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Additional Information

1 **Pathogenesis of intradermal staphylococcal infections: rabbit experimental approach to**  
2 **natural *Staphylococcus aureus* skin infections**

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43  
44

45 **Abstract**

46 Despite the enormous efforts made to achieve effective tools that fight against *Staphylococcus*  
47 *aureus*, the results have not been successful. It is likely that such failure is due to the absence  
48 of truly representative experimental models. To overcome this deficiency, the present work  
49 describes and immunologically characterizes the infection for 28 days, in an experimental low-  
50 dose (300 CFU) intradermal model of infection in rabbits, which reproduces the characteristic  
51 staphylococcal abscess. Surprisingly, when mutant strains in the genes involved in virulence  
52 ( $J\Delta agr$ ,  $J\Delta coa\Delta vwb$ ,  $J\Delta hla$  and  $J\Delta psm\alpha$ ) were inoculated, no strong effect on the severity of  
53 lesions was observed, unlike other models that use high doses of bacteria. The inoculation of a  
54 human “rabbitized” ( $FdltB^r$ ) strain demonstrated its capacity to generate a similar inflammatory  
55 response to a wild-type rabbit strain and, therefore, validated this model for conducting these  
56 experimental studies with human strains. To conclude, this model proved reproducible and may  
57 be an option of choice to check both wild-type and mutant strains of different origins.

58

59 *Keywords:* Pathogenesis; Abscess; Experimental infection; Rabbit; *Staphylococcus aureus*

60

## 61 **Introduction**

62 *Staphylococcus aureus* is a widespread bacterium that has adapted well to humans and animals  
63 which, if provided with a suitable opportunity, can initiate severe infections at various body  
64 sites <sup>1-3</sup>. It can cause a wide range of diseases and syndromes, but one of the most worrying  
65 ones, given community-associated infections, are those that affect both skin and soft tissues <sup>4-</sup>  
66 <sup>9</sup>. Skin and soft tissue infections (SSTIs) can be minor and self-limiting <sup>1</sup>, but if they become  
67 complicated, they can prove life-threatening and be characterized by the formation of large  
68 abscesses <sup>10</sup>.

69 After years of study and millions of dollars invested in research, gaps in our understanding of  
70 *S. aureus* infections remain, and it is not known in detail how staphylococcal infections evolve  
71 in humans. Numerous studies have been conducted on the pathogenesis of this bacterium <sup>1</sup>,  
72 usually for the ultimate objective of developing therapeutic tools against staphylococcal  
73 infections <sup>11</sup>. However, practical results remain frustrating because even today there are no  
74 effective preventive therapies, but resistance to antibiotics is growing and has reached really  
75 worrying levels. The problem could lie in the fact that the results experimentally obtained  
76 in inadequate animal models have been assumed and almost never questioned.

77 There are two important aspects to be taken into account in experimental staphylococcal  
78 infections: the animal species and the strain used. The murine has been the most frequently  
79 studied animal model. However, unlike humans, mice are not natural hosts for *S. aureus*.  
80 Conversely, rabbits (the second most frequently used animal species) usually suffer natural  
81 infections by *S. aureus*. In fact, numerous strains have been reported in commercial rabbitries  
82 <sup>12</sup> ever since a sporadic mutation favored the human-to-rabbit host jump 40 years ago <sup>13</sup>. **It has**  
83 **been described that human skin morphological <sup>14,15</sup> and immunological <sup>16</sup> characteristics are**  
84 **more similar to those of rabbit than to those of mice. For this reason, the skin rabbit model is**

85 more appropriate than the murine one if the objective is to simulate *S. aureus* infection in  
86 humans.

87 In order to develop experimental infections using human *S. aureus* strains in mice, and even in  
88 rabbits <sup>17</sup>, the inoculation of an extraordinary large number of bacteria is necessary as they are  
89 strains that have not adapted to these hosts, which places at a distance the results obtained in  
90 the laboratory from what actually occurs under natural conditions. So in these cases, animal  
91 models can be unreliable predictors of either the potential success of therapeutic or preventive  
92 interventions or the roles played by specific determinants of bacterial virulence in infection <sup>2</sup>.  
93 This is why it is necessary to develop better animal models of colonization that are more  
94 representative of what happens in *in vivo* situations <sup>3</sup>.

95 In this article we present, for the first time, a detailed description of staphylococcal dermal  
96 infection in a rabbit model at low doses to mimic natural infection and to approach what should  
97 occur in natural infections in humans in the most realistic way possible. The specific objectives  
98 of this study are to: (1) describe and characterize a low-dose rabbit *S. aureus* intradermal  
99 infection model using a wild-type reference strain and several mutant strains in different key  
100 pathogenicity factors that somehow contribute in abscess formation; (2) confirm the utility of  
101 this model after infection with a human adapted-to-rabbit (“rabbitized”) *S. aureus* strain and  
102 compare their response with different rabbit strains of known virulence.

103

104

## 105 **Materials and Methods**

### 106 *General approach*

107 This study was designed in two phases: the first intended to (1a) establish a detailed description  
108 of the evolution of lesions and the immune response after infection with low doses of a wild-  
109 type *S. aureus* rabbit strain and (1b) study the effect of different genes on the pathogenesis of  
110 intradermal staphylococcal infections. The second intended to (2) prove the reproducibility of  
111 this experimental model with a human “rabbitized” strain.

112

### 113 *Bacterial strains and growth conditions*

114 The *S. aureus* strains used in this study were: Jwt (a rabbit ST121 wild-type strain isolated from  
115 a natural case of staphylococcosis) and deletion strains isogenic *coa/vwb* ( $J\Delta coa\Delta vwb$ ), *hla*  
116 ( $J\Delta hla$ ), *psma* ( $J\Delta psma$ ) and *agr* ( $J\Delta agr$ ); *Jrot*<sup>+</sup> (Jwt with *rot* gen restored); *JdltB*<sup>h</sup> (Jwt with the  
117 reversion of three identified *dltB* Single Nucleotide Polymorphism –SNPS-); and *FdltB*<sup>r</sup>, a  
118 human ST121 strain expressing *dltB* from rabbit clones <sup>13</sup> (**Table 1**).

119 Bacteria were grown at 37°C overnight on TSA agar medium supplemented with antibiotics as  
120 appropriate. Broth cultures were grown at 37°C in TSB broth with shaking (240 r.p.m.). The  
121 procedures for the preparation and analysis of phage lysates, transduction and transformation  
122 in *S. aureus* were performed essentially as previously described <sup>20 21</sup>.

123

### 124 *DNA methods*

125 General DNA manipulations were performed by following standard procedures <sup>22</sup>.

126 To produce the mutant strains, plasmid pMAD was used <sup>23</sup>, as previously described <sup>24</sup>. The  
127 oligonucleotides used herein are listed in **Table 2**. Briefly, two separate PCR products with  
128 overlapping sequences, including the targeted sequence, were combined. A second PCR was  
129 run with external primers to obtain a single fragment. Specifically, 1 mL of each of the first

130 PCRs was mixed with 10 pM of the outside primers and was PCR-amplified. The fusion  
131 products were purified and cloned at the appropriate sites of shuttle plasmid pMAD, and the  
132 resulting plasmids were transformed into *S. aureus* (RN4220) by electroporation<sup>20</sup>. pMAD  
133 contains a temperature-sensitive origin of replication and an erythromycin-resistance gene. The  
134 plasmid was integrated into the chromosome through homologous recombination at a non-  
135 permissive temperature (43.5°C). From the 43.5°C plate, one to five colonies were picked into  
136 10 mL of TSB and incubated for 24 h at 30°C. Ten-fold serial dilutions of this culture in sterile  
137 TSB were plated on TSA containing X-gal (5-bromo-4-chloro-3-indolyl-B-D-  
138 galactopyranoside; 150 mg/mL). White colonies, which no longer contained the pMAD  
139 plasmid, were tested to confirm replacement by DNA sequencing. Primers were obtained from  
140 Invitrogen Life Technologies (Paisley, UK).

141

#### 142 *Animals and sampling times*

143 Two hundred and twenty 2-month-old albino hybrid rabbits (*Oryctolagus cuniculus*) (10  
144 animals per sampling time) of either gender were inoculated with the eight above-described *S.*  
145 *aureus* strains. Depending on the inoculated strain, samples were taken at different times (**Table**  
146 **1**). After a detailed study of lesions induced by the Jwt strain on different days postinfection  
147 (dpi) (0, 0.5, 1, 2, 3, 7, 14, 21, 28), it was established that 7 dpi was the optimal time to compare  
148 infections with mutants in various toxins (*JΔcoaΔvwb*, *JΔhla*, *JΔpsmα*) and general regulators  
149 (*JΔagr*) as it was the time of maximum lesion development (abscesses opening and emptying)  
150 and when repair phenomena commenced. Then 1, 3 and 7 dpi were selected to compare the  
151 behavior of a human “rabbitized” strain (*FdltB<sup>r</sup>*) with three rabbit strains (Jwt, *Jrot<sup>+</sup>*, *JdltB<sup>h</sup>*) of  
152 well-known virulence in the acute and early chronic phases of infection evolution because, as  
153 before (7dpi), some important milestones were observed: on 1 dpi, externally evident lesions

154 (papules) and the formation of initial abscesses and on 3 dpi, skin necrosis, multiple Splendore-  
155 Hoeppli phenomena and an eosinophilic layer surrounding abscesses.

156 Animals were housed under conventional environmental conditions with an alternating cycle  
157 of 16 h of light and 8 h of darkness in individual cages (600 x 750 x 600 mm) and were fed  
158 with a commercial rabbit diet *ad libitum*.

159 The experimental protocol was approved by the Ethical Committee of the Universidad CEU  
160 Cardenal Herrera and by the Conselleria d'Agricultura, Pesca i Alimentació, Generalitat  
161 Valenciana (permit numbers 2011/010 and 2017/VSC/PEA/00192; date of approval: January  
162 20, 2011). All the animals were handled according to the principles of animal care published  
163 by Spanish Royal Decree 1201/2005 (BOE, 2005).

164

#### 165 *Intradermal infection model*

166 The experimental procedure was performed as previously described <sup>25</sup>, with some  
167 modifications. Briefly, rabbits were intradermally-inoculated in their backs with 300 colony  
168 forming units (CFU) of each studied strain suspended in 0.1 mL of phosphate-buffered saline  
169 (PBS) to inoculate animals with the lowest infective doses of bacteria. The optimal number of  
170 bacteria in the inoculum was empirically determined in the preliminary experiments.  
171 Previously, animals were sedated with a combination of ketamine (Imalgene®, 100 mg/mL,  
172 Merial, Barcelona, Spain) and xylazine (Xilagesic, 200 mg/mL, Calier, Barcelona, Spain) and  
173 a 10x10 cm area of the dorsal-lumbar region was shaved and disinfected with chlorhexidine.  
174 To avoid interactions among strains, each rabbit was infected with only one strain (except in  
175 the phase 1b where Jwt was inoculated as control), which were inoculated in duplicate. The  
176 general status, weight and rectal temperature of animals were recorded daily.

177



178 *Gross, microscopical and microbiological studies*

179 The characteristics of the skin gross lesions (presence of erythema, edema, skin elevation,  
180 nodules, dermo-necrosis and/or ulceration) were recorded daily. Abscess dimensions were  
181 measured with a calliper. The length (L) and width (W) values were used to calculate abscess  
182 areas ( $A = \pi [L \times W]/2$ ) and volumes ( $V = 4/3\pi[L/2]^2 \times [W/2]$ )<sup>26,27</sup>. Macroscopic lesions were  
183 evaluated by their abscess area as follows: Healthy (no apparent lesions), Mild (< 0.5 cm<sup>2</sup>),  
184 Moderate (0.5-5 cm<sup>2</sup>) or Severe (>0.5 cm<sup>2</sup> and gross necrosis).

185 Upon sampling, rabbits were sedated with a combination of ketamine (Imalgene®, 100 mg/mL)  
186 and xylazine (Xilagesic, 200 mg/mL), and were euthanized by an intravenous injection of  
187 barbiturate (T-61, Intervet International GmbH, Unterschleißheim, Germany) and a complete  
188 necropsy was carried out. Skin samples from the inoculation place were used for the  
189 microscopic and microbiological studies and the cytokine assay. For the histological  
190 examination, skin samples were routinely stained with hematoxylin and eosin (H&E), Masson's  
191 trichrome and Gram's stains, and were processed by immunohistochemistry for macrophages,  
192 T- and B-lymphocytes and plasma cell detection by the avidin-biotin-peroxidase complex  
193 method at the dilutions recommended by the manufacturer (**Table 3**). In the  
194 immunohistochemical procedure, positive cells were enumerated in the inoculation areas and,  
195 when lesions were very severe, in the border of the necrotized tissue of twenty 0.08 mm<sup>2</sup>  
196 randomly selected fields per slide. The histological and immunohistochemical findings  
197 observed on the different skin layers were described and recorded in detail.

