1 Evidence for the Modulation of the Immune Response in Peripheral Blood

2 Mononuclear Cells after Stimulation with a High Molecular Weight β-glucan Isolated

3 from *Lactobacillus fermentum* Lf2.

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5 Running title: LF2-HMW-EPS Interaction with PBMC

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27   ABSTRACT

28   *Lactobacillus fermentum* Lf 2 produces large amounts of exopolysaccharides under

29   optimized conditions (~2 g/L, EPS) which have been shown to possess

30   immunomodulatory activity. In this study, the crude EPS was fractionated to give a

31   high molecular weight (HMw) homoglycan and a mixture of medium molecular

32   weight heteroglycans. The HMw EPS was isolated and identified as a β-glucan.

33   Peripheral blood mononuclear cells (PBMC) were pre-treated with purified

34   polysaccharide to determine if the HMw β-glucan is responsible for the

35   immunomodulatory activity. Cells were also stimulated with either lipopolysaccharide

36   (LPS) or phytohemagglutinin (PHA) and their effects, both with and without β-glucan

37   pre-treatment, compared.

38   Exposure of the cells to β-glucan increased their metabolic activity and whilst a small

39   but statistically significant drop in CD14 expression was observed at Day 1, the

40   levels were significantly elevated at Day 2. High levels of CD14 expression were

41   observed in cells initially exposed to the β-glucan and subsequently stimulated with

42   either LPS or PHA. In contrast, reduced levels of TLR-2 expression were observed

43   for cells initially exposed to the β-glucan and subsequently stimulated with LPS.

44   TNF-α levels were elevated in β-glucan treated cells (Day1) with the levels dropping

45   back once the β-glucan had been removed (Day 2). The stimulants LPS and PHA

46   both induced significant rises in TNF-α levels, however, this induction was

47   completely (LPS) or partially blocked (PHA) in β-glucan pre-treated cells.

48   The results indicate a role for the bacterial β-glucan in modulating the immune

49   response following exposure to agonists such as bacterial LPS.

50   IMPORTANCE

51   The use of foods containing probiotics (i.e. live microorganisms providing health

52   benefits) is growing in popularity. Whilst there is empirical evidence identifying

53   specific microorganisms as having beneficial effects, the precise mechanisms by

54   which these strains exert influence is not well understood. One of the problems is

55   that there are a large number of structures presented at the surface of

56   microorganisms which can interact with the host and which can initiate a host

57   response. In the current work, we addressed this issue by choosing a potential

58   probiotic strain of bacteria associated with cheese production, *L. fermentum* Lf2,

59   which produces large quantities of polysaccharides at its surface. From this

60   polysaccharide mixture, we have isolated, purified and characterised a HMw β-

61   glucan and demonstrated that it has immunomodulatory activity. This study

62   demonstrates that the β-glucan produced by *L. fermentum* Lf2 can modify the

63   immune response of PBMC cells when subjected to a bacterial endotoxin.

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1. Introduction

Species of *Lactobacillus fermentum* are obligate heterofermentative Gram-positive bacteria that can be isolated from a wide variety of sources and include members that are normal inhabitants of the human microflora (1; 2). A significant number of strains are closely associated with the production of fermented foods (3-7) and a number have been exploited for their probiotic properties (8; 9). *L. fermentum* Lf2 is a strain that was isolated as a contaminant culture during cheese manufacture (10). The strain produces significant quantities of a mixture of EPS (~2g/L) and it has been demonstrated that the crude EPS has immunomodulatory activity (11). In previous studies, using EPS from lactic acid bacteria (12; 13), we have shown that specific EPS can stimulate production of pro- and anti-inflammatory cytokines. We also provided evidence that the EPS can modulate the immune response and in doing so provide a degree of immunotolerance. It is likely that immunotolerance has evolved as a way of preventing damage that can occur through too robust an immune response and as a mechanism to prevent reaction against non-self but beneficial antigens, such as those produced by commensal microorganisms. Endotoxin tolerance is the ability of an organism to, if previously primed with a bacterial antigen, survive what would under normal circumstances be lethal doses of bacterial infection (14). While this temporary state of unresponsiveness to endotoxin can be detrimental (15) for example in sepsis, equally it can have a positive impact in pathologies that are the consequence of hyperactive immune response such as in inflammatory bowel diseases.

One of the potential mechanisms for inducing immunotolerance is by interaction of EPS with pattern recognition receptors, CD14 and TLR-2, present on the cell surface that are involved in recognising bacterial components including bacterial lipids (16;

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90   17), lipoproteins, peptidoglycans (18) and, relevant to this study, EPS (19). In

91   peripheral blood mononuclear cells (PBMC) CD14 and TLR-2 are present

92   predominantly on monocytes and have an important role in innate host defence and

93   they trigger activation of adaptive immunity. It has been shown that tolerance to

94   bacterial lipoproteins is accompanied by down-regulation of TLR-2 expression (20).

95   However, the role of CD14 in immune tolerance is more complex, with reports

96   showing that development of a tolerant phenotype precedes CD14 down-regulation

97   (21) thus demonstrating a CD14-independent mechanism of tolerance (22; 23).

98   The aim of the current study was to investigate the immunomodulatory activity of the

99   EPS produced by *L. fermentum* Lf2 and more specifically to examine the effect of

100   purified EPS on PBMC. These cells are easily isolated from whole blood and

101   although there are some small differences in the composition and activation status

102   compared with the immune cells of the gastrointestinal tract, they share certain key

103   phenotypic characteristics (24) and have previously been used to study behaviour of

104   mucosal immunity of the GIT (25). PBMC were isolated and pre-treated with different

105   concentration of a purified HMw EPS from *L. fermentum* Lf2. The

106   immunomodulatory activity was evaluated in these cultures as well as those that

107   were subsequently stimulated with known activators of human blood cells such as

108   lipopolysaccharide (LPS) from *E. coli* and the lectin phytohemagglutinin (PHA). The

109   effect was measured by monitoring changes in the expression of the receptors

110   involved in the interaction of bacteria and immune cells such as CD14 and TLR-2,

111   and secretion of TNF-α, a known proinflammatory cytokine important in monocyte

112   and T cell signalling.

113   RESULTS

114   **Composition of the crude exopolysaccharide (EPS) and purification of the**

115   **High Molecular weight exopolysaccharide (HMw EPS).** SEC-MALLS analysis of

116   the crude EPS produced by *L. fermentum* Lf2 identified a complex mixture of

117   biomacromolecules. The three most significant peaks included a HMw EPS (RT= 25

118   min), a mixture of medium molecular weight polysaccharides (average RT= 35 min)

119   and a UV absorbing species (RT= 40 min) (Fig 1A). After preparative size exclusion

120   chromatography a pure sample of the HMw EPS was obtained in fractions 10-19.

121   The combined fractions were concentrated, freeze-dried and redissolved in

122   deuterium oxide (D_2O) before SEC-MALLS analysis. The SEC-MALLS trace (Fig 1B)

123   contained a single peak (RT= 26 mins) with a weight average molecular mass of

124   1.23×10^6 Da and a polydispersity value of 1.104.

125   [Insert Figure 1A and 1B here]

126   **Characterization of the HMw EPS**. Monomer analysis and determination of

127   the absolute configuration of the monosaccharides identified that the HMw EPS was

128   composed entirely of D-glucopyranose. Linkage analysis, using permethylated alditol

129   acetates, confirmed the presence of a 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol

130   (corresponding to a terminal glucose), a 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylglucitol

131   (corresponding to a 1,3-linked glucose) and a 1,2,3,5-tetra-*O*-acetyl-4,6-di-*O*-

132   methylglucitol (corresponding to a 1,2,3-linked glucose).

133   The ^{1}H-NMR spectrum (Fig 2) had two distinct resonances in the anomeric

134   region (4.93 & 4.85 ppm) with an integral ratio very close to 1:2 and both had large

135    $^3J_{H1,H2}$-coupling constants which are indicative of β-linked hexoses.

136   [Insert Figure 2 here]

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137   The location of the ring protons was identified through inspection of a combination of

138   ^{1}H-^{1}H-COSY and ^{1}H-^{1}H-TOCSY spectra (see supplementary information) and that of

139   the ring carbons using a combination of a ^{1}H-^{13}C-HSQC-spectrum and a ^{1}H-^{13}C-

140   HSQC-TOCSY spectrum (see supplementary information). The chemical shifts for

141   the proton and carbons are listed in Table 1.

142   [Insert table 1 here]

143   Finally, the linkage pattern of the monosaccharides in the repeat unit was confirmed

144   by examination of a ROESY and a HMBC spectrum and identification of the inter-

145   residue NOEs and long-range scalar coupling which were compared with data

146   reported in the literature for identical EPS(26; 27) (see supplementary information).

147   U.V. absorption and ^{31}P NMR spectra were recorded for the HMw polysaccharide

148   and these were not able to detect any phosphorous, protein or nucleic acid, and

149   when taken along with the results of the other characterization studies, this

150   confirmed that a high purity polysaccharide had been isolated.

151   **PBMC isolation and cell culture**. In the Day 1 experiment PBMC were

152   activated by treatment with the HMw β-glucan in all the donors ($p < 0.001$ - 0.0001)

153   (Fig 3). The levels of activation, compared to the control, were comparable for both

154   β-glucan concentrations used (50 µg/mL and 100 µg/mL), except for Donor B where

155   higher levels were obtained for 50 µg/mL EPS ($p < 0.0001$).

156   In the Day 2 experiment, levels of activation remained elevated in the β-glucan

157   treated conditions for two donors, regardless of the type of subsequent stimulation (p

158   < 0.05-0.0001). Treatment with LPS alone also showed higher metabolic activity as

159   measured by the MTS assay, and this was true regardless of whether or not they

160   had been pre-treated with β-glucan ($p < 0.0001$).

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161   [Insert Figure 3 here]

162   Apparent anomalies in the MTS results were observed for PHA treatment, where

163   although a statistically significant activation was observed for two out of three donors

164   tested ($p < 0.05$-0.01), the recorded levels did not correspond to the level of cell

165   proliferation observed on microscopic images for this condition (Fig 4). It is possible

166   that the conditions used for PHA stimulation interfered with the results of the MTS

167   assay such that the level of metabolic activity that this assay measured did not

168   accurately correspond to the level of proliferation exhibited after PHA stimulation (28;

169   29).

170   [Insert Figure 4 here]

171   Microscopic inspection of the cells (Fig 4) confirmed the high levels of proliferation

172   and clumping of cells after PHA stimulation when compared to the unstimulated and

173   untreated condition (Fig 4A) and the degree of cell aggregation was attenuated by

174   previous treatment with β-glucan (Fig 4C). Absorbance values measured by MTS

175   assay confirmed the difference between PHA only stimulated condition compared to

176   the EPS pre-treated and PHA stimulated condition, at least in Donors B and C,

177   however, the levels were lower than what would be expected for the higher

178   proliferation visible in the images of PHA stimulated PBMC.

179   **Flow cytometric assessment of pattern recognition receptors CD14 and**

180   **TLR-2.** Changes in expression, presented as median fluorescence intensity (MFI), of

181   the CD14 and TLR-2 receptors that are involved in recognition of microbial-specific

182   molecules are presented in Figures 5 and 6, respectively. In the Day 1 experiment,

183   for two out of three donors CD14 showed lower expression after pre-treatment with

184   EPS compared to the untreated condition, regardless of the concentration used ($p <$

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185   0.0001, Fig 5). In the second experiment, at Day 2, however, initial pre-treatment

186   with EPS led to a robust increase in the CD14 expression, for all conditions,

187   compared to those that had not been pre-treated with EPS (untreated unstimulated

188   vs EPS pre-treated; LPS stimulated vs EPS pre-treated and LPS stimulated, with p <

189   0.0001 in all cases, and in Donor B only, PHA stimulated vs EPS pre-treated and

190   PHA stimulated, p < 0.01; Fig 5B). LPS stimulation led to a moderate decrease in

191   CD 14 expression in two out of three donors (p < 0.0001 for Donor B and p < 0.01 for

192   Donor C), while the levels of CD14 markers were higher in Donor A (p < 0.0001),

193   when compared to the untreated and unstimulated condition at Day 2.

194   Unsurprisingly, PHA consistently attenuated CD14 expression (p <0.0001 for all

195   three donors), while pre-treatment with EPS marginally reduced the decrease in

196   expression caused by PHA, as seen in Donor B (p < 0.01).

