Strain-Specific Features of Extracellular Polysaccharides and Their Impact on *Lactobacillus plantarum*-Host Interactions

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ABSTRACT

Lactobacilli are found in diverse environments and are widely applied as probiotic, health-promoting food supplements. Polysaccharides are ubiquitously present on the cell surface of lactobacilli and are considered to contribute to the species- and strain-specific probiotic effects that are typically observed. Two *Lactobacillus plantarum* strains, SF2A35B and Lp90, have an obvious ropy phenotype, implying high extracellular polysaccharide (EPS) production levels. In this work, we set out to identify the genes involved in EPS production in these *L. plantarum* strains and to demonstrate their role in EPS production by gene deletion analysis. A model *L. plantarum* strain, WCFS1, and its previously constructed derivative that produced reduced levels of EPS were included as reference strains. The constructed EPS-reduced derivatives were analyzed for the abundance and sugar compositions of their EPS, revealing cps2-like gene clusters in SF2A35B and Lp90 responsible for major EPS production. Moreover, these mutant strains were tested for phenotypic characteristics that are of relevance for their capacity to interact with the host epithelium in the intestinal tract, including bacterial surface properties as well as survival under the stress conditions encountered in the gastrointestinal tract (acid and bile stress). In addition, the Toll-like receptor 2 (TLR2) signaling and immunomodulatory capacities of the EPS-negative derivatives and their respective wild-type strains were compared, revealing strain-specific impacts of EPS on the immunomodulatory properties. Taken together, these experiments illustrate the importance of EPS in *L. plantarum* strains as a strain-specific determinant in host interaction.

IMPORTANCE

This study evaluates the role of extracellular polysaccharides that are produced by different strains of *Lactobacillus plantarum* in the determination of the cell surface properties of these bacteria and their capacity to interact with their environment, including their signaling to human host cells. The results clearly show that the consequences of removal of these polysaccharides are very strain specific, illustrating the diverse and unpredictable roles of these polysaccharides in the environmental interactions of these bacterial strains. In the context of the use of lactobacilli as health-promoting probiotic organisms, this study exemplifies the importance of strain specificity.

Lactobacilli are lactic acid bacteria (LAB) found in diverse environments ranging from fermented food to the human gastrointestinal (GI) tract and are widely applied in the food industry (1). In addition, specific *Lactobacillus* strains, such as *Lactobacillus rhamnosus* GG (2), *Lactobacillus plantarum* 299v (3), and *Lactobacillus acidophilus* NCFM (4), are marketed as probiotics. Probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (5). The beneficial effects attributed to probiotic bacteria are often considered strain specific and have been proposed to at least involve cell surface components, including peptidoglycan, teichoic acids, (glyco)proteins, and polysaccharides (6).

Polysaccharides are ubiquitously present on the *Lactobacillus* cell surface (7), exhibiting a wide diversity of structures in terms of sugar compositions, sugar linkages and branching, and nonsugar modifications (8, 9). Based on the sugar composition, extracellular polysaccharides (EPS) are classified into two groups: homopolysaccharides, which are composed of a single type of monosaccharide, and heteropolysaccharides, which are composed of repeating units comprising multiple monosaccharides. The heteropolysaccharides commonly contain D-glucose, D-galactose, and/or L-rhamnose, and in some cases N-acetylgalactosamine, N-acetylglucosamine, or glucuronic acid residues (6). The high EPS diversity is reflected by the variability of the glycosyltransferases in the gene clusters involved in EPS biosynthesis (10, 11). The complexity of the EPS structure is exemplified further by the observation that one bacterial genome might possess multiple polysaccharide biosynthesis-encoding gene clusters. As an example, the *Lactobacillus plantarum* WCFS1 genome carries 4 capsular polysaccharide (CPS) biosynthesis gene clusters (12, 13). EPS, specifically heteropolysaccharides, of lactobacilli have been reported to be involved in various biological functions, such as in the...
as phage absorption (14, 15), adhesion to human cells or to other bacteria (16, 17), and immunomodulation (18, 19). Various studies have employed gene deletion mutation of EPS-associated genes as a strategy to investigate the biological roles of EPS. For example, the deletion of the entire EPS gene cluster in Lactobacillus johnsonii NCC533 resulted in the loss of bacterial (fuzzy) encapsulation and slightly increased gut persistence in a murine model (20). In addition, deletion of the priming glycosyltransferase encoding gene, welE, in the EPS biosynthesis gene cluster of Lactobacillus rhamnosus GG strongly reduced the strain’s capacity to produce high-molecular-weight, galactose-rich polysaccharide while small glucose-rich EPS remained and resulted in enhanced adhesion to mucus and an epithelial cell line (17). However, the enhanced adhesion did not translate to a better gastrointestinal persistence of the welE deletion mutant. A further study found that EPS plays a protective role against intestinal antimicrobial and complement factors (21). In Lactobacillus casei Shirota, deletion of part of the cell wall polysaccharide (WPS)-encoding gene cluster led to a reduction of high-molecular-weight polysaccharides associated with the bacterial cell surface, and the thermally inactivated mutant strain induced higher levels of tumor necrosis factor alpha (TNF-α), interleukin-12 (IL-12), IL-10, and IL-6 production in mouse macrophage and spleen cell lines in vitro. Notably, whereas the wild-type L. casei Shirota strain has been reported to suppress lipopolysaccharide-induced IL-6 production in mouse macrophage-like cells, its WPS deletion derivative lacked this suppressive ability (18). Mutation analysis of the 4 cps gene clusters in L. plantarum WCF51 revealed pronounced differential impacts of the mutation of the different clusters, illustrated by the differential impact of these mutations on the transcriptome profile in the mutant strain and on the surface glycan composition. Moreover, the differential characteristics of the individual and combined cps gene cluster mutants were also observed in their capacity to stimulate Toll-like receptor 2 (TLR2) activation, where cps1 and cps3 deletions did not have an impact on TLR2 activation while cps2 and cps4 deletions led to mild but significant TLR2 activation, but mutation of 3 or all 4 cps gene clusters induced a strong increase in the TLR2 signaling capacity of the strain that also lost most of its surface glycan structure production capacity (13).

