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1                   **Adhesion of *Lactobacillus rhamnosus* GG surface biomolecules to milk**  
2   **proteins**

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15  
16 **Keywords**

17 Milk proteins; *Lactobacillus rhamnosus* GG; Bacterial surface biomolecules; Pili; Adhesion;  
18 Atomic force microscopy

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## 23 **Abstract**

24 Lactic Acid Bacteria (LAB) are not homogeneously located in the dairy matrix and their spatial  
25 distribution seems to be controlled by the establishment of adhesive interactions between matrix  
26 components and bacterial surface biomolecules. However the mechanisms of interaction remain  
27 unknown although they constitute an interesting way of study to appreciate the interactions. The  
28 aim of this work was to understand the role of surface biomolecules in the adhesion of  
29 *Lactobacillus rhamnosus* GG - the most used LAB strain in food products for their health benefits  
30 to the consumer - to milk proteins. Adhesions were probed using atomic force microscopy based  
31 force spectroscopy. To this end, the wild type strain and three of its surface mutants were employed.  
32 The wild type strain interacts with the  $\beta$ -lactoglobulin through the pili SpaCBA. The use of LGG  
33 surface mutants revealed that other surface biomolecules as long / small exopolysaccharides and  
34 proteins are involved in adhesion with milk proteins, in a less pronounced way than pili and in  
35 absence of pili, as all other surface biomolecules are masked in presence of pili. Altogether, this  
36 study demonstrates that adhesive interactions between LGG and milk proteins are governed by the  
37 surface composition of the bacteria.

38

## 39 **1. Introduction**

40 Lactic acid bacteria (LAB) are incorporated in many dairy products due to their fermentative  
41 properties and their probiotic characteristics. LAB are the most important starter cultures used in  
42 all area of dairy and food fermentation (Leroy & De Vuyst, 2004). By causing a rapid acidification  
43 of the raw material through the conversion of lactose into lactic acid, LAB play a central role in  
44 fermentation processes. LAB also produce several compounds (as acetic acid, ethanol, aromatic

45 compounds, bacteriocins, exopolysaccharides or enzymes) often involved in biochemical reactions  
46 during food processing which influence its texture and flavor. This is particularly the case during  
47 cheese ripening (Hickey, Sheehan, Wilkinson, & Auty, 2015; Leroy & De Vuyst, 2004). Many  
48 LAB are also incorporated into food products for their health benefits to the consumer. The strains  
49 *Lactobacillus rhamnosus* GG (LGG) (Valio), *Lactobacillus paracasei* Shirota (Yakult) or  
50 *Bifidobacterium lactis* BB12 (Chr. Hansen) are the most documented LAB used in food industries  
51 (Linares, Ross, & Stanton, 2016). Among these strains, LGG is also an interesting LAB model in  
52 food research because of its wide use, its available genome sequence (Kankainen et al., 2009) and  
53 the availability of numerous surface mutants (Lebeer et al., 2012). The surface composition of LGG  
54 is well-known and contains numerous SpaCBA pili distributed all around the bacterial cells  
55 (Reunanen, von Ossowski, Hendrickx, Palva, & de Vos, 2012; Tripathi et al., 2012), long  
56 galactose-rich exopolysaccharides (EPS), small glucose-rich EPS (Francius et al., 2009) and other  
57 proteins as MBF (von Ossowski et al., 2011) or MabA (Perea Vélez et al., 2010).

58 The spatial distribution of LAB within the dairy matrix plays an essential role during cheese  
59 processing, ripening and storage. This parameter may affect enzymes release and solutes diffusion  
60 in the matrix, which would influences cheese texture, flavor and aroma development (Hickey,  
61 Auty, Wilkinson, & Sheehan, 2015; Hickey et al., 2015). Numerous studies reported that LAB are  
62 not evenly distributed in the dairy matrix. During cheese ripening, various microscopy methods  
63 reported the preferential bacterial location at the fat-protein interface or in direct contact with the  
64 milk fat globule membrane (Laloy, Vuilleumard, El Soda, & Simard, 1996; Lopez, Maillard, Briard-  
65 Bion, Camier, & Hannon, 2006; Oberg, McManus, & McMahon, 1993; Tunick et al., 1993).  
66 Previously published work from the authors demonstrated that bacteria surface biomolecules can  
67 play a key role in bacteria distribution within the food matrix. The presence of the SpaCBA pili, a

68 proteinaceous surface appendages, at the LGG surface leads to a homogeneous location of the  
69 bacteria in the matrix. In absence of pili on their surface, LGG bacteria are closely spaced and  
70 aggregated at the same place in the matrix (**Figure S1**). Therefore, bacteria location in the dairy  
71 matrix seems to be governed both by the matrix composition and the surface composition of the  
72 bacteria.

73 Deciphering adhesive interactions between LAB surface biomolecules and dairy compounds would  
74 allow to better understand -and eventually predict- bacteria location in a food product.  
75 Nevertheless, very few studies focused on this concern. In a recent work, atomic force microscopy  
76 (AFM) was used to reveal specific adhesive interactions between LGG and whey proteins (J.  
77 Burgain et al., 2013), and more particularly with the  $\beta$ -lactoglobulin ( $\beta$ -LG) (Guerin et al., 2016).  
78 Different LGG surface mutants were compared to understand the mechanism occurring during  
79 adhesive interactions and the key role of the SpaCBA pili was underlined (Burgain et al., 2013;  
80 Guerin et al., 2016). However, the role of the other surface biomolecules remains, to the best of  
81 our knowledge, unknown.

82 The aim of this study was to decipher, at the nanoscale, the interactions involved in the adhesion  
83 of LGG to different pure milk proteins: micellar caseins,  $\beta$ -LG,  $\alpha$ -lactalbumin ( $\alpha$ -LA) and bovine  
84 serum albumin (BSA). By comparing the adhesive forces of various LGG cell-wall mutants with  
85 these proteins, the role of different LGG surface biomolecules in the binding mechanisms was  
86 interpreted.

87

## 88 **2. Material and methods**

### 89 **2.1 Material**

90 Micellar caseins (Promilk 872B) are obtained from Ingredia IDI (Arras, France). The  $\beta$ -LG,  $\alpha$ -LA  
91 and BSA are purchased from Sigma-Aldrich (France).