198 Prior to fixation, representative samples were taken from skin lesions and kidneys, and were  
199 weighed under sterile conditions for the microbiological studies. Then they were immersed in  
200 a cold solution of PBS and mechanically homogenized in the presence of ice. One hundred µL  
201 of tissue homogenate were cultivated on blood-agar (BioMérieux, Marcy l'Etoile, France) and  
202 were incubated aerobically at 37°C for 24 h. This allows the results to be expressed in CFU/g.

203 The colonies that grew were identified by coagulase PCR <sup>12</sup> to prove that the strains isolated  
204 from skin lesions were the same as those originally inoculated and to assess the possibility of  
205 septicemia in the samples taken from kidneys.

206

#### 207 *Hematology and flow cytometric analyses*

208 Blood samples (1 mL) in EDTA anticoagulant were obtained from the median artery of the ear.  
209 White blood cell (WBC) counts and lymphocyte proportions were determined using a  
210 hematology analyzer (MEK-6410, Nihon Kohden, Tokyo, Japan). The flow cytometric analysis  
211 of the white blood cells (B, T, T CD4+, T CD8+ and activated T-cells, monocytes and  
212 granulocytes) was performed using specific primary antibodies (**Table 4**) and secondary  
213 antibodies (rat anti-mouse IgG2a+b phycoerythrin conjugate, VMRD; goat anti-mouse IgM  
214 phycoerythrin conjugate, AbD Serotec, Kidlington, UK), as previously described <sup>28,29</sup>.

215 The common leukocyte antigen CD14 and CD45 expression was used for the “lymphogate”  
216 setup, as previously described <sup>28,29</sup>. The calculation of the total lymphocyte and the respective  
217 subset counts were performed as the product of the WBC count and specific population  
218 percentages, as described elsewhere <sup>29,30</sup>.

219

#### 220 *Cytokine assay*

221 The representative interleukins of the immune response (IL-1 $\beta$ , IL-4, IL-17, IL-18 and IFN-  
222 gamma) from plasma and the inoculated skin tissues were analyzed by ELISA kits (CUSABIO,  
223 Wuhan, Hubei Province, P.R. China) according to the manufacturer’s protocol. Previously, the  
224 samples taken from skin abscesses were immersed in a cold solution of PBS with a protease  
225 inhibitor (Complete Mini, Roche, Basel, Switzerland), and were mechanically homogenized for  
226 a 1 min by stopping each 10 s to avoid overheating in a T50 Ultra-Turrax <sup>®</sup> (IKA, Staufen,

227 Germany) in the presence of ice. Next they were centrifuged at 13000 g for 10 min. Aliquots  
228 of the supernatant were stored at -80°C for further processing.

229

### 230 *Statistical analysis*

231 Study 1a. Characterization of the model after infection with Jwt.

232 The data obtained in this study were evaluated by five different models depending on the type  
233 of data recorded for each trait (**Tables 1 and Supplemental Table 1**). The five models included  
234 the postinoculation day (9 levels: 0; 0.5; 1; 2; 3; 7; 14; 21; 28) as the fixed effect. Except for  
235 CFU, the data of quantitative traits with one record per animal were analyzed by a linear model  
236 M1 (proc GLM, SAS, 9.2). CFU usually presents changes in the order of magnitude as  
237 infections evolve. In order to take this situation into account, the CFU data were analyzed by a  
238 mixed model M2 and by considering different variances for the random residual errors on each  
239 postinoculation day. The data of the quantitative traits with more than one record per animal  
240 were analyzed by a mixed model M3 (proc MIXED, SAS, 9.2), which included the effect of  
241 the individual upon infection as random effects [90 levels;  $N \sim (0, \sigma_p)$ ]. The data of the  
242 categorical traits with two levels (e.g.: yes/no, presence/absence, 0/1, etc.) were analyzed by a  
243 generalized linear model M4 (proc GENMOD, SAS, 9.2) after considering that the response  
244 variable followed a binomial distribution and by using logistic transformation [ $\ln(\mu / (1-\mu))$ ]  
245 as a link function. Finally, the ordinal data of the histology traits had more than two levels  
246 because injuries were classified according to a previously described scale (-, - / +, +, + / ++, ++,  
247 ++ / +++, +++, + / +++++, +++++), where ++ was greater (severer) than +. However, this did  
248 not mean that the distance between each class was proportional. To take this situation into  
249 account, the data of the histology traits were analyzed by a generalized linear model M5 (proc  
250 GENMOD, SAS, 9.2) after considering that the response variable followed a multinomial  
251 probability distribution and that the link function followed a cumulative logistic distribution <sup>31</sup>.

252 Study 1b. Effects of the selected genes and the global regulator involved in abscess  
253 development.

254 A pair-wise comparison of the means was performed using Yates correction. The t-student test  
255 was performed for the traits with quantitative data and the chi-square test for the traits with  
256 categorical data [M6].

257 Study 2. Comparison of the lesions caused by a human “rabbitized” strain and different rabbit  
258 strains of known virulence.

259 The data obtained in this study were evaluated by four different models depending on the type  
260 of data recorded per trait (**Supplemental Table 1**). The four models included day  
261 postinoculation (3 levels; 1, 3, 7), the strain (4 levels: Jwt, *FdltB<sup>r</sup>*, *Jrot<sup>+</sup>*, *JdltB<sup>h</sup>*) and their  
262 interaction as fixed effects. Except for CFU, the data of the quantitative traits with only one  
263 record per animal were analyzed by a linear model M7 (proc GLM, SAS, 9.2). CFU was  
264 analyzed by a mixed model M8 after considering the different variances for the random residual  
265 errors on each day postinoculation. The data of the quantitative traits with more than one record  
266 per individual were analyzed by a mixed model M9 (proc MIXED, SAS, 9.2) and included the  
267 effect of the individual at the experimental infection time as random effects [120 levels;  $N \sim (0,$   
268  $\sigma_p)$ ]. Finally, the data of the ordinal categorical traits with more than two levels (histology  
269 variables) were analyzed by a generalized linear model M10 (proc GENMOD, SAS, 9.2) after  
270 considering that the response variable followed a multinomial probability distribution and that  
271 the link function followed a logistic distribution cumulative <sup>31</sup>.

272

273

274

275 **Results**

276 *Study 1a. Characterization of the model after infection with a rabbit wild-type strain (Jwt):*  
277 *infection with low doses of bacteria generates a characteristic and identifiable pathological*  
278 *response over time*

279 After evidencing that rabbits can be infected by a low-dose charge of *S. aureus*<sup>13</sup>, we set out to  
280 make a detailed description, for the first time, of the pathogenesis of staphylococcal infections  
281 in the model that best mimicked staphylococcal disease in humans: the rabbit experimental  
282 model.

283

284 *Health parameters*

285 A slight stagnation of animals' weight on the first 3 days after infection was detected, but they  
286 grew normally during the rest of the experiment. Although an increase in rectal temperature  
287 was observed during the study (38.5 at 0dpi and 39.5 at 28dpi;  $P=0.001$ ), fever was not detected  
288 in any animal and the temperature values fell within physiological ranges.

289

290 *Gross lesions*

291 Lesions of different severities developed in all cases after the inoculation of 300 CFU of *S.*  
292 *aureus* (**Fig. 1A**). Small (< 1 cm in diameter), flat and circumscribed red macules were observed  
293 at the point of inoculation at 0.5 dpi in 100% of the animals. These lesions evolved to slightly  
294 elevated papules (1dpi) and firm nodules (2dpi) in the 100% of the animals and at 100% of the  
295 inoculation points (20 out of 20). The size and intensity of the reddened areas reached its apogee  
296 at 2 dpi ( $2.003\pm 0.51$  vs.  $3.422\pm 0.47$  cm<sup>2</sup> on 1 and 2 dpi, respectively;  $P<0.001$ ) and gradually  
297 disappeared after 3 dpi. At this time (3 dpi), the volume of nodules was maximum ( $4.561\pm 1.15$   
298 cm<sup>3</sup>;  $P<0.001$ ) and 50% of the animals (5 out of 10) at 7 dpi showed epidermal necrosis in the  
299 inoculation zone characterized by darkness, and suppurative to dry (parchment) areas that

300 opened between 7 and 14 dpi in all cases to result in ulceration and discharged a purulent  
301 material. Draining abscesses reduced abscess size. From 14 dpi to 28 dpi, a repair of the ulcers  
302 occurred, and absence of hair and scar retraction were observed at the end of the study (**Fig. 2**).

303

#### 304 *Bacteriology*

305 The bacterial counts increased at 0.5 dpi ( $1.11 \times 10^7$  CFU/g), with the largest number obtained  
306 at 3dpi ( $50.98 \times 10^7$  CFU/g) and, coinciding with the opening of abscesses, the number of  
307 bacteria lowered until the end of the experiment, when the lesions of only four of ten animals  
308 contained bacteria ( $1.16 \times 10^3$  CFU/g at 28dpi) (**Fig. 3**). All the cultured and identified bacteria  
309 recovered from lesions were the same as those inoculated and no bacteria were isolated from  
310 blood or kidneys.

311

#### 312 *Histological findings*

313 Some animals showed early histopathological modifications at 0.5 dpi (**Table 5**). In these cases,  
314 lesions were characterized by moderate edema and minor hemorrhages in the superficial dermis  
315 and a by slight increase in the density of heterophils in the deep dermis, which sometimes  
316 grouped into small clusters of cells (**Figs. 1B and 4A**). The capillary surrounding these  
317 inflammatory foci were hyperemic. The capillaries located near or between cutaneous muscle  
318 cells were also dilated and hyperemic, with the presence of heterophils migrating by diapedesis  
319 to the interstice. Small hemorrhagic foci between adipocytes near the cutaneous muscle were  
320 detected.

321 Vascular phenomena, such as hyperemia (vascular dilatation and presence of numerous  
322 intravascular heterophils), mild to moderate edema and hemorrhages were detected in the  
323 superficial dermis on 1 dpi. The deep dermis showed hyperemia and numerous heterophils  
324 around dilated blood vessels and inflammatory foci structured as a core of eosinophilic necrosis,

325 sometimes with coccoid bacteria surrounded by diffusely distributed heterophils (**Fig. 4B**).  
326 Cutaneous muscle fibers were infiltrated by heterophils, which provoked a mild degeneration  
327 and atrophy of myocytes (**Fig. 1C**).

328 Two days after infection, lesions were similar to the way there were the previous day, but  
329 presented greater severity. It was the first time that clear epidermis thickening (moderate  
330 acanthosis) was detected (10% of animals). The presence of mild vascular dilatation, moderate  
331 edema and some hemorrhages in the superficial dermis was observed. In the deep dermis,  
332 inflammatory foci were better well-defined than they were previously. They were characterized  
333 by a center of eosinophilic necrosis, inside which there were coccoid bacteria surrounded by a  
334 dense layer of heterophils (**Fig. 4C**). Around these foci there were also heterophils. Cutaneous  
335 muscle cells were severely infiltrated by heterophils, which induced their separation and  
336 necrosis (**Figs. 1D and 4D**).

337 On 3 dpi, the percentage of animals with moderate to severe epidermal hyperplasia was higher  
338 than on 2 dpi (46% vs. 10%). The presence of more severe vascular phenomena (vascular  
339 dilatation, edema and hemorrhages) in the superficial dermis induced skin tumefaction, and also  
340 epidermis necrosis when the volume of abscesses was sizeable (**Fig. 4E**). In the deep dermis,  
341 the severity of edemas and hyperemia diminished. The latter was observed only around  
342 suppurative foci, together with diapedesis phenomena of heterophils. Inflammatory foci were  
343 structured by a center with bacterial colonies surrounded by a star-shaped homogeneous  
344 eosinophilic material (Splendore-Hoeppli phenomenon) and an external layer of cellular debris  
345 and inflammatory cells (mainly lymphocytes, macrophages and heterophils) (**Fig. 4F**). When  
346 abscesses were large, this pattern was repeated and they merged with one another to form the  
347 abscess. Externally, abscesses were partially or totally surrounded by a thin eosinophilic layer  
348 of necrosis (**Fig. 4G**). The myocytes of cutaneous muscle were infiltrated by eosinophils and  
349 macrophages, and showed dilatation, degeneration and, sometimes, atrophy (**Figs. 1E and 4H**).