The work related to the species L. plantarum was performed using the type strain WCF51, which does not produce large amounts of EPS and does not have a rory phenotype. In contrast, we found two L. plantarum strains, SF2A35B and Lp90, that have an obvious rory phenotype, implying much higher EPS production levels than strain WCF51. In this work, we set out to identify the genes involved in EPS production in these L. plantarum strains and demonstrate their role in EPS production by mutation analysis. Based on the comparison between the genomes of SF2A35B and Lp90 and those of other sequenced L. plantarum strains, each of the rory strains SF2A35B and Lp90 possesses its own unique polysaccharide gene clusters. The gene clusters were deleted, and the resulting mutants were analyzed for the abundance and sugar compositions of their EPS. Moreover, these mutant strains were tested for phenotype characteristics that are of relevance for their capacity to interact with the host intestinal tract, including bacterial surface properties and survival under acid and bile stresses. In addition, the TLR2 and immunomodulatory capacities of the wild-type and respective mutant strains were compared, illustrating the strain-specific and various impacts of the removal of the EPS in individual strains of the L. plantarum species.

### MATERIALS AND METHODS

**Bacterial strains and culture conditions.** Bacterial strains used in this work are listed in Table 1. Lactobacillus plantarum strains were grown at 37°C in MRS broth (Difco, West Molesey, United Kingdom) without aeration. Escherichia coli strain TOP10 (Invitrogen, Bleiswijk, The Netherlands) was used as an intermediate cloning host and was grown at 37°C in TY broth (22) with aeration (23). Solid media were prepared by adding 1.5% (wt/vol) agar to the broth. Antibiotics were added where appropriate, and the concentrations used for L. plantarum and Escherichia coli strains were 10 µg/ml chloramphenicol (Cm) and 30 and 200 µg/ml erythromycin (Ery), respectively.

**DNA manipulations.** The plasmids and primers used are listed in Table 2. Standard procedures were used for DNA manipulations in E. coli (23). Plasmid DNA was isolated from E. coli using a JetStar kit (Genomed GmbH, Bad Oerhausen, Germany). L. plantarum DNA was isolated and transformed as described previously (24). PCR amplifications were performed using KOD hot start polymerase (Novagen, Madison, WI, USA). Amplicons were purified using a Wizard SV gel and PCR clean-up system (Promega, Leiden, The Netherlands). Restriction endonucleases (Fermentas GmbH, St. Leon-Rot, Germany), MSB Spin PCRapace (Invitrogen),

### TABLE 1 Bacterial strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Characteristic(s)</th>
<th>Reference(s) or source</th>
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</thead>
<tbody>
<tr>
<td>L. plantarum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WCF51</td>
<td>Single-colony isolate of L. plantarum NCIMB8826; originally isolated from human saliva, United Kingdom 12</td>
<td>12</td>
</tr>
<tr>
<td>SF2A35B</td>
<td>Isolate from sour cassava, South America; synonym NIZO1839 36, 60</td>
<td>36, 60</td>
</tr>
<tr>
<td>Lp90</td>
<td>Isolate from a red must, Italy 35, 61</td>
<td>35, 61</td>
</tr>
<tr>
<td>NZ3550Cm</td>
<td>Cm'; derivative of WCF51 containing a lac66-P_3-cat-lox71-tag2.2 replacement of cps1A-3f</td>
<td>This work</td>
</tr>
<tr>
<td>NZ3561BCm</td>
<td>Cm'; derivative of SF2A35B containing a lac66-P_3-cat-lox71-tag9.3 replacement of lpSF_839 to lpSF_853 (lpSF_839-853:lox66-P_3-cat-lox71-tag9.3)</td>
<td>This work</td>
</tr>
<tr>
<td>NZ8220Cm</td>
<td>Cm'; derivative of Lp90 containing a lac66-P_3-cat-lox71-tag10.3 replacement of lp90_1067 to lp90_1077 (lp90_1067-1077:lox66-P_3-cat-lox71-tag10.3)</td>
<td>This work</td>
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<tr>
<td>E. coli</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TOP10</td>
<td>Cloning host; F− mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu)7697 galU galK rpsL (Str') endA1 nupG</td>
<td>Invitrogen</td>
</tr>
</tbody>
</table>

*a* Cm', chloramphenicol resistant; Em', erythromycin resistant; Str', streptomycin resistant.  
*b* Putative subspecies argentoratensis.


### Table 2: Plasmids and primers used in this study

<table>
<thead>
<tr>
<th>Plasmids or primer</th>
<th>Description or sequence</th>
<th>Reference</th>
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<tr>
<td>pNZ5319</td>
<td>Cm&lt;sup&gt;i&lt;/sup&gt; Em&lt;sup&gt;i&lt;/sup&gt;; mutagenesis vector for gene replacements in <em>L. plantarum</em></td>
<td>25</td>
</tr>
<tr>
<td>pNZ5361</td>
<td>Cm&lt;sup&gt;i&lt;/sup&gt; Em&lt;sup&gt;i&lt;/sup&gt;; pNZ5319 derivative containing homologous regions up- and downstream of lpSF_839 to lpSF_853</td>
<td>This work</td>
</tr>
<tr>
<td>pNZ8220</td>
<td>Cm&lt;sup&gt;i&lt;/sup&gt; Em&lt;sup&gt;i&lt;/sup&gt;; pNZ5319 derivative containing homologous regions up- and downstream of lp90_1067 to lp90_1077</td>
<td>This work</td>
</tr>
</tbody>
</table>

