pomegranate by-products, makes it a suitable component of functional food formulations.

Keywords: mesocarp, prebiotic activity, pectin, size exclusion chromatography, ^{1}H-NMR

1. Introduction

The *Punica granatum* L. (Punicaceae) fruit has been extensively used in the folk medicine of many cultures (Viuda-Martos, Fernández-Lóaez, & Pérez-álvarez, 2010), exhibiting a wide range of potential clinical applications (Viuda-Martos et al., 2010) including antitumor properties (Joseph, Aravind, George, Varghese, & Sreelekha, 2013; Joseph, Aravind, Varghese, Mini, & Sreelekha, 2012; Li, Zhang, & Wang, 2014). Up until now, research indicated that the ellagitannins are the principal bioactive constituents of the different extracts obtained from pomegranate fruit. However, there are few studies regarding the extraction and characterization of the polysaccharide fractions recovered from the different parts of the fruit.

To date, natural polysaccharides have been proven to exert antioxidant, antitumor, immunomodulatory, antimicrobial, antiulcer and hypoglycemic activities (Leung, Liu, Koon, & Fung, 2006; Negi, Jayaprakasha, & Jena, 2003; Schepetkin & Quinn, 2006). A polysaccharide extracted from pomegranate peel has shown significant antioxidant, antiglycation and tyrosinase inhibition properties (Rout & Banerjee, 2007). A more recent paper showed that a galactomannan recovered from the fruit rind of *P. granatum*, exerted *in vitro* immunomodulatory and free radical scavenging activities (Joseph et al., 2012), as well as anticancer activity in mice by reducing the tumor either alone or in combination with doxorubicin (Joseph et al., 2013). One of these studies provided evidence of the non-toxic nature of this plant-derived compound, which was also proposed as an adjuvant or as a single agent for the treatment of cancer (Joseph et al., 2013). Polysaccharides from pomegranate peel have also been reported as able to inhibit the proliferation of U-2 human osteosarcoma cancer cells by inducing apoptosis mainly through a mitochondrial signalling pathway (Li et al., 2014).

At the same time, it is known that polysaccharides are an important part of soluble fermentable dietary fiber. They can exhibit prebiotic activity by stimulating the growth of beneficial bacteria in the colon, thereby contributing to the healthy status of the gut (Di Gioia et al., 2014a; Marotti et al.,

2012). A balanced gut microbial composition confers benefits to the host, due to the modulation of metabolic and immune functions, while microbial imbalances are associated with metabolic disorders and/or diseases (Di Gioia, Aloisio, Mazzola, & Biavati 2014b; Tremaroli & Bäckhed, 2012). Therefore, the maintenance of a correct equilibrium between beneficial microorganisms, mainly belonging to the *Bifidobacterium* and *Lactobacillus* genera, and potentially pathogenic strains, is crucial for host health.

The interest in food processing by-products has increased recently. In particular, waste by-products from pomegranate juice production are being considered for the recovery of bioactive compounds, primarily ellagitannins (Akhtar, Ismail, Fraternale, & Sestili, 2015; Goula & Lazarides, 2015), while polysaccharides have not yet been considered. In literature, it is frequently used to indicate the exocarp (the real peel) and mesocarp together, without making a real distinction between these two parts of the fruit(Viuda-Martos et al., 2010). The main by-products of juice production, the mesocarp (40-50 % of the whole fruit) and exocarp, have not been investigated singularly as potential sources of bioactive polysaccharides, and no data are available on the possible prebiotic properties of these polymers recovered from pomegranate. To the best of our knowledge, none of the studies on pomegranate polysaccharides have taken into account the Wonderful and Laffan varieties, the objects of our investigation.

The aim of this research was to study the polysaccharides from the by-products of Laffan and Wonderful, generated in large amount from these two widely diffused pomegranate varieties. Water extraction processes were applied to efficiently recover the polysaccharides separately from the mesocarp and exocarp. Size exclusion chromatography, chemical hydrolysis and proton nuclear magnetic resonance (^{1}H-NMR) were used to analyze the characteristics of the principal polysaccharides. For the first time, the prebiotic properties of crude polysaccharides (CPS) from the Wonderful mesocarp were assessed by *in vitro* by testing them on *Bifidobacterium* and *Lactobacillus* strains. The dry decoction is proposed as source of polysaccharides and ellagitannins.

2. Materials and methods

2.1 Materials

The Laffan cultivar (sour-sweet) was harvested from Rif Idlib, Syria in October 2011; the Wonderful cultivar was purchased from Ortofrutta Grosseto (Italy) in October 2013. About 7-10 kg of fresh ripe fruits of both cultivars were used as the source of the exocarp and mesocarp for extraction of polysaccharides.

2.2 Extraction process for recovery of CPS

The exocarp and mesocarp were manually separated from fresh pomegranate fruits, then cut into small pieces and freeze-dried. Both parts were powdered in a grinder and extracted with distilled water as summarized in Table 1. The term dried matter in the text refers to the dry weight of the mesocarp and exocarp. Each solution, recovered after centrifugation (3900 *g* for 12 min at 5°C) according to the methods listed in Table 1, was supplemented with 2 volumes of ethanol and kept for 3 h at 0°C to induce the precipitation of polysaccharides, which were recovered after a new centrifugation, again at 3900 *g* for 12 min at 5°C. The further addition of ethanol to the supernatants did not induce the formation of new precipitate. The recovered polysaccharides were freeze-dried, then re-dissolved in a minimum water volume and treated again with 2 volumes of ethanol. The precipitate was then freeze-dried to obtain the crude polysaccharides (CPS) which were successively treated to remove the proteins according to the method reported in Joseph et al., (2012). Briefly, CPS were dissolved in water and extracted with 3 volumes of chloroform. The extraction was repeated several times until the water/chloroform inter-phase became clear. The aqueous phase containing the purified polysaccharides was recovered and freeze-dried to obtain CPSp as summarized in Table 3. CPSp were re-dissolved in water, and 500 µL of this solution were transferred into an ultra-filter device with a cut-off of 10,000 Daltons (Amicon, Millipore, Billerica,

MA) and centrifuged at 11,000 *g* for 15 min. The precipitate was re-suspended in its original volume by adding water; the process was repeated up to 7 times, (as indicated by the supplier), to remove about 99% of possible fouling materials (polar low molecular weight molecules and salts) from the sample. After these cleaning steps, the filter device was placed upside down in a clean microcentrifuge tube for 2 minutes at 1,000 *g*; 500 µL of distilled water were then added to dissolve and recover the polysaccharides after filtration.

