

Repeat-dose and local tolerance toxicity of SARS-CoV-2 FINLAY-FR-02 vaccine candidate in Sprague Dawley rats

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ABSTRACT

This study evaluates safety of FINLAY-FR-02, a vaccine candidate against SARS-CoV-2 based on the recombinant receptor binding domain conjugated to tetanus toxoid, in a preclinical, repeat-dose toxicity and local tolerance study. Sprague Dawley rats were randomly allocated to three experimental groups: control (receiving physiological saline solution); placebo (receiving all vaccine components except antigens) and vaccine group (receiving three doses of the vaccine candidate, 37.5 µg of RBD) administered intramuscularly in hind limbs at 24 h intervals during three days. We evaluated physiological condition, pain, food and water consumption, body temperature, dermal irritability, injection site temperature and inflammation, immunological response, blood chemistry, relative organ weight, histopathology and immunotoxicology. The product was well tolerated; no clinically relevant changes, pain, local effects or adverse systemic toxicological changes or deaths were observed. These preliminary results permitted the Cuban regulatory authorities to authorize clinical trials in humans.

1. Introduction

Preclinical studies including preliminary safety evaluation in animal models are mandatory, highly regulated, and time-consuming. Based on a vast experience in conjugate vaccines for over 20 years, the Finlay Vaccine Institute (Instituto Finlay de Vacunas, IFV) in Havana, Cuba, has developed three vaccine candidates against SARS-CoV-2. One is FINLAY-FR-02 whose active ingredient is the recombinant receptor binding domain monomer (mRBD) conjugated to tetanus toxoid. In preliminary preclinical studies, FINLAY-FR-02 elicited strong

neutralizing anti-RBD antibodies (Valdés-Balbín et al., 2021), indicating the product potential as a vaccine candidate. Here we evaluate the safety profile of FINLAY-FR-02 in a repeat-dose toxicity and local tolerance study in Sprague Dawley rats; this versatile and well-characterized model in vaccine toxicology studies (Forster, 2012) has been used in preclinical studies on COVID-19 vaccines (Kandeil et al., 2021; Brian et al., 2020; Wang et al., 2020).

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Table 1
Vaccination design and schedule.

Group	Animals n	Administration hours	Dose/Vol./Route	Animals euthanized (n) at x days after 3 rd dose		
				x	x	x
Finlay-FR-02*	15	0, 24, 72	RBD 12.5 µg + TT 11.5 µg + Al(OH) ₃ 250 µg/0.25 mL/i.m	5	5	5
Placebo	15	0, 24, 72	Al(OH) ₃ 250 µg/0.25 mL + AS/i.m	5	5	5
Control	15	0, 24, 72	PSS 0.9%/0.2 mL/i.m	5	5	5

Legend:: n- Number; PSS- Physiological saline solution; AS- Auxiliary substances: Na CL, NaHPO₄, Na₂HPO₄, Thiomersal and injection water; i.m: Intramuscularly; * - Human dose RBD 25 µg + 20 µg TT + 500 µg Al(OH)₃ in 0.5 mL.

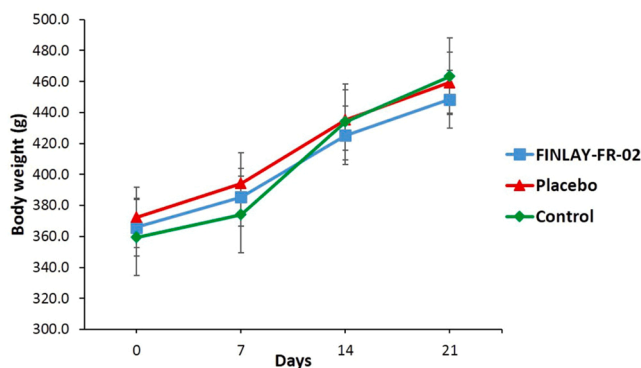


Fig. 1. Time course of Body weight. Values are the average \pm SEM of animals in each group. Kruskal-Wallis test $p \geq 0.05$.

2. Materials and methods

2.1. Product under evaluation

FINLAY-FR-02 is suspension of the active pharmaceutical ingredient, the SARS-CoV-2 RBD protein monomer (sequence: 319–541 residues with a poly-histidine fusion tag at its C-terminus), expressed in CHO cells. Purified RBD is chemically conjugated to the carrier protein tetanus toxoid (TT) and adsorbed on alumina (Valdés-Balbín et al., 2021) (Table 1). The vaccine was manufactured according to Good Manufacturing Practice (GMP) by the IFV and the Centre for Molecular Immunology (CIM) in Havana, Cuba.

Table 2

Average food and water consumption per animal.

Groups	Variables*		Week 2		Week 3	
	Water (mL)	Food (g)	Water (mL)	Food (g)	Water (mL)	Food (g)
FINLAY-FR-02	31.4 \pm 4.9	29.4 \pm 12.8	34.0 \pm 1.3	27.9 \pm 2.2	35.0 \pm 1.8	28.2 \pm 5.1
Placebo	31.5 \pm 3.5	27.7 \pm 10.3	33.3 \pm 1.1	29.1 \pm 0.4	37.6 \pm 4.2	30.2 \pm 5.2
Control	30.8 \pm 4.5	24.2 \pm 3.3	31.4 \pm 3.1	29.0 \pm 0.5	31.2 \pm 3.5	29.7 \pm 5.5
General Average	31.3 \pm 3.1	26.6 \pm 8.2	32.9 \pm 2.3	28.7 \pm 1.4	34.6 \pm 4.2	29.3 \pm 5.1
	$p = 0.6043$	$p = 0.2732$	$p = 0.0232$	$p = 0.8496$	$p = 0.0032$	$p = 0.4771$

Legend: * - Mean \pm SD and p values between groups, data on food and water consumption were evaluated daily for seven days during the first week, then on alternate days, and were grouped by weeks to facilitate statistical analysis. Kruskal-Wallis test.

2.2. Animals and husbandry

Sprague Dawley (SD) males 8–9 old weeks (from the National Center for the Production of Laboratory Animals, CENPALAB, Mayabeque, Cuba) were housed at the IFV Animal Care Facility in Tecniplast® rat cages (2 or 3 animals per cage) at $21 \pm 2^\circ\text{C}$ and a relative humidity of $55 \pm 5\%$ under 12 h light and dark alternating cycles. Food for rodents and water were available ad libitum. Rats were allowed to acclimatize for one week before beginning the experimental protocol. All protocols were approved by the Institutional Committee for Animal Care and Use of IFV (Code: P-05/20).

