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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 β -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 β -lactoglobulin (β -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, β -LG, α -lactalbumin (α -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 β -LG, α -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 NH₂ 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 NH₂
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 32×32 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 nm/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 multi-peaks, 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/ μ 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 ± 0.5 , 3.5 ± 1.5 and 9.0 ± 0.0 % for β -LG, α -
184 LA and BSA, respectively. No adhesive event is observed with caseins. The percentage of adhesive
185 events is very high for β -LG. The force signatures observed with β -LG are very repeatable and
186 correspond to specific events (**Figure 3C**). For α -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 α -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 β -LG reinforces previous hypothesis suggested by
191 Guerin *et al.* (2016) who proposed that the adhesion between LGG WT and β -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 β -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/ μ 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 β -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 β -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 β -LG) possess a rich secondary organization with β -sheet and
215 α -helix structures and the presence of disulfide bonds. Contrary to α -LA and BSA, β -LG
216 monomers are folded into eight stranded antiparallel β -sheets that form a hydrophobic pocket,
217 called calix, with a 3-turn α -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 β -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 β -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 β -LG is confirmed.

228 To confirm the role of SpaCBA pili in adhesion to β -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 β -LG (96.5 ± 1.5 %) are higher than LGG WT (66.5 ± 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 β -LG. A recent work has demonstrated the glycosylated behavior of
234 the stretched biomolecule during interaction between β -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 β -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 β -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 β -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 ± 0.5 and 1.1 ± 0.1 μ m for
242 LGG *welE* and LGG WT, respectively) (**Figure 4C**). As the size of a SpaCBA pilus is around 1
243 μ m (Guerin et al., 2016; Tripathi et al., 2013), the biomolecule stretched at a distance of around
244 2.8 μ 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 β -LG which leads to a stretched distance of 2.8 μm with
250 LGG *welE*.

251 Surprisingly, a lot of adhesive events are observed between LGG *welE* and other milk proteins
252 (caseins, α -LA and BSA). The percentages of adhesive events are of 56.0 ± 6.5 , 98.5 ± 1.5 and
253 88.5 ± 2.5 % for caseins, α -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 β -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, α -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 β -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 ± 5.5 , 13.5 ± 0.5 and 11.0 ± 3.0 % for β -LG, α -LA and BSA, respectively
276 (**Figure 5B**) and are not significantly different from each other. First, results observed with β -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 β -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 μ 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 ± 0.2 , the maximal
292 force is of 1.8 ± 0.1 pN and the maximal stretching distance is of 1.1 ± 0.1 μm (**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 ± 6.7 , 17.5 ± 0.5 and 14.0 ± 2.0 % for β -LG, α -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 β -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

328 **References**

329 Beaussart, A., Rolain, T., Duchêne, M.-C., El-Kirat-Chatel, S., Andre, G., Hols, P., & Dufrêne, Y.
330 F. (2013). Binding Mechanism of the Peptidoglycan Hydrolase Acm2: Low Affinity, Broad
331 Specificity. *Biophysical Journal*, *105*(3), 620–629.
332 <https://doi.org/10.1016/j.bpj.2013.06.035>

333 Burgain, J., Gaiani, C., Francius, G., Revol-Junelles, A. M., Cailliez-Grimal, C., Lebeer, S., ...
334 Scher, J. (2013). In vitro interactions between probiotic bacteria and milk proteins probed
335 by atomic force microscopy. *Colloids and Surfaces. B, Biointerfaces*, 104, 153–162.
336 <https://doi.org/10.1016/j.colsurfb.2012.11.032>

337 Burgain, J., Scher, J., Lebeer, S., Vanderleyden, J., Corgneau, M., Guerin, J., ... Gaiani, C. (2015).
338 Impacts of pH-mediated EPS structure on probiotic bacterial pili–whey proteins
339 interactions. *Colloids and Surfaces B: Biointerfaces*, 134, 332–338.
340 <https://doi.org/10.1016/j.colsurfb.2015.06.068>

341 Çelebioğlu, H. Y., Gudjónsdóttir, M., Meier, S., Duus, J. Ø., Lee, S., & Chronakis, I. S. (2015).
342 Spectroscopic studies of the interactions between β -lactoglobulin and bovine submaxillary
343 mucin. *Food Hydrocolloids*, 50, 203–210. <https://doi.org/10.1016/j.foodhyd.2015.04.026>

344 Dominguez-Ramirez, L., Del Moral-Ramirez, E., Cortes-Hernandez, P., Garcia-Garibay, M., &
345 Jimenez-Guzman, J. (2013). beta-lactoglobulin's conformational requirements for ligand
346 binding at the calyx and the dimer interphase: a flexible docking study. *PLoS One*, 8(11),
347 e79530. <https://doi.org/10.1371/journal.pone.0079530>

