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1 **Adhesive interactions between milk fat globule membrane and *Lactobacillus***  
2 ***rhannosus* GG inhibit bacterial attachment to Caco-2 TC7 intestinal cell**

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## 22 **Abstract**

23 Milk is the most popular matrix for the delivery of lactic acid bacteria, but little is known about  
24 how milk impacts bacterial functionality. Here, the adhesion mechanisms of *Lactobacillus*  
25 *rhamnosus* GG (LGG) surface mutants to a milk component, the milk fat globule membrane  
26 (MFGM), were compared using atomic force microscopy (AFM). AFM results revealed the key  
27 adhesive role of the LGG SpaCBA pilus in relation to MFGM. A LGG mutant without  
28 exopolysaccharides but with highly exposed pili improved the number of adhesive events between  
29 LGG and MFGM compared to LGG wild type (WT). In contrast, the number of adhesive events  
30 decreased significantly for a LGG mutant without SpaCBA pili. Moreover, the presence of MFGM  
31 in the dairy matrix was found to decrease significantly the bacterial attachment ability to Caco-2  
32 TC7 cells. This work thus demonstrated a possible competition between LGG adhesion to MFGM  
33 and to epithelial intestinal cells. This competition could negatively impact the adhesion capacity of  
34 LGG to intestinal cells *in vivo*, but requires further substantiation.

35

## 36 **Keywords**

37 *Lactobacillus rhamnosus* GG; Milk fat globule membrane; pili; Atomic force microscopy; Cellular  
38 adhesion

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## 45 **Introduction**

46 Pili are long and flexible proteinaceous filaments expressed at the surface of many Gram positive  
47 and Gram negative bacteria [1]. Recently, pili were imaged at the surface of the model lactic acid  
48 bacterium (LAB) *L. rhamnosus* GG (LGG) [2,3]. LGG has two pili gene clusters in its genome,  
49 known as the *spaFED* and *spaCBA*, encoding two distinct kinds of pili: SpaFED pili and SpaCBA  
50 pili. Only the *spaCBA* gene cluster is actually expressed in standard laboratory growth conditions  
51 [3,4]. LGG contains between 10 to 50 SpaCBA pili per cell, each with an approximate length of 1  
52  $\mu\text{m}$  [3,5,6]. The SpaCBA pili is composed of three pilin subunits [3]. SpaA is the major subunit  
53 and constitutes the backbone of the pilus. SpaB is mostly found at the base of the pilus, at the  
54 anchorage point with the cell wall, and few of them are also found in the pilus backbone. The SpaC  
55 subunits are present along the pilus and at the pilus tip [3]. SpaCBA pilus glycosylation was  
56 recently demonstrated and it was suggested that the SpaC pilins are the ones carrying glycosylation  
57 [7]. SpaCBA pili are key players in promoting LGG adhesion to a variety of human substrates such  
58 as human mucus [6], collagen [6] or intestinal epithelial cells [8]. Adhesive and mechanical  
59 properties of pili when interacting with extracellular components of host epithelial layers (mucin  
60 or collagen) have been investigated using a non-invasive single cell force spectroscopy method [8].  
61 SpaCBA pili adhesive properties are often attributed to the SpaC subunits. Two adhesive  
62 mechanisms were recently proposed. The first mechanism involves a molecular zipper featuring  
63 low forces with multiple SpaC subunits disposed along the pilus. The second model proposes a  
64 nanospring mechanism implying high forces at the tip of the pilus [6]. Recently, the key role of  
65 SpaCBA pili in LGG adhesion to Caco-2 intestinal epithelial cells was confirmed within a study  
66 involving LGG surface mutants [9]. The exopolysaccharides (EPS) layer of the LGG surface  
67 interfered with the SpaCBA pili during the occurrence of the adhesion phenomenon to epithelial

68 intestinal cells. The use of a LGG mutant without exopolysaccharides but with increasingly  
69 exposed pili improved the adhesion of LGG to epithelial intestinal cells compared to the LGG WT  
70 strain [9].

71 Milk and a wide range of fermented milk products are often used in the industry as LAB delivery  
72 systems, which are likely to feature health benefits for the host [10]. Bacterial adhesion to epithelial  
73 intestinal cells is influenced by the presence of dairy components. Several studies demonstrated  
74 the ability of milk components to modulate pathogenic and probiotic bacteria adhesion to epithelial  
75 intestinal cells [11,12]. The addition of acid-hydrolyzed milk decreases the adhesion of  
76 *Lactobacillus gasseri* R and *Lactobacillus plantarum* S2 to a co-culture of Caco-2 and HT29-MTX  
77 cells [11]. The adhesion of *L. gasseri* R is also negatively affected by the addition of UHT-treated  
78 milk or rennet caseins. On the contrary, the addition of bovine serum albumin or whey proteins  
79 increases the adhesion of both *L. gasseri* R and *L. casei* FMP strains [12]. These studies show that  
80 milk components have the ability to modulate the adhesion of probiotic strains to epithelial  
81 intestinal cells. The same behavior was observed for pathogenic bacteria. Defatted milk fat globule  
82 membrane (MFGM) prevented *E. coli* O157 : H7 infection by inhibiting bacteria attachment to  
83 Caco-2 cells [13]. In another study, bovine mucin extracted from the MFGM inhibited the adhesion  
84 of enteric pathogens to Caco-2 cells [14,15]. Dairy products may thus influence bacterial  
85 attachment to epithelial intestinal cells but to our best knowledge, no mechanism has yet been  
86 described to explain this phenomenon. Interactions between bacterial surface molecule and dairy  
87 matrices may negatively impact the attachment of bacteria to intestinal cells.

88 Adhesive interactions between SpaCBA pili and food matrices, including milk components, are  
89 yet poorly studied. The MFGM has gained considerable attention due to its nutritional and  
90 technological properties [16]. The MFGM is a thin membrane surrounding the milk fat globule.

91 MFGM is composed of phospholipids, sphingolipids, and membrane-specific proteins, mainly  
92 glycoproteins. A recent review describes the potential role of the glycoprotein mucin 1 (MUC1) in  
93 the adhesion phenomenon occurring between bacteria and MFGM [17,18]. In fact, many  
94 similarities are observed between the bovine MFGM mucin and human intestinal mucin [19,20].  
95 Bacterial adhesive properties in relation to intestinal mucin have already been described [21],  
96 whereas surprisingly, the adhesive properties in relation to MFGM have been poorly studied.  
97 Microscopic methods were used to decipher the adhesive phenomena occurring between MFGM  
98 and bacteria [22–25]. Similarly, the role of MUC1 in adhesion with pathogenic bacteria has already  
99 been described [14,15] but the interactive behaviors of MFGM or MUC1 were never studied at the  
100 molecular scale.

101 In this study, a biophysical approach was first implemented to understand how LGG adheres to  
102 MFGM using atomic force microscopy (AFM). Then, a biological study was performed to  
103 understand how the adhesion of LGG to Caco-2 TC7 intestinal epithelial cells can be modulated  
104 by MFGM. For this purpose, LGG surface mutants were employed to decipher how SpaCBA pili  
105 influence LGG adhesion to intestinal epithelial cells in the presence or absence of milk components  
106 (*i.e.* MFGM).

107

## 108 **Materials and Methods**

### 109 **Materials**

110 The wild type strain *L. rhamnosus* GG (ATCC 53103) (LGG WT) and two of its surface mutants  
111 were used in this study. The two mutants were the pili-depleted *spaCBA* mutant CMPG5357 (called

112 LGG *spaCBA* in this study) [9] and the EPS-deficient *welE* mutant CMPG5351 (called LGG *welE*)  
113 [26,27].

114 The isolation of MFGM was adapted from Le *et al.* (2009) with some modifications [28]. For each  
115 experimental run, 40 L of raw bovine milk was collected from a stirred cooling tank from a local  
116 farm (farm of La Bouzule, Laneuvelotte, France). Milk was first heated at 50 °C to favorize milk  
117 creaming. Then, fat globules were separated from skimmed milk using a cream separator  
118 (Elecrem1, Elecrem, Fresnes, France). After one night at 4 °C, the cream was churned using a  
119 mixer to obtain two fractions: butter and buttermilk. The buttermilk containing the MFGM was  
120 added to the final MFGM solution. The butter was melted at 50 °C after adding distilled water (half  
121 of the equivalent volume w / w). The total mixture was centrifuged at 3300 g for 10 min. The  
122 supernatant containing butter oil was eliminated and the butter serum containing the MFGM  
123 fraction was rinsed with 20 g of distilled water and was centrifuged at 3300 g for 10 min. The  
124 suspension of MFGM containing the butter serum was collected and stored in a freezer for one  
125 night. The MFGM powder was obtained after freeze-drying the butter serum.

