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1 **A fast, efficient and easy to implement method to purify bacterial**  
2 **pili from *Lactocaseibacillus rhamnosus* GG based on multimodal**  
3 **chromatography**

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11 **Keywords:** *Lactocaseibacillus rhamnosus* GG, SpaCBA pili, purification, multimodal  
12 **chromatography, light scattering, atomic force microscopy.**

13 **Abstract**

14 Pili are polymeric proteins located at the cell surface of bacteria. These filamentous proteins  
15 play a pivotal role in bacterial adhesion with the surrounding environment. They are found both  
16 in Gram-negative and Gram-positive bacteria but differ in their structural organization.  
17 Purifying these high molecular weight proteins is challenging and has certainly slowed down  
18 their characterization. Here, we propose a chromatography-based protocol, mainly relying on  
19 multimodal chromatography (core bead technology using Capto Core 700 resin), to purify  
20 sortase-dependent SpaCBA pili from the probiotic strain *Lactocaseibacillus rhamnosus* GG  
21 (LGG). Contrary to previously published methods, this purification protocol does not require  
22 specific antibodies nor complex laboratory equipment, including for the multimodal  
23 chromatography step, and provides high degree of protein purity. No other proteins were  
24 detectable by SDS-PAGE and the 260/280 nm ratio (~0.6) of the UV spectrum confirmed the  
25 absence of any other co-purified macromolecules. One can obtain ~50 µg of purified pili,  
26 starting from 1 L culture at OD<sub>600nm</sub> ≈ 1, in 2-3 working days. This simple protocol could be  
27 useful to numerous laboratories to purify pili from LGG easily. Therefore, the present work  
28 should boost specific studies dedicated to LGG SpaCBA pili and the characterization of the  
29 interactions occurring with their protein partners at the molecular level. Moreover, this

30 straightforward purification process might be extended to the purification of sortase-dependant  
31 pili from other Gram-positive bacteria.

## 32 **1. Introduction**

33 Pili, or fimbriae, are length-variable proteinaceous appendages found at the surface of bacteria  
34 and archaea where they play a key role in interaction and/or adhesion with the surrounding  
35 environment (Berne et al., 2018). These proteins exhibit a high structural organization diversity  
36 (Proft and Baker, 2008). Some types of pili are found in both Gram-negative and Gram-positive  
37 bacteria and archaea, such as type IV pili (Hospenthal et al., 2017; Wang et al., 2019). Other  
38 pili are exclusive to Gram-negative bacteria, such as chaperone usher, and others are specific  
39 to Gram-positive bacteria (Hospenthal et al., 2017). Indeed, Gram-positive bacteria possess pili  
40 called sortase-dependent pili, named after the transmembrane enzyme catalyzing the reaction  
41 of polymerization. These pili are composed of three polymerized subunits, called pilin,  
42 covalently bound to each other, contrary to the other types of aforementioned pili. A single  
43 basal pilin allows for attachment to the cell wall, a major pilin is found in multiple copies which  
44 drives the length of the pilus, and a tip pilin located at the end of the pilus is responsible for  
45 interactions with the environment (Krishnan, 2015). Polymerization occurs through the reaction  
46 of a peculiar Lys of a pilin motif (exclusive to the basal and the major pilins) and a specific Thr  
47 found in the LPXTG motif which is common to the three pilins (Krishnan, 2015; von Ossowski,  
48 2017). Remarkably, this kind of pili from *Corynebacterium diphtheriae* has successfully been  
49 rebuilt *in vitro* (Chang et al., 2018) and the reaction occurring between the pilin and the LPXTG  
50 motifs was exploited for protein labelling (McConnell et al., 2018). Sortase-dependent pili were  
51 thought to be exclusive to pathogens, but in the late 2000s, they were observed by transmission  
52 electron microscopy (Kankainen et al., 2009) on the non-pathogenic probiotic bacterium  
53 *Lactobacillus rhamnosus* (LGG) (Capurso, 2019), which was recently reclassified as  
54 *Lacticaseibacillus rhamnosus* GG (Zheng et al., 2020). When overexpressed, SpaCBA pili have  
55 also been detected on a *Lactococcus lactis* strain (Tarazonova et al., 2016). LGG SpaCBA pili  
56 can reach up to 1  $\mu\text{m}$  in length and 10 to 50 copies are found per cell (Kankainen et al., 2009).  
57 They are composed of a basal pilin (SpaB, ~20 kDa), a major pilin (SpaA, ~30 kDa) and a tip  
58 pilin (SpaC, ~90 kDa), whose genes are clustered (Kankainen et al., 2009). The estimated  
59 molecular weight of a single pilus is 1 to 3 MDa, depending on its length. These pilins likely  
60 self-organize according to a the classical way described above to form a functional pilus (von  
61 Ossowski, 2017; Kant et al., 2020). A model of LGG pilus where SpaC is found all along the  
62 pilus was proposed following immunogold labeling and visualization of pilus structure by

63 electron microscopy (Reunanen et al., 2012). This hypothetical model might be erroneous since  
64 polyclonal antibodies might have recognized structural motifs shared by the pilins (von  
65 Ossowski, 2017), *i.e.* the Ig-like fold made of seven  $\beta$ -sheets, of the CnaB domains found in the  
66 three pilins. A recent study appears to remove this ambiguity since when monoclonal antibodies  
67 were used, SpaC was only found at the tip of the pilus according to immunogold labelling in  
68 transmission electron microscopy experiments (Kant et al., 2020). Moreover, SpaCBA pili are  
69 thought to be glycosylated through SpaC, with fucose and mannose residues (Tytgat et al.,  
70 2016). Nonetheless, SpaC harbors a structural homology with a lectin specific to mannose  
71 residues and might also contain some properties specific to fucose binding lectin (Kant et al.,  
72 2020). Therefore, the actual glycosylation of SpaC and/or its lectin capacity still need further  
73 investigation. Interestingly, chemically-induced derivatives of LGG (non-GMO) have been  
74 generated in order to improve adhesion and the associated putative probiotic role through  
75 SpaCBA pili (Rasinkangas et al., 2020).

76 Most studies done on LGG have aimed to decipher the interaction with molecules found in the  
77 gastro-intestinal tract (von Ossowski et al., 2010; Lebeer et al., 2012), where bacteria can exert  
78 their probiotic action. These properties include preventing pathogen adhesion, producing  
79 antimicrobial compounds (*e.g.* bacteriocins), fighting for nutrients, improving the epithelial  
80 barrier through mucin production, and modulating the immune response (Suez et al., 2019).  
81 Over the last decade, the use of probiotics has attracted increasing interest among consumers,  
82 whether consumed within food or as a dietary supplement. This keen interest has increased  
83 incessantly, with the ensuing pros and cons (Suez et al., 2019). One major concern when used  
84 as probiotic is to determine whether pili remain intact after industrial processes including spray-  
85 drying and the subsequent storage (Agudelo et al., 2017; Broeckx et al., 2017; Guerin et al.,  
86 2017; Gomand et al., 2019; Kiekens et al., 2019). One solution to protect bacterial pili during  
87 industrial processes consists to of encapsulating them in a dairy product-based matrix including  
88 whey proteins. This has given rise to recent studies focusing on the interaction of LGG with  
89 dairy components (Guerin et al., 2018a, 2018b; Dos Santos Morais et al., 2020). It was  
90 demonstrated that SpaCBA pilus is capable of establishing interactions with  $\beta$ -lactoglobulin,  
91 while no interactions have been detected with other whey proteins (BSA and  $\alpha$ -lactalbumin) or  
92 with caseins (Burgain et al., 2014; Guerin et al., 2016). A divide-and-conquer approach is often  
93 employed to determine the role of each pilin subunit requiring the production of recombinant  
94 proteins (von Ossowski et al., 2010). *Via* this approach, structural characterizations of SpaCBA  
95 pilus subunits through X-ray crystallography (XRC) aiming to determine the structural

96 organization are ongoing. Indeed, the XRC structure of SpaA has been made available  
97 (Chaurasia et al., 2016), such as that of SpaC (Kant et al., 2020), and efforts are currently being  
98 made to solve the structure of SpaB (Kumar Megta et al., 2019). For a better characterization  
99 of the pili function, it is essential to obtain them in a native state to take into account the  
100 potential effect of adjacent pilins, whatever the context of the gastro-intestinal tract molecules  
101 or food components.

