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Immune Modulation Capability of Exopolysaccharides Synthesised by Lactic Acid Bacteria and Bifidobacteria

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

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Introduction

Bacterial EPS

The ability to produce exocellular 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 characterised 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 colonisation; for example, exocellular 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 exocellular 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 polymerisation 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 organisation 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 characterised 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 [50–52].

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 synthesise 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 the 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.

Biosynthesis of EPS from LAB

To date, a wide variety of EPS synthesised by LAB belonging to both HoPS and HePS types has 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 catalysed 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 polymerisation of α-glucans and *β*-fructans, respectively. They can also catalyse the hydrolysis of sucrose and the synthesis of oligosaccharides when the acceptor molecule is other than glucans [28]. *β*-glucans are less frequent in LAB, and to date, the few that have been described have the same structure: (1 → 3)-*β*-D-glucan with side ramifications of a single (1 → 2)-linked *β*-D-glucose. These *β*-glucans are synthesised through a different mechanism in which another single enzyme type (glucosyltransferase), which does not use sucrose as substrate, is involved [11, 75, 76]. This enzyme belongs to the COG1215 membrane-bound glycosyltransferase 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 *β*-glucan synthesis by LAB is still not known; in *Propionibacterium freudenreichii,* it seems that the homologous polysaccharide-synthase enzyme catalyses the intracellular polymerisation of glucose monomers, from nucleotide sugar precursors, and is able to carry out the export [8].

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

Biosynthesis of EPS from Bifidobacterium Genus

The EPS production phenotype has been less studied in bifidobacteria than in LAB, 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 has 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 produce 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 organised 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 physicochemical 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 A1dOxR, 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 $1 = 1.2 \times 10^6$ Da and EPS $2 = 6.4 \times 10^5$ 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

Table 1 Main monosaccharides present in the HePS synthesised by the *Bifidobacterium* genus

Species	Strain	Monosaccharide ratio				References
		Glc	Gal	Rha	Repeated unit	
B. animalis	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	1	2	3	Hexasaccharide	[31]
B. bifidum	BIM B-465	1.3	1		Heptasaccharide	[81]
	ALM35	1	3	2.4	Unknown	[46]
B. breve	YIT4010	1.5	1		Pentasaccharide	[19]
	NCIMB8807-EPS1	2.5	1	1.4	Unknown	[46]
	NCIMB8807-EPS2	1	1.9		Unknown	[46]
B. catenulatum	YIT4016		1		Trisaccharide	[40]
B. longum subsp. *longum*	ATCC15707^T	1	1		Unknown	[1]
B. longum subsp. *infantis*	ATCC15697^T	[a]	1		Disaccharide	[65]
B. longum	YIT4028		1.5	1	Pentasaccharide	[41]
	BB-79	1	1.5		Unknown	[48]
	JBL05	2	4	1	Heptasaccharide	[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]
B. pseudocatenulatum	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]

Those present in LAB-HePS have been previously published [56]

HMW-IPLA.R1 high molecular weight fraction of EPS IPLA-R1

[a] backbone: → 3)-*β*-D-Gal*f*-(1 → 3)-α-D-Gal*p*-(1 → , which are partially substituted at O-6 with *β*-D-Glc*p*

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.

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

Several works in the literature report the ability of EPS synthesised by LAB and bifidobacteria to elicit immune responses (Table 2). In an attempt to correlate some physicochemical traits of EPS with their

Table 2 Immune responses elicited by the EPS synthesised by bacteria present in foods (updated from Ruas-Madiedo et al. [55])

Producing bacterium	Bacterial strain characteristic	EPS characteristic	Immune response	Study model	References
Lactococcus lactis subsp. *cremoris*	KVS20	HePS, acid (2.2 % PO_4), high M_w	Stimulation	In vitro mice spleen macrophages	[25]
Lactobacillus delbrueckii subsp. *bulgaricus*	OLL-1073R-1	HePS, acid fraction (0.1 % PO_4), high M_w	Strong stimulation	In vitro mice spleen cells and PPL	[26]
		HePS, neutral fraction	Weak stimulation		
Leuconostoc mesenteroides	Commercial dextran	HoPS, α-glucan (dextran)	Weak stimulation	In vitro mice spleen macrophages	[61]
		HoPS, P-dextran (1.7 % PO_4)	Strong stimulation		
Lactobacillus kefiranofaciens	ATCC 46761	HePS (kefiran)	Stimulation	In vivo mice model	[74]
Bifidobacterium longum	BCRC 14634	Unknown	Mild modulator	In vitro macrophage mice J77A.1 cell line	[77]
Lactobacillus casei	YIT 9019 (Shirota, parental)	HePS, high M_w + low M_W	Suppression	In vitro mice spleen cells and mice macrophage cell line	[79]
	KO-mutants	(HePS, low M_W?)	Stimulation		
Lactobacillus rhamnosus	ATCC9595 (parental)	HePS (↑ rham)	Stimulation	In vitro mice macrophages	[4]
	RW-9595M (derivative)	HePS (↑ rham), high M_W	Suppression		
Bifidobacterium animalis subsp. *lactis*	A1 (parental, food origin)	HePS, low M_w	Stimulation	In vitro human PBMC	[32]
	A1dOx (bile adapted)	HePS, low M_w	Stimulation		
	A1dOxR (bile adapted, ropy)	HePS (↑ rham), low M_w + high M_w	Suppression		
Bifidobacterium breve	UCC 2003 (parental EPS^+)	HePS	Suppression	In vivo mice model	[14]
	UCC2003-EPS^- variants	No production	Stimulation	In vitro spleen cells	
Pediococcus parvulus	2.6R (ropy, parental)	HoPS, 2-substituted (1 → 3)-β-glucan	Suppression	In vitro polarised human macrophages	[17]
	2.6NR (isogenic non-ropy mutant)	No production	Stimulation		
Propionibacterium freudenreichii	CB1 (=TL34) (parental)	HoPS, 2-substituted (1 → 3)-β-glucan	Suppression	In vitro human PBMC	[9]
	CB1-KO (*gtf* inactivated)	No production	Stimulation		