#### Primers

<table>
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<tr>
<th>Primers</th>
<th>Description or sequence</th>
<th>Reference</th>
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</thead>
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<tr>
<td>is128 tag-lox66-F</td>
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</tr>
<tr>
<td>is129 tag-lox71-R</td>
<td>5′-CTCAGGCGGGGTATGTAACCG-3′</td>
<td>63</td>
</tr>
<tr>
<td>IS169</td>
<td>5′-TTATCATATCCGGAGGACC-3′</td>
<td>31</td>
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<tr>
<td>87</td>
<td>5′-GGCCAGCTGTCATTTGGACTC-3′</td>
<td>25</td>
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<tr>
<td>CrecF</td>
<td>5′-CGATACCGTGTAGCAAAATTGG-3′</td>
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<td>CrecR</td>
<td>5′-CTTGCCTCATAAGTAACCGTAC-3′</td>
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<tr>
<td>Eryrif</td>
<td>5′-CTCAATACGTCATTAGGTCG-3′</td>
<td>25</td>
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<tr>
<td>EryrifR</td>
<td>5′-ATCACAACAGAATGATGTAAC-3′</td>
<td>25</td>
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<tr>
<td>lpSF-Up-F</td>
<td>5′-AAATCCGGGACACGTAAGG-3′</td>
<td>This work</td>
</tr>
<tr>
<td>lpSF-Up-R</td>
<td>5′-GGATGAGTATGTAACAGTGAC-3′</td>
<td>This work</td>
</tr>
<tr>
<td>lpSF-Down-F</td>
<td>5′-CGGTATACACGGGCGATGTAATGTTAGATTGTC-3′</td>
<td>This work</td>
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<tr>
<td>lpSF-Down-R</td>
<td>5′-GCATAGGATATTCACATGAG-3′</td>
<td>This work</td>
</tr>
<tr>
<td>lpSF-out-F</td>
<td>5′-GTCGCCAGACCACTTACC-3′</td>
<td>This work</td>
</tr>
<tr>
<td>lpSF-out-R</td>
<td>5′-GGCAACAAGGTCAAAACC-3′</td>
<td>This work</td>
</tr>
<tr>
<td>lp90-Up-F</td>
<td>5′-GATATCGGGTGCCGGAGAGG-3′</td>
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<tr>
<td>lp90-Up-R</td>
<td>5′-AGTAGCTATAGACACGCTTAGATTTTGCGAGTACATCATTACCTC-3′</td>
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<tr>
<td>lp90-Down-F</td>
<td>5′-CGGTACAGGCGGCCGATGTAATGTTAGTTGTCG-3′</td>
<td>This work</td>
</tr>
<tr>
<td>lp90-Down-R</td>
<td>5′-GCTATACCCCGTGTAGAATGTCGAGAGG-3′</td>
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<td>lp90-out-F</td>
<td>5′-GCCATATGCTACGTCATGAAAGG-3′</td>
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<tr>
<td>lp90-out-R</td>
<td>5′-CGGCTTACCATATTCATGAC-3′</td>
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</table>

*Abbreviations: Cm<sup>i</sup>, chloramphenicol resistant; Em<sup>i</sup>, erythromycin resistant.

#### Plasmids

- **pNZ5319**
  - Cm<sup>i</sup> Em<sup>i</sup>; mutagenesis vector for gene replacements in *L. plantarum*

- **pNZ5361**
  - Cm<sup>i</sup> Em<sup>i</sup>; pNZ5319 derivative containing homologous regions up- and downstream of lpSF_839 to lpSF_853

- **pNZ8220**
  - Cm<sup>i</sup> Em<sup>i</sup>; pNZ5319 derivative containing homologous regions up- and downstream of lp90_1067 to lp90_1077

#### Primers

- **is128 tag-lox66-F**
  - 5′-AAATCTACGGTGTTAATGTTAG-3′

- **is129 tag-lox71-R**
  - 5′-CTCAGGCGGGGTATGTAACCG-3′

- **IS169**
  - 5′-TTATCATATCCGGAGGACC-3′

- **87**
  - 5′-GGCCAGCTGTCATTTGGACTC-3′

- **CrecF**
  - 5′-CGATACCGTGTAGCAAAATTGG-3′

- **CrecR**
  - 5′-CTTGCCTCATAAGTAACCGTAC-3′

- **Eryrif**
  - 5′-CTCAATACGTCATTAGGTCG-3′

- **EryrifR**
  - 5′-ATCACAACAGAATGATGTAAC-3′

- **lpSF-Up-F**
  - 5′-AAATCCGGGACACGTAAGG-3′

- **lpSF-Up-R**
  - 5′-GGATGAGTATGTAACAGTGAC-3′

- **lpSF-Down-F**
  - 5′-CGGTATACACGGGCGATGTAATGTTAGATTGTC-3′

- **lpSF-Down-R**
  - 5′-GCATAGGATATTCACATGAG-3′

- **lpSF-out-F**
  - 5′-GTCGCCAGACCACTTACC-3′

- **lpSF-out-R**
  - 5′-GGCAACAAGGTCAAAACC-3′

- **lp90-Up-F**
  - 5′-GATATCGGGTGCCGGAGAGG-3′

- **lp90-Up-R**
  - 5′-AGTAGCTATAGACACGCTTAGATTTTGCGAGTACATCATTACCTC-3′

- **lp90-Down-F**
  - 5′-CGGTACAGGCGGCCGATGTAATGTTAGTTGTCG-3′

- **lp90-Down-R**
  - 5′-GCTATACCCCGTGTAGAATGTCGAGAGG-3′

- **lp90-out-F**
  - 5′-GCCATATGCTACGTCATGAAAGG-3′

- **lp90-out-R**
  - 5′-CGGCTTACCATATTCATGAC-3′

GmbH, Berlin, Germany). PCR master mix (Promega), and T4 DNA ligase (Invitrogen) were used as specified by the manufacturers.

**Construction of cps deletion mutants.** The *cps* deletion mutants were constructed as described previously (25), using a double crossing-over strategy to replace the target *cps* gene cluster by a chloramphenicol resistance cassette (*lox66-*P<sub>cat-lox71</sub>) (25). In this study, a derivative of the mutagenesis vector pNZ5319 (25), designated pNZ5319TAG, was used to introduce a unique 42-nucleotide tag into the chromosome during gene deletion, which can be employed for mutant tracking purposes in mixed populations (not relevant for the study presented here). The upstream and downstream flanking regions of each *cps* gene cluster (lpSF_839 to lpSF_853, 1500 bp each) were amplified by PCR using the targets-Up-F/targets-Down-R primer pairs (Table 2). The resulting PCR products were digested with SmaI and Ecl136I and cloned into similarly digested pNZ5319TAG. The mutagenesis plasmids obtained were transformed into *L. plantarum* strains as described previously (24). The resulting transformants were assessed for a double crossover integration event by selecting individual colonies that displayed a Cm-resistant and Ery-sensitive phenotype. The colonies selected were further confirmed by PCR using targets-out-F/targets-R primers (Table 2). For each of the mutant constructions, a single colony displaying the anticipated phenotype and genotype was selected, yielding the mutants NZ3561Cm (<i>ΔlpSF_839 to ΔlpSF_853</i>) and NZ8220Cm (<i>Δlp90_1067 to Δlp90_1077</i>).

