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Physiology, gene expression, and behavior as potential indicators of oxidative stress in piglets

Raúl David Guevara[1,2*], Jose J. Pastor[3], Sergi López-Vergé[3], Xavier Manteca[2], Gemma Tedo[3] and Pol Llonch[2]

Abstract

The goal of the current study was to develop a pig model to investigate oxidative stress with a low negative impact on piglet welfare. Four independent trials (A, B, C, and D) were performed using a single intraperitoneal shot of lipopolysaccharide (LPS) as an immune challenge, aiming to assess the minimal LPS dose for piglets of different age to trigger a measurable acute oxidative stress response in healthy animals. In trial A, piglets received an LPS dose of 25 µg/KgBW at 41 days post-weaning (p.w.). In trial B, piglets received 25 µg/KgBW of LPS at 28 days p.w., in trials C And D, piglets were injected with 50 µg/KgBW of LPS at 21 days p.w., respectively. Piglets were randomly allocated either to the T1) Control group with saline solution (Ctrl), or T2) LPS challenge (LPS). The oxidative stress response was measured through the enzymatic activity of glutathione peroxidase (GPx), glutathione-S-transferase (GST), superoxide dismutase (SOD), and catalase (CAT), in both plasma and intestinal tissues. Intestinal gene expression of oxidative stress and inflammatory markers was assessed. Discomfort behaviors (panting, prostration, trembling, and vomits) were also recorded. Plasmatic and intestinal oxidative stress response was inconsistent across the four trials even when the dose and pig age were similar, possibly due to individual variability. Relative gene expression differences of anti-inflammatory cytokines (IL10), oxidation precursor (iNOS), and antioxidant markers (GPx4, MnSOD, and CAT) were detected between Ctrl and LPS treatment ($P < 0.05$) when assessed. Behavioral observations were sensitive to the LPS dose relative to Ctrl ($P < 0.05$) in all four trials. These results suggest that behavioral observations can be used as a non-invasive methodology to detect the presence of oxidative stress in pigs in challenging conditions. Behavioral observations were more sensitive than other indicators (i.e., biomarkers and gene expression) in the current study. However, a sensitivity scale system needs to be developed to qualify and rank the impact of oxidative stress in pigs.

Keywords Oxidative stress, Antioxidant markers, Gene expression, Pig behavior, Piglets, Lipopolysaccharide

*Correspondence:
Raúl David Guevara
raul.guevara@awec.es
[1]Animal Welfare Education Centre (AWEC) Advisors S.L., , Research Park UAB, Campus UAB, Cerdanyola del Valles 08193, Spain
[2]Department of Animal and Food Science, Universitat Autònoma de Barcelona, Edifici V, Travessera dels Turons, Cerdanyola del Valles 08193, Spain
[3]Animal Science Innovation Division, Lucta, Cerdanyola del Vallès, Spain

Introduction

Oxidative stress occurs when the generation of free radicals overcomes the body's antioxidant capacity [1]. This oxidative imbalance leads to the accumulation of Reactive Oxygen Species (ROS) with the consequent damage to lipids, proteins, and DNA, impairing the physiological condition of the organism. The accumulation of ROS in the intestinal tract is especially relevant and is associated with compromised intestinal function, decreased nutrient transport, and increased susceptibility to infections [2, 3]. Such oxidative threat provokes physiological reactions to control the potential harm to tissues. Physiological responses range from activation of antioxidant proteins [i.e., glutathione peroxidase (GPx), catalase (CAT), or superoxide dismutase(SOD)] and cytokines (i.e., TNF-α, IL-6, IL-8, and IL-1b) [4, 5], to changes in behavior [6].

