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Adhesive interactions between lactic acid bacteria and β -lactoglobulin: specificity and impact on bacterial location in whey protein isolate

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12 **Keywords: adhesion, lactic acid bacteria, dairy, β -lactoglobulin, high-throughput screening,**
13 **bacterial distribution, Atomic Force Microscopy (AFM), Confocal Laser Scanning Microscopy**
14 **(CLSM)**

15 **Abstract**

16 In the last decade, there has been an increasing interest in the potential health effects associated with
17 the consumption of Lactic Acid Bacteria (LAB) in foods. Some of these bacteria such as *Lactobacillus*
18 *rhamnosus* GG (LGG) are known to adhere to milk components, which may impact their distribution
19 and protection within dairy matrices and therefore is likely to modulate the efficiency of their delivery.
20 However, the adhesive behavior of most LAB, as well as its effect on food structuration and on the
21 final bacterial distribution within the food matrix remain very poorly studied. Using a recently
22 developed high-throughput approach, we have screened a collection of 73 LAB strains for their
23 adhesive behavior towards the major whey protein β -lactoglobulin. Adhesion was then studied by
24 genomics in relation to common bacterial surface characteristics such as pili and adhesion-related
25 domain containing proteins. Representative adhesive and non-adhesive strains have been studied in
26 further depth through biophysical measurement using Atomic Force Microscopy (AFM) and a relation
27 with bacterial distribution in Whey Protein Isolate (WPI) solution has been established. AFM
28 measurements have revealed that bacterial adhesion to β -lactoglobulin is highly specific and cannot be
29 predicted accurately using only genomic information. Non-adhesive strains were found to remain
30 homogeneously distributed in solution whereas adhesive strains gathered in flocs. These findings show
31 that several LAB strains are able to adhere to β -lactoglobulin, whereas this had only been previously
32 observed on LGG. We also show that these adhesive interactions present similar characteristics and
33 are likely to impact bacterial location and distribution in dairy matrices containing β -lactoglobulin.
34 This may help with designing more efficient dairy food matrices for optimized LAB delivery.

35 **1 Introduction**

36 Adhesion is a major property of microorganisms which effectively impacts microorganism activities
37 as well as human health, and has been identified as a key factor involved in microorganism ecology.
38 Adhesion enables bacteria to stick to both biotic and abiotic surfaces. Adhesion to abiotic surfaces
39 leads to biofilm formation, which has been widely studied in relation to the food industry (Barnes et
40 al., 2001; Garrett et al., 2008; Notermans et al., 1991; Pontefract, 1991). Adhesion to biotic surfaces
41 enables bacteria to establish direct contact with mucous membranes, and especially the intestinal
42 epithelium, to colonize a host (Conway et al., 1987; Pizarro-Cerdá and Cossart, 2006; Servin and
43 Coconnier, 2003). Adhesion of pathogens is therefore considered to be a virulence factor as it facilitates
44 host invasion (Pizarro-Cerdá and Cossart, 2006; Proft and Baker, 2009). Amongst non-pathogenic
45 bacteria, adhesion is considered essential in order for probiotic bacteria to remain functional and
46 therefore provide health benefits to the host (Ouweland et al., 2001; Quinto et al., 2014; Servin and
47 Coconnier, 2003). In the case of Gram-positive bacteria, bacteria-environment interactions such as
48 bacterial adhesion are mediated by sortase-dependent proteins (Comfort and Clubb, 2004; Maresso and
49 Schneewind, 2008), which are covalently anchored to the cell wall and possess an LPxTG like motif
50 at their C-terminal end (Schneewind and Missiakas, 2014).

51 Bacteria have also been shown to be able to adhere to food components, especially to meat
52 (Firstenberg-Eden, 1981; Piette and Idziak, 1989) and more recently to dairy components (Burgain et
53 al., 2014a; Gomand et al., 2018; Guerin et al., 2016). Bacterial adhesive interactions to food
54 components can compete with bacterial adhesion to the host (Sun and Wu, 2017). Therefore food
55 components such as milk fat globule membrane (Douëllou et al., 2017; Guerin et al., 2018b), milk
56 proteins (Halpin et al., 2008), and milk oligosaccharides (Lane et al., 2012) can play an anti-adhesive
57 role by decreasing bacterial adhesion to the intestine (Guerin et al., 2018b). Some food additives
58 including stabilizers (such as sucrose fatty acid esters) and colors (gardenia yellow, monascus pigment,
59 etc.) have also been found to feature similar effects (Islam et al., 2014).

60 In food matrices, adhesive interactions are likely to play an important part in bacterial spatial
61 distribution and viability during the structuration of the food matrix (Gomand et al., 2019). Adhesive
62 interactions occurring between the model strain *Lactobacillus rhamnosus* GG (LGG) and β -
63 lactoglobulin is mediated by the pili produced by LGG cells on their surface (Guerin et al., 2016).
64 These interactions result in an increased encapsulation efficiency when using dairy components as well
65 as a higher resistance to gastric digestion for this strain (Burgain et al., 2013a, 2014b; Guerin et al.,
66 2017). Adhesive interactions between genetically engineered *Lactococcus lactis* producing pili and
67 dairy components result in texture alteration in fermented milk (Tarazanova et al., 2018a) and can
68 modulate this strain distribution in cheese curd (Tarazanova et al., 2018b). Similarly, during curdling
69 and cheese ripening, bacterial cells mostly co-localize with fat globules or at the casein-fat interface,
70 which suggest adhesive interactions between fat and LAB strains (Laloy et al., 1996; Lopez et al.,
71 2006). This is likely to play a role in lipolysis thus affecting the development of characteristic flavors
72 and textures during ripening (Laloy et al., 1996; Lopez et al., 2006).

73 However, the impact and technological interest of adhesive interactions is yet poorly documented and
74 largely remains to be investigated (Hickey et al., 2015). Adhesive interactions between bacterial
75 surface components and dairy components have only been studied for very few wild type strains,
76 namely LGG (Guerin et al., 2016), *Lactobacillus amylovorus* (Chumphon et al., 2016), and
77 *Lactobacillus paracasei* (De Bellis et al., 2010). This article goes one step forward in that direction by
78 applying the high-throughput screening method recently developed by Gomand et al. (2018) to a
79 collection of 73 LAB strains (for which genome sequence is available) in order to characterize their
80 potential adhesive behavior towards the major dairy protein β -lactoglobulin, to which the adhesive
81 behavior of the model strain LGG is already well-known (Burgain et al., 2013b, 2014b, 2015; Guerin

82 et al., 2016, 2018a). Two strains featuring extreme adhesive and non-adhesive behaviors have then
83 been studied in further depth through AFM. The AFM results were then studied in relation to Confocal
84 Laser Scanning Microscopy (CLSM) experiments, allowing to observe the spatial distribution of these
85 strains in Whey Protein Isolate (WPI) solution.