197   [Insert Figure 5 here]

198   The effect of PBMC stimulation on TLR-2 expression was less consistent and the

199   only result of note is that there was a clear decrease at Day 2 in TLR-2 expression

200   after EPS pre-treatment, followed by LPS stimulation, compared to the LPS

201   stimulated alone (p< 0.05 for Donor C and p < 0.0001 for Donors A and B, Fig 6).

202   [Insert Figure 6 here]

203   **Stimulation of TNF-α secretion**. Initial treatment with EPS resulted in an increase

204   in TNF-α secretion on Day 1 for both concentrations (Fig 7A). However, while in

205   Donors B and C the increase in TNF-α was greater for the lower EPS concentration

206   (50 µg/ml), the opposite was true for Donor A (Fig 7A).

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207   Interestingly, TNF-α levels in the Day 2 experiment for the cells that were pre-treated

208   with EPS at Day 0 and then left unstimulated at Day 1 were comparable to control

209   cells (Fig 7B) indicating a robust decrease in the levels of this cytokine when

210   compared to EPS pre-treated conditions in the Day 1 experiment (Fig 7A). For cells

211   that were treated with EPS on Day 0 and then stimulated on Day 1 with LPS or PHA

212   the pre-treatment with EPS greatly attenuated a robust increase in TNF-α secretion

213   for all donors ($p < 0.01$ between LPS and EPS+LPS in Donor B, and $p < 0.0001$

214   between PHA and EPS+PHA in Donors A and C).

215   [Insert Figure 7 here]

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217   DISCUSSION

218   The aim of this study was determine which component of the bacterial EPS produced

219   by *L. fermentum* Lf2 is responsible for the immunomodulatory properties of the crude

220   EPS and to gain an understanding of the nature of this modulation. Analysis indicated

221   that the crude EPS included both a HMw polysaccharide and a mixture of medium

222   molecular weight polysaccharides that were separated by size exclusion

223   chromatography. The HMw polysaccharide was identified as a β-glucan with a weight

224   average molecular mass (1.23×10^6 Da) which is similar to that of EPS obtained from

225   other strains of lactic acid bacteria (LAB) (30; 31). Using NMR analysis, the ^{1}H and

226   ^{13}C-chemical shifts were recorded for the β-glucan (Table 1) these are identical to

227   those published for the EPS isolated from *Pediococcus damnosus* 2.6 (26),

228   *Oenococcus oeni* I4 (32) and for one of the two EPSs produced by *Lactobacillus sp.*

229   *G-77*(27). The results of the characterisation studies are consistent with the β-glucan

230   having the following trisaccharide repeating unit:

231   →3)-β-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→

232   2

233   ↓

234   1

235   β-D-Glc*p*

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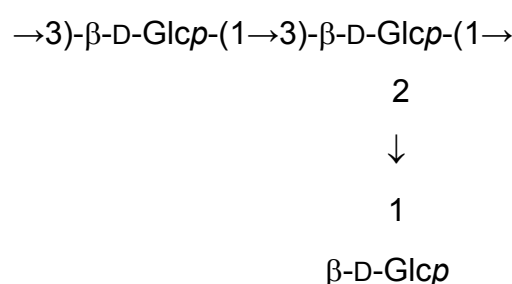
237   It is worth noting that the repeating unit is similar to the type 37 polysaccharide

238   produced by *Streptococcus pneumoniae* which has the same linear backbone (→3)-

239   *β*-D-Glc*p*-(1→) bearing a terminal glucose unit at the 2-position but these appear on

240   each residue in the *S. pneumoniae* EPS, rather than on alternate residues as in the *L.*

241   *fermentum* Lf2 β-glucan (33). All attempts to purify the medium molecular weight

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242   polysaccharide were unsuccessful, the on-going work to characterise this material will

243   be reported elsewhere.

244   Treatment of PBMC with the β-glucan had no impact on cell viability but did increase

245   the metabolic activity of cells that was measured as an increase in mitochondrial

246   dehydrogenase activity. At the concentrations employed, the β-glucan was able to

247   activate PBMC in culture compared to controls at Day 1, indicating a functional

248   interaction between the two. Interestingly, this activation persisted for 24 h after the

249   β-glucan had been removed from solution. This is in agreement with previous reports

250   where cell wall extracts and purified EPS stimulated cell proliferation of different

251   immune cell types: including BALB/c mice splenocytes and mesenteric lymphocytes

252   (34) using EPS from LAB, and mouse lymphocytes by EPS from *Paenibacillus*

253   *jamilae* CP-7 (35). The stimulatory effect of EPS has also been shown to be

254   concentration dependent when two *Lactobacillus rhamnosus* strains (ATCC 9595

255   and RW-9595M), that differ in the amount of EPS they produce, were used to

256   stimulate splenic lymphocytes (36). In contrast, EPS from bifidobacteria has been

257   reported to have anti-proliferative activity on rat PBMC and gut associated lymphoid

258   tissue cells (37). Whilst other authors have reported bifidobacteria EPS stimulates

259   proliferation of the intestinal Caco-2 but not HT-29 (38) cells while the EPS from

260   *Lactobacillus gasseri* strains G10 and H15 had no effect on the proliferation of the

261   cervical cancer cell line HeLa (39). These differences are not surprising since it has

262   previously been demonstrated that EPS can exert a differential effect on the

263   metabolic activity of different cell types (40).

264   The MTS assays gave results in agreement with previous reports, where the optimal

265   PHA concentration for cell stimulation was 2.5μg and where only modest increase in

266   metabolic activity was reported for cultures stimulated with 10μg/ml PHA (28). From

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267 inspection of the microscope images it is clear that pre-treatment with the β-glucan

268 partially reversed the effect of PHA by reducing the amount of cell clumping.

269 Changes in cytokine production in response to the presence of microbial strains and

270 their products is a way of demonstrating immunomodulatory effect on host cells. For

271 example in our previous studies we demonstrated that the EPS obtained from two

272 *Lactobacillus* strains, *L. helveticus ssp. rosyjski* and *L. acidophilus,* both increased

273 IL-8 production in HT29-19A intestinal cell line (41). Other authors have reported

274 changes in a number of pro-inflammatory cytokines, such as IL-12, IL-6 and TNF-α

275 with pre-treatment with EPS from *L. rhamnosus* GG in combination with LPS

276 stimulation when compared to the LPS stimulation alone (42). In the present study,

277 pre-treatment with β-glucan stimulated TNF-α production after Day 1 with the levels

278 falling back to those observed in the control 24 h after the β-glucan had been

279 removed from solution.

280 One of the most significant results was observed for the cells which had been pre-

281 treated with the β-glucan and which were subsequently stimulated by LPS or PHA,

282 this led to large decreases in the levels of this cytokine, when compared to the

283 corresponding stimulation alone (LPS and PHA, respectively). As TNF-α is a key

284 proinflammatory cytokine, this is typical of an immune tolerant phenotype, with

285 potentially important implications given its central role in the aetiology of the

286 inflammatory bowel diseases such as Crohn's disease and ulcerative colitis.

287 To better understand the interaction of the β-glucan with PBMC, expression of CD14

288 and TLR-2, receptors commonly involved in host microbial responses (43), was

289 examined. An initial downregulation of CD14 on Day 1 (observed in two donors)

290 suggests the involvement of this co-receptor in the β-glucan signalling. A similar

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291   small decrease in CD14 MFI on PBMC after 24 h stimulation with LPS has been

292   previously reported (44), unsurprisingly since LPS is known to use CD14 as a co-

293   receptor.   Downregulation of CD14 after LPS stimulation was also observed in

294   alveolar macrophages (23). A typical tolerance phenotype involves a permanent

295   decrease in CD14 expression (23; 45). In contrast, in the present study, pre-

296   treatment with β-glucan reversed any down-regulatory effect of LPS completely and,

297   to a lesser extent, that of PHA. This positive influence on the availability of CD14 on

298   PBMC could have potentially important positive effects: an endotoxin tolerant

299   phenotype can have a detrimental effect on health and survival, such as with sepsis,

300   where the risk of new infections is increased due to the refractory state of the

301   organism (46). Thus the ability of the β-glucan to reverse a tolerance phenotype, at

302   least in respect to CD14 expression, could be beneficial in diseases where

303   continuous downregulation of CD14 reduces an organisms' ability to respond to

304   subsequent infections.

305   The influence of β-glucan treatment of PBMC alone on TLR-2 expression at Day 1

306   was not easy to determine, with both small increases and decreases in expression

307   levels being observed. TLR-2 expression in cells pre-treated with β-glucan and

308   subsequently stimulated with LPS decreased when compared to cells stimulated with

309   LPS only. Similarly, it has been previously reported that treatment with *L.*

310   *rhamnosus* in combination with LPS reduced TLR-2 expression at the mRNA level

311   when compared to LPS stimulation alone (42). By contrast, an increase in cells

312   expressing TLR-2 was observed when immune cells from mouse Peyer patches

313   were stimulated with *Lactobacillus casei* (47). The range of effects that bacterial

314   components exert on immune cells is not unexpected as this is known to be strain

315   dependent (48). Modulation of TLR-2 by different microorganisms and their

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316   components in immunomodulatory phenotypes can cause changes in both pro- and

317   anti-inflammatory cytokine secretion.

318   The data, in combination with recovery of CD14 expression and continuous

319   activation of cells as demonstrated by the MTS assay on Day 2 suggest a difference

320   in mechanism in which this β-glucan exerts tolerance when compared to classical

321   endotoxin tolerance. It is therefore possible that the β-glucan could have a potentially

322   important immunoprotective role. As such further investigations should address the

323   mechanism of these effects and their *in vivo* relevance.

324

325 MATERIAL AND METHODS

326   Unless otherwise stated, all reagents were purchased from Sigma-Aldrich

327 Company Ltd. (Poole, Dorset UK) and were used as supplied.

328 **EPS production and purification.** *L. fermentum* Lf2 was grown at the Instituto de

329 Lactologia Industrial (Santa Fe, Argentina). The growth conditions, as well as the

330 crude EPS extraction and purification methods have previously been reported (11; 49).

331 Size Exclusion Chromatography coupled with Multi Angle Laser Light Scattering (SEC-

332 MALLS- Wyatt technology, Santa Barbara, CA, USA) was used to determine the

333 composition of the crude EPS. EPS samples (1 mg/mL) were prepared in aq. $NaNO_3$

334 (0.1M) and stirred for 16 h to ensure the EPS was completely dissolved. Samples (100

335 µL) were injected in triplicate into a SEC-MALLS system (comprising of three columns

336 connected in series: PL Aquagel-OH 40, 50 and 60 (8 µm, 30 cm x 7.5 mm, Agilent,

337 Cheadle, UK) with a flow rate of 0.7 mL/min. A differential refractometer (Optilab rEX,

338 Wyatt technology, Santa Barbara, CA, USA) was used to determine the concentration

339 of the polysaccharide and a Dawn-EOS MALLS detector (laser operating at 690 nm)

340 was used to determine the weight average molecular mass of the polysaccharide. An

341 in-line UV detector (Shimadzu, Milton Keynes, UK) was used for the detection of

342 proteins and nucleic acids. ASTRA version 6.0.1 software (Wyatt technology, Santa

343 Barbara, CA, USA) was used for the data analysis.

344 The crude EPS was purified by preparative size exclusion chromatography on a

345 Sephacryl S-500 HR column (XK26/60-GE Healthcare, Fisher Scientific, UK) eluting

346 with ultrapure water at a flow rate of 5.0 mL min^{-1} using a FPLC kit (AKTA PRIME-

347 Amersham Pharmacia, Biotech, GE Healthcare Life Sciences, Buckinghamshire, UK)

348 with a set wavelength of 254 nm. In total, sixty 5 mL fractions were collected and the

349 location of polysaccharides in the different fractions was identified by determining the

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350   carbohydrate content of each fraction using the Dubois method (50; 51). Fractions

351   containing EPS were pooled and freeze-dried.