348 Fox, P. F. (2008). Milk: an overview. In A. Thompson, M. Boland, & H. Singh (Eds.), *Milk*
349 *Proteins* (pp. 1–54). San Diego: Academic Press. [https://doi.org/10.1016/B978-0-12-](https://doi.org/10.1016/B978-0-12-374039-7.00001-5)
350 [374039-7.00001-5](https://doi.org/10.1016/B978-0-12-374039-7.00001-5)

351 Francius, G., Alsteens, D., Dupres, V., Lebeer, S., De Keersmaecker, S., Vanderleyden, J., ...
352 Dufrière, Y. F. (2009). Stretching polysaccharides on live cells using single molecule force
353 spectroscopy. *Nature Protocols*, 4(6), 939–946. <https://doi.org/10.1038/nprot.2009.65>

354 Guerin, J., Bacharouche, J., Burgain, J., Lebeer, S., Francius, G., Borges, F., ... Gaiani, C. (2016).
355 Pili of *Lactobacillus rhamnosus* GG mediate interaction with β -lactoglobulin. *Food*
356 *Hydrocolloids*, 58, 35–41. <https://doi.org/10.1016/j.foodhyd.2016.02.016>

357 Hickey, C. D., Auty, M. A. E., Wilkinson, M. G., & Sheehan, J. J. (2015). The influence of cheese
358 manufacture parameters on cheese microstructure, microbial localisation and their
359 interactions during ripening: A review. *Trends in Food Science & Technology*, *41*(2), 135–
360 148. <https://doi.org/10.1016/j.tifs.2014.10.006>

361 Hickey, C. D., Sheehan, J. J., Wilkinson, M. G., & Auty, M. A. E. (2015). Growth and location of
362 bacterial colonies within dairy foods using microscopy techniques: a review. *Food*
363 *Microbiology*, *6*, 99. <https://doi.org/10.3389/fmicb.2015.00099>

364 Kankainen, M., Paulin, L., Tynkkynen, S., von Ossowski, I., Reunanen, J., Partanen, P., ... de Vos,
365 W. M. (2009). Comparative genomic analysis of *Lactobacillus rhamnosus* GG reveals pili
366 containing a human- mucus binding protein. *Proc Natl Acad Sci U S A*, *106*(40), 17193–
367 17198. <https://doi.org/10.1073/pnas.0908876106>

368 Kontopidis, G., Holt, C., & Sawyer, L. (2002). The ligand-binding site of bovine beta-
369 lactoglobulin: evidence for a function? *J Mol Biol*, *318*(4), 1043–1055.
370 [https://doi.org/10.1016/s0022-2836\(02\)00017-7](https://doi.org/10.1016/s0022-2836(02)00017-7)

371 Kontopidis, G., Holt, C., & Sawyer, L. (2004). Invited Review: β -Lactoglobulin: Binding
372 Properties, Structure, and Function. *Journal of Dairy Science*, *87*(4), 785–796.
373 [https://doi.org/10.3168/jds.S0022-0302\(04\)73222-1](https://doi.org/10.3168/jds.S0022-0302(04)73222-1)

374 Laloy, E., Vuilleumard, J.-C., El Soda, M., & Simard, R. E. (1996). Influence of the fat content of
375 Cheddar cheese on retention and localization of starters. *International Dairy Journal*, *6*(7),
376 729–740. [https://doi.org/10.1016/0958-6946\(95\)00068-2](https://doi.org/10.1016/0958-6946(95)00068-2)

377 Lebeer, S., Claes, I. J. J., Verhoeven, T. L. A., Vanderleyden, J., & De Keersmaecker, S. C. J.
378 (2011). Exopolysaccharides of *Lactobacillus rhamnosus* GG form a protective shield
379 against innate immune factors in the intestine. *Microbial Biotechnology*, *4*(3), 368–374.
380 <https://doi.org/10.1111/j.1751-7915.2010.00199.x>

381 Lebeer, S., Claes, I., Tytgat, H. L., Verhoeven, T. L., Marien, E., von Ossowski, I., ...
382 Vanderleyden, J. (2012). Functional analysis of *Lactobacillus rhamnosus* GG pili in relation
383 to adhesion and immunomodulatory interactions with intestinal epithelial cells. *Appl*
384 *Environ Microbiol*, 78(1), 185–193. <https://doi.org/10.1128/aem.06192-11>

