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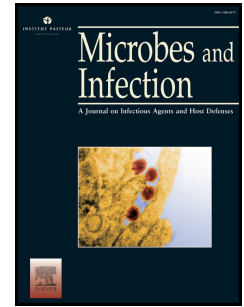
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1 **Biological differences between FIM2 and FIM3 fimbriae of *Bordetella pertussis*: not just**
2 **the serotype**

3

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36 **ABSTRACT**

37

38 **Introduction:** *Bordetella pertussis* still circulates worldwide despite vaccination. Fimbriae are
39 components of some acellular pertussis vaccines. Population fluctuations of *B. pertussis*
40 fimbrial serotypes (FIM2 and FIM3) are observed, and *fim3* alleles (*fim3-1* [clade 1] and *fim3-*
41 *2* [clade 2]) mark a major phylogenetic subdivision of *B. pertussis*.

42 **Objectives:** To compare microbiological characteristics and expressed protein profiles between
43 fimbrial serotypes FIM2 and FIM3 and genomic clades.

44 **Methods:** A total of 23 isolates were selected. Absolute protein abundance of the main
45 virulence factors, autoagglutination and biofilm formation, bacterial survival in whole blood,
46 induced blood cell cytokine secretion, and global proteome profiles were assessed.

47 **Results:** Compared to FIM3, FIM2 isolates produced more fimbriae, less cellular pertussis
48 toxin subunit 1 and more biofilm, but auto-agglutinated less. FIM2 isolates had a lower survival
49 rate in cord blood, but induced higher levels of IL-4, IL-8 and IL-1 β secretion. Global proteome
50 comparisons uncovered 15 differentially produced proteins between FIM2 and FIM3 isolates,
51 involved in adhesion and metabolism of metals. FIM3 isolates of clade 2 produced more FIM3
52 and more biofilm compared to clade 1.

53 **Conclusion:** FIM serotype and *fim3* clades are associated with proteomic and other biological
54 differences, which may have implications on pathogenesis and epidemiological emergence.

55

56 **Keywords:** *Bordetella pertussis*; phylogenetic clade; serotype; fimbriae; proteomics; virulence

57

58 **ABBREVIATIONS**

59 AC-Hly: Adenylate Cyclase Haemolysin

60 aPV: acellular Pertussis Vaccines

61 BGA: Bordet-Gengou Agar

62 *B. pertussis*: *Bordetella pertussis*

63 CFU: Colony-Forming Unit

64 DIA-MS: Data Independent Acquisition Mass Spectrometry

65 FC: Fold-Change

66 FDR: False Discovery Rate

67 FHA: Filamentous Hemagglutinin

68 FIM: fimbriae

69 LOQ: Limit Of Quantification

70 MOI: Multiplicity Of Infection

71 OD: Optical Density

72 PRM: Parallel Reaction Monitoring

73 PRN: Pertactin

74 PT: Pertussis Toxin

75 S1-PT: Pertussis Toxin Subunit 1

76 SS: Stainer and Scholte

77 wPV: whole cell pertussis vaccines

78

79

80 **1. Introduction**

81 Whooping cough is a highly contagious respiratory disease whose main causative agent is the
82 gram-negative bacterium *Bordetella pertussis* [1]. Despite the introduction of whole cell
83 pertussis vaccines (wPV) in the late 1950s, and of acellular pertussis vaccines (aPV) in the mid
84 1990s in many high-income countries, pertussis strains are still circulating worldwide [2].
85 Among the documented evolutionary changes observed in contemporaneous *B. pertussis*
86 populations, variation in the antigens contained in vaccines are of high concern given their
87 potential negative impact on vaccine effectiveness [2, 3].

88 aPVs target 2 to 5 different *B. pertussis* antigens: pertussis toxin (PT), pertactin (PRN),
89 filamentous hemagglutinin (FHA) and the two serologically distinct fimbriae FIM2 and FIM3.
90 FIM2 or FIM3 major subunits have 60 % amino acid sequence identity between each other and
91 these fimbriae contain the common minor tip subunit FIMD [4] but are characterized by
92 different properties, as illustrated by different methods required for their purification [4]. *B.*
93 *pertussis* typically express either FIM2 or FIM3, or much less commonly, both FIM2 and FIM3
94 [4]. The relative frequencies of serotypes FIM2 and FIM3 have fluctuated since the introduction
95 of vaccination: whereas FIM2 serotype was predominant in unvaccinated populations [4], a
96 shift towards FIM3 serotype occurred after the introduction of pertussis vaccines [5, 6]. More
97 recently, a reverse trend of FIM2 reemergence is being observed in France (up to 27.9 %) and
98 other European countries [6, 7].

99 The drivers of the population fluctuations of FIM serotypes remain unclear. At the molecular
100 level, the expression of fimbriae is regulated both by the BvgA/S two component system and
101 by the length of a homopolymeric tract located in the promoter sequence of genes *fim2* and *fim3*
102 [4, 8]. At the population level, one possibility is that vaccine-induced immune pressure might
103 drive evolutionary shifts in the type and level of expression of fimbriae; but as not all acellular
104 pertussis vaccines contain fimbrial proteins[4, 9], recent shifts in serotype may be linked to

105 frequency-dependent selection [4, 9]. Another possibility is that variable biological properties
106 between isolates of the two FIM serotypes would impact colonization, carriage, transmission,
107 and immune escape of *B. pertussis* isolates [10]. In favor of the latter hypothesis, some studies
108 described a role of fimbriae in strain agglutination and biofilm formation properties [11, 12],
109 as well as in the regulation of initial inflammatory responses to pertussis infection [13].

110 *B. pertussis* populations are phylogenetically structured into clades that have emerged
111 successively and are marked by key mutations in genes encoding aPV antigens, such as in the
112 Ptx promotor (from *ptxP1* to *ptxP3*), the genes for subunit A of Ptx (from *ptxA2* to *ptxA1*), and
113 pertactin (from *prn1* to *prn3* and *prn2*) [3]. An important landmark mutation is also observed
114 in the *fim3* gene itself, which displays the two predominant alleles *fim3-1* and *fim3-2* [2, 3].
115 *B. pertussis* isolates of serotype FIM2 are mainly observed in the *fim3-1* clade (clade 1), while
116 FIM3 isolates represent the vast majority of isolates of both *fim3-1* and *fim3-2* (clade 2).
117 Whereas clade 1 is ancestral, clade 2 evolved in the 1980s (wPV period) and, in France, has
118 subsequently increased rapidly in frequency (up to 42 %, observed in year 2019), especially
119 during the aPV period [2, 3, 14]. Differential susceptibility to vaccine-induced immunity, or
120 biological differences between *fim3-1* and *fim3-2* isolates in terms of disease severity and
121 transmission, could be associated with this emergence [3]. However, phenotypic variation
122 between clades 1 and 2 remains to be investigated.

123 To provide novel insights into the drivers of emergence of FIM serotypes and *fim3* clades of *B.*
124 *pertussis*, we investigated the biological variation among them, including *in vitro* vaccine
125 antigen expression, biofilm formation, autoagglutination, global proteomics, and
126 immunological features.

127

128 2. Materials and methods

129

130 2.1 Selection of *B. pertussis* isolates

131 From a set of 1120 *B. pertussis* isolates with available genomic sequences in the National
132 Reference Center (NRC) for Whooping Cough and other Bordetella infections, we selected for
133 this study 19 isolates (e.g., Fig. S1), all from infants under 6-month-old with whooping cough
134 and collected between 2008 and 2019. Isolates had been serotyped to detect the production of
135 fimbrial proteins FIM2 and FIM3 according standard recommendations [15]. The selection
136 process is described in detail in the Supplementary appendix). Selected isolates (e.g., Table S1)
137 corresponded to 8 FIM2 isolates and 11 FIM3 isolates. Among the 11 FIM3 isolates, 6 were
138 from *fim3-1* (clade 1) and 5 were from *fim3-2* (clade 2, e.g., Table S1). All isolates of FIM2
139 serotype (n=8) were from clade 1 (given the absence of FIM2 isolates from clade 2), and their
140 comparators were therefore the 5 FIM3 isolates that also belonged to clade 1. One isolate
141 (CIP1672), which does not produce FHA (a protein involved in auto-aggregation properties),
142 and 3 additional isolates without fimbriae production (FIM-, from clade 1) were also used in
143 auto-agglutination and biofilm assays.