102 Only few publications report the purification of native SpaCBA pili from LGG and it sometimes  
103 requires the use of specific antibodies that are non-commercially available (Reunanen et al.,  
104 2012). Others describe a centrifuge-based simple protocol to purify these pili (Tripathi et al.,  
105 2012), but exopolysaccharides (EPS) were likely co-purified during this procedure. Although  
106 the above protocol was improved by adding a size exclusion chromatography step, the required  
107 equipment is specific and costly (Tytgat et al., 2016). In the present study, an alternative  
108 protocol mainly based on multimodal chromatography (MMC, Capto Core 700) was developed.  
109 This technology combines hydrophobic interaction, ion exchange and size exclusion  
110 chromatographies, and was initially used to purify viruses (Blom et al., 2014), but it can be  
111 extended to very large molecules including pili from LGG. Furthermore, this method is  
112 straightforward and requires minimal laboratory equipment. Once purified, pili were  
113 characterized by mass spectrometry, size exclusion chromatography coupled to triple detection  
114 array (SEC-TDA) and successfully imaged by atomic force microscopy (AFM).

## 115 **2. Material and Methods**

### 116 **2.1. Bacterial culture**

#### 117 **2.1.1. Bacterial strains and pre-culture**

118 The model strain *Lacticaseibacillus rhamnosus* GG (ATCC53103) (LGG WT) and pili  
119 defective derivative mutant LGG  $\Delta spaCBA::Tc^R$  (CMPG 5357, LGG  $\Delta spaCBA$ ) (Lebeer et al.,  
120 2012) were used in this study. A bacterial pre-culture was prepared by adding 500  $\mu$ L of  
121 bacterial glycerol stock (stored at  $-80$  °C) into 50 mL ( $\sim 1/100^\circ$ ) of MRS medium (Biokar) and  
122 placed overnight at  $37$  °C without agitation.

#### 123 **2.1.2. Static culture**

124 LGG WT or LGG  $\Delta spaCBA$  culture was prepared by seeding approximately 1 L of fresh MRS  
125 with the pre-culture ( $\sim 1/50^\circ$ ) and was incubated at  $37$  °C without agitation until the  $OD_{600nm}$   
126 reached  $\sim 1-1.2$  (4-5 h). Bacteria were harvested by centrifugation (3000 g, 10 min,  $20$  °C). The

127 pellets were washed with PBS pH 6.8 (Sigma) and the suspended cells were centrifuged again  
128 (3000 g, 10 min, 20 °C).

### 129 **2.1.3. Bioreactor culture**

130 To increase the biomass and thereby the quantity of pili, LGG WT was grown in a bioreactor  
131 under controlled pH (6.8, adjusted with 2 M NaOH) and temperature (37 °C) under gentle  
132 homogenization. Five liters of MRS were seeded with 150 mL of pre-culture (cf. §2.1.1).  
133 Bacterial growth was monitored by measuring OD<sub>600nm</sub> every hour and by monitoring NaOH  
134 consumption. Cells were harvested by centrifugation (3000 g, 10 min, 20 °C) in the late  
135 exponential phase when OD<sub>600nm</sub> ~ 6-7 (~5 h). They were washed with PBS pH 6.8 (Sigma),  
136 then centrifuged again (3000 g, 10 min, 20 °C).

## 137 **2.2. Protein purification**

### 138 **2.2.1. Cell-wall digestion**

139 For clarity, figure 1 shows a diagram summarizing the extraction and purification protocols.  
140 Washed pellets were suspended in digestion buffer (50 mM Tris pH 6.8, 150 mM NaCl, 2 mM  
141 MgCl<sub>2</sub>, 20% sucrose) supplemented with mutanolysin (50-100 U/mL). The cell wall was  
142 digested overnight at 37 °C under gentle agitation to avoid cell lysis. The measured pH after  
143 enzymatic digestion was 4.2 and was adjusted to 6.8 with 500 mM Tris pH 6.8. The suspension  
144 was supplemented with 5 U/mL of DNase (C-LEcta) to digest potentially released nucleic  
145 acid material in case of eventual cell lysis, and centrifuged (7200 g, 30 min, 20 °C). The  
146 supernatant, containing SpaCBA pili, was carefully collected, diluted in 25 mM Tris pH 6.8,  
147 and filtered through 0.45 µm PES (Polyethersulphone) filter.

### 148 **2.2.2. Diafiltration**

149 All diafiltration steps were performed with Amicon ultra-15 concentrators (100 MWCO,  
150 Millipore) or Amicon stirred-cell (200 mL, 100 MWCO, Millipore) under N<sub>2</sub> pressure (2.5 bar)  
151 to handle larger volumes. Buffer exchanges differed according to the following purification  
152 step.

### 153 **2.2.3. Chromatography**

154 All subsequent chromatography steps were performed with an ÄKTA Start liquid  
155 chromatography system (GE Healthcare).

#### 156 **2.2.3.1. Multimodal chromatography (MMC)**

157 The clarified solution was loaded at 2 mL/min on Capto Core 700 columns (GE Healthcare)  
158 mounted in series (5x 1 mL HiTrap column and 1x 4.7 mL HiScreen column) equilibrated with  
159 25 mM Tris pH 6.8. The flow-through was saved, then the columns were washed with the same  
160 buffer until the Abs<sub>280nm</sub> returned to the initial baseline. The columns were regenerated with 1M  
161 NaOH, 30% isopropanol (cleaning in place or CIP) to desorb captured molecules.

#### 162 **2.2.3.2. Size exclusion chromatography (SEC)**

163 Concentrated fractions (3-5 mL) from the flow-through of the MMC step were filtered through  
164 a 0.22 µm PES filter prior to injection onto a Sephacryl HR 400 column (void volume ~40 mL,  
165 total volume ~120 mL, GE Healthcare) equilibrated with PBS (pH 6.8) buffer at 0.8 mL/min.

#### 166 **2.2.3.3. Ion exchange chromatography (IEX)**

167 The diafiltered solution from MMC was injected onto strong anion exchange columns (2 x 5  
168 mL HiTrap Q HP, GE Healthcare) at 5 mL/min, previously equilibrated with 25 mM Tris pH  
169 6.8 (buffer A). Then the columns were washed with buffer A until the Abs<sub>280nm</sub> returned to the  
170 initial baseline. Captured molecules were eluted with a linear gradient at 2 mL/min reaching  
171 100% 25 mM Tris pH 6.8, 1M NaCl (buffer B) in 50 mL.