Oxidative stress responses are variable depending on the intensity of the stressor. Naito et al. [4] described an organism's different physiological steps to face an oxidative stressor. Oxidative stressors generate ROS, increasing the oxidation of molecules such as lipids, proteins, enzymes, and DNA. If the oxidative threat persists, free radical-trapping antioxidants such as vitamins C and E, carotenoids, coenzyme Q10, and bilirubin are activated. Therefore, the oxidative indicators or biomarkers to assess the oxidative status of pigs are antioxidant enzymes such as the GPx, which is recognized as an antioxidant enzyme, that reduces hydrogen peroxide and organic hydroperoxides, assisting in the control of ROS [5, 7, 8]. GPx activity has been used as an indicator of the antioxidant status and potential [7]. SOD is an important enzyme that catalyzes the dismutation of super peroxide into oxygen and hydrogen peroxide [8, 9]. CAT catalyzes the transformation of hydrogen peroxide into water and oxygen [8, 9]. Finally, GST is a detoxification enzyme that protects cells from unhealthy chemical species [10]. GPx, SOD, and CAT are commonly used as anti-oxidative biomarkers as they are the first response to oppose an oxidative challenge, conforming a preventive type of antioxidant network system [4, 8].

Behavioral responses are considered adaptative responses to cope with stimuli [11–13]. Two major physiological pathways influence animal behavior: the sympathetic-adrenal-medullary axis (epinephrine and norepinephrine, related to the "fight or flight" reaction) and the hypothalamic-pituitary-adrenal axis. The latter controls glucocorticoids to increase protein or fat catabolism, thereby increasing glucose availability [14]. Broom [11] has stated that behavior is related to immunological activity, particularly regarding the action of cytokines, interleukins, enzymes, and other endocrinological agents. This association allows veterinarians and medical doctors to diagnose pathologies accurately [11]. Additionally, behavioral assessment has advantages over the assessment of biomarkers as it can be performed remotely without disturbing the integrity of the animals [15]. Although behavior as a stress indicator has pros such as feasible utilization and the possibility of being automatized, it is not as specific as common biomarker analyses. Nevertheless, through systematic and validated observation protocols, behavioral observations can provide significant information about the status of the animal [11].

In pig production, oxidative stress can be generated by several factors such as nutrition imbalances, negative social interactions due to excessive aggression receptions, or environmental factors that may harm the welfare of animals and their production level [2, 3, 15]. At weaning, piglets are subject to several of these stressors simultaneously, such as separation from their mother, the sudden dietary change from liquid to solid feed, the mixing with new counterparts, and a new environment [16, 17]. Thus, it is necessary to study the physiological responses triggered by these challenges and the possible indicators that might help the pig producers and researchers monitor the stress response.

Experimental stress models help pig production stakeholders to objectively evaluate mitigation strategies to preserve animal welfare and performance. Commonly, current models to study the effects of oxidative stress through immune stimulation with lipopolysaccharide (LPS), which is a component of the gram-negative bacteria membrane and is one of the most potent immune system stimulators [18] commonly used to trigger oxidative stress in experimental conditions [15]. The LPS interacts with the antigen CD14 initiating an inflammatory response including activation of free radical-generating enzymes that provoke host lipid peroxidation [19] increasing the risk of oxidative damage. However, LPS challenges in pigs are not standardized (challenge type, age, dose, etc.) and may severely impact the physiological condition and welfare of the animals [20]. Also, the heterogeneity of experimental conditions might produce a variety of results, making it difficult to elucidate the effectiveness of tested mitigation strategies to deal with oxidative damage. Moreover, the suitable period to measure the oxidative response in piglets remains unclear. Different immune challenges have been reported (e.g. 25–100 μg LPS/Kg BW) to unbalance the oxidative status of animals at different ages (from 10 to 46 days post-weaning) days and diverse housing management [21–24]. These irregular conditions across studies may generate inconsistent results, besides the intense discomfort caused by the challenge to the animals. Hou et al. [24] and Li et al. [25] observed antioxidant activity with lower doses compared to the doses reported in the literature [60–100 μg/kg BW [20–22, 26–31]]. Therefore, it is

Table 1 Summary of the weaning age and weights of the piglets in each trial

Trial	Weaning age (d)	SEM	Weaning weight (Kg)	SEM
Trial $A^{41/25}$	26.05	4.50	5.70	0.85
Trial $B^{28/25}$	26.07	3.10	5.71	0.85
Trial $C^{21/50}$	22.47	2.54	5.13	0.75
Trial $D^{21/50}$	23.60	2.99	5.48	0.65