385 Lebeer, S., Verhoeven, T. L., Francius, G., Schoofs, G., Lambrichts, I., Dufrene, Y., ... De
386 Keersmaecker, S. C. (2009). Identification of a gene cluster for the biosynthesis of a long
387 galactose-rich exopolysaccharide in *Lactobacillus rhamnosus* GG and functional analysis
388 of the priming glycosyltransferase. *Appl Environ Microbiol*, 75, 3554–3563.
389 <https://doi.org/10.1128/AEM.02919-08>

390 Leroy, F., & De Vuyst, L. (2004). Lactic acid bacteria as functional starter cultures for the food
391 fermentation industry. *Trends in Food Science & Technology*, 15(2), 67–78.
392 <https://doi.org/10.1016/j.tifs.2003.09.004>

393 Linares, D. M., Ross, P., & Stanton, C. (2016). Beneficial Microbes: The pharmacy in the gut:
394 Bioengineered: Vol 7, No 1. *Bioengineered*, 7, 11–20.

395 Lopez, C., Maillard, M.-B., Briard-Bion, V., Camier, B., & Hannon, J. A. (2006). Lipolysis during
396 ripening of Emmental cheese considering organization of fat and preferential localization
397 of bacteria. *Journal of Agricultural and Food Chemistry*, 54(16), 5855–5867.
398 <https://doi.org/10.1021/jf060214l>

399 Monaco, H. L., Zanotti, G., Spadon, P., Bolognesi, M., Sawyer, L., & Eliopoulos, E. E. (1987).
400 Crystal structure of the trigonal form of bovine beta-lactoglobulin and of its complex with
401 retinol at 2.5 Å resolution. *J Mol Biol*, 197(4), 695–706.

402 Oberg, C., McManus, W., & McMahon, D. (1993). Microstructure of Mozzarella Cheese During
403 Manufacture. *Food Structure*, 12(2). Retrieved from
404 <http://digitalcommons.usu.edu/foodmicrostructure/vol12/iss2/12>

405 Perea Vélez, M., Petrova, M. I., Lebeer, S., Verhoeven, T. L. A., Claes, I., Lambrichts, I., ... De
406 Keersmaecker, S. C. J. (2010). Characterization of MabA, a modulator of *Lactobacillus*
407 *rhamnosus* GG adhesion and biofilm formation. *FEMS Immunology & Medical*
408 *Microbiology*, 59(3), 386–398. <https://doi.org/10.1111/j.1574-695X.2010.00680.x>

409 Reunanen, J., von Ossowski, I., Hendrickx, A. P., Palva, A., & de Vos, W. M. (2012).
410 Characterization of the SpaCBA pilus fibers in the probiotic *Lactobacillus rhamnosus* GG.
411 *Appl Environ Microbiol*, 78(7), 2337–2344. <https://doi.org/10.1128/aem.07047-11>

412 Salotti de Souza, B. M., Borgonovi, T. F., Casarotti, S. N., Todorov, S. D., & Penna, A. L. B.
413 (2018). *Lactobacillus casei* and *Lactobacillus fermentum* Strains Isolated from Mozzarella
414 Cheese: Probiotic Potential, Safety, Acidifying Kinetic Parameters and Viability under
415 Gastrointestinal Tract Conditions. *Probiotics and Antimicrobial Proteins*.
416 <https://doi.org/10.1007/s12602-018-9406-y>

417 Sullan, R. M. A., Beaussart, A., Tripathi, P., Derclaye, S., El-Kirat-Chatel, S., Li, J. K., ... Dufrene,
418 Y. F. (2014). Single-cell force spectroscopy of pili-mediated adhesion. *Nanoscale*, 6(2),
419 1134–1143. <https://doi.org/10.1039/c3nr05462d>

420 Tripathi, P., Beaussart, A., Alsteens, D., Dupres, V., Claes, I., von Ossowski, I., ... Dufrene, Y. F.
421 (2013). Adhesion and Nanomechanics of Pili from the Probiotic *Lactobacillus rhamnosus*
422 GG. *ACS Nano*, 7(4), 3685–3697. <https://doi.org/10.1021/nn400705u>

423 Tripathi, P., Dupres, V., Beaussart, A., Lebeer, S., Claes, I. J. J., Vanderleyden, J., & Dufrene, Y.
424 F. (2012). Deciphering the Nanometer-Scale Organization and Assembly of *Lactobacillus*
425 *rhamnosus* GG Pili Using Atomic Force Microscopy. *Langmuir*, 28(4), 2211–2216.
426 <https://doi.org/10.1021/la203834d>

