The structure and immunomodulatory activity on intestinal epithelial cells of the EPSs isolated from *Lactobacillus helveticus* sp. Rosyjski and *Lactobacillus acidophilus* sp. 5e2

Daniel A. Patten[a], Shaun Leivers[a], Marcus J. Chadha[a], Mohammed Maqsood[a], Paul N. Humphreys[a], Andrew P. Laws[a*] and Andrew Collett[a*]

Department of Chemical and Biological Sciences, University of Huddersfield, Queensgate, Huddersfield, West Yorkshire, HD1 3DH, UK.

*Corresponding authors:

Laws-Tel: +44-1484-472668; fax: +44-1484-472182 e-mail a.p.laws@hud.ac.uk

Collett- Tel: +44-1484-473587; fax: +44-1484-472182 e-mail A.Collett@hud.ac.uk

Abstract

The Lactic acid bacteria (LAB) *Lactobacillus acidophilus* sp. 5e2 and *Lactobacillus helveticus* sp. Rosyjski both secrete exopolysaccharides (EPSs) into their surrounding environments during growth. A number of EPSs have previously been shown to exhibit immunomodulatory activity with professional immune cells, such as macrophages, but only limited studies have been reported of their interaction with intestinal epithelial cells. An investigation of the immunomodulatory potential of pure EPSs, isolated from cultures of *Lactobacillus acidophilus* sp. 5e2 and *Lactobacillus helveticus* sp. Rosyjski, with the HT29-19A intestinal epithelial cell line are reported here. For the first time the structure of the EPS from *Lactobacillus helveticus* sp. Rosyjski which is a hetropolysaccharide with a branched pentasaccharide repeat unit containing D-glucose, D-galactose and *N*-acetyl-D-mannosamine is described. In response to exposure to lactobacilli EPSs HT29-19A cells produce significantly increased levels of the proinflammatory cytokine IL-8. Additionally, the EPSs differentially modulate the mRNA expression of Toll-like receptors. Finally, the pre-treatment of HT29-19A cells with the EPSs sensitises the cells to subsequent challenge with bacterial antigens. The results reported here suggest that EPSs could potentially play a role in intestinal homeostasis via a specific interaction with intestinal epithelial cells.

Key words: lactobacilli, exopolysaccharides, intestinal epithelial cells, innate immunity, Toll-like receptors

1. Introduction

Exopolysaccharides (EPSs) are long-chain polysaccharides naturally released by a variety of both Gram-positive and Gram-negative bacteria during growth[1-3]. EPSs play a major role in bacterial attachment and biofilm formation[4-6] and it is has been shown that EPS-deficient mutants completely lack the ability to form biofilms *in vitro*[7,8]. Additionally, they are thought to be responsible for enhancing nutrient and water entrapping abilities of bacteria[4]. The inclusion of EPSs and EPS producing bacteria in food and food supplements is becoming more common, originally they were added as biothickening agents[9,10] and as moisture retention agents[11]. However, more recently there have been an increasing number of reports claiming health benefits associated with the consumption of EPS producing bacteria: mostly lactic acid bacteria (LAB) and bifidobacteria[12 13-17].

Those EPSs which are synthesised at the cell membrane and subsequently released into the surrounding media frequently contain multiple repeats of oligosaccharides (repeating units) made up of between 3 and 7 sugar residues. The repeating units typically contain D-glucose, D-galactose and L-rhamnose sugars and occasionally include amino-sugars, such as *N*-acetyl-D-glucosamine and *N*-acetyl-D-galactosamine[9,18]. EPSs are produced by a number of mesophilic and thermophilic bacteria, including LAB[19] and bifidobacteria[2].

A high proportion of bacteria used in the food industry and indeed, naturally residing within the intestine, such as LAB and bifidobacteria, are EPS-producing thus it is surprising that very little research has been undertaken into the immunomodulatory potential of EPSs with the intestinal epithelial layer, particularly as it is known that EPSs can survive transit through the gastrointestinal tract[20]. EPSs have previously been considered in immunomodulatory studies utilising macrophage

cell models, with the up-regulation of both pro- and anti-inflammatory cytokines, such as TNF-α, IL-6 and IL-10, observed in response to challenge with EPSs[13-15,17]. It has been suggested that these differential effects could be correlated with physiochemical properties with large EPSs suppressing the immune response and those with acidic groups stimulating macrophages.[21] Despite this, the mechanism with which EPSs interact with intestinal epithelial cells is unknown; however, Toll-like receptors (TLRs) of the innate immune system, including intestinal epithelial cells have recently been suggested to mediate these responses[22].

Lactobacillus helveticus sp. Rosyjski is a strain of EPS-producing Gram positive bacteria identified by the EU International Scientific Cooperation Project (IC15-CT98-0905) (http://imol.vub.ac.be/IMDO/projects/EPSLAB.html) for which there is no information on its structural and possible biological activities. In this paper, the results of experiments undertaken in order to characterise this novel EPS and to investigate its immune modulation activity in cultured intestinal epithelial cells is described. In addition, its immunomodulatory potential is compared with that of an EPS isolated from *Lactobacillus acidophilus* sp. 5e2 the structure of which has been previously described[23].