2.3 ^{1}H- NMR analysis

The purified polysaccharides from the mesocarp of Wonderful (W-CPSp) and Laffan (L-CPSp) were dialyzed for 48 h at 5°C in a nitrocellulose membrane with a 12-14 kDalton cut-off (Medicell International Ltd, London), and then freeze-dried. The dried samples were dissolved in 1 mL of D_2O and maleic acid was added as internal standard as follows: 6.1 mg W-CPSp and 1.1 mg of maleic acid, 6.3 mg L-CPSp and 1.3 mg of maleic acid, purity grade 98% (Merck, Germany). The ^{1}H-NMR experiments were carried out using a 400 MHz instrument Advance 400 (Bruker, Bremen, Germany). The quantitative evaluation was done according to reference guidelines (Eurolabs, 2014), applying the same protocol previously used for other matrices (Khatib, Pieraccini, Innocenti, Melani, & Mulinacci, 2016).

2.4 Determination of monosaccharide composition

The polysaccharides in Table 2 from Laffan and Wonderful mesocarp and exocarp were hydrolyzed in acidic media (Erbing, Jansson, Widmalm, & Nimmich, 1995). Briefly, 1 mL of a 2 M trifluoroacetic acid water solution was added to 5 mg CPSp, maintained at 120°C for 120 min. Afterwards, samples were cooled on ice, and ultrafiltered at 3500 *g* for 20 min using 3,000 Daltons cut-off centrifuge filter devices (Amicon Ultra-4, Millipore, Billerica, MA). The supernatant was then dried by a rotavapor at 37 °C, and re-suspended in 1 mL MilliQ-grade water. This

139 evaporation/re-suspension process was repeated three times, with the aim of removing the
140 trifluoroacetic acid that could introduce bias into the analysis. The samples were washed twice with
141 MilliQ-grade water, re-dissolved in 1 mL deionized water and then analyzed by ion exchange
142 chromatography using a ICS-2500 ion chromatograph with an ED_{50} pulsed amperometric detector, a
143 gold working electrode and a Carbopac PA1 250x4mm column, all from Dionex (Sunnyvale, CA,
144 USA). The eluents used were MilliQ-grade water (solution A), 0.185 M sodium hydroxide solution
145 (solution B), and 0.488 M sodium acetate solution (solution C). A gradient elution was used
146 consisting of a first stage (injection time up to the 7th min) with the eluent constituted by 84%
147 solution A, 15% solution B, and 1% solution C; a second stage (injection time from the 7th to 13th
148 min) with the eluents constituted by 50% solution B and 50% solution C; and a final stage (injection
149 time from the 13th to the 30th min) with the eluents consisting of 84% solution A, 15% solution B,
150 and 1% solution C. The flow rate was 1 mL min^{-1}. The monosaccharides were detected according to
151 the retention time of pure monosaccharides purchased from Sigma-Aldrich (Milan, Italy) after
152 specific spike injections of the pure monosaccharides; at least three standard injections were
153 repeated in order to obtain a mean retention time of each monosaccharide, and the variance never
154 exceeded 5%.
155
156 *2.5 Size Exclusion Chromatography*

157 The apparent molecular weight of the polysaccharides of the CPS samples was determined
158 according to a previously reported method (Chen et al., 2014; Colica, Li, Rossi, De Philippis, &
159 Liu, 2015), with some modifications. The samples listed in Table 3 were weighed and dissolved in
160 distilled water, at a concentration of roughly 0.14 mg mL^{-1}. The solution was analyzed using a
161 Varian ProStar HPLC chromatograph (Varian, USA) equipped with a 355 refractive index detector
162 and a Biosep s4000 column (Phenomenex, USA). The samples were analyzed with 30 min runs by
163 HPLC-grade water as eluent at 0.6 mL min^{-1} flow rate. Blue dextrans (Sigma-Aldrich, USA) at

different molecular weights (approx. 2000 kDa, 1100 kDa, 410 kDa, 150 kDa and 50 kDa) were used as standards for hydrodynamic volume calculation.

2.6 In vitro evaluation of the prebiotic activity of CPS

The ability of CPS to induce the growth of beneficial bacteria (prebiotic activity) was assayed using two strains previously isolated from human feces: *Bifidobacterium breve* B632 (Aloisio et al., 2012) and *Lactobacillus plantarum* L12. The latter was isolated from a healthy volunteer (unpublished results) and taxonomic characterization was performed via 16S rDNA amplification and sequencing (Gaggìa et al., 2013), this strain is available at the Bologna University, Scardovi Collection of Bifidobacteria. Both strains were stored in lyophilized form. When necessary, they were re-vitalized in de Man Rogosa Sharpe (MRS) medium (Oxoid, Basingstone, UK) supplemented with 0.05% cysteine and incubated in anaerobic conditions at 37°C for 24 h. Anaerobic conditions were created in a capped jar using an anaerobic atmosphere generation system (Anaerocult A, Merck, Darmstadt, Germany).

The MRS medium composition was modified to perform the growth experiment with the pomegranate polysaccharides. The modified medium (m-MRS) did not contain the carbon source (glucose), which was provided by the pomegranate polysaccharides, and had a halved amount of potential growth substrate, such as peptone, yeast extract and meat extract compared to those present in the original medium (peptone, 5 g L^{-1}; yeast extract, 2 g/L, meat extract, 5 g L^{-1}, where the amounts are in m-MRS).