350 The main characteristic on 7 dpi was the presence of local epithelial necrosis and epidermis  
351 ulceration with severe acanthosis in the peripheric epithelium (**Fig. 4I**), together with severe  
352 hyperemia, edema and hemorrhages in the superficial dermis. At greater depths, abscesses  
353 showed several Splendore-Hoepli phenomena, whose peripheral inflammatory reactions  
354 merged with one another. An external thin eosinophilic layer surrounded abscesses. Around  
355 abscesses, heterophils diffusely distributed or a few were seen and some of the capillary vessels  
356 were reactive (presence of endothelial hyperplasia and hypertrophy). The cutaneous muscle was  
357 interrupted by an inflammatory infiltrate (heterophils and round cells) that caused the atrophy  
358 of the affected cells (**Fig. 1F**).

359 On 14 dpi, the severity of some inflammatory phenomena started to decrease and repair  
360 appeared more evident (**Fig. 1G**). In those cases in which the epithelium was lost, epidermis  
361 re-epithelization, characterized by the presence of numerous mitotic figures on the basal and  
362 spinosum layers at the borders of the ulcer, was observed (**Fig. 4J**) together with the absence  
363 of skin adnexa (sweat and sebaceous glands and hair follicles). All the animals showed  
364 acanthosis. The vascular phenomena were milder, but still evident in the superficial dermis like  
365 inflammatory foci with bacteria and, in some cases, Splendore-Hoepli phenomena, all  
366 surrounded by an eosinophilic layer in the deep dermis. In adjacent areas, granulation tissue  
367 (numerous small vessels and abundant fibrous tissue) was observed (**Fig. 4K**). On the muscular  
368 layer, focal fibrosis and atrophy of some cells were observed. Other locations presented  
369 hypertrophy and hyperplasia of muscular fibers.

370 On 21 dpi, mitotic cells and hyperplasia in the peripheral epithelium of lesions were observed,  
371 even in those few rabbits that still had ulcers in the epithelium. When lesions were completely  
372 epithelized, the epidermis was 2-3-fold thicker than in the unaffected areas (**Fig. 5**). In the  
373 dermis (mainly in the deep dermis), abundant granulation tissue was observed with numerous  
374 small blood vessels and heterophils forming small cellular aggregates or diffusely distributed,



375 mainly near cutaneous muscle which, in some cases, was still interrupted by inflammatory cells  
376 and atrophied (**Fig. 1H**).

377 Finally, on 28 dpi, the epidermis of all the animals showed hyperplasia, but with a thinner  
378 epithelium than on 21 dpi (1.5-fold thicker than normal). Skin adnexa were absent in the central  
379 lesion, but the periphery was invaded by hair follicles as a consequence of scar retraction (**Fig.**  
380 **1I**). The dermis presented mature granulation tissue with abundant fibrous tissue and blood  
381 vessels, practically without erythrocytes and with scarce heterophils, as well as some small  
382 hemorrhages (**Fig. 4L**). Cutaneous muscle had completely regenerated.

383

#### 384 *Immunohistochemical studies*

385 Variations in the counts of the studied cells (T-lymphocytes CD3<sup>+</sup>, plasma cells IgG<sup>+</sup> and  
386 macrophages RAM11<sup>+</sup>) were detected locally in the inoculation area during the experiment.  
387 The T-lymphocytes (CD3<sup>+</sup>) counts increased during the experiment until 21 dpi (17.55±1.90  
388 cells/mm<sup>2</sup>) and then abruptly dropped on 28 dpi (**Fig. 6A**). Plasma cells (IgG<sup>+</sup>) appeared in  
389 small groups, mainly in the deep dermis. They were few in number in lesions in the acute  
390 inflammation phase (first 3 days), but significantly increased on 7 dpi and remained high on 14  
391 and 21 dpi (11.03, 11.78 and 11.15±1.93 cells/mm<sup>2</sup>, respectively;  $P<0.001$ ), and once again  
392 abruptly decreased on 28 dpi (**Fig. 6B**). The macrophages counts (RAM11<sup>+</sup>) remained with no  
393 differences compared to 0 dpi until 3 dpi when they significantly increased until 14 dpi. At this  
394 time, the number of macrophages reached the highest level (10.75±1.16 cells/mm<sup>2</sup>;  $P<0.001$ )  
395 before lowering on 21 dpi (still higher than on 0 dpi) and 28 dpi (**Fig. 6C**). Macrophages were  
396 diffusely located in the inoculation area when no abscess was present, but when abscesses  
397 started organizing, macrophages were located around them and formed a palisade on the outer  
398 abscess layer. No significant differences were detected in the number of B-lymphocytes  
399 CD79α<sup>+</sup>.

400

401 *Flow cytometer results*

402 Figure 5 shows the results of the flow cytometric analysis done of the peripheral blood cells in  
403 the rabbits infected by *S. aureus* Jwt. Intradermal inoculation induced modifications in the  
404 counts of most leukocyte populations (granulocytes, monocytes and total B and T CD25<sup>+</sup>  
405 lymphocytes). Total leukocytes (**Fig. 6D**) and granulocytes (**Fig. 6F**) counts showed two peaks  
406 during the study: a first sharp increase on 1 dpi and a second greater increase from 7 to 14 dpi,  
407 which decreased on 21 dpi (more abruptly for granulocytes). The number of monocytes (**Fig.**  
408 **6G**) gradually increased from 3 to 14 dpi, when its highest value was reached ( $869.5 \times 10^6/L$ ;  
409  $P < 0.001$ ). The number of total lymphocytes rose from 0 to 2 dpi, when counts were kept  
410 elevated until the end of the experiment (**Fig. 6E**). Regarding the lymphocyte subpopulations,  
411 statistical differences were observed only in the B (**Fig. 6H**) and CD25<sup>+</sup> (**Fig. 6I**) lymphocytes.  
412 B-cells had higher values at 0.5, and especially at 21 dpi, compared to 0 dpi ( $43.29$  and  $81.66$   
413  $\times 10^6/L$ , respectively;  $P < 0.001$ ). The cell counts of the CD25<sup>+</sup> lymphocytes abruptly increased  
414 on 0.5 dpi and were 11.9-fold higher than on 0 dpi and on later experimental days. No  
415 significant differences were observed in the number of T-lymphocytes, T CD4<sup>+</sup> lymphocytes  
416 and T CD8<sup>+</sup> lymphocytes.

417

418 *Plasmatic and tissue cytokines*

419 Statistical differences were detected only in IL-4 and IL-18 in plasma and in IL-4 and IFN-  
420 gamma in skin samples (**Table 6**). The plasmatic levels of IL-4 and IL-18 oscillated throughout  
421 the experiment. Relative to 0 dpi, statistical differences were only detected for IL-4 on 28 dpi  
422 and IL-18 on 14 and 28 dpi, where these cytokines reached their highest plasmatic levels ( $8.9$   
423  $\text{pg IL-4/mL}$  and  $463.4 \text{ pg IL-18/mL}$ ) on 28 dpi. Similarly to plasma, the IL-4 detected in skin  
424 tissue significantly increased on 28 dpi ( $15.5 \text{ pg/mL}$ ;  $P < 0.05$ ). Conversely, the IFN-gamma

425 level in tissues abruptly increased on 0.5 dpi ( $14015 \pm 1804$  pg/ml;  $P < 0.05$ ) before returning to  
426 previous levels. No significant differences were observed in IL-1 $\beta$  and IL-17 levels.

427

428

429 ***Study 1b. Effects of the selected genes and the global regulator involved in abscess***  
430 ***development: mutants in different key genes for abscess development cause a delay in***  
431 ***lesions, but are still able to infect animals and generate abscesses***

432 Once the lesions and peripheral and local immune responses had been described, the aim of this  
433 section was to evaluate the contribution of different isogenic mutants of the Jwt strain ( $J\Delta agr$ ,  
434  $J\Delta coa\Delta vwb$ ,  $J\Delta hla$  and  $J\Delta psm\alpha$ ) to the development and characteristics of abscesses on 7 dpi  
435 using the proposed rabbit skin model. Unexpectedly, all the mutant strains were able to generate  
436 lesions like the wild-type in terms degree of severity (**Fig. 7**). Whenever present, lesions were  
437 macroscopically indistinguishable from those generated by the wild-type strain.

438 At the histological level, Splendore-Hoeppli phenomena were observed with all the strains, but  
439 not with the same incidence. Only statistically differences were observed with  $J\Delta hla$  (85% Jwt  
440 vs. 68%  $J\Delta hla$ ;  $P < 0.05$ ) (**Fig. 8A**). The percentage of distributed heterophiles surrounding the  
441 abscesses was higher in the mutant strains with respect to Jwt (+20% for  $J\Delta coa\Delta vwb$ , +50%  
442 for  $J\Delta hla$  and +30% for  $J\Delta psm\alpha$  and  $J\Delta agr$ ;  $P < 0.05$ ) (**Fig. 8B**). All the lesions caused by each  
443 studied strain (the wild-type and mutants) presented an eosinophilic layer surrounding  
444 abscesses on 7 dpi. However, there were differences in the characteristics of this band. The  
445 percentage of complete eosinophilic layers always was lower for mutants than for Jwt (-30%  
446 for  $J\Delta coa\Delta vwb$ , -90% for  $J\Delta hla$ , -45% for  $J\Delta psm\alpha$  and -50% for  $J\Delta agr$ ;  $P < 0.05$ ) (**Fig. 8C**).  
447 These histological differences are related to the degree of maturation of the abscess. Thus, the  
448 lesions generated by the mutants at 7 dpi resembled those presented by the wild-type strain in

449 2-3 dpi, which could be interpreted as the absence of these genes delay the development of the  
450 lesions.

451

452

453 **Study 2. Comparison of the lesions caused by a human “rabbitized” strain and different**  
454 **rabbit strains of known virulence: similar, but milder lesions to the rabbit wild-type strain**  
455 **are caused by a human rabbitized strain after intradermal inoculation**

456 Once the infection model had been characterized and that all the rabbit strains were capable of  
457 generating lesions at low doses had been verified, our intention was to test the model with a  
458 human strain. The inconvenience here is that human *S. aureus* strains (e.g., F strain) do not  
459 cause lesions at low doses (300 CFU) in rabbits <sup>13</sup>. In fact to be able to produce them, it is  
460 necessary to use high bacterial doses (more than 10<sup>4</sup> CFU) and, when this occurs, the evolution  
461 of lesions (faster) and the produced characteristics (e.g. presence of extensive coagulative  
462 necrosis, thrombosis, absence of Splendore-Hoeppli phenomena or peripheral eosinophilic  
463 layers on 3 dpi) differ vastly from those observed by rabbit strains at low doses (**JM Corpa,**  
464 **personal communication, Supplemental Figure 1**). Therefore, we had to use a human strain  
465 adapted to rabbits (*FdltB<sup>r</sup>*), which we had previously demonstrated was able to infect this  
466 species at low doses <sup>13</sup>.

467 The specific aim was to compare the response to infection after inoculating the *FdltB<sup>r</sup>* strain  
468 with *S. aureus* strains obtained from rabbits of known virulence, such as Jwt (high virulence),  
469 *Jrot<sup>+</sup>* (medium virulence) and *JdltB<sup>h</sup>* (low virulence), using the previously described model to  
470 demonstrate its utility with strains of different origins.

471

472

473 *Health parameters*

474 No animal suffered fever and no differences in temperature between the studied groups were  
475 observed.

476

477 *Gross lesions*

478 Differences in both the number of infected animals and the characteristics of their lesions after  
479 inoculation with the studied *S. aureus* strains were detected. While 100% of the rabbits were  
480 infected by human *FdltB<sup>r</sup>* and rabbit *Jwt*, 76.7% and 60% of the animals inoculated with *Jrot<sup>+</sup>*  
481 and *JdltB<sup>h</sup>* showed lesions, respectively. The severity of lesions caused by *FdltB<sup>r</sup>* was  
482 intermediate, between *Jwt* (severer lesions) and *Jrot<sup>+</sup>* and *JdltB<sup>h</sup>* (milder lesions) (**Fig. 9**).