92 *L. rhamnosus* GG (ATCC 53103) (LGG WT) and three of its surface mutants are used. The surface  
93 mutants are: the pilus *spaCBA* knockout mutant CMPG5357 (LGG *spaCBA*) (Lebeer et al., 2012),  
94 the *welE* knockout EPS-deficient mutant CMPG5351 (LGG *welE*) (Lebeer et al., 2009; Lebeer,  
95 Claes, Verhoeven, Vanderleyden, & De Keersmaecker, 2011), the double knockout of the *spaCBA*  
96 operon and the *welE* gene CMPG5365 (LGG *welE**spaCBA*) (Lebeer et al., 2012).

97

## 98 **2.2 Microbial Adhesion To Solvent (MATS)**

99 The four strains of LGG are cultivated as described by Guerin et al. (2016). Briefly, the growth of  
100 the four strains is performed at 37 °C in MRS broth until an optical density at 600 nm around 1.2  
101 is reached. The culture is centrifuged at 3,000 g for 10 min at room temperature. The pellet is  
102 washed with PBS and re-suspended in PBS to obtain an optical density of 0.5 at 600 nm. The ability  
103 of LGG cell surface to adhere to solvent was evaluated as described by Salotti de Souza et al.  
104 (2018), with some modifications. For this determination, 1.2 ml of bacterial suspension in PBS is  
105 mixed for 90 s with 0.2 ml of different solvents. The bacterial affinity to a polar solvent  
106 (chloroform) and an apolar solvent (hexadecane) is studied. The mixtures are allowed to stand for  
107 15 min to ensure the good separation of the two phases. Then, the optical density of the aqueous  
108 phase is measured at 600 nm. The percentage of bound cells is calculated with:

$$109 \quad \% \textit{ adhesion} = \frac{A_0 - A}{A_0} \times 100$$

110 Where  $A_0$  is the absorbance of the bacteria suspension measured at 600 nm before mixing and  $A$   
111 is the absorbance of the bacteria suspension measured at 600 nm after mixing.

112

113

## 114 **2.3 Atomic force microscopy**

### 115 *2.3.1 Preparation of bacteria-coated mica*

116 A mica coated with a gold layer functionalized with a NH<sub>2</sub> terminated PEG linker (Novascan,  
117 Ames, Iowa, USA) is used in this study. Culture of LGG is performed as describe by Guerin *et al.*  
118 (Guerin et al., 2016). The bacterial suspension is deposited on mica during 15 h at 4 °C (pH 6.8).  
119 The mica is rinsed with PBS (pH 6.8) before their use. AFM topographic images confirmed the  
120 presence and the good coverage of LGG on the mica surface (**Figure S2**).

### 121 *2.3.2 Preparation of proteins-coated tips*

122 AFM probes with borosilicate glass particle (2 μm), coated with gold and modified with NH<sub>2</sub>  
123 terminated PEG linker are used (Novascan, Ames, Iowa, USA). The nominal spring constant is  
124 0.01 N/m. Milk proteins (micellar caseins, β-LG, α-LA, BSA) are prepared in distilled water at a  
125 concentration of 1 % (w/w). The rehydration is done under stirring for 2 h at room temperature and  
126 overnight at 4 °C. Proteins are adsorbed on the probe by immersion for 15 h at 4 °C. The time left  
127 for adsorption is much higher than time necessary for the proteins to absorb on the tip. Probes are  
128 rinsed with milli-Q-grade water before use.

### 129 *2.3.3 Atomic force microscopy measurements*

130 Force measurements are performed at room temperature in PBS buffer (pH 6.8) using an Asylum  
131 MFP-3D atomic force microscope (Santa Barbara, CA, USA) controlled by the operation software  
132 IGOR Pro 6.04 (Wavemetrics, Lake Oswego, OR, USA) as described by Guerin *et al.* (2016). The  
133 tip coating can slightly modify the cantilever spring constant but the modification is taken into  
134 account by readjusting the value *via* the thermal noise method. For each experiment, the force map

135 is obtained on a  $10 \times 10 \mu\text{m}^2$  surface corresponding to  $32 \times 32$  points, *i.e.* 1024 force curves. AFM  
136 force distance curves are obtained by following the cantilever deflection as a function of the vertical  
137 displacement of the piezoelectric scanner with a scan speed of 400 mm/s.

#### 138 *2.3.4 Curve processing*

139 **Determination of the percentage of adhesive events.** For each AFM force measurement, a force  
140 map containing 1024 force curves is recorded. The presence of specific adhesion between bacteria  
141 and milk proteins are determined by analyzing the retraction curves. The force profiles often show  
142 multipeaks, corresponding to the stretching of biomolecules. For each experiment, 100 force curves  
143 are selected and the corresponding retraction curves are considered to determine the percentage of  
144 adhesive events with specific biomolecule stretching signatures.

145 **Retraction curves analysis.** All retraction curves presenting specific biomolecule stretching  
146 signatures are fitted with two predictive models: FJC (Freely-Jointed Chain) model and WLC  
147 (Worm-Like Chain) model. These two models are used to describe the elongation by statistical  
148 mechanics of ideal chains (Guerin et al., 2016). These predictive models permit to access  
149 parameters such as: number of rupture, maximal rupture force needed to detach the biomolecule,  
150 the maximum extension length of the biomolecule before rupture and the contour length of the  
151 biomolecule. The maximal adhesion force needed to break the contact between bacteria and milk  
152 proteins is calculated through the analysis of the retraction curve. All the information are illustrated  
153 in **Figure 1**.

154 **SpaCBA pili mechanical behavior.** To quantify the mechanical behavior of SpaCBA pili, spring  
155 constant in different loading regimes is estimated as described by Tripathi *et al.* (2013). The pili



156 spring constant ( $k_p$ ) is determined using the slope ( $s$ ) of the linear portion of the curve and the  
157 following equation:

$$158 \quad k_p = (k_c \times s) \div (1 - s)$$

159 with  $k_c$  (pN/ $\mu\text{m}$ ) the spring constant of AFM cantilever. The constant force step values ( $F_p$ , pN)  
160 and the constant length values ( $L_p$ , nm) are determined by measuring the force and the length of  
161 the constant force plateaus on the retraction curve.

162

### 163 **3. Results and Discussion**

#### 164 **Bacterial surface characterization**

165 To better clarify and explain the adhesive interactions occurring between bacteria and milk  
166 proteins, surface properties of the different LGG mutant strains were investigated. Bacterial surface  
167 charge and hydrophobicity may be very important to explain the nature of the interactions. In this  
168 study, adhesion force measurements are performed in PBS. PBS possesses a high molarity  
169 sufficient to mask the charges of both LGG surface and proteins (Burgain et al., 2015). Therefore,  
170 the charge of bacteria should not influence the adhesive interactions with proteins. On the contrary,  
171 the surface hydrophobicity of the four different strains seems important to better highlight the  
172 nature of the interactions between bacteria and milk proteins. With the MATS method, LGG WT,  
173 LGG *welE* and LGG *welEspaCBA* showed a more hydrophilic surface character with a best affinity  
174 to chloroform compared to hexadecane. On the contrary, LGG *SpaCBA* presented a more  
175 hydrophobic surface character with a poorer affinity to chloroform (**Figure 2**).