352   **NMR analysis of the high molecular weight EPS.** NMR spectra of EPSs were

353   recorded in solution in D_2O (5-10 mg in 0.65 mL) and were run either at room

354   temperature or at an elevated temperature (70 °C). All of the NMR spectra were

355   recorded on a Bruker Avance 500.13 MHz spectrometer (Bruker-biospin, Coventry,

356   UK) using Bruker's TOPSPIN 4.0.1 software for analysis. Chemical shifts are

357   expressed in ppm relative to internal acetone, 2.225 for ^1H and 31.55 for ^{13}C. A

358   series of 2D-spectra were recorded including: a 2D gradient-selected double

359   quantum filtered correlation spectrum (gs-DQF-COSY) recorded in magnitude mode

360   at 70°C; a total correlation spectroscopy (TOCSY) experiment recorded with a

361   mixing times of 120 ms; ^1H-^{13}C heteronuclear single quantum coherence (HSQC)

362   spectra (decoupled and coupled); a heteronuclear multiple bond correlation (HMBC)

363   spectrum; and finally, a rotating frame nuclear Overhauser effect spectrum (ROESY,

364   mixing time of 200 ms). The 2D spectra were recorded with 256 experiments of 1024

365   data points. For the majority of spectra, time-domain data were multiplied by phase-

366   shifted (squared-) sine-bell functions. After applying zero-filling and Fourier

367   transformation, data sets of 1024-1024 points were obtained.

368   **Composition of the high molecular weight polysaccharide.** The

369   monosaccharides present were determined after acid hydrolysis either directly using

370   HPAEC-PAD analysis or as their alditol acetates, as previously described(12). The

371   absolute configuration of the sugars was determined by preparation of their

372   respective 2-(S)-butylglycosides using Gerwig's method(52). For linkage analysis,

373   the samples were permethylated using the procedures described by Stellner(53).

374   **PBMC isolation and cell culture.** Venous blood was collected after obtaining

375   informed consent from three healthy volunteers in blood collecting tubes containing

376   citrate-dextrose solution (ACD) at a whole blood to anticoagulant ratio 9:1. The study

377   was approved by the Local Ethics Committee (School of Applied Sciences,

378   University of Huddersfield, UK). Blood samples were layered over Histopaque®-

379   1077 and PBMC isolated using a standard density gradient centrifugation protocol.

380   Enriched PBMC were suspended in RPMI 1640 medium (Gibco, Life Technologies,

381   UK) supplemented with 4 mM L-Glutamine, 50 IU/ml/50 µg/ml/

382   Penicillin/Streptomycin and 20 mM HEPES (all from Gibco, Life Technologies, UK)

383   and 10% heat-inactivated fetal bovine serum (FBS) and $2x10^6$ cells/well were added

384   to 24-well plates (Starlab, UK). The cells were then challenged as shown in Figure

385   8. For the Day 1 experiment (Fig 8A), cells were treated with two different

386   concentrations of EPS (50 µg/mL and 100µg/mL) or left untreated, four replicates

387   were set up for each condition. For the Day 2 experiment (Fig 8B), at Day 0, half of

388   the wells were treated with 100 µg/mL EPS while the other half was left untreated.

389   Cells were incubated for 24 h, at 37 ˚C, 5% CO_2 after which they were washed with

390   supplemented RPMI 1640 and stimulated with either LPS (Lipopolysaccharides from

391   *Escherichia coli* O111:B4) at a final concentration of 100 ng/mL or PHA-M (Lectin

392   from *Phaseolus vulgaris* (red kidney bean)) at 10 µg/ml, or left unstimulated (Fig 8B).

393   (final incubation of 24 h, at 37 ºC, 5% CO_2)Each condition was set up in four

394   replicates.

395   [Insert Figure 8 here]

396   **MTS assay.** In order to measure metabolic activity of the cell cultures under different

397   conditions, the CellTiter 96® AQueous One Solution Cell Proliferation Assay, MTS

398   assay (Promega, UK) was used as per the manufacturer's instructions. At the end of

399 each experiment 1×10^5 cells from individual wells were transferred into separate

400 wells of a 96-well plate and tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-

401 carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS) and an

402 electron coupling reagent (phenazine ethosulfate; PES) were added and the

403 absorbance at 490nm was measured.

404 **Flow cytometry.** To monitor CD14 and TLR-2 expression the remaining PBMC were

405 washed with FACS buffer (PBS, Lonza, UK, 1% Bovine serum albumin, BSA, 0.1%

406 Sodium azide, Sigma Aldrich, UK), and fixed with 1% paraformaldehyde (PFA,

407 Sigma Aldrich, UK). Cells were then washed with permeabilisation solution (Perm

408 Wash, BD Biosciences, UK) and stained with anti-CD14 and anti-TLR-2 antibodies

409 (Biolegend, UK). Following the incubation, cells were washed with Perm Wash

410 buffer and resuspended in 1% PFA until acquisition using The Guava® easyCyte

411 flow cytometer (Merck Milipore, UK). Data were acquired using guavaSoft 2.7

412 software for Windows and analysed using its 3.1.1. version. Data were presented as

413 median fluorescence intensity indicating the level of marker's expression (MFI of

414 CD14$^+$ and MFI of TLR-2$^+$ cells). Doublet and debris exclusion were performed and

415 gating was set based on Fluorescence Minus One (FMO) controls.

416 **Measurement of TNF-α levels by ELISA.** Stored supernatants were used for the

417 analysis of TNF-α concentration by ELISA (TNF alpha (Total) Human ELISA Kit,

418 Invitrogen, UK) following the manufacturer's instructions. Briefly, the plate was

419 coated with TNF-α specific capture antigen and left overnight. Following subsequent

420 washes, non-specific binding was blocked using blocking buffer after which samples

421 were added and incubated for 2 h at room temperature. Plates were then washed

422 and biotinylated detection antibody was added, after which avidin horseradish

423 peroxidase (avidin-HRP) was used to bind to the biotin on the detection antibody.

19

424   Tetra-methyl benzidine was then added as the substrate for the HRP enzyme, and

425   the reaction was stopped with the addition of 1M sulfuric acid. Absorbance was read

426   at 495nm using FLUOstar Optima plate reader (BMG Labtech). The data were

427   analysed using MARS Data Analysis Software, and a 4 parameter logistic regression

428   curve was used as the best fit for the data.

429   **Statistical analyses.** Due to the variability between the donors, each was treated as

430   a separate group and Two-Way analysis of variance (ANOVA) followed by Tukey's

431   posthoc test for multiple comparison were used to examine the differences between

432   the conditions. For each donor, data are presented as mean ± SEM (standard error

433   of the mean) of the replicates of the conditions set up in experiment.

434   References.

435   1.   Grover S, Sharma VK, Mallapa RH, Batish VK. 2013. Draft genome sequence of *Lactobacillus*
436   *fermentum* Lf1, an Indian isolate of human gut origin. *Genome Announcements* 1(6)
437   PMC3828308

438   2.   Jiménez E, Langa S, Martín V, Arroyo R, Martín R, Fernández L, Rodríguez JM. 2010.
439   Complete genome sequence of *Lactobacillus fermentum* CECT 5716, a probiotic strain
440   isolated from human milk. *Journal of Bacteriology* 192:4800

441   3.   Jayashree S, Pooja S, Pushpanathan M, Vishnu U, Sankarasubramanian J, Rajendhran J,
442   Gunasekaran, P. 2013. Genome sequence of *Lactobacillus fermentum* strain MTCC 8711, a
443   probiotic bacterium isolated from yogurt. *Genome Announcements* 1(5) PMC3784788

444   4.   Pereira DIA, McCartney AL, Gibson GR. 2003. An in vitro study of the probiotic potential of a
445   bile-salt-hydrolyzing *Lactobacillus fermentum* strain, and determination of its cholesterol-
446   lowering properties. *Applied and Environmental Microbiology* 69:4743-52

447   5.   Ramos CL, Thorsen L, Schwan RF, Jespersen L. 2013. Strain-specific probiotics properties of
448   *Lactobacillus fermentum*, *Lactobacillus plantarum* and *Lactobacillus brevis* isolates from
449   Brazilian food products. *Food Microbiology* 36:22-9

450   6.   Sanni AI, Morlon-Guyot J, Guyot JP. 2002. New efficient amylase-producing strains of
451   *Lactobacillus plantarum* and *L. fermentum* isolated from different Nigerian traditional
452   fermented foods. *International Journal of Food Microbiology* 72:53-62

453   7.   Verce M, De Vuyst L, Weckx S. 2018. Complete and annotated genome sequence of the
454   sourdough lactic acid bacterium *Lactobacillus fermentum* IMDO 130101. *Genome*
455   *Announcements* 6(19) PMC5946041

456   8.   Maldonado J, Cañabate F, Sempere L, Vela F, Sánchez AR, Narbona, E. López-Huertas E,
457   Geerlings A, Valero A. D, Olivares M, Lara-Villoslada F, 2012. Human milk probiotic
458   *lactobacillus fermentum* CECT5716 reduces the incidence of gastrointestinal and upper
459   respiratory tract infections in infants. *Journal of Pediatric Gastroenterology and Nutrition*
460   54:55-61

461   9.   Mikelsaar M, Zilmer M. 2009. *Lactobacillus fermentum* ME-3 - An antimicrobial and
462   antioxidative probiotic. *Microbial Ecology in Health and Disease* 21:1-27
463   10.   Shi T, Aryantini NP, Uchida K, Urashima T, Fukuda K. 2014. Enhancement of
464   exopolysaccharide production of *Lactobacillus fermentum* TDS030603 by modifying culture
465   conditions. *Biosci Microbiota Food Health* 33:85-90
466   11.   Ale EC, Perezlindo MJ, Burns P, Tabacman E, Reinheimer JA, Binetti AG. 2016.
467   Exopolysaccharide from *Lactobacillus fermentum Lf2* and its functional characterization as a
468   yogurt additive. *Journal of Dairy Res*earch 83:487-92
469   12.   Balzaretti S, Taverniti V, Guglielmetti S, Fiore W, Minuzzo M, Ngo, H. N. Ngere, JB, Sadiq S,
470   Humphreys PN, Laws AP. 2017. A novel rhamnose-rich hetero-exopolysaccharide isolated
471   from *Lactobacillus paracasei DG* activates THP-1 human monocytic cells. *Applied and
472   Environmental Microbiology* 83
473   13.   Patten DA, Leivers S, Chadha MJ, Maqsood M, Humphreys PN, Laws, AP, Collett A. 2014. The
474   structure and immunomodulatory activity on intestinal epithelial cells of the EPSs isolated
475   from *Lactobacillus helveticus* sp. *Rosyjski* and *Lactobacillus acidophilus* sp. 5e2. *Carbohydrate
476   Research* 384:119-27
477   14.   West MA, Heagy W. 2002. Endotoxin tolerance: A review. *Critical Care Medicine* 30:S64-S73
478   15.   Biswas SK, Lopez-Collazo E. 2009. Endotoxin tolerance: new mechanisms, molecules and
479   clinical significance. *Trends Immunology* 30:475-87
480   16.   Pugin J, Heumann ID, Tomasz A, Kravchenko VV, Akamatsu Y, et al. 1994. CD14 is a pattern
481   recognition receptor. *Immunity* 1:509-16
482   17.   Kawai T, Akira S. 2010. The role of pattern-recognition receptors in innate immunity: update
483   on Toll-like receptors. *Nature Immunology* 11:373-84
484   18.   Uematsu S, Akira S. 2008. Toll-Like receptors (TLRs) and their ligands. *Handbook of
485   Experimental Pharmacology*:1-20
486   19.   Watters C, Fleming D, Bishop D, Rumbaugh KP. 2016. Host Responses to Biofilm. *Progress in
487   Molecular Biology and Translational Science* 142:193-239
488   20.   Wang JH, Doyle M, Manning BJ, Di Wu Q, Blankson S, Redmond HP. 2002. Induction of
489   bacterial lipoprotein tolerance is associated with suppression of toll-like receptor 2
490   expression. *Journal of Biological Chemistry* 277:36068-75
491   21.   Labeta MO, Durieux JJ, Spagnoli G, Fernandez N, Wijdenes J, Herrmann R. 1993. CD14 and
492   tolerance to lipopolysaccharide: biochemical and functional analysis. *Immunology* 80:415-23
493   22.   Rajaiah R, Perkins DJ, Ireland DD, Vogel SN. 2015. CD14 dependence of TLR4 endocytosis and
494   TRIF signaling displays ligand specificity and is dissociable in endotoxin tolerance.
495   *Proceedings of the National Academy Science U S A* 112:8391-6
496   23.   Lin SM, Frevert CW, Kajikawa O, Wurfel MM, Ballman K, Mongovin, S. Wong VA, Selk A,
497   Martin TR. 2004. Differential regulation of membrane CD14 expression and endotoxin-
498   tolerance in alveolar macrophages. *American Journal of Respiratory Cell and Molecular
499   Biology* 31:162-70
500   24.   Abbas A, Lichtman AH, Pillai S. 2017. *Cellular and Molecular Immunology*. Elsevier. 608 pp.
501   25.   Kleiveland CR. 2015. Peripheral Blood Mononuclear Cells. In *The Impact of Food Bioactives
502   on Health: in vitro and ex vivo models*, ed. Verhoeckx K, Cotter P, Lopez-Exposito I, Kleiveland
503   C, Lea T, Mackie A, Requena T, Swiatecka D, Wichers H. l:161-7. Spinger, Cham (CH). Chapter
504   15, 161-7.
505   26.   Dueñas-Chasco MT, Rodríguez-Carvajal MA, Mateo PT, Franco-Rodríguez G, Espartero JL,
506   Irastorza-Iribas A, Gil-Serrano AM. 1997. Structural analysis of the exopolysaccharide
507   produced by *Pediococcus damnosus 2.6*. *Carbohydrate Research* 303:453-8
508   27.   Dueñas-Chasco MT, Rodríguez-Carvajal MA, Tejero-Mateo P, Espartero JL, Irastorza-Iribas A,
509   Gil-Serrano AM. 1998. Structural analysis of the exopolysaccharides produced by
510   *Lactobacillus* spp. G-77. *Carbohydrate Research* 307:125-33