HePS heteropolysaccharide, *HoPS* homopolysaccharide, *Rham* rhamnose, *Gal* galactose, M_W molecular weight, *PPL* Payer's patches lymphocytes, *PBMC* peripheral blood mononuclear cells

immune-modulating capability, we have detected two association patterns. First, it seems that acidic HePS, which are characterised as having phosphate (i.e. negative charge) in their composition, are good inducers of the immune response. This fact was illustrated by the HePS synthesised by LAB used as starters in the dairy food industry, such as the strain *Lactococcus lactis* subsp. *cremoris* KSV20 involved in the manufacture of the Scandinavian fermented milk viili. The viilian-HePS was able to induce the synthesis of IFNγ and IL-1α in mouse spleen macrophages cultivated in vitro [25]. The repeating unit of KSV20 HePS is a pentasaccharide composed of glucose–galactose–rhamnose in the ratio of 2:2:1, with the side chain α-D-galacto*pyranose* substituted with PO_4^- [42]. Similarly, *Lb. delbrueckii* subsp. *bulgaricus* OLL-1073-R1 synthesises an HePS composed of two fractions, acidic and neutral, both containing glucose and galactose (ratio 3:2) but the acid fraction additionally having 0.1 % $PO4^-$ [67]. The acid fraction was a strong inducer of proliferation and activity of different macrophages, whereas the neutral fraction was not able to elicit stimulation [26, 44]. Furthermore, the same authors have proved that phosphate was the molecule triggering the immune response, since the chemical dephosphorylation of this HePS encompasses a reduction in the stimulatory effect [26]. The relevant role of PO_4^- in immune stimulation was also proven using the α-glucan HoPS (dextran) synthesised by *Leuconostoc mesenteroides* as a model. When dextran was chemically phosphorylated, the proliferation of lymphocyte subsets from the murine spleen, as well as the gene expression of IFNγ and IL-10, 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 et al. [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 TNFα, 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 TNFα and IL-6, whereas the parental *L. rhamnosus* ATCC9595 strain induced high levels of TNFα, 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 et al. [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 (IFNγ, TNFα 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 physicochemical 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

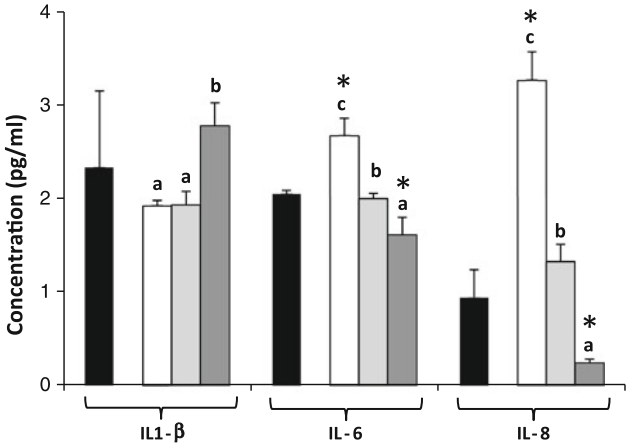

Fig. 1 Concentration (pg/ml) of some cytokines produced by Caco2 cells (in confluent and differentiated monolayer state) co-cultivated (for 8 h at 37 °C, 5 % CO_2) with three closely related *B. animalis* subsp. *lactis* strains (ratio Caco2: bacteria, 1: 10): A1 (*white bar*), A1dOx (*light grey bar*) and A1dOxR (*dark grey bar*). The *black bar* represents the control (Caco2 cultivated with DMEM antibiotic-free medium). The experimental design and material used were similar to that showed in Lopez et al. [32]. Cytokines were measure by means of ELISA tests (R&D Systems Europe Ltd., UK) and values depicted are the mean and SD 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

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 immunosuppressive 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, Fig. 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,