126 The purified gastric mucin was purchased from Sigma-Aldrich (Mucin from porcine stomach,  
127 M1778, France).

128 The Caco-2 TC7 TC7 cells were obtained from Pr. Yves Leroux (team MRCA, laboratoire  
129 URAFPA, Nancy, France).

130

### 131 **Atomic force microscopy**

132 **Bacteria culture.** LGG culture was performed as described by Guerin *et al.* (2016) [29]. Pre-  
133 culture of LGG (WT and its surface mutants) was prepared by inoculating 9 ml of fresh MRS broth

134 with 100  $\mu$ l of a bacterial stock solution and placed overnight at 37 °C without stirring. This pre-  
135 culture was used to inoculate 9 ml of fresh MRS broth and the growth was performed at 37 °C to  
136 reach an optical density of 1.2 at 660 nm. Bacteria were centrifuged at 3000 *g* for 10 min and  
137 resuspended in 1 ml of phosphate-buffered saline (PBS) buffer (pH 6.8).

138 **Preparation of bacterial surfaces.** The bacterial suspension was deposited on a mica coated with  
139 a gold layer functionalized with a NH<sub>3</sub> terminated-PEG linker (PT.BORO.PEG.NH<sub>3</sub>, Novascan,  
140 Ames, Iowa, USA) for 15 h at 4 °C (pH 6.8). The mica was rinsed with PBS before use. AFM  
141 topographic images confirmed the presence and good coverage of LGG on the mica surface (data  
142 not shown).

143 **Preparation of proteins probes.** AFM probes with borosilicate glass particles (2  $\mu$ m) used in this  
144 study were purchased from Novascan (PT.BORO.PEG.NH<sub>3</sub>, Ames, Iowa, USA). These probes  
145 were coated with gold and modified with a NH<sub>3</sub> terminated-PEG linker (PT.BORO.PEG.NH<sub>3</sub>,  
146 Novascan, Ames, Iowa, USA). The spring constant of these probes was 0.01 N/m. MFGM or  
147 purified mucin were prepared in distilled water at a concentration of 1 % (w/w) and adhered on the  
148 probe by immersion at 4°C for 15 h. The time for adsorption was much longer than the time  
149 necessary for the proteins to adsorb on the tip. Preliminary experiments confirmed that the tip was  
150 covered with proteins (data not shown). Tips were rinsed with milli-Q-grade water before use.

151 **Atomic force microscopy measurement.** The bacteria were adhered on a functionalized mica and  
152 the AFM probe was brought in contact with the bacterial surface so as to form a molecule-molecule  
153 complex governed by intermolecular bonds. When the AFM probe retracts from the substrate  
154 surface, the force required for dissociation *i.e.* the unbinding force can be defined as the rupture  
155 force occurring when the probe dissociates from the sample (*i.e.* when the interaction bonds break)  
156 [30].



157 Force measurements were performed at room temperature in PBS buffer (pH 6.8) using an Asylum  
158 MFP-3D atomic force microscope (Santa Barbara, CA, USA) controlled by the operation software  
159 IGOR Pro 6.04 (Wavemetrics, Lake Oswego, OR, USA) as described by Guerin *et al.* (2016) [29].  
160 The cantilever spring constant with borosilicate particle was first determined using the thermal  
161 calibration method which provided a value of 0.01 N/m [31]. For each experiment, the force map  
162 was recorded on a  $10 \times 10 \mu\text{m}^2$  surface corresponding to  $32 \times 32$  points, *i.e.* 1024 force curves.  
163 AFM force-distance curves were obtained a retraction speed of 400 mm/s.

164 **Curves processing.** For each AFM force measurements a force map containing 1024 force curves  
165 was recorded. Retraction curves were analyzed to determine the presence of specific adhesive  
166 occurrences between bacteria and milk proteins. Specific adhesion revealed the presence of  
167 stretching biomolecule signatures on the retraction curve. For each set of experimental conditions,  
168 100 adhesive force curves were selected and the corresponding retractions were analyzed to  
169 determine the percentage of adhesive events with specific biomolecules stretching signatures. In  
170 this study, the percentage of adhesive events represented are the percentage of specific adhesive  
171 phenomena presenting retraction curves which proved to feature specific biomolecules stretching  
172 signatures.

### 173 **Bacterial adhesion to Caco2 TC7 cells**

174 **Epithelial cell lines and culture conditions.** Epithelial cells Caco-2 TC7 were grown in  
175 Dulbecco's modified Eagle's Minimal Essential Medium with 4.5 g/L glucose (DMEM Glutamax,  
176 Fisher Scientific) supplemented with 20 % (v/v) of heat-inactivated fetal calf serum (FCS; Gibco®)  
177 and 1 % (v/v) of penicillin–streptomycin (5 000 U/ml penicillin and 5,000  $\mu\text{g}/\text{ml}$  streptomycin,

178 Gibco®) and 1 % (v/v) non-essential amino acid ×100 (Gibco®). Cells were conserved in DMEM  
179 medium supplemented with FCS and 10 % glycerol in liquid nitrogen.

180 The Caco-2 TC7 culture was adapted from Kebouchi *et al.* (2016) [32]. Briefly, cells were thawed  
181 and seeded in plastic flasks (75 cm<sup>2</sup>, Dutscher). After 4 days, cells were trypsinized with 0.05 % of  
182 trypsin-EDTA (×1, Gibco®). After 5 min of contact with trypsin, DMEM medium with FCS was  
183 added to the culture to inhibit the trypsin action. Cells were recovered after by centrifugation at  
184 1000 rpm for 5 min at 37 °C. The cells pellet was suspended in DMEM (20 % FCS, 1 % antibiotic  
185 and 1 % amino acid) and seeded in six well culture plates (Dutscher) at a final concentration of 6  
186 × 10<sup>4</sup> cell per cm<sup>2</sup>. Cells were maintained at 37 °C in a humidified atmosphere with 10 % CO<sub>2</sub> and  
187 the culture medium was changed every day. The differentiated cell monolayers were obtained after  
188 22 culture days. Two days before the adhesion tests, DMEM without antibiotics was used to avoid  
189 bacteria death during the occurrence of the adhesion phenomenon.

190 **LGG cultures conditions.** Bacterial stocks were stored at -80 °C in MRS broth with 20 % (v / v)  
191 glycerol. Pre-culture of LGG (WT and its surface mutants) were prepared by inoculating 50 ml of  
192 MRS broth with 1 ml of bacterial stock and placed at 37 °C for 8 h without stirring. These pre-  
193 cultures were used to inoculate 200 ml of fresh MRS broth; bacterial growth was led for 12 h at 37  
194 °C. The cultures were centrifuged at 3000 g for 10 min at room temperature. The resulting pellets  
195 were washed with HBSS (Hanks' Balanced Salt Solution, Fisher Scientific) to remove the excess  
196 of medium and centrifuged at 3000 g for 10 min.

197 To study the role of surface components in the adhesion phenomenon occurring between LGG and  
198 Caco-2 TC7, the LGG pellet (WT and its surface mutants) were directly resuspended in DMEM  
199 medium supplemented with only 1 % of amino acid (without FCS and antibiotic) at a bacterial  
200 concentration around 10<sup>9</sup> CFU/ml.

201 To study the effect of MFGM addition on LGG adhesion, the bacterial pellets were resuspended in  
202 a solution of MFGM (5 mg/ml) in DMEM medium supplemented with only 1 % of amino acid, for  
203 1 h at 37 °C without stirring. The bacteria mixtures were then centrifuged at 500 g for 10 min to  
204 obtain bacteria cells pellets. The supernatant containing unbound MFGM was removed and the  
205 pellets containing bacteria cells were resuspended in DMEM media (1% amino acid) at a  
206 concentration around 10<sup>9</sup> CFU/ml.

