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Title: Immune modulation capability of exopolysaccharides synthesised by lactic acid bacteria
and bifidobacteria

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19 Abstract

20 During recent years the exopolysaccharides (EPS) produced by some strains of lactic acid
21 bacteria and bifidobacteria have attracted the attention of researchers, mainly due to their potential
22 technological applications. However, more recently it has been observed that some of these EPS
23 present immunomodulatory properties, which suggest a potential effect on human health. Whereas
24 EPS from lactic acid bacteria have been studied in some detail, those of bifidobacteria largely
25 remain uncharacterized in spite of the ubiquity of EPS genes in *Bifidobacterium* genomes. In this
26 review we have analysed the data collected in literature about the potential immune modulating
27 capability of EPS produced by lactic acid bacteria and bifidobacteria. From this data analysis, as
28 well as from results obtained in our group, a hypothesis relating the physico-chemical
29 characteristics of EPS with their immune modulation capability was highlighted. We propose that
30 EPS having negative charge and/or small size (molecular weight) are able to act as mild stimulators
31 of immune cells, whereas those polymers non-charged and with a large size present a suppressive
32 profile.

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40 **Keywords:** exopolysaccharide, lactic acid bacteria, bifidobacteria, immune response, probiotic

1. Introduction

1.1. Bacterial EPS

The ability to produce exo-cellular polysaccharides is widespread throughout the microbial world, from some algae, fungi and yeasts to prokaryotes. In the last case, polymers produced by Archaea isolated from diverse, extreme environments (extremophiles) have been receiving special attention in recent times due to their potential applications [45]. However, several biopolymers produced by Bacteria, both Gram-positive and Gram-negative, are currently well studied and characterized due to their industrial, medical and biotechnological uses [38, 47, 68]. Besides, it is known that their ecological role, either in Archaea or Bacteria, is similar since these biopolymers are involved in cell protection to fight against harsh environmental conditions [24], and in niche colonization; for example exo-cellular polymers are key players in biofilm formation [22, 36]. In addition, some of these bacterial biopolymers are receiving renewed interest due to their biological functions, particularly their involvement in human health [37, 55].

Most bacteria are often covered by a layer of polysaccharides which is called *glycocalyx* [21]. These polymers can be linked to the cell surface by means of covalent bonds forming a capsule and therefore they are named capsular polysaccharides (CPS). Other polymers are weakly attached to the surface, or are totally released into the surrounding environment, forming slime, and they are referred as exopolysaccharides (EPS). This classification, according to cell location, is not clear [63] and more precise definitions have been collected in the literature [6]. For practical purposes, in the context of this article we will use the term EPS for both exo-cellular layers. In fact, these two types of EPS are carbohydrate polymers built from a reduced number of different monosaccharides (most common: glucose, fructose, galactose, rhamnose and fucose). However the presence of different isomers, linkage types and organic and inorganic monosaccharide substituents that could be combined in repeating units which have, as well, different degrees of polymerization and branching patterns, render a great variety of bacterial EPS. In another practical classification, bacterial EPS are divided into two main groups depending on whether a single monosaccharide type

is present in the polymer or more; they are the homopolysaccharides (HoPS) and heteropolysaccharides (HePS), respectively, which also differ in the number of enzymes and organization of genes involved in their synthesis [6, 56, 62]. Finally, depending on the substituents present in the repeating units of HePS, there are non-charged, charged or neutral polymers. Within the latter, of special mention are the “zwitterionic” EPS characterized to have both positively (e.g. free amine) and negatively (e.g. phosphate or carboxylate) charged moieties within their repeating units [37]. These molecules have been reported to be able to modulate the immune system, both innate and adaptive response, although they are very rare among bacteria. To date, only two Gram-positive pathogens, *Streptococcus pneumoniae* and *Staphylococcus aureus*, as well as the Gram-negative *Bacteriodes fragilis*, a common member of the human intestinal microbiota, were reported as producers of such EPS [36]. Indeed, it has been indicated that the zwitterionic EPS, named polysaccharide A, from *Bact. fragilis* could play a key role in oral tolerance and in balancing the immune status associated with some infections or inflammatory disorders [50, 51, 52].