144

145 2.2 Culture conditions

146 *B. pertussis* isolates were grown at 36 +/- 1 °C for 72 h on Bordet-Gengou Agar plates (BGA)
147 and further grown in bacterial layer on BGA for 24 h [15]. Bacterial suspensions were prepared
148 in the synthetic Stainer and Scholte (SS) liquid medium [16] and calibrated to an optical density
149 (measured at 650 nm) value of 1 +/- 0.1 (OD₆₅₀₋₁). This standardized OD₆₅₀₋₁ suspension was
150 i) immediately used for biological assays (auto-agglutination and biofilm assays) or, ii) 1/10
151 diluted in a new SS medium and cultured at 36°C with 100 RPM agitation; the culture was

152 stopped at a final OD₆₅₀- 1 +/- 0.1 and this suspension was used for cord blood infection or for
153 proteomic analyses. At each stage, the absence of BGA contamination was visually controlled.

154

155 2.3 Auto-agglutination tests

156 For each isolate, 4 ml of the calibrated OD₆₅₀-1 bacterial suspension prepared in SS medium
157 were incubated in static condition at room temperature for 4 hours. Every hour (H0, H1, H2,
158 H3, H4), the OD₆₅₀ of the top fraction of the suspension was measured [17]. Auto-agglutination
159 index was calculated as follows: $(OD_{650} \text{ initial-H}_0 - OD_{650} \text{ final-H}_4) * 100$. In each experiment,
160 one control isolate that does not produce FHA (CIP1672) and which is known to have very low
161 agglutination properties, was used [18].

162

163 2.4 Microplate biofilm assays

164 For each isolate, the calibrated bacterial suspension OD₆₅₀-1 prepared in SS medium was ¼
165 diluted and 200 µL of this dilution were added in triplicate in 96-well plate. For each
166 experiment, negative controls (SS medium) were added. The plates were incubated at 37°C for
167 24 h and 48 h, and then washed with water to eliminate planktonic bacteria. Bacteria on the
168 well surface were stained with 200 µL of Crystal Violet 0.1 %/well (Sigma Aldrich, V5265 1
169 %) for 30 minutes [17]. Plates were washed with water and dried, Crystal Violet was then
170 solubilized with ethanol 95 % and OD₅₉₅ was measured [17]. For each isolate, biofilm formation
171 was assessed by the mean OD values obtained for the triplicates. OD values for each experiment
172 were corrected by subtracting the values deriving from the negative controls.

173

174 2.5 Whole blood ex vivo infection and *B. pertussis* survival

175 Fresh heparinized cord blood instead of whole blood from adult donor was used to mimic an
176 infection in early infancy. Cord blood samples were provided by the biocollection of the Cell

177 Therapy Unit from the Biological Resources Center of Saint-Louis Hospital (Paris, France).
178 200 μ L of whole cord blood was added to each well of a flat bottom 48-well plates and
179 incubated with 200 μ L of bacterial suspension diluted in RPMI medium (RPMI Medium 1640
180 (1X) + GlutaMAXTM-I, Gibco) to the desired concentration, to reach a Multiplicity of infection
181 (MOI, ratio bacteria/cell) of approximately 10. The plates were then incubated at 37°C and 5%
182 CO₂ for 4 h (for bacterial survival assays) or 22 h (for cytokine assays). For survival assays,
183 after 4 h of whole cord blood infection, 50 μ l of the suspension were serial diluted and plated
184 on BGA to estimate *B. pertussis* survival. Survival was expressed as a percentage of the initial
185 *B. pertussis* load. Anti-PT IgG antibody concentrations were measured in all cord blood
186 samples (they were originated from 9 different donors) used in this study (kit Savyon
187 SeroPertussisTM Toxin IgG 1231-01D); all of them had IgG titers < 40 UI/ml and were therefore
188 considered seronegative.

189

190 2.6 Cytokine measurements

191 After 22 h of whole cord blood cells incubation with *B. pertussis*, the plates were centrifuged
192 at 4 °C (1500 rpm) and 150 μ l of serum was collected, aliquoted and then stored at -20 °C. Then
193 19 analytes (cytokines, chemokines, growth factor and adhesins) were measured in the sera
194 (e.g., Table S2), using the Human Magnetic Luminex Assay 20 Plex kit (R&D systems,
195 LXSAHM-20) and the Bioplex 200 technology according to the manufacturer's
196 recommendations. Data analyses are detailed in the Supplementary appendix.

197

198 2.7 Measurement of cellular protein expression by two complementary MS proteomic 199 approaches

200 For each strain, 1 ml of standardized OD₆₅₀-1 suspension was transferred in an Eppendorf tube
201 and centrifuged during 10 min at 4 °C and at 8000 rpm. The pellet was suspended in 100 μ l of

202 a Urea Buffer 8M/Tris HCl 0.1M lysis buffer and then sonicated during 5 cycles of 30 seconds
203 and frozen at -80 °C before use for proteomic characterization.

204 PRM (Parallel Reaction Monitoring) is a proteomic method [19, 20], allowing the absolute
205 quantification of targeted proteins. In PRM a set of unique peptides are used as surrogates and
206 specifically targeted. The method is based on the identification/quantification of endogenous
207 peptides related to their isotopically-labeled counterparts used as standards and spiked into each
208 sample. Here, we targeted the 6 main virulence factors produced by *B. pertussis* (PT, FHA,
209 PRN, FIM2 and FIM3, AC-Hly) all of which except AC-Hly are included in acellular vaccines.
210 Proteins quantities were expressed in nanograms.

211 We then used a large-scale proteome analysis based on Data Independent Acquisition Mass
212 Spectrometry (DIA-MS) [21]. DIA-MS mode consists in consecutive precursor isolation
213 windows which are fragmented to obtain complex product ion spectra used to match with a
214 *Bordetella* reference spectral library that we constituted considering the proteomes of *B.*
215 *pertussis* strains Tohama I (Uniprot: UP000002676) and B1917 (Uniprot: UP000031744). All
216 details regarding proteomic methods used are found in the Supplementary appendix.

217

218 2.8 Data processing and statistical analyses

219 For each isolate, levels of proteins quantified by PRM, and OD values from biofilm, auto-
220 agglutination and survival assays were expressed in median and interquartile range. All graphs
221 and figures were obtained with GraphPad Prism 8.4.3. Dot blots differences between groups of
222 isolates (isolates of FIM2 vs. FIM3 serotypes, and from clade 1 vs. clade 2) were identified
223 with nonparametric Mann Whitney U test or Kruskal-Wallis tests followed by post-hoc multiple
224 comparisons Dunn's test, when appropriate.

225 Cytokine heatmaps were made and analyzed with Qlucore OMICS explorer 3.7 (version
226 1.1.463), and cytokine dot plots with GraphPad Prism 8.4.3. A p-value < 0.05 , or an adjusted
227 p-value value for Luminex assays < 0.05 was considered statistically significant.

228 For DIA-MS, data from three biological samples for each condition (replicates) were analyzed
229 and compared between groups of isolates. When comparing two biological conditions, proteins
230 exhibiting less than six quantified values in at least one biological condition were discarded
231 from the list to ensure a minimum of replicability. Next, proteins without any value in one or
232 the other biological condition have been considered as proteins quantitatively present in a
233 condition and absent in another. They have therefore been set aside and considered as
234 differentially abundant proteins. After \log_2 transformation of the intensities of the leftover
235 proteins, missing values were imputed using the “impute.mle” function of the R package imp4p
236 [22]. Proteins with a fold-change (FC) under 2 (i.e. absolute $\log_2(\text{FC}) < 1$) have been considered
237 not significantly differentially abundant. Statistical testing of the remaining proteins (having a
238 fold-change over 2) was conducted using a limma t-test thanks to the R package limma [23].

239 An adaptive Benjamini–Hochberg procedure was applied on the resulting p values thanks to
240 the function “adjust.p” of R package cp4p [24], using the ‘slim’ method to estimate the
241 proportion of true null hypotheses among the set of statistical tests [25]. The proteins associated
242 to an adjusted p-value inferior to a false discovery rate (FDR) of 1% have been considered as
243 significantly differentially abundant proteins. Finally, the proteins of interest are therefore those
244 which emerge from this statistical analysis supplemented by those which are absent from one
245 condition and present in another. Results of these differential analyses are summarized in
246 volcano plots and heatmaps. Heatmaps display the mean z-scores of proteins with small p-
247 values along the biological samples. A z-score is a $\log_2(\text{intensity})$ value minus the average
248 divided by the standard deviation of the $\log_2(\text{intensity})$ values of a protein in all the samples. A
249 mean z-score have been computed from z-scores of the 3 replicates of a same biological sample.

250 Hierarchical clustering on the left side of heatmaps are estimated from the mean z-scores using
251 a Pearson correlation-based distance and the Ward's method.