207 **LGG survival in DMEM.** Bacterial viability in cells culture media (DMEM + 1 % amino acids)  
208 was determined by comparing the number of LGG initially present in the DMEM with 1 % amino  
209 acids solution and the number of LGG in the medium after incubating for 2 h at 37 °C (**Figure S1**).  
210 In both cases, solutions containing the bacteria were mixed for 1 min using a vortex homogenizer.  
211 Samples were serially diluted in tryptone salt broth and plated on MRS agar. After 48 h incubation  
212 at 37 °C, cell counts were performed and expressed using CFU/ml (Colony Forming Units/ml).

213 **MTT toxicity assay.** The effects of MFGM on Caco-2 TC7 cell viability was determined through  
214 MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) toxicity assays. MTT assays  
215 involve the reduction of the water soluble MTT, a yellow tetrazolium salt, into a blue formazan  
216 dye precipitate, that can be extracted using an organic solvent. The reduction is realized by the  
217 mitochondria of metabolically active cells. MTT assays with 22-day Caco-2 TC7 monolayer  
218 culture were assessed as described by Bu *et al.* (2016) [33]. Briefly, cells were treated with 4 ml of  
219 different MFGM solutions (0.5 and 5 mg/ml in DMEM without FCS and antibiotic) for 2 h at 37  
220 °C. The MFGM solution was then removed and the differentiated Caco-2 TC7 cell monolayers  
221 were gently rinsed with 1 ml of HBSS. Cells were then incubated with 3 ml of DMEM (20% FCS,  
222 1 % amino acid and 1 % antibiotic) and 300 µl of MTT solution (5 mg/ml in PBS) for 3.5 h at 37  
223 °C; the medium was then removed and 2 ml of DMSO per well were added. Solutions containing

224 cells were diluted ten times and transferred on 96-square angled bottom well plates. The plates  
225 were covered from light and incubated with orbital shaking (150 rpm) for 10 min. Absorbance was  
226 then measured using a microplate reader at a wavelength of 570 nm.

227 **Adhesion tests.** Before adhesion, the differentiated Caco-2 TC7 cell monolayers were gently rinsed  
228 with 3 ml of HBSS; 4 ml of bacterial suspension (bacteria alone or bacteria incubated with MFGM)  
229 were then added to the cells at a concentration of  $10^9$  CFU/ml to reach a bacteria cells to epithelial  
230 cells ratio of 1000:1 [32]. The number of bacteria present in the initial solutions were determined  
231 by bacterial enumeration. Bacteria were incubated on epithelial cells during 2 h at 37 °C and the  
232 supernatant of each well was removed. The cell monolayers were gently rinsed with HBSS ( $4 \times 1$   
233 ml) to obtain non-adherent bacterial suspensions. Caco-2 TC7 cells were then scraped with  $3 \times 1$   
234 ml of Triton® ( $\times 100$ , Sigma) at 0.1 % (v / v). The solution containing Caco-2 TC7 cells with  
235 adherent bacteria was passed three times through a needle and then incubated for 30 min at room  
236 temperature. Bacterial enumeration was used to evaluate both the number of non-adherent and  
237 adherent bacteria in the supernatant. In both cases, the solution containing the bacteria was mixed  
238 during 1 min using a vortex homogenizer. Samples were serially diluted in tryptone salt broth and  
239 plated on MRS agar. After a 48 h-incubation period at 37 °C, cell counts were determined and  
240 expressed using CFU/ml. The number of adherent bacteria per cell was determined using the  
241 following equation:

$$242 \quad \text{Number of adherent bacteria/cell} = \frac{\text{Number of total adherent bacteria (CFU/ml)} \times \text{Triton volume (ml)}}{\text{Number of cells in the well}}$$

243 Two independent experiment series were performed in triplicate.

244

## 245 **Statistical tests.**

246 Experimental data obtained in this study were all analyzed using a one-way analysis of variance  
247 (ANOVA) followed by a Tukey's test. Comparisons were performed between LGG WT and the  
248 mutant strains. Results were expressed as means  $\pm$  SEM (Standard Error of the Mean). Differences  
249 were considered statistically significant when P value  $< 0.05$ . In the figures, means  $\pm$  SEM followed  
250 by a different superscript letter indicate a significant difference (P $<0.05$ ).

251

## 252 **Results and Discussion**

### 253 **Key role of pili in the adhesive interactions occurring between LGG and MFGM.**

254 In the present study, adhesive events between LGG and MFGM were measured using AFM to  
255 understand how LGG adheres to MFGM. AFM probes were functionalized with MFGM and  
256 bacteria strains were immobilized on mica. The three strains used in this work are LGG wildtype  
257 (WT), LGG *spaCBA* mutant [9] (pili-depleted), and LGG *welE* mutant [26,27] (EPS-depleted).  
258 Their percentage of adhesive events presenting specific biomolecule stretching signatures with the  
259 MFGM probes were  $75.5 \pm 1.5$ ,  $7.0 \pm 3.5$  and  $99.0 \pm 1.0$  %, respectively (**Figure 1**). The adhesive  
260 events of the pili-depleted *spaCBA* mutant were thus drastically decreased in comparison with  
261 those occurring for the wild type strain. On the contrary, when *SpaCBA* pili were overexposed (for  
262 the EPS-deficient *welE* mutant), the adhesive events significantly increased compared to the wild  
263 type strain. It is known that pili located on the bacterial surface of the LGG *welE* mutant are not  
264 embedded anymore within the EPS layer [9], therefore the pili in this mutant are probably fully  
265 available to establish contact points with MFGM, explaining the increased number of adhesive

266 events [34]. These results highlight the key role of SpaCBA pili in the adhesion phenomenon  
267 occurring between LGG and MFGM.

268 To confirm the role of the SpaCBA pilus in the adhesion phenomenon occurring between LGG and  
269 MFGM, force profiles of AFM retraction curves were then analyzed for each strain (**Figure 2**).  
270 Retraction curves recorded during adhesion between MFGM and LGG WT presented several  
271 rupture peaks with a mean number of ruptures of  $1.9 \pm 0.0$ , a mean maximal rupture force of  $0.5 \pm$   
272  $0.0$  nN, and a maximal rupture distance of  $5.9 \pm 0.1$   $\mu\text{m}$  (**Figure 2A**). These signatures are  
273 characteristic of stretched biomolecules, which would be present on the bacterial surface [35]. To  
274 identify the biomolecule stretched during the adhesion with MFGM, LGG surface mutants were  
275 used. The specific signatures recorded for LGG *spaCBA* mutant on retraction curves were rare (7  
276 %) and totally different from those observed with LGG WT (**Figure 2B**), thus confirming a key  
277 role of the SpaCBA pili in the adhesion phenomenon occurring between LGG and MFGM. In the  
278 absence of pili, the mean number of ruptures was  $1.0 \pm 0.0$ , the mean maximal rupture force was  
279  $0.1 \pm 0.0$  nN, and the mean maximal rupture distance was  $0.3 \pm 0.1$   $\mu\text{m}$ . Signatures recorded during  
280 biomolecules stretching between the LGG *spaCBA* mutant and MFGM appear similar to saccharide  
281 molecules signatures; they could therefore correspond to the EPS [36] stretching behavior, which  
282 are more accessible in the absence of pili. In contrast, the specific signatures recorded on the  
283 retraction curves for the LGG *welE* mutant contained multiple rupture peaks. The number of  
284 rupture peaks and the force parameters increased compared to those recorded for LGG WT. These  
285 signatures have a mean number of ruptures of  $5.8 \pm 0.7$ , a mean maximal rupture force of  $1.2 \pm 0.1$   
286 nN, and a mean maximal rupture distance of  $8.4 \pm 1.1$   $\mu\text{m}$ . Since the SpaCBA pili are overexposed  
287 in the absence of EPS for the LGG *welE* mutant [9], the increase number of adhesion peaks and  
288 the increased force may be explained by the better accessibility of the MFGM adhesion sites to the

289 pili. All these results thus point towards the SpaCBA pilus being the key macromolecule engaged  
290 in the adhesion of LGG to MFGM.