2.3. Experimental design

The study followed the recommendations and guidelines issued by WHO, FDA and ICH for vaccine evaluation (World Health Organization WHO, 2005; World Health Organization WHO, 2013; International Committee of Harmonization ICH, 1992; International Committee of Harmonization ICH, 1997; International Committee of Harmonization ICH, 1997; Food & Drug Administration FDA, 1997). A total of 45 SD male rats were randomly allocated to three experimental groups: control (receiving physiological saline solution 0.9%, PSS); placebo (receiving the vaccine excipients including alumina) and vaccine group (receiving three doses of the vaccine candidate, FINLAY-FR-02) (composition is detailed in Table 1). Doses were administered intramuscularly in hind limbs at 24 h intervals during three days. Animals received 12.5 µg of the vaccine antigen in a volume of 0.25 mL (representing 50% of human dose), divided in two sites (both legs); the injection volume corresponds to the maximum allowable for this administration route and host species (Verdier, 2002; Diehl et al., 2001).

2.4. Clinical signs, pain and body weigh

Animals were monitored after the first injection, event every 12 h for seven days and then daily until the end of the experiment. The administration site was closely examined, and animals were observed looking for the following signs: limp, piloerection, prostration, involuntary movements, shaking of the head, ataxia, salivation, difficult breathing, tearing, hyperactivity or lethargy, incoordination, diarrhea or any other sign. Pain was measured using the grimace scale (Sotocinal et al., 2011), every 12 h for 72 h after each inoculation. Animals were weighed just before the inoculation, and subsequently on days 7, 14 and 21 post-inoculation and just before euthanasia. Signs, weights and pain data were recorded.

2.5. Water and food consumption

These parameters were evaluated on day 0, daily for one week, and then every two days until the end of the experiment. Mean daily water consumption per animal was calculated dividing the consumed water (initial minus final volumes) by the number of animals in the box. Daily, 500 g of ALYco® rat feed was provided per box. Mean daily food

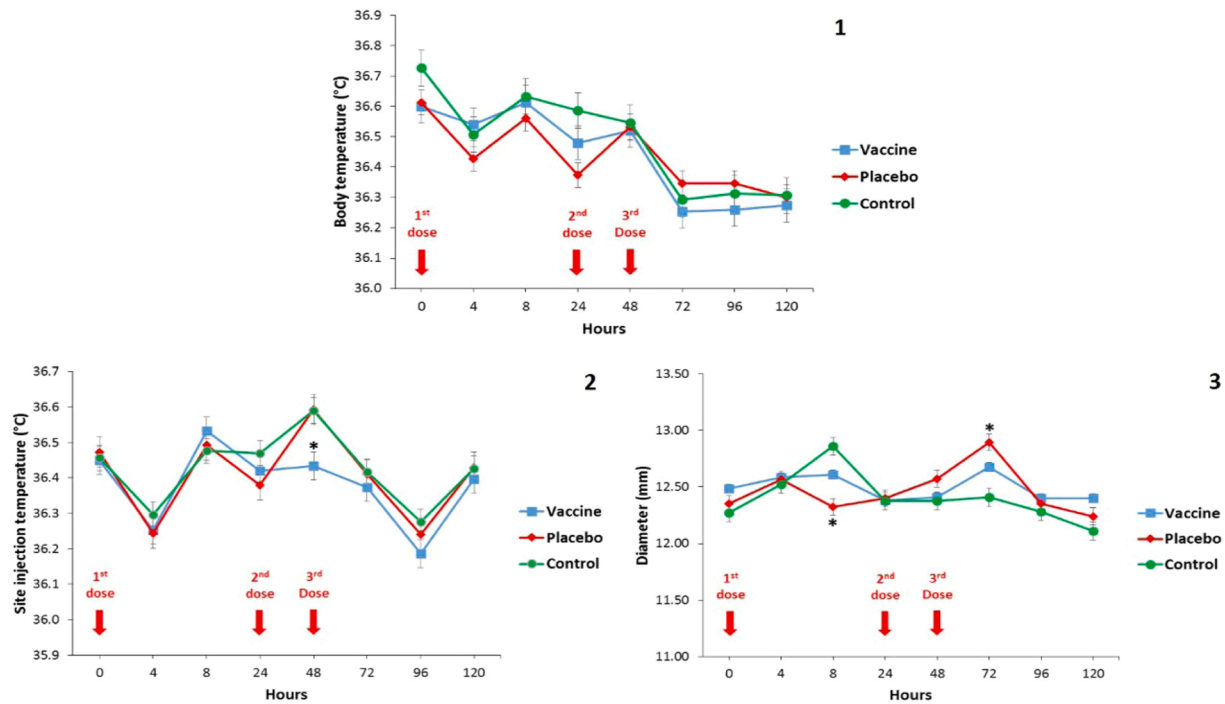


Fig. 2. Time course of 1- Body temperature, 2- Site injection temperature and 3- Muscle diameter of male SD rats. Values are the average \pm SEM of animals in each group. Kruskal-Wallis test * - $p \leq 0.05$.

Table 3
Blood chemistry and hemoglobin analysis of rats vaccinated with FINLAY-FR-02 to COVID-19.