176

177 **Key role of SpaCBA pili in milk proteins adhesion**

178 Some of the biomolecules present at the surface of LGG WT comprise the SpaCBA pili, the long  
179 galactose-rich EPS, the small glucose-rich EPS and other proteins as MBF (Mucus Binding Factor)  
180 or MabA (**Figure 3A**). AFM-based force spectroscopy is used with milk proteins coated probes to  
181 decipher how LGG WT cells interact with these individual proteins. The percentage of adhesive  
182 events presented in **Figure 3B** reveals major differences between the four milk proteins tested.

183 The percentage of specific adhesive events is of  $66.5 \pm 0.5$ ,  $3.5 \pm 1.5$  and  $9.0 \pm 0.0$  % for  $\beta$ -LG,  $\alpha$ -  
184 LA and BSA, respectively. No adhesive event is observed with caseins. The percentage of adhesive  
185 events is very high for  $\beta$ -LG. The force signatures observed with  $\beta$ -LG are very repeatable and  
186 correspond to specific events (**Figure 3C**). For  $\alpha$ -LA and BSA, the percentage of adhesive events  
187 is very low (**Figure 3B**). The force signatures corresponding to these low adhesive interactions  
188 with  $\alpha$ -LA and BSA (**Figures 3E and 3F**) are poorly repeatable which suggest that they are  
189 unspecific events.

190 The specific adhesion between LGG WT and  $\beta$ -LG reinforces previous hypothesis suggested by  
191 Guerin *et al.* (2016) who proposed that the adhesion between LGG WT and  $\beta$ -LG is mediated by  
192 SpaCBA pili. To support this result, the retraction curves recorded between these two entities are  
193 analyzed in details (**Figure 3C and 3D**). Remarkable adhesion signatures are detected on retraction  
194 curves between LGG WT and  $\beta$ -LG. Large adhesion force peaks with linear shape and  
195 characteristic horizontal force steps are observed (**Figure 3C**). These adhesion signatures are  
196 comparable to those observed by Tripathi *et al.* (2013), who demonstrate, using AFM tips  
197 decorated with SpaC proteins to pull on pili at the surface of LGG cells, that the appendages behave  
198 like a nanospring under mechanical stretching. Superimposition of multiple forces profiles reveals

199 a high reproducibility of the events (**Figure 3D**). As described by Tripathi *et al.* (2013), the specific  
200 signatures repetition are most-likely due to the intrinsic mechanical properties of SpaCBA pili. The  
201 different linear slopes and plateau values observed on the ‘step curves’ were then analyzed. The  
202 slopes of the linear segments are found to increase with increasing the applied pulling force. This  
203 behavior describes the stiffening of the stretched pilus. The spring constant of pili ( $k_p$ ) in the  
204 different loading rate is then calculated and found to be  $k_{p1} = 5.6 \pm 1.0$ ,  $k_{p2} = 11.7 \pm 0.8$  and  
205  $k_{p3} = 19.1 \pm 1.1$  pN/ $\mu$ m. Besides, the constant force steps values ( $F_{p1} = 129 \pm 8$  and  $F_{p2} = 310 \pm 8$   
206 pN) and the constant step length values ( $L_{p1} = 91 \pm 6$  and  $L_{p2} = 109 \pm 10$  nm) are highly  
207 reproducible. The values variation follow the same trend that those measured by Tripathi *et al.*  
208 (2013) and Sullan *et al.* (2014). These characteristics are reproducible and confirm the structural  
209 changes of the SpaCBA pilus induced during the retraction of the  $\beta$ -LG tip, leading to a more rigid  
210 conformation of the pilus. These results demonstrated the nanospring-like behavior of pilus  
211 SpaCBA during adhesion with the  $\beta$ -LG.

212 Caseins and whey proteins structures differ. Caseins are organized in spherical and voluminous  
213 superstructures called micelles and present a lack of secondary organization (Fox, 2008). On the  
214 contrary, whey proteins (including  $\beta$ -LG) possess a rich secondary organization with  $\beta$ -sheet and  
215  $\alpha$ -helix structures and the presence of disulfide bonds. Contrary to  $\alpha$ -LA and BSA,  $\beta$ -LG  
216 monomers are folded into eight stranded antiparallel  $\beta$ -sheets that form a hydrophobic pocket,  
217 called calix, with a 3-turn  $\alpha$ -helix on the outer surface (Monaco *et al.*, 1987), able to bind multiple  
218 hydrophobic molecules (Çelebioğlu *et al.*, 2015; Dominguez-Ramirez, Del Moral-Ramirez, Cortes-  
219 Hernandez, Garcia-Garibay, & Jimenez-Guzman, 2013; Kontopidis, Holt, & Sawyer, 2002, 2004).  
220 The difference observed in the percentage of adhesive events between LGG WT and the proteins  
221 may be due to a difference of structure. It worth consider that LGG WT interact preferentially with

222  $\beta$ -LG due to the presence of a hydrophobic calix. However the calix is probably not able to interact  
223 with a larger molecule such as the LGG pili SpaCBA protein. Moreover the SpaCBA pili is a  
224 glycosylated proteins (Tytgat et al., 2016), so the interaction between LGG WT and the  $\beta$ -LG  
225 would be more hydrophilic rather than hydrophobic. MATS method confirms the hydrophilic  
226 character of the LGG WT surface (**Figure 2**). Therefore, the hydrophilic nature of the interaction  
227 occurring between the LGG WT and the  $\beta$ -LG is confirmed.

