Effect of selection for growth rate on the rabbit (*Oryctolagus cuniculus*) immune system and its response after experimental *Staphylococcus aureus* infection

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Abstract

The aim of the work was to evaluate if genetic selection for daily gain may affect the immune system. Two experiments were performed. The first one involved 80 rabbit females and their first two litters to explore the effect of selection on the ability of animals to maintain immune competence. Two generations ~~with different degrees of selection~~from a line selected for average daily gain (ADG) were evaluated (VR19 generation 19th, n=43; VR37 generation 37th, n=37). In females, the effect of selection and its interaction with physiological state were not significant for any trait. In litters, the selection criterion increased the granulocyte to lymphocyte ratio~~, which could be related more to the more selected animals' greater consumption and weight than to the selection criterion itself~~. The second experiment involved 73 19-week-old females (VR19, n=39; VR37, n=34) to explore the effect of genetic selection on immune response after *S. aureus* infection. The VR37 rabbit females had lower counts for total lymphocytes, CD5$^+$, CD4$^+$, CD8$^+$, CD25$^+$, monocytes, the CD4$^+$/CD8$^+$ ratio and platelets than those of VR19 (-14, -21, -25, -15, -33, -18, -11 and -11%, respectively; P<0.05). VR37 had less erythema (-8.4 percentage points; P<0.05), fewer nodules (-6.5 percentage points; P<0.05) and a smaller nodule size (-0.65 cm^3 on 7 day post-inoculation; *P*<0.05) compared to VR19. Our study suggests that genetic selection for average daily gain does not negatively affect the maintenance of a competent immune system or the ability to establish immune response. It seems that such selection may improve the response to *S. aureus* infections.

Keywords: immunologic challenge, immune response, rabbit, genetic selection

Introduction

In the last 50 years, animal productivity has considerably increased due to genetic selection (Hill 2008). However, this selection has sometimes been accompanied by undesired side effects (Rauw et al. 1998). By way of example, ascites syndrome in chicken has usually been associated with genetic progress (Wideman et al. 2013). In dairy cattle, selection to increase milk is normally associated with worse health, fertility and functional longevity (Veerkamp et al. 2009). In rabbits, some examples of these side effects have been reported from paternal lines selected for growth rate. The females from such lines present poorer reproductive performance and ~~stayability~~ lifespan on farms (Penadés et al. 2018) than the females from maternal lines. The young rabbits from these lines are more sensitive to digestive disorders, especially when antimicrobials are not used in feed (García-Quirós et al. 2014). However, it is unclear whether these problems are the effect of the genetic selection criterion or are intrinsic to lines as a result of the animals selected to create them. To elucidate between both options, it is necessary to perform selection experiments to evaluate the indirect effects of the selection criterion. In rabbits, four methodologies have been described (Khalil and Al-Saef 2008): (i) comparing a selected population to a non-selected control population; (ii) divergent selecting two populations; (iii) estimating genetic trends with statistical methods like a mixed model theory or Bayesian approaches; (iv) comparing the contemporaries of two different generations by using vitrified embryos from the same line. The last method offers the advantages of using a control population, which avoids problems with the chosen statistical model ~~or genetic drift in long-term experiments~~ (Piles and Blasco 2010). However, some authors have shown that the vitrification process can have long-term effects that should be considered when comparing vitrified to non-vitrified populations (Dulioust et al. 1995).

63 The immune system constitutes animals' defence system against any pathogenic infection or
64 disease. In light of the above, selection might impair animal immunity (Rauw 2012) because
65 prioritising resources to selected productive functions may dwindle available resources for other
66 functions (Pascual et al. 2013) by increasing animals' sensitivity to environmental stressors
67 (Kolmodin et al. 2003). The consequences of selection can act in two ways by affecting the way that
68 animals (Lochmiller and Deerenberg 2000): (i) maintain a basal competent immune system; (ii)
69 establish the immune response. When evaluating the former, it would seem that the basal level of
70 immune system blood cells evolves with animals' age and physiological state (Wells et al. 1999;
71 Guerrero et al. 2011; Ferrian et al. 2012). As a consequence, maintaining a competent immune system
72 at some key life cycle points might be most relevant. Indeed weaning young rabbits and females' first
73 parturitions have been reported as the most challenging moments (Jeklova et al. 2007, 2009; Penadés
74 et al. 2018). To evaluate the way animals establish the immune response, an immunological challenge
75 using one pathogen under experimentally controlled conditions is frequently applied. Here
76 *Staphylococcus aureus (S. aureus)* presents interesting features. The pathogen is the causal agent of
77 several pathological processes in growing rabbits, especially in female rabbits. It has been associated
78 with mastitis of lactating does (Corpa et al. 2010), which is one of the main reasons to cull females
79 (Segura et al. 2007; Rosell and de la Fuente 2009). It is also possible to infect animals by this pathogen
80 under controlled standard conditions (Viana et al. 2015; Muñoz-Silvestre et al. 2020; Penadés et al.
81 2020) and the literature reports such infections with different strains of the bacterium, at several
82 animal ages, etc. The use of flow cytometry to evaluate rabbits' immune system is a much less
83 widespread approach than in other species because monoclonal antibodies (mAbs) for this species
84 are limited. However, increasingly more studies show its usefulness in rabbit research (Davis and
85 Hamilton 2008), to study the diseases that affect rabbits (Guerrero et al. 2015) and to compare
86 different lines of selected rabbits based on distinct production parameters (Penadés et al. 2018). In

this context, we have the appropriate tools to assess whether selection for growth rate really affects animals' immune status.

Therefore, the present work aims to evaluate the effect of genetic selection for growth rate on the immune system to both maintain basal immune competence under conventional conditions and to establish the immune response to an immunological challenge with *S. aureus*.

Materials and Methods

In this study, two different experiments were carried out. Experiment 1 aimed to evaluate the effect of genetic selection on the immune system on commercial farms. The aim of Experiment 2 was to evaluate the effect of genetic selection on the immune response to an immunological challenge.

Experiment 1

Animals and experimental procedure

Experiment 1 involved 80 rabbit females and their litters (*Oryctolagus cuniculus*), which were evaluated from the first artificial insemination (1IA) of females to their second weaning. Rabbit females came from a long-term line selected for average daily gain (ADG) during the growing period (between weeks 4 and 9 of life) (line R; Estany et al. 1992). The experiment was conducted between November 25th, 2016 and October 9th, 2017 under usual climatic conditions for Valencia during that time of year (12-27ºC and 60-75% RH). Two experimental genetic types~~, which differed in degree of selection,~~ were used in Experiment 1. Forty-three females (VR19) belonged to generation 19 of this line, whereas 37 females (VR37) corresponded to generation 37. To prevent a possible vitrification effect of former generation, all the animals from both genetic types were obtained after completely

restoring both populations from the vitrified embryos, and using only their progeny to avoid direct vitrification effects (Dulioust et al. 1995). From each of the lines, embryos were devitrified until 35 healthy females and 12 healthy males (from 10 different origins) were obtained to reconstitute both populations. Although the experiment was not designed for it, the effect of genetic selection on the ADG of the females used in this experiment could be measured (2.54±1.24g, *P*=0.056; unpublished data). Data on this trait with experiments designed for this purpose will be published soon. Females were housed in individual cages (700 x 500 x 320 mm) and raised under conventional environmental conditions by alternating 16 h of light and 8 h of darkness. At the age of 19 weeks, all the females were inseminated for the first time with pooled semen from the males that belonged to their respective generation. On gestation day 28, cages were provided with a nest for litters. Upon parturition, litters were standardised to 5-8 kits per litter (on av. 5.4 for the first reproduction cycle, and 6.9 for the second). This low value was obtained due to the poor prolificacy of R line (Baselga 2002). Litters were weaned on lactation day 28. Females were inseminated again 11 days after parturition. If females were not pregnant, they were re-inseminated every 21 days until a maximum for three consecutive times, when they were culled for reproductive failure. During the whole experiment, all the animals had free access to water, with commercial diet for reproductive rabbit females. Blood samples were taken from three random kits per litter at weaning in both female reproductivon cycles (samples were only taken from those litters in which at least 3 rabbits survived until weaning and the blood from the three kits per litter was mixed and processed as a single sample) and from females in five different physiological states: first artificial insemination (1IA); first parturition (1P); first weaning (1W); second parturition (2P); second weaning (2W). To prevent diurnal variations in the haematological parameters, blood samples were collected at approximately the same time (9 am to 10 am). Three millilitres of blood were collected from the central artery of the ear in two 1 mL tubes with EDTA as anticoagulant (AQUISEL Tube EDTA K3) and one 1 mL tube for serum extraction (AQUISEL Tube

Clotting activator).~~Three millilitres of blood were taken from the central ear artery in two 1-mL tubes with EDTA as an anticoagulant (AQUISEL Tube EDTA K3)~~.