### 172 **2.3. SDS-PAGE analysis and protein quantification**

173 Aliquots of each step of purification were collected and acetone-precipitated if required.  
174 Purification steps were analyzed by SDS-PAGE (precast 4-15% or 4-20% gradient gel, Biorad)  
175 in reducing condition (50 mM dithiothreitol, DTT) and stained with Coomassie Blue (Instant  
176 Blue, Expedon). Protein concentration was estimated spectrometrically (Nanodrop 2000c,  
177 Thermo Scientific) considering the SpaA molar extinction coefficient (39,880 M<sup>-1</sup>.cm<sup>-1</sup>) and  
178 MW (29.2 kDa) without the signal peptide and the C-term part after the <sup>301</sup>LPXT<sup>304</sup>G motif  
179 (<sup>34</sup>DTN...LPHT<sup>304</sup>). Protein purity towards other types of macromolecules was estimated  
180 considering the 260/280 ratio and visual inspection of the UV-spectrum.

### 181 **2.4. Mass spectrometry**

182 Two different setups were employed in this study: LC-MALDI and LC-ESI.

#### 183 **2.4.1. LC-MALDI**

##### 184 **2.4.1.1. Peptide extraction**

185 Gel bands from SDS-PAGE analysis were processed by successive washes at 20 °C under  
186 agitation in a 50 µL volume. For cysteine reduction and alkylation, bands were incubated in  
187 100 mM ammonium bicarbonate (AB), then in AB containing 50 mM DTT for 45 min, washed  
188 once in AB, and finally incubated in AB containing 50 mM IAA (iodoacetamide) for 45 min.  
189 Bands were washed through two cycles as follows: 15 min in AB/ACN (acetonitrile), 1:1, 15  
190 min in AB. Finally, they were dehydrated twice in ACN and dried in a vacuum concentrator for  
191 one hour. Bands were digested with 50 ng trypsin (sequencing grade, Promega) overnight in 7  
192 µL AB/water, 1/1. The next day, the peptides were extracted twice in 10 µL ACN, 80%, TFA  
193 1% for 7 min under sonication. This procedure was done twice. Peptide extracts were pooled  
194 and dried in a vacuum concentrator, resuspended in 10 µl 2% ACN, 0.1% TFA and processed  
195 for fractionation by nanoHPLC.

196 NanoHPLC was carried out with a UltiMate 3000 system (Thermo Scientific) equipped with a  
197 20 µL sample loop, a pepMap 100 C18 desalting precolumn (Dionex) and a 15 cm pepMap  
198 RSLC C18 fractionation column (Dionex). Samples (5 µL) were injected using the µL pickup  
199 mode and eluted by a 2 to 45% ACN gradient over 30 min at 300 nl/min. Fractions (170, 9  
200 seconds each) were collected on a ProteinerFcII (Bruker) over 25.5 min and eluates were  
201 directly mixed on MTP-1536 TF target (Bruker) spots to  $\alpha$ -cyano-4-hydroxycinnamic acid  
202 (Bruker). LC-MALDI runs were processed using dedicated automatic methods piloted by  
203 WARP-LC software on an Autoflex speed MALDI-TOF/TOF mass spectrometer (Bruker) in  
204 the 800-3,500 mass range, using next-neighbor external calibration for all MALDI spots, using  
205 2000 random laser shots per spot at a 2000 Hz frequency. Masses detected with S/N above 20  
206 were selected for TOF/TOF fragmentation in LIFT mode.

207 Peptide assignments were performed from TOF/TOF spectra by Mascot Server interrogation  
208 (v2.4.1, Matrix Science) piloted and compiled by Proteinscape. The database search parameters  
209 were as follows: mass tolerance for precursors = 50 ppm; mass tolerance for fragments = 0.8  
210 Da; enzyme = trypsin with one missed cleavage allowed; protein modifications =  
211 carbamidomethylation of cysteines (variable) and oxidation of methionines (variable). The  
212 specific database was (Uniprot proteome UP000000955, 2019/06/28, 2877 sequences,  
213 (Kankainen et al., 2009)). Proteins were considered as identified when at least two peptides  
214 passed the Mascot score with  $p < 0.05$  threshold.

#### 215 **2.4.2. LC-ESI**



216 Gel pieces from SDS-PAGE were excised and cut into small cubes and processed with the in-  
217 gel digestion kit (Thermo Scientific). Briefly, the gels pieces were destained with acetonitrile  
218 30%, reduced with TCEP (tris(2-carboxyethyl)phosphine), alkylated with IAA and digested  
219 with trypsin overnight at 37 °C. The peptides were extracted according to a previously  
220 published method (Lavigne et al., 2012).

221 Peptides were analyzed on a Vanquish quaternary UHPLC system (Thermo Scientific) in-line  
222 with a photodiode array detector (PDA) and an Orbitrap ID-X Tribrid mass spectrometer  
223 (Thermo Scientific) equipped with an atmospheric pressure ionization interface operating in  
224 electrospray mode (ESI). Sixteen microliters of peptides were separated on an Acclaim 120  
225 C18 column (100 mm \* 2.1 mm – 2.2 µm, Thermo Scientific) maintained at 30 °C. The flow  
226 rate was set at 200 µl/min and mobile phases consisted in water modified with formic acid  
227 (0.1%) for A and acetonitrile modified with formic acid (0.1%) for B. Peptides were eluted  
228 using a gradient step of 5% to 95% B for 40 min, then an isocratic step was applied at 95% B  
229 for 10 min to wash the column, before returning to the initial composition of 5% B for 5 min to  
230 realize the equilibrium. Mass analysis was carried out in ESI positive ion mode (ESI+) and  
231 mass spectrometry conditions were as follows: spray voltage was set at 3.5 kV; source gases  
232 were set (in arbitrary units/min) for sheath gas, auxiliary gas and sweep gas at 35, 7 and 0,  
233 respectively; vaporizer temperature and ion transfer tube temperature were both set at 300 °C.  
234 Survey scans of peptide precursors from 150 to 2,000 m/z were performed at 60 K resolution  
235 (full width of the peak at its half maximum, fwhm, at 200 m/z) with MS parameters as follows:  
236 RF-lens, 35%; maximum injection time, 50 ms; data type, profile; internal mass calibration  
237 EASY-IC™ activated; custom AGC target; normalized AGC target: 25%. A top speed (0.6  
238 s) data-dependent MS2 was performed by isolation at 1.5 Th with the quadrupole, HCD  
239 fragmentation with a stepped collision energy (25, 35 and 50) and MS analysis in the Orbitrap  
240 at 15 K resolution (high resolution MS/MS analysis). Only the precursors with intensities above  
241 the threshold of 2.104 were sampled for MS2. The dynamic exclusion duration was set to 2.5 s  
242 with a 10 ppm tolerance around the selected precursor (isotopes excluded). Other MS2  
243 parameters were as follows: data type, profile; custom AGC target; normalized AGC target:  
244 20%. Mass spectrometer calibration was performed using the Pierce FlexMix calibration  
245 solution (Thermo Scientific). MS data acquisition was carried out utilizing the Xcalibur v. 3.0  
246 software (Thermo Scientific).