252

253 3. Results

254

255 3.1 *FIM2 isolates produce more fimbriae and less cellular S1-PT than FIM3 isolates*

256 To investigate whether the cellular abundance of key virulence factors (S1-PT, FHA, PRN and
257 AC-Hly) was variable according to the FIM serotype, we quantified their absolute levels as well
258 as the major subunits of fimbriae FIM2 and FIM3, using a targeted proteomics approach by
259 PRM (e.g., Table S3). For this, 6 FIM3 and 8 FIM2 isolates (total n=14, all genotyped as *ptxP3*,
260 *fim3-1*) were used (e.g., Fig. S1). All isolates presented a similar median generation time (hence
261 growth rate) in SS medium: 7.04 hours for FIM3 and 7.46 hours for FIM2 isolates (p=0.23,
262 e.g., Fig. S2), even though we noticed a higher dispersion for FIM2 isolates, with generation
263 times ranging from 5.65 to 8.15 hours.

264 As expected, FIM2 and FIM3 isolates produced almost exclusively FIM2 and FIM3,
265 respectively. When compared to FIM3 isolates, median levels of fimbriae produced by FIM2
266 isolates were much higher (2.80 ng of FIM2 vs. 0.75 ng of FIM3, p=0.0012, e.g., Fig. S3),
267 whereas the median levels of S1-PT produced by FIM2 isolates were lower (1.30 ng vs. 2.10
268 ng, p=0.004) (e.g., Fig. 1). No significant difference in levels of the three other tested virulence
269 factors (FHA, PRN and AC-Hly) was observed (e.g., Fig. 1). Of note, a residual production of
270 FIM2 was observed in FIM3 isolates, as well as FIM3 in FIM2 isolates ($<10^{-11}$ ng) as already
271 reported [8].

272

273 3.2 *FIM2 isolates are characterized by a lower auto-agglutination and a higher biofilm*

274 *formation compared to FIM3 isolates*

275 As *B. pertussis* can adapt to its environment through a higher persistence via agglutination or

276 biofilm formation [26], we evaluated these properties on the same 14 isolates. Three FIM
277 deficient (FIM-) isolates were used as controls. At 4 hours of incubation, we observed a much
278 lower auto-agglutination in FIM2 isolates compared to the FIM3 ones, with a median Auto-
279 Agglutination Index (IAA) of 7.00 vs. 91.67, respectively ($p=0.002$; e.g., Fig. 2A). A significant
280 IAA difference between FIM2 and FIM3 isolates was already observed as of H2 (IAA-H2 of
281 2.71 vs 89.11, $p=0.002$, data not shown). The IAA of FIM2 isolates was comparable to control
282 (FHA-) isolates ($p > 0.99$). Measurement of biofilm formation by Crystal Violet revealed that
283 FIM2 isolates produced more biofilm than FIM3 isolates, with a median OD595nm at 24 hours
284 of 1.11 vs. 0.35 ($p=0.007$), and at 48 hours 2.30 vs. 0.79 ($p=0.01$), respectively (e.g., Fig. 2A
285 and 2C). The 3 FIM- isolates had comparable auto-agglutination and biofilm formation
286 properties as the FIM3 ones (e.g., Fig. 2A-C).

287

288 3.3 *FIM2 isolates induce a distinct cytokine release profile, when compared to FIM3* 289 *isolates*

290 To analyze bacterial resistance to whole blood bactericidal activity and immunological ex vivo
291 processes induced by *B. pertussis* according to serotype, we used human cord blood, considered
292 to reflect the neonatal conditions. The survival rate was lower for FIM2 isolates than for FIM3
293 isolates (median survival rate: 3.62 % vs. 22.00 %, respectively, $p=0.04$; e.g., Fig. 2D).

294 Levels of cytokines and chemokines were also measured in sera following ex vivo cord blood
295 infection by using FIM2 or FIM3 isolates (Fig. 3). After infection, levels of inflammatory
296 markers were elevated for 16/19 measured proteins (adjusted p -value=0.049) as compared to
297 non-infected cord blood (e.g., Fig. S4). Among these 16 markers, we observed markedly higher
298 levels of IL-4 and higher levels of IL-8 and IL-1 β and almost no IL-2 secretion and lower levels
299 of IL-6 following infection with FIM2 isolates, when compared with FIM3 isolates (adjusted
300 p -value=0.049, e.g., Fig. 3).

301

302 *3.4 Isolates from clade 2 produce more FIM3 and more biofilm*

303 To investigate biological differences between *B. pertussis* genomic clades, we selected 11 FIM3
304 isolates: 6 from clade 1 and 5 from clade 2 (n=11). These isolates presented a similar median
305 generation time (hence growth rate) in SS medium: 7.04 hours and 6.86 hours for isolates from
306 clade 1 and 2, respectively (p=0.43, e.g., Fig. S2). Absolute quantification of proteins by PRM
307 revealed that isolates from clade 2 expressed more FIM3 than those from clade 1 (median levels
308 of FIM3: 1.06 ng vs. 0.59 ng, respectively, p=0.004, e.g., Fig. 4). No difference in protein
309 abundance of other tested proteins (S1-PT, FHA, PRN and AC-Hly) was observed between
310 isolates from clade 1 and 2 (e.g., Fig. 4). Auto-agglutination properties were similar between
311 isolates from clade 1 and 2 (p=0.19, e.g., Fig. 5A). Levels of biofilm production were higher in
312 clade 2 than in clade 1 isolates after 24 hours of incubation (median OD595nm at 0.86 vs. 0.35,
313 respectively, p=0.011; e.g., Fig. 5B), but this did not reach significance at 48 hours (p=0.14,
314 e.g., Fig. 5C).

315 We also proceeded to an in vitro cord blood infection by 4 FIM3 *B. pertussis* isolates (2 from
316 clade 1 and 2 from clade 2). A difference in terms of survival was observed between clades but
317 without reaching significance (median survival rates: 3.40 % vs. 22.0 % vs. for clades 2 and 1,
318 respectively, p=0.09, e.g., Fig. 5D). Levels of cytokines and chemokines were also measured
319 in sera following in vitro cord blood infection. As previously observed, levels of inflammatory
320 markers were overall elevated, with 14/19 measured proteins significantly elevated compared
321 to non-infected cord blood (adjusted p-value<0.05, e.g., Fig. S5). However, no significant
322 difference was observed between isolates from genomic clades 1 and 2.

323

324 *3.5 Large-scale proteome analysis of B. pertussis circulating isolates*

325 To test whether the abundance of *B. pertussis* proteins, beyond the main virulence factors, could
326 vary according to serotype (FIM2 vs FIM3) and to genomic clade (clade 2 vs clade 1), we
327 performed a large-scale proteomics analysis using DIA-MS to obtain a comprehensive view of
328 the proteome [21, 27]. In all samples, we detected a total of 2,482 different proteins (Table S4).
329 The median number of proteins detected per isolate was 2,407 (ranging from 2,298 to 2,440,
330 which represents 64.6 to 68.6% of the entire *B. pertussis* proteome that contains a median value
331 of 3,555 encoded proteins [28]. 2,111 proteins (85.05%) were detected in all the samples (in all
332 the 3 replicates of all the strains) with similar distribution of intensities suggesting relatively
333 consistent quantification of proteins. Based on the pre-defined stringent selection criteria
334 ($|\log_2(\text{FC})| > 1$, $p\text{-value} < 1\%$), 15 proteins were found differentially abundant when comparing
335 FIM2 and FIM3 isolates. In addition, one protein, the replication-associated recombination
336 protein A (WP_003814028.1), was only detected in FIM2 isolates. Of note, 26.7 % of proteins
337 represented in Fig 6B were known to be *bvg*-regulated [29]. Among the 15 differentially
338 expressed proteins, 12 were more abundant in FIM2 isolates, among which FIM2, a
339 molybdenum transport system permease ModC (Q7VUJ4), a ferredoxin (Q7VS61), a putative
340 zinc-binding dehydrogenase (Q7VT48) and the ferric siderophore receptor BfrC (Q7VT87)
341 (e.g., Fig 6., Table S5). The three less abundant proteins in FIM2 isolates were a putative
342 exported protein (Q7VUI9), a urease accessory protein UreD (P0A4R4), and a ribosomal
343 subunit interface protein (WP_019247685.1). We did not observe a differential expression
344 among proteins involved in *B. pertussis* biofilm formation [30, 31]. Of note, the *Bordetella*
345 intermediate protein A BipA (Q7VZ27), involved in *B. pertussis* colonization and biofilm
346 formation [30], tended to be slightly more abundant in FIM2 than in FIM3 isolates
347 ($|\log_2(\text{FC})| = 0.68$; $p\text{-value} = 0.09$, e.g., Table S5). Concerning proteins represented in Figure 6B,
348 we found interactions among some of them using STRING interaction network (<https://string->
349 [db.org/](https://string-db.org/)) [32]. Proteins of these networks could be associated with virulence patterns.