86 **2 Material and methods**

87 **2.1 High-throughput screening**

88 Adhesive interactions between bacteria and β -lactoglobulin were screened using the method recently
89 developed by Gomand et al. (2018) using an automated liquid handling system for 96-well microplates.
90 Briefly, this method consists in immobilizing the biomolecules of interest on the surface of 96 well
91 adherent microplates. Microplates are then washed with a blocking agent in order to remove all
92 unbound molecules and to block the remaining empty sites. The bacterial suspension is then added into
93 the wells and incubated for 1 h at 37 °C in order to allow bacterial adhesion to the immobilized
94 biomolecules. Non-adherent bacteria are removed by successive washes using the same blocking agent.
95 The amount of immobilized bacteria is measured through bacterial growth monitoring (turbidity
96 measurements at 595 nm) after the addition of MRS culture growing medium (De Man et al., 1960) in
97 the wells. The higher the initial quantity of bound bacteria, the earlier the growth starts. Adjustments
98 made to this protocol are listed below.

99 **2.1.1 Bacterial strains and cultures**

100 A list of the 73 screened LAB strains is given in **Supplementary data 1 (S1)**. This collection of strains
101 has previously been studied for their genomics and surface properties (Sun et al., 2015). The model
102 strain *Lactobacillus rhamnosus* GG ATCC53103 (LGG wild type, “WT”) and the mutant strain LGG
103 *spaCBA* CMPG 5357 impaired in pili synthesis, which adhesive properties of both are well-known
104 (Guerin et al., 2016; Lebeer et al., 2012; Tripathi et al., 2012, 2013) were respectively used as positive
105 (adherent) and negative (non-adherent) control strains.

106 For each series of experiments, a 96-well microplate previously stored at -80 °C was thawed and
107 replicated on working microplates using 50 μ L of bacterial suspension to inoculate 150 μ L of MRS by
108 well. The working microplates were incubated at 30 °C two days before the adhesion assay. During
109 the adhesion assay, microplates were only centrifuged once at 1,642 *g* for 20 min, emptied and the
110 resulting cell pellets were resuspended in 200 μ L of PBS adjusted at pH 6.8. Triplicates on independent
111 cultures were performed as well as duplicates by strain on each plate (6 repetitions for control strains).

112 **2.1.2 Preparation of the β -lactoglobulin solution and microplate coating**

113 Beta-lactoglobulin (Sigma-Aldrich Co. LLC, St Louis, MO, USA) was prepared in solution (1% w/w)
114 as described by Gomand et al. (2018).

115 **2.1.3 Bacterial growth monitoring**

116 Adhesion and growth monitoring were done according to Gomand et al. (2018). The incubation
117 temperature was changed to 30 °C in order to match the diversity of the growing conditions for all
118 strains (Gomand et al., 2018). Bacterial growth was monitored through OD_{595nm} measurements over 48
119 h.

120 **2.1.4 Data processing**

121 **2.1.4.1 Strain growth comparison**

122 The times at which the apparent bacterial growth starts (t_{start}) were monitored such as described by
 123 Gomand et al. (2018). The higher these time values are, the later the growth starts *i.e.* the fewer bacteria
 124 have adhered *i.e.* the lower the affinity. These values were averaged on all series of experiments and
 125 standard deviations are computed. Strains were compared to one another based on their Minimum
 126 Adhesion Value (MAV) corresponding to the difference between the smallest t_{start} (highest adhesion)
 127 obtained on a control without β -lactoglobulin and the highest t_{start} (lowest adhesion) obtained on β -
 128 lactoglobulin:

129 **Minimum Adhesion Value (MAV)** = $\left[(t_{start})_{average} \right] - \sigma_{CONTROL} - \left[(t_{start})_{average} \right] - \sigma_{\beta-LAC}$

130 Where σ stands for standard deviation. A strain is considered to adhere to β -lactoglobulin if its MAV
 131 is significantly superior to zero for all three series of experiments.

132 **2.1.4.2 Functional domain prediction for the bacterial surface proteome**

133 Bacterial surface proteins featuring LPxTG motif were predicted using the InterPro resource, that
 134 provides functional analysis of protein sequences by classifying them into families and predicting the
 135 presence of domains and important sites (Finn et al., 2017). Protein sequences were obtained from Sun
 136 et al. (2015) and were scanned against InterPro's signatures using the software package InterProScan
 137 (Jones et al., 2014). Gene sequence resemblance with known domains was performed using the Basic
 138 Local Alignment Search Tool resource (BLAST), according to Altschul et al. (1990).

139 **2.1.4.3 Statistical analysis**

140 Statistical analysis were performed via t-tests and Tukey tests (parametric) for normal data and
 141 Wilcoxon-Mann Whitney and Steel-Dwass tests (non-parametric) for data that did not fit normal
 142 distribution using Kyplot software (Kyens Lab Inc.).

143 **2.2 Adhesive interactions between bacteria and β -lactoglobulin characterized through Atomic**
 144 **Force Microscopy**

145 Protocols used in this part have been adapted from Guerin et al. (2018a). Briefly, this method consists
 146 in immobilizing the bacterial strains of interest on functionalized gold-coated mica by depositing the
 147 bacterial suspension during 15 h at 4 °C (pH 6.8). The mica is rinsed with PBS (pH 6.8) before use.
 148 Milk proteins are prepared in distilled water (1% w/w) and adsorbed on modified AFM probes (gold
 149 coated and with NH₂-terminated PEG linker) by immersion for 15 h at 4 °C and then rinsed with milli-
 150 Q-grade water before use. Force measurements are performed at room temperature in PBS buffer (pH
 151 6.8). AFM force distance curves are obtained by following the cantilever deflection as a function of
 152 the vertical displacement of the piezoelectric scanner with a scan speed of 400 mm/s. Adjustments to
 153 this protocol are listed below.

154 **2.2.1 Bacterial cultures**

155 Cultures were prepared according to Guerin et al. (2018a). Precultures of *Lactobacillus aquaticus* DSM
 156 21051 and *Lactobacillus sharpeae* DSM 20505 were prepared by inoculating 9 mL of MRS broth with
 157 100 μ L of bacterial stock and grown overnight at 37 °C. These precultures were used to inoculate 9
 158 mL of fresh MRS broth the next day and the growth was performed at 37 °C until an optical density of

159 1.2 was reached at 660 nm (for about 8 h). Cultures were then centrifuged at 3,000 *g* for 10 min at
160 room temperature. Pellets were suspended in 1 mL of PBS (pH 6.8).