The prebiotic activity was evaluated using CPS at 0.5% (w/v) in m-MRS. A positive growth control was performed using m-MRS with 0.5% glucose and a negative control in m-MRS with no added carbon source. The medium containing CPS as the carbon source was prepared as follows: the m-MRS ingredients were weighed in a flask, dissolved in water and the medium was autoclaved at 120°C for 15 min. A 0.5% (w/v) fiber or glucose at the same concentration were added to the hot

189 medium, stirred, and sterilized again at 102°C for 10 min. This procedure allowed the fiber to
190 dissolve in the medium and, at the same time, to prevent risk of growth of undesirable
191 microorganisms. The *B. breve* B632 and *L. plantarum* M12 strains were grown overnight in the
192 respective media, centrifuged, washed in phosphate buffered saline (PBS) and re-suspended in PBS
193 to obtain a solution having an absorbance of 0.7 at 600 nm. This suspension was used to inoculate at
194 2% (v/v) the flasks containing the m-MRS medium plus the fiber, the m-MRS medium plus glucose
195 (positive control) and the m-MRS medium with no additional carbon source (negative control). The
196 flasks were incubated at 37°C in anaerobic conditions for 48 h and a 1 mL culture was sampled
197 from each flask for viable bacterial counts at pre-established times (0, 6, 24, 30 and 48 h of
198 incubation). The sampled amount was mixed with 9 mL of PBS, serially diluted in the same
199 solution and plated on agarized MRS supplemented with cysteine. Following incubation of the
200 plates at 37°C in anaerobic conditions for 24 h, the number of colonies, corresponding to the
201 number of viable cells, was counted. The number of cells expressed as CFU mL^{-1} were transformed
202 into Log_{10} value (Log CFU mL^{-1}).
203
204 *2.7 Proximate composition and dietary fiber analyses*
205 The proximate composition was determined for the decoction from mesocarp of the Wonderful
206 variety. Protein content (PC) was evaluated using the Kjeldhal method: PC (g/100g) = N*6.25,
207 where N is total nitrogen. The total fat content was determined by Soxhlet extraction, and
208 gravimetrically determined according to ISS protocol (1996/34). Ash content was evaluated by
209 gravimetric assay, according to ISS protocol, (1996/34 method b). Dietary fibers (both soluble and
210 insoluble) were quantified according to AOAC method 991.43 (Determination of soluble, insoluble
211 and total dietary fiber in foods and food products, final approval 1991).
212
213 2.8 *Statistical analysis*

All data in Figure 1, Tables 1 and 3, are presented as mean±SD from triplicate measurements of each measuring point. Statistical significance for evaluating the prebiotic properties of CPS from the mesocarp of Laffan and Wonderful cultivars was calculated within each evaluation time (T6, T24, T30, T48) with a t-test, using the MEANS procedure (SAS).

3. Results and Discussion

3.1 Recovery of polysaccharides: preliminary evaluation on Laffan

The decision to study the by-products from Laffan and Wonderful pomegranate varieties, was mainly determined by the high amount produced because the diffusion of the two varieties. The Laffan pomegranate is widely present in Syria but also in Southern Turkey and Israel, while the Wonderful is one of the principal variety cultivated in the Western world. Although a valorisation of the by-products derived from juice production, requires better knowledge of their composition, so far little attention has been addressed to polysaccharides from pomegranates, and the fruits are mainly known for their juice rich in anthocyanins and ellagitannins. Water extraction (sometimes coupled with increased temperature), and subsequent precipitation by adding ethanol, is the most commonly utilized method for recovering polysaccharides from different sources (Huie & Di, 2004; Joseph et al., 2012; Zhu & Liu, 2013). We used a similar procedure on the mesocarp and exocarp separately, to evaluate the polysaccharide content of Wonderful and Laffan. First of all, to select the most efficient extractive procedure, we used the Laffan mesocarp as reference material.

To increase the extractive yields, (Table 1), different extraction times, extraction temperatures and dried matter/water ratios, were evaluated. Firstly, 30 and 60 min were set, applying a single or two successive extraction steps, and varying the extractive ratio from 1:15 w/v to 1:40 w/v. The extraction was firstly performed at 25±2 °C as previously proposed for pomegranate (Rout & Banerjee, 2007). A second approach was to pre-treat the dried material with a hydroalcoholic solution to remove the ellagitannins, and then extract the polysaccharides by hot water.

To remove part of the impurities co-precipitated after the first ethanol addition, the polysaccharides were re-dissolved in water and precipitated again, adding ethanol to get the CPS listed in Table 1. To verify if this latter step was effective in cleaning the polysaccharides, we evaluated the amount of the impurities by weighing the dried supernatant recovered after the second ethanol addition. The impurities were 5.4% and 7.4% of dried mesocarp for Wonderful and Laffan, respectively, and close to 3% of the dried exocarp for both varieties. These results indicate that the second addition of ethanol was necessary to obtain a cleaner polysaccharide fraction (CPS).

As shown in Table 1, the yield in CPS increased from 5% to 8% with a longer extraction time (from 30 to 60 min) by applying the same extractive ratio (1:15 w/v). The yield further increased up to 10% by applying a single extraction of 60 min and a higher extractive ratio (1/40 w/v). A successive extractive step of 60 min, as well as previous contact of the dried material with water before the decoction, did not increase the recovery of CPS.

Overall, the best result in terms of yield and reproducibility, was obtained with a single decoction of 60 min and an extractive ratio of 1/40 p/v (Table 1). Similar recoveries of polysaccharides (10-13%) were reported by Zhu et al., for a pomegranate purchased from a local Chinese market and extracted by hot water (Zhu and Liu, 2013). The same authors have successively proposed an ultrasound-assisted hot water extraction, but obtained similar yields (Zhu, Zhai, Li, Wu, & Li, 2015). In both these studies, and as frequently reported in literature, the authors cited the pomegranate peel but did not specify if the raw material was comprised only of the exocarp or of the mesocarp plus exocarp. Lastly, and in agreement with a previous report (Rout & Banerjee, 2007), we confirmed that extraction with water at a temperature close to 25°C, even when applying long extraction times, gave considerably lower CPS yields (Table 1).

Since there are about 12% of ellagitannins in the decoction of Laffan mesocarp (Khatib, 2015), a pretreatment with ethanol 70% v/v was also tested to remove these polar compounds before precipitation of the CPS. Even if the CPS yields are lower than those obtained without applying this

264 treatment, this latter approach can be useful when the objective is to efficiently recover the
265 ellagitannins before precipitation of polysaccharides (Table 1).
266
267 *3.2 CPS recovered from the two cultivars*

268 After the pre-screening carried out only on the Laffan mesocarp, only the more efficient
269 methods were selected to extract the CPS from the Laffan and Wonderful exocarp and mesocarp
270 (Table 2). Overall, by applying the same extractive method, we obtained similar results from the
271 exocarp and mesocarp of the two varieties. Again, a one-step decoction gave the highest % yields,
272 and the hot water is determinant for maximizing the extractability of CPS because it increases
273 polysaccharide solubility. On the other hand, the extraction carried out at room temperature, was
274 confirmed as the worst. Despite the low yields, this latter method was tested again to verify the
275 effect of temperature on the characteristics of CPS. To this aim, we analyzed the recovered
276 polysaccharides by size exclusion chromatography and compared their profiles with those from
277 CPS obtained by the hot extraction. As shown in Table 2, CPS were mainly located in the mesocarp,
278 with lower values in the exocarp (4.5-4.7%) for both varieties. There was some variability in the
279 method with the CPS amount recovered from exocarp having higher standard deviation values,
280 from 11% to 25%. This finding is attributable to the non-homogeneous thickness of the removed
281 exocarp, still containing residual parts of mesocarp, which is hard to completely remove. Lastly, the
282 decoction of the exocarp, carried out after a previous extraction with ethanol/water (7:3v/v),
283 provided CPS amounts close to 4% and similar to those derived without using the hydroalcoholic
284 solution pre-treatment.