28. Norian R, Delirezh N, Azadmehr A. 2015. Evaluation of proliferation and cytokines production by mitogen-stimulated bovine peripheral blood mononuclear cells. *Veterinary Research Forum* 6:265-71

29. Molaee N, Mosayebi G, Pishdadian A, Ejtehadifar M, Ganji A. 2017. Evaluating the Proliferation of human peripheral blood mononuclear cells using MTT Assay. *International Journal of Basic Science in Medicine.* 2:25-8

30. Laws A, Gu Y, Marshall V. 2001. Biosynthesis, characterisation, and design of bacterial exopolysaccharides from lactic acid bacteria. *Biotechnology Advances* 19:597-625

31. Ruas-Madiedo P, De Los Reyes-Gavilán CG. 2005. Invited review: Methods for the screening, isolation, and characterization of exopolysaccharides produced by lactic acid bacteria. *Journal of Dairy Science* 88:843-56

32. Ibarburu I, Soria-Díaz ME, Rodríguez-Carvajal MA, Velasco SE, Tejero-Mateo P, Gil-Serrano A, M, Irastorza A, Dueñas MT. 2007. Growth and exopolysaccharide (EPS) production by *Oenococcus oeni* I4 and structural characterization of their EPSs. *Journal of Applied Microbiology* 103:477-86

33. Adeyeye A, Jansson PE, Lindberg B, Henrichsen J. 1988. Structural studies of the capsular polysaccharide from *Streptococcus pneumoniae* type 37. *Carbohydrate Research* 180:295-9

34. Hwang EN, Kang SM, Kim MJ, Lee JW. 2015. Screening of Immune-Active Lactic Acid Bacteria. *Korean Journal for Food Science of Animal Resoucesr* 35:541-50

35. Ruiz-Bravo A, Jimenez-Valera M, Moreno E, Guerra V, Ramos-Cormenzana A. 2001. Biological response modifier activity of an exopolysaccharide from *Paenibacillus jamilae* CP-7. *Clinical and Diagnostic Laboratory Immunology* 8:706-10

36. Bleau C, Monges A, Rashidan K, Laverdure JP, Lacroix M, Van Calsteren MR, Millette M, Savard R, Lamontagne L. 2010. Intermediate chains of exopolysaccharides from *Lactobacillus rhamnosus* RW-9595M increase IL-10 production by macrophages. *Journal of Applied Microbiol*ogy 108:666-75

37. Hidalgo-Cantabrana C, Nikolic M, Lopez P, Suarez A, Miljkovic M, Kojic M, Margolles A, Golic N, Ruas-Madiedo P. 2014. Exopolysaccharide-producing *Bifidobacterium animalis* subsp. *lactis* strains and their polymers elicit different responses on immune cells from blood and gut associated lymphoid tissue. *Anaerobe* 26:24-30

38. López P, Monteserína DC, Meimondea M, de los Reyes-Gavilána C, Margollesa A, Sárez A, Ruas-Madiedoa P. 2012. Exopolysaccharide-producing *Bifidobacterium* strains elicit different *in vitro* responses upon interaction with human cells. *Food Research International* 46

39. Sungur T, Aslim B, Karaaslan C, Aktas B. 2017. Impact of exopolysaccharides (EPSs) of Lactobacillus gasseri strains isolated from human vagina on cervical tumor cells (HeLa). *Anaerobe* 47:137-44

40. Zhou X, Hong T, Yu Q, Nie S, Gong D, Xiong T, Xie M. 2017. Exopolysaccharides from *Lactobacillus plantarum* NCU116 induce c-Jun dependent Fas/Fasl-mediated apoptosis via TLR2 in mouse intestinal epithelial cancer cells. *Scientific Reports* 7:14247

41. Patten DA, Leivers S, Chadha MJ, Maqsood M, Humphreys PN, Laws AP, Collett A. 2014. The structure and immunomodulatory activity on intestinal epithelial cells of the EPSs isolated from *Lactobacillus helveticus* sp. *Rosyjski* and *Lactobacillus acidophilus* sp. 5e2. *Carbohydrate Research* 384:119-27

42. Gao K, Wang C, Liu L, Dou X, Liu J, Yuan, L. Zhang W, Wang Hl. 2017. Immunomodulation and signaling mechanism of *Lactobacillus rhamnosus* GG and its components on porcine intestinal epithelial cells stimulated by lipopolysaccharide. *Journal of Microbiology, Immunology and Infecti*on 50:700-13

43. van Bergenhenegouwen J, Plantinga TS, Joosten LA, Netea MG, Folkerts G, Kraneveld AD, Garssen J, Vos AP. 2013. TLR2 & Co: a critical analysis of the complex interactions between TLR2 and coreceptors. *Journal of Leukocyte Biology* 94:885-902

561   44.   Landmann R, Knopf HP, Link S, Sansano S, Schumann R, Zimmerli W. 1996. Human monocyte
562   CD14 is upregulated by lipopolysaccharide. *Infection and Immunity* 64:1762-9
563   45.   Dobrovolskaia MA, Vogel SN. 2002. Toll receptors, CD14, and macrophage activation and
564   deactivation by LPS. *Microbes and Infection* 4:903-14
565   46.   Angus DC, van der Poll T. 2013. Severe sepsis and septic shock. *New England Journal of*
566   *Medicine* 369:2063
567   47.   Galdeano CM, Perdigon G. 2006. The probiotic bacterium Lactobacillus casei induces
568   activation of the gut mucosal immune system through innate immunity. *Clinical and Vaccine*
569   *Immunol*ogy 13:219-26
570   48.   Zeuthen LH, Fink LN, Frokiaer H. 2008. Toll-like receptor 2 and nucleotide-binding
571   oligomerization domain-2 play divergent roles in the recognition of gut-derived *lactobacilli*
572   and *bifidobacteria* in dendritic cells. *Immunology* 124:489-502
573   49.   Ale, EC, Perezlindo M J, Pavón Y, Peralta GH, Costa S, Sabbag N, Bergamini C, Reinheimer J A,
574   Binetti A G. 2016(a). Technological, rheological and sensory characterizations of a yogurt
575   containing an exopolysaccharide extract from Lactobacillus fermentum Lf2, a new food
576   additive. *Food Research International,* 90:259-267
577   50.   Dubois M, Gilles K, Hamilton JK, Rebers PA, Smith F. 1951. A colorimetric method for the
578   determination of sugars. *Nature* 168:167
579   51.   Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. 1956. Colorimetric Method for
580   Determination of Sugars and Related Substances. *Analytical Chemistry* 28:350-6
581   52.   Gerwig GJ, Kamerling JP, Vliegenthart JFG. 1978. Determination of the d and l configuration
582   of neutral monosaccharides by high-resolution capillary g.l.c. *Carbohydrate Research* 62:349-
583   57
584   53.   Stellner K, Saito H, Hakomori SI. 1973. Determination of aminosugar linkages in glycolipids
585   by methylation. Aminosugar linkages of ceramide pentasaccharides of rabbit erythrocytes
586   and of Forssman antigen. *Archives of Biochemistry and Biophysics* 155:464-72
587
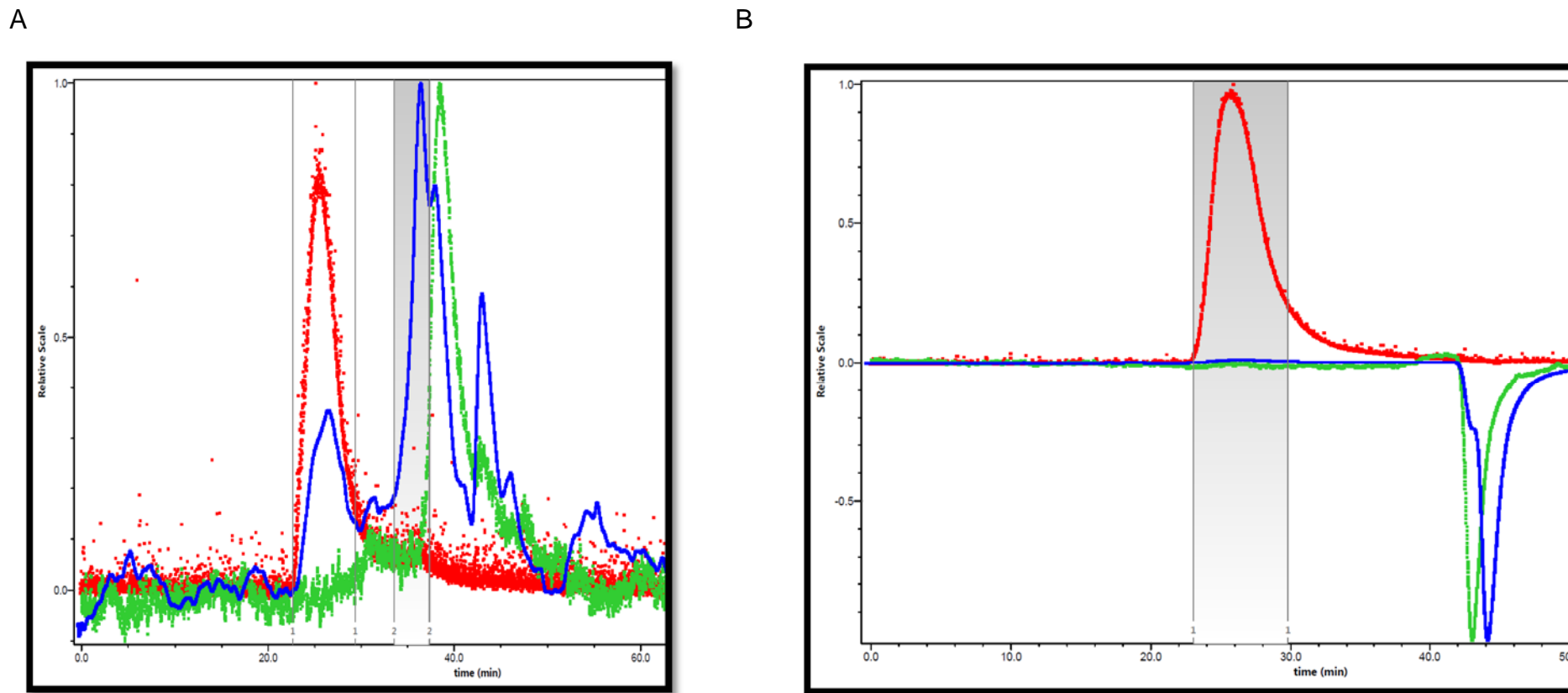
Figure 1 Analytical SEC-MALLS traces; Red trace = molecular weight dependent light scattering, Blue trace = concentration dependent refractive index, Green trace= UV absorption at 260 nm A) Chromatograph for the crude EPS from *Lactobacillus fermentum* Lf2 ((1 mg/mL) in aq. NaNO₃ (0.1M). B) Chromatograph for the HMw EPS from *Lactobacillus fermentum* Lf2 ((1 mg/mL) in aq. NaNO₃ (0.1M).
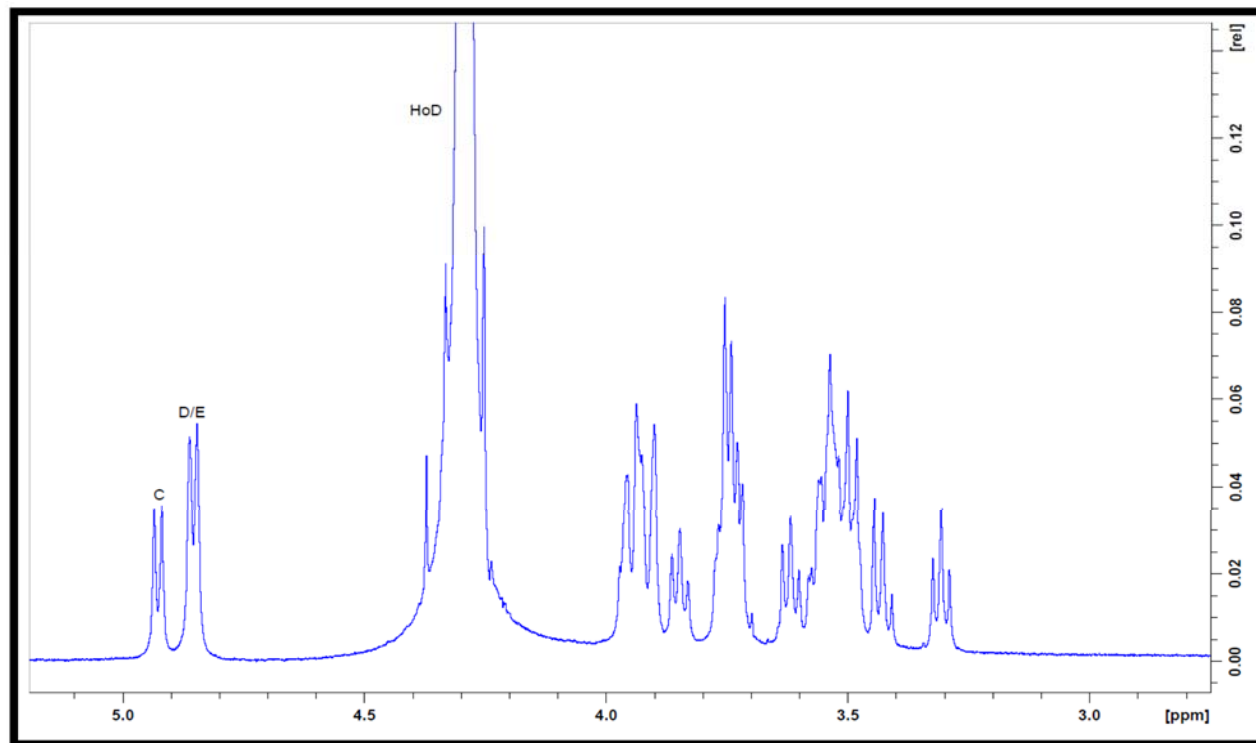
Figure 2 ¹H-NMR for the HMw EPS recorded in solution in D₂O (5-10 mg in 0.65 mL) at 70 °C on a Bruker 500MHz spectrometer.