161 **2.2.2 Preparation of bacteria-coated mica and protein-coated tips**

162 According to Guerin et al. (2018a), a mica coated with a gold layer functionalized with a NH₂-
163 terminated PEG-linker (Novascan, Ames, Iowa, USA) was used, as well as AFM probes with
164 borosilicate glass particle (2 μ m), coated with gold and modified with NH₂ terminated PEG linker
165 (Novascan, Ames, Iowa, USA). The bacterial suspension is deposited on mica at 4 °C and left overnight
166 (pH 6.8). Preparation of the β -lactoglobulin and Bovine Serum Albumine (BSA) 1% (w/w) solutions
167 (Sigma-Aldrich Co. LLC, St Louis, MO, USA) was done according to Guerin et al. (2018a). Probes
168 tips were left to incubate overnight at 4°C in wells containing 1 mL of the β -lactoglobulin or BSA
169 solutions to maximize protein adsorption. β -lactoglobulin was the candidate protein tested and BSA
170 was the negative control.

171 **2.2.3 AFM measurements**

172 Protocol followed is described by Guerin et al. (2018a). Force-volume measurements are performed at
173 room temperature in PBS buffer (pH 6.8) using a Bruker Bioscope Resolve atomic force microscope
174 (Bruker corporation, Santa Barbara, CA) mounted on an inverted microscope (DMi8, Leica
175 microsystems). The spring constants of the cantilevers was measured using the thermal noise method
176 and found to be 0.01 N m⁻¹. Force distance curves were recorded between the bacteria deposited on
177 functionalized mica and the probe coated with β -lactoglobulin or BSA. Three adhesion force maps (20
178 μ m \times 20 μ m, 256 force curves) were recorded for each protein-bacteria interaction analysis. Data
179 analysis was performed using the Nanoscope Analysis software from Bruker (Santa Barbara, CA,
180 USA) and the last peak was calculated for each curve before plotting adhesion forces and last rupture
181 length histograms. The last peak is used for analysis instead of the maximum peak in order to
182 characterize the last interacting point between the β -lactoglobulin and the cell receptor and not the
183 unfolding of a biomolecular domain.

184 **2.3 Adhesive interactions imaged by confocal microscopy**

185 The cultures were prepared as described in 2.2.1., then centrifuged at 3,000 *g* for 10 min at room
186 temperature. Pellets were suspended in 10 mL of WPI solution (15 %, w/w). The WPI solution was
187 prepared using PRODIET 90 S (Ingredia, Arras, France) that is a soluble milk protein isolate containing
188 native whey proteins including β -lactoglobulin. One milliliter of resuspended cells was stained with
189 the LIVE/DEAD BacLight viability kit (1:200 v/v; LIVE/DEAD BacLight viability kit was prepared
190 according to the procedure described for the kit L13152 by ThermoFisher Scientific). Two hundred
191 microliters of LAB suspension (same conditions as 2.2.1) were introduced on chambered glass slides
192 (Nunc Lab-Tek, ThermoFisher Scientific). Confocal Laser Scanning Microscopy (CLSM) images were
193 taken using a Leica TCS SP5-X-AOBS confocal laser scanning microscope (Leica Microsystems CMS
194 GmbH, Mannheim, Germany) equipped with WLL lasers. The objective lens used was a HCX PL APO
195 CS 100 \times 1.40 (oil immersion). The excitation wavelength was 488 nm and emission bandwidth was
196 of 495-510 nm for SYTO 9 and 600-620 nm for propidium iodide. Two independent repetitions were
197 performed and approximately 20 representative images were acquired for each sample.

198 **3 Results**

199 **3.1 Identification of strains adhesive to β -lactoglobulin**

200 Most strains were found not to be adhesive to β -lactoglobulin as the average MAV calculated on the
 201 73 strains was negative (-180 ± 22) although higher than the MAV of the negative control LGG *spaCBA*
 202 (-386), known to be non-adhesive to β -lactoglobulin (Guerin et al., 2016). The microplate adhesive
 203 assays revealed four adhesive candidates to β -lactoglobulin amongst the 73 strains tested:
 204 *Lactobacillus aquaticus* DSM 21051 (MAV = 61.5), *Lactobacillus murinus* DSM 20452 (MAV =
 205 12.8), *Lactobacillus plantarum* DSM 13273 (MAV = 12.6), *Lactobacillus brantae* DSM 23927 (MAV
 206 = 6.97), although these strains were still less adhesive than the positive control LGG WT (MAV =
 207 104). Nine strains were also found to have a MAV inferior to the one of the negative control LGG
 208 *spaCBA*: *Lactobacillus sharpeae* DSM 20505 (MAV = -857), *Lactobacillus kefir* DSM 20587 (MAV
 209 = -787), *Lactobacillus similis* DSM 23365 (MAV = -780), *Lactobacillus pobuzihii* DSM 28122 (MAV
 210 = -617), *Lactobacillus namurensis* DSM 19117 (MAV = 516), *Lactobacillus satsumensis* DSM 16230
 211 (MAV = -490), *Pediococcus parvulus* DSM 20332 (MAV = -477), *Lactobacillus senmazukei* DSM
 212 21775 (MAV = -404), *Lactobacillus lindneri* DSM 20690 (MAV = -387). The MAV for all strains are
 213 listed as supplementary material (S1).

214 3.2 Biophysical deciphering of bacterial adhesive interaction with β -lactoglobulin through 215 AFM