285 It must be emphasized that boiling is a suitable method not only for polysaccharides but also
286 for co-extraction of the ellagitannins in amounts close to 120 mg/g dried decoction (Khatib, 2015).
287 Furthermore, the drying process of the decoction did not require the addition of excipients such as
288 the maltodextrins, commonly used to reduce the final hygroscopicity of the dried herbal extract.

289 This advantage, not frequently observed during the management of herbal products, can be
290 attributable to the presence of CPS in a relatively high amounts.

291 Due to the difficulty of procuring enough fresh Laffan pomegranate during the civil war in
292 Syria, we only determined the nutritional composition of the Wonderful mesocarp decoction. In
293 summary, the total dietary fiber determined by the AOAC.993.41 method was 9.66 % comparable to
294 the CPS content. Moreover, the main fraction was soluble fiber, (6.67 %) fermentable by human
295 microbiota. There was 2.3 % of total proteins and 5.6 % ash, indicating an appreciable amount of
296 minerals.

297

298 *3.3 Sugar composition by hydrolysis*

299 To verify the purity grade of polysaccharides in terms of the co-presence of oligosaccharides
300 and inorganic salts, the efficiency of the ultrafiltration devices was tested on CPSp from the
301 mesocarp of the two varieties (Table 2). The hydrolysis of CPSp samples before and after the cut-
302 off filtration provided the same results in terms of molar percentage of monosaccharides suggesting
303 that the samples listed in Table 2 did not need further purification by this filter device.

304 The CPSp samples listed in Table 3 were treated with TFA acid to hydrolyze the polysaccharide
305 strands and subsequently determine sugar composition by ionic exchange chromatography,
306 according to a previous method (Erbing et al., 1995). The CPSp samples from both the mesocarp
307 and exocarp showed a very similar composition for both the varieties (Table 3). Hexoses galactose
308 and glucose, dehoxysugar rhamnose, and galacturonic acid, were the most abundant monomers,
309 while the main aldopentoses were xylose and arabinose. From our findings it emerges that these two
310 varieties, Laffan from Syria and Wonderful widely diffused throughout the western world, have a
311 very similar compositional profile in terms of polysaccharides. This result is not completely
312 unexpected, and in agreement with a previous work in which it was hypothesized that the
313 Wonderful is derived from the more antique Laffan variety (Goor, 1967).

Overall, other reports on pomegranate by-products did not include the varieties selected in this study. In regard to polysaccharides, the literature indicates there is a wide variability in terms of sugar composition depending on the variety, growth site and purity grade of the polysaccharide itself (Normakhtov, Rakhmanberdyeva, & Rakhimov, 1999; Jahfar, Vijayan, & Azadi, 2003) .

3.4 Characterization of the polysaccharide fractions

The CPSs from the mesocarp and exocarp of the two cultivars were analyzed by size exclusion chromatography to determine their apparent molecular weight. Since these polymers may be characterized by a branched structure, often related to the presence of arabinose, galactose and xylose, their size was calculated in terms of hydrodynamic volume, and not in terms of actual molecular weight. The CPS samples were compared to dextrans standards, considering that a 2000 kDa fraction has the same hydrodynamic volume as dextran at 2000 kDa molecular weight. The analyzed fractions throughout the text are identified as molecular weight, although with approximation.

The data in Figure 1 highlighted that CPS of Laffan and Wonderful mesocarp and exocarp, were of similar molecular weight, since no significant differences were found; all the CPS were characterized by a predominant fraction of about 2000 kDa, accounting for 75.4% of the total. The remaining 24.6% was represented by five minor fractions, the most common being: i) a fraction having a molecular weight between 410 kDa, and 150 kDa (7.4% of total CPS); ii) a fraction having a molecular weight lower than 50 kDa, accounting for 8.9% of total CPS. As expected, more variability was observed for the fractions having small molecular weights (much lower than 50 kDa).

Few reports are available to date on polysaccharide structure from pomegranate fruit. A first report described a glucofructan extracted from the peel, having 31 kDa molecular weight, that was separated using Sephadex G100 column (Jahfar, et. al., 2003). Sun described a polysaccharide extracted from the rind of a non-specified variety, having a molecular weight of 110 kDa

339 determined by gel filtration on a Sephadex G200 column and dextrans at different molecular
340 weights as reference standards (Sun, Li, Yan, & Liu, 2010). More recently, a glucomannan was
341 extracted from the rind of a ripe pomegranate fruit and the authors indicated a molecular weight of
342 110 kDa (Joseph et al., 2012). None of these studies specified which cultivar or variety was
343 investigated.

344 The present work shows the molecular weight distribution of pomegranate polysaccharides
345 obtained from the Laffan and Wonderful cultivars by using size exclusion chromatography for the
346 first time. We demonstrated that the CPS samples have similar apparent molecular weight
347 distribution with overlapping profiles of the two cultivars and the two parts of the fruit. We also
348 verified that hot extraction (100°C, 1 h) did not modify the CPS composition as demonstrated by
349 the complete overlap of size exclusion chromatography profiles obtained after extraction with cold
350 water and boiling water (Table 2).