350 A first network grouped some proteins encoded by genes localized within the “*his*” operon, and
351 involved in histidine biosynthesis (Q7VZ1,2,3 &4 and Q7VSY6,8 &9). Histidine biosynthesis
352 and transport genes can be regulated by a variety of mechanisms including a repressor protein
353 and various RNA structures, such as transcriptional attenuators and T-box riboswitches [33].
354 Observed variations in histidine biosynthesis could affect bacterial fitness. A second network
355 included genes of 2 proteins (Q79GQ2 &5) from T3SS named Bcr4 and BcrH1 and a third one
356 contained 2 proteins (Q7VT48 &50), one of them being a putative zinc binding
357 deshydrogenase. Not all the proteins from *his* or T3SS operons were found differentially
358 expressed in our proteomic analysis: some were not detected, and some were just below
359 significance. For all these proteins related either to T3SS or to iron metabolism, metal transport
360 metabolic pathways can be connected as expression of type III secretion system can be affected
361 by several factors such as iron starvation, which leads to an upregulation of T3SS genes in the
362 ancestral species *B. bronchiseptica* [34]. Targeted transcriptomic and proteomic analyses
363 focused on all the proteins contained in these operons could help solving this issue.

364 When comparing FIM3 isolates from clades 1 and 2, 22 proteins were differentially produced
365 ($|\log_2(\text{FC})| > 1$, $p\text{-value} < 0.1\%$), e.g., Fig. S6, Table S6). Of note, 27.3% of proteins represented
366 in Fig S6B were known to be bvg-regulated according to Moon et al [29]. Among them, 12
367 proteins were more abundant in isolates from clade 2, including FIM3 (as P17835 and as
368 WP_019248658.1; e.g., Table S6). We also identified the ferric siderophore receptor BfrC
369 (Q7VT87), a putative zinc-binding dehydrogenase (Q7VT48), a putative membrane protein
370 (Q7VYP6) and a putative cold shock-like protein (Q7VSQ7). Ten proteins were less abundant
371 in clade 2 isolates (e.g., Fig. S6), including a multidrug resistance protein NorM (Q7VYS9), a
372 putative exported protein (Q7VUI9), a putative inner membrane efflux protein (Q7VWD9), a
373 putative 2-hydroxyacid dehydrogenase (Q7VU57), and two proteins involved in T3SS (proteins
374 Q79GQ1 and Q7VWI3). We did not observe between clades a differential expression of

375 proteins involved in *B. pertussis* biofilm formation [30, 31]. However, BipA (Q7VZ27) was
376 slightly more abundant in clade 2 than clade 1 isolates ($|\log_2(\text{FC})|=0.42$; p-value=0.09). FIM2
377 was also detected in isolates from the two clades without significant difference.

378

379 4. Discussion

380 Here, we analyzed the abundance of major virulence factors, whole cell proteomes, and other
381 biological differences, of *B. pertussis*. We used a combination of two highly innovative
382 proteomic approaches, PRM and DIA-MS. Previous proteomic studies on *B. pertussis* used
383 rather gel-based proteomics approaches [35, 36]. Whereas the most recent proteomics studies
384 based on mass spectrometry obtained 20 to 30% coverage of *B. pertussis* theoretical proteome
385 [37, 38], we reached more than 60% coverage highlighting the power of our technology to
386 analyze bacterial proteomes. Such high coverage was reported in previous DIA analysis of the
387 *E. Coli* proteome [39]. Furthermore, the previous studies on *B. pertussis* proteome aimed to
388 compare recently circulating isolates to historical ones, or recent isolates according to PRN
389 expression status and *ptxP* and *fhaB* alleles [37, 40-42], whereas here we compared expression
390 levels of vaccine antigens as well as whole proteome according to FIM serotype and *fim3* clade,
391 using a large set of isolates. Our study design enables us to address the association of these
392 variations with precise phenotypic features, enabling the formulation of specific hypotheses on
393 the impact of recent molecular changes.

394 4.1 Differences in fimbriae production and its association with biofilm formation

395 The recent increase of FIM2 serotype observed in some European countries [6, 7], raised the
396 question of possible differences between serotypes at the global proteome level and their
397 implications on specific biological properties. The variations of FIM2 and FIM3 production we
398 observed for each isolate were assessed from five highly reproducible biological replicates,
399 indicating that possible heterogeneities among individual cells within cultures were negligible.

400 Here, we found that one prominent difference between FIM serotypes was the higher
401 production of fimbriae by FIM2 isolates. This result confirms but also quantifies, previous
402 findings observed with electron microscopy [4, 43]. In addition, our phenotypic experiments
403 showed that FIM2 isolates produced more biofilm, suggesting a correlation between levels of
404 fimbriae and biofilm formation. This correlation has already been observed in other pathogens
405 [44], but a direct causality still needed to be investigated in *B. pertussis*. Here, the observed
406 variations in biofilm formation could also be due to proteins other than fimbriae that are
407 differentially produced or organized at the cell surface in isolates of different FIM serotype;
408 Our whole proteome analyses showed slightly higher levels of BipA proteins in FIM2 isolates
409 compared to FIM3 ones. BipA, an intermediate phase protein, was described as the most
410 abundant surface-exposed protein in *B. pertussis* biofilms [30] and reported to prevent auto-
411 agglutination and to promote biofilm formation by *B. holmesii* isolates [45]. BipA could
412 therefore explain the variable auto-agglutination and biofilm formation properties of FIM2 and
413 FIM3 isolates. Interestingly, we also observed that FIM3 isolates from the recently emerged
414 clade 2 also produces more fimbriae than those from clade 1; this property of clade 2 is also
415 associated with more biofilm formation and a tendency for higher BipA production. Further
416 investigations are needed to determine whether the difference in biofilm formation is a
417 consequence of the expression levels of fimbriae or an indirect effect of other proteins such as
418 BipA. Overall, this work puts forwards the hypothesis that the drivers of emergence of both
419 FIM2 and clade 2 may be linked to adhesion and biofilm properties.

420 In our study, we observed a much lower auto-agglutination of FIM2 isolates compared to FIM3
421 ones. FHA is the main protein involved in auto-agglutination in *B. pertussis* [46]. Protein
422 quantification by PRM revealed that all tested *B. pertussis* isolates produce similar amounts of
423 FHA. Fimbriae proteins are also known to be involved in autoagglutination of *B. pertussis* [12],
424 with a higher autoagglutination observed in strains producing high FIM3 levels. This suggests

425 that either the nature of serotype 2 fimbriae, or their high expression levels at the bacterial
426 surface, may interfere with FHA agglutination properties. Cooperation between fimbriae and
427 FHA for adhesion and immune modulation has been suggested in *B. bronchiseptica* [47]. The
428 FIMD adhesin might also be of importance in these possible interactions, as its involvement on
429 human cells adhesion has been demonstrated [48]. We did not identify any difference in FIMD
430 production between FIM2 and FIM3 isolates by the DIA proteomic approach, however future
431 studies are needed to explore cell surface conformational modifications associated with FIM
432 production levels and their potential impact on bacteria-bacteria adhesion rather than on human
433 cell adhesion.

434 4.2 Differences in other vaccine antigens production

435 Concurrently with the change in FIM serotype, the recent emergence of isolates that do not
436 produce PRN (PRN-) has been reported in many countries with high acellular vaccine coverage
437 (e.g., in France since 2007 to 2019 from 5.6 to 52.1%) [6, 49]. To avoid interference with this
438 effect, our study only included isolates that produce PRN, and we did not observe any difference
439 in terms of PRN levels between isolates from FIM2 and FIM3 serotypes. This suggests a similar
440 vaccine-driven selective pressure for PRN loss on all *B. pertussis* isolates, regardless of FIM
441 serotype. Indeed, although PRN deficiency was first observed in FIM3 isolates, more recently
442 this was also identified within FIM2 isolates, in Spain [50] and Slovenia [51].