2. Results and Discussion

2.1 Structure of the exopolysaccharide isolated L. helveticus sp. Rosyjski- The EPS isolated from strain *L. helveticus* sp. Rosyjski eluted as a single relatively sharp peak from a size exclusion column and has a mass average molecular mass (M_w) of about 1.0 x 10^6 u. Monomer analysis and determination of the absolute configuration of sugars as their 2-butyl glycosides identified D-glucose, D-galactose and *N*-acetyl-D-mannosamine in the ratio of 2:2:1 as the component of the repeating unit. The presence of *N*-acetyl-D-mannosamine is unusual as there have been no previous reports of LAB EPSs containing this monomer[18]. The linkage pattern of the monomers in the repeating unit was determined using methylation analysis which showed the presence of a terminal hexosamine, a terminal hexose, a 1,4-substituted hexose and two 1,3,4-substituted hexoses. Finally, the sequence of monomers and the establishment of the anomeric configuration of the sugars were determined by a detailed analysis of a number of 1D and 2D-NMR.

Analysis of the anomeric region of the ^{1}H-NMR (Fig. 1 δ =4.5-6 ppm) identified six proton resonances: five are anomeric H-1 signals (labelled **A** to **E** with decreasing chemical shift) and the remaining signal was identified as H-2 of monomer **B** (identified through inspection of cross peaks in the ^{1}H-^{1}H COSY spectrum-not shown). In a number of batches of the EPS a small anomeric signal was observed at δ =5.31; it is believed that this is due to the loss of the N-acetyl group from *N*-acetyl-D-mannosamine during the isolation procedure. The presence of five major anomeric protons suggests that the repeating unit is a pentasaccharide. The ^{13}C 135 DEPT spectrum (Fig. 1b) contains five signals in the anomeric region confirming that the EPS has a pentasaccharide repeat unit. The presence of a carbonyl carbon in the ^{13}C spectrum (not shown), a high field ring carbon at 53.86 ppm and an acetyl-

methyl group at 21 ppm confirmed the presence of an *N*-acetyl-aminosugar. From inspection of the negative peaks in the DEPT 135 spectrum it is clear that all five sugars are hexoses and that there are no 1-6 links present in the repeating unit. The position of the remaining ring protons was established through analysis of the scalar coupling observed in the corresponding ^{1}H-^{1}H COSY spectrum and ^{1}H-^{1}H TOCSY spectrum (mixing times 30-250 ms -not shown).

Figure 1 here.

The assignment of individual carbon resonances to their position (1-6) within monomers **(A-E)** was based on inspection of both the ^{1}H-^{13}C HSQC (Fig 2) and ^{13}C ^{1}H-HSQC-TOCSY spectra (not shown) and the assignments are tabulated in Table 1.

Table 1 here.

The location of C2 of **B** at 53.86 ppm confirms that residue **B** is the terminal *N*-acetyl-D-mannosamine, the high chemical shifts of both C3 and C4 of residues **A** and **C** confirm that these are the 1,3,4-linked hexoses. Similarly, the high chemical shift of C4 in residue **D** suggests that this is the 1,4-linked hexose. Finally, the resonance position for all the carbons of residue **E** are those expected for a terminal hexose. One way of differentiating between galacto- and glucopyranoses is from inspection of the chemical shifts for their H-4 resonances: for a galactose H-4 is shifted substantially to lower field than that of glucose, regardless of the anomeric configuration and linkage. Data collected from assignments for LAB EPS structures show that the H-4 resonances for galactose lie in the range 4.30-3.85 δ whilst those

for glucose lie in the range 3.45 -3.75δ. The latter result would strongly suggest that residues **A & C** are galactose and **D & E** are glucose; this assignment is supported by the failure to get transmission of the scalar coupling beyond H-4 in both residues A and C and by the intra-residue NOEs observed in the ROESY spectrum (not shown).

The anomeric configuration of each of the monomers was determined by measurement of the magnitude of the $^{1}J_{C1\text{-}H1}$ coupling constant measured using a coupled HSQC spectrum (not shown): **A** (172 Hz)**, B** (166 Hz)**, C** (177 Hz) **D** (157Hz) and **E** (164Hz)**.** With values greater than 170 Hz for **A** & **C** represent monomers having α-linkages whilst, with values less than 170 Hz for monomers **B**, **D** & **E** will have β-linkages. Finally, the sequence of the monomers in the repeating unit was determined by examination of the HMBC (Fig. 3) and ROSEY spectra (not shown); the most significant intra- and inter-residue cross-peaks are highlighted on the HMBC spectrum.

Figures 3

The combined results of the chemical and spectroscopic analysis identify that the repeating unit of the EPS from *L. helveticus* sp. Rosyjski has the following novel structure:

Figure 4 here:

2.2 Purity of the isolated EPS-Before measuring the biological activity of the EPSs from *Lactobacillus acidophilus* sp. 5e2 and *Lactobacillus helveticus* sp. Rosyjski it

was necessary to confirm the purity of the samples. The first step in the isolation procedure involved the removal of cells by ultracentrifugation to provide a clear supernatant containing only material that had been secreted into the fermentation media. Careful collection of the EPS from the fermentation media during the log growth phase was done deliberately to minimise contamination by cell wall components. The carbohydrate content of batches of EPS was measured and those having greater than 80% carbohydrate content were used in the biological assays. The vast majority of the remaining mass of the samples was determined to be water of hydration; the measured protein content was less than 2% (typically 0.5%) and the nucleic acids content was less than 0.5%. The samples were analysed by SEC-MALLS which demonstrated that a single high mass polysaccharide was being isolated and the in-line UV detector confirmed the very low levels of protein and nucleic acid contamination. Proton, carbon and phosphorus NMR indicated that the EPS was the only macromolecule present. The absence of any phosphorus signals in the ^{31}P NMR identifies that cell wall components are not present even at low concentrations.