Immunophenotypical characterisation of peripheral blood by flow cytometry and haematology studies

One millilitre of each blood sample was used to carry out the immunophenotypical characterisation by flow cytometry. Another millilitre was employed to conduct haematology studies. A third millilitre was centrifuged at 2,500 g to obtain serum and was used to determine the haptoglobin concentration. The millilitre ~~1mle~~ of whole blood from each sample was utilised for the flow cytometry analyses and was lysed by adding 45 ml of ammonium chloride lysing solution. After spinning, the pellet of leucocytes was resuspended in 1 millilitre of Dulbecco's Phosphate-Buffered Saline (DPBS) suspension (Sigma-Aldrich®). This cell suspension was divided into six sample tubes per animal. Primary mAbs (Table 1) were added to the cell suspension following manufacturers' technical specifications, and were incubated for 25 minutes at room temperature. Afterwards, samples were washed and secondary antibodies (Rat anti-mouse IgG2ak or IgG2bk Phycoerythrin [Nordic-MUbio] and Goat anti-mouse IgM: R-Phycoerythrin [Biorad]) were added following manufacturers' technical specifications to be incubated for 25 minutes at room temperature. Then samples were washed again and 1 ml of DPBS was added before analysing the samples by the flow cytometer. The outcome leucocyte (WBC) suspensions were analysed in a Cytomics FC500 flow cytometer (Beckman Coulter, Brea, CA, USA). Common leucocyte antigen CD14 and CD45 expressions were used for the "lymphogate" setup as previously described Guerrero et al. (2011) and Jeklova et al. (2007). The total lymphocyte count was calculated from the WBC count and the lymphocyte percentage, and the lymphocyte subset counts as described by Guerrero et al. (~~Guerrero et al.~~ 2011).

155 **Table 1.** Monoclonal antibodies used in this study.

Monoclonal antibodies	Isotype	Specificity	Cell labelling	Clone	References	Company
Mouse anti-rabbit T lymphocytes: FITC[a]	IgG1	CD5	T-cell	KEN-5	Kotani et al. (1993a)	Abd Serotec
Mouse anti-rabbit α-pan B	IgM	IgM	B-cell	MRB143A	Davis and Hamilton (2008)	VMRD Inc.
Mouse anti-rabbit CD4	IgG2a	CD4	T-cell subset	KEN-4	Kotani et al. (1993a)	Abd Serotec
Mouse anti-rabbit α-CD8	IgG2a	CD8	T-cell subset	ISC27A	Davis and Hamilton (2008)	VMRD, Inc.
Mouse anti-rabbit CD25	IgG2b	CD25	Activated T-cells	KEI-ALPHA1	Kotani et al. (1993b)	Abd Serotec
Mouse anti-human CD14: FITC	IgG2a	CD14	Monocytes and granulocytes	TÜK 4	Jacobsen et al. (1993)	Abd Serotec
Mouse anti-rabbit α-CD45	IgM	CD45	All the leucocytes	ISC76A	Davis and Hamilton (2008)	VMRD Inc.

156 [a] Clone KEN-5 recognises rabbit T-lymphocytes and immunoprecipitates. This antibody recognises rabbit CD5, but does not bind to rabbit CD5
157 transfectants. Known rabbit CD5 antibodies also show binding to most B-lymphocytes, which are not labelled by this clone (information obtained from
158 the datasheet).

159 The following blood parameters were evaluated using a haematological counter (MEK-6410, Nihon
160 Kohden, Japan): leucocytes (WBC), haematocrit (HCT), haemoglobin (HGB), platelets (PLT) and
161 red blood cell count (RBC). The serum from rabbits was sent to a reference laboratory for haptoglobin
162 determinations by colorimetric assays (Phase Range; Tridelta Developments Ltd., Maynooth, Ireland)
163 according to the manufacturer's protocol.

164 Statistical analysis

165 Before the statistical analysis, variables were submitted to a preliminary analysis to detect outliers and
166 asymmetrical distributions. When asymmetrical distributions were found, the original data were

transformed using logarithmic transformation. The data from all the variables were analysed using linear mixed models (Proc MIXED; SAS, 2009). For the parameters from females' blood, the model included the generation of selection (2 levels; VR19, VR37), physiological state (5 levels; 1IA, 1P, 1W, 2P, 2W), and their interaction as fixed effects. As random effects, all the analyses included the permanent effect of female [80 levels ~ N (0; σ^2_p)] and error term [315 levels ~ N (0; σ^2_e)]. For the parameters from litters' blood, the model included the generation of selection (2 levels; VR19, VR37), the reproduction cycle (RC) of the females when kits were born (2 levels; 1st RC, 2nd RC), and their interaction as fixed effects. As random effects, all the analyses included the permanent effect of litters [58 levels ~ N (0; σ^2_l)] and error term [99 levels ~ N (0; σ^2_e)].

Experiment 2

Animals and experimental procedure

To evaluate the effect of selection on the immune response to immunological challenge, 73 other females from the two populations generated in Experiment 1 were used: VR19 (n=39) and VR37 (n=34). The experiment was conducted between July 4th, 2017 and April 17th, 2018 with a temperature range between 22 and 26°C and a relative humidity between 60 and 70%. Animals were housed under conventional environmental conditions with free access to water and commercial diet. Immunological challenge was carried out when females were 19 weeks old. To perform infections, animals were sedated with a combination of ketamine (Imalgene®, 100 mg/mL, Merial, Barcelona, Spain) and xylazine (Xilagesic, 200 mg/mL, Calier, Barcelona, Spain). A 10x10 cm area of the dorsal-lumbar region was shaved and disinfected with chlorhexidine. Later rabbits were intradermally-inoculated on their backs with 300 *S. aureus* colony-forming units (CFU) from two rabbit strains of

189 different virulences, namely Jwt (high virulence) and J*rot*$^{+}$ (low virulence) (Viana et al. 2015),
190 suspended in 0.1 mL of phosphate-buffered saline (PBS). Each rabbit was infected at four points (2
191 per strain). After inoculation, skin gross lesion characteristics (presence of erythema, nodules,
192 dermonecrosis and ulceration) were daily recorded for 7 days. This infection model was widely
193 described by Muñoz-Silvestre et al. (2020). Erythema and abscess dimensions were measured daily
194 with a calliper [length (L) and width (W)]. These values were used to calculate erythema area ($A = \pi \cdot \frac{L}{2} \cdot \frac{W}{2}$)
195 and nodule volume ($V = \frac{4\pi}{3} \cdot \left(\frac{L}{2}\right)^2 \cdot \frac{W}{2}$) as suggested by Muñoz-Silvestre et al. (2020).

196 **Immunophenotypical characterisation of peripheral blood by flow cytometry and**
197 **haematology studies**

198 Two millilitres of blood were taken from the central ear in two 1 ml tubes using EDTA as an
199 anticoagulant (AQUISEL Tube EDTA K3) at 0, 1, 3 and 7 days post-inoculation (dpi). One millilitre
200 of blood was used to carry out the immunophenotypic characterisation by flow cytometry and the
201 other millilitre to conduct the haematology studies. Both analyses were run in the same way, as
202 explained in the same point of Experiment 1.

203 **Study of phagocytosis of polymorphonuclear leucocytes and macrophages measured**
204 **by flow cytometry**

205 The week before inoculation started (age, 19 weeks), blood samples were taken from the central ear
206 artery of each animal included in the study to purify polymorphonuclear leucocytes and monocytes
207 separately on different days. Next 16 ml of blood were taken from each rabbit to carry out this
208 experiment, of which 8 ml were used for the purification of polymorphonuclear leucocytes and 8 ml

for the purification of macrophages. Blood samples were left inside heparin tubes (BD Vacutainer® Heparin Tubes).

Purification of polymorphonuclear leucocytes (PMN) and macrophages: PMN and macrophage purification were carried out according to previously described protocols (Siemsen et al. 2007; Yamane and Leung 2016). and in the same way as Penadés et al. (2020) describe.

Phagocytosis of PMN: Fluorescent yellow-green latex beads in aqueous suspension (2.0 µm mean particle size; Sigma-Aldrich®) were used to measure phagocytosis. The inoculum of beads for cells was done by adding 10-fold more latex beads than cells. For each animal, three replicates and a negative control (without beads) were performed. Phagocytosis was left for 30 minutes at 37ºC and was stopped with ice. Cells were labelled with mAb CD11b-PE (Thermo Fisher®) following the manufacturer's technical specifications. Cells were fixed with paraformaldehyde (4%). Finally, cells were resuspended in 1 ml of DPBS and transferred to cytometer tubes. Phagocytosis was measured by an FC500 cytometer (Beckman Coulter). All the cells labelled with the CD11b-PE antibody were taken as the granulocyte population. Cells were considered phagocytosed if they showed FITC fluorescence of latex beads. Those that had not phagocytosed were the cells lacking this fluorescence.

Phagocytosis of macrophages: Fluorescent yellow-green latex beads in aqueous suspension (2.0 µm mean particle size; Sigma-Aldrich®) were used to measure phagocytosis. The animals with less than 5% confluence of cells in wells were discarded. Three replicates and a negative control (without beads) were performed for each animal. Phagocytosis was left for 30 minutes at 37ºC and was stopped with ice. Cells were collected from wells to be placed in cytometer tubes using Trypsin solution from porcine pancreas (1x) (Sigma-Aldrich®). Then cells were labelled with the mAb CD11b-PE (Thermo Fisher®) following the manufacturer's technical specifications. The phagocytosis data were obtained by measuring the phagocytosis of cells in an FC500 flow cytometer (Beckman Coulter). All the cells

labelled with the CD11b-PE antibody were taken as the macrophage population. The cells that had phagocytosed were those showing FITC fluorescence of latex beads. Those that had not phagocytosed were the cells lacking this fluorescence.