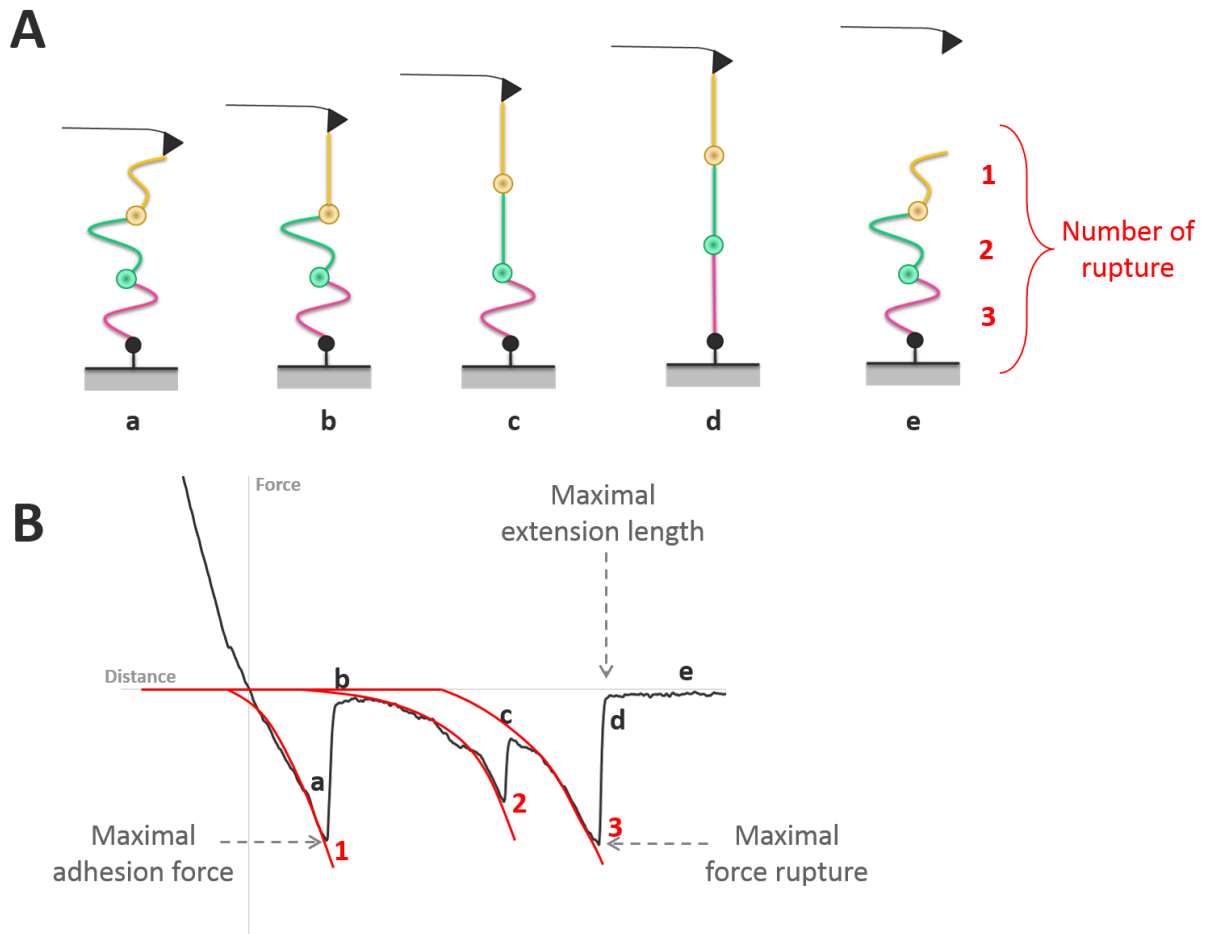
427 Tunick, M. H., Mackey, K. L., Shieh, J. J., Smith, P. W., Cooke, P., & Malin, E. L. (1993).
428 Rheology and microstructure of low-fat Mozzarella cheese. *International Dairy Journal*,
429 3(7), 649–662. [https://doi.org/10.1016/0958-6946\(93\)90106-A](https://doi.org/10.1016/0958-6946(93)90106-A)

430 Tytgat, H. L. P., van Teijlingen, N. H., Sullan, R. M. A., Douillard, F. P., Rasinkangas, P., Messing,
431 M., ... Lebeer, S. (2016). Probiotic Gut Microbiota Isolate Interacts with Dendritic Cells
432 via Glycosylated Heterotrimeric Pili. *PloS One*, 11(3), e0151824.
433 <https://doi.org/10.1371/journal.pone.0151824>