Some strains of Gram-positive bacteria such as *Lactobacillus*, a member of lactic acid bacteria (LAB) group, and *Bifidobacterium* are considered probiotics; that is “live microorganisms which when administered in adequate amounts confer a health benefit on the host” [15]. Worldwide, research on probiotics is one of the most interesting scientific fields due to its implication on human health, also representing a good opportunity for the industrial development of new products [3]. Some bifidobacteria and lactobacilli species share common habitats, for example animal mucosa such as the oral-gastrointestinal tract [34, 73]. However, the genomic G+C content separates *Bifidobacterium* (G+C > 50%) and *Lactobacillus* (G+C < 50%) in two taxonomic phyla: *Actinobacteria* and *Firmicutes*, respectively [16, 34, 66]. Nowadays, the capability of lactobacilli to synthesize EPS is well documented; in fact, some strains isolated from foods have been used for the manufacture of fermented dairy products from a long time ago [55]. The research interest in bifidobacterial EPS is more recent and there are data in literature that attribute some of the beneficial health effects produced by probiotic lactobacilli and bifidobacteria to their EPS.

Therefore, in this article we will review some traits of the EPS-producing phenotype in LAB and bifidobacteria and we will gather, as well, evidence that could give us clues to understand the capability of EPS to modulate immune response.

1.2. Biosynthesis of EPS from LAB

To date, a wide variety of EPS synthesized by LAB belonging to both HoPS and HePS types have been described (for greater comprehension, read [55, 56]). The HoPS are composed either of D-glucose (α -glucans and β -glucans) or D-fructose (β -fructans), which can be divided into subtypes depending on the position of the carbon involved in linkage. The synthesis of α -glucans and β -fructans requires the presence of sucrose as substrate, which donates the corresponding glycosyl moiety to a polymer in formation in a reaction catalyzed by a single type of enzyme named glycansucrase [39, 72]. These extracellular enzymes are members of the glycoside hydrolase (GH) family, and are referred to as glucansucrases (family GH70) and fructansucrases (family GH68) involved in the polymerization of α -glucans and β -fructans, respectively. They can also catalyze the hydrolysis of sucrose and the synthesis of oligosaccharides when the acceptor molecule is other than glycans [28]. β -glucans are less frequent in LAB and, to date, the few that have been described have the same structure: (1 $\rightarrow$ 3)- β -D-glucan with side ramifications of a single (1 $\rightarrow$ 2)-linked β -D-glucose. These β -glucans are synthesized through a different mechanism in which another single enzyme type (glucosyltransferase), that does not use sucrose as substrate, is involved [11, 75, 76]. This enzyme belongs to the COG1215 membrane-bound glucosyltransferase family and topology predictions show that a conserved cytosolic domain is flanked by two and four transmembrane segments [76]. The sequences of the glucosyltransferases from a few LAB are homologous to that of the polysaccharide synthase (GenBank accession no. CAB51329) from *S. pneumoniae* serotype 37 which produce the same structural capsular β -glucan. In addition, by means of agglutination experiments using pneumococci serotype 37 anti-serum, the presence of this type of polymer was detected in some LAB [75, 76] as well as in propionibacteria [8, 11]. However, the mechanism of

116 β -glucan synthesis by LAB is still not known; in *Propionibacterium freudenreichii* it seems that the
117 homologous polysaccharide-synthase enzyme catalyzes the intracellular polymerization of glucose
118 monomers, from nucleotide sugar precursors, and is able to carry out the export [8].