Whilst we cannot completely rule out that small levels of impurities are responsible for some of the biological activity that is observed; it is highly unlikely that this is the case. The choice of Gram positive bacteria lacking flaggellin, the method of isolation of the EPS and the processes used for its purification have been developed in order to reduce as far as possible the contamination of the EPS by bacterial cell wall antigens.

2.3 Immunomodulatory activity of Lactobacilli EPS the effect of exposure on chemokine production- The enteric microflora is thought to play a key role in

intestinal homeostasis[24], with non-pathogenic bacteria, such as lactobacilli and bifidobacteria, characterised by their differential stimulation of intestinal epithelial cells [25-27] and intestinal-associated immune cells, such as dendritic cells and macrophages[28,29]. In addition to being part of the normal enteric microflora specific strains of *lactobacilli* and *bifidobacteria* are the most commonly used probiotic bacteria. Previously, EPSs isolated from such bacteria have shown immunomodulatory activity with immune cells such as macrophages and lymphocytes[13-15,17]. However, the first line of defence in the intestinal tract is the epithelial layer, a one-cell thick barrier which lines the lumen of the gut; however, the potential of EPSs to interact with these cells has been neglected. A recent review article, considered the potential of EPS interacting with the epithelial layer[21] and presented preliminary data showing that bifidobacteria EPSs can stimulate the release of inflammatory cytokines, such as IL-6 and IL-8, in an intestinal epithelial cell line (Caco-2)[21].

In the experiments reported here lactobacilli EPS were applied directly to HT2919A cells. This cell line was chosen because it has previously been shown that these cells expresses a full complement of pattern recognition receptors (PRR) and that these are coupled to cytokine responses[30,31]. It is important to note that HT2919A cells are not 'professional immune cells' and that they require a challenge from a substantially higher concentrations of bacterial antigens before a biological response is observed. Despite this the EPSs from *L. helveticus* sp. Rosyjski stimulated a small, yet statistically significant ($p \leq 0.01$), increase in expression of the proinflammatory chemokine, IL-8, (Fig. 5a), and increased levels of IL-8mRNA (up-regulated ~4-fold, $p \leq 0.05$, Fig. 5b). In addition, despite the recently discovered potential of HT29 cells to secrete the anti-inflammatory cytokine, IL-10[32], the *L.*

helveticus sp. Rosyjski EPS did not stimulate either mRNA or protein expression of IL-10 (data not shown).

Figures 5a and 5b here

As a direct comparison, we studied the effect on intestinal epithelial cells of the EPS from a separate strain of lactobacilli, *L. acidophilus* sp. 5e2 the structure of which has been previously described[23] and reproduced here (Fig. 6). It is also a neutral EPS but with a smaller molecular mass of 4.5 x 10^5 u and has a heptasaccharide repeating unit composed of D-glucose, D-galactose and *N*-acetyl-*D*-glucosamine in the molar ratio 3:3:1 but its biological activity was unknown. A similar effect on the immune response of intestinal epithelial cells was found for the EPS isolated from *L. acidophilus* sp. 5e2 EPS as occurred with *L. helveticus* sp. Rosyjski (Fig. 5). Both EPSs were used in the cell assays at a final concentration (20 µg/ml) similar to that which has been reported to accumulate in the culture media of a number of EPS producing strains of lactobacillus[33]. As the concentration of EPS is relatively high, it could be argued that the levels of impurities will be above those where they might be expected to contribute to the observed biological activity; indeed this would be true for macrophages, however, much higher concentrations of protein and nucleic acid antigens would be needed before a response from epithelial HT2919A cells could be expected.

Previously, evidence has been presented that the immunomodulatory effects of EPSs could be related to their physiochemical properties with a tendency for those that are charged to stimulate an immune response in highly immunogenic cells types such as macrophages and splenic cells and conversely, large neutral structures

suppress immunity[21]. In contrast to this the two novel neutral EPSs that we have studied both stimulate a small increase in the proinflammatory cytokine IL-8 in an in vitro model of the intestinal epithelia. Intestinal epithelial cells represent the frontline innate immune defence against the enteric microflora and have previously been shown to release IL-8 in response to bacteria[34] and their products[35], however, they are not professional immune cells and this could account for the discrepancy.

If the results presented here that indicate EPSs can stimulate low-levels of proinflammatory IL-8 from intestinal epithelial cells are reproduced *in vivo*, it would suggest a potential beneficial effect for gut homeostasis. Because the EPSs could compete with more active inflammatory mediators for host pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), and thus reduce excessive inflammation. Additionally, the low level of IL-8 secretion reported here could, somewhat paradoxically, be beneficial, priming the intestine and thus allowing a more controlled and rapid response to pathogenic bacteria.