228 To confirm the role of SpaCBA pili in adhesion to  $\beta$ -LG, the LGG *welE* strain, deprived of long  
229 rich-galactose EPS layer, is used. This mutant is known to overexpose SpaCBA pili, due to the  
230 EPS removal (**Figure 4A**) (Lebeer et al., 2012). The percentages of adhesive events between LGG  
231 *welE* and  $\beta$ -LG ( $96.5 \pm 1.5$  %) are higher than LGG WT ( $66.5 \pm 0.5$  %) (**Figure 4B**). In LGG *welE*,  
232 pili located on bacterial surface are not embedded within the EPS layer and are entirely free to  
233 establish contact points with  $\beta$ -LG. A recent work has demonstrated the glycosylated behavior of  
234 the stretched biomolecule during interaction between  $\beta$ -LG and LGG WT and LGG *welE* (Guerin  
235 et al., 2016). It appears that the SpaC pilins of the SpaCBA pili are the pilins carrying the  
236 glycosylation (Tytgat et al., 2016), suggesting that SpaC subunits is implicated in  $\beta$ -LG interaction.  
237 In LGG *welE*, the increased exposure of SpaC pilins at the basal part of each pilus could allow  
238 SpaCBA pili to establish more contact points with  $\beta$ -LG resulting in an increased number of rupture  
239 events in the retraction curve (Lebeer et al., 2012). The specific biomolecules stretching signatures  
240 between  $\beta$ -LG and LGG *welE* differed from LGG WT with the apparition of more irregular and  
241 jerky signatures and a more important maximal rupture distance ( $2.8 \pm 0.5$  and  $1.1 \pm 0.1$   $\mu$ m for  
242 LGG *welE* and LGG WT, respectively) (**Figure 4C**). As the size of a SpaCBA pilus is around 1  
243  $\mu$ m (Guerin et al., 2016; Tripathi et al., 2013), the biomolecule stretched at a distance of around  
244 2.8  $\mu$ m with LGG *welE* might not be an individual pilus. In fact, the pilus SpaCBA is able to

245 interact each other with a zipper-like adhesion mechanism involving multiple SpaC molecules  
246 distributed along the pilus length. In LGG *welE*, SpaCBA pili are not embedded in EPS layer and  
247 are totally free to interact with each other. SpaCBA pili may form a network entirely engaged in  
248 the interaction and leading to the observed numerous ruptures and the presence of jerky signatures  
249 during the retraction of the tip containing  $\beta$ -LG which leads to a stretched distance of 2.8  $\mu\text{m}$  with  
250 LGG *welE*.

251 Surprisingly, a lot of adhesive events are observed between LGG *welE* and other milk proteins  
252 (caseins,  $\alpha$ -LA and BSA). The percentages of adhesive events are of  $56.0 \pm 6.5$ ,  $98.5 \pm 1.5$  and  
253  $88.5 \pm 2.5$  % for caseins,  $\alpha$ -LA and BSA, respectively (**Figure 4B**). It was previously shown that  
254 these three proteins poorly interact with LGG WT. In contrast, a lot of specific signatures, similar  
255 to those observed with  $\beta$ -LG, are observed with LGG *welE* (**Figure 3C, E and F**). In LGG *welE*,  
256 the over-exposition of the SpaCBA pili in absence of long EPS layer could allowed caseins,  $\alpha$ -LA  
257 and BSA to interact with SpaCBA pili. An explanation would be that, for LGG *welE*, the absence  
258 of long EPS lead to uncover pili subunits initially buried in the EPS structure. It can be the case of  
259 the SpaB pilins, which are located at the pilus base. In the absence of long-EPS layer, these SpaB  
260 subunits would be accessible to promote interaction between LGG *welE* and these three milk  
261 proteins. To verify this hypothesis, adhesion force measurements should be made between milk  
262 proteins and purified SpaCBA pili or purified subunits SpaB of the pilus. The rupture distance  
263 observed on retraction curve was too long to believe that other surface biomolecules such as Maba  
264 and MBF proteins or small EPS governed interaction between LGG *welE* and milk proteins.

265 In conclusion, the SpaCBA pili environment seems to determine LGG ability to interact with milk  
266 proteins. In presence of long-EPS layer, the SpaCBA pili interact preferably with  $\beta$ -LG. In absence  
267 of long-EPS layer, SpaCBA pili possess the ability to adhere with all the studied milk proteins.

**269 Role of EPS and other surface proteins in adhesion to milk proteins in absence of SpaCBA**

270 **pili.** To study the role of others LGG surface biomolecules (*i.e.* other than SpaCBA pili), two LGG  
271 mutants depleted in SpaCBA pili are compared: LGG *spaCBA* and LGG *welEspaCBA*. LGG  
272 *spaCBA* surface is composed of a long rich-galactose EPS layer, small glucose-rich EPS and  
273 proteins as MBF or MabA (**Figure 5A**) (Francius et al., 2009; Perea Vélez et al., 2010; von  
274 Ossowski et al., 2011). The percentages of adhesive events between LGG *spaCBA* and the different  
275 milk proteins are of  $13.5 \pm 5.5$ ,  $13.5 \pm 0.5$  and  $11.0 \pm 3.0$  % for  $\beta$ -LG,  $\alpha$ -LA and BSA, respectively  
276 (**Figure 5B**) and are not significantly different from each other. First, results observed with  $\beta$ -LG  
277 demonstrated a dramatic decrease of adhesive events in absence of SpaCBA pili on LGG surface,  
278 which reinforce the key role of pili in adhesion between LGG and  $\beta$ -LG. In contrast, specific  
279 biomolecules stretching signatures recorded on retraction curves are rare but highly reproducible  
280 for all whey proteins (**Figure 5C, D, E**). The rupture number is ranging from 1 and 2, the maximal  
281 force varies between 100 and 200 pN and the maximal stretching distance is ranging from 0.6 and  
282 1.4  $\mu$ m. These signatures are similar to those observed by Francius *et al.* (2009) when they used  
283 lectin-functionalized AFM tips. Francius *et al.* (2009) obtained the same multiple force peak with  
284 a magnitude ranging from 50 to 100 pN and up to 1000 nm length during the stretching of EPS. In  
285 another work, similar results are obtained during the LGG *spaCBA* adhesion to hydrophobic  
286 surfaces. The observed forces profiles resemble to those obtained from stretching of EPS on LGG  
287 cells (Sullan et al., 2014). Signatures recorded with LGG *spaCBA* during biomolecules stretching  
288 seem to be those of sugars which should correspond to the stretching behavior of EPS (Francius et  
289 al., 2009). Thus, in the absence of SpaCBA pilus, all whey proteins may be able to interact with  
290 LGG via EPS. With the casein-modified tip, interaction with bacterial surface reveals specific

291 signatures of biomolecules stretching (**Figure 5F**). The rupture number is of  $2.8 \pm 0.2$ , the maximal  
292 force is of  $1.8 \pm 0.1$  pN and the maximal stretching distance is of  $1.1 \pm 0.1$   $\mu\text{m}$  (**Figure 5F**). With  
293 caseins, the particular shape of the retraction curves does not allow the determination of the  
294 biomolecule involved in the interaction with LGG SpaCBA.