119 HePS from LAB result from the polymerization of repeating units which are composed of
120 monosaccharides and of organic- and inorganic-substituted monosaccharides. Most common sugars
121 are D-glucose, D-galactose and L-rhamnose and, to a lesser extent, N-acetyl-glucosamine and N-
122 acetyl-galactosamine [56]. Fucose and ribose have been described in the repeating units of a few
123 HePS [12, 33] and organic and inorganic molecules can also be present, for example, glucuronic
124 acid [49], acetyl [71], glycerol-phosphate [78] and phosphate [70]. At the time of writing,
125 combinations of 2 to 8 monomers have been described rendering more than 45 different structural
126 repeating units determined by means of nuclear magnetic resonance (NMR) techniques. These
127 different NMR structures are obtained from the variation in monomers, linkage type (α or β),
128 position of the carbon involved in linkage, presence of different side chains, etc. [56, 58]. The
129 complexity of the chemical and structural composition of the HePS repeating units is reflected in
130 the organization of genes encoding proteins involved in their synthesis, which are organized in *eps*
131 clusters. In general, *eps* clusters from LAB have an operon structure with most genes oriented in
132 one direction and having a high coding density and a highly conserved structural-functional
133 organization. In general, LAB-*eps* clusters harbour genes coding for glycosyltransferases (GTF),
134 which build the HePS repeating units, and proteins involved in the export-polymerization of the
135 repeating units, in the HePS chain length determination, and in regulation of the full HePS
136 biosynthesis process. Very often, mobile elements are located bordering these *eps*-clusters, which
137 could explain the instability of HePS-production phenotype in some LAB [5, 10, 23, 56].

138 139 **1.3. Biosynthesis of EPS from Bifidobacterium genus**

140 The EPS-production phenotype has been less studied in bifidobacteria than in LAB,
141 probably due to the lack of known technological use for bifidobacterial polymers. However, in the

context of probiotics, research in EPS field is gaining interest since these polymers, as components of bacterial envelope, could be directly related with probiotic-host interactions, and therefore with a potential health benefit. Indeed, the first question to arise is whether bifidobacteria and non-food origin lactobacilli are able to synthesise EPS. Using culture-dependent techniques and a combination of phenotypic and genotypic screening, our group has demonstrated that lactobacilli and bifidobacteria from human origin (faeces) harbour *eps* genes and they are able to produce HePS under laboratory conditions [53, 59]. Similar results have recently been reported with another collection of bifidobacteria from human origin [46]. On the contrary, and as far as we know, synthesis of HoPS-type polymers have not been reported yet in strains of these genera isolated from human sources. Nevertheless, lactobacilli species isolated from duck and pig intestines were able to produced both glucan and fructan HoPS-types from sucrose [64]. By using animal models, it was inferred that HePS could be synthesised *in vivo* under the gastrointestinal conditions, either by lactobacilli or bifidobacteria [7, 14, 29]. Finally, a recent *in silico* analysis of bifidobacterial genomes underlines the ubiquitous presence of genes involved in the synthesis of HePS, which seems to be organized in cluster-like structures [30, 58]. However, the remaining, and challenging, task is to purify the *in vivo* synthesised EPS in order to understand their physico-chemical properties, which could help to answer why only some specific polymers have biological and functional properties.

Up to now, only a few bifidobacterial polymers have been purified and fully characterised [58]. The EPS described in bifidobacteria are HePS-type, their monosaccharide constituents being those found for LAB-EPS; that is, glucose, galactose and rhamnose (Table 1). In *B. animalis* subsp. *lactis*, 3 out of 4 strains showed polymers with the same glucose- galactose- rhamnose ratio (about 2.5: 2: 1, respectively). Only the EPS synthesised by strain IPLA-R1 was different, since the rhamnose content increased; this was due to the production of another EPS-fraction of high molecular weight (HMW, about 3×10^6 Da) having 50% of rhamnose in its repeating unit structure [31]. The strain IPLA-R1, also named AldOxR, was obtained from strain A1 by adaptation to