Trial $A^{41/25}$: LPS challenge on day 41 post-weaning, p.w. with LPS dose 25 µg/kg BW

Trial $B^{28/25}$: LPS challenge on day 28 post-weaning, p.w. with LPS dose 25 µg/kg BW

Trial $C^{21/50}$: LPS challenge on day 21 post-weaning, p.w. with LPS dose 50 µg/kg BW

Trial $D^{21/50}$: LPS challenge on day 21 post-weaning, p.w. with LPS dose 50 µg/kg BW

Trial D was a repetition of trial C conditions (LPS dose and the piglets' age) aiming to observe the repeatability of the oxidative stress model

reasonable to subject the piglets to a mild oxidative challenge in order to measure the oxidative response while minimizing the negative impacts on their welfare.

The main goal of this research was to develop a model capable of triggering a measurable oxidative stress response aiming to provoke minimal harm to piglets and avoid clinical illness. This study assessed four trials describing the piglets' physiological response toward an immune stimulator to trigger a physiological oxidative challenge. Additionally, the present study aims to assess behavior as a more sensitive oxidative stress indicator compared to other measurement methods used in the study. The present study will contribute to identifying different less or non-invasive biomarkers and improve the *Refinement* (*three Rs: Replacement*, *Reduction*, *and Refinement*) [32] of experimental protocols for pig oxidative stress research.

Materials and methods

Animals, housing and diets

All procedures were approved by the Laboratory Animal Care Advisory Committee of the Universitat Autònoma de Barcelona (CEA/9310/P1). Four consecutive trials were performed at an Experimental Swine Unit farm "El Castell" (Sant Aniol de Finestres, Girona, Spain) following the same housing and management routines.

All crossbred piglets [(Largewhite × Landrace) × Pietrain] were purchased, weaned, and transported to the nursery unit from the farrowing farm of origin ("La Sala", Sant Bartomeu del Grau, Barcelona, Spain) on the same day. Healthy piglets, weighing between 3.5 and 6.5 kg of body weight (BW), were carefully chosen from sows with the most uniform litters. They were checked for clean skin, healthy joints, and no lesions or injuries. Weaning ages and weights at each trial are shown in Table 1. At arrival, piglets were housed in four rooms with 12 pens (6 piglets/pen) in each room. Except for trial A, where only two piglets were housed together in a pen with a division allowing visual contact, to increase the number of experimental units in the trial. Therefore, the piglets in trial A were considered individually housed because they did not have physical contact with the piglets beside them. Each pen (2.64 m^2) had two nipple drinkers covered with a mobile metal lid to prevent external contamination (feces and urine) and to decrease water waste. Pens had one feeder per pen, with four separations per feeder covered with a metal lid. All experimental pens were equipped with completely slatted plastic floors. Piglets were randomly distributed into pens according to their initial BW and sex to homogenize the animals in each treatment group.

Table 2 Summary of the nutrient composition of the diets supplied during the phases of the trials. Creep feed diet (from day 0 to day 7 post-weaning, p.w.), pre-starter diet (from day 7 to day 21 p.w.), and starter diet (from day 21 to day 41 p.w.)

Nutrient	Creep feed diet	Pre-starter diet	Starter diet
Digestible energy pigs (Mcal/kg)	3.58	3.56	3.51
Crude protein (%)	18.49	18.06	16.99
Lysine (%)	1.39	1.40	1.33

All pigs were fed a standard commercial diet in mash form formulated to meet or exceed the National Research Council [33] nutrient requirements (Table 2). Feed and water were offered *ad libitum* throughout the trials.