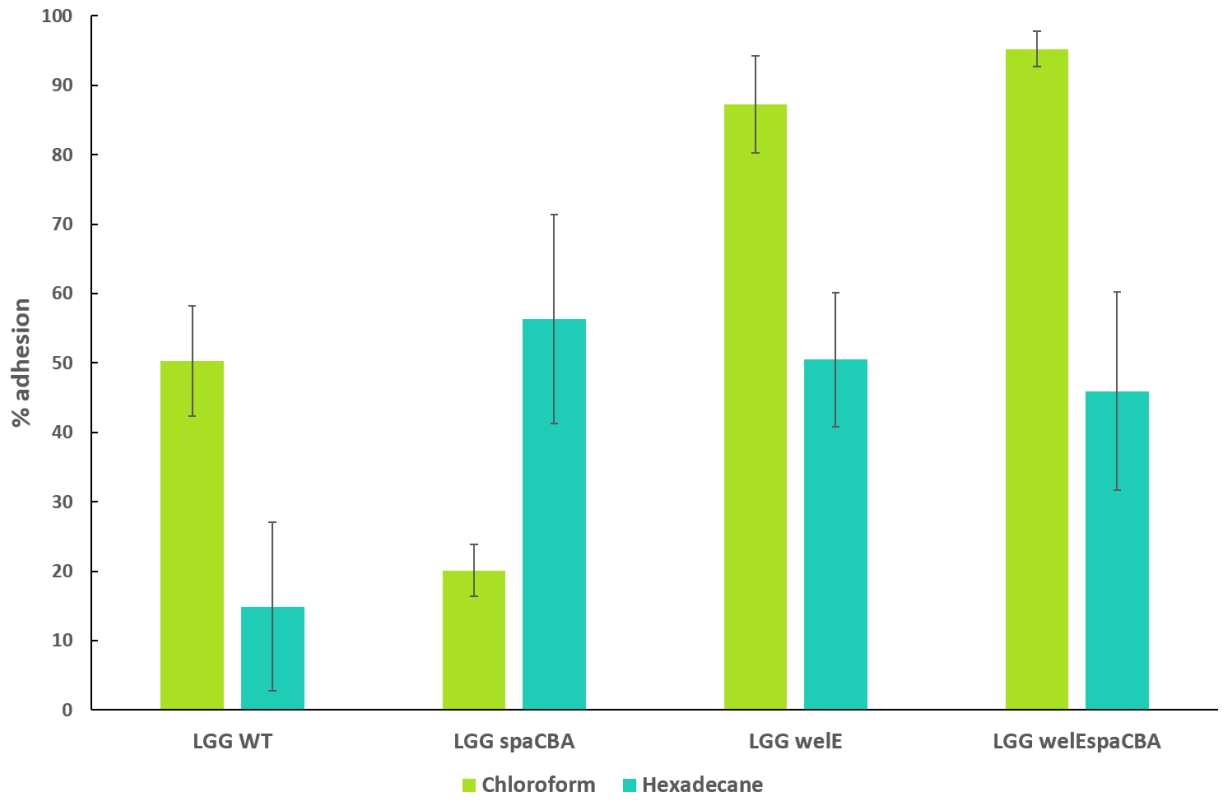
434 von Ossowski, I., Satokari, R., Reunanen, J., Lebeer, S., De Keersmaecker, S. C. J., Vanderleyden,
435 J., ... Palva, A. (2011). Functional characterization of a mucus-specific LPXTG surface
436 adhesin from probiotic *Lactobacillus rhamnosus* GG. *Applied and Environmental*
437 *Microbiology*, 77(13), 4465–4472. <https://doi.org/10.1128/AEM.02497-10>