216 The adhesive interactions between β -lactoglobulin and the strains at the extremes of the adhesion
 217 spectrum, *L. aquaticus* DSM 21051 (the most adhesive strain) and *L. sharpeae* DSM 20505 (the least
 218 adhesive strain) were studied through AFM, in order to characterize them in further depth. Only two
 219 strains were chosen to precise our understanding of the interaction mechanism of the LAB surface with
 220 β -lactoglobulin since AFM is not a suitable method for screening of large populations. This is why we
 221 decided to select only the two strains at the extreme of the adhesion spectrum for this analysis. BSA
 222 was used as a negative control as LAB strains have previously been found to feature low adhesion to
 223 it (Gomand et al., 2018; Guerin et al., 2016). The percentages of adhesive events (frequencies) observed
 224 between *L. aquaticus* DSM 21051 and the two proteins, β -lactoglobulin and BSA, were respectively
 225 of $82.6 \pm 7.1\%$ and $27.6 \pm 10.4\%$ (**Figure 1A₁**). The frequencies of adhesive events observed between
 226 *L. sharpeae* DSM 20505 and the same two proteins were respectively of $3.4 \pm 1.5\%$ for β -lactoglobulin
 227 and $2.5 \pm 0.6\%$ for BSA (**Figure 1B₁**). Typical force-distance curves obtained for the interactions
 228 occurring between the two strains and the AFM probes functionalized with the two proteins are
 229 presented, *i.e.* *L. aquaticus* DSM 21051 and β -lactoglobulin (**Figure 1A₂**), *L. aquaticus* DSM 21051
 230 and BSA (**Figure 1A₃**), *L. sharpeae* DSM 20505 and β -lactoglobulin (**Figure 1B₂**), and *L. sharpeae*
 231 DSM 20505 and BSA (**Figure 1B₃**). During the withdrawal of functionalized β -lactoglobulin-coated
 232 probe from the surface of *L. aquaticus* DSM 21051 several specific adhesive events occur (**Figure**
 233 **1A₂**), whereas more than 70% of the curves observed for BSA-coated probes did not feature any
 234 adhesive event (**Figure 1A₃**). Moreover, the few adhesive events observed between BSA and *L.*
 235 *aquaticus* DSM 21051 appeared to be random and therefore could not be associated to any specific
 236 interaction (**Figure 1A₃**). Almost no adhesive event was observed for both BSA- and β -lactoglobulin-
 237 coated probes on *L. sharpeae* DSM 20505 cells (**Figures 1B₁, B₂, B₃**). These results are consistent with
 238 those obtained using the screening method: *L. aquaticus* DSM 21051 significantly adheres to β -lac
 239 whereas poor adhesion was observed for *L. sharpeae* DSM 20505. Retraction curves recorded between
 240 *L. aquaticus* DSM 21051 and β -lactoglobulin attest the specificity of occurring adhesive interactions,
 241 which would happen according to a lock and key mechanism (**Figure 2A**). 3D-AFM images recorded
 242 on mica attest of the good coverage of *L. aquaticus* DSM 21051 and therefore that adhesive events
 243 recorded did occur between *L. aquaticus* DSM 21051 cells and β -lactoglobulin-coated probes (**Figure**
 244 **2B**). The biophysical properties of the adhesion between *L. aquaticus* DSM 21051 and β -lac were
 245 analyzed using additional force parameters including adhesion forces (**Figure 2C**) and final rupture
 246 length (**Figure 2D**). Retraction curves exhibited adhesion forces averaging around 1.43 ± 0.03 nN.

247 Final rupture length averaged around $0.90 \pm 0.03 \mu\text{m}$. These results will be compared with those of
248 LGG WT and the mutant strains LGG *spaCBA* and *welE* in the discussion section.

249 **3.3 Impact of adhesive interactions on bacterial distribution in whey protein isolate probed by** 250 **confocal microscopy**

251 *L. aquaticus* DSM 21051, *L. sharpeae* DSM 20505, LGG WT and LGG *spaCBA* were first imaged in
252 MRS to make sure that they were originally homogeneously distributed (**Figure 3A₁, 3B₁ and 4A₁,**
253 **4B₁**). Live cells of *L. aquaticus* DSM 21051 were found to aggregate in the WPI solution whereas *L.*
254 *sharpeae* DSM 20505 live cells remained homogeneously distributed (**Figure 3A₂ and 3B₂**). This is
255 consistent with the adhesive properties of the control strains: LGG WT (positive control) aggregate in
256 the WPI solution whereas LGG *spaCBA* remained homogeneously distributed (**Figure 4A₂ and 4B₂**).
257 Dead bacterial cells or cells with a damaged membrane gathered in flocs for all 4 strain types (data not
258 shown).

259 **3.4 Relation between bacterial adhesion to β -lactoglobulin and predicted bacterial surface** 260 **characteristics**

261 **3.4.1 Presence of pili genes clusters (PGCs)**

262 Predicted bacterial surface characteristics were analyzed in relation to the results of the adhesive assays
263 in order to delineate gene candidates predicted to encode surface proteins that could be involved in
264 bacterial adhesion to β -lactoglobulin. Amongst the 73 strains tested, 32 of them possessed at least one
265 sortase-dependent pilus gene cluster (PGC) and therefore were predicted to express pili on their surface
266 (Sun et al., 2015). The average MAV of these 32 strains was -163 ± 33.2 whereas the average MAV
267 of the 41 non-piliated strains was -194 ± 30.1 . Amongst the 32 strains presenting PGCs, 16 possessed
268 PGCs similar to LGG pilus clusters in terms of gene order, that is, a cluster of three pilin genes and
269 one pilin-specific sortase gene (Sun et al., 2015). The MAV of these 16 strains was -165 ± 53.8 whereas
270 the MAV of the 16 strains with PGCs different from LGG was -160 ± 38.8 . Although a mean
271 comparison of the MAV for strains featuring PGCs compared to non-piliated strains would suggest
272 that the presence of PGCs fosters adhesion to β -lactoglobulin, this was not supported statistically. No
273 difference could be observed between strains featuring PGCs similar to LGG WT's and PGCs different
274 from LGG WT's. The number of PGCs, sortase enzymes or proteins with LPxTG motif (listed for all
275 strains in **S1**) were not found either to impact strain adhesion to β -lactoglobulin (data not shown).

276 **3.4.2 Predicted protein domains candidates for mediating bacterial adhesion to β -lactoglobulin**

277 More predicted surface characteristics were analyzed for the four strains found to be adhesive to β -
278 lactoglobulin. Predicted protein domains featuring LPxTG motif found for each strain are listed in
279 **Table 1**. Strains were analyzed for gene sequence resemblance with the *spaCBA* domain, known to be
280 responsible for adhesion to β -lactoglobulin for LGG WT (Guerin et al., 2016) but no homologue
281 sequence could be identified for any of the four adhesive strains. All strains are predicted to feature
282 immunoglobulin-like (Ig-like) fold domains, which are usually involved in binding or molecular
283 recognition processes (Bodelón et al., 2013). Other and more specific adhesion-related domains present
284 on the four adhesive strains studied as well as on LGG WT include MucBP (mucin-binding),
285 CBME/CBM3 (carbohydrate-binding), fibrinogen- and collagen-binding domains, cysteine- and
286 leucine-rich domains, and SD-repeat B-domain. Most of these domains are present once in the genome
287 of the adhesive strains (*L. plantarum* DSM 13273 is the only adhesive strain presenting 3 MucBP
288 domains) and are not repeated within a given protein.