Groups	a)						
	Glucose (mmol/L)	Cholesterol (mmol/L)	Triglycerides (mmol/L)	TP (g/dL)	Urates (μ mol/L)	Alb (g/L)	Creatinine (μ mol/L)
3 days after 3rd doses							
FINLAY-FR-02	9.46 \pm 0.81	1.62 \pm 0.08	1.42 \pm 0.42	5.88 \pm 0.30	56.46 \pm 10.00	36.66 \pm 1.39	55.82 \pm 10.10
Placebo	10.16 \pm 1.47	1.68 \pm 0.29	1.24 \pm 0.32	5.71 \pm 0.17	60.61 \pm 20.93	37.02 \pm 1.03	58.55 \pm 15.67
Control	10.78 \pm 0.32	1.93 \pm 0.23	1.49 \pm 0.45	5.83 \pm 0.32	61.02 \pm 17.95	37.88 \pm 2.12	57.18 \pm 15.67
7 days after 3rd doses							
FINLAY-FR-02	6.91 \pm 0.78	1.50 \pm 0.21	0.96 \pm 0.65	5.81 \pm 0.28	56.30 \pm 20.5	35.36 \pm 1.27	21.72 \pm 17.44
Placebo	7.44 \pm 0.62	1.40 \pm 0.19	1.14 \pm 0.27	5.74 \pm 0.24	48.52 \pm 11.94	35.42 \pm 1.01	22.53 \pm 10.87
Control	7.83 \pm 1.15	1.52 \pm 0.10	0.92 \pm 0.19	5.91 \pm 0.33	47.60 \pm 16.53	35.68 \pm 1.52	18.50 \pm 8.34
21 days after 3rd doses							
FINLAY-FR-02	8.39 \pm 1.58	1.42 \pm 0.07	0.69 \pm 0.14	6.08 \pm 0.31	50.26 \pm 16.24	38.67 \pm 1.98	48.68 \pm 11.99
Placebo	8.79 \pm 1.05	1.37 \pm 0.38	0.77 \pm 0.31	6.01 \pm 0.21	56.37 \pm 24.07	38.62 \pm 1.12	40.93 \pm 7.42
Control	8.65 \pm 1.06	1.51 \pm 0.18	0.70 \pm 0.39	5.94 \pm 0.43	52.14 \pm 26.33	39.18 \pm 1.66	43.14 \pm 16.78
Groups	b)						
	AST (U/L)	CPK (U/L)	LDH (U/L)	ALP (U/L)	ALT (U/L)	Urea (mmol/L)	HB
3 days after 3rd doses							
FINLAY-FR-02	43.53 \pm 10.66	272.66 \pm 101.03	183.21 \pm 41.44	333.48 \pm 66.76	26.07 \pm 5.10	6.61 \pm 0.57	12.62 \pm 0.76
Placebo	56.34 \pm 14.94	327.68 \pm 51.94	204.55 \pm 16.15	338.43 \pm 23.77	25.26 \pm 4.59	7.32 \pm 1.17	11.99 \pm 1.06
Control	52.85 \pm 15.54	273.21 \pm 61.19	182.55 \pm 25.20	300.12 \pm 65.63	30.26 \pm 8.57	6.99 \pm 1.66	12.02 \pm 0.30
7 days after 3rd doses							
FINLAY-FR-02	55.99 \pm 5.57	472.14 \pm 186.35	–	252.63 \pm 82.51	23.05 \pm 4.97	8.79 \pm 1.43	13.54 \pm 1.13
Placebo	59.01 \pm 9.85	637.95 \pm 66.95	–	259.05 \pm 88.37	20.25 \pm 3.98	7.46 \pm 0.83	12.91 \pm 0.48
Control	57.50 \pm 10.98	695.74 \pm 288.47	–	224.22 \pm 26.56	19.21 \pm 3.47	7.40 \pm 1.53	13.33 \pm 1.59
21 days after 3rd doses							
FINLAY-FR-02	60.18 \pm 8.69	506.95 \pm 255.05	107.37 \pm 5.40	192.68 \pm 23.17	28.98 \pm 6.83	8.05 \pm 0.35	12.30 \pm 0.54
Placebo	76.01 \pm 11.21	669.80 \pm 420.52	115.25 \pm 14.84	206.43 \pm 38.87	30.85 \pm 8.76	6.83 \pm 0.31	12.40 \pm 1.16
Control	72.75 \pm 16.57	611.36 \pm 361.37	112.62 \pm 10.42	224.77 \pm 44.36	32.36 \pm 7.57	7.35 \pm 1.25	11.80 \pm 1.16

Legend: Values represent the mean \pm SEM of the 5 animals in each group per time-point. No statistical differences were found between the groups for any parameter. (a) TP: total Protein, ALB: albumin, (b) AST: aspartate aminotransferase, CPK: creatine phosphokinase, LDH: lactate dehydrogenase, ALP: Alkaline phosphatase, ALT: alanine aminotransferase, HB: hemoglobin.

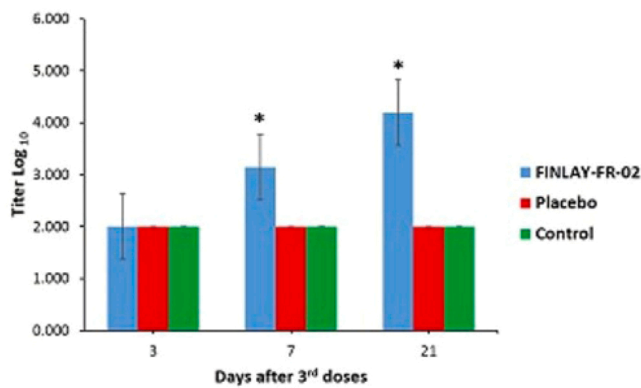


Fig. 3. anti-RBD IgG. Values are the average \pm SEM of animals in each group. Kruskal-Wallis test * - $p \leq 0.05$.

consumption per animal was measured similarly; the residual food was weighed daily.

2.6. Thermometry, temperature and muscular diameter at the injection site

These parameters were measured as reported (Oliva-Hernández et al., 2018; Oliva et al., 2019; Fraleigh et al., 2019), on day 0 (before and after injection), at 4, 8, 24, 48 and 72 h after the first dose and 72 h after last dose (in total, during 120 h). Body and injection site temperatures were measured with a laser clinical thermometer (Equate, non-contact forehead thermometer, model #10857, Mississauga, ON, Canada) on the thorax previously depilated and on the internal part of the legs. Muscle diameter was evaluated with a digital caliper (electronic caliper with digital display, 6', 150 mm, Mastercraft, Toronto, ON, Canada) by measuring the diameter of the inoculated limb at the center of the thigh as indicated by the fabricants.

2.7. Dermal irritability

Dermal irritability was evaluated following WHO and OECD recommendations (World Health Organization WHO, 2013; Organization for Economic Cooperation and Development, 2004) as previously described (Oliva-Hernández et al., 2019). Two hours after each injection, the presence of erythema, edema, eschar, and papules was evaluated (Draize et al., 1944). The dermal irritability index (DII) was calculated by adding all values determined during observations (0, 4, 8, 24, 48 and 72 h post-inoculation) and dividing them by the number of observations (6). DII was compared with that reported in the corresponding IFV standard operating procedures to classify the product as irritating or not irritating and thus, recommending its approval or rejection according to OECD standards.

2.8. Euthanasia, blood collection, blood chemistry and immunological evaluation

All animals were subjected to 4 h of fasting before euthanasia and blood collection. On days 3, 7, and 21 after the last vaccination, rats were euthanized (five animals per day per group, according to the study design) by an intravenous overdose of sodium thiopental (80 mg/kg of animal weight, AICA Laboratories, Havana, Cuba) and bled out, following the Canadian Council on Animal Care, the American Veterinary Medical Association guidelines (Committee for the Update of the Guide for the Care and Use of Laboratory Animals, 2011; American Veterinary Medical Association, 2013), and Morton's recommendations on the humanitarian endpoint (Morton, 1999). Hemoglobin and blood chemistry, including glucose, urea nitrogen, uric acid, creatinine, alkaline phosphatase, total protein, triglycerides, cholesterol, direct

bilirubin, creatine phosphokinase, alanine aminotransferase, and aspartate aminotransferase were analyzed using diagnostic kits (Helfa Diagnostics, CIE, Havana, Cuba) and a spectrophotometer (Genesys 10 S, China). One normal and one pathological control serum samples (Helfa Diagnostics) were analyzed simultaneously with every group of 10 samples following manufacturer's instructions.