247 **2.5. LC-MSMS data were processed and analyzed with Mascot Distiller (v2.7, Matrix**  
248 **Science) and Mascot Server (v2.7, Matrix Science). The database search**

249 parameters were as follows: mass tolerance for precursors = 10 ppm; mass  
250 tolerance for fragments = 20 ppm; enzyme = trypsin with two missed cleavages  
251 allowed; protein modifications = carbamidomethylation of cysteines (fixed) and  
252 oxidation of methionines (variable). The databases were (#1: Contaminants  
253 2016/01/29, 247 sequences, #2: SwissProt 2019/11, 561 568 sequences, and #3:  
254 Uniprot\_LGG\_UP000000955, 2020/02/25, 2877 sequences, (Kankainen et al.,  
255 2009)). Proteins were considered as identified when at least two peptides passed  
256 the Mascot score with  $p < 0.01$  threshold scores. **Dynamic light scattering (DLS)**

257 The polydispersity and the hydrodynamic radius ( $R_h$ ) of the pili were estimated by DLS using  
258 a Zetasizer instrument (Nano ZS, Malvern Panalytical). The sample was filtered through a 0.22  
259  $\mu\text{m}$  PES-filter just before analysis. Measurements were performed at 20 °C in PBS buffer (pH  
260 7.4) using a quartz cell. Data were processed with the Zetasizer software (v7.13, Malvern  
261 Panalytical) with default parameters. The size distribution by intensity was obtained from 6  
262 successive experiments to ensure sample stability.

## 263 **2.6. Size-exclusion chromatography coupled to triple detection array (SEC-TDA)**

264 SEC experiments were performed with a HPLC pump (LC10AD, Shimadzu) coupled to an  
265 autosampler (Viscotek VE 2001, Malvern Panalytical) and a multi-detector system recording  
266 light scattering (RALS/LALS,) intrinsic viscosity and refractive index signals (Viscotek  
267 TDA305, Malvern Panalytical). The HPLC SEC column (BioSec5 500 Å, 5  $\mu\text{m}$ , 7.8 mm ID x  
268 300 mm, void volume ~6 mL, total volume ~ 12.5 mL, Agilent) was equipped with a post-  
269 column nylon filter (0.22  $\mu\text{m}$ ). The column was equilibrated with PBS (pH 6.8) supplemented  
270 with sodium azide 0.02%. The flow rate and the temperature were 0.35 mL/min and 30 °C  
271 respectively. Data were processed with the Omniseq software (v5.12, Malvern Panalytical). The  
272 calibration procedure was done with BSA (Sigma) and cross-validation was performed with  $\beta$ -  
273 lactoglobulin (Sigma). The refractometer was used as the concentration detector and the  
274 refractive index increment value ( $dn/dc$ ) used to determine the molecular weight was  
275 0.185 mL/g. Samples were diafiltered in the aforementioned buffer and filtered through a 0.22  
276  $\mu\text{m}$  PES-filter just before injection.

## 277 **2.7. Atomic Force Microscopy (AFM) imaging.**

278 Two hundred microliters of LGG WT cell suspension cultivated as in §2.1.1 in MRS or two  
279 hundred microliters of purified pili (diluted at 50 mg/L and 2.5 mg/L) in 25 mM Tris pH 6.8,  
280 150 mM NaCl were deposited on freshly cleaved mica substrates and allowed to settle for 2 h.

281 Then the surfaces were gently rinsed by immersion in 3 baths of ultrapure water and dried  
282 overnight at 30 °C before imaging. Images were obtained in peak force tapping mode with a  
283 Bioscope Resolve AFM (Bruker corporation, Santa Barbara, CA), using SNL cantilevers  
284 (Bruker corporation, nominal spring constant of  $\sim 0.24 \text{ N.m}^{-1}$ ) and with a maximum applied  
285 force of 5 nN.

### 286 **3. Results and Discussion**

#### 287 **3.1. Purification protocols available in the literature**

288 The literature was investigated to determine available protocols to purify pili from LGG. First,  
289 (Tripathi et al., 2012) developed a simple centrifuged-based protocol. The bacterial culture was  
290 subjected to two successive centrifugation steps, one at 8000 g to remove pili from bacteria due  
291 to shear stress and a second one at 20000 g to pellet them. The authors characterized the pili by  
292 AFM and admitted that exopolysaccharides (EPS) could have co-precipitate during the 20000  
293 g centrifuge step, meaning that EPS contaminants could interfere with other techniques.  
294 Moreover, other cell wall proteins (CWPs) could have been released during the centrifugation  
295 steps and probably altered the pili purity even if they were not visible by AFM, due to their  
296 relative small sizes.

297 In the study by (Tytgat et al., 2016) LGG was grown in an industrial whey permeate, as  
298 previously done (Laakso et al., 2011), and cells were concentrated by microfiltration. Cells were  
299 then centrifuged at 20000 g, and the authors stated that the pili were found in the supernatant  
300 instead of the pellet, contrary to (Tripathi et al., 2012). Then they added a second step of  
301 purification consisting of a size exclusion chromatography step. Pili were presumably found in  
302 the void volume of the column. In this case, EPS might have co-eluted with the pili during this  
303 step and SEC requires specific equipment.

304 Instead of simply centrifuging bacterial cells, (Reunanen et al., 2012) digested the cell-wall  
305 with mutanolysin and lysozyme in order to release CWPs including pili. Then CWPs were  
306 injected onto a column functionalized with polyclonal antibodies anti-SpaCBA, allowing them  
307 to remove contaminants. It is worth noting that to destabilize the specific antibody-antigen  
308 bound, it is necessary to elute the bound proteins at low pH ( $\sim 3$ ). This could damage the native  
309 state of the pili even if pH was neutralized directly in collection tube. Although efficient, this  
310 protocol requires the use of specific antibodies that are not commercially available. Moreover,  
311 the intermediates of polymerization were still present in the purified fractions, as shown by  
312 western blots.

### 313           **3.2. Extraction of SpaCBA pili via enzymatic digestion**

314   In order to purify the pili, the simplest protocol available (*i.e.* the centrifuge-based protocol)  
315   developed by (Tripathi et al., 2012) and improved by (Tytgat et al., 2016) was tested.  
316   Unfortunately, for us it was not possible to extract any detectable CWPs in the supernatant or  
317   in the pellet of each centrifuge steps based on Coomassie blue staining after SDS-PAGE  
318   analysis. The same result was obtained after resuspending the cells vigorously with a vortex  
319   during 1 min before the 8000 g centrifuge step (Figure S1A).

320   As a second option, it was decided to employ a double enzymatic digestion of the CW with  
321   mutanolysin and lysozyme, as done by (Reunanen et al., 2012) (Figure S1B). Mutanolysin  
322   being quite expensive, the enzyme concentration was lowered to 50-100 U/mL and the digestion  
323   time increased from 2 h to overnight (~12 h) under gentle agitation to avoid protoplast lysis.  
324   Later, only mutanolysin was employed as it was not necessary to use lysozyme to obtain a  
325   satisfactory CW digestion (data not shown). As expected, the enzymatic digestion of LGG CW  
326   released CWPs, as previously done to purify pili from *Streptococcus pneumoniae*  
327   (Hilleringmann et al., 2008).

