Title: Exopolysaccharides synthesized by *Bifidobacterium animalis* subsp. *lactis*
interact with TLR4 in intestinal epithelial cells

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14 **Abstract**

15 The toll-like receptors involved in recognition of the exopolysaccharide produced by
16 two isogenic, ropy and non-ropy, *Bifidobacterium animalis* subsp. *lactis* strains were
17 investigated. Both strains interact with human embryonic kidney (HEK)-293 cells via
18 TLR2, whereas purified EPSs specifically stimulate TLR4 regardless their molar mass.

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Exopolysaccharides (EPSs) are carbohydrate polymers surrounding the cell envelope of bacteria, and as such, they help for their interaction with the environment. EPSs synthesized by probiotics are involved in protection against the adverse gastrointestinal conditions and can play a role in the beneficial effect attributed to the EPS-producing probiotic [1]. These polymers are one type of microbial associated molecular patterns (MAMPs) that are ligands of pattern recognition receptors (PRRs) expressed in intestinal epithelial cells [2]. Toll-like receptors (TLRs) are one of the important families of PRRs expressed in immune and somatic cells including intestinal epithelial cells. TLRs are located in cytoplasmic membranes or endosomes and play important roles in the homeostasis of tolerance and immunity in the intestinal mucosa [3]. Extracellular activation of membrane associated TLRs by binding of microbial ligands leads to activation of the nuclear translocation of NF- κ B pathway and secretion of cytokines and chemokines [4]. TLRs are specialized in the recognition of distinct ligands. In bacteria TLR2 recognized lipoproteins from cell wall or lipoteichoic acids, TLR4 bounds the lipid A of LPS of Gram-negatives, the ligands for TLR5 are flagellin proteins and for TLR9 is un-methylated CpG DNA [5]. It is known that probiotic bifidobacteria and lactobacilli are able to interact with TLRs through different MAMPs and induce differential immune responses in the host [6-8]. Genetic differences in strains of the same species, for example glycosylation of cell wall teichoic acids, can lead to differential cellular responses [9].

Previous results obtained in our group showed that EPS-producing *Bifidobacterium animalis* subsp. *lactis* strains elicited different cytokine release by immune cells isolated from gut associated lymphoid tissue (GALT) and from peripheral blood mononuclear cells (PBMCs) of naïve rats [10], but also from *ex vivo* cultures of human colonic tissue [11]. The strains having a “ropy” phenotype due to the synthesis

of a high molar mass EPS (about 10^6 Da), induced a different response than those non-ropy, which produced a smaller molar mass EPS (about 10^4 Da); this suggests that the physical characteristics of the polymers might play different roles in immune modulation [10, 11]. In the current work, we investigated whether intestinal TLRs are involved in the recognition of ropy and non-ropy EPSs.

Here we analysed two isogenic strains: *B. animalis* subsp. *lactis* DMS10140 (parental, non-ropy) and S89L (mutant, ropy), differing in a single nucleotide in the gene involved in the chain length determination [12]. The EPSs of these strains were isolated using a standard procedure followed by additional purification steps, with DNase and Pronase E, as previously described [13].

The *in vitro* TLR assays were performed using commercial human embryonic kidney (HEK)-293 cells harbouring human TLR 2, TLR2/1, TLR2/6, TLR 4 or TLR 5 combined with the reporter plasmid pNiFty2-Luc (ZeozinTM resistant) containing the luciferase gene under the control of the NF- κ B promoter (InvivoGen Europe, France). The HEK-293 cells were seeded at 6×10^4 cells per well in 96-well black plates with clear bottom, and incubated at 37 °C, 5% CO₂ overnight using the selective medium recommended by the manufacturer. Bifidobacterial suspensions (at concentrations ranging from 3 to 9 Log₁₀ CFU/ml), purified EPSs (at 10, 50 and 100 μ g/ml), and the corresponding positive controls (specific stimuli for each TLR and TNF α for NF- κ B activation) as well as the negative control (DMEM without additives) were added. All assays were carried out in triplicate in two independent experiments (n=6) and the NF- κ B activation was measured using the Bright-GloTM luciferase assay (Promega, The Netherlands) after 3 h of co-cultivation [14]. Results obtained were expressed as Log₁₀ bioluminescence units and they were compared by means of ANOVA followed by SNK (Student–Newman–Keuls) mean comparison test using the IBM-SPSS Statistics 25.Ink.