2.8.1. Anti-RBD IgG ELISA

ELISA plates (96 wells, NUNC, Maxisorp) were coated with the dimeric RBD antigen (50 μ L per well, 3 μ g/mL in carbonate-bicarbonate buffer pH 9.6) for 1 h at 37°C. Then, plates were blocked in 5% BSA-PBS for 1 h at 37 °C. After five washes with PBS-0.5% Tween 20 (PBS-T), serial dilutions of serum samples (1:3, in 1% BSA-PBS, pH 7.2) were added starting from 1/50. Plates were incubated for 1 h at 37°C, and washed with PBS-T. Goat anti-rat IgG-HRP antibody (Sigma Aldrich) diluted 1/5000 in 1% BSA-PBS, pH 7.2 was added and plates were incubated for another hour at 37°C. Following five washes with PBS-T, plates were incubated for 20 min after adding 3',3',5',5'-tetramethylbenzidine (TMB) substrate. The reaction was stopped with 2 N H₂SO₄ and the absorbance measured at 450 nm in a microplate reader ELISA (Multiskan EX, ThermoScientific). The endpoint titer was defined as the highest reciprocal dilution of serum giving an absorbance > 4-fold the value for pre-immune serum diluted 1/50.

Hematology, blood chemistry, histopathology and organ weight were studied according to FDA and WHO indications (World Health Organization WHO, 2012; Food and Drug Administration FDA, 2020). New elements of immunotoxicity evaluation included systemic inflammation by measuring the total area of the spleen, popliteal and deep inguinal nodes, using ImageJ software ver. 1.43 on photos taken with a professional digital camera (Canon, EUA) (Batistia-Duharte et al., 2011; Tamargo et al., 2019).

2.9. Anatomopathological studies and organ weights

Euthanized animals were immediately subjected to gross necropsy, inspecting all the organs and the inoculation sites. Samples were taken from all the anatomic locations where alterations were detected. Tissue samples (4–6 μ m thickness) were taken using a microtome (Histolide 2000, Leica Biosystems) and fixed in 4% formaldehyde neutralized with calcium carbonate until embedding in paraffin. Tissue slices were stained with hematoxylin–eosin (QUIMEFA, Cuba) and observed using a conventional microscope (CH-2, Olympus, Tokyo, Japan). Weights of parenchymal organs (brain, heart, thymus, lung, spleen, liver, kidneys and adrenal glands) were expressed as relative organ weight (ROW), calculated by the following equation: ROW = (OW x 100)/EEW, where OW is the organ weight and EEW (euthanasia end weight) is the animal weight on the day of euthanasia.

2.10. Statistical analysis

Statistical analyses were performed using GraphPad Prism 5. Statistical differences were set for $p \leq 0.05$. Data were expressed as central tendency values with dispersion (means plus/less standard deviation, lower and upper values). Normality assumptions (Kolmogorov-Smirnov and Shapiro-Wilk tests) and homogeneity of variances (Levene test) were verified. When satisfied, a parametric analysis of variance (ANOVA) was applied. If they did not meet these criteria, the nonparametric alternative was used (Kruskal-Wallis test). Data resulting from the histopathological study were analyzed through the construction of the cross-classification tables, with the associated independence test (Fisher's exact test).

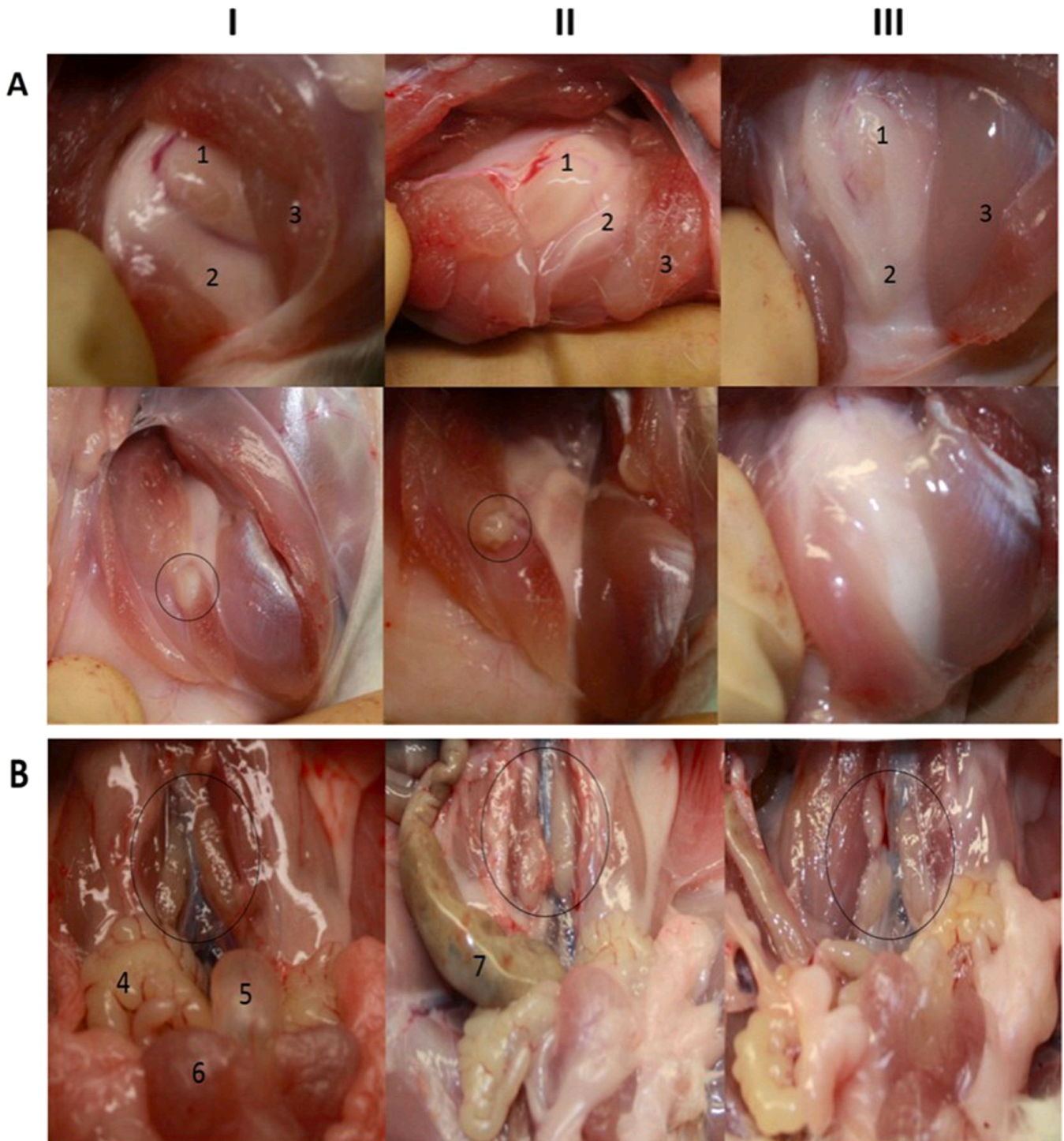


Fig. 4. Macroscopic findings related to the injection site. **I-** FINLAY-FR-02, **II-** Placebo, **III-** Control; **A-** Both legs of experimental groups, 1- Popliteal lymph nodes, 2- Fatty tissue, 3- Muscular tissue., Small black circles frame possible granulomatous formation diffuse of white-gray color; **B-** Abdominal cavity: black circles frame deep inguinal lymph nodes, 4- Seminal vesicles, 5- Urinary bladder, 6- Prostate, 7- Large intestine.