438

439



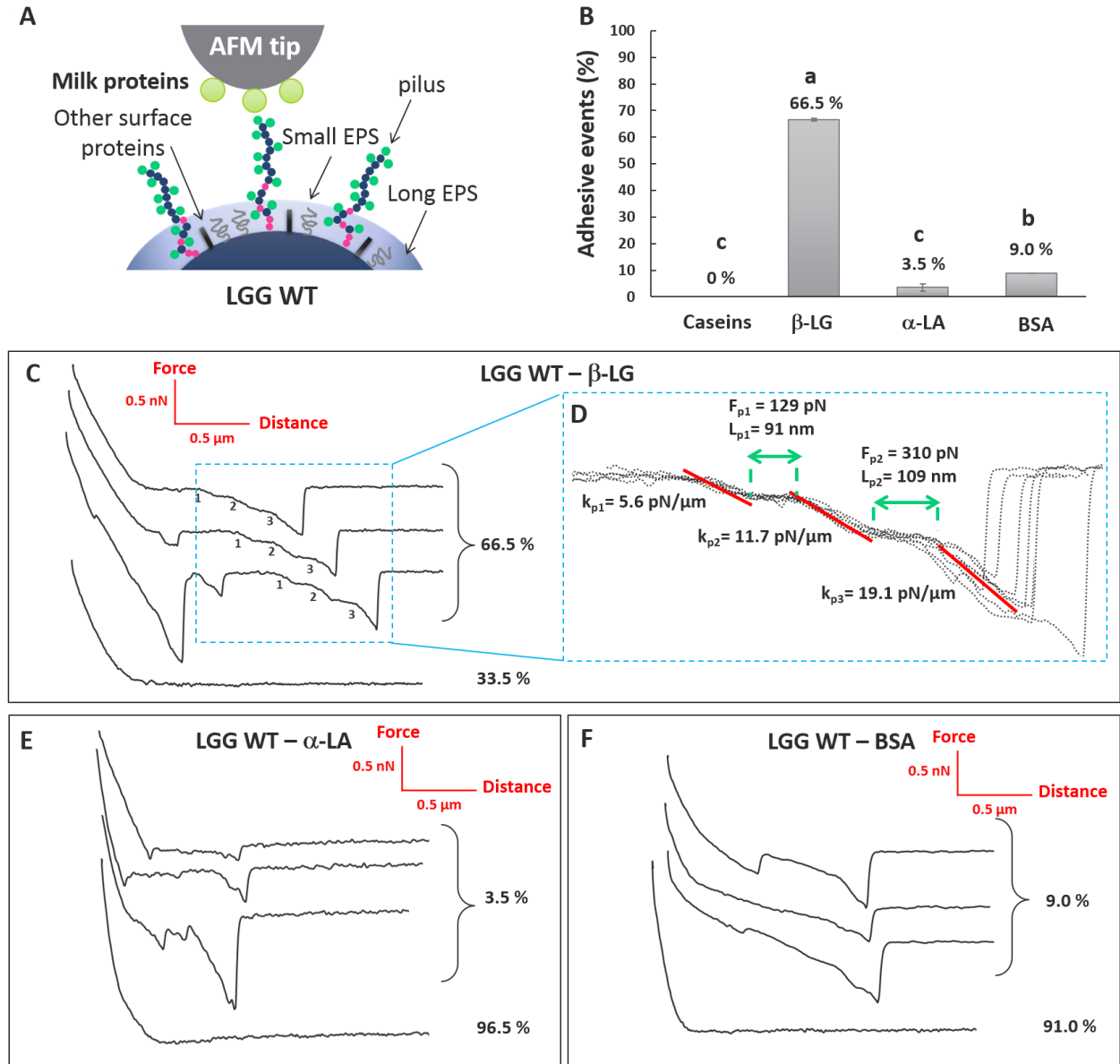
443 **Figure 2**



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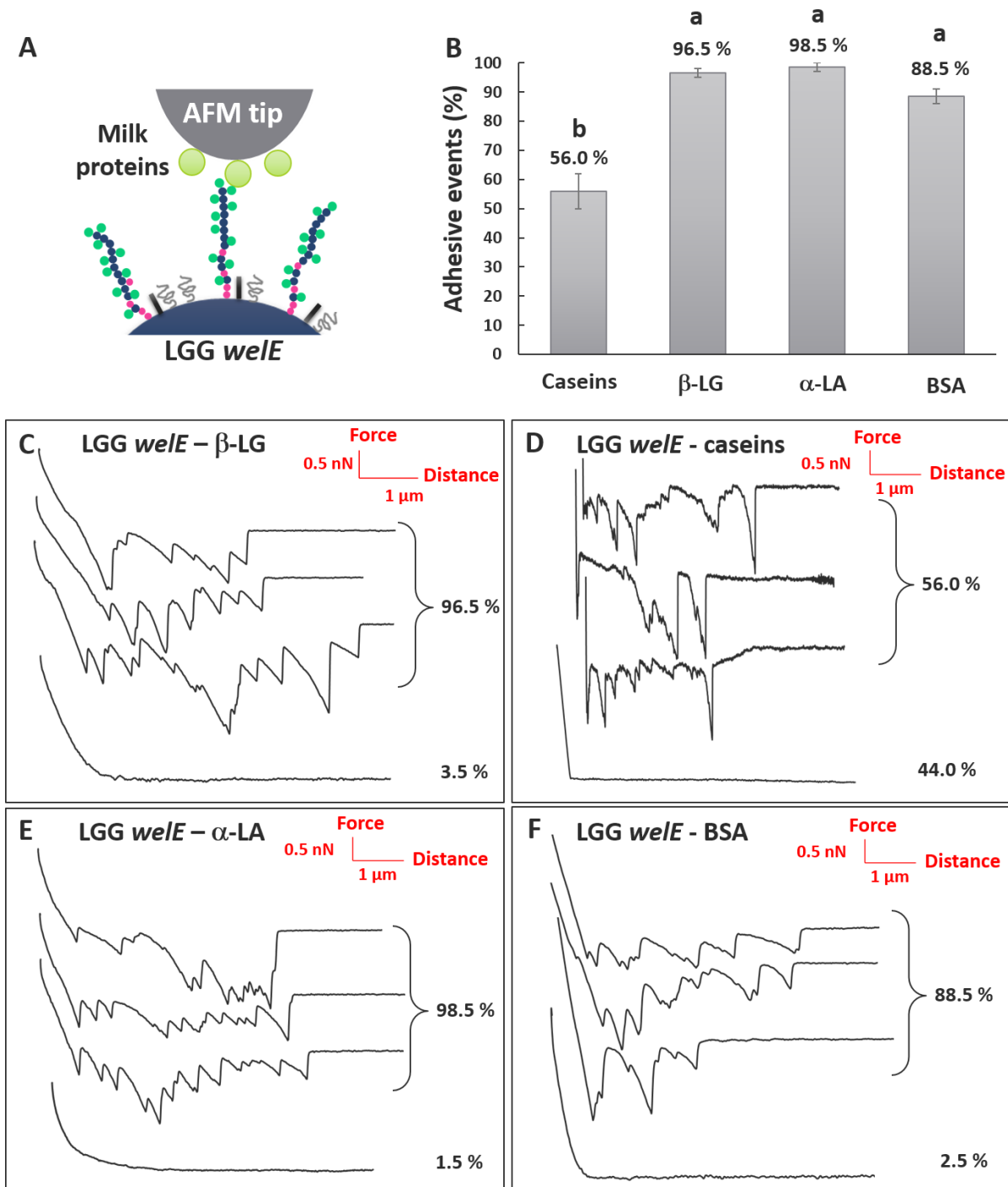