328   For the development of the protocol of purification, LGG WT was cultivated in MRS medium  
329   in static condition under uncontrolled pH, and LGG  $\Delta spaCBA$  was used as a negative control.  
330   The difference in migration in SDS-PAGE, only visible at the top of the gel, clearly indicated  
331   the absence of pili on LGG  $\Delta spaCBA$  (Figure 2A). This approach would allow for a rapid  
332   identification of pili at the protein level for other lactic acid bacteria. Currently, the  
333   presence/absence of pili is limited to genome mining and analysis, without any guarantee of  
334   gene expression and protein production. To increase the biomass and the quantity of pili, LGG  
335   WT was grown in a bioreactor under controlled pH (6.8) and gentle homogenization. For both  
336   static and bioreactor cultures, cells were harvested by centrifugation in the late-exponential  
337   growth phase where pili are still expressed (Laakso et al., 2011).

### 338           **3.1. Purification of SpaCBA pili via chromatography**

339   Having a CWP-enriched fraction, the chosen approach to separate pili from other protein  
340   contaminants was ratiocinated based on their size. Considering the length of SpaA (~9.3 nm)  
341   (Chaurasia et al., 2016), and taking into account the fact that the size of pili ranges from ~300  
342   nm to up to 1  $\mu$ m, it was roughly estimated that a pilus is composed of ~33-100 SpaA subunits.  
343   The MW of SpaA being ~30 kDa, it can be reasonably assumed that a single pilus has a MW  
344   of 1-3 MDa. With this information in mind, the use of multimodal chromatography (MMC,

345 Capto Core 700) to purify the pili was chosen. This core bead technology was initially  
346 developed for virus purification (Blom et al., 2014), but should be adapted for the purification  
347 of high molecular weight (HMW) proteins including bacterial pili (Figure 1). This technique is  
348 a flow-through-based method. Molecules having a MW > to 700 kDa cannot enter the beads  
349 while molecules having MW < to 700 kDa should enter the beads and be captured *via*  
350 electrostatic and/or hydrophobic interactions *via* the octyl amine ligand that is positively  
351 charged at neutral pH. Positively charged molecules that are not retained by the hydrophobic  
352 part of the ligand will also go through the beads. Up to 10 column volume (CV) can be loaded  
353 onto the column, much more than the 0.5-4% CV of sample volume that is recommended to be  
354 loaded on SEC columns. This unequivocally increases the amount of samples as well as the  
355 rapidity of the purification. Notably, the technology is available for 400 kDa molecules (Capto  
356 Core 400).

357 The supernatant of digestion (SOD) was injected on Capto Core 700 columns and the flow-  
358 through was saved. The column was washed with PBS and regenerated with NaOH/isopropanol  
359 mixture to desorb simultaneously molecules bound *via* hydrophobic and/or electrostatic  
360 interactions (Figure 2B). Following SDS-PAGE analysis (Figure 2D), one smeared band in the  
361 HMW part on the gel (>260 kDa) was observed and other bands from 30 to 60 kDa were noticed  
362 in the flow-through fraction. In the cleaning-in-place fraction, a smear was noted down to 10  
363 kDa, meaning that plenty of CWPs were successfully bound to Capto Core 700 resin.  
364 Importantly, it was noted that running SDS-PAGE at low current (~100V) improved the  
365 resolution at HMW and gave a smeared band as expected, corresponding to pili with different  
366 but close sizes. Interestingly, the major pilin SpaA harbors two isopeptide bonds between K47  
367 and N172 and K184 and D295, one in each CnaB domain (Chaurasia et al., 2016). If those  
368 bonds are maintained during SDS-PAGE analysis, it could drastically decrease the apparent  
369 MW of the pili since the proteins might not be fully unfolded by SDS. Indeed, CnaB domains  
370 are predicted to be inextensible, contrary to CnaA domains, due to the difference in position of  
371 the isopeptide bonds (Echelmann et al., 2016). From one purification batch, a band at HMW was  
372 excised, trypsin digested and analyzed by mass spectrometry (LC-MALDI) (Table S1). Six  
373 peptides matching with SpaC sequence (UniParc: UPI0001B5E4D6) were identified,  
374 confirming the presence of the pilin and therefore of pili. These peptides are found either in the  
375 CnaA or the vWFA domains (PDB: 6M48). Moreover, two peptides belonging to a protein of  
376 unknown function (matrix-binding protein, UniParc: UPI0001B5E7B8) were also identified.  
377 Surprisingly, only one peptide from SpaA (UniParc: UPI0001B5E4D4) was found in a  $\beta$ -sheet

378 from one CnaB domain (PBD: 5F44). However, it did not pass the conditions confirming the  
379 presence of the protein, *i.e.* identifying at least two peptides having a score higher than the  
380 threshold ( $p < 0.05$ ). This result was quite surprising, since SpaA is the major pilin and should  
381 be the most represented in the pilus. One explanation might be the resistance to trypsin digestion  
382 since the presence of the two isopeptide bonds could drastically affect digestion efficiency if  
383 they maintain a large portion of intact secondary structures, and so a resistance to SDS  
384 unfolding similarly to mechanical unfolding (Baker et al., 2015; Chaurasia et al., 2016). One  
385 peptide belonging to a murein DD-endopeptidase (UniParc: UPI0001B5E883) was also  
386 identified. Not surprisingly, no peptides from SpaB were detected since it is the least  
387 represented pilin within the pilus.

388 From another purification batch after MMC, it was observed that most of the CWPs were again  
389 efficiently captured by Capto Core 700 resin and only four bands were detected by SDS-PAGE:  
390 the band found at HMW, likely the pili, and four bands at 35, 50, 60 and 65 kDa (**Figure 2D**).  
391 In order to eliminate the low molecular weight contaminants, concentrated samples were  
392 injected from the MMC step onto a SEC column (Sephacryl HR 400, separation range for  
393 globular proteins: 20-8000 kDa). Only one peak was observed as having an elution volume ( $V_e$ )  
394 of ~40 mL. After SDS-PAGE analysis, contaminants were still present (data not shown). Thus,  
395 it was suggested that these contaminants might co-elute with the pili, meaning that they interact  
396 with each other, or that they just have the same elution volume. To answer the question, the  
397 same protocol was used to purify CWPs from LGG  $\Delta spaCBA$  mutant, which is devoid of pili.  
398 After CW digestion, MMC and SEC, bands at ~35, 50, 60 and 65 kDa were still observed but  
399 no longer the band at HMW, confirming that this band corresponds effectively to the pili (data  
400 not shown). Moreover, these contaminants had the same elution volume on SEC, meaning that  
401 they do not bind pili but are certainly a complex or aggregate of HMW. To obtain more  
402 information about these proteins, they were analyzed by mass spectrometry (LC-ESI). All of  
403 them were successfully identified as ABC transporter substrate binding-related proteins (**Table**  
404 **S2**).