291 The MFGM is composed of many glycoproteins; one of them is a kind of mucin called MUC1.  
292 Many similarities in the structure and composition of mucin MUC1 and purified mucin [19,20]  
293 have been found. The signatures observed during the retraction of the MFGM-functionalized probe  
294 from the LGG *welE* mutant's surface are similar to those observed during the gastrointestinal mucin  
295 stretching in another study [37]; this indicates that MUC1 (from MFGM) may be implied in the  
296 adhesion phenomenon with LGG. Mucins are highly glycosylated proteins, with multiple lectin-  
297 adhesive sites. Gunning *et al.* (2013) explained the multiple adhesion peaks by repeated  
298 attachment-detachment cycles of the lectin-functionalized AFM tip present on the mucin chains  
299 [37]. These events are achieved with the presence of multiple binding sites along the mucin  
300 molecule. During adhesion between MFGM and the LGG *welE* mutant, the relative large number  
301 of adhesion peaks observed on the retraction curve may be the result of repeatedly attachment and  
302 detachment of mucin MUC1 on the LGG *welE* mutant cells due to the increased number of  
303 adhesive sites on pili for this mutant. [37] used the term “glycocode” to talk about signatures with  
304 multiple adhesion peaks, typical of glycosylated molecule-stretching as for mucin.

305 To support our hypothesis concerning the role of mucin MUC1 in the adhesion phenomenon  
306 involving MFGM and LGG, forces measurements with purified mucin from porcine stomach were  
307 performed using AFM. The adhesive events percentages were  $58.5 \pm 11.5$ ,  $13.0 \pm 1.0$ , and  $86.0 \pm$   
308  $2.0$  % with LGG WT, LGG *spaCBA* mutant and LGG *welE* mutant, respectively (**Figure 3**).  
309 Similar as MFGM, adhesive events decreased for the LGG *spaCBA* mutant compared to LGG WT.  
310 On the contrary, adhesive events significantly increased with the LGG *welE* mutant compared to  
311 LGG WT. These results suggest a key role for the SpaCBA pilus in LGG adhesion with purified

312 intestinal mucin. Retraction curves recorded during the occurrence of the adhesion phenomenon  
313 between the purified mucin and LGG WT (**Figure 4A**), the LGG *spaCBA* mutant (**Figure 4B**), or  
314 the LGG *welE* mutant (**Figure 4C**) confirmed the role of the SpaCBA pilus in adhesion.

315 The retraction curves recorded for LGG WT presented the specific signatures of stretching  
316 biomolecules with a mean number of ruptures of  $2.2 \pm 0.1$ , a mean maximal rupture force of  $0.8 \pm$   
317  $0.1$  nN, and a mean maximal distance of rupture of  $0.7 \pm 0.0$   $\mu\text{m}$  (**Figure 4A**). The similar  
318 signatures observed on the retraction curves of LGG WT when interacting with both MFGM or  
319 purified mucin (**Figure 5A**) suggest that the mucin MUC1 may be the biomolecule implicated in  
320 the adhesion between LGG and MFGM. For the pili-depleted strain, the LGG *spaCBA* mutant, the  
321 specific signatures on retraction curves are rare (13.0 %) and totally different from those observed  
322 with LGG WT (**Figure 4B**); this indicates that SpaCBA pili are essential for adhesion to occur  
323 between purified mucin and LGG. Retraction curves observed between the LGG *welE* mutant and  
324 purified mucin contained multiple rupture peaks similar to those observed for MFGM (**Figure 4C**),  
325 yet the maximal rupture forces was higher for purified mucin ( $1.4 \pm 0.0$  nN of mean value). This  
326 could probably due to differences in purity levels; purified mucin is indeed highly purified, whereas  
327 MFGM contains milk mucin but also many other biomolecules such as glycolipids and other  
328 glycoproteins. Some part of the retraction curves recorded for MFGM and the purified mucin were  
329 similar and correspond to the “glycocode” (**Figure 5B**), meaning that the adhesion between LGG  
330 SpaCBA pili and MFGM may be governed by the mucin MUC1. The maximal distance of rupture  
331 was very important during adhesion between the LGG *welE* mutant and MFGM with a value of  
332  $8.4 \pm 0.2$   $\mu\text{m}$ . The adhesion between the LGG *welE* mutant and purified mucin lead to a maximal  
333 distance of rupture of only  $1.9 \pm 0.2$   $\mu\text{m}$  and ruptures observed on retraction curves which returned  
334 several times to the baseline. Because MFGM is composed of many different biomolecules, this



335 could mean that different biomolecules, including MUC1, are successively stretched during the  
336 retraction of the MFGM-functionalized AFM probe from the LGG *welE* mutant surface.

337  
338 **SpaCBA pilus play a key role in LGG adhesion to epithelial intestinal cells.** A recent study  
339 based on the LGG surface mutants suggests that SpaCBA pili play a key role in LGG adhesion to  
340 Caco-2 epithelial cells [9]. To validate our culture cells model and confirm the role of bacterial  
341 surface components in LGG adhesion to epithelial intestinal cells, the adhesion phenomena  
342 occurring between LGG (LGG WT and two of its surface mutants) and Caco-2 TC7 cells was first  
343 checked (**Figure 6**).

344 All results are obtained by counting the number of adhering bacteria per intestinal cell. Our data  
345 revealed that the adhesion of LGG to Caco-2 TC7 cells was drastically reduced for the LGG  
346 *spaCBA* mutant compared to the WT strain ( $9.1 \pm 1.1$  bacteria per cell, *versus*  $19.7 \pm 3.8$ ,  $P < 0.05$ ).  
347 On the contrary, the adhesion capacity of the LGG *welE* mutant to Caco-2 TC7 cells was  
348 significantly increased when compared to the WT strain capacity ( $42.6 \pm 4.7$  bacteria per cell,  
349 *versus*  $19.7 \pm 3.8$ ,  $P < 0.05$ ). Lebeer *et al.* (2012) demonstrated and explained the improved  
350 adhesive capacities of the EPS-deficient *welE* mutant by the increased exposure of the SpaC  
351 subunits from the pili SpaCBA [9]. Our results validate the culture cell adhesion experiment and  
352 confirm the key role of pilus SpaCBA in LGG adhesion to Caco-2 TC7 epithelial intestinal cells  
353 which has been described in the literature [9].

354

355 **Impact of MFGM on LGG adhesion to Caco 2 TC7 epithelial cells**

356 When comparing proteins-pilus SpaCBA interactions vs. cell-pilus SpaCBA interactions, the  
357 stability of proteins during the gastrointestinal digestion is assumed. It was indeed recently  
358 demonstrated that some MFGM proteins are resistant to human enzymatic gastrointestinal  
359 digestion [38]. Some glycoproteins such as the mucin MUC1, the cluster of differentiation 36, and  
360 a significant part of the periodic acid Schiff 6/7 presented a high resistance to proteases thanks to  
361 their glycosylation level. Therefore, MFGM has been chosen to study the impact of food matrix  
362 composition on bacterial adhesion.

363 The impact of MFGM addition on LGG adhesion capacity to Caco-2 TC7 epithelial intestinal cells  
364 was investigated by comparing the adhesive capacities of LGG in the presence or the absence of  
365 MFGM (**Figure 7**), after first validating the nontoxic effect of MFGM on Caco-2 TC7 epithelial  
366 intestinal cells viability via a cell integrity MTT assay (**Figure S2**). The key role of the pilus  
367 SpaCBA on LGG adhesion to Caco-2 TC7 cells was confirmed by reproducible and repeatable  
368 experiments. The number of adherent bacteria per cell was  $19.4 \pm 2.9$ ,  $8.4 \pm 0.5$  and  $29.2 \pm 3.1$  for  
369 LGG WT, the LGG *spaCBA* mutant and the LGG *welE* mutant, respectively. The presence of  
370 MFGM drastically decreased the adhesion capacity of LGG WT and the LGG *welE* mutant with a  
371 number of adherent bacteria per cell of  $8.5 \pm 0.2$  and  $9.0 \pm 1.2$ , respectively. On the contrary, the  
372 adhesion capacity of the LGG *spaCBA* mutant was not impacted by the presence of MFGM ( $8.1 \pm$   
373  $1.6$  adherent bacteria per cell).