446 **Figure 3**



447

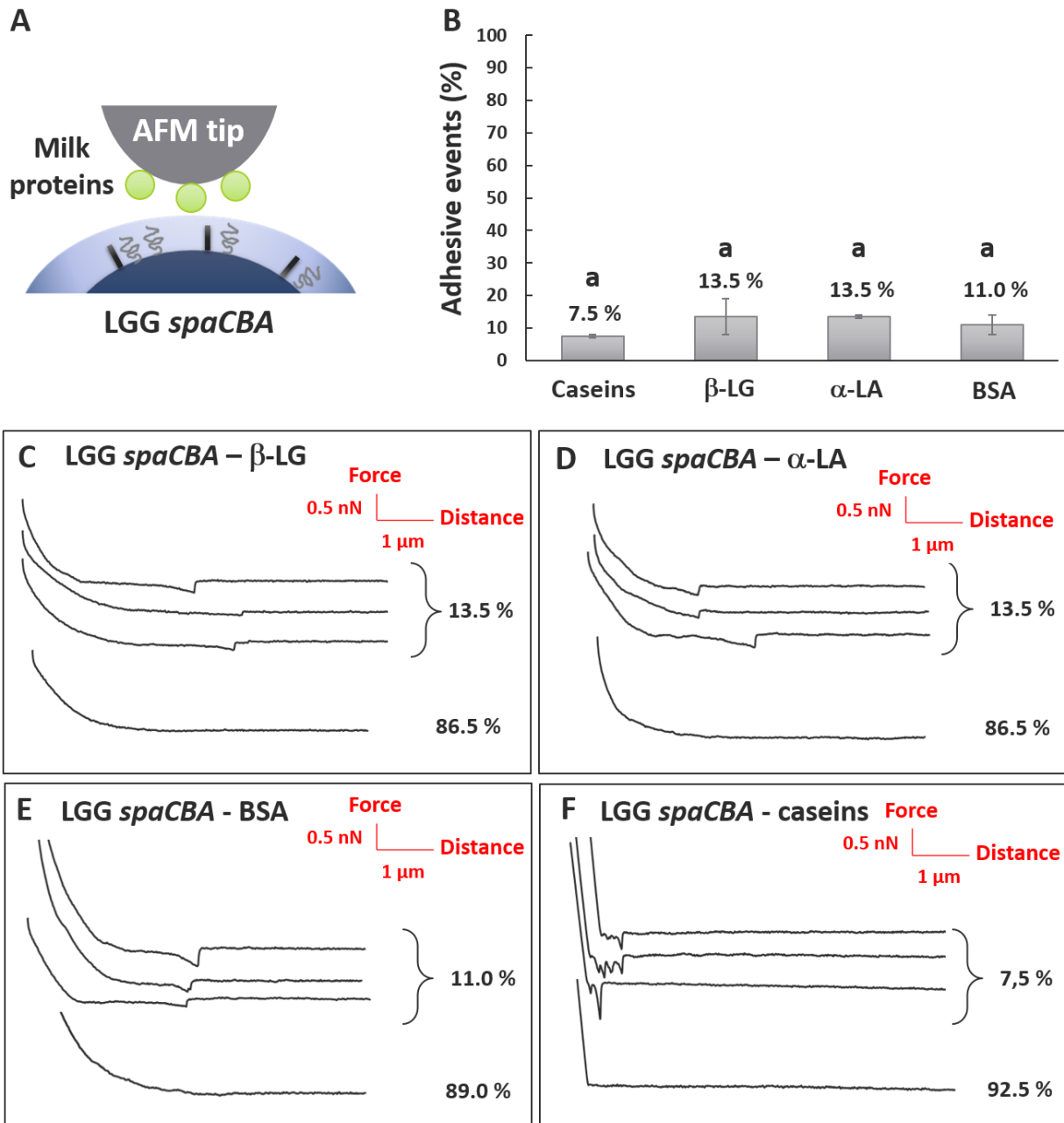
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449 **Figure 4**