405 To get rid of these protein contaminants and to concentrate SpaCBA pili, IEX chromatography  
406 was used. SpaA has a predicted pI of 4.7 in its mature form, *i.e.* without the signal peptide and  
407 without the C-term part after the LPXTG motif. Therefore, pili should bind to an anion-  
408 exchange column, while the contaminants have a  $pI > 9$  and should not bind to the resin. Before  
409 injecting the flow-through from MMC, the ionic strength of the sample was lowered by  
410 diafiltration in Tris 25 mM pH 6.8 buffer to ensure a proper binding of the pili. The diafiltered

411 sample was injected on strong anion exchange chromatography bearing a quaternary  
412 ammonium group. Once the sample was completely loaded and the columns washed, proteins  
413 were eluted with a linear gradient to 1M NaCl in 10 CV. Two properly separated peaks (**Figure**  
414 **2C**) were observed, one eluting at ~15 mS/cm and the other at 50 mS/cm (top of the peaks).  
415 The first peak contained the pili and the second one the contaminants, as shown in SDS-PAGE  
416 analysis (**Figure 2D**). The contaminants were not expected to remain bound to the resin since  
417 the theoretical pI is > 9 and those proteins should have been positively charged. Positive charges  
418 may be hidden while negative charges are accessible in the conformation state adopted by these  
419 ABC transporter-related complex/aggregates. Although no other protein other than pili were  
420 detected on SDS-PAGE stained with Coomassie blue, UV-vis quantification showed an unusual  
421 260nm/280nm ratio > 1, showing that non-protein biomolecules were present with the purified  
422 pili. A simple last step of diafiltration was added to obtain highly purified pili to get rid of these  
423 contaminants.

424 Although the IEX steps were performed with an automatic liquid chromatography device, peaks  
425 were well separated, and operating with a syringe in step elution mode, instead of gradient,  
426 should not interfere with pili purity. Interestingly, keeping the ionic strength high after the  
427 MMC step (~15 mS/cm) allowed the pili to flow through the column, but the contaminants were  
428 still captured. In this case, the pili were highly diluted but could easily be concentrated  
429 afterwards (data not shown). Other conditions were tested during the development of this  
430 protocol including running MMC in PBS pH 7.4 with or without 300 mM NaCl, but this did  
431 not change the results. Similarly, running MMC in Tris buffer 25 mM with 150 mM NaCl did  
432 not capture more protein contaminants and a second step of IEX was mandatory to obtain  
433 purified pili. To sum up, a simple protocol based mainly on MMC that allows for the  
434 purification of SpaCBA pili from LGG was developed. In 2-3 working days, and starting from  
435 1 L culture at  $OD_{600nm} \approx 1$ , ~50  $\mu$ g of pili (~ $2 \cdot 10^{13}$  molecules, 500 nm long, 1.6 MDa) can be  
436 purified.  $OD_{600nm} \approx 1$  corresponds to a bacterial concentration of  $\sim 1.25 \cdot 10^8$  CFU/mL  
437 (considering 5 LGG per bacterial chain (Gomand et al., 2020)). This suggests that ~30 pili are  
438 purified per bacterium.

### 439 **3.2. Characterization of the purified SpaCBA pili**

440 SpaCBA pili, purified thanks to this method, were characterized by DLS (**Figure 3A**). The  
441 mean  $R_h$  is centered at 82 nm and the distribution is broad since a polydispersity index (PDI) >  
442 0.7 was obtained, as expected. Indeed, pili could harbor different hydrodynamic volumes  
443 according to the number of SpaA found in one pilus and/or they may adopt a conformation that

444 is more or less straight or bent in solution. The purified pili were then analyzed by HPLC, more  
445 precisely by SEC coupled to multidetectors (TDA) to determine their MW, radius of gyration  
446 ( $R_g$ ) and hydrodynamic radius ( $R_h$ ) (Figure 3B). Buffer exchange was performed by  
447 diafiltration with the same buffer as the eluent to avoid buffer mismatches that could interfere  
448 with the refractometer signal at the end of the column. The first observation was that the pili  
449 eluted close to the void volume (~6 mL) of the column designed to separate globular proteins  
450 up to 5 MDa, confirming their high MW. The MW value at the top of the peak was ~25 MDa  
451 while  $R_g$  and  $R_h$  values were ~70 and ~65 nm respectively. Henceforth, the shape factor  $\rho$   
452 ( $R_g/R_h$ ) was approximately 1.1, a value in line with the elongated form of the pili. From these  
453 data, it can be concluded that pili are not monomeric and might form aggregates as previously  
454 observed by (Tripathi et al., 2012), who observed by AFM the formation of bundles.  
455 Differences in  $R_h$  could be due to a small proportion or larger pili present in DLS analysis, but  
456 not in SEC-TDA analysis, since the latter is equipped with a post-column nylon filter.  
457 Moreover, SEC-TDA data are from the top of the peak only and did not include the analysis of  
458 the whole peak.

### 459 3.2.1. AFM imaging of dry pili

460 AFM was employed for further characterization and imaging. Imaging pili is quite challenging  
461 and only a few publications have reported AFM imaging of these filamentous proteins. Indeed,  
462 LGG pili were successfully imaged directly on bacteria or after purification (Tripathi et al.,  
463 2012). Recently, the resolution serving to distinguish SpaA all along the pilus and SpaC at the  
464 tip was even reached (Kant et al., 2020). In the present work, pili were imaged on bacteria as  
465 well (Figure 4A). As expected, they appear to be protruding, long and thin filaments having a  
466 length around 500 nm. The height measured on a pilus on a cross-section from the base to the  
467 tip corresponds to a thickness of 2 nm all along the pilus and a value of around 4-5 nm at the  
468 top of the pilus. These values are in line with those of the aforementioned recently published  
469 work (Kant et al., 2020). Purified pili were also imaged by AFM (Figure 4B). Pili were analyzed  
470 either at 50 mg/L or at 2.5 mg/L. At the higher concentration, the pili formed a network bound  
471 and no bundles were observed as previously reported (Tripathi et al., 2012). Such bundles could  
472 have been formed due to the co-purification of EPS following the centrifugation-based protocol  
473 (Tripathi et al., 2012) (Figure S1A). Nonetheless, a thickness of ~2 nm was measured. This  
474 value is in line with the XRC 3D structure of SpaA, the major pilin (Chaurasia et al., 2016). At  
475 this concentration and taking into account drying, this network formation might occur *via* SpaC  
476 since SpaC-SpaC interactions were highlighted by single molecule force spectroscopy with



477 recombinant SpaC (Tripathi et al., 2013). At the lower concentration, isolated purified pili are  
478 easily detectable and, similarly to cell-attached pili, the average length was 500 nm and the  
479 thickness 2 nm (Figure 4B). No measures at the top of the purified pilus were made since the  
480 N-term C-term orientation might be tricky and could have led to mistakes.

#### 481 **4. Conclusion**

482 In the present study, we have developed a more versatile and easy to implement protocol than  
483 those available in the literature to purify SpaCBA pili from *Lactobacillus rhamnosus* GG, one  
484 of the most widely studied probiotic bacteria, whose probiotic properties might partially occur  
485 through these pili. This novel protocol is mainly based on multimodal chromatography (core  
486 bead technology using Capto Core 700 resin), requires minimal laboratory equipment, is not  
487 expensive and can be used to obtain SpaCBA pili with a high degree of purity. Although done  
488 with a basic chromatographic system, purification might be performed with a peristaltic pump  
489 or even a simple syringe and would not require complex lab equipment nor the use of specific  
490 antibodies that are not commercially available. A custom antibody production might be  
491 expensive and only dedicated to the targeted pilins. This work could allow for the expansion of  
492 studies dedicated to SpaCBA pili at the molecular level, and the characterization of interactions  
493 with their protein partners would also be facilitated. Finally, we believe that this new  
494 purification method might be a solid basis for further purification of other sortase-dependant  
495 pili.