351 Recently, some authors (Moorthy, Maran, Surya, Naganyashree, & Shivamathi, 2015; Pereira et
352 al., 2016) observed the presence of pectin in pomegranate fruit but no spectral data, particularly ^{1}H-
353 NMR spectra, are reported or discussed. Taking into account these data, we searched for the
354 presence of pectin by analysing of the proton spectra of CPSp from Laffan and Wonderful mesocarp
355 (Figure 2). According to the literature (Bédouet, Courtois, & Courtois, 2003), specific signals
356 indicate the presence of O-methyl and O-acetyl groups typical of pectin and their intensity can be
357 associated with the degree of methylation and acetylation. As shown in Figure 2, the two spectra
358 obtained dissolving comparable amounts of CPSp from the two varieties, clearly revealed signals
359 attributable to O-acetyl groups in the region (of δ 1.98-2.15) and an intense signal ascribable to a
360 singlet of O-methyl groups close to δ 3.7. Both these data and the high percentage of galacturonic
361 acid after the acidic hydrolysis (Table 3), confirm the presence of pectin in Laffan and Wonderful.
362 The singlet at δ 6.31 is due to maleic acid, that added as internal standard permits a preliminary
363 comparison of the degree of methylation and acylation in CPSp from the two varieties. In other

words, the addition of an accurately weighed internal standard can be usefully applied for quantitative purposes. Particularly, the higher intensity observed for the signal at δ 2.15 in the Wonderful spectrum, indicates a higher degree of acylation compared to that of Laffan. The opposite behavior is observed for the signal at δ 1.98 ppm that was more intense in Laffan sample. Analogously, a different degree of methylation is indicated by the signal at δ 3.73, ascribable to a singlet of O-methyl groups (Cui, 2005) at higher intensity in the Laffan sample. Finally, the presence of less intense signals close to δ 1.1 is in agreement with the presence of low amounts of rhamnose units according to hydrolysis results (Table 3). Overall, this rapid measurement, obtained without the need of high magnetic field spectrometer, was able to point out structural differences between W-CPSp and L-CPSp, not highlighted by the size exclusion chromatography technique, showing the same profile for these samples.

Although further studies are needed to elucidate the structure of these polysaccharides, the ^{1}H-NMR spectra and the sugar composition derived by acidic hydrolysis suggest that the main polysaccharides of pomegranate mesocarp are pectin with different degree of methylation and acetylation. This applied hydrolysis method was recently confirmed as being suitable to guarantee a complete hydrolysis of pectin (Wikiera, Mika, Starzyńska-Janiszewska, & Stodolak, 2015).

We also carried out preliminary tests to evaluate the water-absorption ability of some dried polysaccharide fractions: LM-CPS, WM-CPS and WE-CPS. The adsorbed water ranged from 98.6 to 99.1% of the dried material. Adsorption was rapid and the final samples appeared as clumps with a gel consistency, exhibiting the well-known pectin behaviour.

3.5 *In vitro evaluation of prebiotic properties*

The decoction from mesocarp, was used to recover the CPS for *in vitro* tests of prebiotic properties. We investigated the ability of *B. breve* B632 and *L. plantarum* L12 strains to use crude polysaccharides from pomegranate exocarp and mesocarp as their carbon source and compared this

389 to their growth on glucose, *i.e.* an easily fermentable carbon source. Bifidobacteria and Lactobacilli
390 are able to compete for nutrients with enteric pathogens, to adhere strongly to the intestinal mucosa,
391 thus preventing pathogen adhesion, and to stimulate the development of the mucosal immune
392 system. Moreover, they are known to provide some protection against incoming enteric pathogens
393 in man (Jankowska, Laubitz, Antushevich, Zabielski, & Grzesiuk, 2008; Symonds et al., 2012).

394 Figure 3 shows that both strains grow well on CPS from Laffan and Wonderful mesocarp, being
395 significantly higher ($p<0.01$) than the negative controls (i.e. with no added carbon source) and
396 comparable to that of an easily fermentable carbon source such as glucose added at the same
397 concentration. Growth on the Laffan variety at 24 h was only 0.6 and 0.1 Log CFU/mL lower than
398 that on glucose for *L. plantarum* L12 and *B. breve* B632, respectively. Growth on the Wonderful
399 variety at 24 h was 1.0 and 0.2 Log CFU/ml lower, respectively, than that on glucose for the same
400 strains. After the 24^{th} h of incubation, both strains grown on glucose entered the steady phase,
401 whereas a small decrease in cell survival was observed with CPS as the carbon source. The results
402 shown in Figure 3 clearly indicate that CPS and/or the products of their degradation are not toxic
403 for the assayed strains and, on the contrary, are good growth substrates for them. Growth on the
404 medium with no added carbon source reached only a 1 Log CFU/mL increase at the 24^{th} h
405 compared to the beginning of incubation, thus showing that the m-MRS medium used in the
406 experiments is a valid choice for performing prebiotic activity tests. Furthermore, if we might
407 propose the whole dried decoction for human consumption, due to its easy and rapid preparation,
408 the same is not true for the sub-fractions in Figure 1, whose preparation was longer and more
409 complex. Evaluation of the prebiotic activity of a single fraction from CPS was outside of the scope
410 of our research, but could be object of future investigations.

411 In agreement with our results, a high ability to ferment pectin by human gut microbiota
412 associated with an increase of almost 25 % of *Bifidobacterium* has been demonstrated *in vitro*
413 (Yang, Martinez, Walter, Keshavarzian, & Rose, 2013). Moreover, several studies in the literature
414 (as reviewed by Koropatkin, Cameron, & Martens, 2012) show that the degradation of complex

carbohydrates (glycans and polysaccharides) is a major symbiotic function carried out by microorganisms that inhabit the human distal gut, which increases host nutrition by digesting glycans that the host cannot degrade, providing the host with usable metabolic products such as short-chain fatty acids. Therefore, glycans shape the composition of the gut microbiota. Members of the Firmicutes and Actinobacteria phyla, to which *Lactobacillus* and *Bifidobacteria* spp. belong, possess different glycan acquisition strategies that also involve glycan-degrading enzymes (Mahowald et al., 2009).

4. Conclusions

This work improves the knowledge of the chemical and physical properties of polysaccharides recovered from the typical wastes of the pomegranate fruit, and reveals future perspectives for adding value to these food by-products, produced in large amount but currently discarded. The use of hot water maximized solubility and extractability of the crude polysaccharides from the Laffan and Wonderful varieties. The maximum recovery of polysaccharides was obtained from mesocarp, by a single-step water decoction. At the same time, the boiling process did not modify the molecular size distribution of the polysaccharides as demonstrated by their profiles in size exclusion chromatography, comparable with those obtained by a cold-water extraction. For the first time, the size exclusion chromatography was applied to evaluate the polysaccharides from mesocarp and exocarp of Laffan and Wonderful. A very similar distribution of the apparent molecular weights of the main polysaccharides was highlighted for the two varieties, with chromatographic profiles characterized by a predominant polymer with a hydrodynamic volume close to 2000 kDa, and five other minor fractions. The ^{1}H-NMR spectra, the sugar composition and the high gelling capacity of some purified polysaccharide fractions of mesocarp, confirmed the presence of pectin as primary component. The use of maleic acid as internal standard was proposed to evaluate the acylation and methylation degree of the main purified polysaccharide fractions. Finally, the crude polysaccharides

from Laffan and Wonderful pomegranate mesocarp showed prebiotic properties *in vitro* by serving as an excellent substrate for the growth of potentially probiotic bacteria such as *Lactobacillus* and *Bifidobacterium* strains.