289 The MucBP domain is the only domain with a known adhesive-related function (apart from the Ig-like
290 fold domain) which could be identified on *L. aquaticus* DSM 21051, the most adhesive strain to β -
291 lactoglobulin. MucBP domains have been found predominantly in lactobacilli found naturally in
292 intestinal niches, which suggests that they play an important role in establishing host-microbial
293 interactions in the gut by binding mucus (Roos and Jonsson, 2002; Tassell and Miller, 2011). *L.*
294 *plantarum* DSM 13273 is the strain featuring the highest number of adhesion-related domains in its
295 genome (**Table 1**). This is also the only strain out of the four presenting fibrinogen- and collagen-
296 binding domains. The fibrinogen-binding domain has been found to accommodate linear peptides with
297 a certain degree of ligand sequence variability (Ponnuraj et al., 2003) and therefore might be able to
298 interact with β -lactoglobulin. *L. brantae* DSM 23927 features leucine-rich repeats (LRRs) and SD-
299 repeat (Sdr) domains (**Table 1**), both of them susceptible to play a role in adhesive interactions to β -
300 lactoglobulin. LRRs have been found to provide a structural framework for the formation of protein-
301 protein binding and interactions (Gay et al., 1991; Kobe and Kajava, 2001) and are likely to allow a
302 broad range of ligands (Kobe and Kajava, 2001). Sdr-repeat domains are surface proteins that play an
303 important role in *Staphylococcus aureus* adhesion and pathogenesis (McCrea et al., 2000; Wang et al.,
304 2013). The protein containing Sdr-repeat domains may therefore be a good candidate for mediating
305 adhesion to β -lactoglobulin for the strain *L. brantae* DSM 23927. No other adhesion-related domain
306 than the Ig-like fold domain was identified on *L. murinus* DSM 20452 (**Table 1**), which would suggest
307 that the protein containing this domain would likely be the one involved in adhesive interactions with
308 β -lactoglobulin.

309 **4 Discussion**

310 The aim of this study was to evaluate and characterize adhesive interactions occurring between lactic
311 acid bacteria and β -lactoglobulin. A collection of 73 LAB strains was screened for their adhesive
312 behavior towards β -lactoglobulin and strains at the extreme of the adhesion spectrum *i.e.* a highly
313 adhesive and a poorly adhesive strains were studied in further depth.

314 Only four strains out of 73 were found to present adhesive affinities towards β -lactoglobulin. Therefore,
315 adhesion to β -lactoglobulin appears not to be a common characteristic of the LAB group. The
316 consequences of these adhesive interactions, when they occur, are not fully understood. However, it
317 could be hypothesized that strains featuring adhesive affinities towards whey proteins would be lost
318 during the drainage step of cheese manufacturing processes, alongside with whey expulsion from the
319 cheese network. It would be interesting to test the affinity of this same strain collection to other food
320 components in future work, in order to dispose of more comparison points to our study and to get a
321 better understanding of the importance of adhesion to β -lactoglobulin compared to adhesion to other
322 food components. Currently, the rare existing studies discussing bacterial adhesion to food components
323 other than β -lactoglobulin concern up to four strains at most at a time (Chumphon et al., 2016; De
324 Bellis et al., 2010; Tarazonova et al., 2017, 2018b, 2018a; Utratna et al., 2017), therefore failing to
325 provide an overview of adhesion to food components amongst wide bacterial groups such as the LAB
326 group.

327 The study performed by Tarazonova et al. (2017) is the only one to our knowledge that compares the
328 adhesion level of a wide number of strains (55) to food (casein-derived) components, however these
329 strains are all of the same species, *Lactococcus lactis*. Out of 55, 30 to 40 strains presented adhesive
330 affinities towards casein-derived components, depending on their growth phase, and strains isolated
331 from a dairy environment presented much stronger binding of milk proteins versus strains isolated from
332 plants, suggesting a selective advantage (Tarazonova et al., 2017). However, this was not confirmed in
333 our case, as the four strains out of 73 that were originally isolated from dairy products, *i.e.* *Lactobacillus*

334 *casei* DSM 20011, *Lactobacillus paracasei* subsp. *tolerans* DSM 20258, *Lactobacillus bif fermentans*
335 DSM 20003, and *Lactobacillus kefir* DSM 20587, did not present more adhesive affinities towards β -
336 lactoglobulin in average than the strains isolated from nondairy sources (data not shown).

337 The strain found to be the most adhesive to β -lactoglobulin, *L. aquaticus* DSM 21051, exhibited a
338 specific adhesive behavior when studied by AFM. The signature of the observed retraction curves was
339 identified as specific of biomolecules stretching, suggesting that the surface of *L. aquaticus* DSM
340 21051 features a strong affinity towards β -lac. This has also been shown previously for the model strain
341 LGG WT by our team as well as for the mutant strain LGG *welE*, expolysaccharide-depleted and
342 known to adhere more to β -lactoglobulin than LGG WT due to its increased pili exposure (Guerin et
343 al., 2016, 2018a). *A contrario*, *L. sharpeae* DSM 20505 which screening results show not to adhere to
344 β -lactoglobulin presented retraction curves characteristic of a lack of adhesion to β -lac when studied
345 by AFM (frequency of adhesive events was inferior to 5 %). Similarly, our team demonstrated
346 previously this same fact for the model strain non-adhesive to β -lactoglobulin, LGG *spaCBA* (Guerin
347 et al., 2016). Comparative results are presented in **Table 2**.

348 The adhesive behavior of *L. aquaticus* DSM 21051 towards β -lactoglobulin appears relatively close to
349 the one of LGG *welE* in terms of frequency of adhesive events. The high specificity of the adhesion
350 phenomenon occurring between *L. aquaticus* DSM 21051 and β -lactoglobulin is highlighted by the
351 fact that the frequency of adhesion is almost twice as high as the one characterizing adhesive
352 interactions between LGG WT and β -lactoglobulin, whereas the frequency of adhesion of *L. aquaticus*
353 DSM 21051 on BSA is almost 4 times lower than the one occurring between LGG *welE* and BSA. The
354 mean adhesion force recorded on the last peak is also 3 times higher than the mean adhesion force
355 recorded for LGG WT and β -lactoglobulin, and higher than the highest adhesion force recorded on the
356 last peak for LGG *welE* and β -lactoglobulin, reaffirming the idea of a very strong specificity and
357 adhesion strength. When comparing the length of biomolecules stretched by adhesive interactions with
358 β -lactoglobulin, *L. aquaticus* DSM 21051 and LGG *welE* both exhibit molecules stretched up to 1 μ m
359 *i.e.* 3 times longer than the molecule stretched in the case of LGG WT (**Table 2**). The molecule
360 mediating adhesive interactions with β -lactoglobulin in the case of *L. aquaticus* DSM 21051 is
361 therefore comparable in length to LGG pili when stretched, which may explain the higher specificity
362 and adhesion strength found for *L. aquaticus* DSM 21051 compared to LGG WT, which pili are
363 partially hidden within the expolysaccharides layer (Guerin et al., 2016).

364 On the other hand, the frequency of adhesive events observed between *L. sharpeae* DSM 20505 and
365 β -lactoglobulin is inferior to 5 % and similar to the frequency of adhesive events observed on BSA for
366 both this strain and *L. aquaticus* DSM 21051. The frequency of adhesive events recorded when using
367 BSA-coated probes is also 4 times lower for *L. sharpeae* DSM 20505 than for LGG *spaCBA* (negative
368 control). Overall, *L. sharpeae* DSM 20505 has demonstrated very poor adhesive capacities towards β -
369 lactoglobulin. However, when analyzed for predicted adhesion-related protein domains, this strain
370 revealed a total of 23 adhesion-related domains, 8 of which being different, including MucBP and
371 Gram positive pilin subunit D1 N-terminal, although no sequence homologue to the *spaCBA* domain
372 was found (data not shown). The *spaCBA* domain is known to mediate adhesion to β -lactoglobulin for
373 the piliated strain LGG WT (Guerin et al., 2016). This confirms that adhesive interactions with β -
374 lactoglobulin are specific, and cannot be predicted accurately using only genomic predictions (the
375 functions of these domains may not be accurately predicted or they may not be expressed).