295 In conclusion, these results demonstrated the ability of EPS to interact with milk proteins, in a less  
296 pronounced way than pili and only when pili are absent. These results suppose that the presence of  
297 pili masked the other biomolecules and inhibit their ability to mediate adhesion to milk proteins.

298 The surface of LGG *welEspaCBA* mutant is composed of different biomolecules such as small  
299 glucose-rich EPS, other proteins as MBF or MabA, lipoteichoic acids or peptidoglycans (**Figure**  
300 **6A**) (Lebeer et al., 2012). The percentages of adhesive events measured with LGG *welEspaCBA*  
301 are of  $44.0 \pm 6.7$ ,  $17.5 \pm 0.5$  and  $14.0 \pm 2.0$  % for  $\beta$ -LG,  $\alpha$ -LA and BSA, respectively (**Figure 6B**).

302 No specific adhesive events are observed between LGG *welEspaCBA* and caseins. The specific  
303 signatures recorded for whey proteins on retraction curves are presented on **Figures 6C, D and E**.

304 These specific signatures revealed the presence of surface biomolecules, other than SpaCBA pili  
305 and long EPS, implicated in adhesion with whey proteins. The observed signatures may recall the  
306 small EPS stretching (Francius et al., 2009). These signatures may also be due to the detection of  
307 peptidoglycan, accessible in the absence of both long-EPS and SpaCBA pili, with the milk proteins  
308 (Beaussart et al., 2013) and the observed peak will be due to the stretching of milk proteins.

309 Nevertheless, other surface mutants will be required to validate these hypotheses.

310

311 **Conclusion**

312 AFM in force mode was used in this work to underline the role of LGG surface biomolecules in  
313 adhesion to milk proteins. The high frequency of adhesive events demonstrated that the SpaCBA  
314 pili and other LGG surface biomolecules such as EPS and proteins are involved in the adhesion.  
315 However, the ability of each biomolecule to interact with milk proteins depend on their  
316 environment. In LGG WT, only the pili are implicated in adhesive interactions with the  $\beta$ -LG as  
317 other surface biomolecules are masked. When pili are absents, the results demonstrated that EPS  
318 are also able to interact with all milk proteins, in a less pronounced way than pili. This work  
319 revealed the ability of LGG surface biomolecules to interact to different milk proteins. Depending  
320 on the surface biomolecules composition, LAB present different affinity with milk components. In  
321 that respect, it is expected that the media and the growth phase may have an impact on the  
322 preferential location of bacteria in the dairy matrix, and could be extended to other LAB strains.