As previously stated there is very little information on the receptors which mediate the cellular activity of EPSs, although it is suspected that this is via the TLR family, it was therefore decided to study the effect of the two EPSs on TLR mRNA expression. On incubation of HT29-19A cells with 20 µg/ml of *L. acidophilus* sp. 5e2 EPS for 24 h, mRNA expression of TLRs-1, -2, -5 and -9 was significantly ($p \leq 0.05$) increased (Fig. 7). However, despite a ~3-fold increase, the change in expression of TLR-4 mRNA was not statistically different from the control level (Fig. 7). Cells challenged with *L. helveticus* sp. Rosyjski EPS, showed significant ($p \leq 0.05$) up-regulation of TLRs-1, -2, -4 and -5 mRNA, (Fig. 7). In contrast, TLR-9 mRNA expression was down-regulated, such that no detectable levels were observed (Fig. 7). It is very clear from the current results that when challenged with the EPSs, HT29-19A cells

exhibited a very significant up-regulation of TLR-2 mRNA expression (Fig. 7). It has previously been reported that upon binding their cognate receptor, TLR agonists have been shown to cause an up-regulation of the receptor in question [36-38], therefore, we can hypothesise that the up-regulation of TLR-2 mRNA is due to direct association of the EPSs with TLR-2

Figure 6 here

2.4 Monitoring the ability of lactobacilli EPS to prime the immunomodulatory activity of epithelial cells to subsequent challenge by bacterial antigens.

Currently, little is known of the cellular pathways involved in the interaction of bacterial SPSs with cells of host organisms. However, a recent study on a novel EPS (TA-1) isolated from the thermophilic marine bacterium, *Thermus aquaticus*, was shown to stimulate the release of proinflammatory cytokines, TNF-α and IL-6, from murine macrophages via a TLR-2-mediated pathway[22]. This is consistent with the fact that TLR-2 is well characterised in its interactions with a diverse range of microbial components[39,40]. A number of bacterial cell wall components such as lipopolysaccharides (LPS), flagellin and peptidoglycan (PGN) are known to activate the immune response, through their action as ligands for Toll-like receptors on intestinal epithelial cells. The specific receptors mediating cellular responses to the agonists tested here are: LPS for TLR 4, flagellin for TLR 5, and PGN for TLR 2; additionally an agonist for TLR 9, CpG oligodeoxynucleotide (ODN) of bacterial origin was also tested. Aberrant responses to these bacterial antigens are believed to play a key role in the pathogenesis of inflammatory bowel diseases, therefore, we were interested to see if exposure to EPSs could modulate the cellular response to

these. Cells which were pre-treated with 20 µg/ml of EPSs for 24 h and subsequently challenged with peptidoglycan (PGN; 500 µg/ml) exhibited a small but statistically significant ($p < 0.05$) increase in IL-8 release, when compared to cells pre-treated with cell culture media alone (Fig. 8). Additionally, cells pre-treated with *L. helveticus* sp. Rosyjski EPS and challenged with flagellin (100 ng/ml) afterwards, secreted significantly ($p < 0.05$) increased levels of IL-8 (Fig 8).

The results of these experiments suggest that the up-regulation of TLR-2 appears to 'sensitise' the epithelial cells to subsequent challenge with the known TLR-2 agonist, PGN, with a small but measured increase in the secretion of IL-8; a phenomena which again if repeated in vivo could potentially lead epithelial cells to produce a more rapid and significant immune response. Previously, live lactobacilli have been shown to up-regulate expression of non-cognate TLRs in intestinal epithelial cells, thus sensitising them to subsequent challenge with bacterial antigens[25,27]. The same phenomenon was observed in this investigation, with *L. helveticus* sp. Rosyjski EPS sensitising the epithelial cell line to subsequent challenge with bacterial flagellin, causing a significant increase in IL-8 release, despite the fact that no increase in mRNA expression of TLR5, the receptor which is thought to mediate the inflammatory effects of flagellin[41], was observed. Nevertheless, this sensitisation could potentially be of clinical importance because flagellin plays an important role in gut immunity, and is known to be involved in both homeostatic regulation of immune responses to the commensal microflora[42] and the pathogenesis of inflammatory bowel disease[43]. Indeed, Lodes *et al.*[43] showed flagellin to be a dominant antigen in patients with Crohn's disease. The mechanism for the sensitisation of epithelial cells to bacterial products is currently unknown; however, it is thought that stimulation of

the common intracellular pathways involved in transduction of TLR-stimulated signals may be responsible[27]. Through this method, EPSs could potentially play a role in priming the epithelial layer to pathogen attack, heightening the inflammatory response.

Importantly, the present data indicates that exposure to these EPSs result in the increase of mRNA for other TLRs involved in bacterial recognition, and thus they would have the potential to sensitise the intestinal epithelial cells to various PAMP's. However, at the same time they had limited effect on the potential of the range of bacterial products tested here to stimulate IL-8 secretion in HT29 cells. Taken together these data indicate a possible beneficial role for these EPSs in modulating intestinal inflammation via a direct interaction with the intestinal epithelium.