increasing concentrations of bile salts in our laboratory, followed by successive sub-cultivations without selective agent. Indeed we have proved that bile salts trigger the synthesis of EPS in this species [54]. The production of more than one EPS-fraction was also detected in *B. breve* NCIMB8807 cultivated in milk, which synthesised two polymers differing in size (EPS 1 = 1.2×10^6 Da and EPS 2 = 6.4×10^5 Da) and in monosaccharide composition [46]. Similar to *B. animalis* subsp. *lactis*, the EPS synthesised by several strains of *B. pseudocatenulatum* showed similar monosaccharide ratio and composition (Table 1). In contrast, EPS synthesised by *B. longum* strains showed a wide distribution in their monosaccharide ratio. Galactose and glucose were present in most of them in variable proportions, with the exception of EPS synthesised by strain YIT4028; whereas, rhamnose was detected in half of the *B. longum* HePS (Table 1). In this regard, it was found that this monosaccharide was present in 48% of the HePS characterised from bifidobacteria to date. We have previously reported that the rhamnose content in EPS synthesised by lactobacilli and bifidobacteria of human origin was higher than in EPS synthesised by LAB strains of food origin: 52% in 21 human-origin EPS and 28% in 25 food-origin EPS [59]. Besides, to date only two described LAB- or bifido-polymers have an unusually high rhamnose content in their structural repeating unit, as determined by NMR technique; these are the heptasaccharide synthesised by *Lactobacillus rhamnosus* RW-9595M [69] and the HMW hexasaccharide synthesised by *B. animalis* subsp. *lactis* IPLA-R1 [31]. However, to the best of our knowledge, the biological relevance that a high rhamnose content in HePS from non-food origin could have, is still unknown.

2. Immune modulation of EPS from LAB and bifidobacteria

Apart from the ecological relevance that EPS may have for the producing bacteria and their industrial applications, there is increasing evidence to suggest a role for bifido- and LAB-EPS in the interaction between producing-bacteria and the (human) host, and therefore, exerting an impact on human health. It has been claimed that some EPS reduce cholesterol levels, act as fermentable (prebiotic) substrates for intestinal microbiota and modulate the immune response [55]. However, it

194 is well known that not all EPS are able to improve the technological properties of fermented foods
195 [20] or to promote health benefits [2]. Therefore, the physico-chemical characteristics of EPS must
196 be the key parameters determining their biological and functional properties.

197 Several works in literature report the ability of EPS synthesised by LAB and bifidobacteria
198 to elicit immune responses (Table 2). In an attempt to correlate some physico-chemical traits of EPS
199 with their immune-modulating capability, we have detected two association patterns. First, it seems
200 that acidic HePS, which are characterised as having phosphate (i.e. negative charge) in their
201 composition, are good inducers of the immune response. This fact was illustrated by the HePS
202 synthesised by LAB used as starters in the dairy food industry, such as the strain *Lactococcus lactis*
203 subsp. *cremoris* KSV20 involved in the manufacture of the Scandinavian fermented milk viili. The
204 viilian-HePS was able to induce the synthesis of IFN γ and IL-1 α in mouse spleen macrophages
205 cultivated *in vitro* [25]. The repeating unit of KSV20 HePS is a pentasaccharide composed of
206 glucose- galactose- rhamnose in ratio 2:2:1, with the side-chain α -D-galactopyranose substituted
207 with PO $_4^-$ [42]. Similarly, *Lb. delbrueckii* subsp. *bulgaricus* OLL-1073-R1 synthesises an HePS
208 composed of two fractions, acidic and neutral, both containing glucose and galactose (ratio 3:2) but
209 the acid-fraction additionally having 0.1% PO $_4^-$ [67]. The acid fraction was a strong inducer of
210 proliferation and activity of different macrophages, whereas the neutral-fraction was not able to
211 elicit stimulation [26, 44]. Furthermore, the same authors have proved that phosphate was the
212 molecule triggering the immune response, since the chemical dephosphorylation of this HePS
213 encompasses a reduction in the stimulatory effect [26]. The relevant role of PO $_4^-$ in immune
214 stimulation was also proven using the α -glucan HoPS (dextran) synthesised by *Leuconostoc*
215 *mesenteroides* as a model. When dextran was chemically phosphorylated, the proliferation of
216 lymphocyte subsets from the murine spleen, as well as the gene expression of IFN γ and IL-10,
217 directly augmented with the phosphate content [61].