376 The gathering behavior observed by CLSM for the adhesive strains in the WPI solution also pledges
377 in favor of a specific bacterial adhesion to β -lactoglobulin for *L. aquaticus* DSM 21051. CLSM results
378 indicate that the location of bacteria in a dairy matrix strongly depends on bacterial surface properties.

379 These observations are important as it was evidenced recently that physical properties of dairy
380 products, such as viscosity and gel hardness, are affected by bacterial surface properties in the case of
381 surface-engineered strains (Tarazanova et al., 2018a). In light of our results, it would be interesting to
382 see if that is also the case for wild strains presenting different surface properties inducing different
383 adhesive behaviors. Some peptides shown to be linked to bacterial aggregation were also recently
384 evidenced to be able to promote bacterial adhesion to functionalized surfaces and Caco-2-cells (Okochi
385 et al., 2017). This typical behavior was responsible for observed enhanced interactions between LAB
386 and the host intestinal mucosa (Okochi et al., 2017). Adhesive interactions with β -lactoglobulin leading
387 to the aggregation of *L. aquaticus* DSM 21051 and LGG WT cells might therefore be considered for
388 further study in order to determine whether they would promote such kind of behavior as well.

389 This work was performed in the continuity of previous studies, in which a method was developed
390 allowing screening a wide number of strains for their adhesive affinities towards biomolecules such as
391 dairy food components (Gomand et al., 2018), and which identified the bacterial surface molecules
392 (pili) involved in the adhesion of LGG to dairy components using AFM (Guerin et al., 2016). The
393 present study sought to go beyond bacterial species differences in revealing common adhesive
394 characteristics of Lactic Acid Bacteria in relation to dairy food components such as β -lactoglobulin.
395 We first looked for LAB species featuring adhesive affinities for β -lactoglobulin, then focused on the
396 molecular characteristics of this adhesion. We observed adhesion to β -lactoglobulin for few LAB (less
397 than 6% of our collection). However, for those which did feature adhesive affinities, some common
398 characteristics were pointed out that matched the characteristics previously identified on the model
399 strain LGG. These characteristics include the specificity of the affinity, as well as the impact on
400 bacterial spatial distribution in the matrix. The major findings of the present paper are that (i) Adhesion
401 to whey proteins is apparently not a common characteristic to the LAB group (few strains presented
402 adhesive affinities towards β -lactoglobulin), (ii) Strains featuring adhesive affinities towards β -
403 lactoglobulin present common adhesive characteristics (specific β -lactoglobulin-adhesion domains
404 related to the specificity of the AFM signature), and (iii) Adhesion to β -lactoglobulin was shown to
405 strongly influence bacterial distribution in dairy matrices featuring this component (adhesive bacteria
406 gathered in flocs in whey matrices whereas non-adhesive bacteria distribute more homogeneously),
407 and could therefore modulate their accessibility and later delivery when designing functional foods
408 containing LAB with potential associated health effects.

409 According to these findings, food matrices could play a protective role on bacteria by influencing their
410 spatial distribution, which may prove especially useful for probiotic bacteria. Indeed, as bacteria
411 adhering to a component have been found to flocculate in the food matrix containing this component,
412 this could result in later heterogeneous delivery in the gastro-intestinal tract (GIT) which would impact
413 host colonization, but may also better protect bacterial survival until they reach the GIT. These findings
414 also pave the road to future experiments aiming generalizing bacterial adhesion characteristics to broad
415 bacterial groups, thus helping with practical food matrix design. It would therefore be interesting to
416 study the potential protective effect of components to which bacteria are adherent during critical steps
417 of the food manufacturing process, such as spray-drying during probiotic milk powder production.

418 **Author contributions**

419 FG, JG, JB, FB, CG conceived the research. FG, JG, JB, SEKC, DD, GF carried out the experiments.
420 FG, JB, JG, SEKC, DD, GF analyzed the data. FG, JG, JB wrote the manuscript. All authors
421 commented on the manuscript.

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425 The authors declare no competing interest.

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430 Antwerp, Antwerp, Belgium).

431 **Supplementary material**

432 The Supplementary Material for this article can be found online at: ...

433 **Figure captions**

434 Figure 1: Comparison of the adhesive properties of two strains (*Lactobacillus aquaticus* DSM 21051,
435 *Lactobacillus sharpeae* DSM 20505) for whey proteins isolates probed by Atomic Force Microscopy
436 (AFM): frequency of adhesive events occurring between whey proteins and *L. aquaticus* DSM 21051
437 (A₁) and *L. sharpeae* DSM 20505 (B₁) and representative examples of retraction curves obtained for
438 force measurements between *L. aquaticus* DSM 21051 and β -lactoglobulin (A₂), *L. aquaticus* DSM
439 21051 and BSA (A₃), *L. sharpeae* DSM 20505 and β -lactoglobulin (B₂), and *L. sharpeae* DSM 20505
440 and BSA (B₃).

441
442 Figure 2: Schematic description of Atomic Force Microscopy (AFM) with protein-coated tips and
443 bacteria-coated mica (A) 3D-AFM image of *Lactobacillus aquaticus* DSM 21051 recorded in liquid in
444 phosphate buffered saline (B) Interactions between β -lactoglobulin and *L. aquaticus* DSM 21051
445 explored by force measurement using atomic force microscopy: adhesions forces (C) and final rupture
446 length (D). Averages of adhesion forces and rupture lengths are precised in (C) and (D) with standard
447 errors.

448
449 Figure 3: Spatial distribution of *L. aquaticus* DSM 21051 and *L. sharpeae* DSM 20505 in MRS culture
450 medium [A₁ and B₁] and in whey protein isolate (WPI) solution [A₂ and B₂], imaged by confocal laser
451 scanning microscopy (CLSM). Bacterial concentration is 10⁷ u.f.c./ mL. Bacteria cells are represented
452 in green on this figure whether they are viable or damaged (no difference is made here that would
453 depend on bacterial status).

454 Figure 4: Spatial distribution of LGG WT and LGG *spaCBA* in MRS culture medium [A₁ and B₁] and
455 in whey protein isolate (WPI) solution [A₂ and B₂], imaged by confocal laser scanning microscopy
456 (CLSM). Bacterial concentration is 10⁷ u.f.c./ mL. Bacteria cells are represented in green on this figure
457 whether they are viable or damaged (no difference is made here that would depend on bacterial status).