Data obtained with the bifidobacterial suspensions are presented in Table 1 (data from the 293/null cell line used as control, carrying the empty-TLR plasmid, are showed in the supplementary material: Table S1). Both DSM10140 and S89L *B. animalis* subsp *lactis* strains, at doses equal or higher to 8 log CFU/ml units, significantly ($p < 0.001$) activated the three NF- κ B-dependent TLR2 receptors in the same or higher extent than the positive stimuli. However, these strains were not able to activate TLR4 and TLR5 showing results comparable to the negative control. This last result could be expected since LPS and flagellin, the ligands for TRL4 and TLR5 respectively, are not present in *B. animalis* subsp. *lactis*. No differences, at any concentration, between the two strains were found for TLR2 receptors suggesting that the presence of different molar mass polymers surrounding the bacterial surface does not modify their activation. Several studies have reported that bifidobacteria and lactobacilli are able to interact with intestinal epithelial cells by means of TLR2 [15, 16]. When EPSs are present on the surface of the bacteria, such as those tightly attached to the cell surface known as capsular polysaccharides (CPSs), the polymers could reduce the exposure of TLR2-activating molecules as demonstrated with four *Lactobacillus plantarum* strains [17]. On the contrary, the EPS-produced by *Bifidobacterium breve* UCC2003 significantly increased the expression of intestinal epithelial TLR2 in mice feed with this strain and promoted protection against LPS-induced cell shedding which seemed to be mediated by the adaptor protein MyD88. Additionally, an UCC2003 mutant strain lacking EPS production was not able to confer this protection; thus the authors concluded that EPS could be recognized through TLR2 and modulate T regulatory immune function [18]. In this regard the EPS synthesized by *Bifidobacterium longum* 35624 was also able to dampen pro-inflammatory Th17 response in a mice model of colitis [19].

In order to check whether the stimulation of TLR2 induced by our *B. animalis* subsp. *lactis* strains was due to the presence of the polymer, both purified EPS-DSM10140 and EPS-S89L were added at different concentrations to HEK-293 cells (Fig. 1). None of the purified EPSs were able to stimulate the three TLR2 tested since they induced significantly ($p < 0.001$) lower signal than the positive stimuli; this suggests that other molecules from the cell wall of the *B. animalis* subsp. *lactis* strains under study are recognized by these receptors. Interestingly, both purified EPSs at concentrations equal or higher than 10 $\mu\text{g/ml}$ induced NF- κ B activation in HEK-293 cells expressing human TLR4 in the same extent than the positive stimulus (1 $\mu\text{g/ml}$ of LPS) or than the TNF α control, although no differences between the two polymers were detected at any concentration (Fig. 1).

To discard the presence of LPS in the purified EPSs, due to a potential contamination of the material used for purification, two approaches were followed. Firstly, the concentration of LPS in the purified polymers was quantified by means of the EndoZyme® II recombinant factor C endotoxin detection assay (Hyglos GmbH – bioMérieux, Germany) according to the protocol recommended by the manufacturer. EPS stocks (at 1 mg/ml) assayed contained 5.6 ng/ml LPS and 5.0 ng/ml LPS in EPS-DSM10140 and EPS-S89L, respectively. The highest concentration of EPS used for TLR assay was 100 $\mu\text{g/ml}$; thus, the amount of LPS presents was 0.56 ng/ml for EPS-DSM10140 and 0.50 ng/ml for EPS-S89L which was much less than that of the positive LPS control (1000 ng/ml) that induced the same signal as the bifidobacterial EPS. As a second control, both purified EPSs were treated by polymyxin B (100 $\mu\text{g/ml}$ final concentration, for 2 h), which blocks the lipidic part of the LPS avoiding its binding to the TLR4. Results obtained with polymyxin B-treated EPS demonstrated that a significant reduction in TLR4 expression given that statistical differences were detected

between non-treated and treated polymers for all doses tested (supplementary material Table S2). The effect of polymyxin B treatment led to a significant reduction of the activity that could be due to the loss of traces LPS; nevertheless, in all cases (except treated EPS-S89L at 10 µg/ml) the polymers induced higher signal than the control medium (Fig. 2). Finally, for each polymyxin B-treated polymer the three concentrations tested induced significantly increasing levels of NF-κB induction (Fig. 2); however, due to the narrow range of concentrations tested we cannot unequivocally ascertain a dose-dependent effect of bifidobacterial EPS on TLR4 activation.