In conclusion, the structure of a novel EPS isolated from a strain of lactobacillus which uniquely contains *N*-acetyl-D-mannosamine has been determined. In addition, evidence has been presented which suggests that the EPSs isolated from lactobacilli interact with HT29-19A intestinal epithelial cells, stimulating IL-8 expression at both the transcriptional and translational level. It has also been shown that these EPSs modulate TLR mRNA expression in the epithelial cell line, resulting in 'priming' of the cells and an increased release of IL-8 in response to subsequent challenge with the bacterial antigens peptidoglycan and flagellin.

3. Experimental

3.1 Materials- HT29-19A intestinal epithelial cells (passages 41-70) were kindly donated by Prof. G. Warhurst, Salford Royal NHS Foundation Trust & University of Salford and cultured using standard techniques (30). Cell culture media ingredients were all purchased from Gibco® Invitrogen™ Life Technologies Ltd.(Paisley, UK). Cells were cultured in a standard media of high glucose (4500mg/l) Dulbecco's Modified Eagles Media (DMEM), 10% foetal bovine serum (FBS), 4 mM glutamine and a mixture of 50 IU/ml penicillin and 50 µg/ml streptomycin (PenStrep), supplemented with 20mM HEPES. Cells were seeded at a density of 0.5 x 10^5 cells/cm^2 and cultured to confluence (7 days) in 35 mm x 10 mm cell culture dishes. Cultures of the bacteria, *Lactobacillus acidophilus* sp. 5e2 and *Lactobacillus helveticus* sp. Rosyjski, were donated by Rhodia food Biolacta-Texel (Olsztyn, Poland), and were maintained on MRSc agar (Lab M Ltd., Lancashire, UK), at 37℃ in anaerobic conditions. The TLR ligands peptidoglycan (PGN) isolated from *Bacillus subtilis* and deoxyribonucleic acid sodium salt (CpG DNA) from *Escherichia coli* strain B were purchased from Sigma-Aldrich Company Ltd. (Poole, Dorset, UK). Flagellin from *Salmonella typhimurium* strain 14028 was from Enzo Life Sciences Ltd. (Exeter, UK). Lipopolysaccharide (LPS) was isolated from E. coli C25 using the LPS Extraction Kit from Chembio Ltd. (Hertfordshire, UK). All other reagents were purchased from Sigma-Aldrich Company Ltd. (Poole, Dorset, UK) and were used as supplied.

3.2 EPS isolation and purification-The multistep extraction and purification of EPSs from lactobacilli cultures used here has been described previously[10]. Briefly, *L. helveticus sp.* Rosyjski was cultured in 10% (w/v) skimmed milk solution (St. Ivel

Ltd., Swindon, UK) supplemented with 0.166 M glucose for 24 h at 42 ℃. The resultant culture was used to inoculate, at 1% (v/v) concentration, a large volume (between 1 & 2L, depending on batch number) of skimmed milk solution, supplemented with 0.166 M glucose and fermented for 48h at 42℃. Fermentation was stopped with the addition of 80% (w/v) trichloroacetic acid (TCA), giving a final concentration of 14% (v/v) of TCA, and the resulting solution was stored overnight at 4℃. The solution was then centrifuged at 25,000g (Avanti J-26 XPI centrifuge, Beckman Coulter Ltd, High Wycombe, UK) for 35 mins at 4℃ to remove bacteria and precipitated proteins. The solution was then filtered through grade 4 filter paper (Whatman UK Ltd, Kent, UK), and crude EPS was precipitated by the addition of an equal volume of chilled absolute ethanol; this was stored overnight at 4℃. The sample was then centrifuged at 25,000g for 35 mins at 4℃. The recovered pellet was re-dissolved in deionised water (~50 ml). Gentle heating in a water bath at 50℃ was required for complete dissolution of the pellet. The dissolved crude EPS was subjected to another precipitation step followed by subsequent centrifugation as described above. The pellet obtained was then re-dissolved in a minimum of deionised water (~10 mL); again gentle heating in a water bath at 50℃ was required for complete dissolution of the pellet. Small neutral sugars were then removed by dialysis, for 72 h at 4℃, against three changes of deionised water per day. After three days, the contents of the dialysis tubing was collected in a round-bottom flask and lyophilised. The dry mass of the EPS produced was then determined.

3.3 Measurement of purity and mass average molecular mass M_w. the purity of the EPS was determined using a combination of biochemical methods and SEC-MALLS and NMR analysis. The carbohydrate content was determined using the procedures

described by Dubois *et al*[44], the protein content was measured using the Bradford assay[45] and the nucleic acid content was determined spectrophometrically using SEC-MALLS linked to a UV detector (A260 nm) with prior calibration of the instrument using a DNA ladder purchased from Promega UK (Southampton, UK). The water content of solid EPS samples was determined using a Karl-Fischer autotitrator. The phosphorus content of each batch of the EPSs was monitored using ^{31}P-NMR.

For determination of the mass average molecular mass by SEC-MALLS, solutions of EPS in deionised water (1 mg mL^{-1}) were prepared and left for 24 h to completely dissolve. Samples (100 µl) were injected onto an analytical SEC system comprising three columns Aquagel-OH 40, 50 and 60 (15 µm particle size, 25 cm x 4 mm, Varian, Oxford, UK) connected in series. The neutral analytes were eluted with deionised water flowing at 1 mL min^{-1}. The concentration of the EPS fractions eluting from the column were determined by a differential refractometer (Optilab rEX, Wyatt technology, Santa Barbara, USA, fitted with 16 photodetectors at different angles) and the mass average molecular mass was measured using a Dawn-EOS MALLS operating with a 690 nm laser (Wyatt technology, Santa Barbara). The Astra software was used to analyze the light scattering data using the Zimm method.