Table 4

Summary of macroscopic anatomopathological alterations related to the immune system and the site of injection in SD rats.

Euthanasia days*	Observed alterations	FINLAY-FR-02 Animals (n)/Frequency %	Placebo	Control
3	Possible macrophage granulomatous formation	5/80 ^a	5/80 ^a	5/0 ^b
	Popliteal lymph node adenitis.	5/40 ^a	5/100 ^b	40 ^a
	Deep inguinal lymph nodes adenitis.	5/80 ^a	5/60 ^a	5/20 ^b
7	Possible macrophage granulomatous formation	5/80 ^a	5/80 ^a	5/0 ^b
	Popliteal lymph node adenitis.	5/60 ^a	5/60 ^a	5/20 ^b
	Deep inguinal lymph nodes adenitis.	5/100 ^a	5/100 ^a	5/20 ^b
21	Possible macrophage granulomatous formation	5/80 ^a	5/100 ^a	5/0 ^b
	Popliteal lymph node adenitis.	5/100 ^a	5/100 ^a	5/0 ^b
	Deep inguinal lymph nodes adenitis.	5/80 ^a	5/80 ^a	5/10 ^b

Legend: * - days after 3rd dose, different letters stand for statistically significant differences (Fisher's exact test; $p \leq 0.05$).

3. Results

3.1. Clinical signs, pain and body weigh

No clinical symptoms and pain signs were observed among animals in the three experimental groups. All animals gained weight during the 21 days after first inoculation (Fig. 1), with no statistical significant differences among the three experimental groups. The growth curves were similar to other published curves for this animal model (Oliva-Hernández et al., 2018; Oliva et al., 2019; Oliva-Hernández et al., 2019). This is the first evidence of product safety.

3.2. Water and food consumption

The consumption of water and food behaved in a similar way in the three experimental groups and similar to other toxicological studies (Oliva-Hernández et al., 2018; Oliva et al., 2019); however, statistical differences in water consumption were observed in the second and third weeks after last injection. In the second week the average water consumption was lower in the control than in the vaccine group; in the third week, the placebo group had a higher water consumption than the vaccine group; both being higher than in the control group (Table 2). Nevertheless, the values for water consumption (and also for food consumption) are similar to the historical values recorded in our facilities for the species and were in all cases inside the normal range (Oliva-Hernández et al., 2018; Oliva et al., 2019; Oliva-Hernández et al., 2019; López et al., 2011; Oliva et al., 2020).

3.3. Thermometry, temperature and muscular diameter of injection site

During the evaluation period (120 h after the first injection), body temperatures were in the physiological range reported for rats (Lillie et al., 1996; Charles River, 2020) (Fig. 2.1).

The average temperature on injection sites on both hind limbs only presented differences at 48 h after the first injection (this is, 24 h after the second): animals in the Finlay-FR-02 group registered lower local temperatures compared to those in the placebo and the control groups (Fig. 2.2). These values were similar to those recorded for body temperature. Nevertheless, these changes in temperature were in all cases inside the normal range of body temperature for rats (Lillie et al., 1996; Charles River, 2020).

There were statistical differences in the limb diameters at the injection site between the control group and placebo 8 h post first injection, (FINLAY-FR-02 group did not differ from the placebo group.), The placebo and FINLAY-FR-02 groups, both had a larger mean diameter than the control group 72 h after the first injection (24 h post-third injection) (Fig. 2.3).

3.4. Dermal irritability

The dermal irritability test showed the absence of erythema, edema, eschar, or papules, thus the dermal irritation index for the three experimental groups was 0.0.

3.5. Hemoglobin, biochemical and immunological evaluations

No statistical differences were observed between control, placebo and the FINLAY-FR-02 groups for blood parameters (Table 3).

There were statistical differences in anti-RBD IgG titers in vaccinated animals; which were superior with respect to titers in placebo and control group animals at 7 and 21 days after the third dose (three days after the last dose there were no differences) (Fig. 3).

3.6. Anatomopathological studies and organ weights

Organ and systems macroscopic studies did not reveal modifications attributable to toxicity. At the injection site, we observed adenitis in the popliteal and deep inguinal ganglia in both the vaccinated animals and in those in the placebo group (Fig. 4 A and B).

In vaccinated animals and placebos, there were possible diffuse white-gray granulomatous formations in the first euthanasia group that became better defined and more delimited in the subsequent ones. (Fig. 4 A, Table 4). These possible diffuse white-gray granulomatous formations and lymph nodes were verified microscopically (Fig. 5); at the inoculation site they are granulomatous (Table 5).

In the vaccine and placebo groups we observed subcapsular and paracortical secondary follicles, as well as abundant plasma cells in the sinuses and hilum of the deep inguinal and popliteal lymph nodes (Fig. 5). Significant differences were found in both, compared to control group (Table 5). Spleens presented a discrete hyperplasia of the Malpighian corpuscles in the vaccinated and placebo groups that were not observed in the control animals (Fig. 5).

Concerning organs weight, there were significant differences only in lymphoid organs (popliteal and deep inguinal lymph nodes) where both FINLAY-FR-02 and placebo groups registered a higher of weight with the control group (Tables 6a 6b).

3.7. Immunotoxicological evaluation

The macroscopic morphometric analysis of spleen from rats of the vaccine group carried out at 3, 7 and 21 days after the last dose did not show significant differences in the total area of this organ (Fig. 6).

While the morphometric studies of the deep inguinal nodes showed significant differences, the vaccine group registered a higher average total area with respect to the placebo and control groups in the euthanasia on days 3 and 7 after the last dose; no significant differences were observed on day 21. No significant differences at any time were