443 Our PRM analysis showed that FIM2 isolates display lower levels of cellular-associated
444 fraction of S1-PT, when compared with FIM3 isolates. This is a potentially important
445 observation raising the possibility of a differential virulence of FIM2 isolates. The *ptx* and *ptl*
446 genes, encoding PT subunits S1-S5 and PT secretion system respectively, are co-transcribed
447 under the control of the *B. pertussis* *ptx* promoter (*ptxP*) [52]. At the genomic level, *ptx-ptl*
448 locus and *fim* encoding genes are not related. Here, FIM2 and FIM3 isolates are both

449 characterized by a *ptxP3*-type promoter, excluding variations of *ptxP* as an explanatory factor.
450 In a landmark work, Mooi et al. have suggested that *ptxP3* emerging lineages produced more
451 PT than ancestral *ptxP1* ones [53]. However, other teams suggested that *ptxP3* is not an up-
452 promoter and therefore should not affect PT expression (Stibitz S., International Bordetella Lab
453 Meeting 2021. “unpublished”). Here, we note that the FIM serotypes of the 8 isolates used by
454 Mooi et al. (also controlled by the French NRC for Bordetella infections) were not
455 homogeneous: 2 *ptxP1* isolates produced FIM2. Hence, the Mooi et al. *ptxP1-ptxP3* comparison
456 may have been affected in part by the fimbriae serotype of isolates, rather than solely by the
457 *ptxP1-ptxP3* genotypes. Further investigations of isogenic mutants are needed to determine
458 whether fimbriae proteins can interfere with levels of intracellular PT, and whether similar
459 findings are observed at the transcriptome and secretome levels.

460

461 4.3 Differences in cytokine profiles in whole cord blood induced by *B. pertussis* isolates

462 In our study, we showed that FIM2 isolates had lower survival rate in blood and induced higher
463 secretion of IL-4, IL-8 and IL-1 β than FIM3 isolates. Since infants of less than 3-months are
464 the most vulnerable population to pertussis, using this experimental *in vitro* setup [54], which
465 reflects the response immune signatures of young infants, was considered to be relevant [55].
466 IL-4 is involved in the regulation of inflammation and Th2 response [56], IL-8 induces
467 chemotaxis in target cells and stimulates phagocytosis [57] and IL-1 β is a potent pro-
468 inflammatory cytokine that is crucial for host-defense responses to infection [58]. Conversely,
469 FIM3 isolates were associated with more IL-2 and IL-6 secretion; IL-2 is involved in T-cell and
470 Th1 response [59] and IL-6 is a key pro-inflammatory cytokine associated to the stimulation of
471 acute phase proteins [60]. Interestingly, natural infection and immunization with wPVs both
472 induce a pattern of Th1/Th17 response, while aPVs induce a Th2 response [61, 62], similarly

473 to our in vitro results on whole blood stimulation with FIM2 isolates. The differences found
474 here between FIM2 and FIM3 induction are remarkable and suggest a distinct immune reaction
475 profile depending on *B. pertussis* serotype. FIM2 and FIM3 have been copurified and integrated
476 jointly in fimbriae-containing aPVs. It has been shown that FIM2 is more immunogenic than
477 FIM3 [4, 63, 64], in terms of antibody responses which is mostly Th2-dependent [61-63]. Our
478 findings might be of importance in vaccine development, which needs to clearly identify the
479 respective role of FIM2 and FIM3 on induction or modulation of immune responses.
480 Interestingly, Kroes et al. suggested mild immunological activation of human airway
481 epithelium by 3 different *B. pertussis* strains from unknown serotype, associated with the
482 secretion of IL-6, CXCL8/IL-8 and the enrichment of genes involved in bacterial recognition
483 and innate immune processes [65]. Our results suggest that these previously published results
484 should be interpreted with regard to the serotype of the isolates that were studied. Further
485 investigations are needed to make a link between these observations especially because they
486 are using different study models (whole blood and epithelium).

487 4.4 Differences in proteome profiles

488 Most of *B. pertussis* virulence factors are regulated by the master two-component bvgAS
489 system [29]. In our dataset, less than 30% of differential proteins were known as bvg-regulated.
490 Most of the differential proteins identified with our large-scale proteome analysis of *B. pertussis*
491 circulating isolates were related to transport of metal chemicals such as molybdenum, iron or
492 zinc. Molybdenum has been described in other bacterial species such as *Pseudomonas*
493 *aeruginosa*, to participate in anaerobic respiration, virulence, biofilm formation or intracellular
494 survival within macrophages [66]. The higher levels of molybdenum transport system permease
495 ModC (Q7VUJ4) in FIM2 isolates may be related to a greater biofilm production with FIM2
496 isolates [67]. Ferric siderophore receptor bfrC (Q7VT87) and ferredoxin (Q7VS61) were also
497 more abundant in FIM2 isolates; as iron is crucial for *Bordetella* adaptation to nutrient

498 limitation [68], this finding suggests that FIM2 isolates may be more efficient at acquiring iron,
499 which may have an impact on bacterial growth, fitness and survival. In our experiments, we
500 used SS medium that contains iron as ferrous sulfate, which might have impacted our data;
501 additional experiments on iron-restricted SS medium would be necessary to further investigate
502 differential iron-regulated protein expression by FIM2 or FIM3 *B. pertussis* isolates. The
503 proteins found more abundant in clade 2 also include iron or zinc related proteins, providing
504 interesting hypotheses as to the evolutionary success of clade 2 and the impact of metal
505 metabolism in *B. pertussis* evolution: there were some interesting commonalities between the
506 phenotypic novelties observed in the two emerging groups FIM2 and *fim3-2* from their
507 ancestors, in particular the increase in the expression of certain proteins such as FIM, iron or
508 zinc metabolism proteins. This suggests that similar selective pressures are acting, leading to
509 parallel evolution in emerging *B. pertussis* populations. Whereas the role of aPV vaccines that
510 contain FIM proteins may drive the evolution towards higher levels of FIM expression, iron
511 metabolism evolution might be driven by host adaptation, which is still recent for *B. pertussis*
512 [3].

513

514 In conclusion, our study suggests that ongoing evolutionary shifts of *B. pertussis* towards
515 expression of FIM2 rather than FIM3, and through emergence of clade 2, are associated with
516 higher FIM fimbriae production and biofilm formation, potential immune escape, and
517 differential metal metabolism. Furthermore, the lower cellular levels of PT in FIM2 isolates
518 may impact *B. pertussis* virulence. These evolutionary observations raise important hypotheses
519 that await experimental demonstration using FIM isogenic mutants to define the mechanisms
520 behind these important phenotypic differences.

521

522 **Authors' contributions**

523 JT and SB conceived the study, and JT, VB, SM, MM and SB designed the study. SM, PL, NC,
524 AL and SG collected the data, and SM, PL, NC, VB and JT were responsible for data
525 management and analysis. SM, PL, MH were involved in cytokines data analysis. TD, QGG,
526 JCR and MM were responsible for proteomic management and analyses. TD and QGG did the
527 bioinformatic and statistical analyses. SM, VB and JT wrote the first draft of the manuscript,
528 had access to and verified the source data, and were responsible for the decision to submit the
529 manuscript. All authors drafted the manuscript for important intellectual content, contributed
530 to the revision of the final version of the manuscript, and approved the final version submitted.
531 All authors had full access to all the data in the study and had final responsibility for the decision
532 to submit for publication.

533

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538

539 Conflict of interest:

540 The authors declare no conflict of interest

541

542 Ethical approval:

543 This study was approved by the Comité Ethique et Scientifique pour les Recherches, les Etudes
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Figure Legends

Fig. 1: Abundance of 4 proteins involved in virulence by FIM2 and FIM3 isolates based on PRM absolute quantification

Cellular abundance of 4 different virulence factors: (A) S1-PT, (B) PRN, (C) FHA, (D) FIM and (E) AC-Hly, were measured by PRM [in grams (g)] in 8 FIM2 isolates (FR5510, FR5810, FR5862, FR5994, FR6156, FR6440, FR6762, FR6845, blue dot) and 6 FIM3 isolates (FR4887, FR5031, FR5333, FR5782, FR5893, FR6167, black dot). Each dot represents the mean protein amount of 5 independent experiments. Levels of measured proteins are represented in median and interquartile. P-value <0.05 of the Mann-Whitney test between FIM2 and FIM3 serotype group was considered significant.