Statistical analysis

Immunological-blood parameters. Before the statistical analysis, variables were submitted to a preliminary analysis to detect outliers and asymmetrical distributions. When asymmetrical distributions were found, the original data were transformed by logarithmic transformation. The data from all the variables were analysed with linear mixed models (Proc MIXED; SAS, 2009), which included the generation of selection (2 levels; VR19, VR37), day post-inoculation (4 levels; 0, 1, ~~2, 3, 4~~3,7), and their interaction as fixed effects. As random effects, all the analyses included the permanent effect of females [73 levels ~ N (0; σ^2_p)] and error term [292 levels ~ N (0; σ^2_e)].

Parameters from macroscopic lesions. The six evaluated traits were analysed using two different models. Both models included the generation of selection effect (2 levels: VR19, VR37), *S. aureus* strain (2 levels: Jwt, J*rot*$^+$), day post-inoculation (7 levels: 1, 2, 3, 4, 5, 6, 7 dpi), and their interactions as fixed effects. If erythema, nodule, dermonecrosis and ulceration (dichotomic traits) were present, a generalised linear model was used (proc GENMOD, SAS) after considering that the response variable followed binomial distribution and by using logistic transformation [ln(μ / (1- μ))] as a link function. For erythema area and nodule volume, a linear mixed model was performed (proc MIXED, SAS) that included animals' permanent effects [73 levels; N ~ (0, σ_p)] and infection [292 levels; N ~ (0, σ_i)], with residuals [2044 levels; N ~ (0, σ_e)] as random effects. By assuming that measures of the same infection close in time were more correlated than far in time, residuals were considered to decreasingly correlate with an increasing lag.

Results

Experiment 1

The results of the immunological blood parameters of the rabbit females bred on commercial farms are reported in Table 2. Neither the generation of selection for ADG nor its interaction with physiological state had an effect on any parameter. On the contrary, the effect of physiological state was significant for almost any parameter. Most of the results shown below are presented as percentage increase or decrease, and these percentages are expressed in the original scale, never in the logarithmic scale. The total number of lymphocytes lowered at both weaning times in relation to 1IA and parturition counts (on av. -21%; $P<0.05$). A similar pattern was observed for the main lymphocyte subpopulations ($CD5^+$, $CD4^+$, $CD8^+$ and $CD25^+$), which showed a progressive decrease from the 1IA to 1W, recovery at 2P and a new decrease at 2W. The number of B-lymphocytes sharply dropped by 75% throughout the first reproduction cycle (-45% from 1IA to parturition, -55% from parturition to weaning; $P<0.05$). No variation was observed from this point onwards. The number of monocytes increased progressively from 1IA to 2W (+199%; $P<0.05$). Similarly, the number of granulocytes increased from 1IA to 2W (+95%; $P<0.05$), but with a reduction from parturition to weaning in the first cycle (-13%; $P<0.05$), which was not observed in the second. The $CD4^+/CD8^+$ ratio rose during gestation (+49%; $P<0.05$), with no significant differences found from that point onwards. The granulocytes to total lymphocytes ratio progressively increased from 1IA to 2W (+137%; $P<0.05$). The white blood cells count showed an overall 32% increase ($P<0.05$) from 1IA to 2W, but with a significant 16% reduction ($P<0.05$) from 1P to 1W. Red blood cells, haematocrit and haemoglobin followed a similar pattern during the experiment. In all three cases, a progressive overall decrease took place from 1IA to 2W (-12% for red blood cells, -14% for haematocrit, -17% for haemoglobin; $P<0.05$). On the contrary, an overall increase

277 in platelets occurred during the experiment (+10% from 1IA to 2W; $P<0.05$). No differences were
278 observed for serum haptoglobin content throughout the study.

279 The results of young rabbits' immunological-blood parameters, obtained at weaning when bred on
280 commercial farms, are reported in Table 3. The effect of generation of selection for ADG was
281 significant in four of the 10 evaluated parameters. The granulocytes from the VR37 animals were
282 41.25% ($P<0.05$) higher than the VR19 animals. The granulocytes to total lymphocytes ratio for the
283 VR37 animals was 55.28% ($P<0.05$) higher than for the VR19 animals. On the contrary, the values of
284 both haematocrit and haemoglobin were lower in the weaned VR37 rabbits vs. the VR19 ones (-5 and
285 -7%, respectively; $P<0.05$). Total lymphocytes, $CD5^+$, $CD4^+$, $CD8^+$ and monocyte counts in the weaned
286 rabbits were higher at 2W than at 1W (+17%, +43%, +46%, +51% and +100%, respectively; $P<0.05$).

287

288 ## Experiment 2

289 The results for the leucocyte populations and haematological parameters in the peripheral blood of the
290 19-week-old rabbit females after intradermal inoculation with *S. aureus* are reported in Table 4. The
291 female VR37 rabbits had lower counts for total lymphocytes, $CD5^+$, $CD4^+$, $CD8^+$, $CD25^+$, monocytes,
292 the $CD4^+/CD8^+$ ratio and platelets than those of VR19 (-14%, -21%, -25%, -15%, -33%, -18%, -11%
293 and -11%, respectively; $P<0.05$). Conversely, B-lymphocytes and red blood cells were higher in the
294 VR37 than in the VR19 animals (+43% and +4%, respectively; $P<0.05$). No significant effects for
295 genetic type were observed for the other traits.

296 The dpi effect was significant on several parameters (Table 4). Total lymphocytes, B-lymphocytes,
297 monocyte, granulocyte, the granulocytes and total lymphocytes ratio, white blood cells and platelets
298 generally presented increasing values during the experiment (+5%, +186%, +149%, +106%, +127%,

+57% and +12%, respectively; P<0.05). The evolution of the $CD25^+$ counts and the $CD4^+/CD8^+$ ratio diverged slightly from this pattern and varied depending on the group (Fig. 1). The $CD25^+$ counts were almost flat in the VR19 females, with no differences over time, but significantly lowered on 3 dpi (on av. -40%; P<0.05) compared to 1 and 7 dpi in the VR37 females (Fig. 1A). On the contrary, the $CD4^+/CD8^+$ ratio was almost flat in the VR37 females but lowered at between 0 and 3 dpi (-18%; P<0.05) and subsequently recovered at 7 dpi (+11%; P<0.05) in the VR19 females (Fig. 1B).

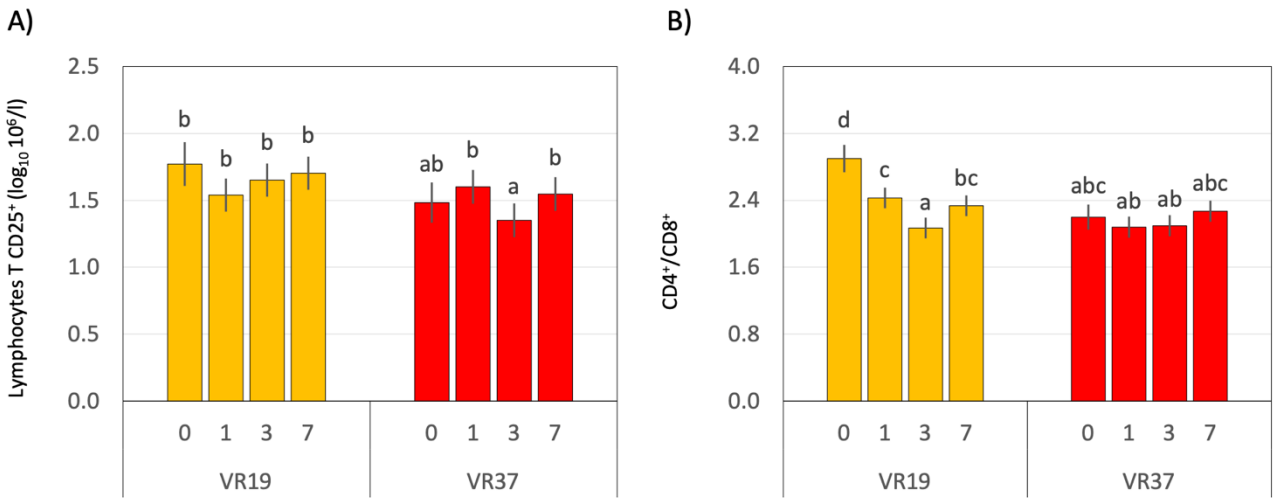

Fig. 1 Effect of generation of selection for growth rate depending on day post-inoculation on T-lymphocytes $CD25^+$ (A) and the $CD4^+/CD8^+$ ratio (B).

LS-means and standard errors. [a-f] The means that do not share a letter significantly differed ($P<0.05$). VR19: vitrified R line in the 19th generation, VR37: vitrified R line in the 37th generation.

The results of the macroscopic lesions of the 19-week-old rabbit females after intradermal inoculation with two *S. aureus* strains are reported in Table 5 (see the P-values for the effects reported in this table in the Supplementary Material; Table S1). ~~The more selected a~~Animals from the modern generarion (VR37) presented less erythema (-8.4 percentage points; P<0.05) and fewer nodules (-6.5

percentage points; P<0.05) than the ~~less selected~~ animals from the former generation (VR19). The lesions from the inoculations with Jwt more frequently presented bigger erythema (+12 percentage points and +0.74 cm^2, respectively; P<0.05) than with J*rot*$^+$. However, the differences between strains for erythema presence varied with generation of selection (Fig. 2). No differences appeared between infections with both strains in ~~the less selected~~ animals from the former generation (VR19). Conversely, erythema presence was 23 percentage points lower in the lesions from the inoculations with J*rot*$^+$ than for the inoculations with Jwt in ~~the more selected~~ animals from the modern generation (VR37). After Jwt infection, bigger nodules appeared more frequently (+51 percentage points and +1.27 cm^2, respectively; P<0.05), and dermonecrosis and ulceration were also more frequent (+6.3 and +3.3 percentage points, respectively; P<0.05) than after J*rot*+ inoculation. Further details about the effect of strain and its evolution over time on erythema area, nodule volume and presence of dermonecrosis or ulceration are reported in the Supplementary Material (Tables S1A, S1B, S2A and S2B, respectively).