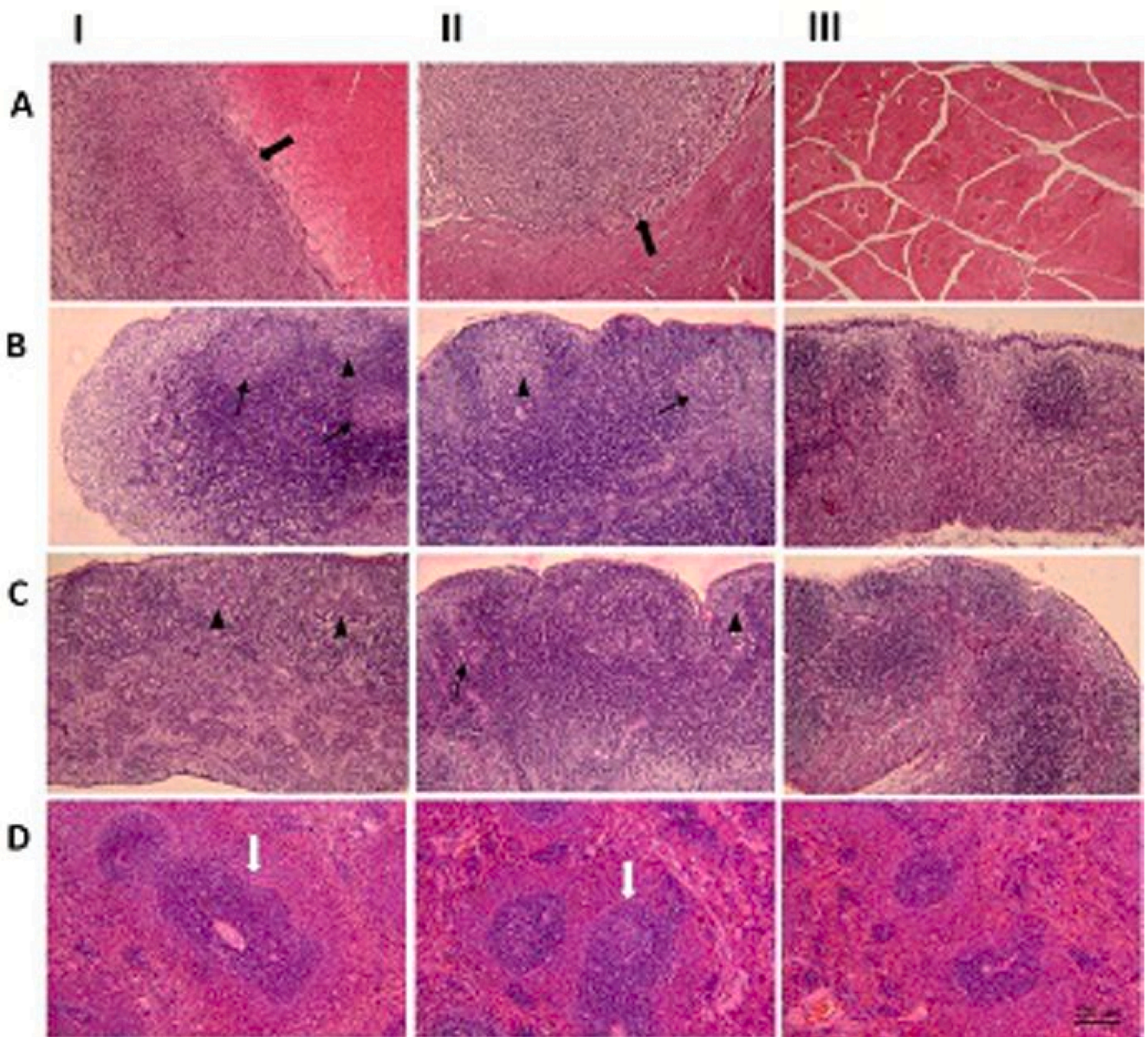


Fig. 5. Histopathological findings in organs related to the immune system. I- FINLAY-FR-02, II-Placebo, III- Control; A- Muscle, B- Popliteal lymph node, C- Deep inguinal lymph node, D- Spleen. I A, II A- Macrophage type granulomatous formation (thick black arrow), III A- Normal muscle, IB, IC, IIB, IIC - Subcapsular secondary lymphoid follicles (arrowhead) and paracortical (thin black arrow), IIIB, IIIC- Normal lymph node. ID, IID- Hyperplasia of the corpuscles of Malpighi. (White arrow), IIID- Normal spleen. HE: hematoxylin–eosin, rod: 200 μ m.

Table 5

Summary of histopathological alterations related to the immune system and the site of injection in SD rats.

Euthanasia days*	Observed alterations	Animals (n)/Frequency %		
		Finlay-FR-02	Placebo	Control
3	Macrophage granulomatous process	5/80 ^a	5/100 ^a	5/0 ^b
	Subcapsular secondary follicles in popliteal lymph nodes	5/100 ^a	5/100 ^a	5/40 ^b
	Paracortical secondary follicles in popliteal lymph nodes	5/80 ^a	5/100 ^a	5/0 ^b
	Subcapsular secondary follicles in deep inguinal lymph nodes	5/100 ^a	5/100 ^a	5/100 ^a
	Paracortical secondary follicles in deep inguinal lymph nodes	5/60 ^a	5/0 ^b	5/80 ^a
	Plasma and mast cells in sinus and hilum	5/80 ^a	5/100 ^a	5/80 ^a
7	Macrophage granulomatous process	5/80 ^a	5/100 ^a	5/0 ^b
	Subcapsular secondary follicles in popliteal lymph nodes	5/100 ^a	5/100 ^a	5/20 ^b
	Paracortical secondary follicles in popliteal lymph nodes	5/80 ^a	5/100 ^a	5/40 ^b
	Subcapsular secondary follicles in deep inguinal lymph nodes	5/100 ^a	5/100 ^a	5/0 ^b
	Paracortical secondary follicles in deep inguinal lymph nodes	5/100 ^a	5/80 ^a	5/40 ^b
	Plasma and mast cells in sinus and hilum	5/100 ^a	5/80 ^a	5/20 ^b
21	Macrophage granulomatous process	5/80 ^a	5/100 ^a	5/0 ^b
	Subcapsular secondary follicles in popliteal lymph node	5/100 ^a	5/60 ^b	5/60 ^b
	Paracortical secondary follicles in popliteal lymph nodes	5/100 ^a	5/60 ^b	5/20 ^c
	Subcapsular secondary follicles deep in inguinal lymph nodes	5/100 ^a	5/80 ^a	5/100 ^a
	Paracortical secondary follicles deep in inguinal lymph nodes	5/40 ^a	5/20 ^a	5/0 ^b
	Plasma and mast cells in sinus and hilum	5/100 ^a	5/60 ^b	5/60 ^b

Legend: *- days after 3rd dose, different letters stand for statistically significant differences (Fisher's exact test; p ≤ 0.05).

observed in the popliteal nodes among the groups (Fig. 7).

4. Discussion

The possible toxicity of a product can be assessed by integrally investigating clinical, physiological and pathological parameters to determine local and systemic issues associated to repeated administration.

FINLAY-FR-02 conjugated vaccine is developed on the platform in use for more than twenty years (eg: Quimi-Hib® and Heber Penta® vaccines) at IFV, using TT for conjugation. The adjuvant aluminum hydroxide is in use in several COVID-19 vaccines formulation (Liang et al., 2020; Danielsson and Eriksson, 2021).