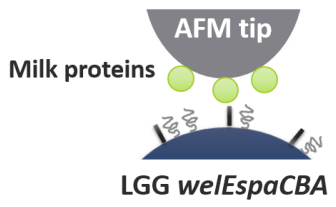
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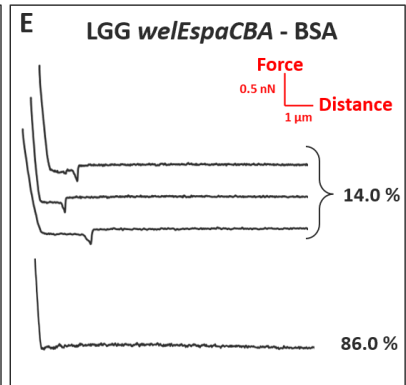
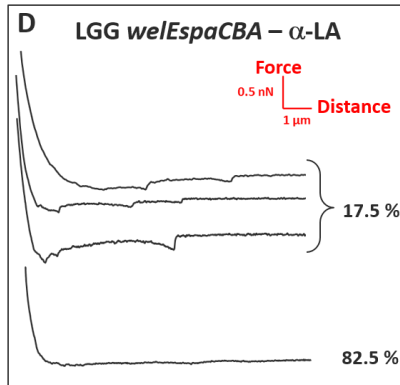
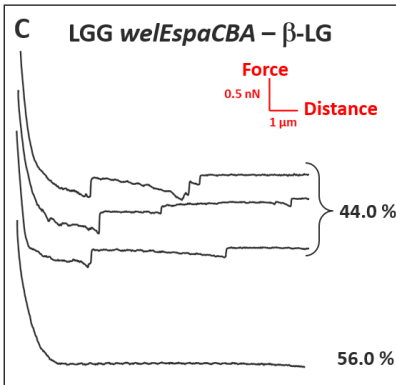
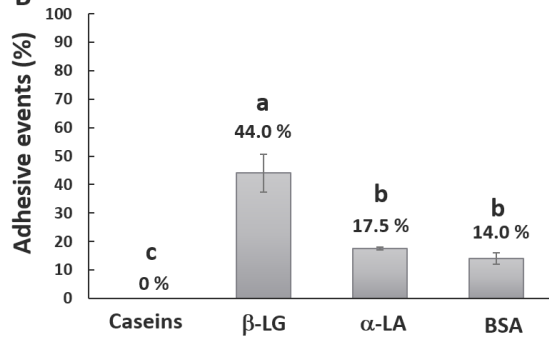


455 **Figure 6**

A



B



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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, β -LG, α -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 β -LG (C). Superimposition of retraction curves and determination of spring-like properties of*
473 *pili *SpaCBA* (D). Representative retraction curves recorded between LGG and α -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 β -LG (C), caseins (D), α -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 β -LG (C), α -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 β -LG (C), α -LA (D) or BSA (E).*
493