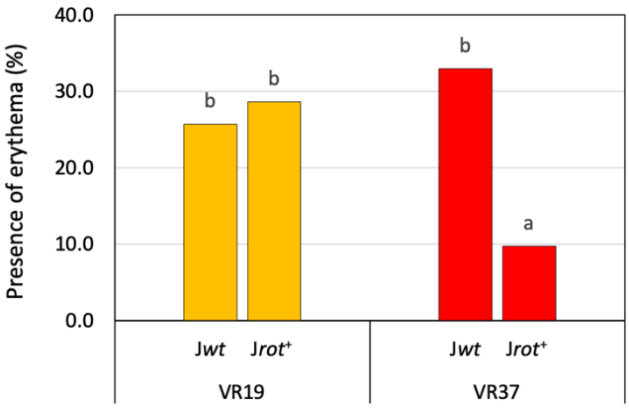

Fig. 2 Effect of generation of selection for growth rate and strain depending on day post-inoculation on erythema area and nodule volume.

LS-means and standard errors. [a-j] The means that do not share a letter significantly differed ($P<0.05$). VR19: vitrified R line in the 19th generation, VR37: vitrified R line in the 37th generation. Strain (Jwt, J*rot*$^{+}$).

The dpi effect was significant for all the parameters recorded to evaluate macroscopic lesions. Erythema presence peaked at 1 dpi. It was present in nearly three of the four inoculations. Afterwards, its presence progressively diminished and it had almost disappeared by the end of the experiment (-74 percentage points; P<0.05). Similarly, erythema area decreased throughout the experiment (-0.66 cm^2; P<0.05), but the maximum size was observed on 2 dpi. Both the presence and volume of nodules increased during the experiment. Nodule presence increased between 1 and 4 dpi (+24 percentage points; P<0.05), with no differences from this point onwards. Nodule volume grew between 1 and 6 dpi (+1.35 cm^3; P<0.05), with no significant differences between the last two days. However, erythema area and nodule volume evolved differently depending on the generation of selection for ADG (Fig. 3). As previously indicated, erythema area evolved for the lesions from VR19, but did not reduce between 1 and 5 dpi for the lesions from VR37 (Fig. 3A). There were no differences among generations for nodules at 1 dpi, but the nodules from the VR37 females were not as large as those from VR19 (-0.65 cm^3 at 7 dpi with Jwt; $P<0.05$; Fig. 3B). Finally, dermonecrosis and ulceration were barely present in lesions on the first 3 dpi (<0.1 percentage points), but the presence of both progressively increased between 3 and 7 dpi (+8.1 percentage points for dermonecrosis; +7.5 percentage points for ulceration; P<0.05).

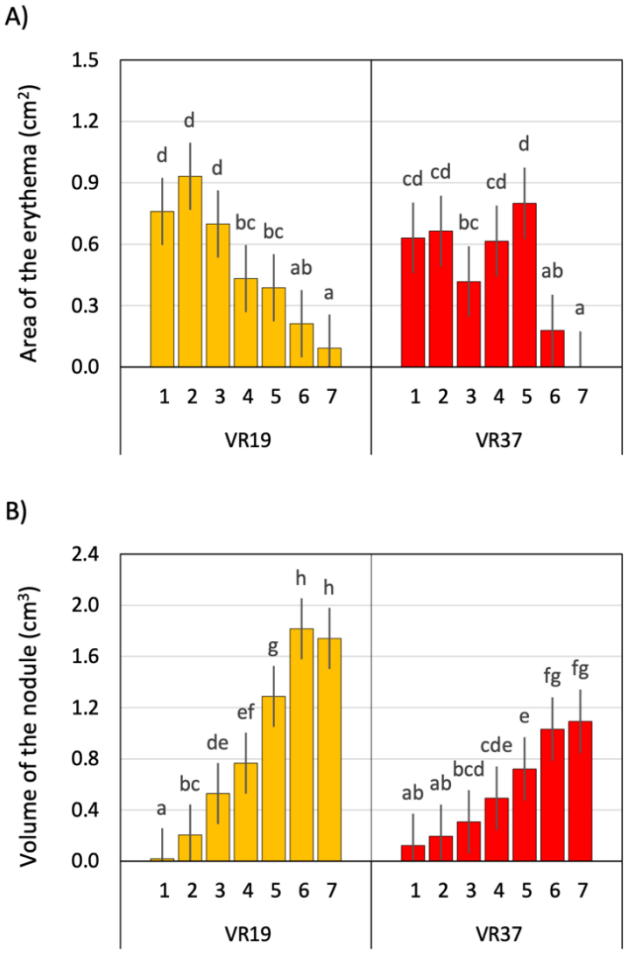

Fig. 3 Effect of generation of selection for growth rate and strain on erythema presence.

LS-means. [a, b] The means that do not share a letter significantly differed ($P<0.05$). VR19: vitrified R line in the 19th generation, VR37: vitrified R line in the 37th generation. Strain (Jwt, J*rot*$^{+}$).

Discussion

In many animal species, including rabbit, the levels of different leucocyte subpopulations and other haematological parameters indicate animals' health status and if their immune system is competent or not (Jeklova et al. 2007; Guerrero et al. 2011). Regarding leucocyte subpopulations, smaller white

359 blood cell counts do not always indicate good immune system conditions, but very high counts
360 suggest pathological problems. So this scenario is worth discussing to evaluate the obtained results.
361 On the one hand, a low white blood cell count indicates that an animal's immune system has not been
362 activated due to an infectious or other agent type or, if it has been activated, a slight reaction of the
363 immune system has sufficed to avoid infection. On the other hand, a simultaneous low leucocyte
364 count may indicate immunosuppression by some animal intrinsic or extrinsic factors, which may
365 make the immune system unable to effectively face an infectious challenge and results in disease. By
366 way of example, it has been described before parturition that physiological immunosuppression
367 occurs in some species, which is a critical time for females. However, the present study found the
368 highest total lymphocyte counts at parturition compared to subsequent weaning. In a previous study
369 carried out by (Penadés et al. 2018), no notable differences were noted in the leucocyte count at 1W
370 and 2P in this paternal line. Therefore, in this study we consider that low leucocyte counts are due to
371 healthy animal status because there are no obvious reasons for it being due to immunosuppression,
372 and high counts are due to infection or disease and, therefore, imply females' worse health status. To
373 support this, collecting macroscopic data from the lesions during infection in Experiment 2 is useful
374 for interpreting them together with the data obtained by the haematological counter and the
375 immunophenotype evaluated by flow cytometry.

376 **Experiment 1**

377 During the rabbit production cycle, two of the most decisive points for animal physiology and health
378 for both females and litters are the parturition and weaning of kits. Firstly, the time of 1P is a crucial
379 point for females, and their health status will affect both females and litters. Secondly, rabbit status
380 at weaning age is decisive for their future health during the growing period (Rashwan and Marai
381 2000; Bivolarski and Vachkova 2014). The first mating is the best time to evaluate the rabbit immune

system in relation to their genetics because it can be later affected by the environment, reproduction path, infections, among other factors (Pascual et al. 2013) and is, therefore, an important point in the rabbit cycle on farms. For this reason, 1IA, 1P and 1W in the first female cycle were herein sampled.

No significant differences were found in this study for any of studied parameters between the two genetic types of rabbit females bred on commercial farms separated by 18 generations of selection for growth rate. Another study has compared different blood leucocyte populations of rabbit females from three rabbit lines selected by different criteria. Parental line R showed lower counts for B-lymphocytes, CD5+ T-lymphocytes, CD4+ T-lymphocytes, CD8+ T-lymphocytes, but higher CD25+ T-lymphocytes, monocytes, granulocytes and the granulocytes to lymphocytes ratio than a line characterised by good robustness and lower counts for CD4+ T-lymphocytes, but higher counts for granulocytes and the granulocytes to lymphocytes ratio than a line characterised by prolificacy (Penadés et al. 2018). From this immunological profile, the above-cited authors indicated that the R animal line seemed to present a more stressful immunological situation than the other selected genetic lines based on other criteria such as prolificacy or robustness. However, the fact that no differences were found in the controlled immunological traits between the females of the genetic types separated by 18 generations of selection suggests that genetic selection was not the cause.