In these toxicology studies, the FINLAY-FR-02 conjugate COVID-19 vaccine candidate demonstrated no overt signs of toxicity when assessing clinical conditions. Signs of toxicity after repeated administration would have resulted in behavioral differences and changes in body weight, food and water consumption (Wang et al., 2020; World

Health Organization WHO, 2005; World Health Organization WHO, 2013; Chakravarty and Herkenham, 2005), which were not seen during the trial. Not surprisingly, we saw transient significant increases in muscle diameter 8 and 72 h after injection with either the placebo (adjuvant) or FINLAY-FR-02 (vaccine). This local, transient increase may be associated to the adjuvant and is due to the recruitment of leukocytes at the site of injection, as reported for other injectable adjuvants (Oliva et al., 2019; Oliva-Hernández et al., 2019; Lu and Hogen Esch, 2013); it is not considered a safety concern issue. During all the evaluation period, animals did not develop fever; a discrete increase in temperature at the injection sites was observed in 48 h after the last dose, that was in the physiological range reported for rats (37.5 ± 0.5 °C) (Lillie et al., 1996; Charles River, 2020).

Immunotoxicity was integrally evaluated by hematology, blood chemistry, morphometric analysis of spleens and regional lymph nodes proximal to injection sites 3, 7, and 21 days after the last vaccination. The hematological and blood chemistry parameters in all groups showed no significant changes, indicating no acute or chronic systemic

Table 6a

Relative organ weights* (%).

Groups	Brain	Thymus	Heart	Left Lung	Right Lung	Liver	Spleen	Left Kidney	Right Kidney
3 days after 3rd doses									
FINLAY-FR-02	0.5473 ± 0.018	0.1427 ± 0.045	0.4223 ± 0.012	0.2002 ± 0.124	0.2647 ± 0.022	4.3407 ± 0.274	0.2082 ± 0.035	0.3108 ± 0.169	0.3903 ± 0.025
Placebo	0.5545 ± 0.036	0.1288 ± 0.026	0.3203 ± 0.103	0.3422 ± 0.125	0.2529 ± 0.022	4.0298 ± 0.122	0.1878 ± 0.014	0.3753 ± 0.041	0.3730 ± 0.029
Control	0.5543 ± 0.033	0.1470 ± 0.036	0.3318 ± 0.108	0.1676 ± 0.084	0.2419 ± 0.026	4.0253 ± 0.261	0.1961 ± 0.022	0.3749 ± 0.040	0.3787 ± 0.031
7 days after 3rd doses									
FINLAY-FR-02	0.5564 ± 0.035	0.1341 ± 0.025	0.3135 ± 0.091	0.2023 ± 0.101	0.2866 ± 0.065	3.0813 ± 0.323	0.2006 ± 0.027	0.3929 ± 0.025	0.3769 ± 0.023
Placebo	0.5156 ± 0.024	0.1389 ± 0.024	0.3020 ± 0.101	0.1624 ± 0.088	0.2356 ± 0.339	3.3174 ± 0.339	0.1784 ± 0.017	0.3889 ± 0.017	0.3785 ± 0.021
Control	0.5523 ± 0.017	0.1747 ± 0.042	0.3711 ± 0.043	0.1446 ± 0.010	0.2733 ± 0.011	3.2954 ± 0.469	0.2054 ± 0.013	0.3795 ± 0.030	0.3837 ± 0.028
21 days after 3rd doses									
FINLAY-FR-02	0.4747 ± 0.014	0.1246 ± 0.020	0.2686 ± 0.129	0.2185 ± 0.122	0.2455 ± 0.028	3.3103 ± 0.262	0.1783 ± 0.018	0.3799 ± 0.037	0.3720 ± 0.043
Placebo	0.4554 ± 0.024	0.1629 ± 0.092	0.2666 ± 0.124	0.2227 ± 0.141	0.2421 ± 0.018	3.1395 ± 0.419	0.1790 ± 0.018	0.3558 ± 0.019	0.3720 ± 0.043
Control	0.4492 ± 0.016	0.1297 ± 0.016	0.3263 ± 0.085	0.1700 ± 0.096	0.2564 ± 0.055	3.0913 ± 0.336	0.1635 ± 0.015	0.3455 ± 0.017	0.3602 ± 0.027

Legend: Values represent the mean ± SEM of the 5 animals in each group per time-point. * - Relative organ weight, ROW= (OW x 100)/EEW, where OW is the organ weight and EEW (euthanasia end weight) is the animal weight on the day of euthanasia.

Table 6b

Relative organ weights* (%).

Groups	LAG	RAG	Adrenals L+R	LDILN	RDILN	L+R DILN	LPLN	RPLN	L+R PLN
3 days after 3rd doses									
FINLAY-FR-02	0.0099 ± 0.003	0.0110 ± 0.005	0.0110 ± 0.005	0.0037 ± 0.002	0.0035 ± 0.001	0.2000 ± 0.201	0.0093 ± 0.002	0.0036 ± 0.002	0.0099 ± 0.003
Placebo	0.0069 ± 0.004	0.0059 ± 0.002	0.0059 ± 0.002	0.0030 ± 0.002	0.0038 ± 0.002	0.1910 ± 0.193	0.0081 ± 0.004	0.0034 ± 0.002	0.0069 ± 0.004
Control	0.0071 ± 0.002	0.0049 ± 0.002	0.0049 ± 0.002	0.0025 ± 0.001	0.0029 ± 0.001	0.1934 ± 0.196	0.0071 ± 0.002	0.0027 ± 0.001	0.0071 ± 0.002
7 days after 3rd doses									
FINLAY-FR-02	0.0088 ± 0.001	0.0093 ± 0.001	0.0035 ± 0.002	0.0102 ± 0.005a	0.0088 ± 0.001	0.0036 ± 0.003a	0.0034 ± 0.003	0.1942 ± 0.196	0.0098 ± 0.004
Placebo	0.0086 ± 0.002	0.0083 ± 0.002	0.0029 ± 0.001	0.0044 ± 0.001b	0.0073 ± 0.003	0.0031 ± 0.001b	0.0028 ± 0.001	0.1935 ± 0.195	0.0063 ± 0.002
Control	0.0102 ± 0.001	0.0100 ± 0.001	0.0035 ± 0.001	0.0049 ± 0.002b	0.0053 ± 0.002	0.0030 ± 0.001b	0.0040 ± 0.002	0.1970 ± 0.198	0.0075 ± 0.003
p = 0.0204									
21 days after 3rd doses									
FINLAY-FR-02	0.0084 ± 0.001	0.0074 ± 0.001	0.1902 ± 0.194	0.0054 ± 0.001	0.0063 ± 0.002	0.0064 ± 0.001	0.0028 ± 0.000	0.0035 ± 0.001	0.0064 ± 0.001
Placebo	0.0071 ± 0.001	0.0062 ± 0.001	0.01843 ± 0.187	0.0057 ± 0.002	0.0035 ± 0.001	0.0059 ± 0.001	0.0039 ± 0.001	0.0024 ± 0.001	0.0059 ± 0.001
Control	0.0068 ± 0.002	0.0053 ± 0.003	0.1835 ± 0.187	0.0065 ± 0.002	0.0047 ± 0.003	0.0061 ± 0.003	0.0020 ± 0.000	0.0022 ± 0.001	0.0061 ± 0.003
p = 0.0076									
p = 0.0413									
p = 0.0174									