374 The presence of MFGM only modified the adhesive capacities of LGG WT and the LGG *welE*  
375 mutant. These two strains express SpaCBA pili on their surface. By interacting with pili, MFGM  
376 may mask SpaCBA pili adhesion sites to epithelial intestinal cells and thus inhibit the adhesion of  
377 LGG WT and the LGG *welE* mutant to Caco-2 TC7 epithelial intestinal cells (**Figure 7**).  
378 Additionally, as expected, MFGM does not influence the adhesion to Caco-2 TC7 epithelial

379 intestinal cells for this mutant, in agreement with the fact that no adhesive events were observed  
380 between MFGM and the LGG *spaCBA* mutant as shown above. The number of adherent bacteria  
381 per cell for the wild type strain and the *welE* mutant in the presence of MFGM was similar to the  
382 basal level of adherent bacteria per cell expressed for strains without pili. MFGM thus appears to  
383 block *SpaCBA* pili adhesion sites to cells but other surface molecules present on LGG WT and the  
384 *welE* mutant surfaces still allow adhesive phenomena to occur between WT and *welE* strains and  
385 the cells. These molecules are probably the same as those expressed on the *spaCBA* strain surface.  
386 Therefore a competition is likely to occur between the dairy matrix and epithelial intestinal cells  
387 for the adhesion to LGG *SpaCBA* pili.

388 Few studies have compared the efficacy of probiotics in different food matrices and little is known  
389 about how the dairy matrix impacts probiotic functionality [39,40]. Dairy products count amongst  
390 the most popular matrices used for probiotic delivery because they provide a physiochemical  
391 barrier against gastric acid [41]. Milk matrices help bacteria to survive when passing through the  
392 stomach thanks to the buffering capacity of milk proteins. Milk proteins are able to create a pH  
393 gradient around bacteria permitting a gradual exposure of bacteria to the external low pH leading  
394 to an increase in bacterial survival [42,43]. The delivery matrix can also modify the functional traits  
395 at the site of delivery in the gut but very few studies look at this aspect. For example, the  
396 functionality of *L. casei* BL23 is improved in milk. When *L. casei* BL23 is added in milk, its effect  
397 on reducing the development of colitis is improved [44]. The adhesion of LGG to Caco-2 TC7  
398 epithelial intestinal cells is also reinforced after incubation in yogurt vs. ice cream and the binding  
399 properties of bacteria are correlated to the product storage time [45]. Our study brought to light  
400 new knowledge on how food matrices impact probiotic efficacy. The consumption of LGG with

401 MFGM may significantly decrease the adhesion of LGG to Caco-2 TC7 cells, possibly lowering  
402 the resulting/associated health impacts.

403

#### 404 **Conclusion**

405 The ability of MFGM to inhibit the attachment of LGG to host cells was demonstrated. By using  
406 two complementary approaches (biophysical and biological), a mechanism was proposed to explain  
407 how MFGM can impact bacterial adhesion to intestinal epithelial cells (**Figure 8**). The biophysical  
408 approach using AFM revealed that LGG is able to interact with MFGM through SpaCBA pili. The  
409 biological approach using cellular adhesion highlighted that LGG SpaCBA pili also play a key role  
410 in the intestinal epithelial cells adhesion phenomenon. When bacteria integrated within a native  
411 dairy matrix (as it is the case for some MFGM glycoproteins) reach the intestine, pili adhesive sites  
412 are thus probably blocked by milk proteins such as MFGM and therefore bacterial adhesion to  
413 intestinal cells can be inhibited or limited. As many lactic acid bacteria are incorporated into dairy  
414 products, much research should be performed in order to check if bacterial functionality is  
415 preserved in the presence of dairy components. This knowledge on adhesive mechanisms may also  
416 be important when formulating new nutraceutical products when the goal is to inhibit the adhesion  
417 of pathogenic bacteria to the intestine.

418

419 **Supporting Information.** The LGG survival in DMEM + 1 % amino acid was determined in Figure  
420 S1. The nontoxic effect of MFGM on Caco-2 TC7 epithelial intestinal cells viability was  
421 determined via a cell integrity MTT assay (Figure S2).

422

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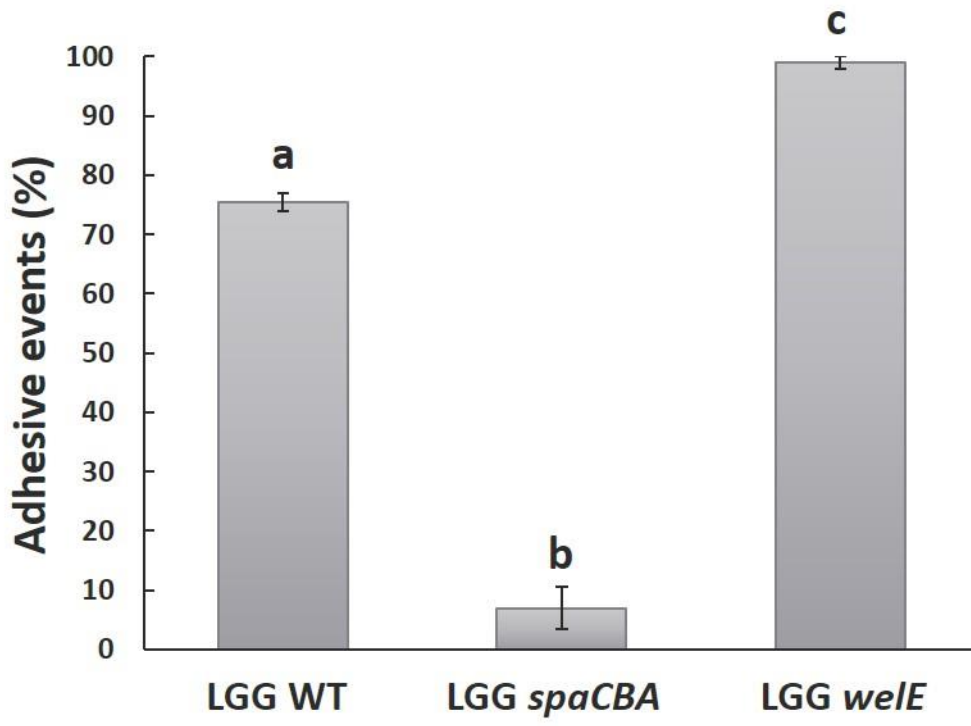
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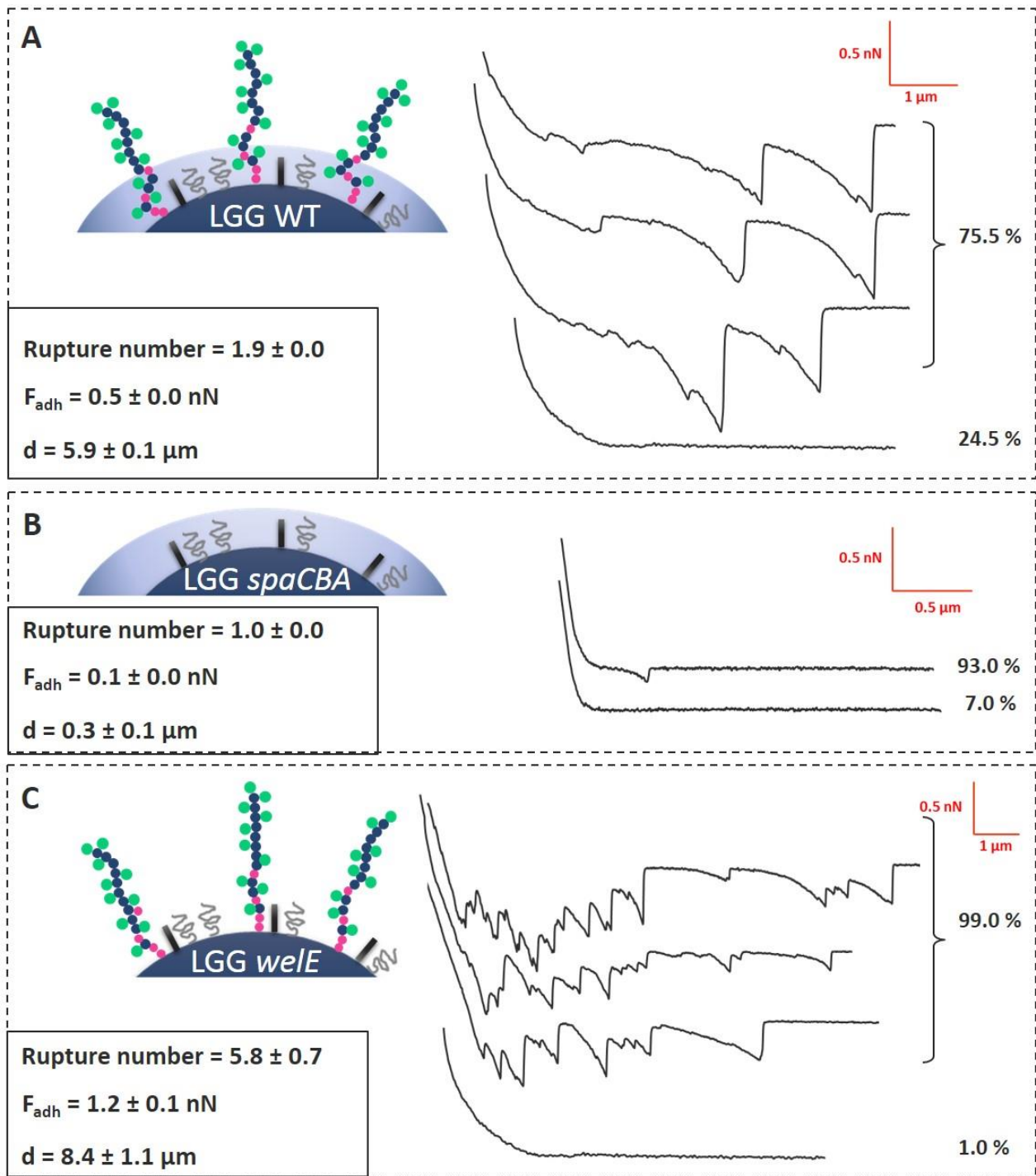