With the weaned rabbits, the granulocytes counts and the granulocytes to lymphocytes ratio for the kits from the ~~more selected~~ modern-generation-females for growth rate were higher, which could be an indicator of the presence of stressors, diseases or infections (Davis et al. 2008). García-Quirós et al. (2014) observed that the weaned rabbits from this R paternal line had lower leucocytes counts, but a higher granulocytes to lymphocytes ratio, than the other lines selected by other criteria at the age of 28 days. This could explain why the rabbits of this paternal line were more sensitive to digestive disorders during the fattening period. However, García-Quirós et al. (2014) did not observe

any relation between rabbits' immune status at weaning and the risk of suffering digestive disorders. In our experiments, mortality during the growing period was very high in both groups due to an Epizootic Rabbit Enteropathy outbreak. Nevertheless, the results are not consistent enough to establish any relation linking the immune system, genetic selection and mortality of weaned rabbits. For these same animals, and as expected, Marín-García et al. (2023) observed that the kits born on the VR37 females showed better milk yields and feed intake during lactation, and, consequently, were heavier upon weaning, than those from the VR19 females. In humans, it is well-known that increased consumption and overweight reduce leucocyte counts and increase the neutrophil to lymphocyte ratio (Kim and Park 2008; Wang et al. 2011). Richardson et al. (2002) observed in cattle a positive correlation between the genetic merit for residual feed intake and the granulocyte to lymphocyte ratio. All these results could indicate that the changes observed in the leucocyte counts in weaned rabbits due to the selection for growth rate could be related more to animal physiology (more consumption and heavier weight of ~~the more selected~~ animals from the modern generation) than to the selection criteria itself.

A previous study carried out by our research group to compare different rabbit generations belonging to line V, and selected for litter size upon weaning, showed that selection for reproductive parameters can affect blood lymphocyte populations (Ferrian et al. 2012). Therefore, ~~it would seem that selection for productive parameters can affect breeding females' immunological status, but this would not be the case in the specific case of genetic selection for ADG~~it seems that genetic selection for productive parameters may affect the immune system, but it depend on the selection criteria.

Interestingly, the values for some of the parameters reported in the present experiment slightly differed from those reported by Penadés et al. (2018) for R females (lymphocytes B: 1.31 vs. 0.51

430 $\log_{10} 10^6$/L; G/L: 1.69 vs. 2.20, respectively). These differences could be associated with the followed
431 experimental procedure because either females nursed 2-3 kits more than in the present experiment,
432 which implies more reproduction effort by females (Elmaghraby et al. 2004) or the females in that
433 experiment did not come from a restored vitrified population, which can have long-term effects on
434 litters (Dulioust et al. 1995). However, a recent study suggests that vitrification would not strongly
435 affect leucocyte counts (Garcia-Dominguez et al. 2020), which rules out the second hypothesis. No
436 difference in the presence of haptoglobin in plasma was observed at any time. Haptoglobin is a plasma
437 protein that can increase during inflammatory processes, but can also rise or lower for other reasons,
438 such as pregnancy or intravascular haemolysis, which makes it quite non-specific. In other studies,
439 this protein increased by inducing an acute phase response in the immune system (Siegel and Honaker
440 2009). Perhaps this is why the study by Ferrian et al. (2013) observed an acute phase immune response
441 that induced, as in this work, the immune system to a basal state and, as there was no inflammatory
442 process, the haptoglobin parameter was not affected. Except for B-lymphocytes, the results obtained
443 for young rabbits were similar to those reported by García-Quirós et al. (2014) for R animals.
444 However, in the present experiment, litters were also compared at 1W and 2W. This comparison
445 found higher total lymphocyte, CD5 + T-lymphocyte, CD4 + T-lymphocyte, CD8 + T-lymphocyte
446 and monocytes counts at 2W than at 1W. Perhaps this was due to the female immune system being
447 more mature at 2W than at 1W when they are still not fully adults. This would affect the transmission
448 of immunity to young rabbits through the placenta before birth and to milk after birth.
449

450 **Experiment 2**

In this experiment, the macroscopic lesions from the inoculations with both *S. aureus* strains evolved similarly to those reported by Muñoz-Silvestre et al. (2020). Consequently, discussion focuses exclusively on the work objectives.

During rabbit production, breeding programmes may affect rabbits' capacity to face immune challenges (Ferrian et al. 2012). This has been previously reported for other species, which have evidenced that immunological capability may differ depending on animals' genetic origin (Rauw et al. 1998; Siegel and Honaker 2009). In Experiment 2, we induced acute infectious challenge on female rabbits' immune system by inoculating pathogen *S. aureus.* The lesions developed by rabbits and their immune response were simultaneously evaluated. The analysis of the flow cytometry data obtained during females' infection at 1IA (19 weeks old) revealed that the peripheral blood of the VR19 females presented significantly higher counts for total lymphocytes, T-lymphocytes, CD25 + lymphocytes, CD4 + and CD8 + than those of the VR37 females. However, the B-lymphocyte values were higher in the VR37 blood than in that of VR19. ~~The more selected a~~Animals from the modern generarion (VR37) showed less erythema and fewer nodules than ~~the less selected~~ animals from the former generation (VR19). These data indicate that the females from generation 19 developed a better inflammatory response based on the cellular counts and severer lesions observed than in the females from generation 37 when an acute inflammatory response was triggered. This is interesting because it has been traditionally assumed that T-cell-mediated immunity is better than humoral response to confer protection against staphylococcal infections (Armentrout et al. 2020). As mentioned earlier, previous studies (García-Quirós et al. 2014) show that the R line generally has lower white blood cell counts, but probably not a worse immune status, than other lines when the immune system is in a basal state (no immune response is triggered). However, when the immune system is challenged with acute infection (as in our Experiment 2), higher white blood cell counts are not always indicative of

a better immune response and might sometimes suggest the immune system's inability to cope with and overcome infection. In fact, the ability of superantigenic toxins secreted by *S. aureus* to activate T-cells has been reported (Dinges et al. 2000). We herein observed that the ~~more selected~~ rabbits from the modern generation for growth rate (VR37) developed fewer lesions and had lower leucocyte counts and were, therefore, better capable of facing infectious challenge than the ~~less selected~~ rabbits from the former generation (VR19). This reveals that if the immune system faces an acute response to infection, it is not negatively affected by selection for ADG. In other words, our study suggests that the higher incidence of diseases in this genetic line vs. others selected by different criteria does not seem to result from the applied strategy while selecting these animals (García-Quirós et al., 2014). Indeed, the fact that the ~~more selected~~ females from the modern generation developed a lower lesion count when facing infectious challenge suggests that selection for ADG even favoured the immune system and its ability to cope with this infection type. We hypothesise that this might be due to an indirect selection in the breeding programme because only the animals that have reached adulthood were selected for the next generation, which may improve their ability to consequently cope with immunological challenges. Moreover, sick animals are not selected for health reasons and because their productivity are worse. This decrease in productivity is due to the fact that during illness, intake is reduced and energy expenditure is increased to activate the immune response (Rauw, 2012). This results in a poorer utilization of resources and lower efficiency. Consequently, health of the animal is prioritised over the selection criteria.

In conclusion, genetic selection for ADG does not seem to affect reproductive rabbit females' ability to maintain a competent immune system under conventional conditions. ~~With~~ On the other hand their weaned kits~~, for which this~~ selection criterion seems to increase the granulocyte to lymphocyte ratio~~, it might be related more to the greater consumption and weight of the more selected animals than to the selection criteria itself~~. Consequently, ~~I~~it seems clear, however, that selection for ADG does not

negatively influence young rabbit females' ability to establish an immune response. In fact, the obtained data indicate that this breeding programme favours the immune system's ability to undergo infectious challenge with *S. aureus*.

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References

Armentrout E, Liu G, Martins G (2020) T Cell Immunity and the Quest for Protective Vaccines against Staphylococcus aureus Infection. Microorganisms 8(12):1936. https://doi.org/10.3390/microorganisms8121936

Baselga M (2002) Line R. In: Khali MH, Baselga M (ed) Rabbit genetic resources in Mediterranean countries. Options Méditerranéennes : Série B. Etudes et Recherches; n. 38, Zaragoza, Spain, pp257-262.

Bivolarski BL, Vachkova EG (2014) Morphological and functional events associated to weaning in rabbits. J Anim Physiol Anim Nutr 98(1):9-18. https://doi.org/10.1111/jpn.12058

Corpa JM, Hermans K, Haesebrouck F (2010) Main pathologies associated with Staphylococcus aureus infections in rabbits: a review. World rabbit Sci 17(3):115-25. https://doi.org/10.4995/wrs.2009.651

Davis AK, Maney DL, Maerz JC (2008) The use of leukocyte profiles to measure stress in vertebrates: a review for ecologists. Funct Ecol 22(5):760-72.

518 Davis WC, Hamilton MJ (2008) Use of flow cytometry to develop and characterize a set of
519 monoclonal antibodies specific for rabbit leukocyte differentiation molecules. J Vet Sci
520 9(1):51. https://doi.org/10.4142/jvs.2008.9.1.51

521 Dinges MM, Orwin PM, Schlievert PM (2000) Exotoxins of Staphylococcus aureus. Clin Microbiol
522 Rev 13(1):16-34. https://doi.org/10.1128/cmr.13.1.16

523 Dulioust E, Toyama K, Busnel MC, Moutier R, Carlier M, Marchaland C, et al (1995) Long-term
524 effects of embryo freezing in mice. Proc Natl Acad Sci USA 92(2):589-93.
525 https://doi.org/10.1073/pnas.92.2.589

526 El Maghraby MMA, Helal MA and El Sheikh AI (2004) Maximum number of kits a rabbit doe
527 should nurse for optimum litter performance up to weaning. In: El Sheikh, Ahmed (ed) The
528 4th Scientific Conference for Vet. Medical Research, Fac. Vet. Med., Alex. Univ. October 2-
529 4, pp 658-672.

530 Estany J, Camacho J, Baselga M, Blasco A (1992) Selection response of growth rate in rabbits for
531 meat production. Genet Sel Evol 24(6):527.