The residual HOD signal is at 4.29 ppm at 70 °C.
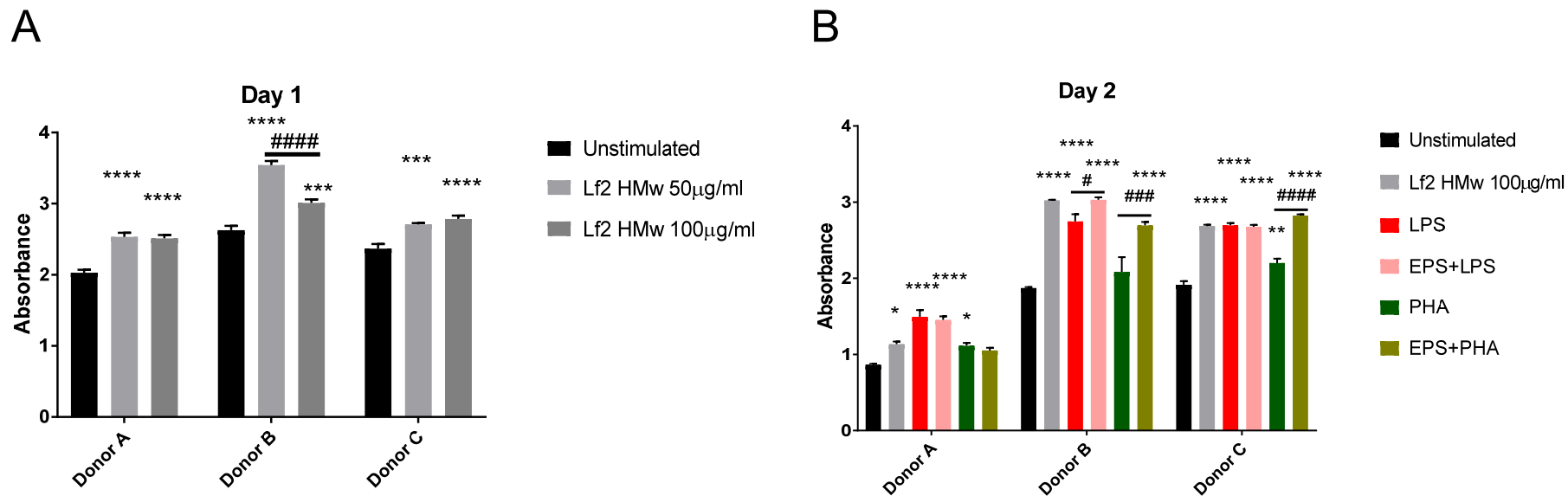
Figure 3. Non-radioactive MTS assay was used for measuring metabolic activity of the cells under different culture conditions as measured after A. Experiment 1, Day 1 and B. Experiment 2, Day 2. For Experiment 1, Unstimulated conditions were left untreated while Lf2 HMw 50μg/ml and Lf2 HMw 100μg/ml were treated with EPS at indicated concentrations on Day 0 and incubated at 37˚C, 5% CO_2. Following 24h incubation, cells were harvested and 1×10^5 cells/well were transferred into 96-well plates for MTS assay performed following the supplier's instructions. For Experiment 2, Day 2, Unstimulated condition was left untreated on Day 0 then washed and left unstimulated on Day 1. Lf2 HMw 100μg/ml were pre-treated at Day 0 with indicated concentration of EPS, then washed and left unstimulated on Day 1. LPS and PHA alone are conditions that were left untreated on Day 0, then washed and subsequently stimulated with 100ng/ml LPS and 10μg/ml PHA, respectively. EPS + LPS and EPS + PHA represent the conditions that were pre-treated with EPS Lf2 HMw on Day 0, washed and stimulated with 100ng/ml LPS and 10μg/ml PHA, respectively on Day 1. Following 24h incubation, cells were harvested and 1×10^5 cells/well were transferred into 96-well plates for MTS assay performed following the supplier's instructions. Two-Way ANOVA was used to compare the conditions for each donor. Data are presented as Mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ when compared to the untreated, unstimulated condition; # $p < 0.05$, ### $p < 0.001$, #### $p < 0.0001$ when compared between two indicated conditions.

A.

B.

C.
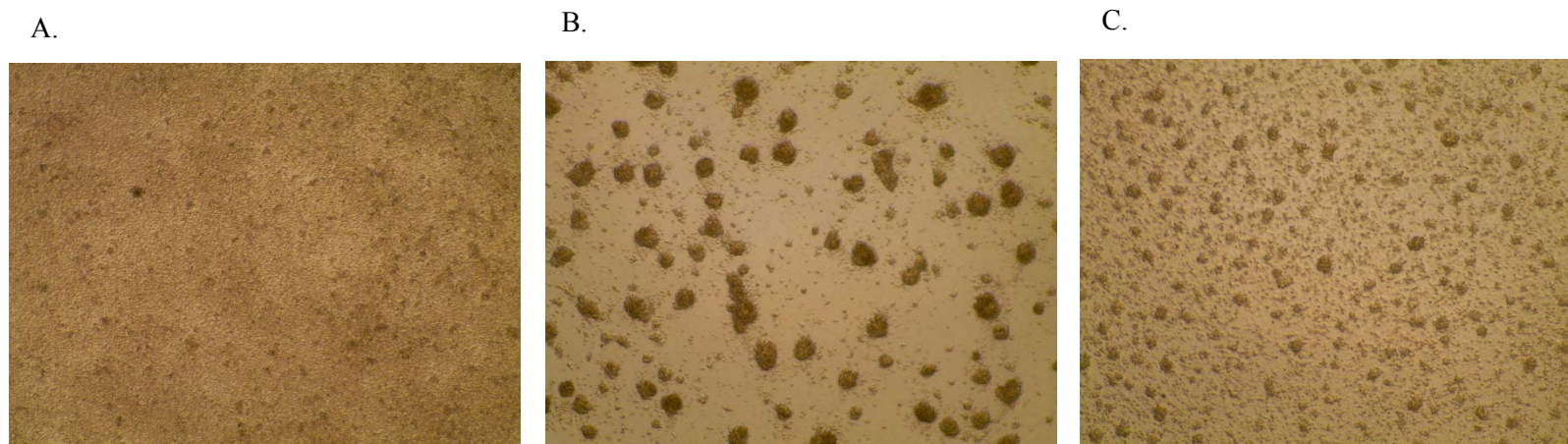
Figure 4. Representative microscopic images showing the effect of PHA with and without EPS pre-treatment on PBMC proliferation as seen at Day 2 of experiment 2. A. Untreated at Day 0, then washed and unstimulated at Day 1; B. Untreated at Day 0, then washed and stimulated with 10μg/ml PHA at Day 1; C. Pre-treated with 100μg/ml Lf2 HMw EPS at Day 0, then washed and stimulated with 10μg/ml PHA at Day 1.
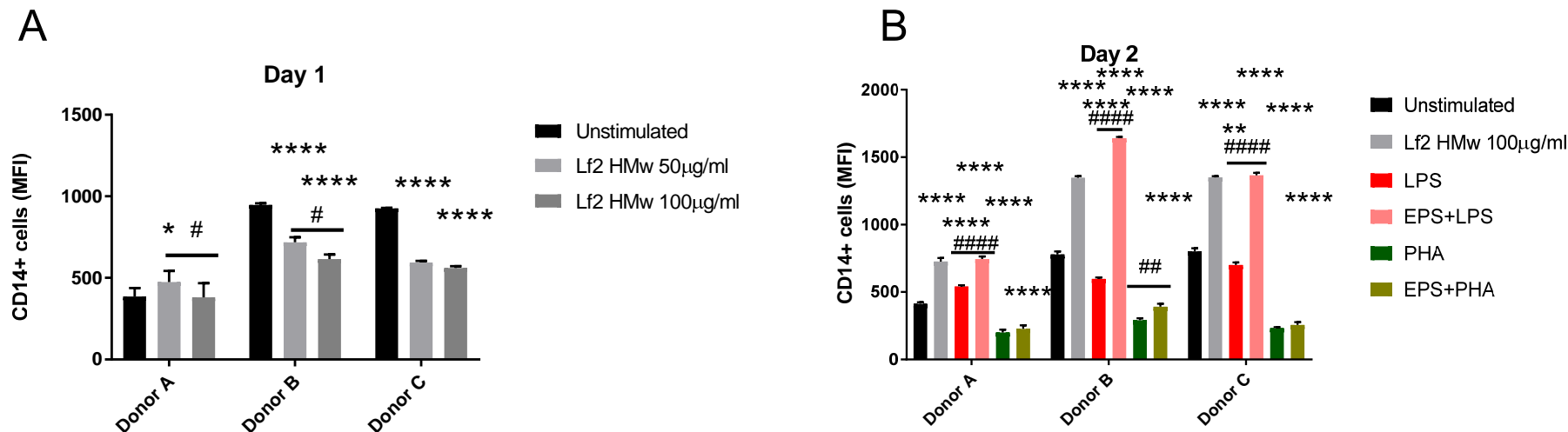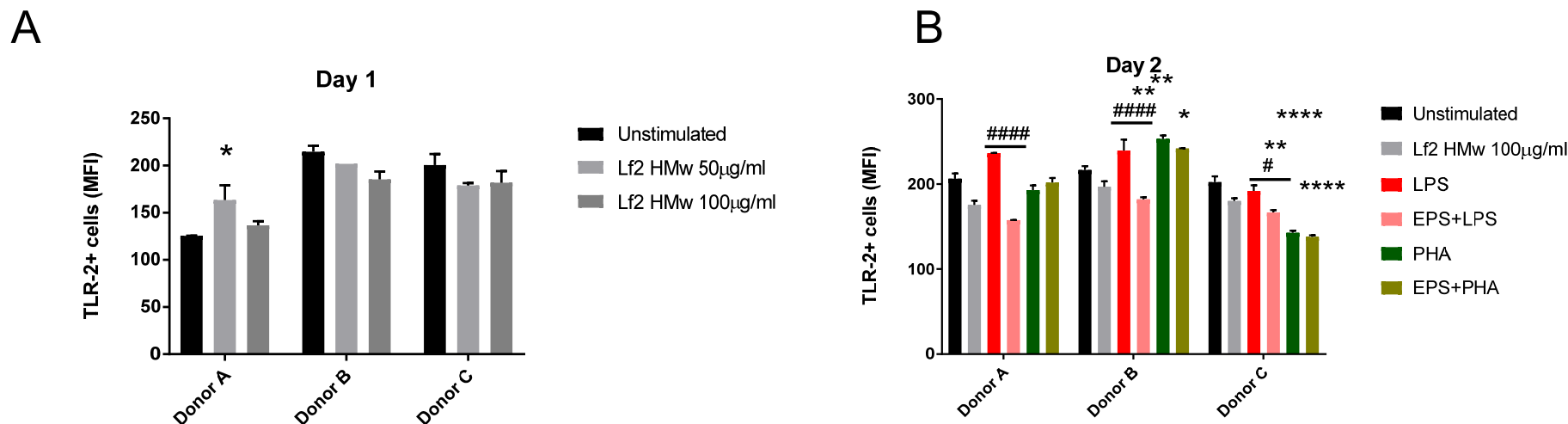
Figure 5. Changes in CD14 expression on PBMC following different conditions presented as median fluorescence intensity (MFI) of CD14$^+$ cells after A. Day 1 and B. Day 2 experiment. For Day 1, Unstimulated conditions were left untreated while Lf2 HMw 50μg/ml and Lf2 HMw 100μg/ml were treated with EPS at indicated concentrations on Day 0 and incubated at 37°C, 5% CO_2. Following 24h incubation, cells were harvested and stained with anti-CD14 antibody and analysed by flow cytometry. For Day 2 experiment, Unstimulated condition was left untreated on Day 0, then washed and left unstimulated on Day 1. Lf2 HMw 100μg/ml were pre-treated at Day 0 with indicated concentration of EPS, then washed and left unstimulated on Day 1. LPS and PHA alone are conditions that were left untreated on Day 0, then washed and subsequently stimulated with 100ng/ml LPS and 10μg/ml PHA, respectively. EPS + LPS and EPS + PHA represent the conditions that were pre-treated with EPS Lf2 HMw on Day 0, then washed and stimulated with 100ng/ml LPS and 10μg/ml PHA, respectively on Day 1. Following 24h incubation, cells were harvested and stained with anti-CD14 antibody and analysed by flow cytometry. Two-Way ANOVA was used to compare the conditions for each donor. Data are presented as Mean ± SEM. * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$ when compared to the untreated, unstimulated condition; # $p < 0.05$, ## $p < 0.01$, #### $p < 0.0001$ when compared between two indicated conditions. Gates were set on single cells and debris was excluded using FSC vs SSC plots. Gating was performed using FMO (Fluorescence Minus One) controls.