Legend: LAG- Left adrenal gland, RAG- Right adrenal gland, LDILN- Left deep inguinal lymph nodes, RDILN- Right deep inguinal lymph nodes, LPLN- Left popliteal lymph node, RPLN- Right popliteal lymph node and L+R- Average of both. Values represent the mean ± SEM of the 5 animals in each group per time-point. Different letters stand for statistically significant differences. Kruskal-Wallis test.

immunological changes as compared to the controls. Hematological and blood chemistry parameters in rats are sensitive to immunotoxicity and are used to assess evolution of animal health over time (Batistia-Duharte et al., 2011; Tamargo et al., 2019; Nygaard and Løvil, 2002). This evaluation is also complemented by the morphometric analysis and histology of the spleen and lymph nodes. A systemic inflammation would have modified spleen size; there were no significant changes as compared to the control and the adjuvant groups at any time-point. The changes seen in the lymph nodes 3 and 7 days after the last administration in vaccine group in size and the presence of histological structures, such as the subcapsular and paracortical follicles, showed a progressive reversion over time, returning to their physiological state on day 21, suggesting no immunotoxicological effects compared to the controls.

Blood chemistry parameters are sensitive to toxicity related to heart, liver, gall bladder, pancreas, kidneys, bones, and muscles. There were no changes in the organs resulting in changes in relative weight, surface lesions, or measured blood chemistry parameters. This was also in line with the histopathology of vital parenchymal organs (brain, heart, lungs, liver, kidneys), which showed no clinically relevant differences between the controls and the FINLAY-FR-02 or placebo groups.

In addition of dermal irritability and local temperature, local toxicity was assessed through the histological examination of the injection sites (muscle and nodes), a crucial aspect for evaluating vaccine reactogenicity. The histology of the nodes in vaccine group showed no signs of irreversible inflammation, abnormal infiltrate, lymphocyte recruitment or additional damage as compared to the controls, considering to FINLAY-FR-02 as a tolerable product.

This integrated analysis of clinical signs, behavior, food and water consumption, animal and organ weight, hematology, and blood chemistry allows assessing toxicity associated with repeated exposure to drugs (Oliva et al., 2019; Fraleigh et al., 2019; Park et al., 2010; Baldrick et al., 2002). We incorporated morphometric studies of organs related to the immune system; this additional evaluation complements and increases the predictive value of conventional toxicology studies on

product safety.

Previous COVID-19 vaccine candidates, such as ChAdOx1 nCoV-19 and BBIBP-CorV have been evaluated for their humoral immunogenicity and toxicity in preclinical studies using different animals models including SD rats (van Doremalen et al., 2020; Wang et al., 2020). Toxicity for these vaccines focused on systemic and local toxicity. Certainly, antibody response to vaccine antigen needs a longer evaluation time, which could vary from weeks to months, depending on the vaccine and number of doses. Previous investigations demonstrated that vaccines induce anti-SARS-CoV-2 antibodies in SD rats (Forster, 2012; Kandeil et al., 2021; Brian et al., 2020). We found that FINLAY-FR-02 induces strong anti-SARS-CoV-2 antibodies in mice (Valdés-Balbín et al., 2021) and now, in SD rats, as shown here. In previous preclinical studies (Fig 8, supplementary material), we saw that SD rats responded satisfactorily to the vaccine candidate, the differences between groups

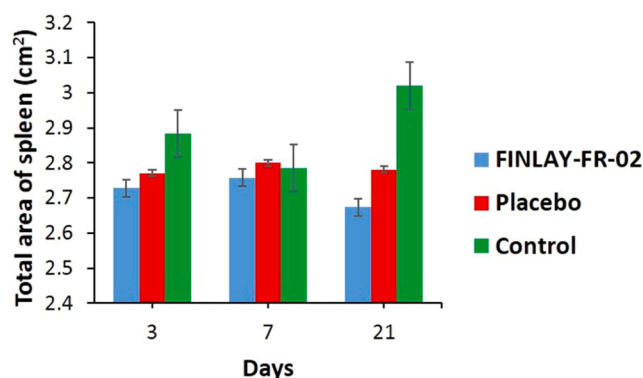


Fig. 6. Time course of morphometric macroscopic area of spleen of male SD rats. Values are the average ± SEM of the 5 animals in each group. Kruskal-Wallis test $p \geq 0.05$.

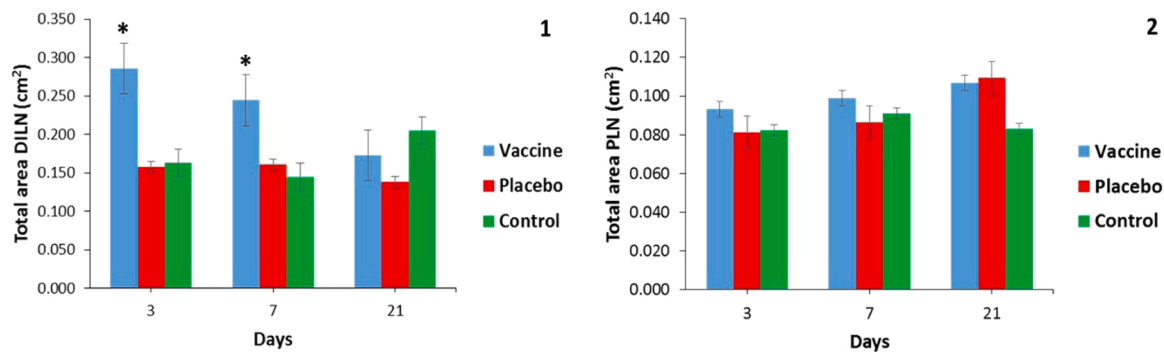


Fig. 7. Time course of morphometric macroscopic area of 1- Deep inguinal lymph node (DILN) and 2- Popliteal lymph node (PLN) of male SD rats. Values are the average \pm SEM of the 5 animals in each group. Kruskal wallis test * - $p \leq 0.05$.

are clear after three weeks, where the vaccinated animals double the anti-RBD antibody titers.

We evaluated the antibody response at each time of scheduled euthanasia (3, 7 and 21 days after the last administration); an antibody response was detected on days 7 and 21 (Fig. 3), suggesting that FINLAY-FR-02, in addition to being safe, would be capable of inducing an immune response even in the adverse scenario of repeated administration, not saturating the immune system.

This toxicological study in SD rats demonstrated the preclinical safety of the FINLAY-FR-02 Cuban conjugated vaccine candidate obtained using established platforms at the Finlay Vaccine Institute and opened the route to its clinical evaluation in humans.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.tox.2022.153161](https://doi.org/10.1016/j.tox.2022.153161).

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