532 Ferrian S, Guerrero I, Blas E, García-Diego FJ, Viana D, Pascual JJ, et al (2012) How selection for
533 reproduction or foundation for longevity could have affected blood lymphocyte populations of
534 rabbit does under conventional and heat stress conditions. Vet Immunol Immunopathol 150(1-
535 2):53-60.

536 Ferrian S, Blas E, Larsen T, Sánchez JP, Friggens NC, Corpa JM, et al (2013) Comparison of
537 immune response to lipopolysaccharide of rabbit does selected for litter size at weaning or

538 founded for reproductive longevity. Res Vet Sci 94(3):518-25.
539 https://doi.org/10.1016/j.rvsc.2013.01.008

540 Garcia-Dominguez X, Vicente JS, Marco-Jiménez F (2020) Developmental Plasticity in Response
541 to Embryo Cryopreservation: The Importance of the Vitrification Device in Rabbits. Animals
542 (Basel) 10(5):804. https://doi.org/10.3390/ani10050804

543 García-Quirós A, Arnau-Bonachera A, Penadés M, Cervera C, Martínez-Paredes E, Ródenas L, et
544 al (2014) A robust rabbit line increases leucocyte counts at weaning and reduces mortality by
545 digestive disorder during fattening. Vet Immunol Immunopathol 161(3-4):123-31.
546 https://doi.org/10.1016/j.vetimm.2014.07.005

547 Guerrero I, Ferrian S, Blas E, Pascual JJ, Cano JL, Corpa JM (2011) Evolution of the peripheral
548 blood lymphocyte populations in multiparous rabbit does with two reproductive management
549 rhythms. Vet Immunol Immunopathol 140(1-2):75-81.
550 https://doi.org/10.1016/j.vetimm.2010.11.017

551 Guerrero I, Ferrian S, Penadés M, García-Quirós A, Pascual JJ, Selva L, et al (2015) Host responses
552 associated with chronic staphylococcal mastitis in rabbits. The Veterinary Journal 204(3):338-
553 44. https://doi.org/10.1016/j.tvjl.2015.03.020

554 Hill WG (2008) Estimation, effectiveness and opportunities of long term genetic improvement in
555 animals and maize. Lohmann information 43(1):3-20.

556 Ingvartsen KL, Moyes K (2013) Nutrition, immune function and health of dairy cattle. Animal
557 7:112-22. https://doi.org/10.1017/s175173111200170x

558 Jacobsen CN, Aasted B, Broe MK, Petersen JL (1993) Reactivities of 20 anti-human monoclonal
559 antibodies with leucocytes from ten different animal species . Vet Immunol Immunopathol
560 39(4):461-6. https://doi.org/10.1016/0165-2427(93)90075-f

561 Jeklova E, Leva L, Faldyna M (2007) Lymphoid organ development in rabbits: Major lymphocyte
562 subsets. Dev Comp Immunol 31(6):632-44. https://doi.org/10.1016/j.dci.2006.10.002

563 Jeklova E, Leva L, Knotigova P, Faldyna M (2009) Age-related changes in selected haematology
564 parameters in rabbits. Res Vet Sci 86(3):525-8. https://doi.org/10.1016/j.rvsc.2008.10.007

565 Khalil MH, Al-Saef AM (2008) Methods, criteria, techniques and genetic responses for rabbit
566 selection: a review. Proceedings of the 9th World Rabbit Congress, June 10-13, 2008, Verona,
567 Italy, pp 1-22.

568 Kolmodin R, Strandberg E, Jorjani H, Danell B (2003) Selection in the presence of a genotype by
569 environment interaction: response in environmental sensitivity. Anim Sci 76(3):375-85.

570 Kotani M, Yamamura Y, Tamatani T, Kitamura F, Miyasaka M (1993a) Generation and
571 characterization of monoclomal antibodies against rabbit CD4, CD5 and CD11a antigens. J
572 Immunol Methods 157(1-2):241-52. https://doi.org/10.1016/0022-1759(93)90093-m

573 Kotani M, Yamamura Y, Tsudo M, Tamatani T, Kitamura F, Miyasaka M (1993b) Generation of
574 Monoclonal Antibodies to the Rabbit Interleukin-2 Receptor a Chain (CD25) and Its
575 Distribution in HTLV-1-transformed Rabbit T Cells. Jpn J Cancer Res 84(7):770-5.
576 https://doi.org/10.1111/j.1349-7006.1993.tb02042.x

577 Lochmiller RL, Deerenberg C (2000) Trade-offs in evolutionary immunology: just what is the cost
578 of immunity? .Oikos 88(1):87-98.

579 Madsen SA, Weber PSD, Burton JL (2002) Altered expression of cellular genes in neutrophils of
580 periparturient dairy cows. Vet Immunol Immunopathol 86(3-4):159-75.
581 https://doi.org/10.1016/s0165-2427(02)00020-x

582 Marín-García PJ, Martínez-Paredes E, Ródenas L, Larsen T, Cambra-López M, Blas E, et al (2023)
583 Changes in resource allocation strategy resulting from selection for growth rate after weaning
584 affect survival and reproductive performance of rabbit females. World Rabbit Sci Submitted.

585 Muñoz-Silvestre A, Penadés M, Selva L, Pérez-Fuentes S, Moreno-Grua E, García-Quirós A, et al
586 (2020) Pathogenesis of Intradermal Staphylococcal Infections. The American Journal of
587 Pathology 190(6):1188-210. https://doi.org/10.1016/j.ajpath.2020.01.019

588 Pascual JJ, Savietto D, Cervera C, Baselga M (2013) Resources allocation in reproductive rabbit
589 does: a review of feeding and genetic strategies for suitable performance. World Rabbit Sci
590 21(3):123-44. https://doi.org/10.4995/wrs.2013.1236

591 Penadés M, Arnau-Bonachera A, García-Quirós A, Viana D, Selva L, Corpa JM, et al (2018) Long-
592 term implications of feed energy source in different genetic types of reproductive rabbit
593 females. II. Immunologic status. Animal 12(9):1877-85.
594 https://doi.org/10.1017/s1751731117003299

595 Penadés M, Viana D, García-Quirós A, Muñoz-Silvestre A, Moreno-Grua E, Pérez-Fuentes S, et al
596 (2020) Differences in virulence between the two more prevalent Staphylococcus aureus clonal
597 complexes in rabbitries (CC121 and CC96) using an experimental model of mammary gland
598 infection. Vet Res 51(1):11. https://doi.org/10.1186/s13567-020-0740-1

599 Piles M, Blasco A (2010) Response to selection for growth rate in rabbits estimated by using a
600 control cryopreserved population. World rabbit Sci 11(2):53-62.

601 Rashwan AA, Marai IFM (2000) Mortality in young rabbits: a review. World rabbit Sci 8(3).
602 https://doi.org/10.4995/wrs.2000.427

603 Rauw WM, Kanis E, Noordhuizen-Stassen EN, Grommers FJ (1998) Undesirable side effects of
604 selection for high production efficiency in farm animals: a review. Livest Prod Sci 56(1):15-
605 33.

606 Rauw WM (2012) Immune response from a resource allocation perspective. Front Gene 3:267.
607 https://doi.org/10.3389/fgene.2012.00267

608 Richardson EC, Herd RM, Colditz IG, Archer JA, Arthur PF (2002) Blood cell profiles of steer
609 progeny from parents selected for and against residual feed intake. Aust J Exp Agric
610 42(7):901.

611 Rosell JM, de la Fuente LF (2009) Culling and mortality in breeding rabbits. Prev Vet Med
612 88(2):120-7. https://doi.org/10.1016/j.prevetmed.2008.08.003

613 Siegel PB, Honaker CF (2009) Impact of genetic selection for growth and immunity on resource
614 allocations ,. J Appl Poult Res 18(1):125-30.

615 Siemsen DW, Schepetkin IA, Kirpotina LN, Lei B, Quinn MT (2007) Neutrophil Isolation From
616 Nonhuman Species. In: Quinn MT, DeLeo FR, Bokoch GM (ed) Neutrophil Methods and
617 Protocols. Totowa, NJ: Humana Press, pp 21-34.

618 Sordillo LM, Contreras GA, Aitken SL (2009) Metabolic factors affecting the inflammatory
619 response of periparturient dairy cows. Anim Health Res Rev 10(1):53-63.
620 https://doi.org/10.1017/s1466252309990016

621 Veerkamp RF, Windig JJ, Claus MPL, Ouweltjes W, de Haas Y, Beerda B (2009) Selection for
622 high production in dairy cattle. In: W M Rauw (ed) Resource allocation theory applied to
623 farm animal production. CABI Publishing, Wallingford, UK, pp 243-60.