Study design

Oxidative stress was induced by the intraperitoneal (i.p.) administration (in the upper left area of the intraperitoneal cavity) of *Escherichia coli* LPS (serotype 055: B5 Sigma Chemical, San Luis, USA) to healthy animals based on the experimental oxidative stress model from Hou et al., [24]. The LPS was selected as an immune stimulator because it is commonly and widely used as an oxidative challenge [15]. The i.p. administration was intended to enhance the effectiveness of the oxidative challenge in the organism, especially in the intestinal tissues. The four trials in the current study included two experimental treatment groups (i) control (Ctrl); and (ii) LPS challenge (LPS). The experimental conditions (LPS dose administered, and the age of the piglets) for each trial were adjusted based on the results of the previous one (Table 3). Pigs were randomly allocated to one of the two treatments from weaning until the challenge day. All the treatment groups were homogenized by sex and body weight. LPS doses of 25 and 50 µg/kg BW were chosen to determine if it was possible to induce an acute oxidative stress response with a milder challenge. Pigs from the LPS challenge group received a single intraperitoneal dose of an LPS agent, whereas Ctrl pigs received an equivalent intraperitoneal injection of saline solution.

Table 3 Summary of the four trials conditions to assess the pig oxidative stress model

	Trial $A^{41/25}$	Trial $B^{28/25}$	Trial $C^{21/50}$	Trial $D^{21/50}$
n	20	24	24	24
Age (d p.w.)	41	28	21	21
LPS dose (µg/KgBW)	25	25	50	50
Housing	Individual (1.32 m^2)	6 pigs/pen (2.64 m^2)	6 pigs/pen (2.64 m^2)	6 pigs/pen (2.64 m^2)

The LPS dose and final injection volume were determined based on the pigs' body weight (LPS µg/KgBW). The goal of each subsequent trial was to refine the oxidative stress model by adjusting the conditions of the LPS challenge (such as the housing type, the dose of LPS, and the age of piglets) based on the piglets' response from the previous trial. This aimed to achieve a measurable response with less impact on the piglets' health. Similarly, the sampling procedures (i.e., blood sampling and collection of intestinal tissue) varied across the trials to identify measurement methodologies with higher sensitivity to the piglets' oxidative response. The efficiency of the oxidative model in each trial was assessed by comparing the differences in oxidative response between the challenged piglets and non-challenged piglets.

Sample collection

Sampling protocols were the same in all the trials of the current study. Piglets were in their pens during the blood sampling. Blood samples were collected from the *cava cranialis* vein pre-sacrifice (4 h post LPS i.p injection) using a vacutainer into pre-labeled tubes with EDTA anticoagulant. The blood tubes were immediately centrifuged (1200 g x 10 min, 4 °C) and the supernatant was separated and frozen immediately in dry ice. After blood sampling, 4 h post-LPS i.p. injection, piglets were individually moved to the sacrifice room. LPS and Ctrl pigs (10 piglets/treatment in trial A; 12 piglets/treatment in trials B, C, and D) were euthanized to collect the intestinal tissues. Piglets were stunned by a captive bolt shot in the piglet's front and then sacrificed exsanguinating by severing the jugular and carotid veins. After cessation of any sign of consciousness (i.e., no pain and corneal reflexes, and absence of rhythmic breathing) the abdomen was sectioned to extract and dissect the intestine into jejunum, ileum, and colon. Sections of the mid jejunum, ileum, and colon were washed with 0.9% saline to remove content and longitudinally opened to collect mucosa. All samples were flash-frozen (in liquid N_2 or dry ice) and stored at -80 °C.

Biological parameters

The activity of antioxidant enzymes in intestinal mucosa and plasma was measured using commercial ELISA kits following manufacturers' protocol (Cayman Chemical, Ann Arbor, MI). The activity of antioxidant enzymes in intestinal mucosa was measured in Trials $A^{41/25}$ and $B^{28/25}$ but not in Trials $C^{21/50}$ and $D^{21/50}$.