483 On 1 dpi, the inoculation points showed erythema in 100% of the animals infected by *FdltB<sup>r</sup>*,  
484 *Jwt* and *Jrot<sup>+</sup>* and 80% in the rabbits inoculated with *FdltB<sup>r</sup>*. On 3 dpi, lesions evolved to nodules  
485 in 100% of the animals infected by the *FdltB<sup>r</sup>* and *Jwt* strains, but only in 30% of the rabbits  
486 inoculated with *Jrot<sup>+</sup>*, and 10% with *JdltB<sup>h</sup>*. At the end of the experiment (7 dpi), while all the  
487 animals infected with *FdltB<sup>r</sup>* and *Jwt* presented nodules, even some developed dermonecrosis  
488 (10% and 50%, respectively), only 50% of the rabbits inoculated with *Jrot<sup>+</sup>* and *JdltB<sup>h</sup>* showed  
489 lesions. The animals infected with *Jrot<sup>+</sup>* exhibited erythema and nodules (40%), but erythema  
490 was observed only in the rabbits infected with *JdltB<sup>h</sup>* at this time point.

491

492 *Bacteriology*

493 The bacterial counts obtained from the animals inoculated with *FdltB<sup>r</sup>* and *Jrot<sup>+</sup>* followed the  
494 same dynamics as the *Jwt* strain on 1, 3 and 7 dpi (**Fig. 10**), but the average number of bacteria  
495 obtained from their lesions was smaller ( $6.18 \pm 32.21 \times 10^6$  and  $1.10 \pm 47.54 \times 10^6$  CFU/g,  
496 respectively,  $P < 0.05$ ) than from the lesions caused by *Jwt* ( $244.36 \pm 36.98 \times 10^6$  CFU/g).  
497 Bacteria were isolated only in one animal infected by *JdltB<sup>h</sup>* on 3 dpi ( $2.26 \times 10^3$  CFU/g) at

498 3dpi. All the cultured and identified bacteria recovered from lesions were the same as those  
499 inoculated, and no bacteria were isolated from blood or kidneys.

500

### 501 *Histological findings*

502 Different degrees of severity were observed in the histological lesions depending on the type of  
503 inoculated strain (**Table 7**). In general, *FdltB<sup>r</sup>* caused more similar (but milder) lesions to the  
504 *Jwt* strain than to the other two. Vascular dilatation and edema in the superficial dermis  
505 observed in the animals infected by *FdltB<sup>r</sup>* were severer than *Jrot<sup>+</sup>* and resembled *Jwt*.

506 The abscesses caused by *FdltB<sup>r</sup>* were evident on 1 dpi and were more numerous and severer on  
507 3 dpi, similarly to *Jwt*. Conversely, the severity of the abscesses (heterophils organized, **Table**  
508 **7**) in the animals infected by *Jrot<sup>+</sup>* and *JdltB<sup>h</sup>* reduced on 3 dpi. In this last case, abscesses were  
509 classified mainly as “mild” (**Fig. 11A**).

510 The abscesses produced by the three mutant strains were histologically more immature  
511 compared to *Jwt* as they did not show a complete eosinophilic peri-abscess layer. On 3 dpi, all  
512 the *Jwt*-related abscesses had a well-developed eosinophilic layer, only 55% of *FdltB<sup>r</sup>* and 20%  
513 of *Jrot<sup>+</sup>* had one, and no band was detected in the *JdltB<sup>h</sup>* abscesses. At the end of the experiment  
514 (7 dpi), percentages varied, but differences remained (100% *Jwt*; 36% *FdltB<sup>r</sup>*; 30% *Jrot<sup>+</sup>* and  
515 0% *JdltB<sup>h</sup>*).

516 It was noteworthy that Splendore-Hoeppli phenomena were seen only in the abscesses from the  
517 rabbits infected by *Jwt* and *Jrot<sup>+</sup>* on 3 and 7 dpi. There were fewer Splendore-Hoeppli  
518 phenomena in *Jrot<sup>+</sup>* (1 on 3 dpi and 2 on 7 dpi) than in *Jwt* (5 on 3 dpi and 8 on 7 dpi), although  
519 the number of abscesses also lowered with *Jrot<sup>+</sup>* (2 on 3 dpi and 3 on 7 dpi) than with *Jwt* (10  
520 at both time points).

521 The degree of dilated blood vessels in the deeper dermis close to cutaneous muscle was higher  
522 in the animals inoculated with strains *FdltB<sup>r</sup>* and *JdltB<sup>h</sup>* for all the studied times, but the  
523 incidence of perivascular inflammation was higher with Jwt ( $P<0.05$ ) (**Table 7**).

524 The pathological findings (inflammation and degeneration) observed in cutaneous muscle were  
525 similar between strains *FdltB<sup>r</sup>* and Jwt, and were severer than those caused by strains *Jrot<sup>+</sup>* and  
526 *JdltB<sup>h</sup>* ( $P<0.05$ ) (**Table 7**).

527

#### 528 *Immunohistochemical studies*

529 The number of positive cells was related to lesion severity, with no positive cells in the animals  
530 without lesions regardless of the inoculated strain. The number of studied cells in the lesions  
531 caused by *FdltB<sup>r</sup>* strain infection was lower to those of the wild-type strain (Jwt) and more  
532 similar to those of strain *Jrot<sup>+</sup>* and *JdltB<sup>h</sup>* (**Fig. 11B-E**). The T-lymphocytes (CD3<sup>+</sup>) counts were  
533 significantly higher during the three moments analyzed in the Jwt compared to *FdltB<sup>r</sup>*. The  
534 macrophages counts (RAM11<sup>+</sup>) were significantly higher on 3 and 7 dpi in the animals infected  
535 with Jwt regarding to *FdltB<sup>r</sup>*, and the counts of B-lymphocytes (CD79 $\alpha$ <sup>+</sup>) and plasma cells  
536 (IgG<sup>+</sup>) were significantly higher on 3 and 7 dpi, respectively. The lesions produced by the  
537 inoculation of *Jrot<sup>+</sup>* were characterized mainly by the absence of T-lymphocytes (CD3<sup>+</sup>) on all  
538 the analyzed days and high number of B-lymphocytes on 3 dpi, similar to Jwt. The lesions  
539 produced by the inoculation of *JdltB<sup>h</sup>* were characterized mainly by the absence of B-  
540 lymphocytes (CD79 $\alpha$ <sup>+</sup>) on all the sampling times.

541

#### 542 *Flow cytometer results*

543 The differences detected in the peripheral blood cell counts in the rabbits infected by *S. aureus*  
544 *FdltB<sup>r</sup>*, Jwt, *Jrot<sup>+</sup>* and *JdltB<sup>h</sup>* are shown in **Fig. 12**. The animals infected by the *FdltB<sup>r</sup>* strain had

545 fewer leukocytes (-15.2%) and total lymphocytes (-26.5%) than Jw<sup>t</sup>, *Jrot*<sup>+</sup> and *JdltB*<sup>h</sup>, with  
546 similar counts to one another (**Fig. 12A and D**).

547 In the B, T, CD4<sup>+</sup>, and CD8<sup>+</sup> lymphocyte counts, no significant differences were observed  
548 between the rabbits inoculated with the *FdltB*<sup>r</sup> strain and Jw<sup>t</sup>. Both animal groups had more B-  
549 lymphocytes and fewer T, CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes than *Jrot*<sup>+</sup> and *JdltB*<sup>h</sup> (**Fig. 12E, F, G**  
550 and **H**). *FdltB*<sup>r</sup> and Jw<sup>t</sup> strains had less CD4<sup>+</sup>/CD8<sup>+</sup> ratio than *Jrot*<sup>+</sup> (**Fig. 12J**).

551 The animals infected by the mutant strains presented fewer granulocytes (-34.5%;  $P < 0.05$ ) and  
552 monocytes than those inoculated with Jw<sup>t</sup> (**Fig. 12B and C**). For monocytes, the animals  
553 inoculated with *Jrot*<sup>+</sup> and *JdltB*<sup>h</sup> had an intermediate level (-23.9% *Jrot*<sup>+</sup> and *JdltB*<sup>h</sup>, -50.5%  
554 *FdltB*<sup>r</sup>;  $P < 0.05$ ) (**Fig. 12C**). The CD25<sup>+</sup> lymphocytes showed significantly higher counts in the  
555 animals infected by mutant strains *FdltB*<sup>r</sup>, *Jrot*<sup>+</sup> and *JdltB*<sup>h</sup> (+50.4%, +61.2% and +65%,  
556 respectively;  $P < 0.05$ ) than the rabbits infected by Jw<sup>t</sup> (**Fig. 12I**). Finally, strains *FdltB*<sup>r</sup> and Jw<sup>t</sup>  
557 had a higher granulocytes/lymphocytes ratio than *Jrot*<sup>+</sup> and *JdltB*<sup>h</sup> (**Fig. 12K**).

558

## 559 **Discussion**

560 Staphylococcal infections have spread worldwide in hospitals and communities in the last  
561 decades of the 20<sup>th</sup> century, are multi-resistant to antibiotic strains (especially methicillin-  
562 resistant *S. aureus* – MRSA-) and have become a major health challenge in all industrialized  
563 countries today<sup>1</sup>. This problem has also extended to farm animals since 2005, when a livestock-  
564 associated MRSA (LA-MRSA) was isolated in pigs<sup>32</sup> and extended to other animal species,  
565 including horses, cattle, poultry or rabbits<sup>33-35</sup>. Among the wide range of diseases associated  
566 with community infections that can be caused by *S. aureus*, SSTIs are one of the most worrying  
567 because they can be a life-threatening process if they are complicated<sup>4-9</sup>. SSTIs are  
568 characterized by the formation of bacterial abscesses in dermis, epidermis or subcutaneous  
569 tissues<sup>1</sup>.



570 In this context, it would be valuable to have a definitive experimental model that allows the  
571 pathogenesis of the infection to be studied or new therapeutic tools to be tested. Mice are  
572 commonly used for staphylococcal colonization and infection models, mainly for its small size,  
573 easy handling and the abundance of research facilities compared to other larger species like  
574 rabbits <sup>16,36</sup>. However, these characteristics alone do not justify its use if the results are not truly  
575 representative. Traditionally, rabbit models of *S. aureus* infection have been considered optimal  
576 to investigate virulence and host-pathogen interactions, mainly due to certain similarities with  
577 the human species: (1) similar thickness of their skin <sup>14,15</sup>; (2) similar immune response to  
578 infection <sup>16</sup>; (3) both are naturally infected by *S. aureus*. For these reasons, we present a rabbit  
579 model because this animal species has its own adapted *S. aureus* strains <sup>12,35,36</sup>, which easily  
580 allow to naturally study staphylococcal infections.

581 The experimental model was quite respectful of animal health as no animal displayed fever,  
582 loss of weight, septicemia or external signs of disease, except for the expected local lesions at  
583 inoculation points. The model was reproducible, and previously described characteristic  
584 abscesses developed <sup>37</sup>, but it also provided more detailed characteristics of lesion development  
585 over time than previously described in the bibliography. Although lesions were observed at all  
586 the studied time points, staphylococcal infection with a Jwt strain was evaluated with this model  
587 at three key time points, 1, 3 and 7 dpi. On 1 dpi it was possible to observe small slightly raised  
588 reddish papules that were histologically characterized by the presence of acute vascular  
589 changes, small organized abscesses, sometimes with inner bacteria and surrounded by  
590 heterophils that were diffusely distributed. One interesting finding was the sharp and one-time  
591 increase in the CD25<sup>+</sup> lymphocytes 12 h before (0.5 dpi). As these cells are considered T-  
592 regulatory cells <sup>38</sup>, it would be interesting to verify to what extent the inflammatory response  
593 described in this intradermal infection is modulated by this cell population. T-regulatory cells  
594 are essential for preventing exacerbation of inflammatory response, but they have been found

595 to lose their activity in the presence of *S. aureus*, as shown in children with atopic skin<sup>39,40</sup>. On  
596 3 dpi superficial lesions lost their initial reddish color and became nodular lesions, and it was  
597 when they acquired their largest volume and had more bacteria. Microscopically, vascular  
598 changes increased in severity, abscesses were surrounded by an eosinophilic necrotic layer in  
599 whose core Splendore-Hoeppli phenomena with inner bacteria were observed. Surrounding the  
600 abscess there were increasing number of T-lymphocytes (CD3<sup>+</sup>) and macrophages (RAM11<sup>+</sup>)  
601 compared to previous days, which remained high until 21 dpi., and were related with the  
602 increased number of blood monocytes and lymphocytes on 2 dpi, as previously described<sup>41</sup>.  
603 On 7 dpi, 50% of the nodules suffered epidermal necrosis and opened, which triggered a  
604 discharge of pus, and a reduction in both lesion size and the number of isolated bacteria. These  
605 epidermal lesions generated a new inflammatory episode, peripherally characterized by an  
606 increased number of heterophils in blood, which lasted until 14 dpi. In histological terms,  
607 lesions reached their severest point at all the studied locations, with several Splendore-Hoeppli  
608 phenomena surrounded by many T-lymphocytes (CD3<sup>+</sup>), plasma cells (IgG<sup>+</sup>) and macrophages  
609 (RAM11<sup>+</sup>).