624 Viana Martín D, Selva L, Penadés M, Corpa JM (2015) Screening of virulence genes in
625 Staphylococcus aureus isolates from rabbits. World Rabbit Sci 23(3):185.
626 https://doi.org/10.4995/wrs.2015.3961

627 Wang R, Chen P, Chen W (2011) Diet and Exercise Improve Neutrophil to Lymphocyte Ratio in
628 Overweight Adolescents. Int J Sports Med 32(12):1-5. https://doi.org/10.1055/s-0031-
629 1283185

630 Wells MY, Decobecq CPM, Decouvelaere DM, Justice C, Guittin P (1999) Changes in Clinical
631 Pathology Parameters During Gestation in the New Zealand White Rabbit. Toxicol Pathol
632 27(3):370-9. https://doi.org/10.1177/019262339902700315

633 Wideman RF, Rhoads DD, Erf GF, Anthony NB (2013) Pulmonary arterial hypertension (ascites
634 syndrome) in broilers: A review. Poult Sci 92(1):64-83. https://doi.org/10.3382/ps.2012-
635 02745

636 Yamane K, Leung K (2016) Rabbit M1 and M2 macrophages can be induced by human
637 recombinant GM - CSF and M- CSF. FEBS Open Bio 6(9):945-53.
638 https://doi.org/10.1002/2211-5463.12101

639 Kim JA, Park HS (2008) White blood cell count and abdominal fat distribution in female obese
640 adolescents. Metabol 57(10):1375-9. https://doi.org/10.1016/j.metabol.2008.05.005

641 Segura P, Martinez J, Peris B, Selva L, Viana D, Penades JR, et al (2007) Staphylococcal infections
642 in rabbit does on two industrial farms. Veterinary Record 160(25):869-72.
643 https://doi.org/10.1136/vr.160.25.869

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647 **List of abbreviations**

648 Average daily gain (ADG)
649 Colony-forming units (CFU)
650 Days post-infection (dpi)
651 Dulbecco's Phosphate-Buffered Saline suspension (Sigma-Aldrich®) (DPBS)
652 First artificial insemination (1IA)
653 First parturition (1P)
654 First weaning (1W)
655 Haematocrit (HCT)
656 Haemoglobin (HGB)
657 Length (L)
658 Monoclonal antibodies (mAbs)
659 Phosphate-buffered saline (PBS)

660 Platelets (PLT)

661 Polymorphonuclear leucocytes (PMN)

662 Red blood cell count (RBC)

663 Reproductive cycle (RC)

664 Second parturition (2P)

665 Second weaning (2W)

666 Volume (V)

667 White blood cells (WBC)

668 Width (W)

669

670 Statements & Declarations

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676 Competing Interests

677 The authors declare that they have no competing interests

678 Authors' contributions

JMC, JJP, DV and LS designed the experiment and got the funding. EMP and PJMC raised the animals prepare them for the experiments and got the blood samples. EMG and SPF obtained cytometry data and evaluated infections. AAB performed the statistical analysis, coordinated the approach of the paper and elaborated the first draft of the work. All authors read all the drafts, contributed to the submitted version and approved the final manuscript.

Ethics approval

The experimental protocols were approved by the Animal Welfare Ethics Committee of the Universitat Politècnica de València (authorisation code: 2018/VSC/PEA/0116) and the Ethical Committee of the Universidad CEU Cardenal Herrera, and by the Conselleria d'Agricultura, Pesca i Alimentació, Generalitat Valenciana (permit numbers 2011/010 and 2017/VSC/PEA/00192; approval date: 20 January 2011). All the animals were handled according to the principles of animal care published by Spanish Royal Decree 1201/2005 (BOE, 2005; BOE = Official Spanish State Gazette).

Consent for publication

Not applicable

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704

Figures

Fig. 1 Effect of generation of selection for growth rate depending on day post-inoculation on T-lymphocytes $CD25^+$ (A) and the $CD4^+/CD8^+$ ratio (B).

LS-means and standard errors. [a-f] The means that do not share a letter significantly differed ($P<0.05$). VR19: vitrified R line in the 19th generation, VR37: vitrified R line in the 37th generation.

Fig. 2 Effect of generation of selection for growth rate and strain depending on day post-inoculation on erythema area and nodule volume.

LS-means and standard errors. [a-j] The means that do not share a letter significantly differed ($P<0.05$). VR19: vitrified R line in the 19th generation, VR37: vitrified R line in the 37th generation. Strain (Jwt, J*rot*$^+$).

Fig. 3 Effect of generation of selection for growth rate and strain on erythema presence.

LS-means. [a, b] The means that do not share a letter significantly differed ($P<0.05$). VR19: vitrified R line in the 19th generation, VR37: vitrified R line in the 37th generation. Strain (Jwt, J*rot*$^+$).

718 # Tables

Table 2. Effect of generation of selection for growth rate and physiological state on the immunological-blood parameters of the rabbit females bred on commercial farms.

	Generation (G)[1]			State (S)[2]						Contrasts[3]		*P-values*		
	VR19	**VR37**	**SEM**	**1AI**	**1P**	**1W**	**2P**	**2W**	**SEM**	**RC**	**WP**	**G**	**S**	**G x S**
Leucocyte counts (log_{10} 10^6/L):														
Total lymphocytes (L)	3.391	3.371	0.019	3.441^{b}	3.395^{b}	3.309^{a}	3.434^{b}	3.328^{a}	0.021	0.029	-0.096^{***}	0.453	<0.001	0.139
B-lymphocytes	0.406	0.508	0.070	0.847^{c}	0.586^{b}	0.238^{a}	0.289^{a}	0.324^{a}	0.074	-0.106	-0.157^{**}	0.309	<0.001	0.434
T-lymphocytes $CD5^+$	3.069	3.046	0.020	3.191^{c}	3.062^{b}	2.971^{a}	3.096^{b}	2.966^{a}	0.026	0.014	-0.11^{***}	0.426	<0.001	0.432
$CD4^+$	2.827	2.815	0.020	2.894^{d}	2.829^{bc}	2.742^{a}	2.872^{cd}	2.768^{b}	0.025	0.034	-0.096^{***}	0.681	<0.001	0.483
$CD8^+$	2.352	2.291	0.033	2.519^{c}	2.280^{ab}	2.234^{a}	2.342^{b}	2.232^{a}	0.035	0.03	-0.077^{*}	0.190	<0.001	0.927
$CD25^+$	1.298	1.221	0.059	1.277^{ab}	1.198^{a}	1.169^{a}	1.369^{b}	1.285^{ab}	0.068	0.144^{*}	-0.057	0.359	0.166	0.266
Monocytes	2.487	2.500	0.044	2.228^{a}	2.501^{b}	2.465^{b}	2.570^{bc}	2.704^{c}	0.063	0.153^{*}	0.049	0.837	<0.001	0.889
Granulocytes (G)	3.621	3.626	0.017	3.445^{a}	3.658^{c}	3.598^{b}	3.684^{cd}	3.734^{d}	0.020	0.081^{***}	-0.005	0.822	<0.001	0.912
$CD4^+/CD8^{+4}$	3.310	4.019	0.280	2.814^{a}	4.181^{b}	3.806^{b}	3.830^{b}	3.692^{b}	0.316	-0.233	-0.257	0.078	0.004	0.228
G/L[4]	2.073	2.200	0.138	1.213^{a}	2.187^{b}	2.156^{b}	2.249^{b}	2.878^{c}	0.162	0.393^{*}	0.299^{*}	0.516	<0.001	0.158
White blood cells (log_{10} 10^9/L)	0.969	0.959	0.010	0.900^{a}	0.993^{bc}	0.915^{a}	1.019^{c}	0.994^{c}	0.011	0.052^{***}	-0.052^{***}	0.465	<0.001	0.797
Red blood cells (10^{12}/L)	5.230	5.312	0.059	5.740^{c}	5.241^{b}	5.334^{b}	4.983^{a}	5.055^{a}	0.070	-0.269^{***}	0.083	0.328	<0.001	0.521
Haematocrit (%)	34.61	34.37	0.373	37.91^{d}	34.46^{b}	35.82^{c}	31.55^{a}	32.70^{a}	0.446	-3.015^{***}	1.26^{***}	0.646	<0.001	0.099
Haemoglobin (g/L)	11.41	11.27	0.149	12.42^{c}	11.50^{b}	11.79^{b}	10.36^{a}	10.61^{a}	0.175	-1.156^{***}	0.271	0.504	<0.001	0.298
Platelets (10^9/L)	293.1	282.6	9.233	278.4^{a}	280.9^{a}	286.7^{ab}	285.6^{ab}	307.5^{b}	9.999	12.745	13.825	0.420	0.158	0.956
Haptoglobin (log_{10} 10^9/L)	-0.443	-0.467	0.025	-0.491	-0.471	-0.456	-0.386	-0.471	0.044	0.035	-0.034	0.507	0.507	0.719

[a,b,c,d] The means in a row within an effect not sharing a superscript were significantly different (P<0.05); SEM: Pooled standard error of means;[1] VR19: vitrified R line in the 19th generation, VR37: vitrified R line in the 37th generation. [2] 1AI: first artificial insemination, 1P: first parturition, 1W: first weaning, 2P: second parturition, 2W: second weaning. [3] RC, reproduction cycle 1 vs. reproductive cycle 2: [(2P+2W)/2-(1P+1W)/2]; WP, weaning vs. parturition: [(1W+2W)/2-(1P+2P)/2]; *P<0.05; **P<0.01; ***P<0.001. [4] Ratios were directly obtained from the counts with no logarithmic transformation.

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Table 3. Effect of generation of selection for growth rate and reproduction cycle on the immunological-blood parameters of young rabbits at weaning when bred on commercial farms.