Table 4 Sequence of primers used in the RNA isolation

Gene	Forward	Reverse	Annealing (°C)	Accession Number
IL-8	GGTCTGCCTG GACCCCAAG GAA	TGGGAGCCACG GAGAATGGGTT	60	NM_213867.1
IL-10	GTCCGACTCAA CGAAGAAGG	GCCAGGAAGAT CAGGCAATA	60	NM_214041.1
IL-1b	GCAGTGGAG AAGCCGATG AAG	GGCCAGCCA GCACTAGAGA TTTG	64	NM_214055.1
iNOS	GTCCAGCGCTA CAACATCCT	TCCATGATGGTC ACGTTCTG	60	U59390
GPx1	TGGGGAGATCC TGAATTG	GATAAACTTGG GGTCGGT	60	NM_214201.1
GPx4	GATTCTGGCCT TCCCTTGC	TCCCCTTGGGC TGGACTTT	60	NM_214407.1
MnSOD	GGACAAATCTG AGCCCTAACG	CCTTGTTGAAA CCGAGCC	60	NM_214127
CAT	CGAAGGCGAA GGTGTTTG	AGTGTGCGATC CATATCC	60	XM_021081498.1
β-actin	GCCAACCGTGA GAAGATGAC	ATCCCCAGAGT CCATGACAA	60	XM_003357928.2
TBP	AACAGTTCAG TAGTTATGAGC CAGA	AGATGTTCTCAA ACGCTTCG	63	DQ845178.1
TNFα	CGTGAAGCTG AAAGACAAC CAG	GATGGTGTGA GTGAGGAAA ACG	60	NM_214022.1

Interleukin (IL-), nitric oxide synthase (iNOS), Glutathione peroxidase (GPx-), manganese superoxidase dismutase (MnSOD), catalase (CAT), beta-actin (β -actin), TATA-Box Binding Protein (TBP), and tumor necrosis factor alfa (TNFα)

Total RNA was isolated from 100 mg mucosal samples automatically in a KingFisher Duo Prime (ThermoFisher Scientific, Waltham, USA) with the MagMax™ mirVana™ Total RNA Isolation kit (ThermoFisher Scientific), following manufacturers' instructions. Final RNA concentration and quality were spectrophotometrically assessed in a NanoDrop system (ThermoFisher Scientific), and cDNA synthesis was performed with the High-Capacity cDNA Reverse Transcription Kit. RT-PCR was finally performed on a StepOne Plus system (ThermoFisher Scientific Inc.) with the Fast SYBR® Green Master Mix and the primers sequences detailed in Table 4. Specific product amplification was checked by the melting curve analysis. Gene efficiencies were evaluated by generating standard curves using cDNA from a pool of samples and calculated according to the equation $E=10\ (-1/\text{slope})$. Relative expression was calculated according to the $2^{-\Delta\Delta Ct}$ method using TBP and β-actin as housekeeping

genes. Relative expression was only measured in Trials $C^{21/50}$, and $D^{21/50}$.

Behaviors indicating discomfort triggered by the immune challenge (Table 5) were counted through direct observation at four time points: 60, 120, 180, and 240 min after intraperitoneal injection on Trials $C^{21/50}$ and $D^{21/50}$. Behavioral observations were performed by the same observer, counting the number of piglets in each pen displaying the selected behaviors at each time point.

A summary of the samples collected and measurements performed in the different trials is presented in Table 6.

Statistical analyses

All statistical analyses were performed with the statistical software SAS (version 9.4, SAS Institute Inc., Cary, NC). Oxidative markers activity and genetic expression analyses were performed by T-test (PROC TTEST) with treatment as the main effect without any adjustment for multiple comparisons. LSMEANS compared the main effect treatment group. Behavioral observations were analyzed through a Logistic model (PROC LOGISTIC) within each observation time (60, 120, 180, and 240 min after intraperitoneal injection) comparing the frequency of each behavior among the treatments. In all the parameters measured, the pen was considered the experimental unit, and the piglet was considered the observational unit. Significant differences were declared at $P \leq 0.05$ whereas near-significant trends were considered at $0.05 < P \leq 0.10$.