323

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327

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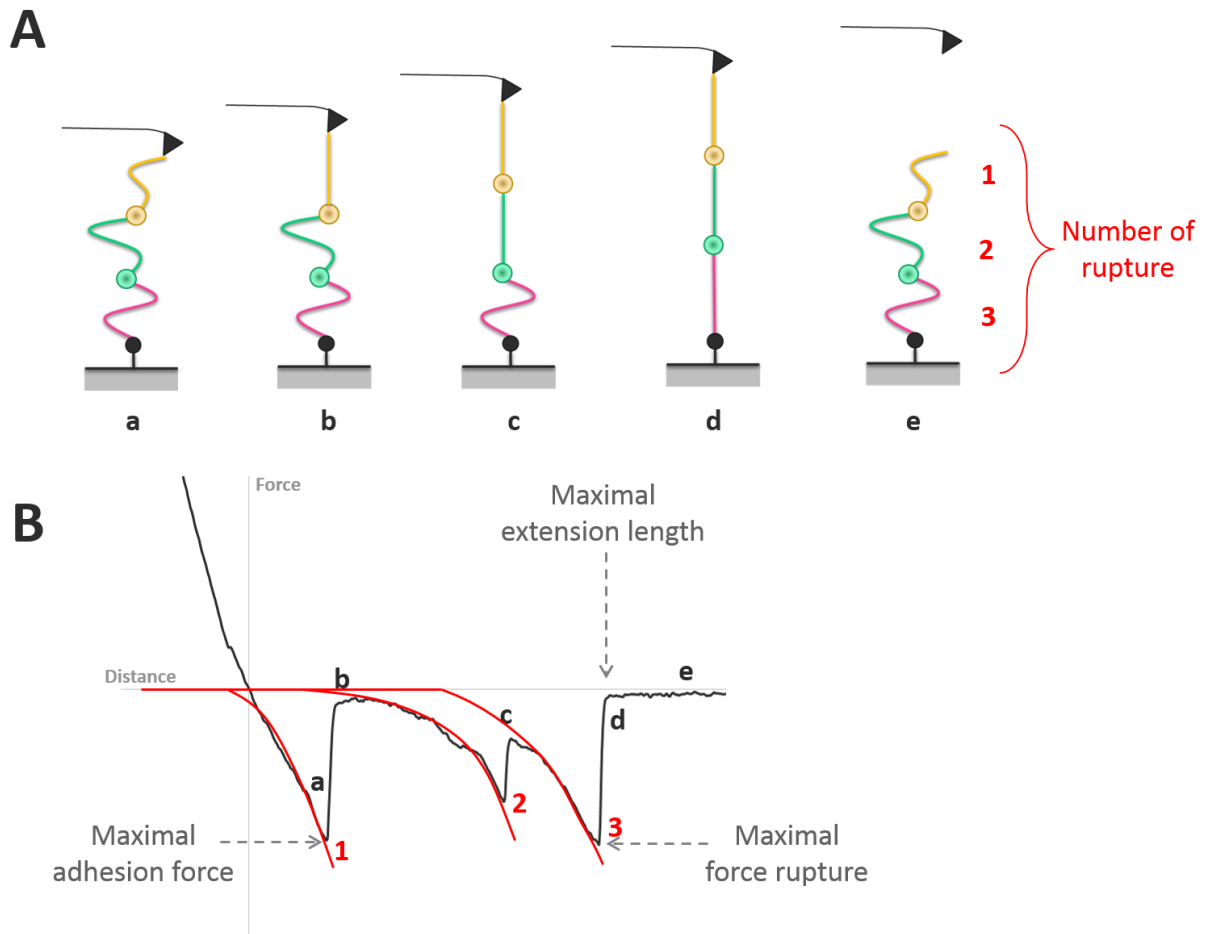
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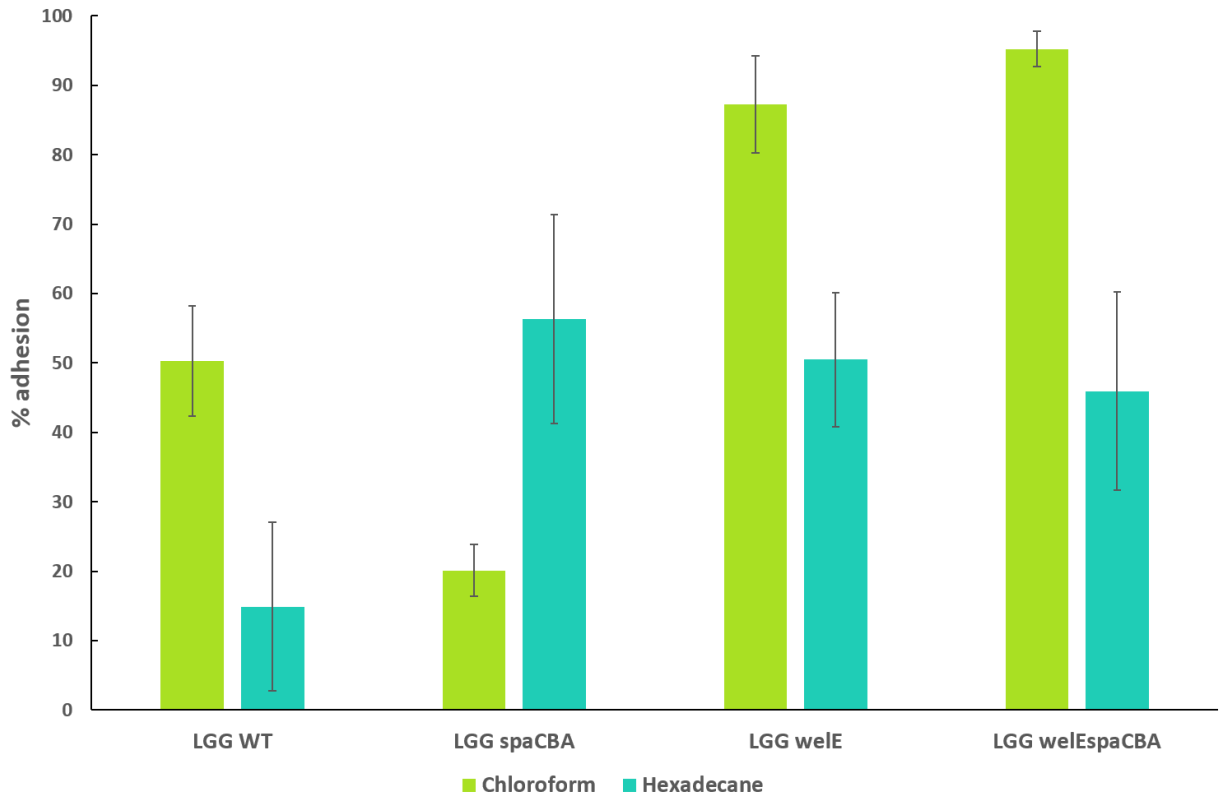
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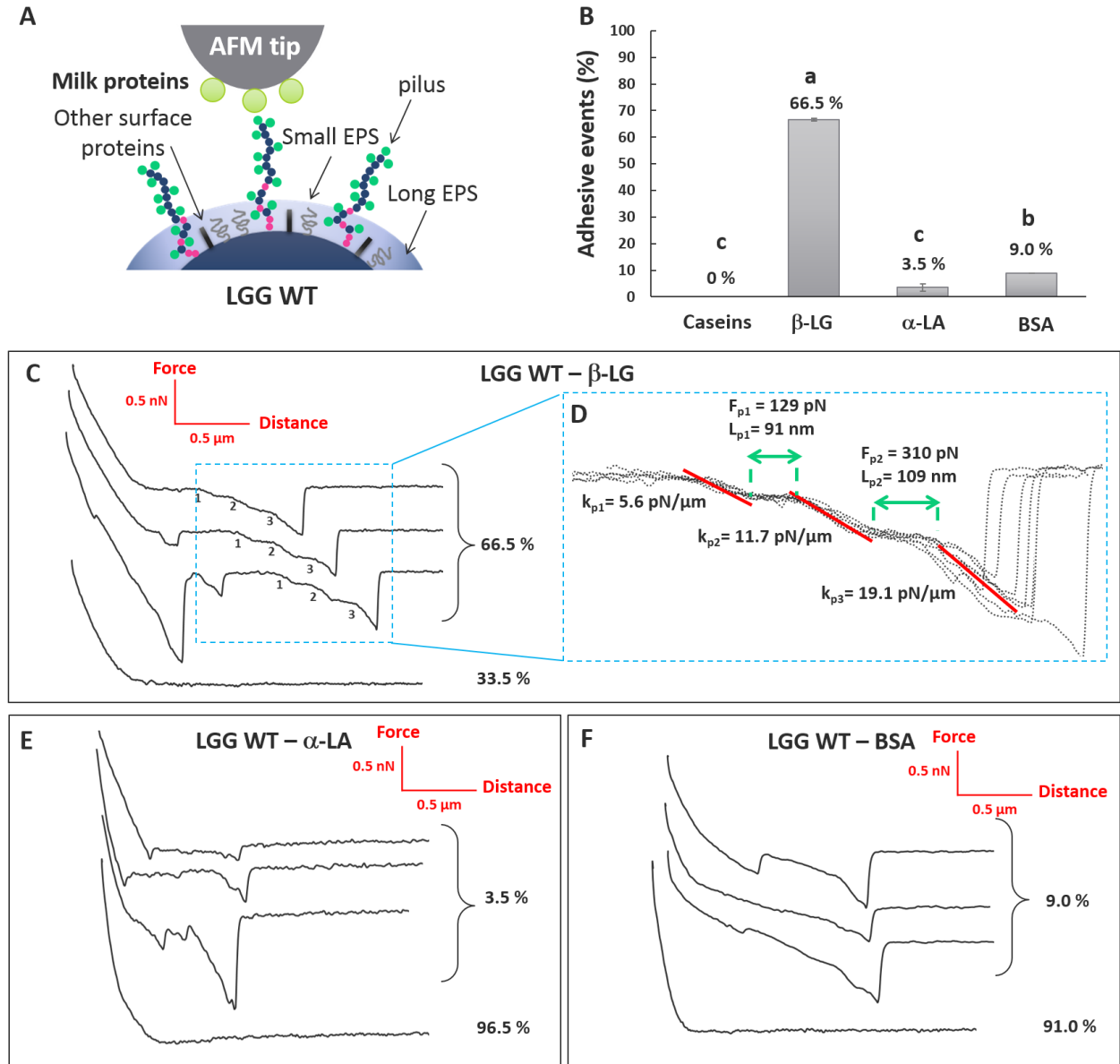
443 **Figure 2**