Fig. 2: Auto-agglutination, biofilm formation and survival in blood of FIM2 and FIM3 isolates

Biological phenotypes of 8 FIM2 isolates (FR5510, FR5810, FR5862, FR5994, FR6156, FR6440, FR6762, FR6845, blue dot), 6 FIM3 isolates (FR4887, FR5031, FR5333, FR5782, FR5893, FR6167 black dot), were compared in terms of: (A) Auto-agglutination property based on an auto-agglutination index ($OD_{650\text{ nm}} \text{ at H0} - OD_{650\text{ nm}} \text{ at H4} \times 100$), (B) Biofilm formation after 24 hours and (C) 48 hours of incubation (Crystal Violet 0.1% $OD_{595\text{ nm}}$). The 3 FIM- isolates (FR4922, FR4925, FR5167, orange dot) and one isolate that do not produce FHA (CIP1672, Ctrl, grey dot) were used as controls. (D) Bacterial survival rate (%) after 4 hours in whole cord blood (MOI=10) was quantified in CFU/ml for a subset of 2 different FIM2 (FR5862, FR6440, in blue) and 2 FIM3 (FR4887, FR5333, in black) isolates. Each dot represents the mean of 3 independent experiments for each isolate. Measures of IIA, Biofilm and survival are represented in median and interquartile. P<0.05 of the Kruskal Wallis followed by Dunn's comparison for auto-agglutination and biofilm and Mann Whitney test between FIM2 and FIM3 for survival, was considered significant.

Fig. 3: Cytokine release after infection of whole cord blood cells by FIM2 and FIM3 isolates

Cord blood [28] from healthy donors (n=9) was infected ex vivo with FIM2 isolates (FR5862 and FR6440, expected MOI of 10, 6.55×10^7 to 1.84×10^8 CFU/ml) or FIM3 isolates (FR5333, expected MOI of 10, 7.7×10^7 to 3.5×10^8 CFU/ml). Analytes were measured in whole blood supernatants after 22 hours of infection using a panel of 19 cytokines/chemokines (Human Magnetic Luminex Assay 20 Plex kit) (A) The heatmap represents a hierarchical clustering of all the cytokines, chemokines and growth factors differentially secreted (adjusted p-value<0.05) upon stimulation with FIM2 isolates (in blue) and by FIM3 isolates (in black). Cytokines are expressed as pg/ml and log transformed with blue to red colors representing lower to higher expression respectively. Levels (in pg/ml) of (B) IL-4, (C) IL-8, (D) IL-2 and (E) IL-6 in whole cord blood following infection with FIM2 (in blue) and FIM3 (in black) isolates are represented in median and interquartile. Each dot represents an experiment. P-value <0.05 of the Mann-Whitney test between FIM2 and FIM3 serotype group was considered significant.

Fig. 4: Abundance of 4 proteins involved in virulence by FIM3 isolates from clade 1 and 2 based on PRM absolute quantification

Cellular abundance of 4 different virulence factors: (A) FIM3, (B) S1-PT, (C) PRN, (D) AC-Hly, (E) FHA, were measured by PRM [in grams (g)] in 6 FIM3 isolates from clade 1 (FR4887, FR5031, FR5333, FR5782, FR5893, FR6167) and 5 isolates from clade 2 (FR3977, FR4930, FR5468, FR5730, FR5771). Each dot represents the mean of 5 independent experiments for each isolate. Levels of measured proteins are represented in median and interquartile. P-value <0.05 of the Mann-Whitney test between FIM2 and FIM3 serotype group was considered significant.

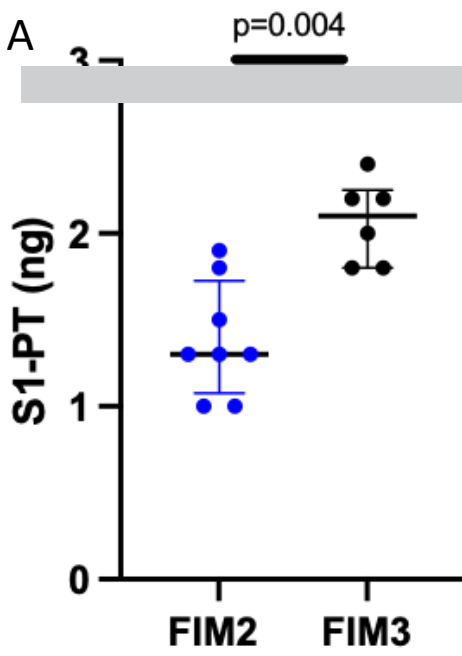
Fig. 5: Auto-agglutination, biofilm formation and survival in blood of *B. pertussis* isolates from clade 1 and 2.

Biological phenotypes of 6 FIM3 isolates from clade 1 (FR4887, FR5031, FR5333, FR5782, FR5893, FR6167, black dot) and 5 FIM3 isolates from clade 2 (FR3977, FR4930, FR5468, FR5730, FR5771, green dot) were compared in terms of: (A) Auto-agglutination property based on an auto-agglutination index ($OD_{650\text{ nm}} \text{ at H0} - OD_{650\text{ nm}} \text{ at H4} \times 100$), (B) Biofilm formation at 24 hours and (C) 48 h of incubation (Crystal Violet 0.1% $OD_{595\text{ nm}}$). One isolate that do not produce FHA (CIP1672, Ctrl, grey dot) was used as control. (D) Bacterial survival rate (%) after 4 h in whole cord blood (MOI=10) was quantified in CFU/ml for a subset of 2 FIM3 isolates from clade 1 (FR4887, FR5333, in black) and 2 FIM3 isolates from clade 2 (FR4930, FR5730, in green) and expressed in %. Each dot represents the the mean of 3 independent experiments for each isolate. Levels of each measure are represented in median and interquartile. P<0.05 of the Kruskal Wallis followed by Dunn's comparison test for auto-agglutination, and Mann Whitney test between clade 1 and clade2 for biofilm and survival, was considered significant.

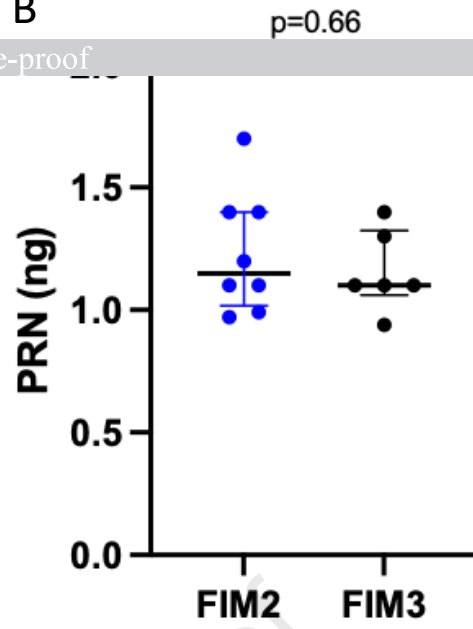
Fig. 6: Large-scale proteomic analysis according to FIM serotype

8 FIM2 isolates (FR5510, FR5810, FR5862, FR5994, FR6156, FR6440, FR6762, FR6845) and 6 FIM3 isolates (FR4887, FR5031, FR5333, FR5782, FR5893, FR6167) were used for the DIA-MS acquisition. Our pre-defined selection criteria were a $|\log_2(\text{FC})| > 1$ with an adjusted p-value<0.01 and a FDR<1%. (A) Volcano plot showing the differential proteins between FIM2 and FIM3 isolates. Each dot represents the mean protein amount of 3 independent replicates. The red dots represent proteins that are significantly more abundant and the blue dots proteins that are significantly less abundant in FIM2 compared to FIM3 isolates. (B) Heatmap display the mean z-scores of proteins with small p-values (p-value<1 %) along FIM2 and FIM3 isolates. Hierarchical clustering on the left side of the heatmap is estimated from the mean z-scores using a Pearson correlation-based distance and the Ward's method.