The second association pattern was detected for those HePS having big size or HMW, which seem to act as suppressors of the immune response (Table 2). One of the first examples illustrating this fact was reported by Yasuda and co-workers [79] using *Lactobacillus casei* Shirota as a model. This strain harbours an *eps*-cluster of 10 genes which is involved in the synthesis of a HMW (cell-wall) polysaccharide; knockout-mutants of *eps* genes were able to induce the production of TNF α , IL-12, IL-10 and IL-6, by mouse macrophage (RAW-264.7) cell line or by mouse spleen cells, to a higher extent than the wild-type bacterium. Thus, the authors concluded that the EPS synthesised by *L. casei* Shirota act by reducing excessive reaction of immune cells not only against its own stimulating components, but also against other inducers such as LPS. Similarly, murin peritoneal macrophages stimulated with the HMW-EPS-producing *L. rhamnosus* RW-9595M, an isogenic variant of the strain ATCC9595, induced low levels of TNF α and IL-6; whereas, the parental *L. rhamnosus* ATCC9595 strain induced high levels of TNF α , IL-6 and IL-12 and showed decreased IL-10 production [4]. We have also detected immune suppression upon human peripheral blood mononuclear cells (PBMC) induced by the HMW-EPS-producing *Lactobacillus paraplantarum* BGCG11, in comparison with its isogenic EPS⁻ mutants [43]. Recently, Fanning and co-workers [14] reported similar behavioural patterns in *Bifidobacterium* genus, using the approach of wild / KO-mutants of *B. breve* UCC2003 in murin *in vitro* and *in vivo* models. Naïve spleen cells stimulated with the EPS⁺ (wild strain) had significantly lower levels of pro-inflammatory cytokines (IFN γ , TNF α and IL-12) compared with those of the isogenic *B. breve* EPS⁻ mutants. Additionally, the type and percentage of immune spleen cells examined from mice fed with the EPS⁺ strain were similar to that of untreated mice. On the contrary, mice fed with EPS⁻ strains increased the number and the percentage of different immune-subset cells in comparison with untreated mice. The expression of the cytokines produced by these cells also differed among the mice groups (feed placebo, EPS⁺ or EPS⁻). The authors concluded that the strain *B. breve* UCC2003, which synthesises an EPS, whose physico-chemical composition has not been reported yet but seems to have big size, was able to evade some adaptive B-cell responses.

The studies indicated above report the immune effect of the EPS-producing bacteria, but only a few works have effectively demonstrated the ability of purified HePS to elicit immune response. Our group has recently shown that human PBMC cultivated in the presence of EPS isolated from 10 bifidobacteria strains display specific cytokine profiles; that is, chemically different EPS were able to elicit different immune responses [32]. Especially relevant are the results obtained with the HePS purified from the three closely related *B. animalis* subsp. *lactis* strains mentioned above: A1 (parental), A1dOx (bile-adapted derivative) and A1dOxR (bile-adapted derivative having ropy phenotype). The EPS A1dOxR (at 1 µg/ml) elicited lower production of cytokines by human PBMC than the EPS A1 or A1dOx, therefore suggesting an immune-suppressive profile of the former. Additionally, when these HePS-producing *B. animalis* subsp. *lactis* strains were co-cultivated with colonocyte-like cells (Caco2) they elicited different cytokine patterns (unpublished results, Figure 1). The ropy A1dOxR strain induced significantly lower levels of pro-inflammatory IL-6 and IL-8 cytokines than the control (Caco2 cultivated in DMEM), whereas A1 induced the secretion of elevated amounts of these molecules, suggesting an immune activating role of the parental strain. On the other hand, Caco2 cells did not modify significantly IL-1β production after culture with any bacterial strain; however, treatment with A1 and A1dOx showed a tendency to decrease its levels whereas an inverse trend was observed with A1dOxR, the production being significantly higher when compared with the other two strains. Given the relevance of IL-1β in the generation of Th17 cells, involved in mucosal defence and Treg/Th17 plasticity, these data could suggest a role of the ropy A1dOxR strain in the immune homeostasis in the gut. In this regard, the EPS A1dOxR is a polymer composed of three EPS-fractions of different size: HMW (3.5×10^6 Da), middle weight (3.0×10^4 Da) and low weight (4.9×10^3 Da) [31]; whereas, the other two related EPS (A1 and A1dOx) lack the HMW fraction [57]. Therefore, it seems that the presence of the HMW fraction could be responsible for the immune suppression capability of EPS A1dOxR. In this regard, the findings of Bleau and co-workers [4] also reinforce this idea since intermediate polymer chains (16-30 units, molecular weight $< 10^4$ Da) obtained after hydrolysis of