610 Therefore, by studying on 1 dpi and 3 dpi, it would be possible to evaluate the acute  
611 inflammatory response, as well as the subacute inflammatory reaction and initial repair  
612 mechanisms on 7 dpi, which could be useful for the majority of experimental studies on this  
613 topic. For chronic inflammation studies, other later study times (e.g., 14, 21 or 28 dpi) would  
614 be more recommendable, when regenerative modifications were predominant (re-epithelization  
615 and acanthosis in the epidermis, granulation tissue and fibrosis in the dermis, and regeneration  
616 of the cutaneous muscle), and when blood monocytes (2 to 14 dpi) and tissue lymphocytes and  
617 macrophages (3 to 21 dpi) significantly increased, as previously described for chronic  
618 inflammatory processes<sup>41</sup>.

619 Several findings in human and animal models have suggested a primarily role for T-cells in  
620 immunity to *S. aureus* skin infections by enhancing the recruitment of phagocytes <sup>42-44</sup>. *S.*  
621 *aureus* antigens induce different Th1, Th2 and Th17 cell pathways. Therefore, the role of T-  
622 cells in immunity to *S. aureus* skin infections likely involves multiple T-cell effector cytokines.  
623 As there is a paucity of reagents available to analyze panels of rabbit immune mediators, in this  
624 work it was not possible to accurately characterize the immune response type, but significant  
625 differences were observed in some cytokines after the Jwt strain inoculation. Significant  
626 changes were found in cytokines INF-gamma and IL-18. Cytokine IFN-gamma is a potent  
627 activator of monocytes by increasing their phagocytic activity in tissues. <sup>41</sup>. Despite this  
628 cytokine increasing on 21 dpi, it became significant only on 0.5 dpi. IL-18 has been associated  
629 with atopic dermatitis expelled by *S. aureus* <sup>45</sup>. Although *S. aureus* is capable of causing the  
630 release of IL-18 from keratinocytes <sup>46</sup>, in this work an increase was detected only in plasma,  
631 but not in skin. An increase in IL-4 in plasma and skin was noted, especially after 21 days. *S.*  
632 *aureus* is able to inhibit the response of T-cell responses and to induce Th2 cell responses by  
633 producing IL-4 <sup>47</sup> in relation to chronic inflammations <sup>41</sup>.

634 The difficulty in developing an experimental model that reproduces an immune response under  
635 natural conditions might be related to the effect of the origin of both the bacteria and animal  
636 species used as hosts on the infective dose <sup>48</sup>. In general, a higher dose is necessary to infect  
637 animals with the *S. aureus* strains obtained from a different species (e.g. human bacteria CA-  
638 MRSA USA300 in mice:  $\geq 5.0 \times 10^8$  CFU <sup>49</sup>). In such cases, animal models can be unreliable  
639 predictors of either the potential success of therapeutic or preventive interventions or the roles  
640 played by specific determinants of bacterial virulence in infection <sup>2</sup>. Only a few standardized  
641 studies about the number of bacteria needed to begin infection can be found <sup>50</sup>. Schmid-Hempel  
642 and Frank <sup>50</sup> proposed an infective *S. aureus* dose of  $10^5$ - $10^6$  bacteria and classified it as a high-  
643 infective dose microorganism. In fact it has been reported that inoculation with less than  $5 \times 10^8$

644 CFU of USA300 does not result in reproducible abscesses in rabbits <sup>17,49</sup>. Our results agree with  
645 this observation when we used human strains (not adapted to rabbit), but disagree when rabbit  
646 strains were employed, which can generate dermal lesions with only 300 CFU by simulating  
647 natural infection. Moreover, when high doses of human strain Fwt ( $> 10^6$  CFU) were used with  
648 this model, the generated lesions differed from those observed for low doses of rabbit wild-type  
649 strains: lesions rapidly evolved and skin necrotic phenomena (dermonecrosis) predominated  
650 and, in histological terms, the presence of thrombosis and the absence of Splendore-Hoeppli  
651 phenomena or a peripheral eosinophilic layer were observed on 3 dpi (**JM Corpa, personal  
652 communication, Supplemental Figure 1**). Similar results have been reported by other authors  
653 <sup>17</sup>, who recognized that using such high doses would result in rapidly progressive infection,  
654 which would vastly differed from that typically encountered in clinical situations, and the  
655 results could not be extrapolated to humans. These skin necrotic phenomena are probably  
656 caused by the mass production of bacterial toxins, which is far removed from what actually  
657 happens in natural infection. This hypothesis would support the results observed in the present  
658 study with the mutants (*J $\Delta$ coa $\Delta$ vwb*, *J $\Delta$ hla*, *J $\Delta$ psm $\alpha$* , *J $\Delta$ agr*) employed to evaluate their  
659 contribution to the development and characteristics of abscesses. Studies carried out in rabbits,  
660 and mainly in mice, using  *$\Delta$ hla* mutants have resulted in reduced virulence in animal models of  
661 dermonecrotic skin infection. Moreover, immunization with Hla-specific antisera significantly  
662 reduces the size of skin lesions and prevents dermonecrosis <sup>11,17,51,52</sup>, and similar results have  
663 been obtained with mutants in *psm- $\alpha$*  <sup>53</sup>. Surprisingly, when the herein described skin infection  
664 model was used, mutants  *$\Delta$ hla* and  *$\Delta$ psm $\alpha$*  produced the same percentage of lesions as the wild-  
665 type strain, and even became macroscopically severer for  *$\Delta$ hla* regarding Jwt. Toxins and other  
666 virulence factors, such as  $\alpha$ -toxin, PSM- $\alpha$  and PSM- $\beta$ , are produced as a result of increased  
667 bacterial population density and environmental conditions, partly under a quorum-sensing  
668 control through different regulators <sup>54-56</sup>. Therefore, the effect observed in previous works with

669 either the  $\Delta hla$  or the  $\Delta psm\alpha$  mutant could be due to the use of high doses of bacteria to produce  
670 lesions, which would favor the rapid production of numerous toxins, and would make the effect  
671 of the toxin more evident. The absence of an effect on the  $\Delta agr$  mutant would follow the same  
672 hypothesis. In *S. aureus*, the majority of quorum-sensing components are coded by the  
673 accessory gene regulator (Agr) system. This regulation is important for acute disease to develop  
674 <sup>37</sup>, but we showed that Agr was dispensable in intradermal infections of rabbits. Agr suppresses  
675 the expression of the repressor of toxins (*rot*) at a high cell density <sup>57</sup>, which could also influence  
676 the virulence of the  $\Delta agr$  mutant. However, rabbit strains have a natural loss-of-function  
677 mutation in *rot*, which increases the severity and infectivity of the rabbit strain. This could  
678 justify the *FdltB<sup>r</sup>* strain (with functional *rot*) producing milder lesions than the wild-type strain.  
679 A histological characteristic observed from 3 dpi in the lesions produced by the strain wild-type  
680 was an eosinophilic necrotic layer in whose core Splendore-Hoeppli phenomena with inner  
681 bacteria were observed. These phenomena have been previously described as an electron-dense,  
682 granular and amorphous pseudocapsule. This pseudocapsule constitutes a barrier for immune  
683 cells by preventing them from penetrating the staphylococcal abscess community (SAC), where  
684 bacteria replicate without interference and are partially composed of prothrombin and  
685 fibrinogen that separate bacteria from leukocytes <sup>37,58,59</sup>. As the  $\Delta coa\Delta vwb$  mutant was able to  
686 produce lesions like Jw<sub>t</sub>, the absence of these two clotting factors secreted by *S. aureus* does  
687 not seem essential for abscesses to develop in rabbits. Despite all the employed mutants  
688 ( $J\Delta coa\Delta vwb$ ,  $J\Delta hla$ ,  $J\Delta psm\alpha$ ,  $J\Delta agr$ ) being able to generate similar lesions to the wild type in  
689 size and degree of severity terms, albeit in different percentages, they all triggered a delay in  
690 the evolution of histological lesions compared to the wild type. This could indicate that their  
691 mutations affected abscess development, but other factors also had to be involved in their  
692 development. More studies are needed to learn the specific role of these and other virulence  
693 factors in *S. aureus* infection development.

694 The utility of this model was corroborated when a human strain, modified to infect rabbits  
695 (“rabbitized”), was inoculated (*FdltB<sup>r</sup>*). This adaptation to the host seemed essential because  
696 neither macro- nor microscopic lesions developed when the human Fwt strain was inoculated  
697 with 300 CFU <sup>13</sup>, while the modified strain (*FdltB<sup>r</sup>*) was able to produce similar lesions to the  
698 wild strain adapted to rabbit (Jwt).

699 Finally, although mouse models have been presented as the “gold standard” to study  
700 staphylococcal infections in humans, along with their therapy and prevention <sup>60</sup>, we propose a  
701 low-infective-dose rabbit model as a more representative and realistic model, in which the effect  
702 of both homologous and heterologous strains can be proven.

703

#### 704 **Author contributions**

705 Conceptualization: Laura Selva, Juan M. Corpa, David Viana. Formal analysis: Juan J. Pascual,  
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712 editing: Alberto Arnau-Bonachera, Juan J. Pascual, Juan M. Corpa, David Viana. Juan M.  
713 Corpa and David Viana are the guarantors of this work and, as such, had full access to all of the  
714 data in the study and take responsibility for the integrity of the data and the accuracy of the data  
715 analysis.

716

717

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722

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901

902 **Figure Legends**

903 **Figure 1.** Representation of abscess evolution after experimental infection with *S. aureus* (Jw  
904 strain) with time. **A: 0 days postinoculation (dpi).** Intradermal inoculation of 300 CFU of *S.*  
905 *aureus*. Colored bars and numbers indicate the different histological parts of skin (**1:** epidermis;  
906 **2:** superficial dermis; **3:** deep dermis; **4:** cutaneous muscle). **B: 0.5 dpi. 1:** Externally, reddish  
907 macule. **2:** Mild vascular phenomena (edema and hemorrhages). **3:** Migration of heterophils  
908 mainly from deep vessels (arrows) and grouping at the inoculation point. **4:** Small hemorrhages.  
909 **C: 1 dpi. 1:** External slightly elevated reddish papule. **2:** Mild to moderate edema and  
910 hemorrhages. **3:** Initial abscess structured as a center of eosinophilic necrosis, with some  
911 bacteria surrounded by diffusely distributed heterophils. **4:** Muscular cells moderately  
912 infiltrated by heterophils. **D: 2 dpi. 1:** External reddish and elevated nodule and mild epidermal  
913 acanthosis, **2:** Moderate edema and hemorrhages. **3:** Well-defined abscess composed of a center  
914 of eosinophilic necrosis with bacteria, surrounded by a dense layer of heterophils. **4:** Muscle  
915 cells infiltrated by numerous heterophils that induce their separation and necrosis. **E: 3 dpi. 1:**  
916 Big dark nodule with epidermal necrosis and acanthosis. **2:** Severe vascular phenomena. **3:**  
917 Abscess with the presence of multiple Splendore-Hoeppli phenomena and an external layer of  
918 eosinophilic necrosis. **4:** Myocytes severely infiltrated by heterophils causing their  
919 degeneration. **F: 7 dpi. 1:** Necrosis and skin ulceration allowing the outflow of purulent content  
920 and acanthosis in the periphery to the ulcer. **2:** Very severe vascular phenomena. **3:** Splendore-  
921 Hoeppli phenomena merging with one another and the presence of a thin eosinophilic layer of  
922 necrosis surrounding the entire abscess. **4:** Cutaneous muscle interrupted by the presence of  
923 numerous heterophils and round cells. **G: 14 dpi. 1:** External lack of hair (alopecia), ulcer  
924 reepitelization and moderate to severe acanthosis. **2:** Absence of skin adnexa and moderate  
925 vascular phenomena. **3:** Presence of immature granulation tissue (numerous small neo-vessels  
926 and abundant connective tissue) and some scattered Splendore-Hoeppli phenomena. **4:** Atrophy  
927 of myocytes. **H: 21 dpi. 1:** External alopecia, very severe acanthosis. **2 and 3:** Abundant mature  
928 granulation tissue. **4:** Interstitial fibrosis and atrophy of myocytes. **I: 28 dpi. 1:** External small  
929 area of alopecia and moderate acanthosis. **2:** Invasion of skin appendages to the periphery of the  
930 scar. **3:** Mature granulation tissue with abundant fibrous tissue. **4:** Completely regenerated  
931 cutaneous muscle.