We showed that, after a simple decoction of these pomegranate fruit by-products, it was possible to obtain a dry extract rich in polysaccharides with prebiotic activity, associated with a pool of bioactive ellagitannins. This combination of natural compounds can help to valorize these by-products and to enhance the use of pomegranate dry decoction in functional food formulations.

Acknowledgments.

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565

Figure Captions

Figure 1. Apparent molecular weight distribution by size exclusion chromatography (abscissa) of the CPSp samples from mesocarp and exocarp, data expressed as peak area % of total areas, as mean from triplicate measurements. *W, Wonderful; L Laffan*.

Figure 2. ^{1}H-NMR spectra in 1mL of D_2O, at room temperature (23±2 °C) for: Laffan mesocarp - CPSp (6.2 mg + 1.3 mg maleic) and Wonderful mesocarp-CPSp (6.05 mg + 1.12 mg maleic acid). *O-Acetyl, singlet of the acetyl groups; Gal A-OCH_3, singlet of the methoxyl group of galacturonic acid units*

Figure 3. Evaluation of prebiotic properties of CPS (5 % in m-MRS medium) from the mesocarp of Laffan and Wonderful cultivars on **(a)** *L. planctarum* L12 and **(b**) *Bifidobacterium breve* B632. *C-: growth on m-MRS with no added carbon source; C+: growth on m-MRS with 0.5% glucose; CFU, colony forming units*

Table 1. Applied methods to recover CPS from Laffan mesocarp and corresponding extraction yields (*mean values* as weight/dried matter); all the determinations were carried out in triplicate except for of **1a** and **1b** methods that were in single.
§ *24 hrs pretreatment with water before decoction;* ***** *24 hrs pretreatment with 70% ethanol at 25°C.*

Table 2. Polysaccharide content in the mesocarp and exocarp of Wonderful and Laffan varieties; the values are a mean of triplicates. ***** *24 hrs pretreatment with 70% ethanol at 25°C*

Table 3: Sugar composition by acidic hydrolysis , *W, Wonderful; L , Laffan.*

Table(s)

Methods	DM (g)/ solvent (mL)	T (°C)	Time (min)	Yield (%)
1a	1/15	100	30	5
1b	1/15	100	30+30	8
***2a**	1/40	100	60	10
***2b**	1/40	100	60+ 60	9.8
***2c**	1/40	100	60+ 60	9.1
***3a**	1/40	25	720	2.0
***3b**	1/40	25	1440	3.3
***4a**	1/25	100	60	7.8
***$4b**	1/25	100	60	7.2

Table 1. Applied methods to recover CPS from Laffan mesocarp and corresponding extractive yields (*mean values* as w/w DM); *tests carried out in triplicate. **$**, *pretreatment with ethanol 70% at 25 °C for 12 h before boiling.*

Table(s)

Extractive methods	Samples	Yield (%) *mesocarp*	Yield (%) *exocarp*
2a	Laffan	9.80±0.28	4.47±0.50
	Wonderful	8.0±0.10	4.7±1.15
3b	Laffan	3.7±0.42	1.93±0.23
	Wonderful	3.33±1.15	1.99±0.02
4a	Laffan	7.80±0.28	4.20±0.20
	Wonderful	5.67±0.58	4.13±0.31
4b	Laffan	7.15±0.21	3.93±0.12
	Wonderful	6.70±0.66	4.07±0.31

Table 2. Crude polysaccharides (CPs) content in mesocarp and exocarp of Wonderful and Laffan. The values are a mean of triplicates and expressed as % on DM.

Table(s)

	Molar %			
Sugars	**WM-CPSp**	**LM-CPSp**	**WE-CPSp**	**LE-CPSp**
Rhamnose	10.4	7.2	10.8	10.1
Arabinose	4.52	4.04	4.88	4.08
Galactose	5.91	7.31	7.34	7.05
Glucose	14	10.3	11.5	10.9
Xylose	11.2	7.87	9.36	9.3
Fructose	0.41	0.29	0.17	0.2
Galacturonic acid	53.8	63.1	56	58.4

Table 3: Sugar composition of different CPSp samples obtained by acidic hydrolysis

Figure(s)