**TEM.** The production of EPS in WCF81, SF2A35B, and Lp90 was analyzed by transmission electron microscopy (TEM). One-half milliliter samples of overnight cultures of *L. plantarum* strains were centrifuged at 12,000 × g for 2 min at 20°C and resuspended in 100 μl of phosphate-buffered saline (PBS) at pH 7.0 before the analysis. For the analysis, electron microscopy grids (commercial Formvar- and carbon-coated copper grids, 300 mesh; Electron Microscopy Sciences, Hatfield, PA) were used. Immediately before use, the grids were subjected to an ion discharge for 1 min to make the carbon film more hydrophilic, thus favoring adsorption of the sample. Subsequently, 10 μl of sample was added on the grid for 1 min. The excess solution was removed using filter paper, followed by negative staining by 2% uranyl acetate for 40 s. Finally, excess staining agent was removed, and the samples were observed under a transmission electron microscope (Jeol JEM1011) stabilized at 100 kV.

**Surface polysaccharide isolation and sugar composition determination.** EPS was isolated and analyzed according to previously described methods (13). *L. plantarum* strains and their mutant derivatives were grown in 50 ml 2X chemically defined medium (CDM) until late stationary phase. After growth, cultures were incubated at 55°C for 1 h, followed by pelleting of the bacterial cells (6,000 × g, 15 min, room temperature). The supernatants were supplemented with erythromycin and lincomycin (10 μg/ml each) to avoid growth during dialysis, which was performed in dialysis tubes (molecular mass cutoff of 12,000 to 14,000 Da; Fisher Scientific, Landsmeer, The Netherlands) incubated overnight against running tap water, followed by dialysis for 1 h against deionized water. The dialyzed samples were freeze-dried, weighed, and stored at −20°C until further analysis.

The abundance and sugar compositions of EPS were analyzed as described previously (13). Freeze-dried samples were dissolved in eluent (100 mM NaNO₃, plus 0.02% NaN₃) and then were separated by size exclusion chromatography (SEC) with refractive index (dRI), UV (280 nm), viscosity, and multiangle laser light scattering (MALLS) detection (ViscoStar; Wyatt Technologies, Santa Barbara, CA, USA), using columns of TSK gel PWXL Guard, TSK gel G6000 PWXL, and TSK gel G5000 PWXL. During SEC, polysaccharide peaks were collected and hydrolyzed with 2 M trifluoroacetic acid (TFA) at 120°C for 75 min, which removes possible modifications on sugars (e.g., acetylations), and then were dried and dissolved in water. The quantitative monosaccharide composition of the polysaccharide fractions was analyzed using high-performance liquid chromatography (HPLC) with refractive index (dRI) and UV (280 nm) detection.
anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) with a PA-1 column. The monosaccharides were eluted isocratically with 16 mM sodium hydroxide followed by the elution of the acid monosaccharides starting at 20 min with a linear gradient to 200 mM sodium hydroxide plus 500 mM sodium acetate in 20 min. Data analysis was performed with Dionex Chromelone software version 6.80. Quantitative analyses were carried out using standard solutions of the monosaccharides (rhamnose, galactosamine, glucosamine, galactose, glucose, mannose, xylose, galacturonic acid, and glucuronic acid) (Sigma-Aldrich, St. Louis, MO, USA).

**Zeta potential and hydrophobicity.** Overnight cultures were washed twice with 10 ml PBS, and bacteria were resuspended in PBS at an optical density at 600 nm (OD600) of 1. The zeta potential was measured at 20°C using Zetasizer cuvettes (DST1070; Malvern Instruments, Malvern, St. Louis, MO, USA).

Surface hydrophobicity was determined using the microbial adhesion to solvents (MATS) method (27). Briefly, overnight cultures of _L. plantarum_ strains were harvested by centrifugation at 5,000 × _g_ for 10 min, washed twice, and resuspended to an OD600 of 1 (_A_600) in PBS. Five milliliters of bacterial suspension was mixed by 2 min of vortexing with 2 ml petroleum ether (the solvent) in a 10-ml glass tube. The tubes were incubated statically for 15 min at room temperature to allow phase separation of the mixture. The aqueous phase was collected, and its OD600 was measured (_A_600). The cell surface hydrophobicity (CSH) is presented as the percentage of microbial cells retained in the solvent and calculated as [1 – (_A_600/ _A_600)] × 100.

**In vitro Caco-2 adhesion assay.** For adhesion tests, Caco-2 cells were seeded in 96-well tissue culture plates (Falcon Microtest, Becton Dickinson, NJ, USA) at a concentration of 1.6 × 10^5 cells per well and cultured for 12 to 15 days as previously described (28). The _L. plantarum_ cells were harvested at late stationary phase, pelleted by centrifugation, and resuspended in antibiotic-free Dulbecco's modified Eagle's medium (DMEM). The obtained monolayers (about 5.0 × 10^4 cells per well as counted in a Bürker chamber) of differentiated cells that mimic small intestine mature enterocytes (29, 30) were overlaid with stationary-phase cells of _L. plantarum_ (OD600 of 5.0), at a multiplicity of exposure (MOE) of 1:1,000 (Caco-2 cells to bacteria).

After 1 h of incubation at 37°C, under a 5% CO₂ atmosphere, adhesion determination wells were washed three times with PBS (pH 7.4) to remove unbound bacteria. No washing was performed on control wells, with the aim of recovering both adherent and nonadherent bacteria. Caco-2 cells and adherent bacteria were then detached by trypsin-0.05% EDTA (Gibco) for 10 min at 37°C and resuspended in sterile PBS (Gibco). Serial dilutions of samples were plated onto MRS agar plates to determine the number of cell-bound bacteria (viable counts) expressed as CFU, which were determined by Kruskal-Wallis ANOVA on ranks and Dunn’s test for multiple comparisons.

**Gi Tract survival assay.** The assay was performed as described previously (31). Briefly, stationary-phase bacterial cultures were harvested and treated for 60 min in a stomach-like environment at pH 2.4, followed by neutralization and 60 min under intestine-like conditions. Samples were collected before treatments and after stomach-like and intestine-like conditions to determine CFU counting using spot plating (32). The survival results are presented as relative survival, which is the percentage of microbial cells retained in the solvent and calculated as [1 – (_A_600/ _A_600)] × 100.