270 the HMW-EPS (size 5.3×10^5 Da) RW-9595M showed increased production of IL-10 by
271 macrophages, in comparison with the native polymer synthesised by *L. rhamnosus*. It is worth
272 noting that another common trait between the HMW-EPS A1dOxR (from *B. animalis* subsp. *lactis*)
273 and the native EPS RW-9595M (from *L. rhamnosus*) is the high presence of rhamnose (more than
274 50%) in the composition of their repeating units [31, 69]. At the moment, it is unknown if this
275 characteristic could also be relevant to regulate the immune response.

276 Finally, some works have been carried out regarding the immune activity of β -glucans
277 (HoPS-type) produced by LAB [17] and *Propionibacterium*;; this last genus belonging to
278 *Actinobacteria* phylum, as bifidobacteria, and specific species are relevant for the sensorial
279 properties of some cheeses [9]. Bacterial β -glucans, which have a common structure of branched
280 $(1 \rightarrow 3)$ - β -D-glucose substituted at C2 with β -D-glucose (Table 2), act by modifying the response of
281 PBMC since it seems that KO-mutants are able to relieve the immune suppression elicited by the
282 wild type strains. Similarly, β -glucans from fungus *Sclerotium*, having the same monomer
283 composition, but differing in size and spatial conformation, are able to elicit variable proliferation in
284 human monocyte cultures [13]. Therefore, it seems that the size of EPS polymers, either HePS or
285 HoPS is of special relevance for their immune properties.

286 Information about immune mechanism(s) elicited by bacterial EPS at molecular level is still
287 scarce and, as far as we know, at present it is unknown for bifido- or LAB-EPS. In relation with
288 other microbial EPS, it seems that some fungi glucuronoxylomannans (GXM) interact with the
289 immune system through different Toll-like Receptors (TLR), also inducing production of nitric
290 oxide by phagocytes [18]. In these fungal EPS the diameter of the molecule may potentially
291 influence the inflammatory response against the GXM-producing fungi *Cryptococcus* [18, 80], as
292 previously stated for *Sclerotium* β -glucans [13]. Regarding bacteria, it has recently been
293 demonstrated that the zwitterionic polysaccharide A (or PSA) produced by *Bact. fragilis* can
294 suppress *in vivo* the pro-inflammatory IL-17 production by intestinal immune cells induced by
295 *Helicobacter hepaticus* [35]. This PSA has the ability to induce Foxp3⁺ T_{reg} cells, which produce an

increase of anti-inflammatory cytokines such as IL-10, and it seems that this induction is mediated by TLR-2 [51]. These authors propose that the immune system can discriminate between pathogens and predominant members of the commensal microbiota via recognition of molecules such as EPS [52]. Based on our findings and those reported in the literature, we propose a hypothetical mechanism(s) of action for HMW-EPS synthesized by bifidobacteria (Figure 2). The presence of HMW-EPS A1doXR at the mucosal surfaces could induce the generation of dendritic cells with a tolerogenic function by different mechanisms, not well known, but which could include the production of specific cytokines. These tolerogenic dendritic cells also secreted immunosuppressive cytokines and then the resultant cytokine network could mediate the induction and/or the expansion of regulatory T cells (probably FOXP3⁺CD25^{high} T_{reg}) which in turn will control an excessive effector T cell (T_{eff}) response or, in the presence of a specific cytokine environment, might trans-differentiate into Th17 cells, thus maintaining mucosal defence and homeostasis.

3. Conclusion

In summary, base on literature data we propose that EPS having negative charge (phosphate in its composition) and/or having small size are able to act as mild stimulators of different immune cells; whereas, neutral and big-size (near 10⁶ Da) polymers have a suppressive profile, or could attenuate an excessive response, thus helping the producing bacteria to evade the immune response of the host. Nevertheless, although there is some evidence supporting this hypothesis, further research is needed in order to demonstrate which are the key physico-chemical parameters determining the ability of bifido- and LAB-EPS to modulate the immune response. Also, the mechanism(s) of action through the putative mediation of Foxp3⁺ T_{reg} cells induction, or whether other pathways could be plausible, deserves further investigation.