	Generation (G)[1]			Reproduction cycle (RC)[2]			*P-values*		
	VR19	**VR37**	**SEM**	**1W**	**2W**	**SEM**	**G**	**RC**	**G x RC**
Leucocyte counts (log_{10} 10^6/L):									
Total lymphocytes (L)	3.306	3.281	0.023	3.259[a]	3.328[b]	0.022	0.451	0.026	0.167
B-lymphocytes	0.141	0.186	0.124	0.230	0.097	0.105	0.796	0.259	0.456
T-lymphocytes $CD5^+$	2.992	2.995	0.026	2.916[a]	3.071[b]	0.024	0.950	0.001	0.686
$CD4^+$	2.795	2.753	0.024	2.692[a]	2.857[b]	0.022	0.227	0.001	0.571
$CD8^+$	2.462	2.470	0.033	2.376[a]	2.555[b]	0.031	0.863	0.001	0.388
$CD25^+$	1.243	1.129	0.068	1.219	1.153	0.065	0.242	0.443	0.876
Monocytes	2.466	2.332	0.067	2.249[a]	2.549[b]	0.065	0.167	0.002	0.067
Granulocytes (G)	3.189[a]	3.339[b]	0.036	3.269	3.259	0.036	0.005	0.834	0.742
$CD4^+/CD8^{+3}$	2.105	1.911	0.072	2.085	1.930	0.069	0.063	0.097	0.384
G/L[3]	0.890[a]	1.382[b]	0.101	1.250	1.022	0.101	0.001	0.117	0.390
White blood cells (log_{10} 10^9/L)	0.760	0.809	0.024	0.772	0.797	0.022	0.155	0.358	0.344
Red blood cells (10^{12}/L)	4.326	4.389	0.067	4.311	4.404	0.062	0.511	0.243	0.940
Haematocrit (%)	30.89[b]	29.35[a]	0.52	29.95	30.29	0.46	0.041	0.549	0.723
Haemoglobin (g/l)	9.485[b]	8.853[a]	0.197	9.106	9.231	0.177	0.027	0.568	0.693
Platelets (10^9/L)	407.0	405.8	20.5	401.6	411.2	17.3	0.966	0.613	0.856

[a,b] The means in a row within an effect not sharing a superscript were significantly different (P<0.05); SEM: Pooled standard error of means ; [1] VR19: vitrified R line in the 19th generation, VR37: vitrified R line in the 37th generation. [2] Reproduction cycle of young rabbits' mother; 1W: first weaning, 2W: second weaning. [3] Ratios were directly obtained from the counts with no logarithmic transformation.

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Table 4. Effect of generation of selection for growth rate and post-inoculation time on leucocyte populations and haematological parameters in the peripheral blood of 19-week-old rabbit females after intradermal inoculation with *S. aureus*.

	Generation (G)[1]			Days post-inoculation (dpi)					*P-values*		
	VR19	**VR37**	**SEM**	**0**	**1**	**3**	**7**	**SEM**	**G**	**tdpi**	**G x dpi**
Leucocyte counts (log_{10} 10^6/L):											
Total lymphocytes (L)	3.572^{b}	3.504^{a}	0.026	3.550^{ab}	3.485^{a}	3.547^{b}	3.572^{b}	0.028	0.013	0.001	0.688
B-lymphocytes	1.245^{a}	1.401^{b}	0.085	1.057^{a}	1.369^{b}	1.353^{b}	1.513^{c}	0.093	0.032	0.001	0.938
T-lymphocytes $CD5^{+}$	3.302^{b}	3.197^{a}	0.028	3.295	3.219	3.235	3.247	0.031	0.000	0.219	0.266
$CD4^{+}$	3.113^{b}	2.990^{a}	0.028	3.109^{b}	3.018^{a}	3.029^{a}	3.050^{ab}	0.031	0.001	0.089	0.435
$CD8^{+}$	2.745^{b}	2.672^{a}	0.033	2.724^{ab}	2.678^{a}	2.724^{b}	2.708^{ab}	0.036	0.032	0.237	0.135
$CD25^{+}$	1.666^{b}	1.495^{a}	0.112	1.627	1.570	1.501	1.625	0.123	0.002	0.201	0.017
Monocytes	2.567^{b}	2.480^{a}	0.057	2.377^{a}	2.386^{a}	2.558^{b}	2.773^{c}	0.061	0.035	0.001	0.227
Granulocytes (G)	3.611	3.618	0.052	3.443^{a}	3.644^{b}	3.616^{b}	3.756^{c}	0.055	0.832	0.001	0.202
$CD4^{+}/CD8^{+2}$	2.433^{b}	2.161^{a}	0.091	2.549^{c}	2.254^{b}	2.083^{a}	2.302^{bc}	0.099	0.025	0.003	0.013
G/L[2]	1.382	1.688	0.151	0.863^{a}	1.855^{c}	1.464^{b}	1.958^{c}	0.169	0.063	0.001	0.205
White blood cells (log_{10} 10^9/L)	1.020	0.990	0.022	0.909^{a}	0.997^{b}	1.008^{b}	1.104^{c}	0.023	0.148	0.001	0.613
Red blood cells (10^{12}/L)	5.573^{a}	5.813^{b}	0.124	5.82^{ab}	5.775^{b}	5.559^{a}	5.620^{ab}	0.141	0.011	0.065	0.148
Haematocrit (%)	38.37	39.16	0.849	39.61^{b}	39.16^{b}	37.88^{a}	38.42^{ab}	0.957	0.122	0.085	0.326
Haemoglobin (g/l)	120.4	123.0	2.888	123.9^{b}	123.2^{b}	118.6^{a}	120.9^{ab}	3.252	0.144	0.082	0.135
Platelets (10^9/L)	322.9^{b}	287.9^{a}	9.621	292.7^{ab}	289.7^{a}	311.7^{b}	327.7^{c}	10.35	0.001	0.001	0.969
Phagocytosis (%)											
Macrophages	42.35	39.97	8.694						0.353		
Heterophils	47.15	46.43	4.821						0.732		

^{a,b,c,d} The means in a row within an effect not sharing a superscript were significantly different (P<0.05); * Significantly differed from 0 (P<0.05) .** (P<0.01). *** (P<0.001); SEM: Pooled standard error of means; [1] VR19: vitrified R line in the 19th generation, VR37: vitrified R line in the 37th generation. [2] Ratios were directly obtained from the counts with no logarithmic transformation.

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Table 5. Effect of generation of selection for growth rate, strain and post-inoculation time on the parameters from the macroscopic lesions of the 19-week-old rabbit females after intradermal inoculation with two *S. aureus* strains.

	Generation[1]			Strain			Days post-inoculation							
	VR19	**VR37**	**SEM**	**Jwt**	**J*rot*$^+$**	**SEM**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**SEM**
Erythema														
Presence (%)	27.1^{b}	18.7^{a}	-	29.2^{b}	17.2^{a}	-	77.3^{g}	51^{f}	32.9^{e}	22.3^{d}	12.3^{c}	6.4^{b}	3.8^{a}	-
Area (cm^2)	0.502	0.469	0.138	0.853^{b}	0.118^{a}	0.137	0.695^{e}	0.798^{f}	0.558^{cd}	0.524^{c}	0.594^{d}	0.195^{b}	0.033^{a}	0.141
Nodule														
Presence (%)	29.2^{b}	22.7^{a}	-	59.0^{b}	7.8^{a}	-	8.8^{a}	20.4^{b}	29.7^{c}	33.1^{d}	32.5^{cd}	33.2^{d}	33.2^{d}	-
Volume (cm^3)	0.909	0.566	0.226	1.372^{b}	0.103^{a}	0.220	0.071^{a}	0.200^{b}	0.418^{c}	0.629^{d}	1.004^{e}	1.424^{f}	1.417^{f}	0.217
Presence of dermonecrosis (%)	2.7	2.5	-	7.2^{b}	0.9^{a}	-	0.8^{a}	0.8^{a}	1.2^{a}	2.6^{b}	4.9^{c}	6.9^{cd}	9.3^{d}	-
Presence of ulceration (%)	2	1.9	-	4.2^{b}	0.9^{a}	-	0.8^{a}	0.8^{a}	0.8^{a}	1.3^{a}	2.9^{b}	5.5^{c}	8.7^{d}	-

$^{a\text{-}g}$ The means in a row within an effect not sharing a superscript were significantly different (P<0.05). SEM: Pooled standard error of means. [1] VR19: vitrified R line in the 19th generation, VR37: vitrified R line in the 37th generation.

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Additional files

Additional file 1 Table S1

Format: Word

Title: *P-values* of the effects for each evaluated trait from macroscopic lesions

Description: In this table*, P-Values* for the effects for each evaluated trait from macroscopic lesions are presented. The effect of group was significant for the presence of erythema and nodule. The effect of strain was significant for all the traits. The effect of DPI was significant for the presence of erythema. Interaction between group and strain was significant in all the traits. Interaction between strain and DPI was significant for the area of the erythema and volume of nodule. Interaction between group and DPI was significant for all the traits except for presence of erythema and nodule

Additional file 2 Fig. S1

Format: TIFF

Title: Effect of strain (Jwt, J*rot*$^{+}$) and day post-inoculation (1-7) on erythema area and nodule volume. LS-means and standard errors.

Description: The area of the erythema and the volume of the nodule was lower in infections coming from inoculations with J*rtot*$^{+}$ than those observed in infections coming from inoculations with JWT. These results denoted a lower virulence of J*rot*$^{+}$

747 **Additional file 3 Fig. S2**

748 Format: TIFF

749 Title: Effect of strain (Jwt, J*rot*$^{+}$) and day post-inoculation (1-7) on the presence of dermonecrosis
750 and ulceration.

751 Description: The presence of the erythema and the nodule was lower in infections coming from
752 inoculations with J*rtot*$^{+}$ than those observed in infections coming from inoculations with JWT. These
753 results denoted a lower virulence of J*rot*$^{+}$