932

933

934 **Figure 2.** Correspondence between the gross (external and internally) and histological lesions  
935 during the experiment of the wild-type (Jwt) *S. aureus* strain inoculation. A: external aspect; B:  
936 sagittal cut of formalin fixed tissue; C: microscopically low magnification view stained with  
937 Hematoxilin-Eosin (H&E).

938

939 **Figure 3.** Colony-forming units after intradermal inoculation with Jwt (n = 10 animals per  
940 time). <sup>a-c</sup> The means not sharing superscript significantly differ ( $P < 0.05$ ). Error bars correspond  
941 to the standard error for each least square mean.

942

943 **Figure 4.** Different histological characteristics of lesions throughout infection with the Jwt *S.*  
944 *aureus* strain. **A** (0.5 dpi): Slight increase in the number of heterophils. Deep dermis. H&E.  
945 Scale bar = 200  $\mu\text{m}$ . **B** (1dpi): Abscess surrounded by numerous diffusely distributed  
946 heterophils. Skin. H&E. Scale bar = 1 mm. **C** (2dpi): Well-defined abscess characterized by a  
947 center of eosinophilic necrosis, with numerous coccoid bacteria inside (insert). Skin and  
948 bacteria (insert). H&E. Scale bar = 1 mm (skin) and 50  $\mu\text{m}$  (insert). **D** (2pi): Muscular  
949 degeneration caused by the intense interstitial infiltration of heterophils. Cutaneous muscle.  
950 H&E. Scale bar = 100  $\mu\text{m}$ . **E** (3dpi): Necrosis of epidermis. Epidermis. H&E. Scale bar = 200  
951  $\mu\text{m}$ . **F** (3dpi): A bacterial colony surrounded by star-shaped homogeneous eosinophilic material  
952 and an external layer of inflammatory cells. Splendore-Hoeppli phenomenon. H&E. Scale bar  
953 = 20  $\mu\text{m}$ . **G** (3dpi): Abscess internally composed of the fusion of several Splendore-Hoeppli  
954 phenomena, surrounded by a thin eosinophilic layer of necrosis. Skin. H&E. Scale bar = 1 mm.  
955 **H** (3dpi): Severe atrophy of myocytes, infiltrated by connective tissue (left) *versus* normal  
956 muscular tissue (right). Cutaneous muscle. Masson's trichrome stain. Scale bar = 200  $\mu\text{m}$ . **I**  
957 (7dpi): Significant increase in the number of cells with numerous mitosis (white arrows) on the  
958 basal layer of the epidermis (epidermal hyperplasia or acanthosis), compared to normal  
959 epidermis (insert). Epidermis. H&E. Scale bars = 100  $\mu\text{m}$  (acanthosis) and 50  $\mu\text{m}$  (insert). **J**  
960 (14dpi): Severe inflammatory reaction associated with an ulcer and epidermal hyperplasia on  
961 the border. Epidermis. H&E. Scale bar = 200  $\mu\text{m}$ . **K** (14dpi): Repair of the lesion characterized  
962 by acanthosis, absence of skin adnexa (left) and presence of abundant granulation tissue  
963 composed of numerous vessels and fibrous tissue (right). Skin (left) and deep dermis (right).  
964 H&E. Scale bars = 500  $\mu\text{m}$  (left) and 200  $\mu\text{m}$  (right). **L** (28dpi): Epithelial hyperplasia (arrow  
965 heads) and dermis with abundant fibrous tissue and blood vessels and scarce heterophils and  
966 some small hemorrhages (asterisk). The periphery of the lesion is invaded by hair follicles  
967 (arrows). Epidermis and dermis. H&E. Scale bar = 200  $\mu\text{m}$ .



968

969 **Figure 5.** Hyperplasia of the epithelium with the presence of numerous mitosis in the basal  
970 layer (white arrows) on 21 days postinfection with the Jwt *S. aureus* strain. Insert: Normal  
971 epithelium. H&E. Scale bars: 100  $\mu$ m.

972

973 **Figure 6.** Evolution of the counts of CD3<sup>+</sup>(A), IgG<sup>+</sup>(B) and RAM11<sup>+</sup>(C) cells  
974 immunochemically marked in the skin samples and total leukocytes (D), total lymphocytes (E),  
975 granulocytes (F), monocytes (G), lymphocytes B (H) and lymphocytes T CD25<sup>+</sup> (I) ( $\times 10^6/L$ )  
976 in peripheral blood after intradermal inoculation with the Jwt strain (n = 10 animals per day).<sup>a-</sup>  
977 <sup>c</sup> The means that do not share a superscript in the same figure significantly differ ( $P < 0.05$ ) for  
978 each postinoculation time. Error bars correspond to the standard error for each least square  
979 mean.

980

981 **Figure 7.** Severity assessment of gross lesions on day 7 after intradermal inoculation with  
982 different mutants (*J $\Delta$ coa $\Delta$ vwb*, n=16; *J $\Delta$ hla*, n=15; *J $\Delta$ psma* n=8; *J $\Delta$ agr* n=15) vs. Jwt (high  
983 virulent) and *Jrot*<sup>+</sup> (low virulent) strains.<sup>a, b</sup> Within an experiment, the groups not sharing letters  
984 above the bar significantly differ ( $P < 0.05$ ).

985

986 **Figure 8.** Presence of the main histological characteristics (Splendore-Hoeppli phenomena, A;  
987 heterophils surrounding the abscesses, B; and complete eosinophilic layers, C) on day 7 after  
988 intradermal inoculation with different mutant strains (*J $\Delta$ coa $\Delta$ vwb*, *J $\Delta$ hla*, *J $\Delta$ psma* and *J $\Delta$ agr*).  
989 Evaluated as variation in relation to intradermal inoculation with Jwt for: *J $\Delta$ coa $\Delta$ vwb* (n=16),  
990 *J $\Delta$ hla* (n=15), *J $\Delta$ psma* (n=8), *J $\Delta$ agr* (n=15). \* Significant variation in relation to intradermal  
991 inoculation with Jwt ( $P < 0.05$ ).

992

993 **Figure 9.** Mean severity assessment of gross lesions after intradermal inoculation with different  
994 strains (*FdltB*<sup>r</sup>, Jwt, *Jrot*<sup>+</sup> and *JdltB*<sup>h</sup>; n= 30 animals per strain). Assessment is represented as  
995 the percentage at four levels: Absence, Mild, Moderate, Severe.<sup>a, b</sup> The groups not sharing  
996 letters above the bar significantly differ ( $P < 0.05$ ).

997

998 **Figure 10.** Colony-forming units after intradermal inoculation with different strains of *S.*  
999 *aureus* [*FdltB*<sup>r</sup>, Jwt, *Jrot*<sup>+</sup>; n=10 animals per strain and day].<sup>a-d</sup> The means not sharing

1000 superscript significantly differ ( $P<0.05$ ). Error bars correspond to the standard error for each  
1001 least square mean.

1002

1003 **Figure 11.** Severity assessment of abscesses (heterophils organized) histologically (A) after  
1004 infections with strains *FdltB<sup>r</sup>*, *Jwt*, *Jrot<sup>+</sup>* and *JdltB<sup>h</sup>* (n=30 animals per strain). Assessment is  
1005 represented at four levels: Absence, Mild, Moderate, Severe. The groups not sharing letters (a,  
1006 b, c, d, e) above the bar significantly differ ( $P<0.05$ ). dpi: days postinfection. Evolution of the  
1007 counts of T-lymphocytes CD3<sup>+</sup>(B), B-lymphocytes CD79α<sup>+</sup> (C), plasma cells IgG<sup>+</sup>(D) and  
1008 macrophages RAM11<sup>+</sup>(E) immunochemically marked in skin samples after infection with  
1009 strains *FdltB<sup>r</sup>*, *Jwt*, *Jrot<sup>+</sup>* and *JdltB<sup>h</sup>*. <sup>a-d</sup> The means in a graph not sharing superscript  
1010 significantly differ ( $P<0.05$ ). Error bars correspond to the standard error for each least square  
1011 mean.

1012

1013 **Figure 12.** Effect of *S. aureus* strains on leukocyte population counts (A-I) in peripheral blood  
1014 and CD4<sup>+</sup>/CD8 (J) and granulocytes/lymphocytes (K) ratios after intradermal inoculation with  
1015 strains *FdltB<sup>r</sup>*, *Jwt*, *Jrot<sup>+</sup>* and *JdltB<sup>h</sup>*; n=30 animals per strain. <sup>a, b, c</sup> The means in a same graph  
1016 not sharing superscript significantly differ at  $P<0.05$ . G/L: Granulocytes/Lymphocytes.

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1019 **Table 1.** Number of rabbits inoculated with different *S. aureus* strains, sampling days, expected  
 1020 outcomes after infection and the study where they were involved.

Strain	No. rabbits	Sampling days	Objective	Study
Jwt	90	0, ½, 1, 2, 3, 7, 14, 21, 28	To describe the model in detail after the intradermal inoculation of a virulent <i>S. aureus</i> strain of rabbit origin.	1a
JΔcoaΔvwb	10	7	Both coagulases Coa and vWbp are required for abscesses to form. A reduction in the virulence of JΔcoaΔvwb and the presence of differences in the morphology of abscesses were expected.	1b
JΔhla	10	7	α-Hemolysin is a cytolytic toxin that promotes dermonecrosis in animal skin infections <sup>1</sup> . A reduction in abscess size and fewer and milder dermonecrotic lesions were expected.	1b
JΔpsma	10	7	PSMα performs cytolytic (lysis of erythrocytes and neutrophils) and immunomodulation (induces tolerogenic phenotypes in dendritic cells and inhibits T <sub>H</sub> 1 differentiation) activities <sup>3</sup> . Diminished reduction in the severity of dermal lesions was expected.	1b
JΔagr	10	7	Agr regulates most <i>S. aureus</i> toxins and exoenzymes <sup>18</sup> . A dysfunctional strain in this global virulence regulator should reduce the high toxicity in strongly aggressive <i>S. aureus</i> strains.	1b
FdltB <sup>r</sup>	30	1, 3, 7	F is a human ST121 strain that does not infect rabbits <sup>13</sup> . After its “rabbitization” (introduction of most 5’ nonsynonymous SNP at the <i>dltB</i> * locus from rabbit strains), the virulence of FdltB <sup>r</sup> was expected to be similar to that of Jwt.	2
Jrot <sup>+</sup>	30	1, 3, 7	<i>rot</i> is a global virulence regulator, a repressor of toxins <sup>5</sup> , which is altered in rabbits. Its restoral is expected to make Jrot <sup>+</sup> less virulent than Jwt.	2
JdltB <sup>h</sup>	30	1, 3, 7	After the reversion of the three identified <i>dltB</i> SNPs ( <i>dltB</i> <sup>h</sup> ) in a rabbit strain J (Jwt), loss of infectivity was expected <sup>13</sup> .	2

1021 \*The *dltB* gene is involved in the resistance of *S. aureus* to positively charged antimicrobial peptides, such  
 1022 as defensins and other host defense peptides <sup>19</sup>.