#### 496 **5. Data availability**

497 All the mass spectrometry proteomics data were deposited into the ProteomeXchange  
498 Consortium via the PRIDE (Perez-Riverol et al., 2019) partner repository with the dataset  
499 identifier PXD020875 and 10.6019/PXD020875". Reviewer account details: **Username:**  
500 [reviewer\\_pxd020875@ebi.ac.uk](mailto:reviewer_pxd020875@ebi.ac.uk); **Password:** UmSe6CUz.

#### 501 **6. Authors' contributions**

502 R.D.S.M., JB, FB and CG designed the study. R.D.S.M., B.S. and S.B. prepared the sample.  
503 R.D.S.M., S.E.K.C., B.S and C.P. acquired the data. R.D.S.M. and S.E.K.C. analyzed/or  
504 interpreted the data. R.D.S.M drafted the manuscript. R.D.S.M., JB, FB and CG wrote the  
505 manuscript. All authors reviewed the manuscript and approved the final version

#### 506 **7. Conflict of interest statement**

507 The authors declare no conflicts of interests.

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661 **10. Figures' legends**

662 **Figure 1:** Overview of the purification process of bacterial SpaCBA pili from *Lactocaseibacillus*  
663 *rhamnosus* GG (LGG). Protocol develop in the present study relying mainly on multimodal  
664 chromatography and the core bead technology using Cpto Core 700 resin. SN: supernatant, P:  
665 pellet, DF: diafiltration, CIP: cleaning in place, CWP: cell wall protein, MMC: multi modal  
666 chromatography, IEX: ion exchange chromatography.

667 **Figure 2:** SpaCBA pili purification from LGG. (A) SDS-PAGE analysis highlighting the  
668 absence of pili production in LGG  $\Delta spaCBA$  (B) Multimodal chromatogram (Cpto Core 700)  
669 and (C) ion exchange chromatogram (HiTrap Q HP). Blue lines corresponds to Abs<sub>280nm</sub> signal  
670 and red lines to conductivity. (D) SDS-PAGE analysis throughout the purification process.  
671 SOD: supernatant of digestion, DF: diafiltration, MMC: multimodal chromatography, CIP:  
672 cleaning in place, IEX: ion exchange chromatography.

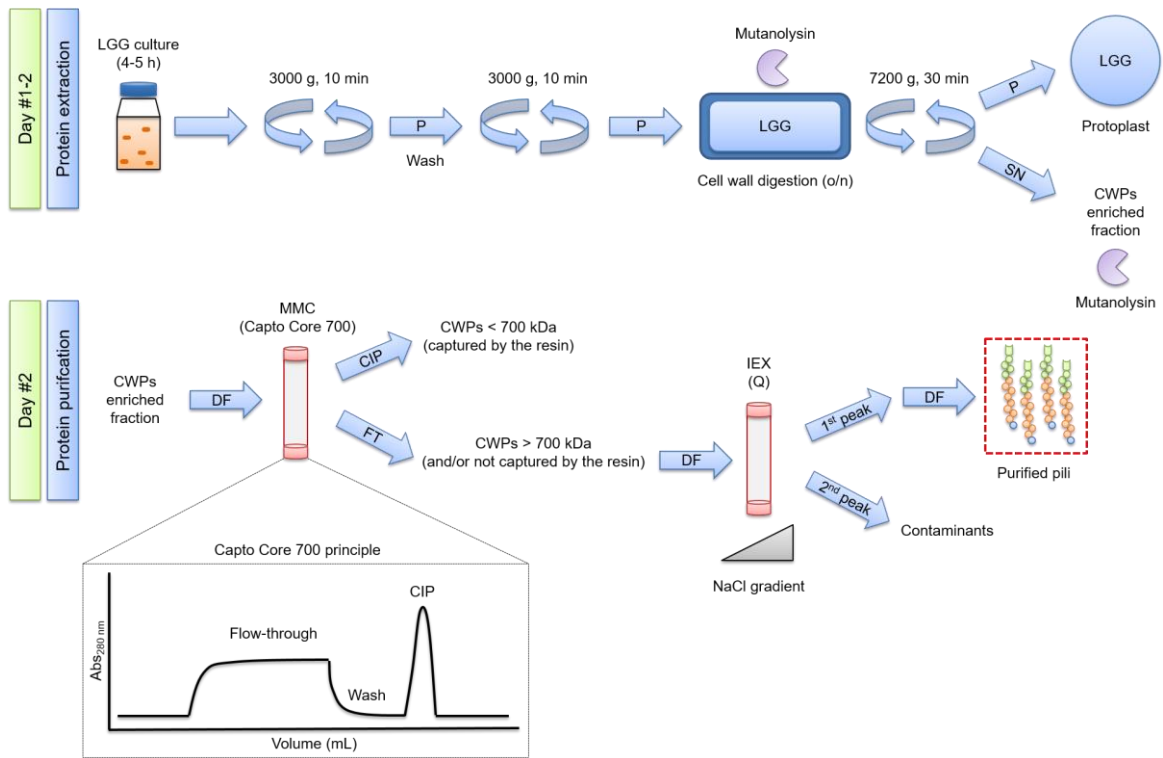
673 **Figure 3:** Characterization of the purified pili. (A) DLS analysis showing a large distribution of  
674 the  $R_h$  centered on  $\sim 85$  nm. (B) SEC-TDA analysis of SpaCBA pilus giving a MW (red) at the  
675 top of the peak of  $\sim 25$  MDa and  $R_g$  (pink) and  $R_h$  (blue) values of 70 and 65 nm. Refractive  
676 index signal is represented in black. The void volume and the total volume of the SEC column  
677 are  $\sim 6$  and 12.5 mL respectively.

678 **Figure 4:** AFM imaging of SpaCBA pili. (A) Low and high resolution images of LGG cells  
679 presenting pili. The vertical cross sections were taken along the dashed lines on the  
680 corresponding height images. (B) Purified pili imaged at two concentrations (top: 50 mg/L,  
681 bottom: 2.5 mg/L).

682 **Figure S1:** A (Tripathi et al., 2012) and B (Reunanen et al., 2012): protocols available in the  
683 literature and tested in this work. In the present study, no proteins were detected by SDS-PAGE  
684 following Coomassie staining (InstantBlue, Expedeon) for protocol A, but pili were  
685 successfully imaged by AFM in the original publication. SN: supernatant, P: pellet.