**PBMC assay.** The assay was performed as described previously (34) and was approved by the Wageningen University Ethical Committee and performed according to the principles of the Declaration of Helsinki. Peripheral blood samples of healthy donors were from the Sanquin Blood Bank, Nijmegen, The Netherlands. Peripheral blood mononuclear cells (PBMCs) were separated from the blood using Ficoll–Paque Plus gradient centrifugation according to the manufacturer’s description (Amersham Biosciences, Uppsala, Sweden). The mononuclear cells were collected, washed in Iscove’s modified Dulbecco’s medium (IMDM) plus GlutaMAX (Invitrogen, Breda, The Netherlands) and adjusted to 1 × 10^6 cells/ml in IMDM plus GlutaMAX supplemented with penicillin (100 U/ml) (Invitrogen), streptomycin (100 μg/ml) (Invitrogen), and 1% human AB serum (Lonza, Basel, Switzerland). PBMCs (1 × 10^6 cells per well) were seeded one night prior to the experiment in 48-well tissue culture plates and incubated at 37°C in 5% CO₂. Bacteria from late-stationary phase were added to PBMCs at a MOE of 1:10 (PBMCs to bacteria). PBMCs from 3 different donors were used in the assay. Following a 24-h incubation at 37°C in 5% CO₂ culture supernatants were collected and stored at −20°C until cytokine analysis. Cytokines were measured using a FACSCanto II flow cytometer (BD Biosciences, NJ, USA) and BD cytometric bead array flex sets (BD Biosciences) for interleukin-10 (IL-10) and IL-12p70 (henceforth referred to as IL-12), TNF-α, IL-6, IL-1β, and IL-8 according to the manufacturer’s procedures. Concentrations of cytokines were calculated based on the standard curves in the BD Biosciences FCAP software.

**Statistical analysis.** All analyses were performed in triplicate except that zeta potential measurements were done in quadruplicate. The one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test was used to compare the means of adhesion, biofilm formation, and TLR2 activations between strains. The cytokine production levels in PBMC assays were transformed to log values to compare the stimulation between wild-type and mutant strains using the paired _t_ test with respect to individual donors. The differences in surface properties among strains were determined by Kruskal–Wallis ANOVA on ranks and Dunn’s test for multiple comparisons. GraphPad Prism 5 software (GraphPad Software, San Diego, CA) was used for all determinations, and a _P_ value of <0.05 was considered significant.

**RESULTS**

Identification of genes responsible for the ropy phenotype of _SF2A35B_ and _Lp90_. Under normal laboratory conditions of growth, the strains _SF2A35B_ and _Lp90_ display an obvious ropy phenotype in comparison to that of the model strain _WCF51_ (see Fig. S1 in the supplemental material), which implies a substantially higher level of surface polysaccharide production in these strains than in _WCF51_. To explore the gene cluster(s) that is responsible for this ropy phenotype, the genomes of both strains were sequenced (35, 36), and the genes that could be related to capsular polysaccharide (CPS) biosynthesis were annotated in de novo. The genomes of the _SF2A35B_ (36) and _Lp90_ (35) strains carry 3 and 4 recognizable and apparently complete _cps_ clusters, respectively. A comparison of the genomes of the ropy strains with that of strain _WCF51_ indicates that the polysaccharide synthesis cluster assigned _cps_ cluster 4 in strain _WCF51_ is most conserved among the _L. plantarum_ strains (data not shown), and in particu-
The gene cluster assigned cps cluster 2 in strain WCFS1 appears to be variable and contains genes that are present only in the ropy strains SF2A35B and Lp90 (Fig. 1). In addition, strain SF2A35B appears to lack the entire cps cluster 1 (nomenclature according to the strain WCFS1 genome) but contains 5 unique genes within its cps cluster 2, which were assigned the gene identifiers lpSF_846, lpSF_849 to lpSF_851. While both LpSF_847 and LpSF_850 are hypothetical proteins with unknown function, the other 3 proteins have functions related to polysaccharide biosynthesis, including a putative polysaccharide polymerase protein (LpSF_846) and two glycosyltransferases (LpSF_849 and LpSF_851). Analogously, the cps cluster 2 of strain Lp90 also encompassed 4 genes that are specific for that strain, i.e., Lp90_1073 to Lp90_1075 and Lp90_1077. Lp90_1073 is annotated as a hypothetical membrane protein. Moreover, Lp90_1074 is a family 2 glycosyltransferase, Lp90_1075 is a polysaccharide pyruvyl transferase, and Lp90_1077 is a putative mannosyltransferase and thus are possibly involved in polysaccharide biosynthesis.

Thereby, the cps cluster 2 assigned in strain WCFS1 appears to be highly diverse among the different strains of the species L. plantarum, which is corroborated by the observation that the genes specifically present in the cps clusters of strains SF2A35B and Lp90 are not orthologs of each other and each represents the typical cps gene cluster mosaic-like reorganization.

Deletion of the SF2A35B and Lp90 unique cps clusters abolishes the ropy phenotype. In order to investigate whether the
unique cps cluster 2-associated gene sets are responsible for the ropy phenotype in strains SF2A35B and Lp90, the cps clusters containing these genes were deleted using a double crossover gene replacement strategy, resulting in the strains NZ3561BCm (ΔlpSF_837 to ΔlpSF_853 derivative of SF2A35B) and NZ8220Cm (Δlp90_1067 to Δlp90_1077 derivative of Lp90). Both cps cluster mutations led to a loss of the ropy phenotype. Moreover, transmission electron micrographs clearly established that the deletion mutants constructed produce much less surface-associated polysaccharide-like structures (gray net-like substances) around the cells than their parental strains (Fig. 2). Taken together, these data illustrate that the unique cps clusters found in SF2A35B and Lp90 are responsible for the ropy phenotype and encode surface polysaccharide production, both of which are much less apparent in cps cluster mutant derivatives.

Abundance and sugar compositions of EPS are diverse in three L. plantarum strains and their cps deletion mutants produce much less EPS. The abundance and sugar compositions of EPS in the ropy strains, SF2A35B and Lp90, and their cps2-like gene cluster deletion mutants, NZ3561BCm and NZ8220Cm, were further analyzed. The model strain L. plantarum WCFS1 and its cps deletion mutant NZ3550Cm (Δcps1A-3J) were also included as references. First, to confirm the reduction in EPS production in the deletion mutants, the polysaccharides were isolated for quantification and determination of the sugar compositions by high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD). SF2A35B and Lp90 both produce large amounts of EPS, which are approximately 15- and 20-fold higher, respectively, than the amount of EPS isolated from the WCFS1 strain (Table 3). Besides large differences in abundance, the sugar compositions of EPS are also diverse in the 3 L. plantarum strains. The EPS from WCFS1 is composed of a high percentage of glucose, while the EPS from SF2A35B is composed of high percentages of galactose and galactosamine but low percentages of glucose and glucosamine. The EPS from Lp90 consists of similar percentages of glucosamine, galactose, galactosamine, and rhamnose but has a low percentage of glucose (Table 3). Moreover, the EPS isolated from WCFS1 has a molecular mass of >5,500 kDa; the EPS from SF2A35B is about 500 kDa, while the EPS of Lp90 is about 280 kDa, the smallest in molecular mass (Table 3).