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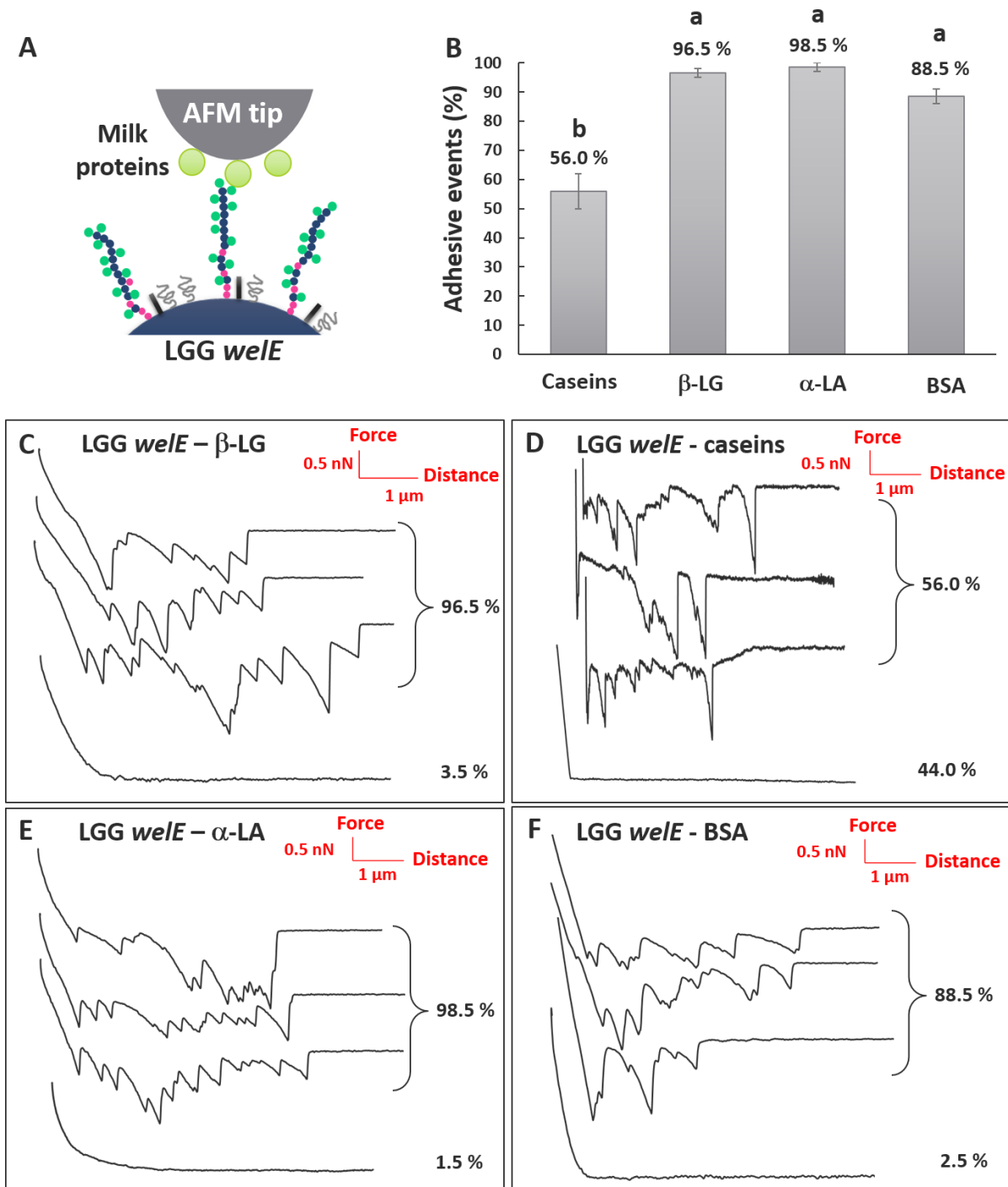
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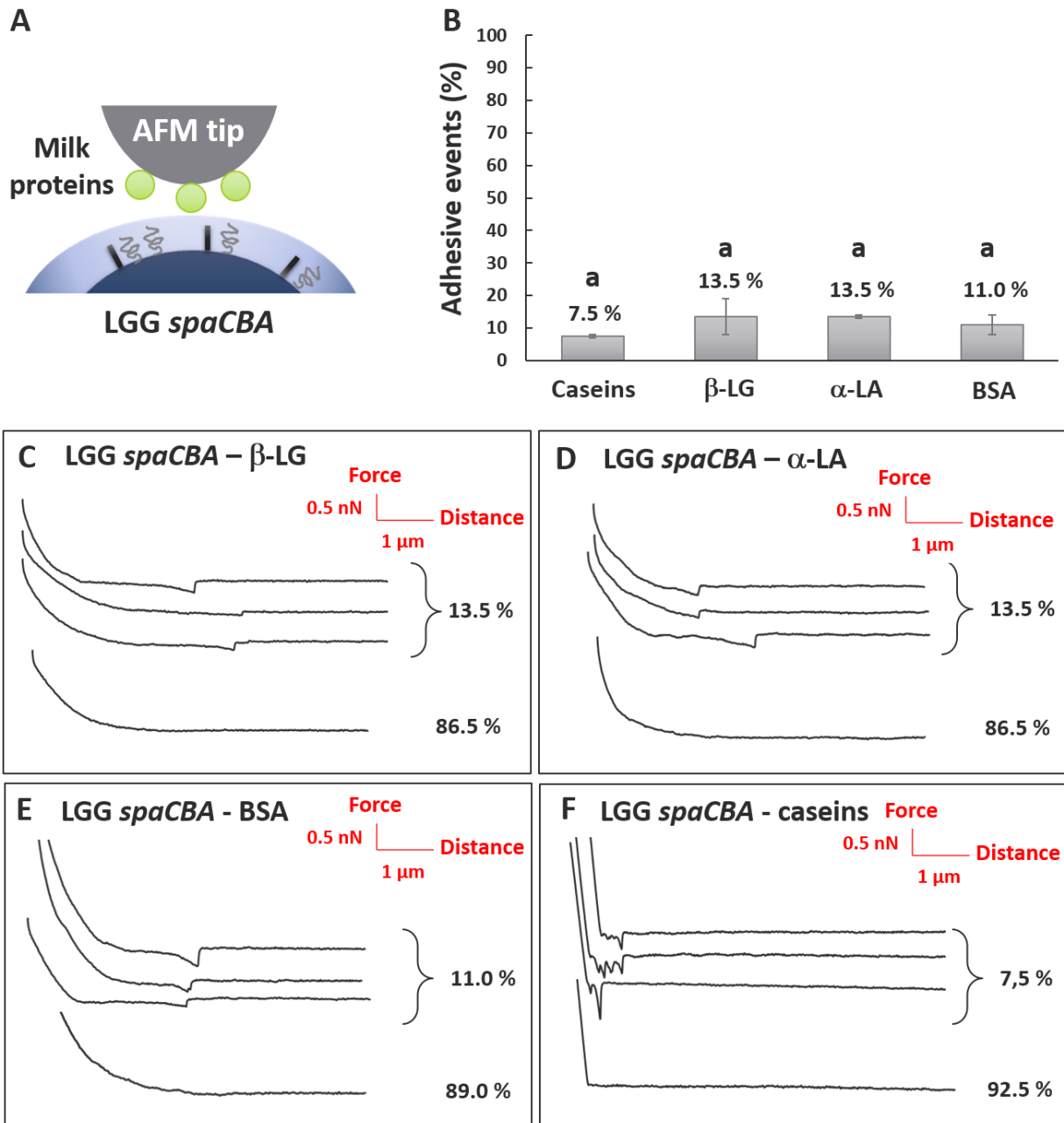