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1028 **Table 2.** The oligonucleotides used in this study.  
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Oligonucleotides	Description	Sequence
Coa-1m	Checking the <i>coa</i> mutants in Jwt and <i>FdltB</i> <sup>r</sup> strains by inside-inside PCR.	5'–ATAGAGATGCTGGTACAGG–3'
Coa-2c	Checking the <i>coa</i> mutants in Jwt and <i>FdltB</i> <sup>r</sup> strains by inside-inside PCR.	5'–GCTTCCGATTGTTTCGATGC–3'
Coa-5mB	Deletion of <i>coa</i> ( $\Delta coa$ ) in Jwt and <i>FdltB</i> <sup>r</sup> strains. 5' flanking region.	5'–cgggatccTCATGACGATACTTTCAGAGG–3'
Coa-6c	Deletion of <i>coa</i> ( $\Delta coa$ ) in Jwt and <i>FdltB</i> <sup>r</sup> strains. 5' flanking region.	5'–CCCAATCTACATTAAGAAAC–3'
Coa-7m	Deletion of <i>coa</i> ( $\Delta coa$ ) in Jwt and <i>FdltB</i> <sup>r</sup> strains. 3' flanking region.	5'–GGTAATTACATTTTGGAGGAAAACCTCTATC CATAGACATACAG–3'
Coa-8cE	Deletion of <i>coa</i> ( $\Delta coa$ ) in Jwt and <i>FdltB</i> <sup>r</sup> strains. 3' flanking region.	5'–ccggaattcGTACGAATGTGAATGGTGGC–3'
Coa-11m	Checking the <i>coa</i> mutants in Jwt and <i>FdltB</i> <sup>r</sup> strains by outside-outside PCR.	5'–CATACTTCGATCGTTCTATAAG–3'
Coa-12c	Checking the <i>coa</i> mutants in Jwt and <i>FdltB</i> <sup>r</sup> strains by outside-outside PCR.	5'–TTGCATCTATTAAAGAAGTAGG–3'
PSM $\alpha$ -1mB	Deletion of <i>psm<math>\alpha</math></i> locus ( $\Delta psm\alpha$ ) in Jwt and <i>FdltB</i> <sup>r</sup> strains. 5' flanking region.	5'–cgggatccACATGTTGACCATGAATACC–3'
PSM $\alpha$ -2c	Deletion of <i>psm<math>\alpha</math></i> locus ( $\Delta psm\alpha$ ) in Jwt and <i>FdltB</i> <sup>r</sup> strains. 5' flanking region.	5'–gtattcaattcgcttaaattattCATTAAGATTACCTCCT TTGC–3'
PSM $\alpha$ -3m	Deletion of <i>psm<math>\alpha</math></i> locus ( $\Delta psm\alpha$ ) in Jwt and <i>FdltB</i> <sup>r</sup> strains. 3' flanking region.	5'–AATAATTTAAGCGAATTGAATAC–3'
PSM $\alpha$ -4cE	Deletion of <i>psm<math>\alpha</math></i> locus ( $\Delta psm\alpha$ ) in Jwt and <i>FdltB</i> <sup>r</sup> strains. 3' flanking region.	5'–ccggaattcAATTGTGGCTTAATTGTTTGC–3'
PSM $\alpha$ -5m	Checking the <i>psm<math>\alpha</math></i> locus mutants in Jwt and <i>FdltB</i> <sup>r</sup> strains by inside-inside PCR.	5'–GCAAAGGAGGTAATCTTAATG–3'
PSM $\alpha$ -6m	Checking the <i>psm<math>\alpha</math></i> locus mutants in Jwt and <i>FdltB</i> <sup>r</sup> strains by outside-outside PCR.	5'–TACAAAGCCAGCTAATAACC–3'
PSM $\alpha$ -7c	Checking the <i>psm<math>\alpha</math></i> locus mutants in Jwt and <i>FdltB</i> <sup>r</sup> strains by outside-outside PCR.	5'–AATGGCAAATTAGACCAGC–3'
PSM $\alpha$ -8c	Checking the <i>psm<math>\alpha</math></i> locus mutants in Jwt and <i>FdltB</i> <sup>r</sup> strains by inside-inside PCR.	5'–CGAATTCCATGTGAATGGC–3'
vwb-1mB	Deletion of <i>vwb</i> ( $\Delta vwb$ ) in Jwt and <i>FdltB</i> <sup>r</sup> strains. 5' flanking region.	5'–cgggatccTCAGGTTCTAACAATAATGTAG–3'
vwb-2c	Deletion of <i>vwb</i> ( $\Delta vwb$ ) in Jwt and <i>FdltB</i> <sup>r</sup> strains. 5' flanking region.	5'–GCTCCCAATGATAAAACTAGC–3'
vwb-3m	Deletion of <i>vwb</i> ( $\Delta vwb$ ) in Jwt and <i>FdltB</i> <sup>r</sup> strains. 3' flanking region.	5'–gctagttttatcattgggagcAAGCAAATAATGAGTTT GCCG–3'
vwb-4cE	Deletion of <i>vwb</i> ( $\Delta vwb$ ) in Jwt and <i>FdltB</i> <sup>r</sup> strains. 3' flanking region.	5'–ccggaattcTTTGTGTGTCAGCTAAACTTCC–3'
vwb-7m	Checking the <i>vwb</i> mutants in Jwt and <i>FdltB</i> <sup>r</sup> strains by outside-outside PCR. 3' flanking region.	5'–ACCAATTCCAGAGGATTCAG–3'
vwb-8c	Checking the <i>vwb</i> mutants in Jwt and <i>FdltB</i> <sup>r</sup> strains by outside-outside PCR. 5' flanking region.	5'–TATTCAATTTATCTTCAGAAGC–3'
vwb-11m	Checking the <i>vwb</i> mutants in Jwt and <i>FdltB</i> <sup>r</sup> strains by outside-outside PCR. 3' flanking region.	5'–GGCTGAAGATGAGGCTTTG–3'
vwb-12c	Checking the <i>vwb</i> mutants in Jwt and <i>FdltB</i> <sup>r</sup> strains by outside-outside PCR. 5' flanking region.	5'–TAAGTCGCACTTTAATTGC–3'

The sequences recognized by the restriction enzymes used in cloning are denoted **lower case** letters.

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**Table 3.** The monoclonal antibodies used in the immunohistochemical studies.

Primary antibodies (dilution)	Isotype	Reactivity	Antigen retrieval	Reference & Company	Secondary antibodies (dilution)	Reference & Company
Mouse anti-canine CD3 (1:20)	IgG1	T-cells	Enzymatic treatment	CA17.2A12, UC Davis, USA	Biotinylated anti-rat IgG (H+L) (1:400)	BA-9400, Vector Laboratories, USA
Mouse anti-human CD79 $\alpha$ (1:50)	IgG1	B-cells	Heat treatment	MCA2538H, AbD Serotec, UK	Biotinylated anti-mouse IgG (1 drop into 10mL of diluent)	PK 6102, Vector Laboratories, USA
Mouse anti-rabbit macrophage clone RAM11 (1:50)	IgG1k	Macrophages	Enzymatic treatment	M0633, Dako, USA	Biotinylated anti-mouse IgG (1 drop into 10mL of diluent)	PK 6102, Vector Laboratories, USA
Mouse anti-rabbit light chain, HRP conjugated (1:250)	IgG1k	Plasma cells	Enzymatic treatment	MAB201P, Merck, Germany	None	-

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**Table 4.** The monoclonal antibodies used in the flow cytometry studies.

Monoclonal antibodies	Isotype	Specificity	Reactivity	Clone	Reference & Company
Mouse anti-rabbit IgM	IgG1	IgM	B-cells	NRBM	MCA812GA, AbD Serotec, UK
Mouse anti-rabbit T-lymphocytes: FITC*	IgG1	CD5	T-cells	KEN-5	MCA800F, AbD Serotec, UK
Mouse anti-rabbit CD4	IgG2a	CD4	T CD4 cells	KEN-4	MCA799, AbD Serotec, UK
Mouse anti-rabbit CD8	IgG2a	CD8	T CD8 cells	ISC27A	WS0796U-100, Kingfisher Biotech, USA
Mouse anti-rabbit CD25	IgG2b	CD25	Activated T-cells	KEI- $\alpha$ 1	MCA1119G, AbD Serotec, UK
Mouse anti-human CD14: FITC	IgG2a	CD14	Monocytes and granulocytes	TÜK4	MCA1568F, AbD Serotec, UK
Mouse anti-rabbit $\alpha$ -CD45	IgM	CD45	All leucocytes	ISC76A	ISC76A, VMRD Inc., USA

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\*Clon KEN-5 recognizes rabbit T-lymphocytes and immunoprecipitates. This antibody recognizes rabbit CD5 but does not bind to rabbit CD5 transfectants. Known rabbit CD5 antibodies also show binding to most B-lymphocytes, which are not labeled by this clone (information obtained from datasheet).

**Table 5.** The histopathological findings observed after the inoculation of a Jwt *S. aureus* strain and their evolution during the experiment (n = 10 animals per day).

	Days postinoculation (dpi)								P value
	0.5	1	2	3	7	14	21	28	
<i>Epidermis*</i>									
Hyperplasia	100/0/0/0 <sup>a</sup>	100/0/0/0 <sup>a</sup>	30/60/10/0 <sup>b</sup>	27/27/36/10 <sup>b</sup>	0/0/0/100 <sup>d</sup>	0/0/0/100 <sup>d</sup>	0/8/50/42 <sup>c</sup>	0/16/46/38 <sup>c</sup>	0.001
Reepithelization (mitosis)	100/0/0/0 <sup>a</sup>	100/0/0/0 <sup>a</sup>	100/0/0/0 <sup>a</sup>	100/0/0/0 <sup>a</sup>	100/0/0/0 <sup>a</sup>	58/8/17/17 <sup>b</sup>	25/25/0/50 <sup>b</sup>	0/0/8/92 <sup>c</sup>	0.001
<i>Superficial dermis</i>									
Vascular dilatation	80/20/0/0 <sup>a</sup>	30/60/10/0 <sup>ab</sup>	30/60/0/0 <sup>ab</sup>	18/55/27/0 <sup>bc</sup>	0/10/40/50 <sup>d</sup>	0/50/50/00 <sup>c</sup>	17/42/33/8 <sup>c</sup>	23/46/31/0 <sup>bc</sup>	0.001
Hemorrhages	89/0/0/11 <sup>a</sup>	10/80/10/0 <sup>bc</sup>	40/50/10/0 <sup>abc</sup>	27/27/36/10 <sup>c</sup>	10/40/30/20 <sup>c</sup>	50/25/17/8 <sup>ab</sup>	67/33/0/0 <sup>a</sup>	31/23/38/8 <sup>c</sup>	0.001
Edema	80/0/20/0 <sup>ab</sup>	70/10/20/0 <sup>ab</sup>	80/0/20/0 <sup>ab</sup>	18/10/36/36 <sup>d</sup>	0/60/20/20 <sup>cd</sup>	33/17/33/17 <sup>bc</sup>	67/25/8/0 <sup>ab</sup>	85/15/0/0 <sup>a</sup>	0.001
<i>Deep dermis</i>									
Vascular dilatation	80/20/0/0 <sup>a</sup>	0/100/0/0 <sup>a</sup>	30/70/0/0 <sup>a</sup>	18/55/27/0 <sup>ab</sup>	0/60/40/0 <sup>b</sup>	0/100/0/0 <sup>ab</sup>	17/42/33/8 <sup>ab</sup>	23/46/23/8 <sup>a</sup>	0.132
Perivascular inflammation	30/50/10/10 <sup>b</sup>	0/20/10/70 <sup>e</sup>	10/10/30/50 <sup>de</sup>	0/36/55/9 <sup>c</sup>	0/20/60/20 <sup>cd</sup>	0/40/30/30 <sup>cd</sup>	0/66/17/17 <sup>bc</sup>	75/15/8/0 <sup>a</sup>	0.001
Heterophiles diffuses	40/50/10/0 <sup>a</sup>	10/40/10/40 <sup>bc</sup>	10/20/50/20 <sup>bc</sup>	0/27/63/0 <sup>bc</sup>	0/30/30/40 <sup>c</sup>	8/33/42/17 <sup>b</sup>	0/41/42/17 <sup>bc</sup>	69/15/16/0 <sup>a</sup>	0.001
Heterophiles organized (abscess)	80/10/0/10 <sup>a</sup>	20/10/10/60 <sup>b</sup>	20/10/10/60 <sup>bc</sup>	0/0/0/100 <sup>c</sup>	0/0/0/100 <sup>c</sup>	20/0/0/80 <sup>bc</sup>	75/0/0/25 <sup>a</sup>	92/8/0/0 <sup>a</sup>	0.001
Eosinophilic peri-abscess layer	100/0/0/0 <sup>a</sup>	50/30/20/0 <sup>b</sup>	40/10/0/50 <sup>cd</sup>	0/0/18/82 <sup>d</sup>	0/0/50/50 <sup>cd</sup>	20/0/40/40 <sup>c</sup>	75/0/17/8 <sup>b</sup>	92/8/0/0 <sup>ab</sup>	0.001
Hemorrhages	100/0/0/0 <sup>a</sup>	40/60/0/0 <sup>cd</sup>	30/70/0/0 <sup>d</sup>	0/100/0/0 <sup>d</sup>	30/70/0/0 <sup>d</sup>	75/17/0/8 <sup>bc</sup>	100/0/0/0 <sup>a</sup>	85/15/0/0 <sup>b</sup>	0.001
Granulation tissue	100/0/0/0 <sup>a</sup>	100/0/0/0 <sup>a</sup>	100/0/0/0 <sup>a</sup>	100/0/0/0 <sup>a</sup>	100/0/0/0 <sup>a</sup>	75/8/17/0 <sup>b</sup>	0/9/8/83 <sup>c</sup>	15/0/0/85 <sup>c</sup>	0.001
Regeneration (hair follicles)	100/0/0/0 <sup>a</sup>	100/0/0/0 <sup>a</sup>	100/0/0/0 <sup>a</sup>	100/0/0/0 <sup>a</sup>	100/0/0/0 <sup>a</sup>	100/0/0/0 <sup>a</sup>	83/17/0/0 <sup>a</sup>	9/76/15/0 <sup>b</sup>	0.001
<i>Cutaneous muscle</i>									
Interstitial inflammation	40/40/0/20 <sup>b</sup>	10/10/60/20 <sup>cde</sup>	10/50/20/20 <sup>bcd</sup>	0/28/36/36 <sup>de</sup>	0/0/30/70 <sup>e</sup>	0/50/10/40 <sup>cd</sup>	25/33/25/17 <sup>bc</sup>	75/25/0/0 <sup>a</sup>	0.001
Atrophy/degeneration	90/0/10/0 <sup>a</sup>	70/20/10/0 <sup>ab</sup>	70/20/10/0 <sup>ab</sup>	35/55/10/0 <sup>ab</sup>	0/30/70/0 <sup>c</sup>	8/50/8/34 <sup>c</sup>	33/42/8/17 <sup>bc</sup>	75/8/0/15 <sup>a</sup>	0.001
Hypertrophy	100/0/0/0	100/0/0/0	100/0/0/0	100/0/0/0	100/0/0/0	75/25/0/0	92/8/0/0	69/31/0/0	0.204
Interruption	90/10/0/0 <sup>a</sup>	100/0/0/0 <sup>a</sup>	100/0/0/0 <sup>a</sup>	70/20/10/0 <sup>ab</sup>	40/30/20/10 <sup>b</sup>	50/33/0/17 <sup>ab</sup>	76/8/8/8 <sup>ab</sup>	100/0/0/0 <sup>a</sup>	0.010
Fibrosis	100/0/0/0 <sup>a</sup>	100/0/0/0 <sup>a</sup>	100/0/0/0 <sup>a</sup>	100/0/0/0 <sup>a</sup>	40/60/0/0 <sup>a</sup>	25/50/17/8 <sup>c</sup>	58/42/0/0 <sup>b</sup>	92/8/0/0 <sup>b</sup>	0.001