The cps deletion mutants of the ropy strains produce much smaller amounts of polysaccharides around the cells. The deletion of the cps2-like cluster in SF2A35B and Lp90 resulted in a >95% reduction in the total amount of EPS, yielding an overall amount of EPS that is comparable to that isolated from the nonropy WCFS1. The reduction in EPS production in the mutants was confirmed by microscopy observations that establish much fewer surface-associated polymer structures in the mutants than in their cognate wild types. Unfortunately, the EPS levels in cps deletion mutants of SF2A35B and Lp90 appeared too low to determine the sugar compositions. The CPS-negative derivative of WCFS1 pro-

**TABLE 3** Sugar compositions of surface polysaccharides isolated from L. plantarum strains and their cps cluster deletion derivatives

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Results for L. plantarum strain:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WCFS1</td>
</tr>
<tr>
<td>Sugar (% of total sugars)</td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>65.8</td>
</tr>
<tr>
<td>Glucosamine</td>
<td>21.7</td>
</tr>
<tr>
<td>Galactose</td>
<td>12.5</td>
</tr>
<tr>
<td>Galactosamine</td>
<td>ND</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>ND</td>
</tr>
<tr>
<td>M_w (thousands)</td>
<td>5,652</td>
</tr>
<tr>
<td>Total polysaccharide isolated (mg/liter)</td>
<td>4.0</td>
</tr>
</tbody>
</table>

*ND, not detected.*

**FIG 2** TEM analysis of L. plantarum strains and their deletion mutant derivatives. L. plantarum strains were negatively stained with 2% uranyl acetate to visualize surface polysaccharides that are visible as the gray net-like substances surrounding the cells and were directly observed using TEM without sectioning. The top panels show the overview (bar, 5 μm), and the bottom panels show higher magnification (bar, 1 μm).
duced an amount of EPS similar to that of the wild type, and the sugar composition percentages between the two strains are also comparable. However, the molecular weight of the cps mutant is reduced by 60%, suggesting the impact of cps deletion in polysaccharide structures (Table 3).

These results clearly establish that the cps2-like clusters in both SF2A35B and Lp90 play a dominant role in EPS production in both strains, and these clusters support a substantially higher polysaccharide production than the related gene cluster in strain WCFS1 (cps2 gene cluster). Variations in abundance, glycan composition, and molecular weight support the typical mosaic-like genetic organization of the cps clusters, which are apparently subject to high-frequency evolutionary adaptation (37).

Impact of the cps2-like gene cluster on surface physicochemical properties. The effect of EPS in relation to properties that may influence the interactions of the bacteria with their environment and host cells (38) was further studied. The influence of deletion of the cps2-like gene cluster in terms of surface charge and hydrophobicity were investigated. The surface charges were assessed by the measurement of the zeta potential, which determines the mobility of cells in an electric field. The SF2A35B strain is the most negatively charged among the wild-type strains tested, whereas the WCFS1 and Lp90 strains have moderate negative surface charges (Fig. 3A). Comparative analysis of the cps deletion mutants and their corresponding wild-type strains revealed quite distinct consequences in the 3 strains. Deletion of cps2-associated functions in SF2A35B led to a significant reduction of the negative cell surface charge. Conversely, deletion of the cps1 to cps3 clusters in WCFS1 led to an increased negative surface charge, whereas the mutation of cps2-associated functions in Lp90 did not significantly affect the surface charge in this strain (Fig. 3A).

Cell surface hydrophobicity is another physicochemical feature of the cell surface that can be readily determined. Although the hydrophobicities of the different L. plantarum strains were somewhat variable, there was no significant difference observed between the 3 wild-type strains tested (Fig. 3B). Mutation of the cps2-like clusters in the 3 genetic backgrounds only elicited a significant effect in surface hydrophobicity in strain SF2A35B, where the deletion of the cps2-associated genes led to a strong increase in surface hydrophobicity (Fig. 3B). Notably, the analogous mutations in the WCFS1 and Lp90 genetic backgrounds did not appear to influence the surface hydrophobicities in a significant manner.

Taken together, these measurements underpin the impact of EPS on the physicochemical surface properties of bacteria. However, the impact of cps mutation on surface charge and hydrophobicity appears to strongly vary between strains.

Impact of the cps2-like gene cluster on adhesion and gastrointestinal survival. Adhesion and colonization are considered important aspects of probiotics for delivering their health-beneficial functions, such as pathogen exclusion and immunomodulation (39, 40). We further investigated the impact of cps deletion on adhesion and biofilm formation of the same set of L. plantarum strains. The adhesive capacity was tested in vitro using the human intestinal cell line Caco-2. Among the 3 L. plantarum strains tested, WCFS1 had higher adhesion to the intestinal cells, while SF2A35B and Lp90 have similar levels of adhesion, which are about 2-fold lower than that of WCFS1 (Fig. 4A). The impact of the cps2-like gene cluster on adhesion was investigated by comparing the cps mutant with the corresponding parental strains. The cps mutant of Lp90, the NZ8820Cm strain, has improved adhesion to Caco-2 compared to that of the Lp90 wild-type strain (Fig. 4A), suggesting that EPS hinders the adhesion of Lp90 to Caco-2 cells. On the other hand, the deletion of cps clusters in the WCFS1 and SF2A35B strains has no significant influence on adhesion (Fig. 4A), which again demonstrates that the impact of the cps gene clusters on adhesion follows a strain-dependent manner.