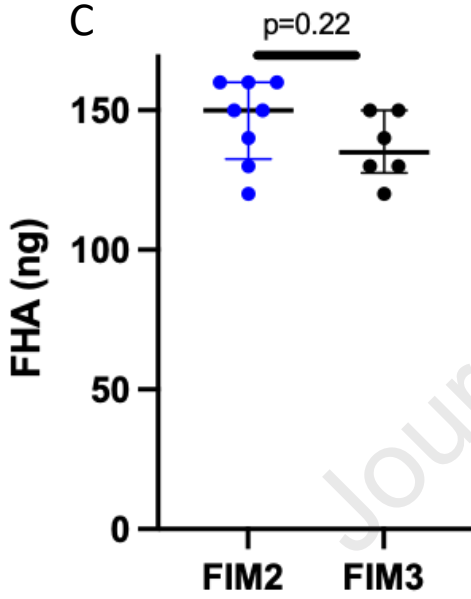
A



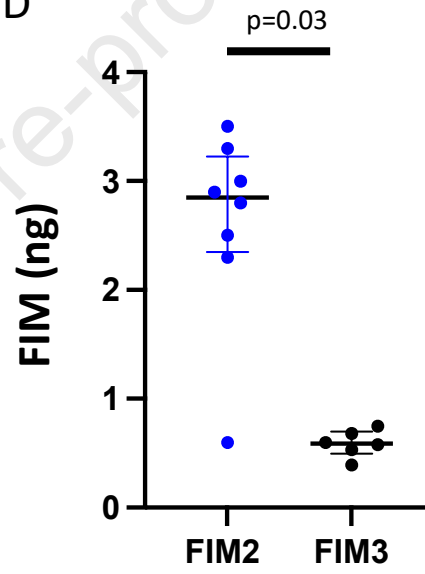
B



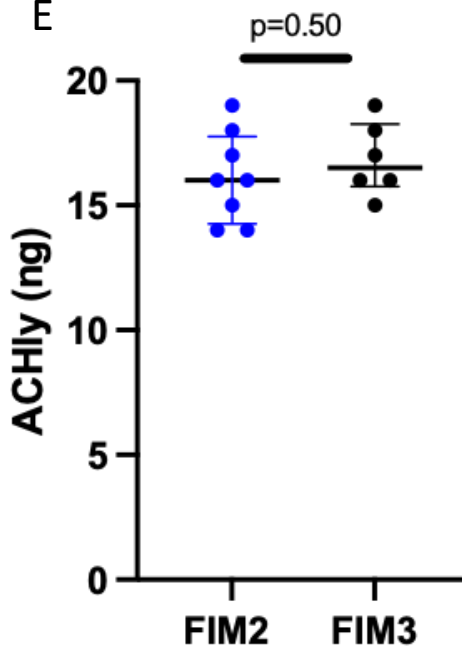
C



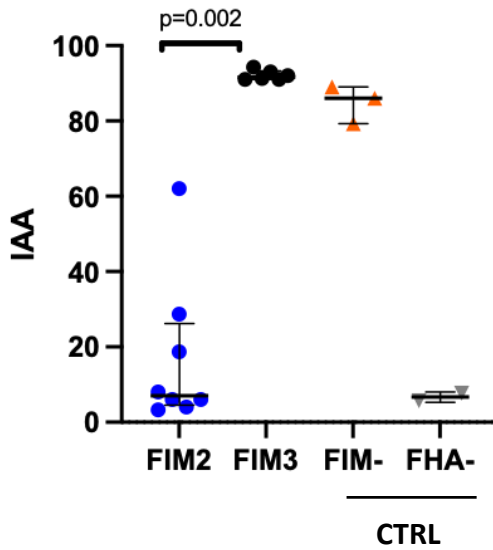
D



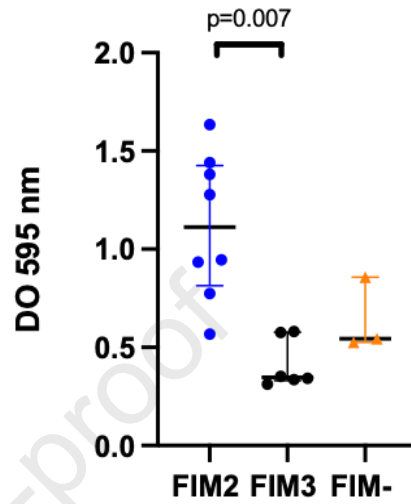
E



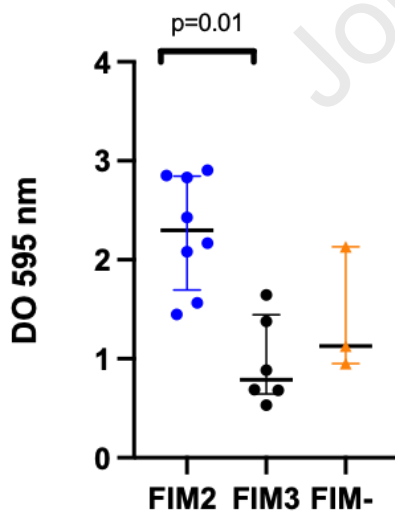
A-Auto-Agglutination



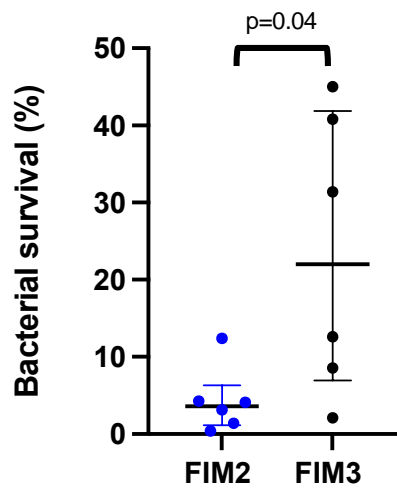
B-Biofilm H24



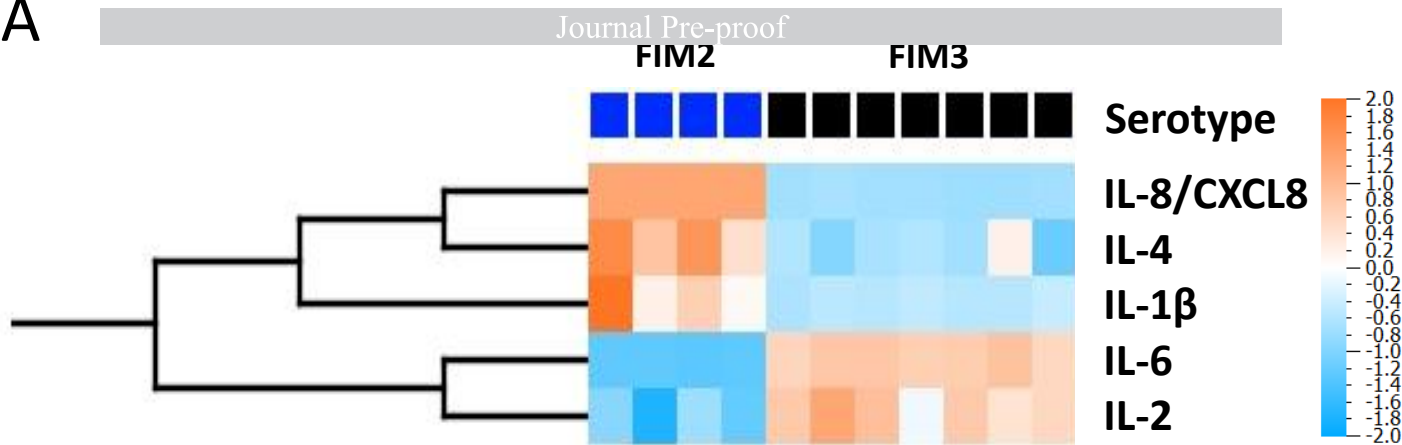
C-Biofilm H48



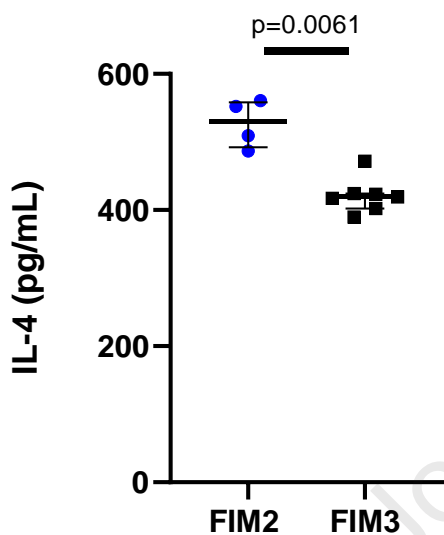
D-Survival rate



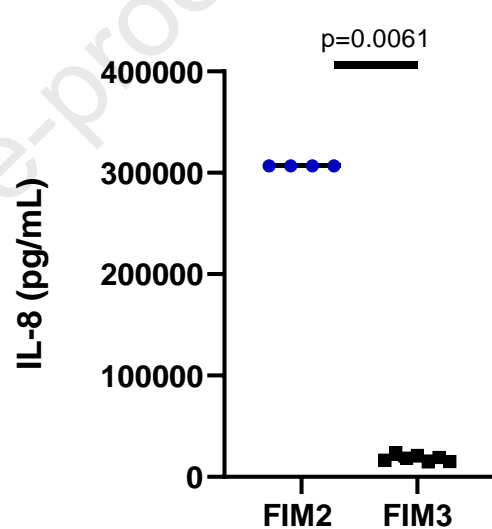