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Figure Legends

Figure 1 Concentration (pg/ml) of some cytokines produced by Caco2 cells (in confluent and differentiated monolayer state) co-cultivated (for 8 h at 37°C, 5% CO₂) with three closely related *B. animalis* subsp. *lactis* strains (ratio Caco2: bacteria, 1: 10): A1 (white bar), A1dOx (light grey bar) and A1dOxR (dark grey bar). The black bar represents the control (Caco2 cultivated with DMEM antibiotic-free medium). The experimental design and material used was similar to that showed in Lopez and co-workers [32]. Cytokines were measured by means of ELISA tests (R&D Systems Europe Ltd., UK) and values depicted are the mean and standard deviation of three independent co-culture replicates. Data were analysed by means of one-way ANOVA (SPSS/PC 15.0 software package, SPSS Inc., USA). For each cytokine, differences ($p < 0.05$) between each strain with respect to the control are represented by an asterisk; differences among the three strains were assessed by a mean comparison test LSD (less significant difference) and bars that do not share a common letter are significantly ($p < 0.05$) different.

Figure 2 Hypothesis for immune suppression elicited by the high molecular weight (HMW)-EPS from *B. animalis* subsp. *lactis* A1dOxR. The capability to activate naïve CD4⁺ T cells through dendritic cells (DC) presenting EPS molecules has been demonstrated for specific (“zwitterionic”) polymers [50, 51, 52], although this way seems to be less plausible for non-charged EPS (a). In the *B. animalis* subsp. *lactis* strain, and in accordance with our preliminary *in vitro* [32] and *in vivo* (unpublished) data, the presence of HMW-EPS A1dOxR could induce the activation and differentiation of DC, resulting in a cytokine (CK) production pattern that promotes the generation of tolerogenic DC. This tolerogenic DC and the CK environment could mediate the differentiation of naïve T cells into regulatory T cells (probably FOXP3⁺CD25^{high} T_{reg}) that would drive the control of an excessive effector T cells (T_{eff}) response or, in the presence of specific CK environment, might trans-differentiate into Th17 cells (b). Additionally, this strain was able to reduce the release of pro-inflammatory cytokines (IL-6 and IL-8) by enterocyte-like cells (c, Figure 1).

Table 1 Main monosaccharides present in the HePS synthesised by the *Bifidobacterium* genus. Those present in LAB-HePS have been previously published [56].

Species	Strain	Monosaccharide ratio			Repeated unit	Reference
		Glc	Gal	Rha		
<i>B. animalis</i>	C64MRa	2.5	1.5	1	Unknown	[59]
	E43	2.5	2	1	Unknown	[59]
	A1	2.5	2	1	Unknown	[57]
	IPLA-R1 (A1dOxR)	1	1	1.5	Unknown	[57]
	HMW-IPLA-R1 ^a	1	2	3	Hexa-saccharide	[31]
<i>B. bifidum</i>	BIM B-465	1.3	1		Hepta-saccharide	[81]
	ALM35	1	3	2.4	Unknown	[46]
<i>B. breve</i>	YIT4010	1.5	1		Penta-saccharide	[19]
	NCIMB8807-EPS1	2.5	1	1.4	Unknown	[46]
	NCIMB8807-EPS2	1	1.9		Unknown	[46]
<i>B. catenulatum</i>	YIT4016		1		Tri-saccharide	[40]
<i>B. longum</i> subsp. <i>longum</i>	ATCC15707 ^T	1	1		Unknown	[1]
<i>B. longum</i> subsp. <i>infantis</i>	ATCC15697 ^T	^b	1		Di-saccharide	[65]
<i>B. longum</i>	YIT4028		1.5	1	Penta-saccharide	[41]
	BB-79	1	1.5		Unknown	[48]
	JBL05	2	4	1	Hepta-saccharide	[27]
	H73	2.5	1	2	Unknown	[59]
	L55	4	1	1	Unknown	[59]
	H67	4	1	4	Unknown	[59]
	E44	1	1		Unknown	[59]
	CCUG52486	1.3	1		Unknown	[46]
	NCIMB702205	1	2.5		Unknown	[46]
	NB667	1	2.2	1	Unknown	[60]
	N667dCo	1.2	2.3	1	Unknown	[60]
<i>B. pseudocatenulatum</i>	A102	2	1		Unknown	[59]
	C52	1	1		Unknown	[59]
	E515	2	1		Unknown	[59]
	E63	1	1		Unknown	[59]
	H34	1.5	1		Unknown	[59]