To our knowledge, there are no reports of on the interaction of purified EPSs from bifidobacteria with TLR4 from intestinal epithelial cells. Polymers purified from *Lactobacillus rhamnosus* LOCK 0900, having different molar mass and composition/structure, were not able to interact with TLR4 from HEK-293 cells [20]. However, it was also reported that EPSs purified from other lactobacilli acted as ligands for TLR4, as in the case of the acidic (phosphorylated) EPS purified from *L. plantarum* N14 [21] or from *Lactobacillus delbrueckii* OLL1073R-1 [22]. Besides, it was reported that the EPS synthesized by *B. animalis* RH was able to interact with TLR4 of mouse RAW 264.7 macrophage cells [23]. In this study we demonstrated that the EPS-DSM10140 and EPS-S89L produced by isogenic *B. animalis* subsp. *lactis* strains had the same capability to stimulate TLR4, pointing that the differences in molar mass do not influence the binding capability of the polymers. However, “ropy” strains producing neutral high molar mass EPS were able to attenuate inflammation in a murine DSS-induced colitis model more effectively than those “non-ropy” strains producing smaller chain length EPS, probably favoring a Treg response [24]. Therefore, additional signaling pathways must be involved in the capacity of the non-charged, high molar mass EPS to elicit protective effects in the mouse colitis models.

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Table 1. Activation of the luciferase gene under the control of NF- κ B reporter (harboured in the plasmid pNiFty2-Luc selectable with the antibiotic ZeocinTM) in HEK (human embryonic kidney)-293 cells expressing human TLR2, TLR2/1, TLR2/6, TLR 4 or TLR5 in the presence of different concentrations of the exopolysaccharide producing *Bifidobacterium animalis* subsp. *lactis* strains DSM10140 (no ropy) and S89L (ropy).