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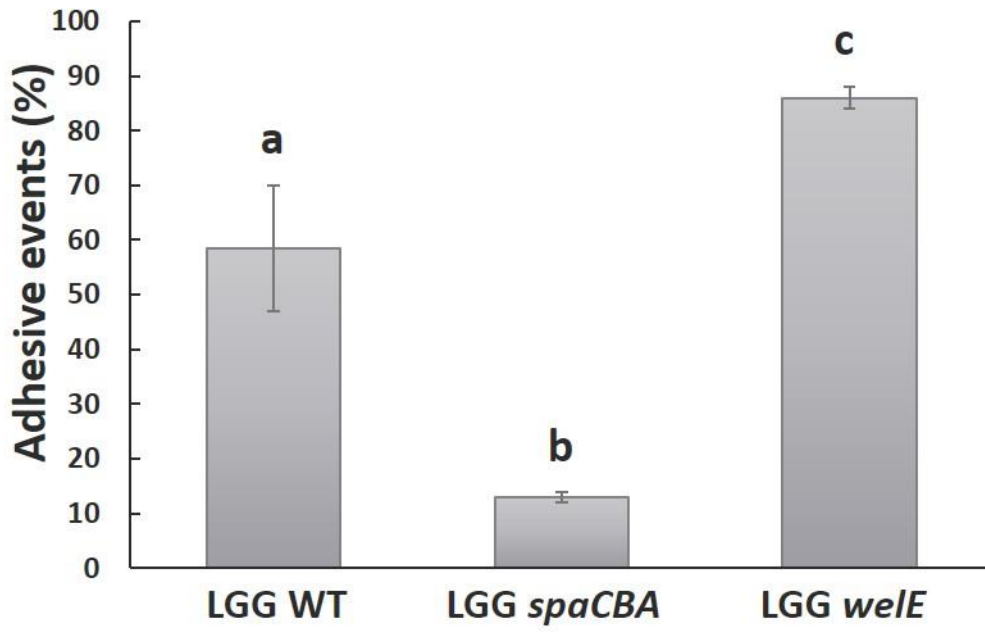


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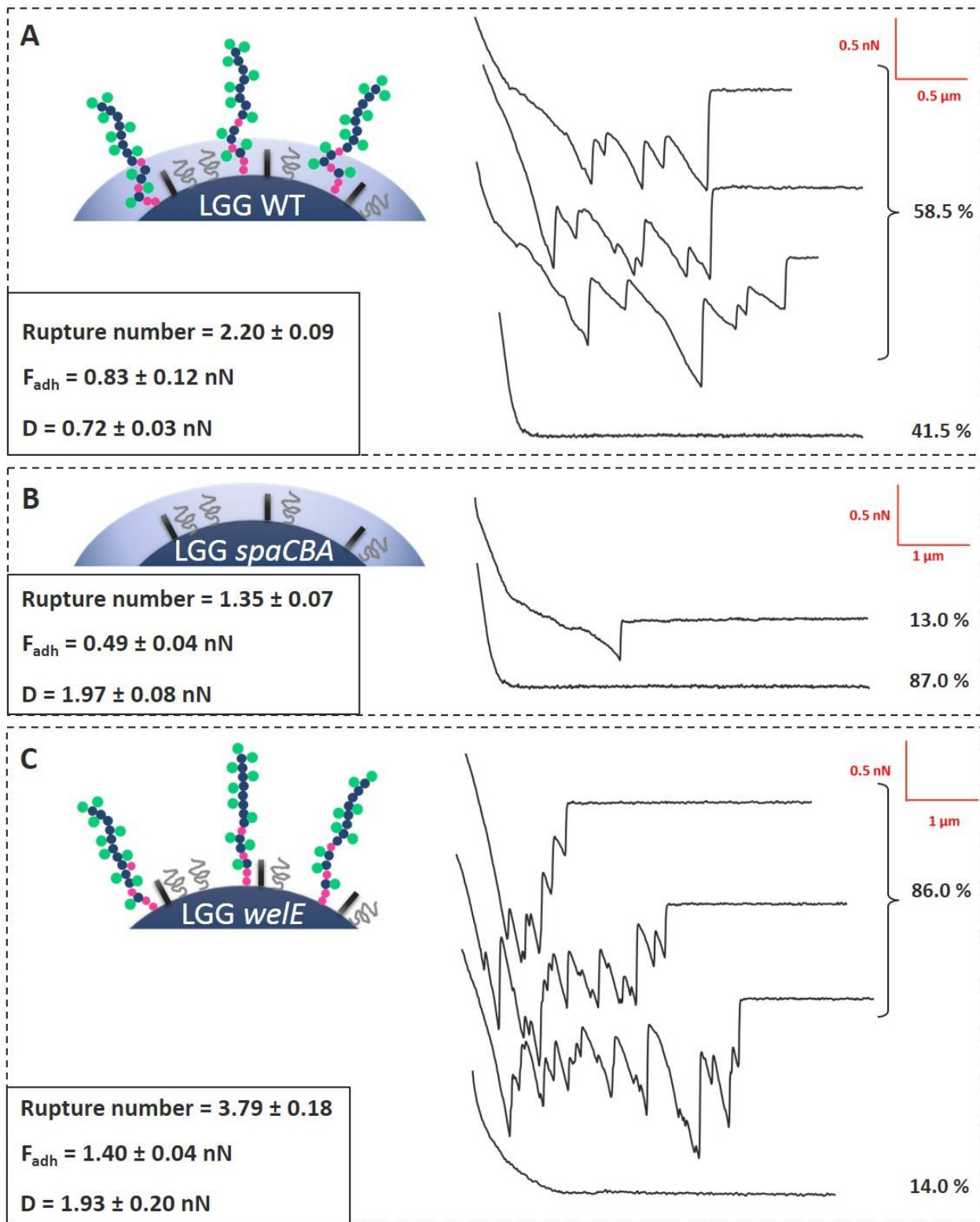