^a HMW-IPLA.R1: high molecular weight fraction of EPS IPLA-R1

^b backbone: →3)-β-D-Galp-(1→3)-α-D-Galp-(1→, which are partially substituted at O-6 with β-D-Glcp.

Table 2 Immune responses elicited by the EPS synthesised by bacteria present in foods (updated from Ruas-Madiedo et al., 2009b).

Producing-bacterium	Bacterial strain characteristic	EPS characteristic ^a	Immune response	Study model ^b	Reference
<i>Lactococcus lactis</i> subsp. <i>cremoris</i>	• KVS20	• HePS, acid (2.2% PO ₄), high M _w	• Stimulation	<i>In vitro</i> mice spleen macrophages	[25]
<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i>	• OLL-1073R-1	• HePS, acid fraction (0.1% PO ₄), high M _w • HePS, neutral fraction	• Strong stimulation • Weak stimulation	<i>In vitro</i> mice spleen cells and PPL	[26]
<i>Leuconostoc mesenteroides</i>	• Commercial dextran	• HoPS, α-glucan (dextran) • HoPS, P-dextran (1.7% PO ₄)	• Weak stimulation • Strong stimulation	<i>In vitro</i> mice spleen macrophages	[61]
<i>Lactobacillus kefirano</i> faciens	• ATCC 46761	• HePS (kefiran)	• Stimulation	<i>In vivo</i> mice model	[74]
<i>Bifidobacterium longum</i>	• BCRC 14634	• Unknown	• Mild modulator	<i>In vitro</i> macrophage mice J77A.1 cell line	[77]
<i>Lactobacillus casei</i>	• YIT 9019 (Shirota, parental) • KO-mutants	• HePS, high M _w + low M _w • (HePS, low M _w ?)	• Suppression • Stimulation	<i>In vitro</i> mice spleen cells and mice macrophage cell line	[79]
<i>Lactobacillus rhamnosus</i>	• ATCC9595 (parental) • RW-9595M (derivative)	• HePS (↑ rham) • HePS (↑ rham), high M _w	• Stimulation • Suppression	<i>In vitro</i> mice macrophages	[4]
<i>Bifidobacterium animalis</i> subsp. <i>lactis</i>	• A1 (parental, food origin) • A1dOx (bile adapted) • A1dOxR (bile adapted, ropy)	• HePS, low M _w • HePS, low M _w • HePS (↑ rham), low M _w + high M _w	• Stimulation • Stimulation • Suppression	<i>In vitro</i> human PBMC	[32]
<i>Bifidobacterium breve</i>	• UCC 2003 (parental EPS ⁺) • UCC2003-EPS ⁻ variants	• HePS • no production	• Suppression • Stimulation	<i>In vivo</i> mice model <i>In vitro</i> spleen cells	[14]
<i>Pediococcus parvulus</i>	• 2.6R (ropy, parental) • 2.6NR (isogenic non-ropy mutant)	• HoPS, 2-substituted (1→3)-β-glucan • no production	• Suppression • Stimulation	<i>In vitro</i> polarized human macrophages	[17]
<i>Propionibacterium freudenreichii</i>	• CB1 (=TL34) (parental) • CB1-KO (<i>gtf</i> inactivated)	• HoPS, 2-substituted (1→3)-β-glucan • no production	• Suppression • Stimulation	<i>In vitro</i> human PBMC	[9]

^a HePS, heteropolysaccharide; HoPS, homopolysaccharide; Rham, rhamnose; Gal, galactose; M_w, molecular weight;^b PPL, Payer's Patches Lymphocytes; PBMC, peripheral blood mononuclear cells

Figure 1

Figure 2