The ability to survive digestive tract passage is an important characteristic for probiotic bacteria (31, 41). To test whether the reduction in EPS impacts the survival of the SF2A35B and Lp90 strains under GI tract conditions, an in vitro GI survival assay was performed, which was shown to qualitatively reflect the actual in vivo persistence in the human intestine (31). This assay monitors the relative survival of bacteria under conditions that mimic those encountered in the stomach, including acid pH and enzyme exposure, followed by those resembling the small intestine, i.e., neutral pH, exposure to pancreatic digestive enzymes, and bile (31).
Comparative evaluation of the survival characteristics of the strains revealed no difference in GI survival between cps deletion mutants and their parental wild-type (Fig. 4B), suggesting that the reduction of EPS does not alter the survival under in vitro GI stresses. However, the GI survival is significantly different for the 3 wild-type L. plantarum strains (Fig. 4B). Strain SF2A35B (and its corresponding cps mutant) displays the poorest survival among the three strains and is approximately 1,000-fold more sensitive for the stomach-mimicking conditions than WCFS1, which corroborates previous results reported by van Bokhorst-van de Veen et al. (42). The Lp90 strain displays survival characteristics that are very similar, suggesting that the WCFS1 and SF2A35B strains possess MAMPs, which can interact with the 3 receptors. These MAMPs were undistinguishably recognized by TLR2, TLR1_2, and TLR2_6 or the TLR2-, TLR1_2-, and TLR2_6-specific signaling is TLR2 specific (see Fig. S3C in the supplemental material). The effect of cps gene cluster deletions in L. plantarum alters TLR2 signaling in WCFS1 and SF2A35B strains. The effect of cps gene cluster deletions in the WCFS1, SF2A35B, and Lp90 strains on host cell signaling was investigated using an established TLR2 signaling assay (13) that employs HEK-293 reporter cell lines. HEK-293 reporter cells expressing TLR4 were employed as a negative control to illustrate that the signaling is TLR2 specific (see Fig. S3C in the supplemental material). The signaling of TLR1_2 (Fig. 5), TLR2_6, and TLR2 (see Fig. S3A and S3B, respectively) was determined and is displayed as log values of bioluminescence units, and the experiments were performed in triplicate. The results obtained from the 3 TLR reporter cell lines are consistent. The wild-type L. plantarum strains elicited different TLR2-mediated signaling intensities, and strain SF2A35B consistently showed the lowest signaling among the three strains tested (Fig. 5). The cps deletion derivatives of WCFS1 and SF2A35B displayed approximately 3-fold increased TLR1_2 signaling compared to that of their respective wild-type strains (Fig. 5), implying that removal of EPS led to more effective exposure of microorganism-associated molecular patterns (MAMPs), molecules that are recognized by TLR2. Notably, deletion of the cps2-like gene cluster in the SF2A35B strain’s background (strain NZ3561BCm) led to levels of TLR2 signaling resembling those of the WCFS1 strain (Fig. 5). In contrast, cps deletion in the Lp90 strain did not affect TLR2-mediated signaling, and both the wild-type and cps-derivative strains stimulate moderate TLR2 signaling compared to that of the other strains (Fig. 5). The relative NF-κB pathway activations measured in the three TLR2-expressing reporter cell lines (TLR1_2, TLR2_6, and TLR2) were very similar, suggesting that the WCFS1 and SF2A35B strains possess MAMPs, which can interact with the 3 receptors. These MAMPs were undistinguishably recognized by TLR2, TLR1_2, and TLR2_6 or the TLR2-, TLR1_2-, and TLR2_6-specific MAMPs are present in similar amounts in the WCFS1 and SF2A35B strains (Fig. 5; see also Fig. S3 in the supplemental material). Taken together, these results indicate that although both SF2A35B and Lp90 produce large amounts of EPS, their influence

FIG 4 Adhesion to Caco-2 cells (A) and relative survival (B) during an in vitro gastrointestinal assay of L. plantarum strains and the deletion mutant derivatives. Data points for the adhesion are shown, and bars indicate medians. Closed symbols represent wild-type strains, while open symbols represent cps deletion mutants. The survival was measured in 3 independent assays, each using triplicate enumerations for CFU. Data shown are means ± standard deviations. Statistically significant differences between wild-type and corresponding cps deletion mutant, as well as between the three wild-type strains, in adhesion and relative survival were determined by one-way ANOVA followed by Tukey’s multiple comparison test: ***, P ≤ 0.001 (overall ANOVA); ***, P ≤ 0.001; **, P ≤ 0.01; *, P ≤ 0.05.

FIG 5 Effects of L. plantarum strains and the deletion mutant derivatives on TLR1_2 signaling using TLR expressing HEK cell lines containing an NF-κB-responsive luciferase reporter system. Measurements were performed in triplicate and are presented as log luminescence units, individually displayed (n = 3); the bars indicate medians. PBS serves as the negative control, and Pam3CysSK4 is the positive stimulant of TLR1_2. Data comparison of the wild-type strains and their corresponding cps deletion derivatives as well as the 3 wild-type strains were tested for significant differences using one-way ANOVA followed by Tukey’s multiple comparison correction and significant differences are indicated: ***, P ≤ 0.001; *, P ≤ 0.05.
on human TLR2 signaling is not the same, suggesting that the possible TLR2 ligand-shielding effect of EPS is not generic and appears to be strain or polysaccharide specific. In addition, the impact of cps gene cluster deletions on general immune responses was also investigated by determining cytokine production by human peripheral blood mononuclear cells (PBMCs) following stimulation with the different bacterial strains. These experiments also illustrate the strain-dependent impact of EPS removal on the immunomodulation of different \textit{L. plantarum} strains (see Fig. S4 in the supplemental material). However, the substantial variations of these responses measured in PBMCs from individual donors do not allow us to draw very firm conclusions concerning the precise immunomodulatory impact of the EPS molecules in these strains that go beyond the generic conclusion that the impact of EPS removal appears highly strain specific.

\section*{DISCUSSION}

Throughout the studies of the mechanisms underlying probiotic activity, it became apparent that the probiotic effects are often strain specific (43–46). This situation has led more researchers to focus on the molecular characteristics of probiotic strains, intending to link specific molecular structures to specific probiotic functions and thereby deduce the mechanisms of the molecular communication of probiotics. Molecules on the bacterial cell surface, such as peptidoglycan, teichoic acids, proteins, and EPS not only play important roles in bacterial physiology where they are important to maintain cell integrity and morphology but also are considered to be key players in the interaction with the host mucosa through pattern recognition receptors (PRRs) expressed by the host cells (46–48). These molecules are commonly present in Gram-positive bacteria, yet their structural properties can be substantially diverse between species and also between strains of the same species, raising the basic question: to what extent can these structural differences influence the interaction between these bacteria and the host cells. To date, this question remains largely unanswered, and the majority of our working models of host-microbe molecular interaction are built on the basis of research performed with exemplary strains of a species rather than with consideration of the diversity among strains as an important factor of variation.