Figure 6. Changes in TLR-2 expression on PBMC following different conditions presented as median fluorescence intensity (MFI) of TLR-2^{+} cells after A. Day 1 and B. Day 2 experiment. For Day 1, Unstimulated conditions were left untreated while Lf2 HMw 50μg/ml and Lf2 HMw 100μg/ml were treated with EPS at indicated concentrations on Day 0 and incubated at 37°C, 5% CO_2. Following 24h incubation, cells were harvested and stained with anti-TLR-2 antibody and analysed by flow cytometry. For Day 2 experiment, Unstimulated condition was left untreated on Day 0, then washed and left unstimulated on Day 1. Lf2 HMw 100μg/ml were pre-treated at Day 0 with indicated concentration of EPS, then washed and left unstimulated on Day 1. LPS and PHA alone are conditions that were left untreated on Day 0, then washed and subsequently stimulated with 100ng/ml LPS and 10μg/ml PHA, respectively. EPS + LPS and EPS + PHA represent the conditions that were pre-treated with EPS Lf2 HMw on Day 0, then washed and stimulated with 100ng/ml LPS and 10μg/ml PHA, respectively on Day 1. Following 24h incubation, cells were harvested and stained with anti-TLR-2 antibody and analysed by flow cytometry. Two-Way ANOVA was used to compare the conditions for each donor. Data are presented as Mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ when compared to the untreated, unstimulated condition; # $p < 0.05$, #### $p < 0.0001$ when compared between two indicated conditions. Gates were set on single cells and debris was excluded using FSC vs SSC plots. Gating was performed using FMO (Fluorescence Minus One) controls.
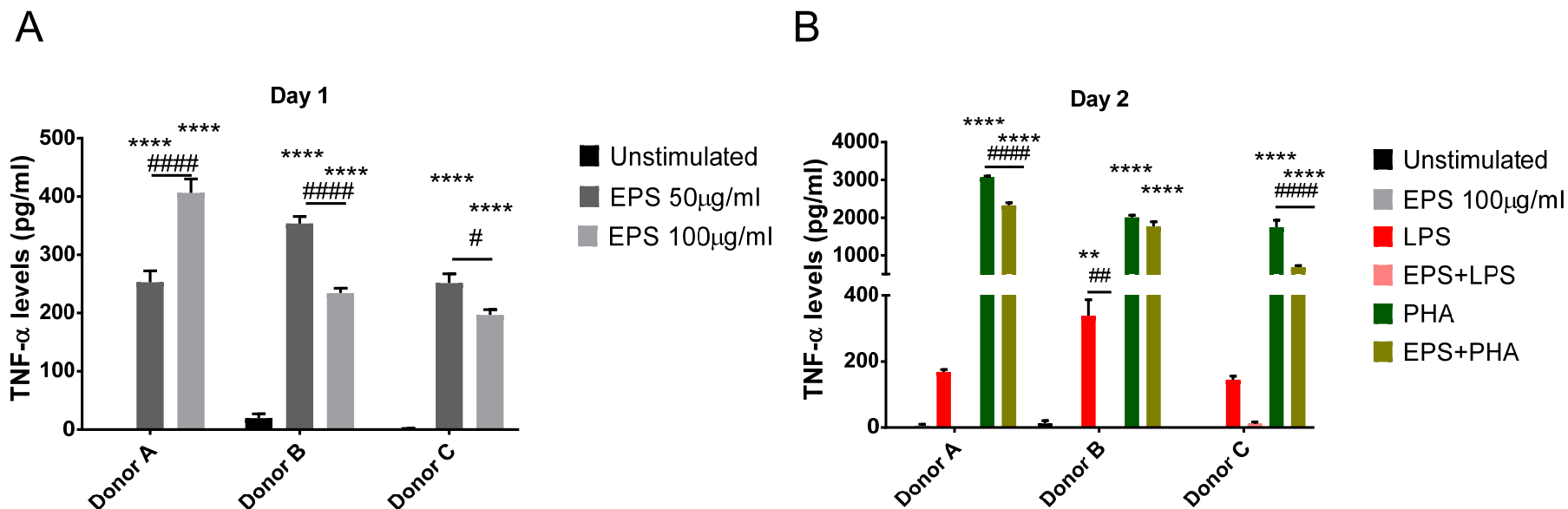
Figure 7. Changes in TNF-α levels in cell culture supernatants following different conditions after A. Day 1 and B. Day 2 experiment. For Day 1, Unstimulated conditions were left untreated while Lf2 HMw 50µg/ml and Lf2 HMw 100µg/ml were treated with EPS at indicated concentrations on Day 0 and incubated at 37˚C, 5% CO_2. Following 24h incubation, supernatants were stored at -80˚C until subsequent analysis for TNF-α levels by ELISA. For Day 2 experiment, Unstimulated condition was left untreated on Day 0, then washed and left unstimulated on Day 1. Lf2 HMw 100µg/ml were pre-treated at Day 0 with indicated concentration of EPS, then washed and left unstimulated on Day 1. LPS and PHA alone are conditions that were left untreated on Day 0, then washed and subsequently stimulated with 100ng/ml LPS and 10µg/ml PHA, respectively. EPS + LPS and EPS + PHA represent the conditions that were pre-treated with EPS Lf2 HMw on Day 0, then washed and stimulated with 100ng/ml LPS and 10µg/ml PHA, respectively on Day 1. Following 24h incubation, supernatants were stored at -80˚C until subsequent analysis for TNF-α levels by ELISA. Two-Way ANOVA was used to compare the conditions for each donor. Data are presented as Mean ± SEM. ** $p < 0.01$, **** $p < 0.0001$ when compared to the untreated, unstimulated condition; # $p < 0.05$, ## $p < 0.01$, #### $p < 0.0001$ when compared between two indicated conditions.
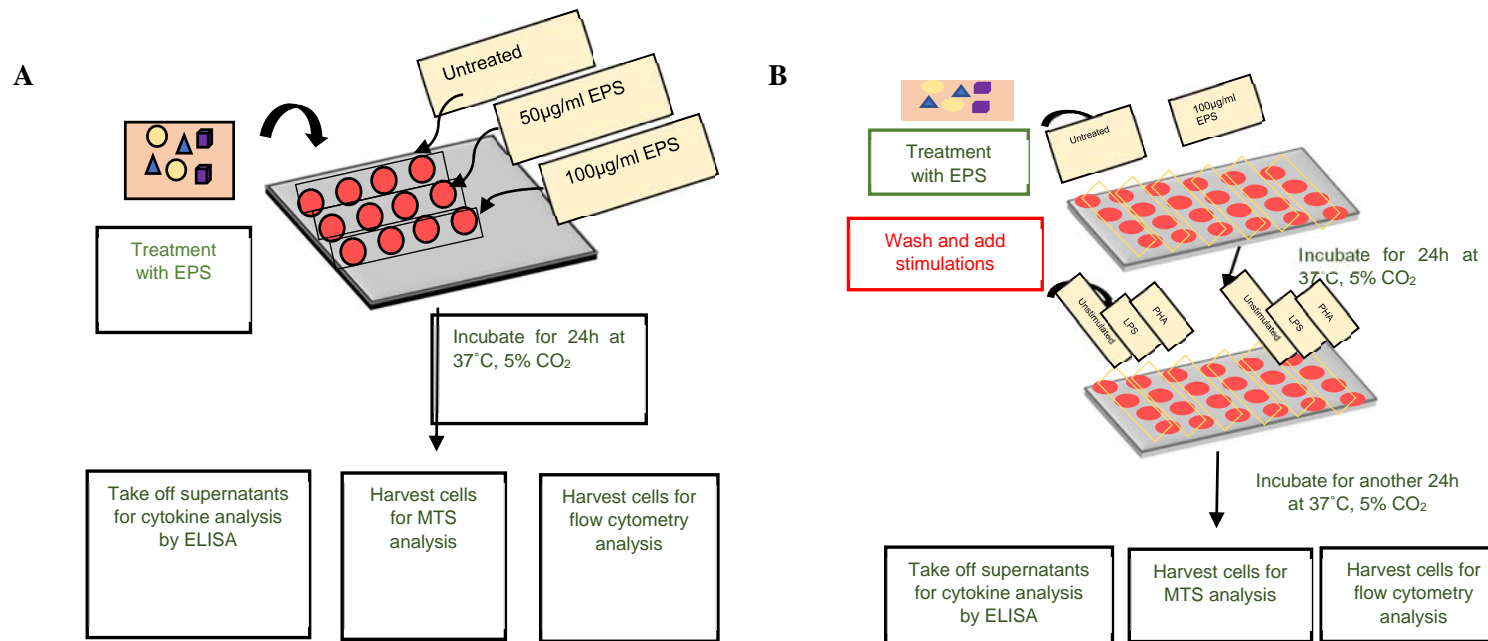
Figure 8. Schematic representation of the cell culture protocol completed on A. Experimet 1 and B. Experiment 2. A. On Day 0, cells were treated with two different Lf2 HMw EPS concentrations (50μg/ml and 100μg/ml) or left untreated at 37°C, 5% CO_2. Following 24h incubation, supernatants were taken off and stored at -80°C for subsequent analysis by ELISA. Cells were harvested and 1×10^5 cells/well were transferred into 96-well plates for MTS assay performed following the supplier's instructions. The remaining cells were stained with antibodies for CD14 and TLR-2 and analysed for the expression of these markers by flow cytometry. B. on Day 0 cells were pre-treated with 100μg/ml EPS or left untreated and incubated for 24h at 37°C, 5% CO_2. On Day 1, cells were washed to remove EPS and were stimulated with LPS (100ng/ml) or PHA (10μg/ml) or left unstimulated and incubated for another 24h at 37°C, 5% CO_2. On Day 2, supernatants were then stored at -80°C for subsequent cytokine analysis by ELISA. As before 1×10^5 of harvested cells/well were transferred into 96-well plate for MTS assay while remaining cells were stained for flow cytometric analysis of CD14 and TLR-2 expression.