Treatment	Doses	Log luminescence units (Mean $\pm$ SD)				
		TLR2	TLR2/1	TLR2/6	TLR4	TLR5
<i>B. animalis</i> subsp. <i>lactis</i> DSM10140	9 Log CFU/ ml	5.37 $\pm$ 0.01 ^e	5.77 $\pm$ 0.04 ^d	4.97 $\pm$ 0.02 ^e	3.66 $\pm$ 0.09 ^a	-
	8 Log CFU/ ml	5.02 $\pm$ 0.13 ^d	5.30 $\pm$ 0.14 ^{cd}	4.62 $\pm$ 0.09 ^d	3.79 $\pm$ 0.21 ^a	2.45 $\pm$ 0.11 ^a
	7 Log CFU/ ml	4.53 $\pm$ 0.11 ^{cd}	4.59 $\pm$ 0.13 ^b	4.13 $\pm$ 0.08 ^c	3.75 $\pm$ 0.20 ^a	2.60 $\pm$ 0.36 ^a
	6 Log CFU/ ml	3.89 $\pm$ 0.11 ^b	3.70 $\pm$ 0.18 ^a	3.49 $\pm$ 0.11 ^b	3.78 $\pm$ 0.19 ^a	2.69 $\pm$ 0.43 ^a
	$\leq$ 5 Log CFU/ ml	3.47 $\pm$ 0.04 ^a	3.33 $\pm$ 0.07 ^a	3.17 $\pm$ 0.16 ^a	3.76 $\pm$ 0.23 ^a	2.49 $\pm$ 0.03 ^a
	Positive control [#]	4.81 $\pm$ 0.60 ^c	4.01 $\pm$ 0.82 ^c	4.55 $\pm$ 0.60 ^d	4.59 $\pm$ 0.33 ^b	3.97 $\pm$ 0.08 ^b
	TNF α -control	5.39 $\pm$ 0.21 ^e	5.66 $\pm$ 0.25 ^d	5.32 $\pm$ 0.16 ^f	4.57 $\pm$ 0.05 ^b	4.30 $\pm$ 0.05 ^b
	Medium	3.34 $\pm$ 0.09 ^a	3.20 $\pm$ 0.05 ^a	3.94 $\pm$ 0.85 ^a	3.77 $\pm$ 0.20 ^a	2.46 $\pm$ 0.06 ^a
		***	***	***	***	***
<i>B. animalis</i> subsp. <i>lactis</i> S89L	9 Log CFU/ ml	5.38 $\pm$ 0.01 ^e	5.81 $\pm$ 0.02 ^d	4.95 $\pm$ 0.02 ^d	3.62 $\pm$ 0.07 ^a	-
	8 Log CFU/ ml	5.04 $\pm$ 0.21 ^{de}	5.33 $\pm$ 0.30 ^{cd}	4.60 $\pm$ 0.22 ^c	3.74 $\pm$ 0.22 ^a	2.59 $\pm$ 0.06 ^a
	7 Log CFU/ ml	4.65 $\pm$ 0.11 ^c	4.62 $\pm$ 0.36 ^b	4.16 $\pm$ 0.16 ^b	3.73 $\pm$ 0.18 ^a	2.53 $\pm$ 0.04 ^a
	6 Log CFU/ ml	4.00 $\pm$ 0.09 ^b	3.66 $\pm$ 0.23 ^a	3.41 $\pm$ 0.17 ^a	3.73 $\pm$ 0.15 ^a	2.49 $\pm$ 0.07 ^a
	$\leq$ 5 Log CFU/ ml	3.48 $\pm$ 0.06 ^a	3.35 $\pm$ 0.04 ^a	3.21 $\pm$ 0.16 ^a	3.77 $\pm$ 0.24 ^a	2.47 $\pm$ 0.09 ^a
	Positive control [#]	4.81 $\pm$ 0.60 ^{cd}	5.01 $\pm$ 0.82 ^{bc}	4.55 $\pm$ 0.60 ^c	4.59 $\pm$ 0.33 ^b	3.97 $\pm$ 0.08 ^b
	TNF α -control	5.39 $\pm$ 0.21 ^e	5.66 $\pm$ 0.25 ^d	5.32 $\pm$ 0.16 ^e	4.58 $\pm$ 0.05 ^b	4.30 $\pm$ 0.05 ^c
	Medium	3.34 $\pm$ 0.09 ^a	3.20 $\pm$ 0.05 ^a	3.13 $\pm$ 0.19 ^a	3.77 $\pm$ 0.20 ^a	2.46 $\pm$ 0.06 ^a
		***	***	***	***	***

[#] Positive controls: 25 ng/ ml Pam3CSK4 for TLR2/1 and TLR2; 25 ng/ ml Pam2CSK4 for TLR2/6 and TLR2; 1 μ g/ ml lipopolysaccharide for TLR 4; 0.04 μ g/ ml flagellin for TLR5. Also, 2.5 ng/ml TNF α was used as positive control to stimulate pNiFty2-Luc plasmid. The medium DMEM used for the HEK-293 cells was the negative control. *** p<0.001 according to ANOVA (analysis of variance) test; for each strain, values that within the same column do not share a common superscript letter are statistically (p<0.05) different according to the SNK (Student–Newman–Keuls) mean comparison test.

Figure legend

Fig 1. Activation of the luciferase gene under the control of NF- κ B reporter in HEK-293 cells expressing human TLR2, TLR2/1, TLR2/6, TLR 4 or TLR5 in the presence of different concentrations of the EPS-DSM10140 (no ropy, blue bar) and EPS-S89L (ropy, red bar) purified from two strains of *B. animalis* subsp. *lactis*. Stimuli, TNF α and medium controls are described in Table 1. For each cell line expressing different receptors, values that do not share a common letter are statistically ($p < 0.05$) different according to the SNK mean comparison test.

Fig 2. Activation of the luciferase gene under the control of NF- κ B reporter in HEK-293 cells expressing human TLR 4 in the presence of different concentrations of the bifidobacterial EPS-DSM10140 (no ropy, blue bars) and EPS-S89L (ropy, red bars) treated with polymyxin B or non-treated. Stimuli (1 μ g/ml LPS) and culture medium were used as controls. Values that withing each treatment do not share a common letter are statistically ($p < 0.05$) different according to the SNK mean comparison test. Values showing an asterisk are statistically ($p < 0.05$) different with respect to the control medium, acoding to ANOVA test.

Log NF- κ B activation
(Log luminescence units)

Log NF- κ B activation
(Log luminescence units)