458

459

Strain	MAV	Predicted adhesion-related protein domains*
<i>Lactobacillus aquaticus</i> DSM 21051	61.5	Immunoglobulin-like fold, MucBP
<i>Lactobacillus murinus</i> DSM 20452	12.8	Immunoglobulin-like fold
<i>Lactobacillus plantarum</i> DSM 13273	12.6	Immunoglobulin-like fold, MucBP, CMBE-CBM3 (carbohydrate-binding), fibrinogen-binding, collagen-binding
<i>Lactobacillus brantae</i> DSM 23927	6.97	Immunoglobulin-like fold, Collagen-binding surface protein Cna-like (type B), Leucin-rich repeat, SD-repeat (type B)
<i>Lactobacillus rhamnosus</i> GG WT	104	Immunoglobulin-like fold, MucBP, Leucin-rich repeat, Fn3-like (frequently found in the adhesin/invasin streptococcal C5a), Gram positive pilin subunit D1 N-terminal (containing <i>spaCBA</i> domain, responsible of adhesion to β-lactoglobulin)

460 *proteins containing-domains written in **bold characters** for the four adhesive strains are likely to
 461 mediate adhesive interactions with β -lactoglobulin

462 **Table 1:** Predicted proteins domains with LPxTG motif which may play a role in bacterial adhesion to
 463 β -lactoglobulin. Domains present on *L. rhamnosus* GG (known to be adhesive to β -lactoglobulin) are
 464 included as a reference. Proteins sequences used were those provided by Sun et al. (2015).

		Adhesive events (%)		Adhesion forces to β -lac (nN)	Length of the stretched biomolecule (μ m)	Reference
		To β -lac	To BSA			
Strains highly adhesive to β -lac	<i>L. aquaticus</i>	82.6 \pm 7.1	27.6 \pm 10.4	1.43 \pm 0.03	0.90 \pm 0.01	/
	LGG WT	51.4 \pm 9.9	13.1 \pm 0.8	[0.13 ; 0.81] \pm 0.01	0.39 \pm 0.02	Guerin et al., 2016
	LGG <i>welE</i>	84.1 \pm 3.0	88.5 \pm 2.5	[0.58 - 1.31] \pm 0.01	0.93 \pm 0.03	Guerin et al., 2016 Guerin et al., 2017
Strains poorly adhesive to β -lac	<i>L. sharpeae</i>	3.4 \pm 1.5	2.5 \pm 0.6	NS*	/	/
	LGG <i>spaCBA</i>	NS*	/	NS*	/	Guerin et al., 2016

466 *Frequency of adhesive events as found to be inferior to 5%.

467 **Table 2:** Comparison of the adhesive capabilities of five strains to β -lactoglobulin when studied by
 468 Atomic Force Microscopy: *L. aquaticus* DSM 21051, *L. sharpeae* DSM 20505, and the model strains
 469 LGG WT, LGG *spaCBA* (pili-depleted) and LGG *welE* (expolysaccharides-depleted).

470

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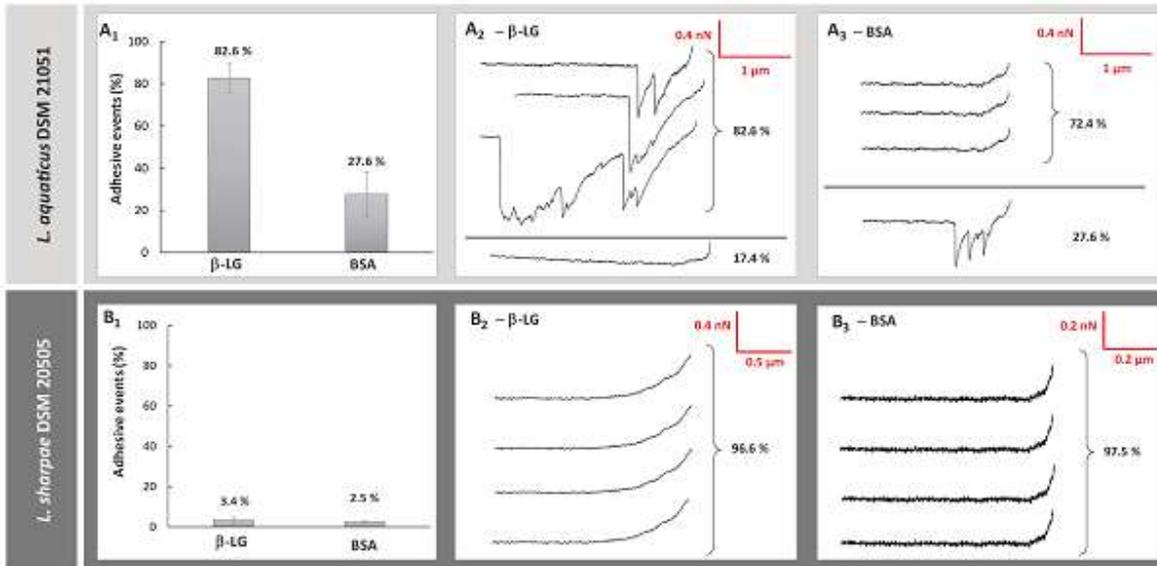
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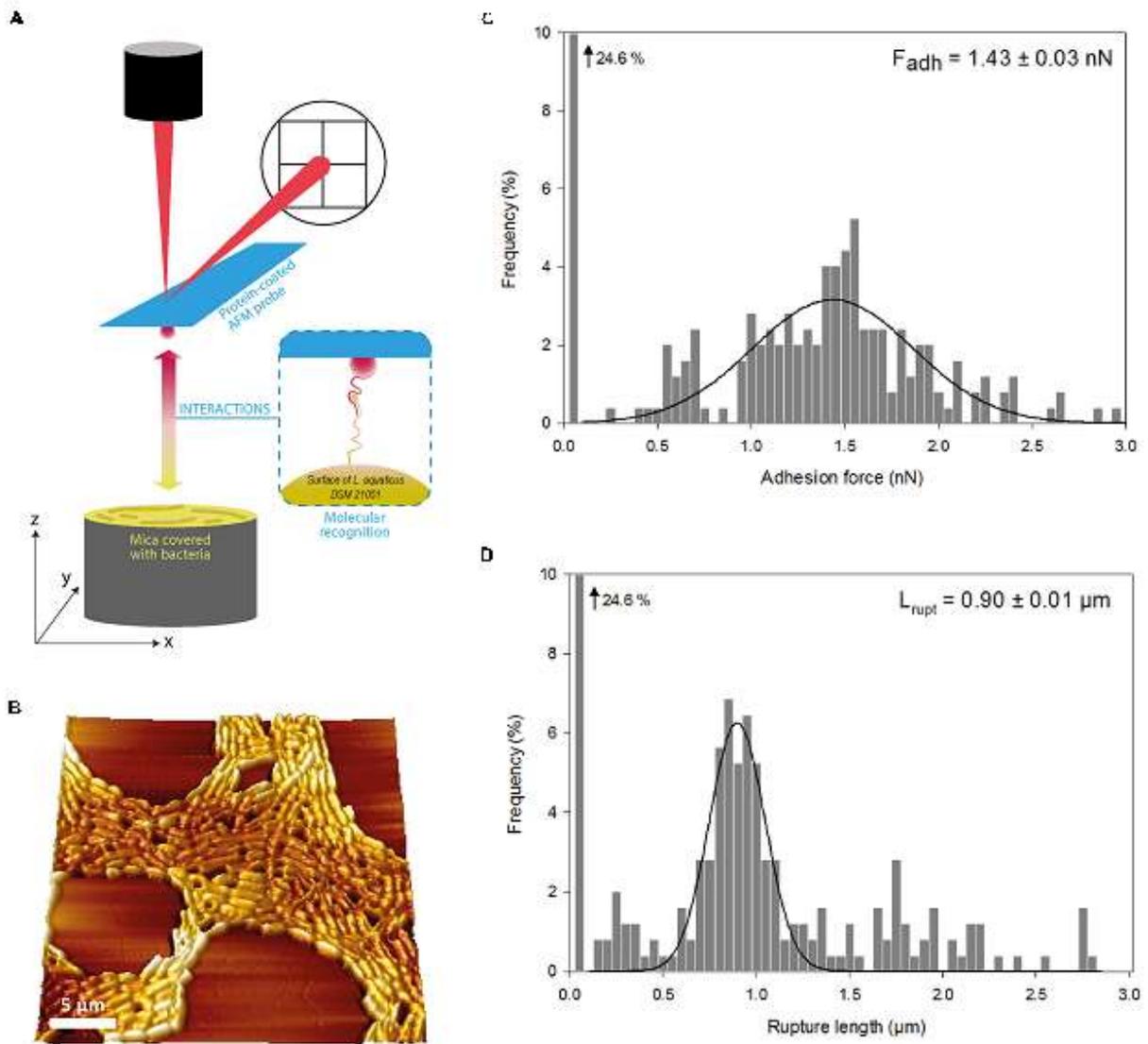
638 **Figure 1**