\* Each histopathological finding presents 4 values w/x/y/z that indicate the percentage of animals with microscopic findings classified as absent (w), mild (x), moderate (y) and severe (z).

For a given trait presented in a row, P-Value tests hypothesis H0: there was no effect of treatment to explain the observed trait variance.

<sup>a-e</sup> The means in a same row not sharing superscript significantly differ for each inoculation time (P<0.05).

1041 **Table 6.** Evolution of the concentration (pg/mL) of cytokines on plasma and skin tissue after the intradermal inoculation with the Jwt strain (n =  
 1042 10 animals per day).

	Postinoculation time (days)									SEM	P-value
	0	0.5	1	2	3	7	14	21	28		
<i>Plasma</i>											
IL-4	3.587 <sup>ab</sup>	2.317 <sup>a</sup>	3.375 <sup>a</sup>	5.701 <sup>b</sup>	3.780 <sup>b</sup>	3.751 <sup>b</sup>	3.886 <sup>b</sup>	5.552 <sup>b</sup>	8.904 <sup>c</sup>	0.807	<0.001
IL-18	126.8 <sup>ab</sup>	316.4 <sup>bc</sup>	263.0 <sup>bc</sup>	245.9 <sup>b</sup>	328.2 <sup>bc</sup>	87.2 <sup>a</sup>	350.0 <sup>c</sup>	307.2 <sup>bc</sup>	463.4 <sup>d</sup>	39.8	<0.001
<i>Skin</i>											
IL-4	8.998 <sup>a</sup>	6.419 <sup>a</sup>	7.080 <sup>a</sup>	7.780 <sup>a</sup>	9.392 <sup>a</sup>	7.307 <sup>a</sup>	8.774 <sup>a</sup>	9.951 <sup>a</sup>	15.479 <sup>b</sup>	1.554	0.001
IFN-gamma	643 <sup>a</sup>	14015 <sup>b</sup>	412 <sup>a</sup>	401 <sup>a</sup>	547 <sup>a</sup>	558 <sup>a</sup>	619 <sup>a</sup>	3879 <sup>a</sup>	2588 <sup>a</sup>	2282	<0.001

<sup>a-d</sup> The means in a same row not sharing superscript significantly differ at  $P < 0.05$  for day postinoculation.

For a given trait presented in a row, P-Value tests hypothesis H0: there was no effect of treatment to explain the observed trait variance.

SEM: standard error of the mean.

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**Table 7.** The histopathological findings observed after the inoculation of different *S. aureus* strains (n = 10 animals per day and strain).

	<i>S. aureus</i> strain				<i>P</i> value	
	<i>FdltB</i> <sup>r</sup>	<i>Jwt</i>	<i>Jrot</i> <sup>+</sup>	<i>JdltB</i> <sup>h</sup>	Strain	Time*Strain
<i>Epidermis</i> <sup>*</sup>						
Hyperplasia	74/2/21/3 <sup>b</sup>	42/10/13/35 <sup>c</sup>	83/8/6/3 <sup>a</sup>	90/10/0/0 <sup>a</sup>	0.001	0.001
Reepithelization (mitosis)	100/0/0/0	100/0/0/0	100/0/0/0	100/0/0/0	1.000	1.000
<i>Superficial dermis</i>						
Vascular dilatation	44/52/4/0 <sup>b</sup>	16/42/26/16 <sup>c</sup>	40/57/3/0 <sup>a</sup>	33/67/0/0 <sup>ab</sup>	0.001	0.001
Hemorrhages	58/26/16/0 <sup>a</sup>	16/48/26/10 <sup>b</sup>	50/37/13/0 <sup>a</sup>	50/43/7/0 <sup>a</sup>	0.001	0.001
Edema	23/48/29/0 <sup>b</sup>	29/26/26/19 <sup>b</sup>	40/40/17/3 <sup>a</sup>	50/47/3/0 <sup>a</sup>	0.001	0.005
<i>Deep dermis</i>						
Vascular dilatation	2/46/52/0 <sup>b</sup>	0/84/13/3 <sup>a</sup>	0/77/13/10 <sup>a</sup>	0/41/56/3 <sup>b</sup>	0.001	0.012
Perivascular inflammation	2/46/52/0 <sup>a</sup>	0/26/42/32 <sup>b</sup>	77/7/16/0 <sup>a</sup>	3/47/50/0 <sup>a</sup>	0.001	0.001
Heterophiles diffuses	4/54/34/8 <sup>ab</sup>	0/36/39/25 <sup>b</sup>	0/64/23/13 <sup>a</sup>	0/47/47/6 <sup>a</sup>	0.016	0.060
Heterophiles organized (abscess)	58/4/2/36 <sup>b</sup>	6/4/3/87 <sup>c</sup>	60/7/3/30 <sup>a</sup>	67/17/13/3 <sup>a</sup>	0.001	0.001
Eosinophilic peri-abscess layer	75/17/4/4 <sup>b</sup>	16/10/29/45 <sup>c</sup>	83/4/7/6 <sup>ab</sup>	100/0/0/0 <sup>a</sup>	0.001	0.095
Hemorrhages	100/0/0/0 <sup>a</sup>	23/77/0/0 <sup>b</sup>	100/0/0/0 <sup>a</sup>	100/0/0/0 <sup>a</sup>	0.001	0.845
Granulation tissue	100/0/0/0	100/0/0/0	100/0/0/0	100/0/0/0	1.000	1.000
<i>Cutaneous muscle</i>						
Interstitial inflammation	15/46/27/12 <sup>b</sup>	3/14/42/41 <sup>c</sup>	21/63/3/13 <sup>a</sup>	17/53/27/3 <sup>a</sup>	0.001	0.008
Atrophy/degeneration	71/19/10/0 <sup>b</sup>	35/35/30/0 <sup>b</sup>	86/8/3/3 <sup>a</sup>	100/0/0/0 <sup>a</sup>	0.001	0.029
Hypertrophy	100/0/0/0	100/0/0/0	97/3/0/0	100/0/0/0	0.415	1.000
Interruption	96/2/2/0	68/19/10/3	97/0/0/3	97/3/0/0	0.191	0.525
Fibrosis	100/0/0/0	81/19/0/0	100/0/0/0	100/0/0/0	0.066	0.144

<sup>\*</sup> Each histological parameter presents 4 values (w/x/y/z) that indicate the average percentage of animals for the three sampling times (1, 3 and 7 days postinfection). The microscopic findings are classified as absent (w), mild (x), moderate (y), severe (z).

For a given trait presented in a row, P-Value tests hypothesis H0: there was no effect of treatment to explain the observed trait variance.

<sup>a-c</sup> The means in a same row not sharing letters significantly differ ( $P < 0.05$ ).

1 **Supplemental Figure 1.** Severe dermonecrosis (A: external aspect; B: sagittal cut of formalin fixed  
2 tissue; C: microscopically low magnification view stained with H&E) characterized by a peripheral halo  
3 of inflammatory cells (arrows) and a center of coagulative necrosis (asterisk) at 3 days postinoculation  
4 of  $10^7$  CFU of a human wild-type strain (Fwt).

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1 **ONLINE SUPPLEMENTAL MATERIAL**

2

**Supplemental Table 1.** The statistical models used in this work based on the trait and the study.

Group of traits Trait	Study 1*		Study 2 <sup>†</sup>
	1a. Jwt	1b. Mutants	<i>FdltB</i> <sup>r</sup> vs. J
Animal weight	M1		
Body temperature	M1		M7
CFU	M2		M8
Gross lesions			
Erythema (area)	M3		M9
Nodule (volume)	M3		M9
Histological findings			
Epidermal thickening	M5		M10
Reepithelization	M5		M10
Vascular dilatation	M5		M10
Perivascular inflammation	M5		M10
Edema	M5		M10
Hemorrhages	M5		M10
Heterophiles diffuses	M5	M6	M10
Heterophiles organized (abscess)	M5		M10
Eosinophilic peri-abscess layer	M5	M6	M10
Granulation tissue	M5		M10
Splendore-Hoeppli phenomena		M6	
Regeneration (mitosis)	M5		M10
Interstitial inflammation	M5	M6	M10
Atrophy/degeneration	M5	M6	M10
Hypertrophy	M5		M10
Interruption	M5		M10
Fibrosis	M5		M10
Immunohistochemistry			
Macrophages	M1		M7
Plasma cells	M1		M7
T-lymphocytes	M1		M7
B-lymphocytes	M4		M7
Hematology and flow cytometry			
Total leukocytes	M1		M7
Granulocytes	M1		M7
Total lymphocytes	M1		M7
B-lymphocytes	M1		M7
T-lymphocytes	M1		M7
T CD4 <sup>+</sup>	M1		M7
T CD8 <sup>+</sup>	M1		M7
T CD25 <sup>+</sup>	M1		M7
Monocytes	M1		M7
Cytokines			
IL-1 $\beta$	M1		
IL-4	M1		
IL-17	M1		
IL-18	M1		
IFN-gamma	M1		

\* Study 1. Characterization of the model after infection with a Jwt (1a. Jwt) and mutant strains (1b. Mutants: *J $\Delta$ coa $\Delta$ vw**b***, *J $\Delta$ hla*, *J $\Delta$ psma* and *J $\Delta$ agr*).

<sup>†</sup> Study 2. Comparison of lesions caused by a human “rabbitized” strain (*FdltB*<sup>r</sup>) with different-virulence rabbit strains (J: Jwt, *Jrot*<sup>+</sup> and *JdltB*<sup>b</sup>).

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