Table 1.

	H-1 C-1	H-2 C-2	H-3 C-3	H-4 C-4	H-5 C-5	H-6a C-6a	H-6b C6b
A	4.93 102.7	3.31 74.7	3.50 76.3	3.50 76.3	3.50 76.3	3.96 61.6	3.75 61.6
B	4.85 102.2	3.84 80.1	3.95 84.2	3.55 68.8	3.55 68.8	3.94 61.6	3.74 61.4
C	4.85 102.2	3.62 73.2	3.75 86.6	3.42 70.6	3.42 70.6	3.90 61.6	3.73 61.4

Proton and carbon chemical shifts (ppm) for the Lf2 HMW-EPS recorded in D_2O at 70 °C on a Bruker Avance 500MHz NMR spectrometer. Chemical shifts are reference to external acetone 2.225 ppm for ^1H and 31.55 ppm for ^{13}C

Supplementary Data:
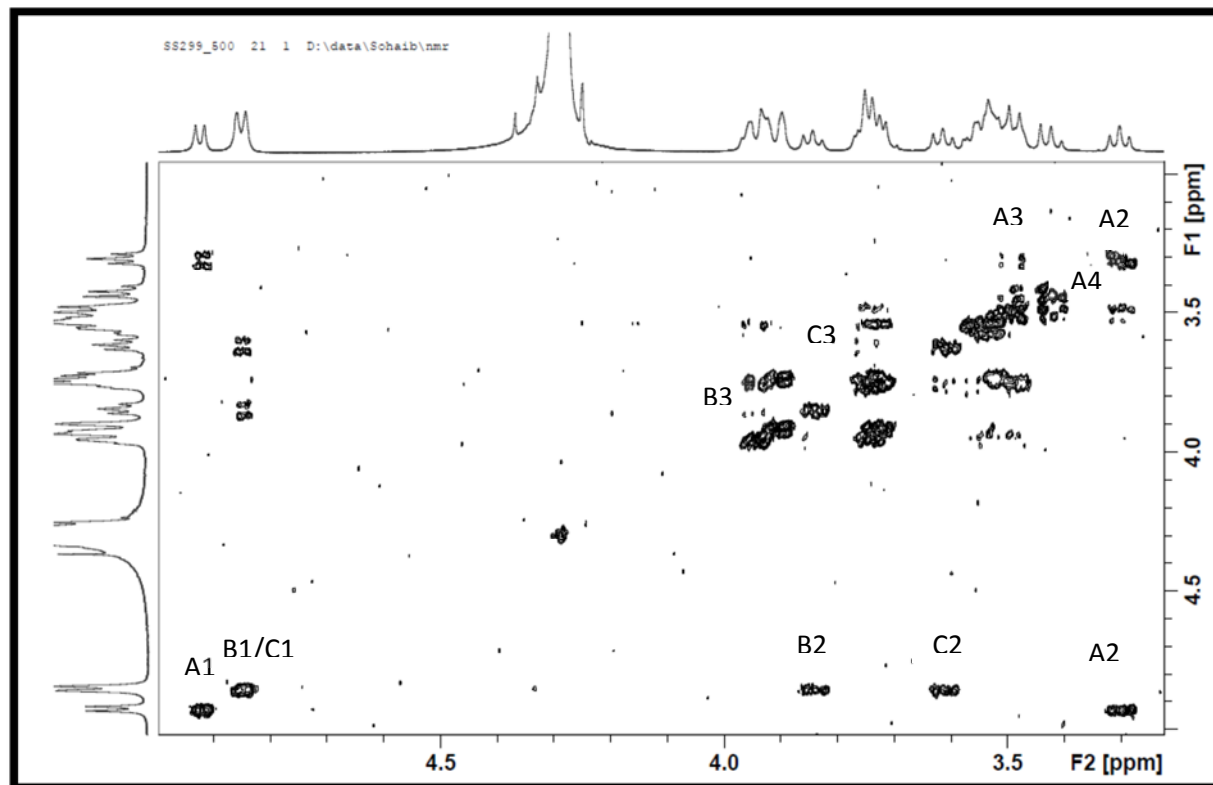
Figure S1: ^{1}H-^{1}H COSY spectrum for the HMw β-glucan recorded in solution in D_2O (5-10 mg in 0.65 mL) at 70 ºC on a Bruker 500MHz spectrometer, Labels (A-C) identify the different monosaccharides and the numbers(1-3) identify the respective proton/carbon.
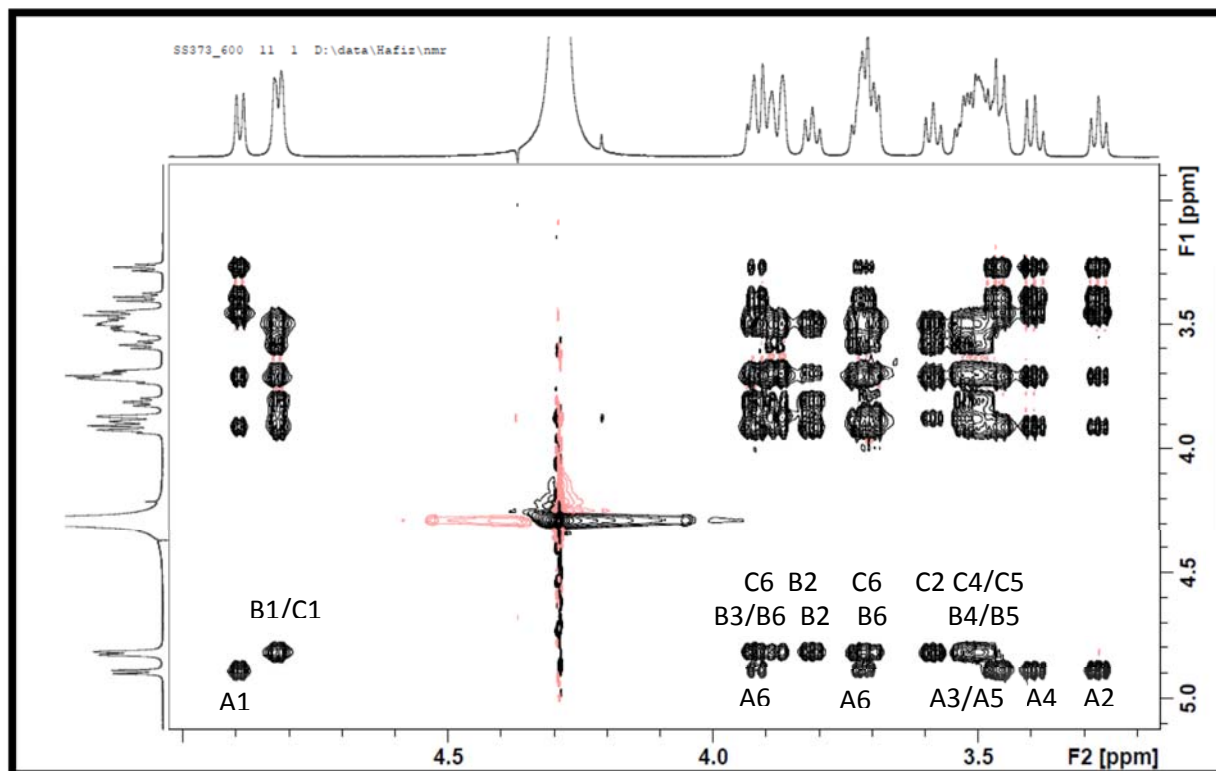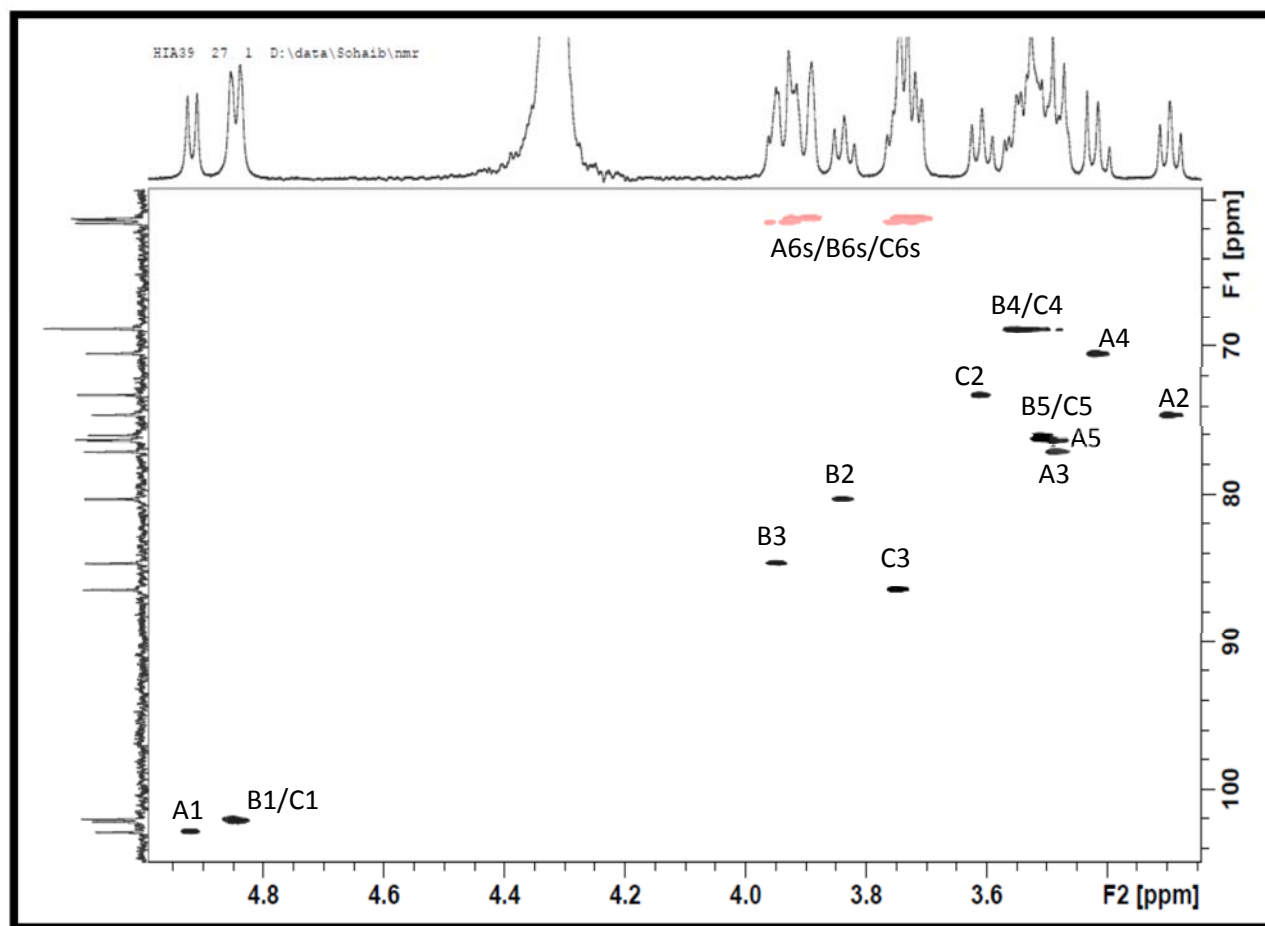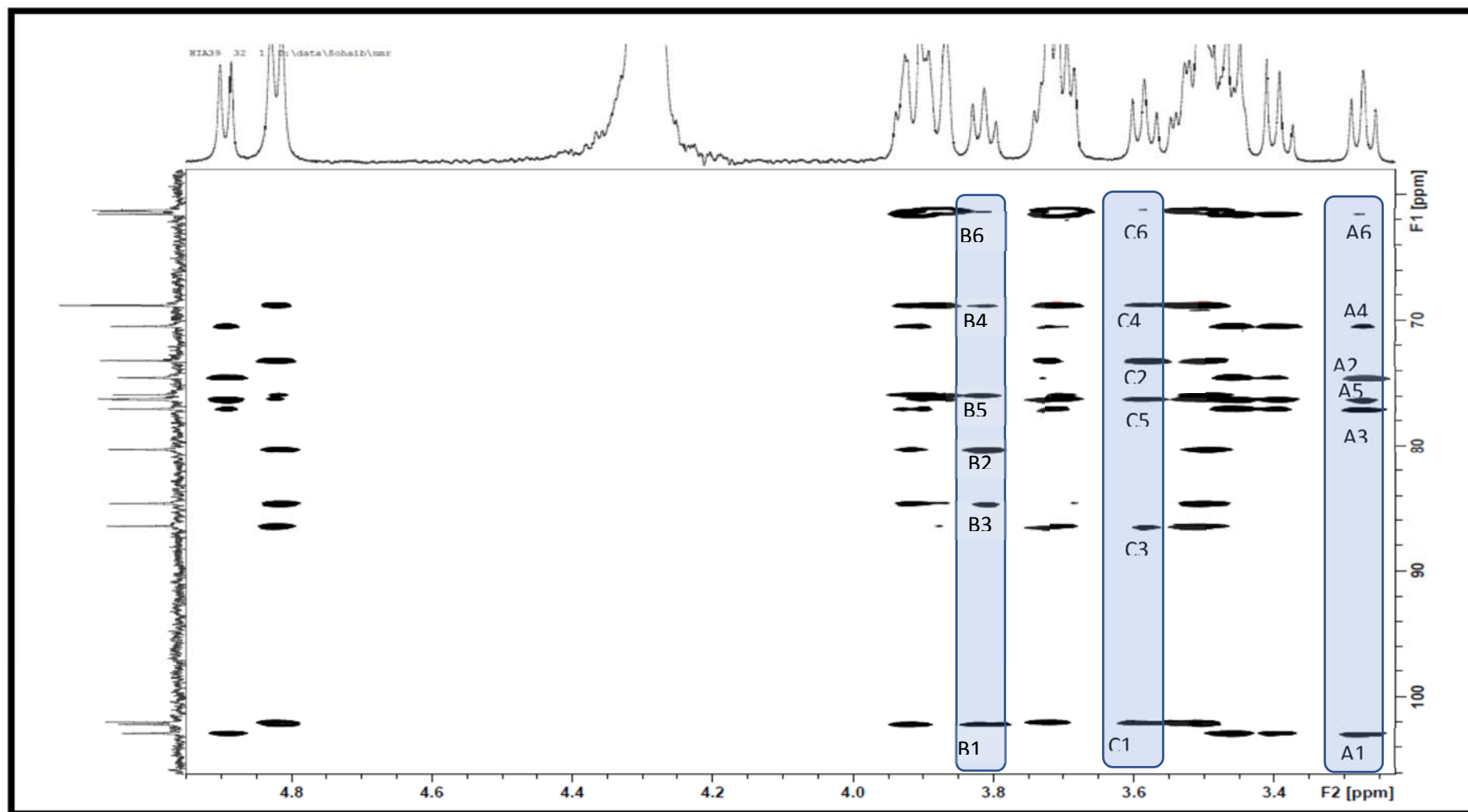
Figure S2: ^1H-^1H TOCSY spectrum for the HMw β-glucan recorded in solution in D_2O (5-10 mg in 0.65 mL) at 70 °C on a Bruker 600MHz spectrometer, Labels (A-C) identify the different monosaccharides and the numbers (1-6) identify the respective proton/carbon.