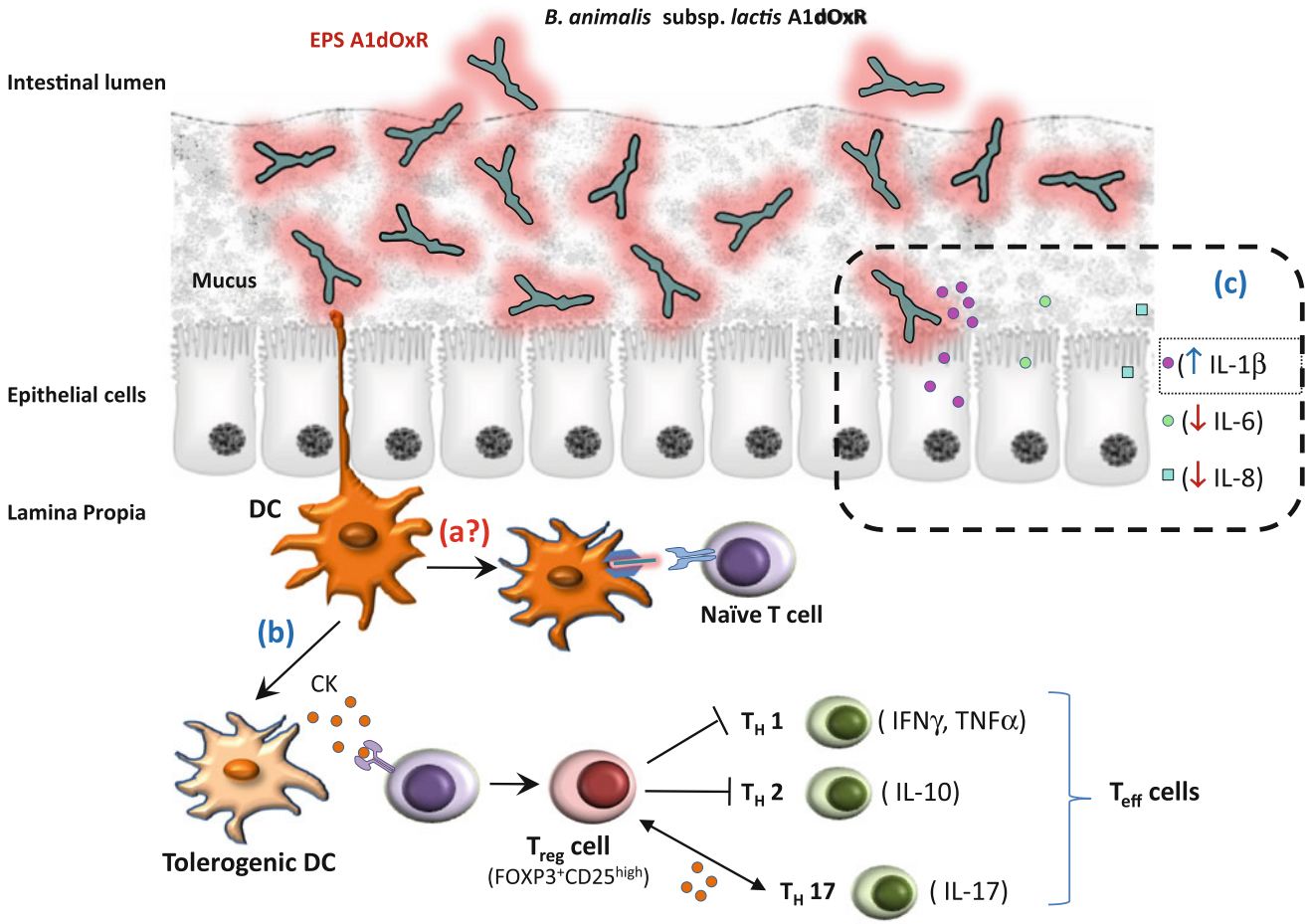

Fig. 2 Hypothesis for immune suppression elicited by the high molecular weight (HMW)-EPS from *B. animalis* subsp. *lactis* A1dOxR. The capability to activate naïve $CD4^+$ T cells through dendritic cells (DC) presenting EPS molecules has been demonstrated for specific ("zwitterionic") polymers [50–52], 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**, Fig. 1)

these data could suggest a role of the ropy A1dOxR strain in the immune homoeostasis 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 et al. [4] also reinforce this idea since intermediate polymer chains (16-30 units, molecular weight $<10^4$ Da) obtained after hydrolysis of the HMW-EPS (size 5.3×10^5 Da) RW-9595M showed increased production of IL-10 by macrophages, in comparison with the native polymer synthesised by *L. rhamnosus*. It is worth noting that another common trait between the HMW-EPS A1dOxR (from *B. animalis* subsp. *lactis*) and the native EPS RW-9595M (from *L. rhamnosus*) is the high presence of rhamnose (more than 50 %) in the composition of their repeating units [31, 69]. At the moment, it is unknown whether this characteristic could also be relevant to regulate the immune response.

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

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

Conclusion

In summary, based on the 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 10^6 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 physicochemical 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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