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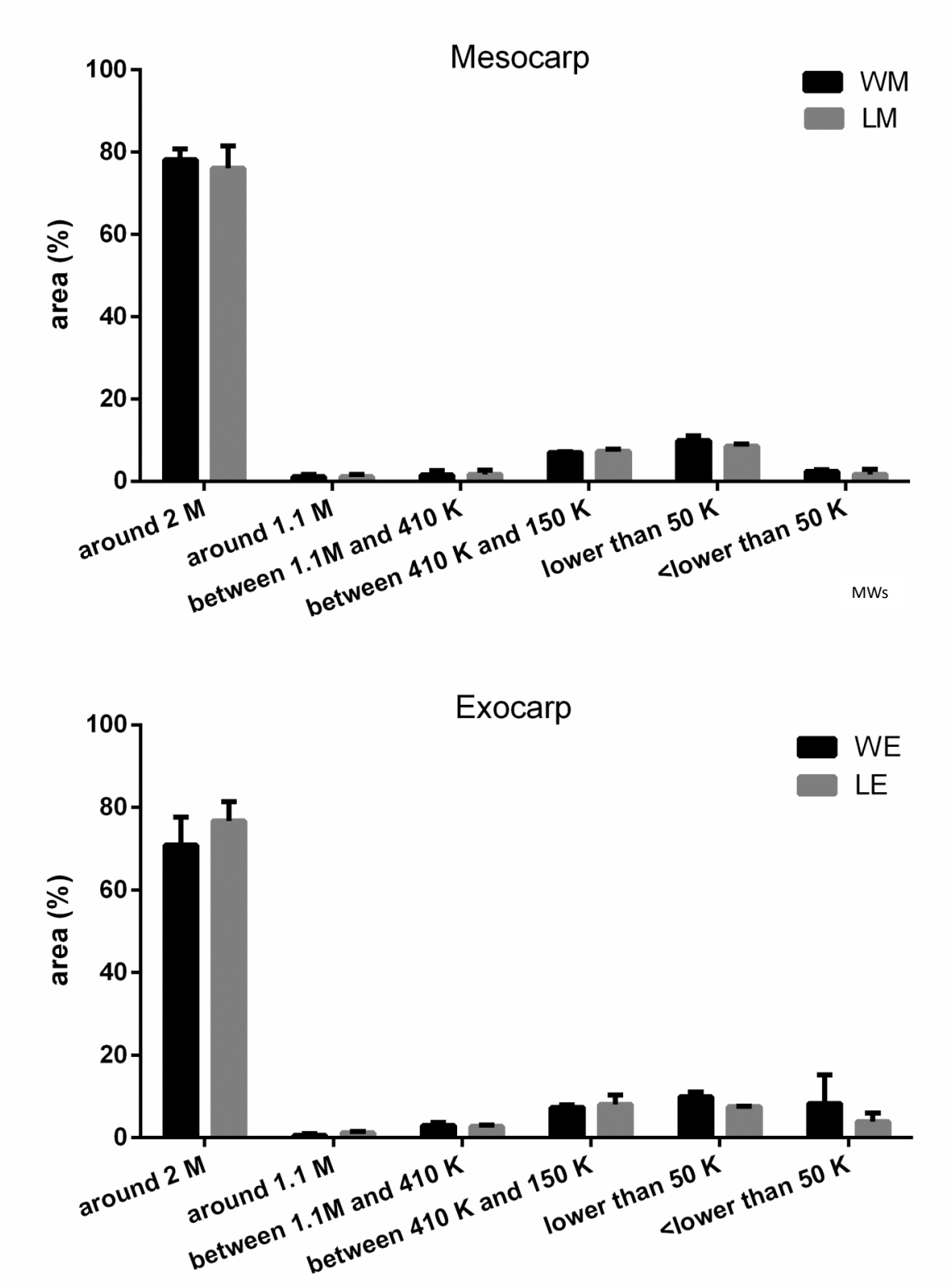

Figure 1.

Figure(s)

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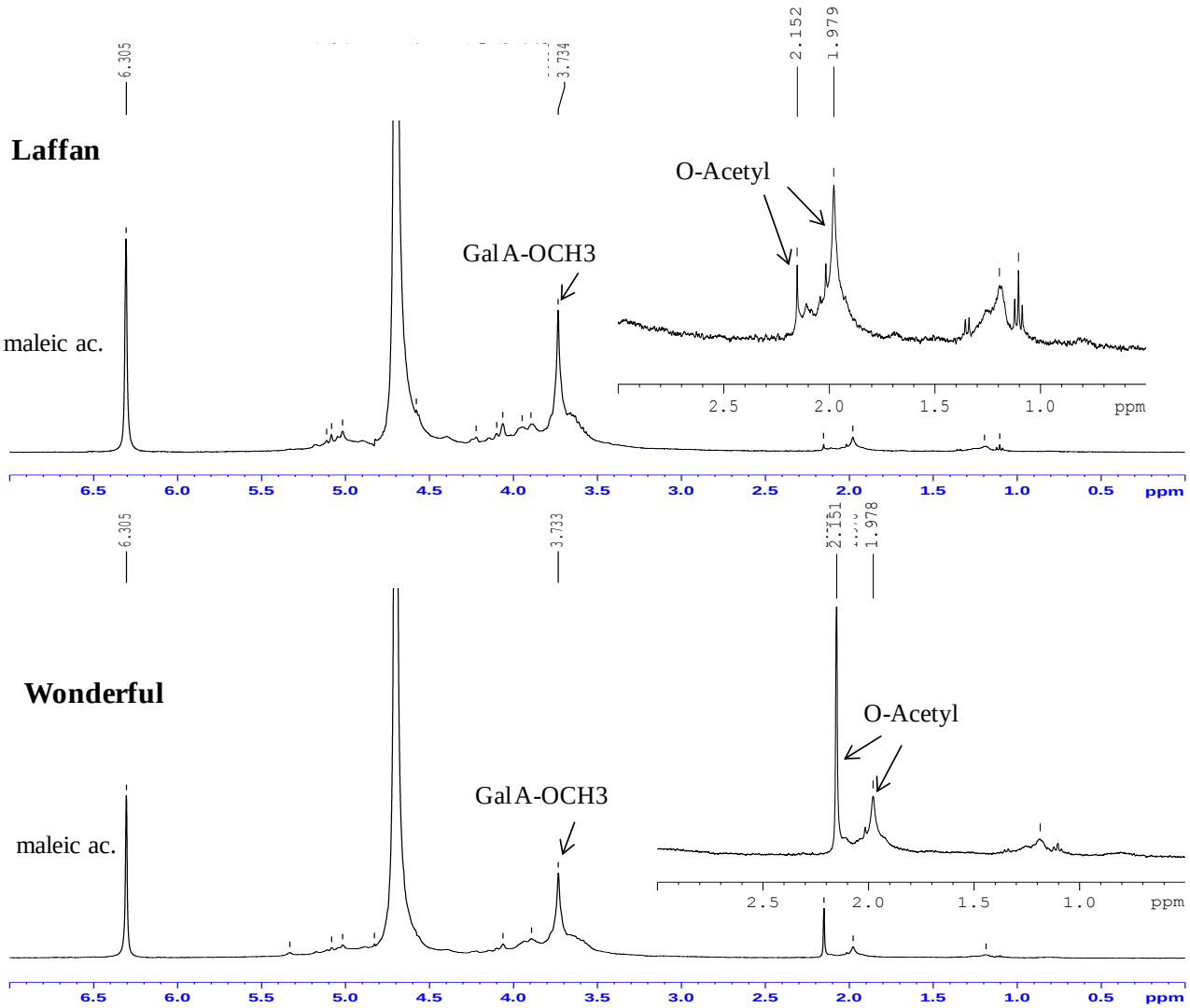