3.4 Monomer composition and linkages of the -EPS For sugar composition determinations polysaccharides were hydrolyzed by treatment with 2M TFA (120 °C for 2 h); the released sugars were converted to their alditol acetates and analyzed by GC-MS. The relative proportions of the different sugars were determined by consideration of the total ion count for the different alditol acetates and by comparison with the ion count determined for a mixture of alditol acetates. GC-MS analyses were performed on a Thermo (Finnigan) Polaris Q-Trace 2000 GC-MS,

(Hemel Hempstead, UK). The samples were eluted from a SGE column (BPX5, 25 m x 0.32 mm-id, 0.5 μm film; HP5, 15m x 0.32mm-id) eluting with helium (9 psi, flow rate 1ml.min^{-1}) and using a temperature programme (start temperature 150 °C, hold time 4 min., and a final column temperature of 250 °C reached via a rising gradient of 4 °C/min.). Under the standard conditions for the analysis of monomers the integral area for the amino-sugar was very small this is a consequence of on column thermal decomposition, problems with the analysis of amino sugars has been well documented; the final monomer ratio was taken from integration of the NMR peak integrals for the respective anomeric protons. The absolute configurations of monosaccharides were determined by conversion to their butyl glycosides using the procedure described by Gerwig *et al*[46].

For linkage analysis, the EPS was permethylated using the procedures described by Stellner *et al*[47]. The methylated-polysaccharide was hydrolysed by treatment with 2M TFA (120 °C for 2 h) and the methylated monosaccharides converted to their corresponding methylated alditol acetates. The identity of the variously methylated alditol acetates was determined by GLC-MS and by analysis of the individual fragmentation patterns observed in the MS. GLC-MS analyses were performed on an Agilent 7890A GC system (Santa Clara, CA, USA) coupled to an Agilent 5675c quadrupole MS. The samples were eluted from a HP-5 column (30 m x 0.25 mm-id, 0.25 μm film) using helium as carrier (9 psi, flow rate 1 mL min^{-1}) and using the following temperature programme: start temperature 155°C, hold time 1 min, and a final column temperature of 195°C reached via a rising gradient of 0.75°C min^{-1}.

3.5 NMR analysis of the EPS from L. helveticus sp. Rosyski - NMR spectra were recorded for EPS samples that were dissolved (10 mg mL^{-1}) directly in D_2O (Goss

Scientific Instruments Ltd., Essex, UK). NMR spectra were recorded at a probe temperature of 70°C. The elevated temperature was initially chosen as it shifted the HOD signal to higher field, into a clear region of the spectrum. The higher temperature also increased spectral resolution by reducing the sample viscosity. All of the NMR spectra were recorded on a Bruker Avance 500.13 MHz ^{1}H (125.75 MHz ^{13}C) spectrometer (Bruker-biospin, Coventry, UK) operating with Z-field gradients where appropriate, and using Bruker's pulse programs. Chemical shifts are expressed in ppm relative to either internal or external acetone; δ 2.225 for ^{1}H and δ 31.55 for ^{13}C. The 2D gs-DQF-COSY spectrum was recorded in magnitude mode at 70°C. TOCSY experiments were recorded with variable mixing times (60, 90, 120 ms). The 2D-heteronuclear ^{1}H-^{13}C HSQC, and phase sensitive HSQC-TOCSY spectra were recorded using Bruker pulse sequences and 256 experiments of 1024 data points. The NOESY spectrum was recorded using a Bruker pulse sequence and 256 experiments of 1024 data points using a mixing time of 200 ms. For the majority of spectra, time-domain data were multiplied by phase-shifted (squared-) sine-bell functions. After applying zero-filling and Fourier transformation, data sets of 1024-1024 points were obtained.

3.6 Biological Assays- IL-8 stimulation and analysis EPSs were reconstituted in ultrapure water to give a final concentration of 1 mg/ml. HT29-19A cells were challenged with 20 µg/ml EPSs for 24h. Supernatants were collected and frozen at -80°C until assayed for IL-8 and IL-10 by ELISA (IL-8 and IL-10 Human Antibody Pairs, Invitrogen, Paisley, UK.). Analysis was carried out according to the manufacturer's instructions.

For investigation of TLR ligand stimulation with EPS-pre-treatment, HT29-19A cells were challenged 20 µg/ml EPSs for 24 h, at 37℃, 5% CO_2 and constant humidity. Supernatants were removed and cells were subsequently challenged with either PGN (500 µg/ml), LPS (1000 ng/ml), flagellin (100 ng/ml) or CpG DNA (50µg/ml) for 24 h, at 37℃, 5% CO_2 and constant humidity. Supernatants were collected and frozen at -80℃ until assayed for IL-8 by ELISA.