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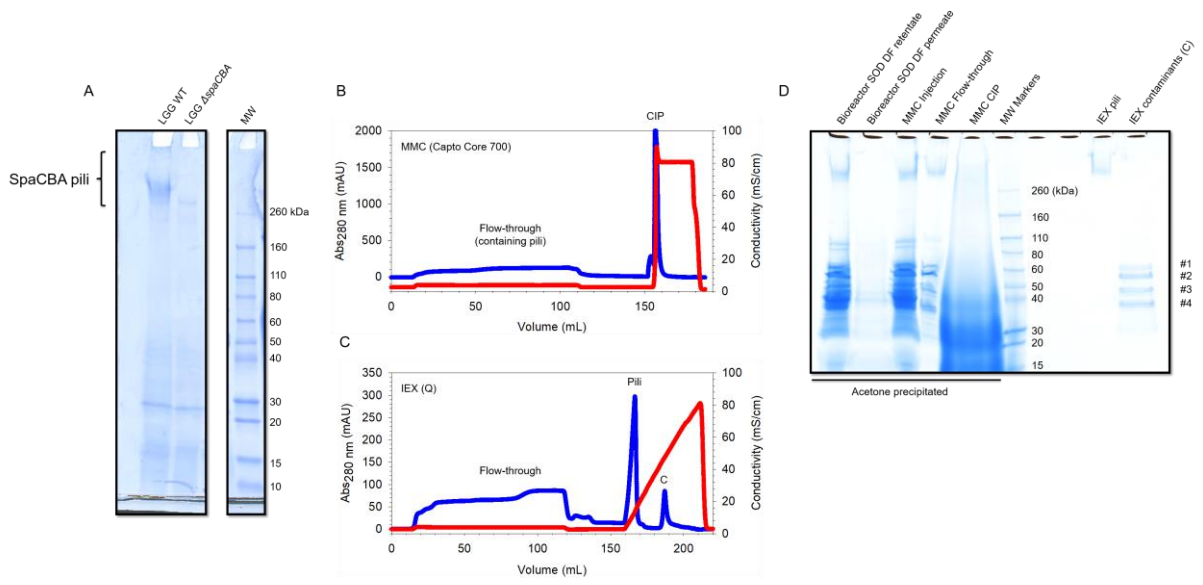
687 Figure 1



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690 Figure 2

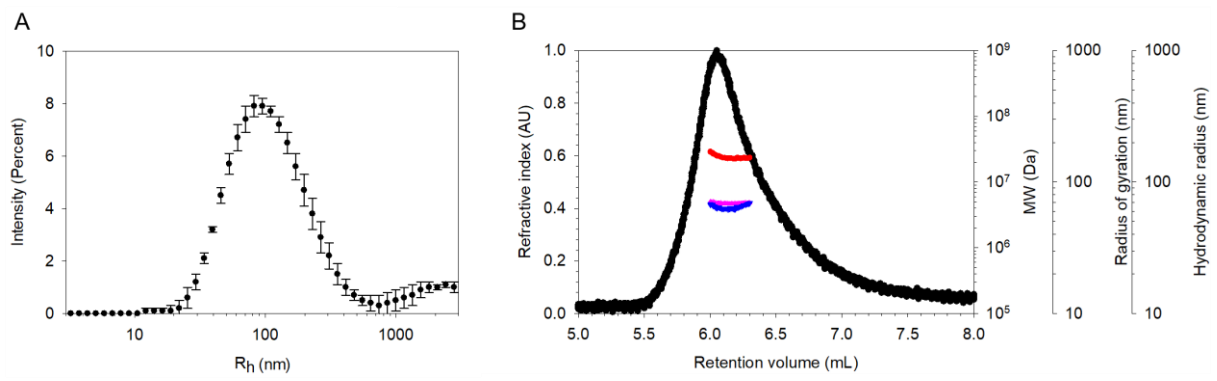


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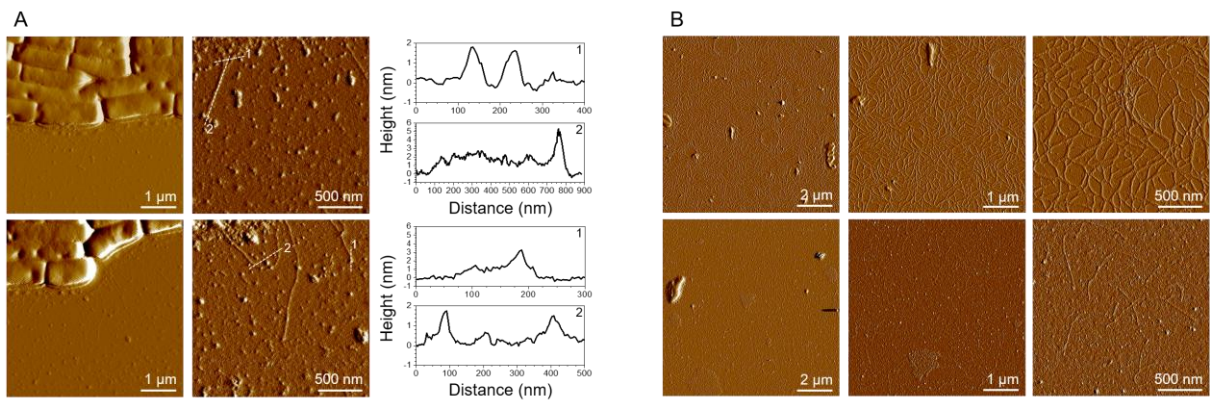
693 Figure 3



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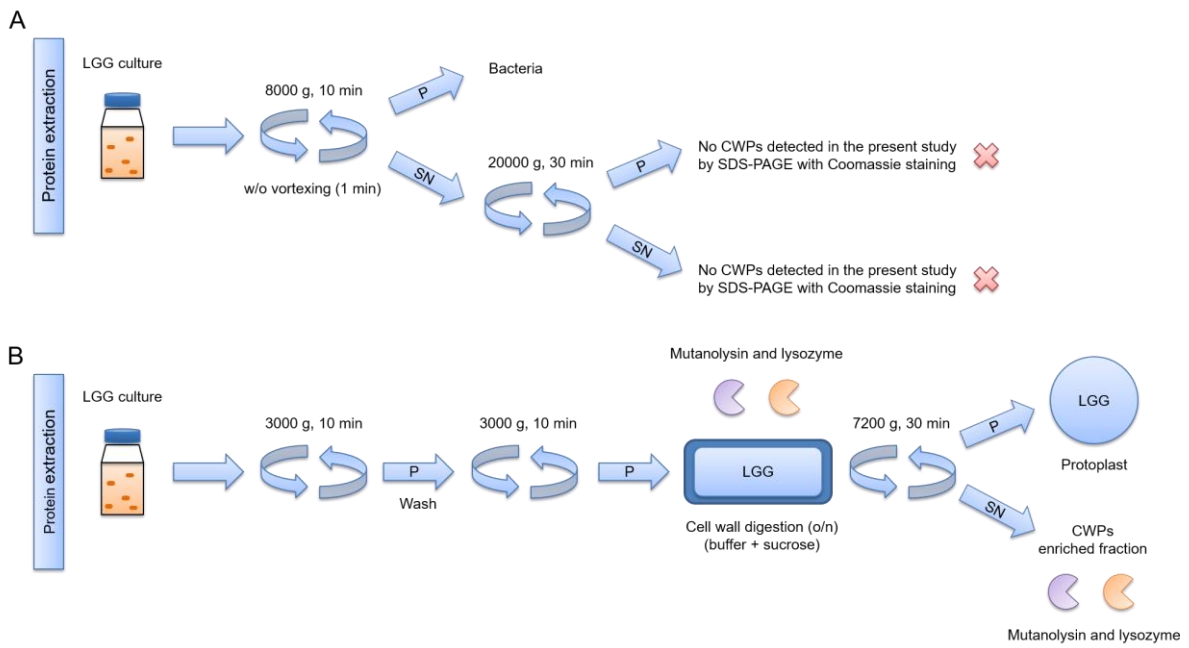
696 Figure 4



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699 Figure S1



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