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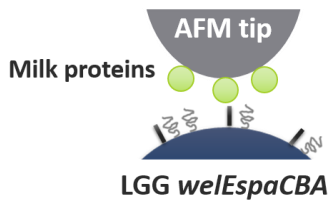




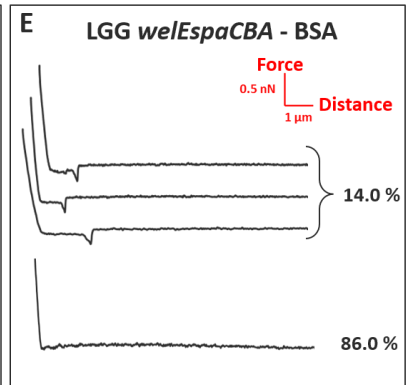
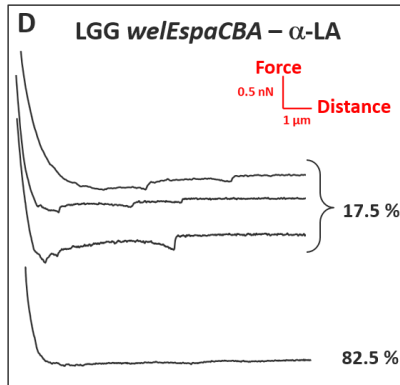
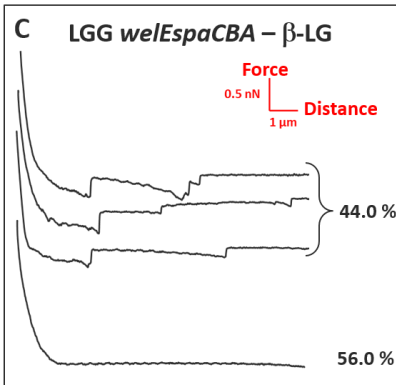
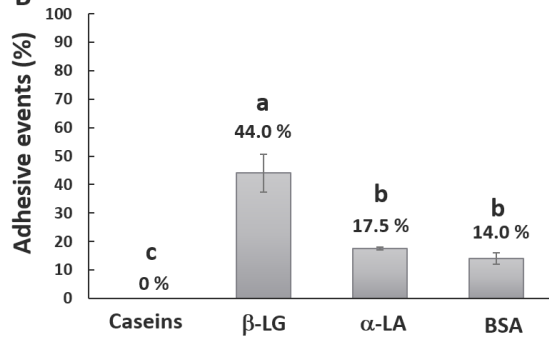


455 **Figure 6**

**A**



**B**



456

457

458

## Figure captions

459

460 **Figure 1:** Illustration of a biomolecule displaying force-induced structural modification (A).  
461 Example of data obtained by adjustment of the retraction curve with the FJC or WLC models (red  
462 lines) (B).

463

464 **Figure 2:** Microbial adhesion to solvents test to determine the surface hydrophobicity of LGG WT  
465 and the three mutants: LGG *spaCBA*, LGG *welE* and LGG *welEspaCBA*.

466

467 **Figure 3:** Adhesive interactions between LGG WT and milk proteins (caseins,  $\beta$ -LG,  $\alpha$ -LA and  
468 BSA).

469 *Schema of the principle of AFM-based force spectroscopy to measure the interaction between milk*  
470 *proteins coated probes and the surface of LGG WT (A). Frequency of adhesive events between*  
471 *LGG and individual milk proteins (B). Representative retraction curves recorded between LGG*  
472 *and  $\beta$ -LG (C). Superimposition of retraction curves and determination of spring-like properties of*  
473 *pili *SpaCBA* (D). Representative retraction curves recorded between LGG and  $\alpha$ -LA (E) or BSA*  
474 *(F).*

475

476 **Figure 4:** Adhesion between LGG *welE* and milk proteins.

477 *Schema of the principle of AFM-based force spectroscopy to measure the interaction between milk*  
478 *proteins coated probes and the surface of LGG *welE* (A). Frequency of adhesive events between*  
479 *LGG and individual milk proteins (B). Representative retraction curves recorded between LGG*  
480 *and  $\beta$ -LG (C), caseins (D),  $\alpha$ -LA (E) or BSA (F).*

481

482 **Figure 5:** Adhesion between LGG *spaCBA* and milk proteins.

483 *Schema of the principle of AFM-based force spectroscopy to measure the interaction between milk*  
484 *proteins coated probes and the surface of LGG *spaCBA* (A). Frequency of adhesive events between*  
485 *LGG and individual milk proteins (B). Representative retraction curves recorded between LGG*  
486 *and  $\beta$ -LG (C),  $\alpha$ -LA (D), BSA (E) or caseins (F).*

487

488 **Figure 6:** Adhesion between LGG *welEspaCBA* and milk proteins.

489 *Schema of the principle of AFM-based force spectroscopy to measure the interaction between milk*  
490 *proteins coated probes and the surface of LGG *welEspaCBA* (A). Frequency of adhesive events*

491 *between LGG and individual milk proteins (B). Representative retraction curves recorded between*  
492 *LGG and  $\beta$ -LG (C),  $\alpha$ -LA (D) or BSA (E).*  
493