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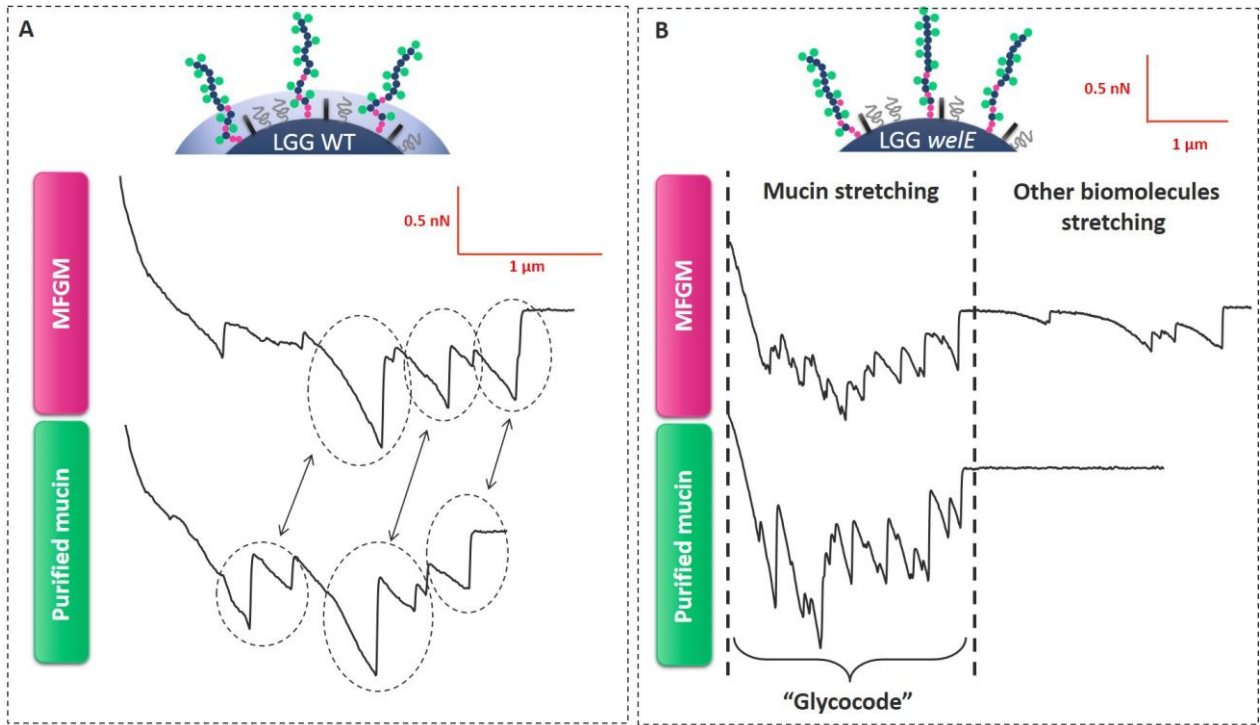


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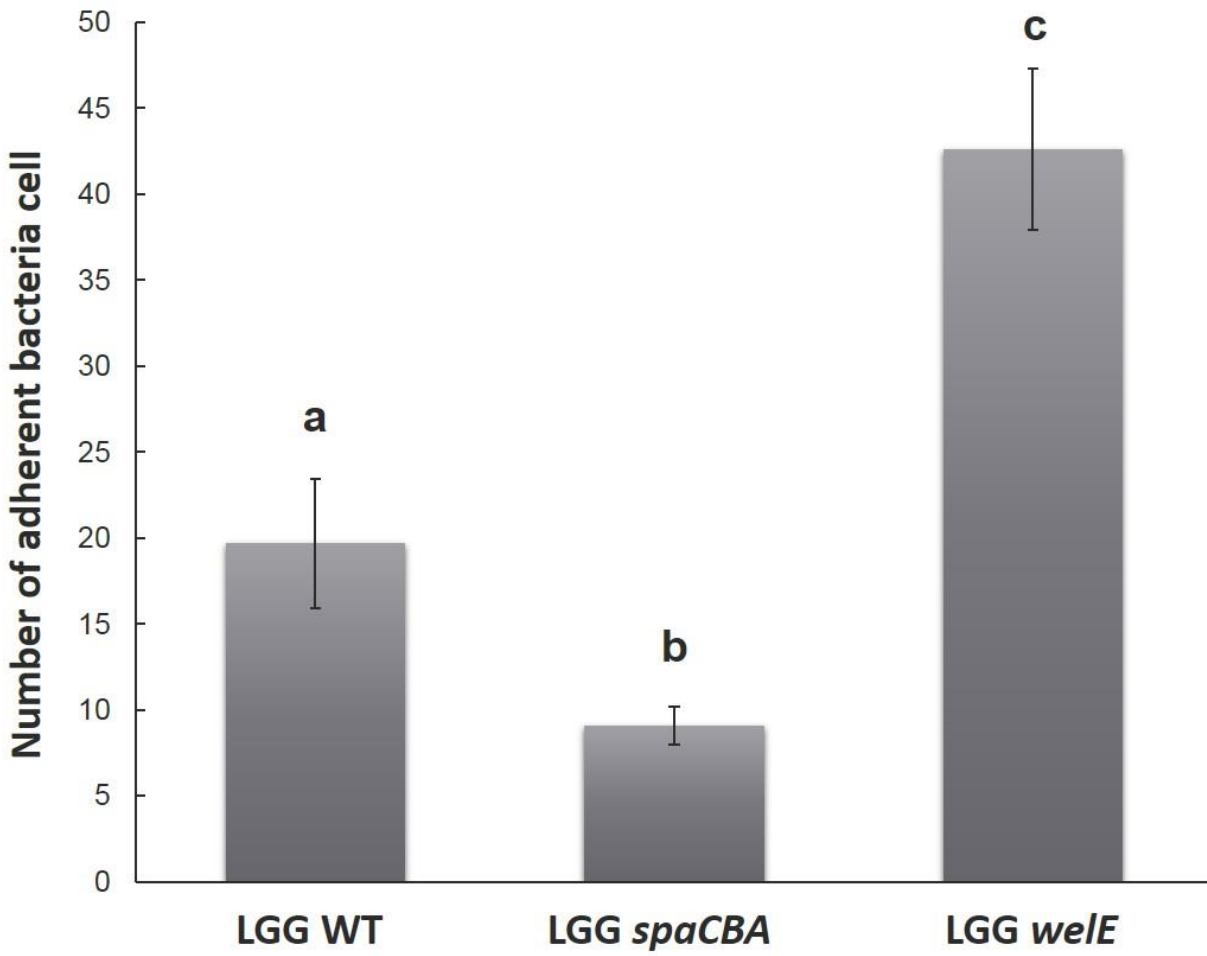
589 Figure 5



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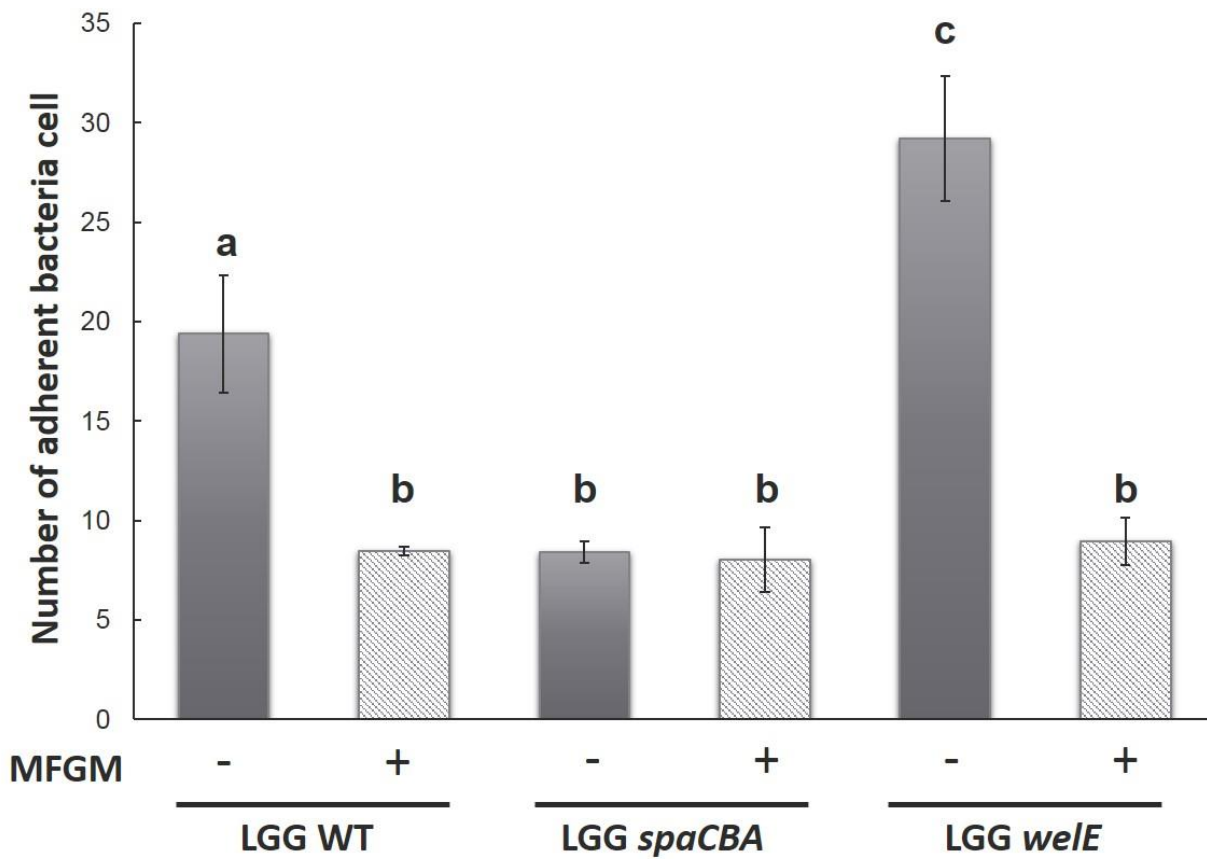
592 Figure 6