Figure 2

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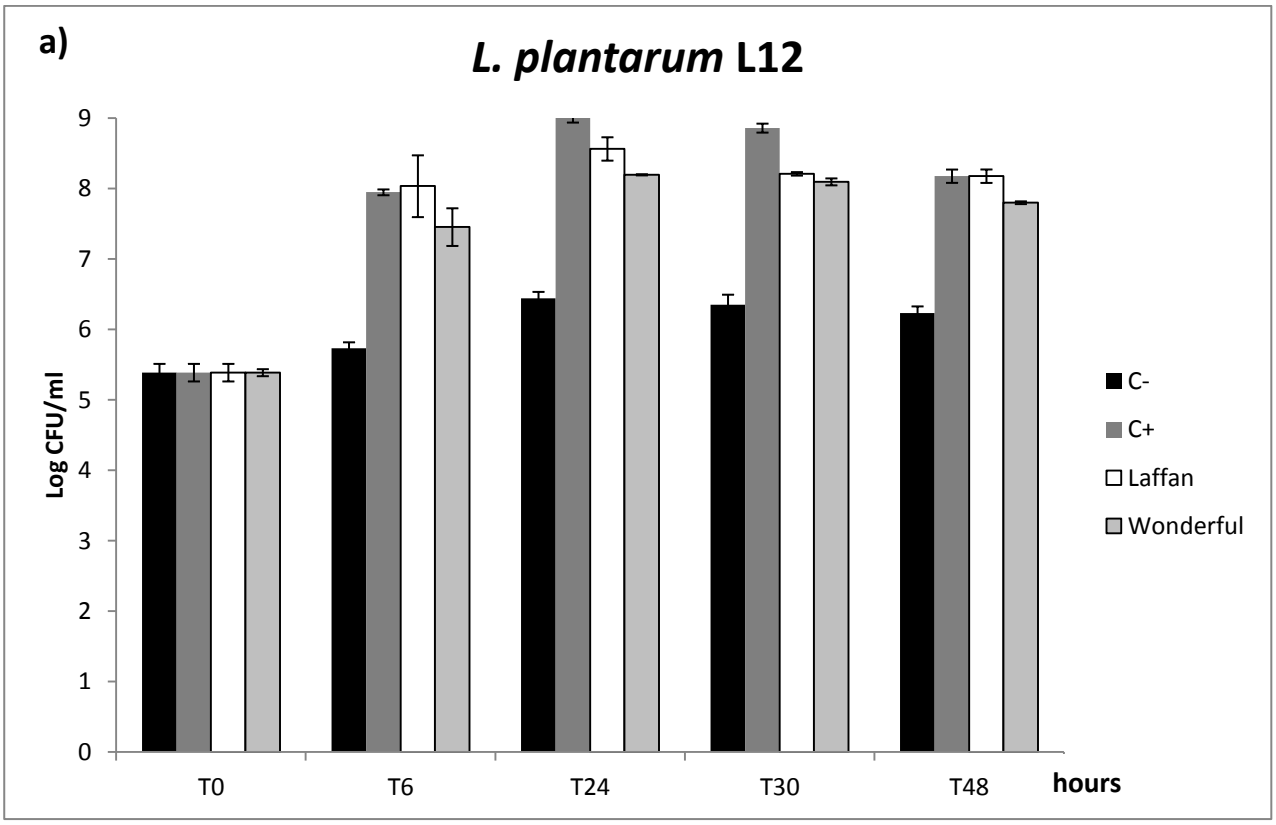

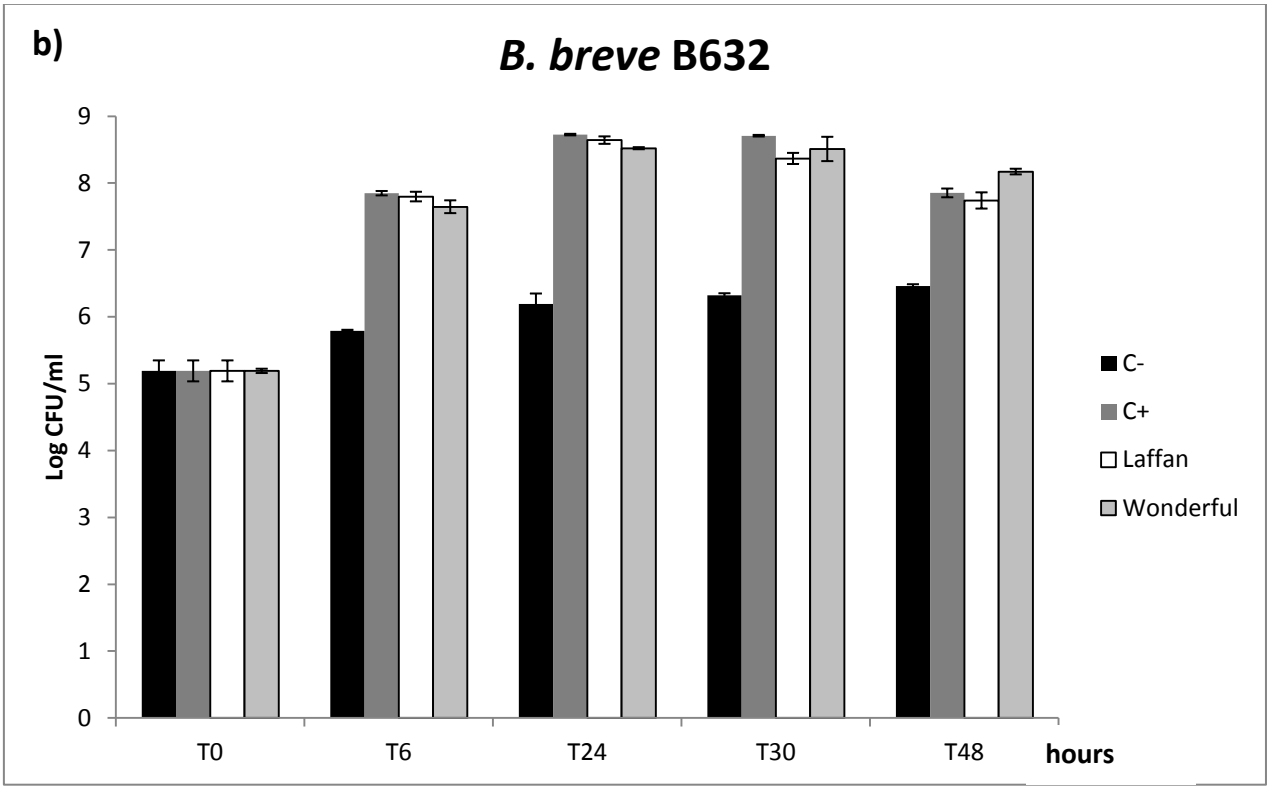

Figure 3