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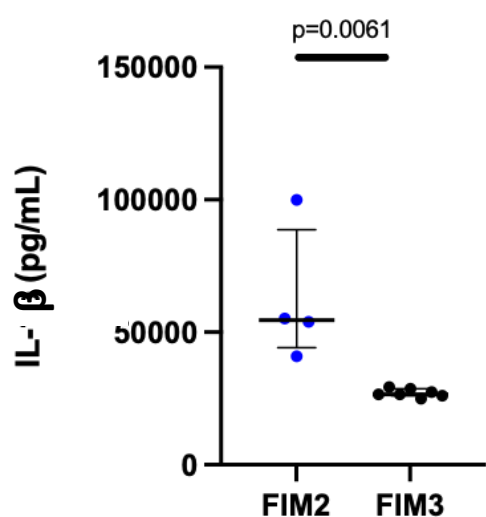
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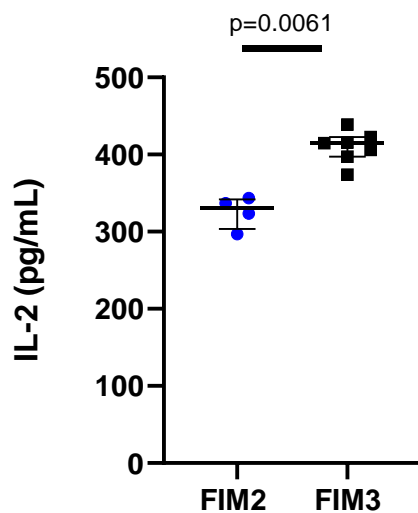
C



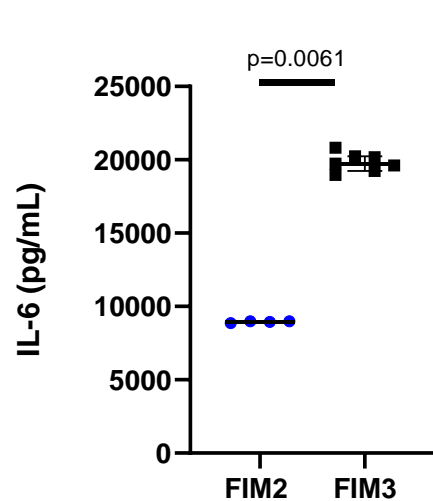
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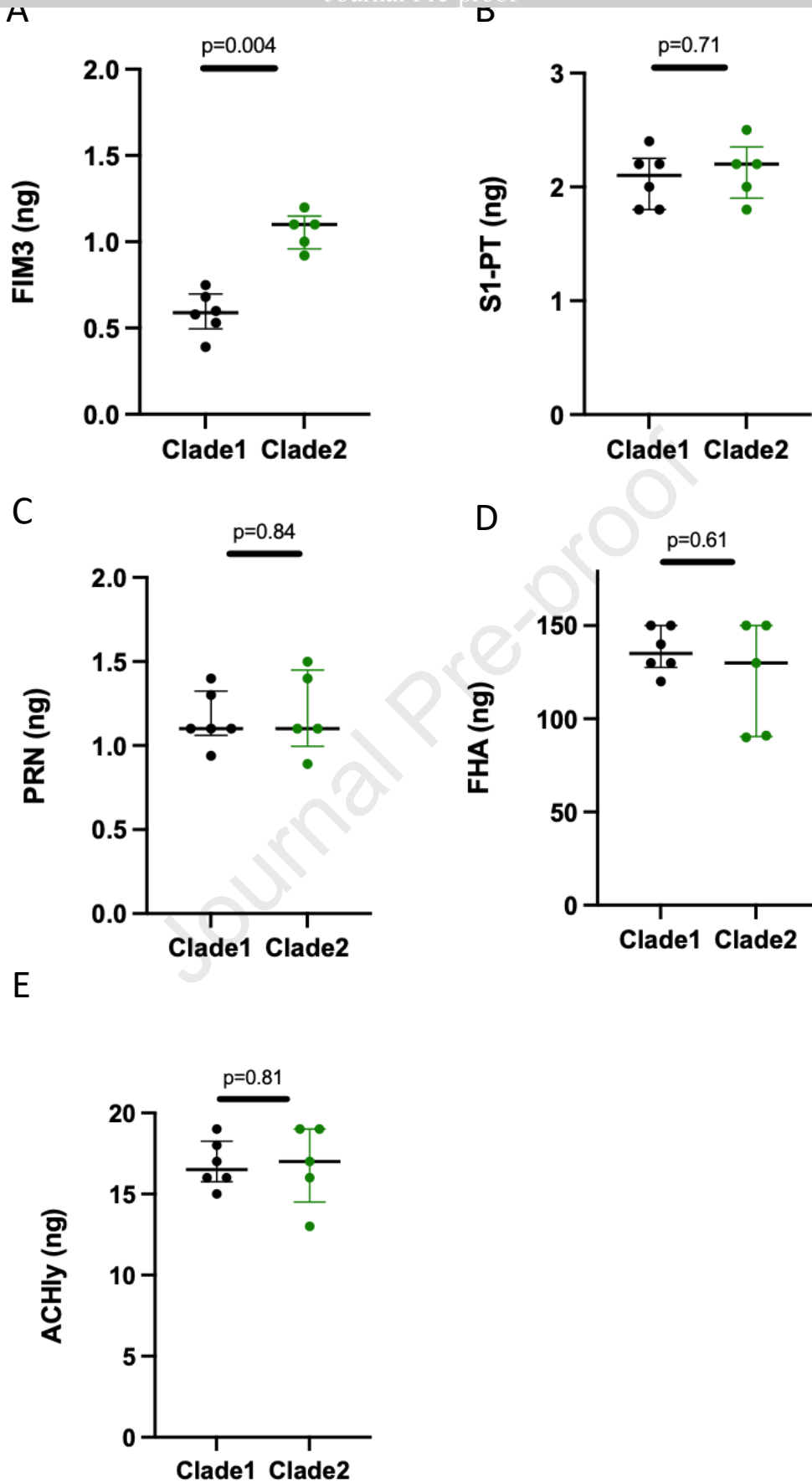


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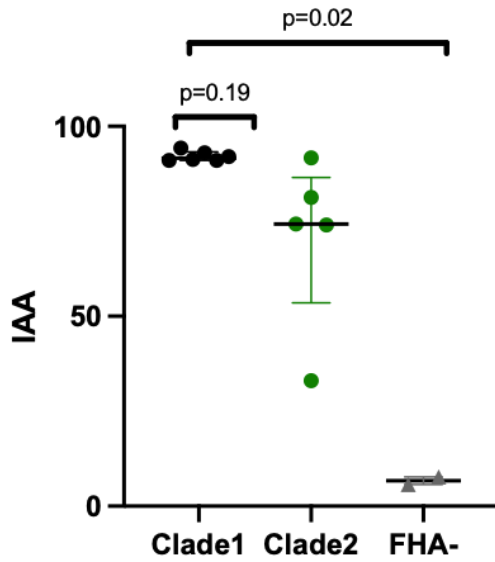


F

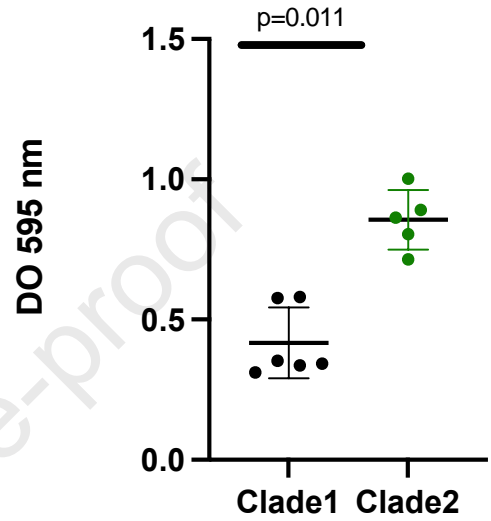




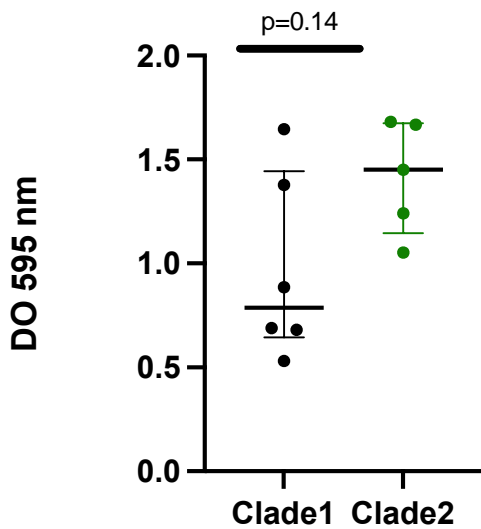
A-Auto-Agglutination



B-Biofilm H24



C-Biofilm H48



D-Survival rate