EPS has been studied to a much lesser extent in probiotic bacteria such as lactobacilli. Although the EPS production, composition, genes involved in EPS biosynthesis, and biological functions of EPS have been reported, so far less attention has been paid to EPS as a determinant for the strain-specific features of probiotics. A previous study has investigated the impact of deletion of individual or multiple \textit{cps} clusters in \textit{L. plantarum} strain WCFS1 on EPS sugar composition, transcriptome, and TLR2 signaling (13). In this work, we have expanded the investigation to 3 \textit{L. plantarum} strains and to different features related to probiotic-host interactions. The probiotic function is conveyed through probiotic-host interactions, which are affected by many factors, including bacterial surface physicochemical (charge and hydrophobicity), adhesion and GI survival, and immunomodulatory properties. The surface charges of Gram-positive bacteria arise from various components, e.g., ionized phosphate, amino, and carboxylate groups of surface polymers, mainly teichoic acids but also proteins and polysaccharides (49). Notably, our sugar composition analysis is not designed to detect the presence of charged glycans, such as glucuronic and galacturonic acids, in EPS isolated from the 3 \textit{L. plantarum} strains (Table 3), suggesting that the effect of EPS on surface charges may result in more exposure of other charged molecules, most likely teichoic acids, on the bacterial cell surface, which may be reflected in the increased zeta potential measurement observed in the mutant relative to that in the parental strain (Fig. 3A). Additionally, the deletion of the \textit{cps} gene clusters in strain WCFS1 has previously been shown to have global effects on gene expression in this strain, including altered expression levels of genes encoding extracellular proteins and teichoic acid decarboxylation (13). These transcriptional changes may alter surface molecules and thereby contribute to the changes in the surface charges. These pleiotropic changes may also contribute to the impact of EPS on surface hydrophobicity. As for EPS, other surface molecules can also differ between strains. For example, six different structures of wall teichoic acids have been reported among \textit{L. plantarum} strains (50). The combination of differences in different surface molecules may determine the strain-specific impact of EPS on surface properties.

Adhesion is one of the factors proposed to be involved in host interaction and in the antagonistic effects of gastrointestinal pathogens against adhesion (48, 51). There are conflicting data in the literature on whether there is a correlation between the ability to adhere to host mucosal tissues and surface hydrophobicity (52, 53) or not (54, 55). In our experiments, we did not correlate hydrophobicity and cell adhesion, e.g., \textit{cps} deletion in SF2A35B increased hydrophobicity but did not affect adhesion to Caco-2 cells (Fig. 3B and 4A). Moreover, \textit{cps} deletion in strain Lp90 did not have a significant impact on hydrophobicity and, if anything, appeared to slightly reduce it in this strain background, whereas it drastically increased the capacity of the strain to adhere to Caco-2 cells (Fig. 3B and 4A). The latter finding may imply that adhesion to Caco-2 cells of strain Lp90 is mediated by a specific ligand-receptor interaction rather than by generic hydrophobic interactions and that the ligand–receptor interactions are shielded or prevented by the large amounts of surface polysaccharides produced in this strain. Further studies are required to confirm these specific ligand-receptor interactions between the Lp90 strain and epithelial cells, which may reveal specific adhesion factors that are of interest in the field of host-microbe interactions.

Immunomodulation is one important mechanism underlying the proposed health-beneficial effects of probiotic bacteria (56, 57). EPS have been suggested to affect the immune responses of lactobacilli. For example, the EPS-deficient mutant of \textit{L. casei} Shiroti stimulates more proinflammatory cytokine production in mouse macrophages and spleen cells than in the parental strain (18). Ghandimi et al. also suggested that the presence of genes to synthesize polysaccharide might potentially modulate the immunomodulatory property of \textit{Lactobacillus fermentum} strains toward Th1-inducing cytokine secretion in PBMCs, while the strain lacks the genes that lead to Th1/Th2-suppressing cytokine production (19). Interestingly, \textit{L. plantarum} strain SF2A35B was shown previously to elicit very limited immune responses in PBMCs and blood monocyte-derived dendritic cells (34, 58). The observation that this phenotypic trait is combined with the strain’s ropy phenotype might imply that the high level of polysaccharide production by this strain impacts its immunomodulatory capacity, e.g., by shielding specific cell envelope-associated signaling molecules and thereby suppressing its immunomodulatory stimulation. The shielding effect of EPS has been reported also in \textit{L. plantarum} WCFS1 (13) and \textit{L. rhamnosus} GG (21); we confirmed this obser-
viation in WCFS1 with the cps deletion mutant, NZ3550Cm, which stimulates higher TLR2 signaling than the wild-type strain (Fig. 5). The data presented here appear to be at least partially in agreement with this proposed role of EPS, in the sense that the SF2A35B strain stimulates low TLR2 signaling (Fig. 5) and induces the production of small amounts of IL-10 and IL-12 in PBMCs compared to the WCFS1 strain (see Fig. S5A and B, respectively, in the supplemental material) (34). One of these earlier studies also reported induction of small amounts of IL-10 and IL-12 in PBMCs stimulated with SF2A35B compared to that in those stimulated by other L. plantarum strains, including WCFS1 (34), which has been suggested as a marker indicative of the prediction of in vivo inflammation-suppressive effects of different probiotics in a mouse colitis model (39). However, the PBMC stimulation data obtained here do not corroborate this finding, since the IL-10/IL-12 ratio elicited in PBMCs was not significantly different after stimulation with either WCFS1 or SF2A35B, which is in agreement with the results in a previous study (34). In addition, the higher TLR2-elicited in PBMCs was not significantly different after stimulation with either WCFS1 or SF2A35B, which is in agreement with the stimulation data obtained here appear to be at least partially in agreement with this proposed role of EPS, in the sense that the IL-10/IL-12 ratio elicited in PBMCs was not significantly different after stimulation with either WCFS1 or SF2A35B, which is in agreement with the results in a previous study (34). In addition, the higher TLR2-

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We thank Michiel Wels and Luigi Orrù for the annotation of the cps gene clusters of the SF2A35B and Lp90 strains, respectively, and Mariya Tarazanova for technical assistance for the zeta potential and hydrophobicity measurements. We also thank Guido Staring (NIZO Food Research, Ede, the Netherlands) for sugar composition analysis.

ACKNOWLEDGMENTS

We thank Michiel Wels and Luigi Orrù for the annotation of the cps gene clusters of the SF2A35B and Lp90 strains, respectively, and Mariya Tarazanova for technical assistance for the zeta potential and hydrophobicity measurements. We also thank Guido Staring (NIZO Food Research, Ede, the Netherlands) for sugar composition analysis.
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