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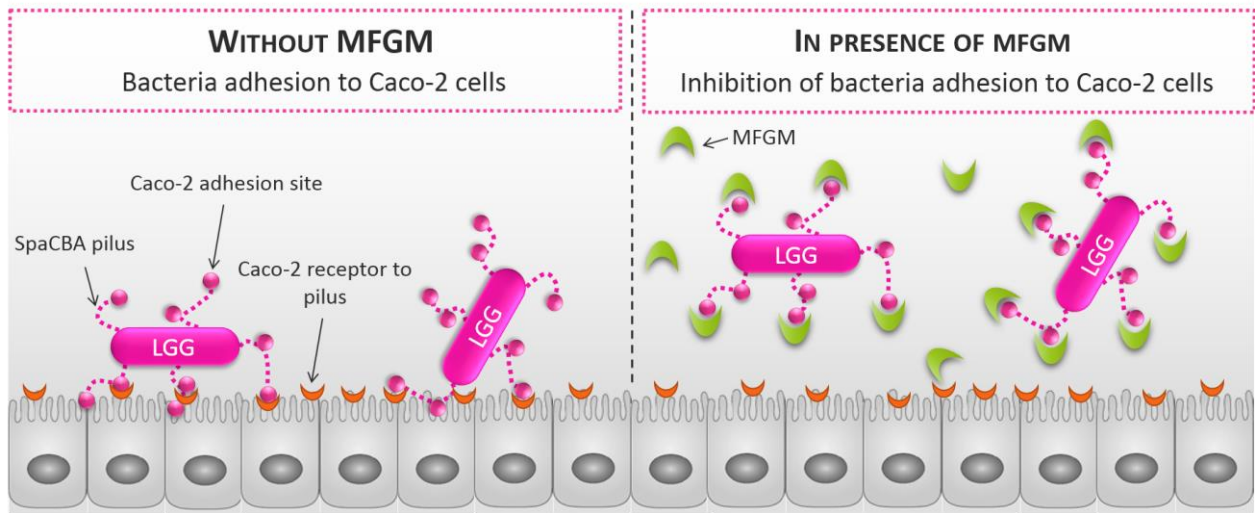
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595 Figure 7



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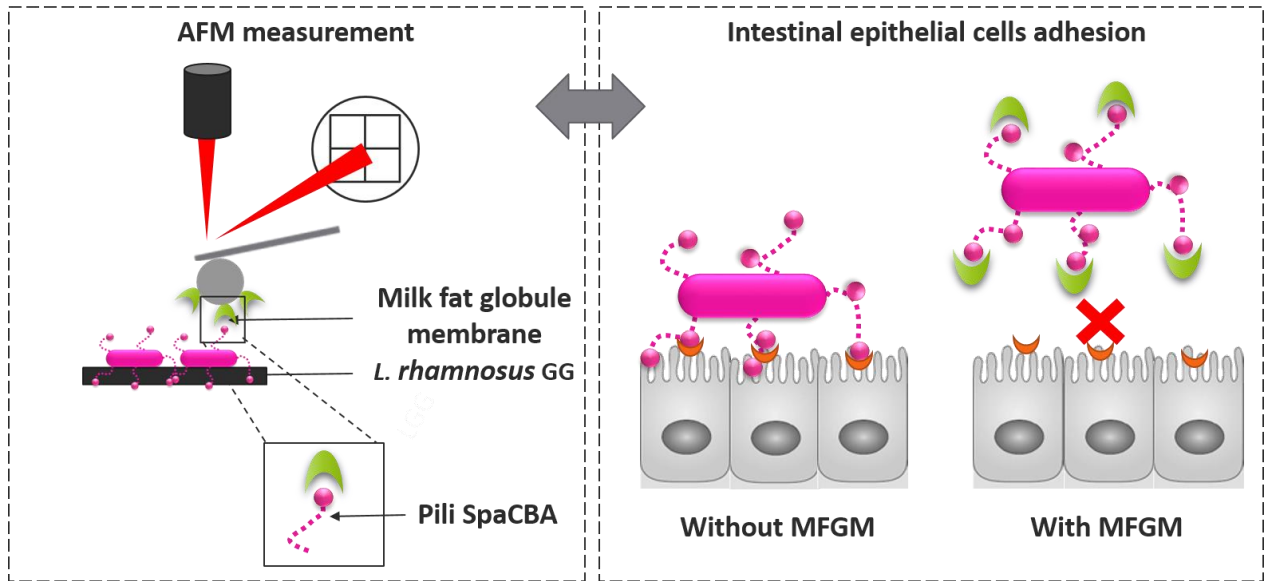
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## Figure captions

604  
605  
606 **Figure 1:** Adhesive events quantification between MFGM and LGG WT and two surface mutants  
607 (LGG *spaCBA* mutant and LGG *welE* mutant). Means  $\pm$  SEM followed by a different superscript  
608 letter indicate a significant difference ( $P < 0.05$ )

609  
610 **Figure 2:** Adhesion between MFGM AFM probes and LGG WT, LGG *spaCBA*, and LGG *welE*  
611 surface mutants. Representative retraction curves observed between MFGM and LGG WT (A),  
612 LGG *spaCBA* (B) and LGG *welE* (C) are presented.  $F_{adh}$ : maximal rupture force; d: maximal  
613 distance of rupture

614  
615 **Figure 3:** Adhesive events quantification between purified mucin and LGG WT, LGG *spaCBA*  
616 mutant, and LGG *welE* mutant surface mutants. Means  $\pm$  SEM followed by a different superscript  
617 letter indicate a significant difference ( $P < 0.05$ )

618  
619 **Figure 4:** Adhesion between purified mucin and LGG WT and LGG *spaCBA* and LGG *welE*  
620 surface mutants. Representative retraction curves observed between mucin AFM probes and LGG  
621 WT (A), LGG *spaCBA* (B) or LGG *welE* (C) are presented.  $F_{adh}$ : maximal rupture force; d: maximal  
622 distance of rupture

623

624 **Figure 5** : Representative retraction curves observed during adhesion between LGG WT (A) or  
625 LGG *welE* (B) and purified mucin; comparison with representative retraction curves recorded with  
626 MFGM

627  
628 **Figure 6:** Adhesion of LGG WT and its derivative mutants (LGG *spaCBA* and LGG *welE*) to a  
629 Caco-2 TC7 cell line after 2 h of co-incubation. Data represent means  $\pm$  SEM from at least two  
630 independent experiments performed with each strain in triplicate ( $n = 2 \times 3$ ). Means  $\pm$  SEM  
631 followed by a different superscript letter indicate a significant difference ( $P < 0.05$ )

632  
633 **Figure 7:** Impact of MFGM addition on the adhesion of LGG WT and its derivative mutants (LGG  
634 *spaCBA* and LGG *welE* mutant) to a Caco-2 TC7 cell line. The symbols “+” and “-” mean the  
635 presence or the absence of MFGM during the adhesion, respectively. Data represent means  $\pm$  SEM  
636 from at least two independent experiments performed with each strain in triplicate ( $n = 2 \times 3$ ).  
637 Means  $\pm$  SEM followed by a different superscript letter indicate a significant difference ( $P > 0.05$ )

638  
639 **Figure 8:** Mechanism proposed for the inhibition of the adhesion phenomenon between LGG and  
640 Caco-2 TC7 cells in presence of MFGM

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