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641 **Figure 2**

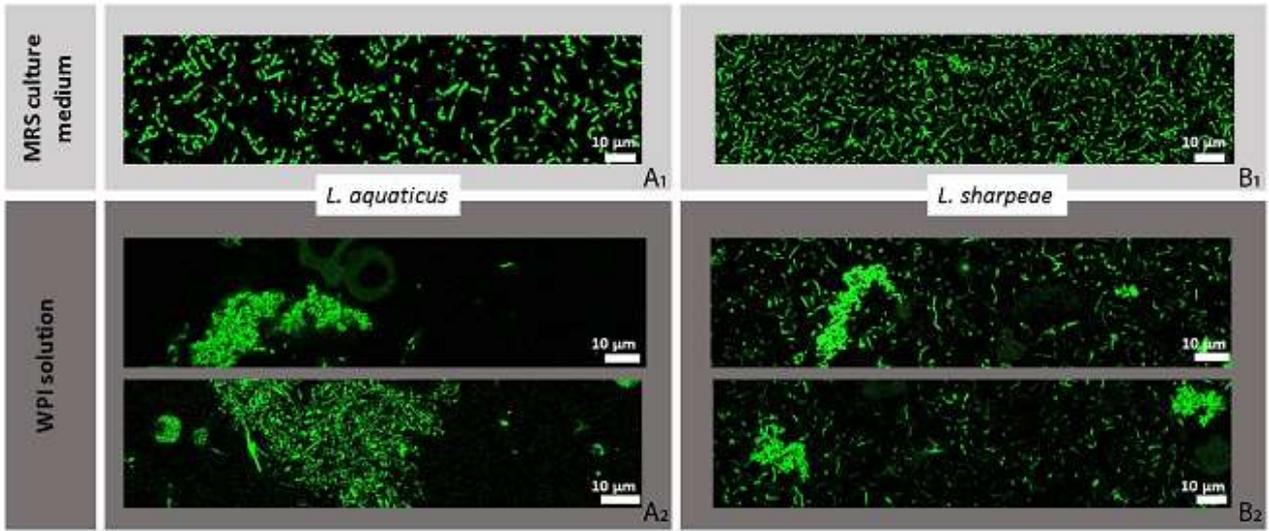


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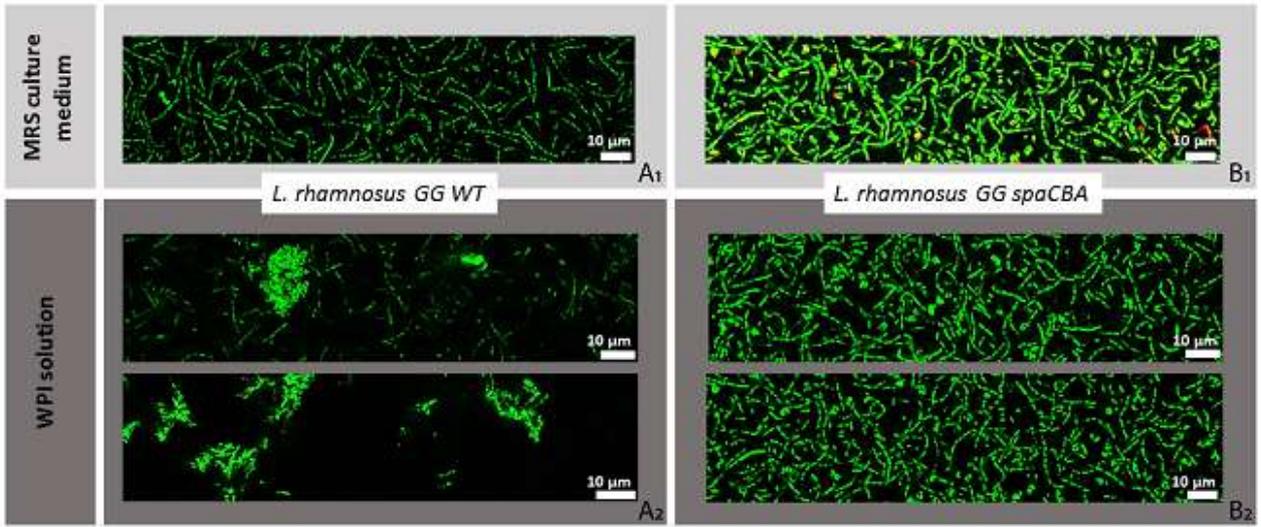
645 Figure 3



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648 Figure 4



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