For Quantitative real-time (qRT)-PCR total RNA was extracted from cell layers previously challenged with 20 µg/ml EPSs for 24 h using the RNeasy® Mini Kit and RNase-free DNase Set (Qiagen, Crawley, UK). The concentration was measured using the absorbance at 260 nm (A260) x 44 µg/ml x dilution factor and the purity was measured using A260/A280. mRNA was converted to cDNA by the iScript™ cDNA Synthesis Kit from Bio-Rad Laboratories Ltd., (Hemel Hempstead, UK). All PCR primers (Table 1) were purchased from Eurofins MWG Operon (Ebersberg, Germany). Universal ProbeLibrary probes and Lightcycler® Taqman® Master Mix were purchased from Roche Diagnostics Ltd.(West Sussex, UK). Amplification was carried out in 20 µl final volume, containing 1.5µl cDNA , 0.5 µl F-primer and R-primer (0.4 µM), 0.5 µl Universal probe, 4 µl Mastermix (5x) and 13 µl DNase/RNase-free water. The following program was used: (i) activation of the PCR polymerase at 95 ℃ for 10 min (ii) amplification included 45 cycles of denaturation at 95 ℃ for 10 s, annealing at 60 ℃ for 30 s and extension at 72 ℃ for 1 s (iii) cooling at 40 ℃ for 30 s. Target gene expression was normalised to the housekeeping genes GAPDH and β-actin and fold difference of expression from the control was calculating using the 2-ΔΔCt method.

3.7 Statistical Analysis- Results are expressed as mean ± SEM for the specified number of experimental repeats (n). Statistical significance is resolved using

unpaired Student's t-test or ANOVA with Tukey's post hoc analysis and defined as a p value ≤ 0.05 and/or p value ≤ 0.01. All statistical analyses were undertaken using PASW® Statistics 17 (IBM, Armonk, NY, USA).

Acknowledgements: The authors wish to acknowledge funding from the University of Huddersfield and the EU under the EU International Scientific Cooperation Project (IC15-CT98-0905) and for the assistance of partner institutions in (IC15-CT98-0905).

References:

(1) Badel, S.; Bernardi, T.; Michaud, P. *Biotechnol. Adv.,***2011,** *29*, 54-66.
(2) Ruas-Madiedo, P.; Medrano, M.; Salazar, N.; de los Reyes-Gavilan, C. G.; Perez, P. F.; Abraham, A. G. *J.of Appl. Microbiol.,* **2010**, *109*, 2079-2086.
(3) Whitfield, C.; Valvano, M. A. *Adv.in Microb.Physiol.,* **1993**, *35*, 135-246.
(4) Poulsen, L. V. *Lebensm.Wiss. Technol.* **1999**, *32*, 321-326.
(5) Ruas-Madiedo, P.; Gueimonde, M.; Margolles, A.; De los Reyes-Gavilan, C. G.; Salminen, S. *J. Food Prot.,* **2006**, *69*, 2011-2015.
(6) Sutherland, I. W. *Microbiol.-UK,* **2001**, *147*, 3-9.
(7) Danese, P. N.; Pratt, L. A.; Kolter, R. *J. Bacteriol.,* **2000**, *182*, 3593-3596.
(8) Watnick, P. I.; Kolter, R. *Mol. Microbiology,* **1999**, *34*, 586-595.
(9) De Vuyst, L.; Degeest, B. *FEMS Microbiol. Rev.,* **1999**, *23*, 153-177.
(10) Marshall, V. M.; Rawson, H. L. *Int. J. Food Sci. and Technol.,* **1999**, *34*, 137-143.
(11) Bhaskaracharya, R. K.; Shah, N. P. *Aust. J. Dairy Technol.* **2000**, *55*, 132-138.
(12) Marco, M. L.; Pavan, S.; Kleerebezem, M. *Curr.Opin. Biotechnol.,* **2006**, *17*, 204-210.
(13) Chabot, S.; Yu, H.-L.; De Leseleuc, L.; Cloutier, D.; Van Calsteren, M.-R.; Lessard, M.; Roy, D.; Lacroix, M.; Oth, D. *Lait* **2001**, *81*, 683-697.
(14) López, P.; Monteserín, D. C.; Gueimonde, M.; de los Reyes-Gavilán, C. G.; Margolles, A.; Suárez, A.; Ruas-Madiedo, P. *Food Res. Int.,* **2012**, *46*, 99-107.
(15) Wu, M.-H.; Pan, T.-M.; Wu, Y.-J.; Chang, S.-J.; Chang, M.-S.; Hu, C.-Y. *Int. J.Food Microbiol.,* **2010**, *144*, 104-110.
(16) Kleerebezem, M.; Hols, P.; Bernard, E.; Rolain, T.; Zhou, M. M.; Siezen, R. J.; Bron, P. A. *FEMS Microbiol. Rev.,* **2010**, *34*, 199-230.
(17) Bleau, C.; Monges, A.; Rashidan, K.; Laverdure, J. P.; Lacroix, M.; Van Calsteren, M. R.; Millette, M.; Savard, R.; Lamontagne, L. *J. Appl. Microbiol.,***2010**, *108*, 666-675.
(18) Laws, A. P.; Gu, Y.; Marshall, V. *Biotechnol.Adv.,* **2001**, *19*, 597-625.
(19) Cerning, J. *FEMS Microbiol. Rev.,* **1990**, *87*, 113-130.
(20) Marco, M. L.; Bongers, R. S.; de Vos, W. M.; Kleerebezem, M. *Appl. Environ. Microbiol.* **2007**, *73*, 124-132.
(21) Hidalgo-Cantabrana, C.; López, P.; Gueimonde, M.; de los Reyes-Gavilán, C. G.; Suárez, A.; Margolles, A.; Ruas-Madiedo, P. *Probiotics Antimicrob. Proteins.,* **2012**, *Online*, 1-11.