Table 5 Ethogram and definitions of the behaviors recorded to assess the impact of oxidative challenge on piglets in different trials

Behavior	Definition
Panting	Piglets exhibit a high frequency of rapid inhalation and exhalation characterized by open-mouth respiration
Prostration	Piglets are lying down with their chest on the floor and their limbs pulled towards their body
Trembling	Piglet's body shivers
Vomiting	Piglets regurgitant intestinal content

Table 6 Samples and measurements for the trials

Enzyme activity (Plasma & Intestinal tissues)	Gene expression (Intestinal mucosa)	Behavior incidence
GPx	IL10	Panting
GST	iNOS	Prostration
SOD	GPx4	Trembling
CAT*	MnSOD	Vomiting
	CAT	

Glutathione peroxidase (GPx), glutathione-S-transferase (GST), superoxide dismutase (SOD), catalase (CAT), Interleukin 10 (IL10), nitric oxide synthase (iNOS), GPx4, and manganese SOD (MnSOD). *Only in plasma samples

Results

Antioxidant enzyme activity

Plasma

Table 7 summarizes the plasmatic activities of the antioxidant enzymes. No differences in the activity of GPx ($P=0.3138$), SOD ($P=0.4713$), GST ($P=0.2456$), and CAT ($P=0.2936$) were observed for Trial $A^{41/25}$. In trial

Table 7 Antioxidant enzyme activities in plasma from trials $A^{41/25}$, $B^{28/25}$, $C^{21/50}$, and $D^{21/50}$

	GPx, mU/ml		SOD		GST, mU/ml		CAT, mU/ml	
	Mean	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**
Trial $A^{41/25}$			**Inhibition %**					
Ctrl	1066.12	77.016	57.03	2.610	30.03	1.536	46.19	6.474
LPS	1226.24	77.016	56.03	2.610	32.99	1.629	30.47	6.824
P value	0.1531		0.7892		0.1995		0.1067	
Trial $B^{28/25}$			**Inhibition %**					
Ctrl	**791.08^{b}**	**44.912**	47.63	3.541	**17.96^{b}**	**1.088**	52.09	6.002
LPS	**931.73^{a}**	**55.005**	46.34	3.541	**22.05^{a}**	**1.088**	45.07	6.002
P value	**0.0476**		0.7981		**0.0122**		0.4140	
Trial $C^{21/50}$			**mU/ml**					
Ctrl	732.15	41.480	3.76	0.534	23.91	1.066	46.10^{y}	7.069
LPS	747.42	41.480	4.82	0.534	24.83	1.066	65.52^{x}	7.069
P value	0.7964		0.1696		0.5475		0.0609	
Trial $D^{21/50}$			**mU/ml**					
Ctrl	471.65	32.429	**2.92^{b}**	**0.508**	21.04	2.382	22.59	4.538
LPS	522.29	32.429	**5.03^{a}**	**0.508**	23.91	2.382	30.21	4.142
P value	0.2783		**0.006**		0.4039		0.2243	

Different letters (a & b) indicate significant differences ($P<0.05$). Different letters (x & y) indicate a statistical trend ($0.05 \leq P \leq 0.10$)

Trial $A^{41/25}$: LPS challenge on day 41 post-weaning, p.w. with LPS dose 25 µg/kg BW

Trial $B^{28/25}$: LPS challenge on day 28 post-weaning, p.w. with LPS dose 25 µg/kg BW

Trial $C^{21/50}$: LPS challenge on day 21 post-weaning, p.w. with LPS dose 50 µg/kg BW

Trial $D^{21/50}$: LPS challenge on day 21 post-weaning, p.w. with LPS dose 50 µg/kg BW

$B^{28/25}$, the GST ($P=0.0122$) and GPx ($P=0.0476$) activity were increased in the LPS group relative to the Ctrl. SOD ($P=0.5166$), and CAT ($P=0.6452$) activities remained unchanged between groups.

No differences in the activity of GPx ($P=0.7964$), GST ($P=0.5475$), and SOD ($P=0.1696$) were observed for Trial $C^{21/50}$, but CAT activity of the LPS piglets tended to increase ($P=0.0611$). In Trial $D^{21/50}$, with piglets of the same age at the same LPS dose of Trial C, a significant increase in plasmatic SOD activity was detected in the LPS group, compared to the Ctrl group ($P=0.0060$). In contrast, GPx ($P=0.2783$), GST ($P=0.4039$), and CAT ($P=0.2252$) activities remained unaffected.