Figure S3: ^1H-^1H HSQC spectrum for the HMw β-glucan recorded in solution in D_2O (5-10 mg in 0.65 mL) at 70 °C on a Bruker 500MHz spectrometer, Labels (A-C) identify the different monosaccharides and the numbers (1-6) identify the respective proton/carbon.
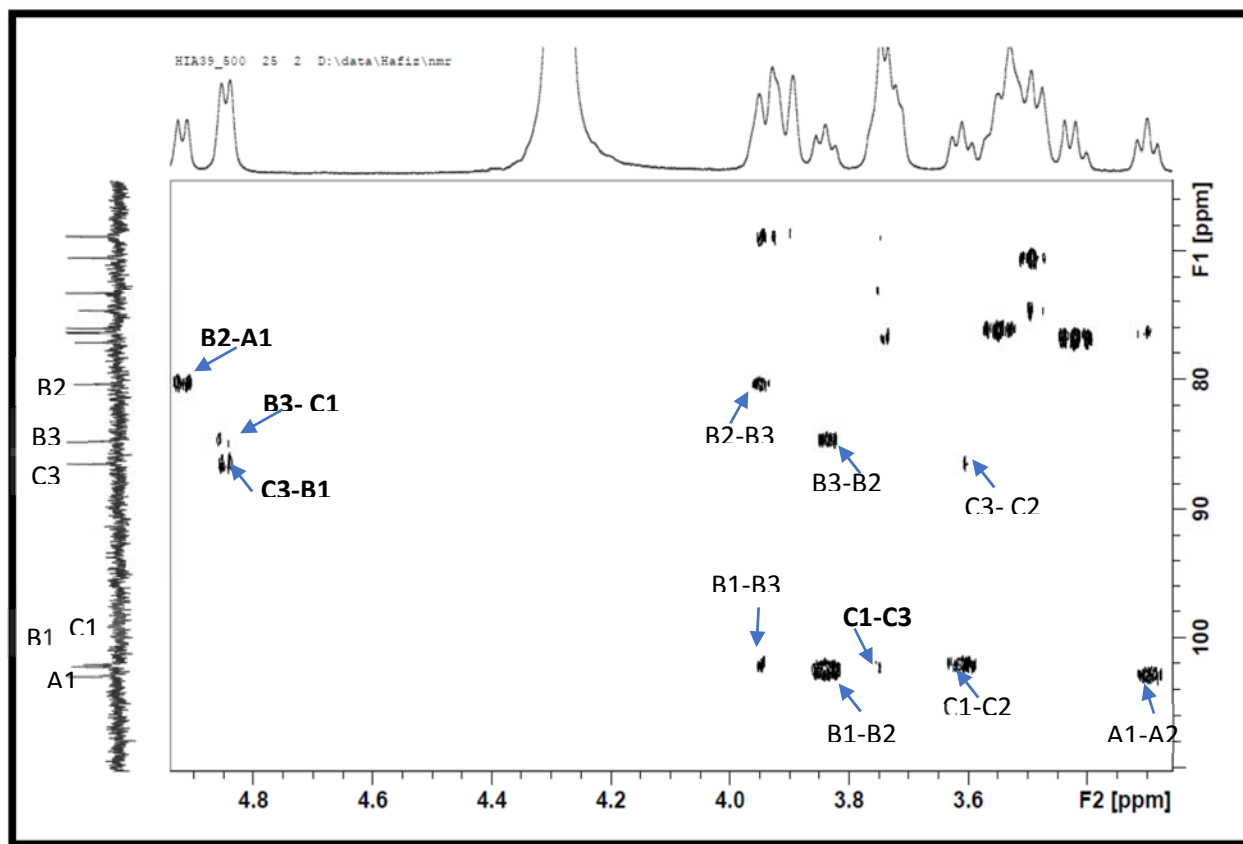
Figure S4: ^{13}C-^1H HSQC-TOCSY spectrum for the HMw β-glucan recorded in solution in D_2O (5-10 mg in 0.65 mL) at 70 °C on a Bruker 400MHz spectrometer, Labels (A-C) identify the different monosaccharides and the numbers (1-6) identify the respective proton/carbon.

Figure S5: ^{13}C-^{1}H HSQC-HMBC spectrum for the HMw β-glucan recorded in solution in D$_2$O (5-10 mg in 0.65 mL) at 70 °C on a Bruker 500MHz spectrometer, Labels identify the significant inter and intra residue scalar coupling, those involved in linkages are highlighted in bold; (A-C) identify the different monosaccharides and the numbers (1-6) identify the respective proton/carbon.
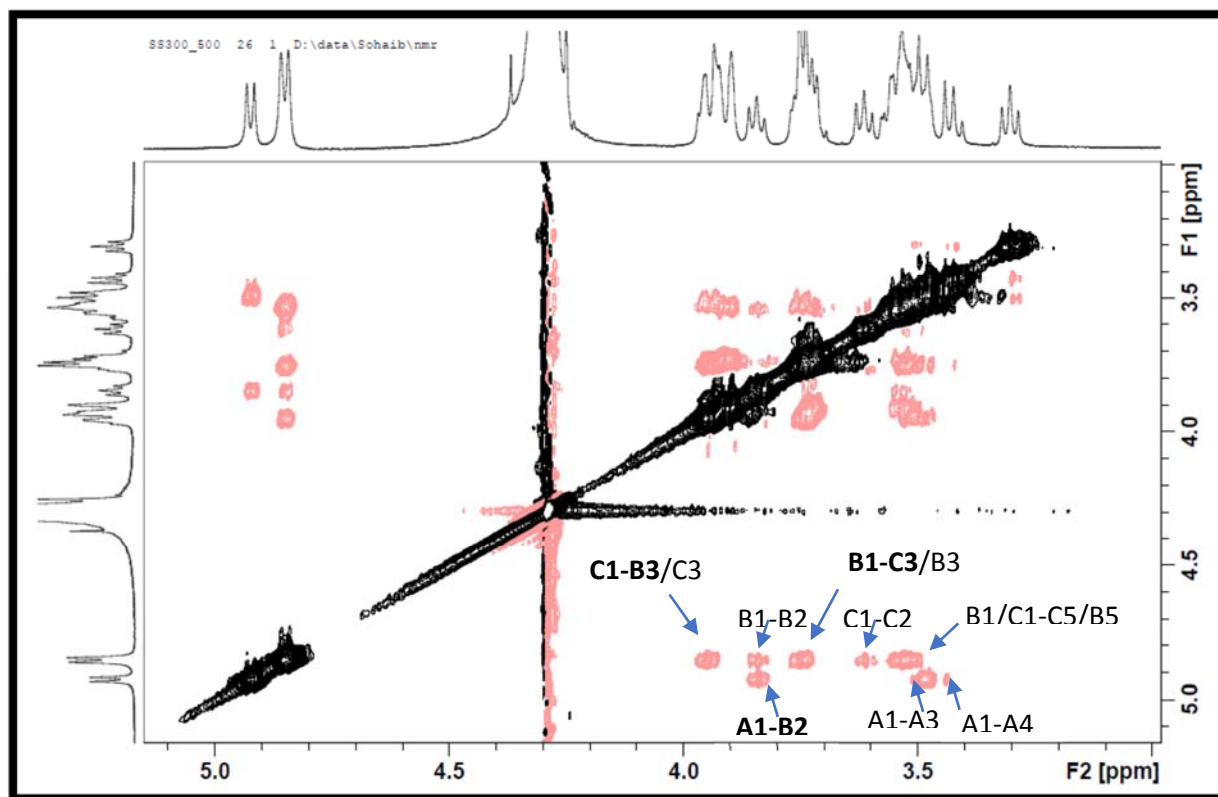
Figure S6: ^{1}H-^{1}H ROESY spectrum for the HMw β-glucan recorded in solution in D$_2$O (5-10 mg in 0.65 mL) at 70 °C on a Bruker 500MHz spectrometer, Labels the significant inter and intra residue NOEs-those locating linkages are highlighted in bold involved (A-C) identify the different monosaccharides and the numbers (1-6) identify the respective proton/carbon.