(22) Lin, M.-H.; Yang, Y.-L.; Chen, Y.-P.; Hua, K.-F.; Lu, C.-P.; Sheu, F.; Lin, G.-H.; Tsay, S.-S.; Liang, S.-M.; Wu, S.-H. *J. Biol. Chem.,* **2011**, *286*, 17736-17745.
(23) Laws, A. P.; Chadha, M. J.; Chacon-Romero, M.; V.M., M.; Maqsood, M. *Carbohydr .Res.* **2008**, *343*, 301-307.
(24) Artis, D. *Nat. Rev. Immunol.,* **2008**, *8*, 411-420.
(25) Seifert, S.; Gomez, M. R.; Watzl, B.; Holzapfel, W. H.; Franz, C. M. A. P.; Vizoso Pinto, M. G. *Probiotics Antimicrob.Proteins,* **2010**, *2*, 211-217.
(26) Vinderola, G.; Matar, C.; Perdigon, G. *Clin. Diagn. Lab. Immunol.,* **2005**, *12*, 1075-1084.
(27) Viszoso Pinto, M. G.; Rodriguez Gomez, M.; Seifert, S.; Watzl, B.; Holzapfel, W. H.; Franz, C. M. A. P. *Int. J. Food Sci. and Technol.,* **2009**, *133*, 86-93.
(28) Christensen, H. R.; Frokiaer, H.; Pestka, J. J. *J. Immunol.,***2002**, *168*, 171-178.
(29) Miettinen, M.; Veckman, V.; Latvala, S.; Sareneva, T.; Matikainen, S.; Julkunen, I. *J. Leukocyte Biol.,***2008**, *84*, 1092-1100.
(30) Patten, D. Doctoral, University of Huddersfield, 2013.
(31) Bannon, C.; Davies, P.J.; Collett, A.; Warhurst, G.; 2009, *Biochem. J.*, 423(1) 119-28.
(32) Gao, Q.; Qi, L.; Wu, T.; Wang, J. *BMC Microbiol.,* **2012**, *12:*100.
(33) Ruas-Madiedo, P.; de los Reyes-Gavilan, C. G. *J. Dairy Sci.,* **2005**, *88*, 843-856.
(34) Eckmann, L.; Kagnoff, M. F.; Fierer, J. *Infect. Immun.,* **1993**, *61*, 4569-4574.
(35) Schuerermaly, C. C.; Eckmann, L.; Kagnoff, M. F.; Falco, M. T.; Maly, F. E. *Immunology* **1994**, *81*, 85-91.
(36) Hornung, V.; Rothenfusser, S.; Britsch, S.; Krug, A.; Jahrsdorfer, B.; Giese, T.; Endres, S.; Hartmann, G. *J. Immunol.,* **2002**, *168*, 4531-4537.
(37) Poltorak, A.; He, X.; Smirnova, I.; Liu, M.-Y.; Van Huffel, C.; Du, X.; Birdwell, D.; Alejos, E.; Silva, M.; Galanos, C.; Freudenberg, M.; Ricciardi-Castagnoli, P.; Layton, B.; Beutler, B. *Science* **1998**, *282*, 2085-2088.
(38) Visintin, A.; Mazzoni, A.; Spitzer, J. H.; Wyllie, D. H.; Dower, S. K.; Segal, D. M. *J. Immunol.,* **2001**, *166*, 249-255.
(39) Kawai, T.; Akira, S. *Immunity*, **2011**, *34(5)* 637-50.

(40) Kawai, T.; Akira, S. *Nat. Immunol*., **2010,** *11(5)* 373-84.

(41) Hayashi, F.; Smith, K. D.; Ozinsky, A.; Hawn, T. R.; Yi, E. C.; Goodlett, D. R.; Eng, J. K.; Akira, S.; Underhill, D. M.; Aderem, A. *Nature* **2001**, *410*, 1099-1103.
(42) Vijay-Kumar, M.; Aitken, J. D.; Gewirtz, A. T. *Semin. Immunopathol.,***2008**, *30*, 11-21.
(43) Lodes, M. J.; Cong, Y.; Elson, C. O.; Mohamath, R.; Landers, C. J.; Targan, S. R.; Fort, M.; Hershberg, R. M. *J.Clin. Invest.,* **2004**, *113*, 1296-1306.
(44) DuBois, M.; Gilles, K. A.; Hamilton, J. K.; Rebers, P. A.; Smith, F. *Anal.Chem.,* **1956**, *28*, 350-356.
(45) Bradford, M. M. *Anal. Biochem.,* **1976**, *72*, 248-254.
(46) Gerwig, G. J.; Kamerling, J. P.; Vliegenthart, J. F. G. *Carbohydr. Res.***1978**, *62*, 349-357.
(47) Stellner, K.; Saito, H.; Hakomori, S. I. *Arch. Biochem. Biophys.,* **1973**, *155*, 464-72.