Intestinal tissues

Table 8 summarizes the activities of the antioxidant enzymes measured in the mucosa of the different tissue sections (jejunum, ileum, and colon). Oxidative markers measured in trial $A^{41/25}$ were not different between Ctrl and LPS treatments in any of the intestinal tissues analyzed ($P\geq0.1626$). In trial $B^{28/25}$, piglets in the LPS group presented higher SOD activity in the ileum ($P<0.05$). Trial $B^{28/25}$ oxidative markers in other intestinal tissues were not different between Ctrl and LPS groups ($P\geq0.33$). Antioxidant activity in the intestinal mucosa was not measured in trials $C^{21/50}$ and $D^{21/50}$.

Gene expression results

Piglets on trial $C^{21/50}$ from the LPS group showed a higher jejunum gene expression of the anti-inflammatory cytokine IL-10 (149.09%) the defense protein iNOS (384.67%) and the antioxidant enzymes GPx4 (22.54%), MnSOD (73.58%), and CAT (152.42%), compared to Ctrl ($P<0.05$, Fig. 1A). On the other hand, the jejunal gene expression of LPS piglets in trial $D^{21/50}$, using the same LPS dose and piglets age, only showed significant increases of the oxidative markers iNOS (227.60%), MnSOD (147.05%), and CAT (107.84%), (Fig. 1B) compared to Ctrl ($P<0.05$). Gene expression was not performed on trials $A^{41/25}$ and $B^{28/25}$.

Other inflammatory and antioxidant markers such as IL-8 ($P\geq0.4796$), IL-1b ($P\geq0.9090$), TNFα ($P\geq0.1832$), and GPx1 ($P\geq0.6648$) were not different between Ctrl and LPS treatment groups trial $C^{21/50}$. In trial $D^{21/50}$ Ctrl

Table 8 Activity of the antioxidant enzymes in intestinal tissues (jejunum, ileum, and colon) from the trials $A^{41/25}$, and $B^{28/25}$

		GPx, mU/ml		SOD, Inhibition %		GST, mU/ml	
		Mean	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**
Jejunum	Trial $A^{41/25}$						
	Ctrl	250.60	17.008	59.94	1.792	61.06	4.446
	LPS	285.13	17.008	62.41	1.792	69.86	4.446
	P value	0.1626		0.3394		0.1730	
	Trial $B^{28/25}$						
	Ctrl	35.10	3.523	55.88	1.410	66.85	7.393
	LPS	29.97	3.523	55.06	1.410	77.33	7.393
	P value	0.3104		0.6849		0.3233	
Ileum	Trial $A^{41/25}$						
	Ctrl	210.44	27.984	47.24	4.062	34.75	5.699
	LPS	249.66	27.984	44.65	4.062	35.41	5.331
	P value	0.3305		0.9338		0.6547	
	Trial $B^{28/25}$						
	Ctrl	147.28	8.748	**36.02[b]**	**1.628**	23.82	1.916
	LPS	136.63	8.748	**41.06[a]**	**1.628**	26.71	1.916
	P value	0.3955		**0.0358**		0.2937	
Colon	Trial $A^{41/25}$						
	Ctrl	431.56	32.458	46.10	1.287	82.63	4.797
	LPS	417.68	32.458	48.77	1.287	81.54	5.088
	P value	0.7647		0.1540		0.8768	
	Trial $B^{28/25}$						
	Ctrl	381.78	37.463	49.51	2.572	26.52	3.198
	LPS	345.08	35.868	47.26	2.572	24.63	3.062
	P value	0.4842		0.5416		0.6729	

Different letters (a & b) indicate significant differences ($P<0.05$). Different letters (x & y) indicate a statistical trend ($0.05\leq P\leq0.10$)

Trial $A^{41/25}$: LPS challenge on day 41 post-weaning, p.w. with LPS dose 25 µg/kg BW

Trial $B^{28/25}$: LPS challenge on day 28 post-weaning, p.w. with LPS dose 25 µg/kg BW

Trial $C^{21/50}$: LPS challenge on day 21 post-weaning, p.w. with LPS dose 50 µg/kg BW

Trial $D^{21/50}$: LPS challenge on day 21 post-weaning, p.w. with LPS dose 50 µg/kg BW

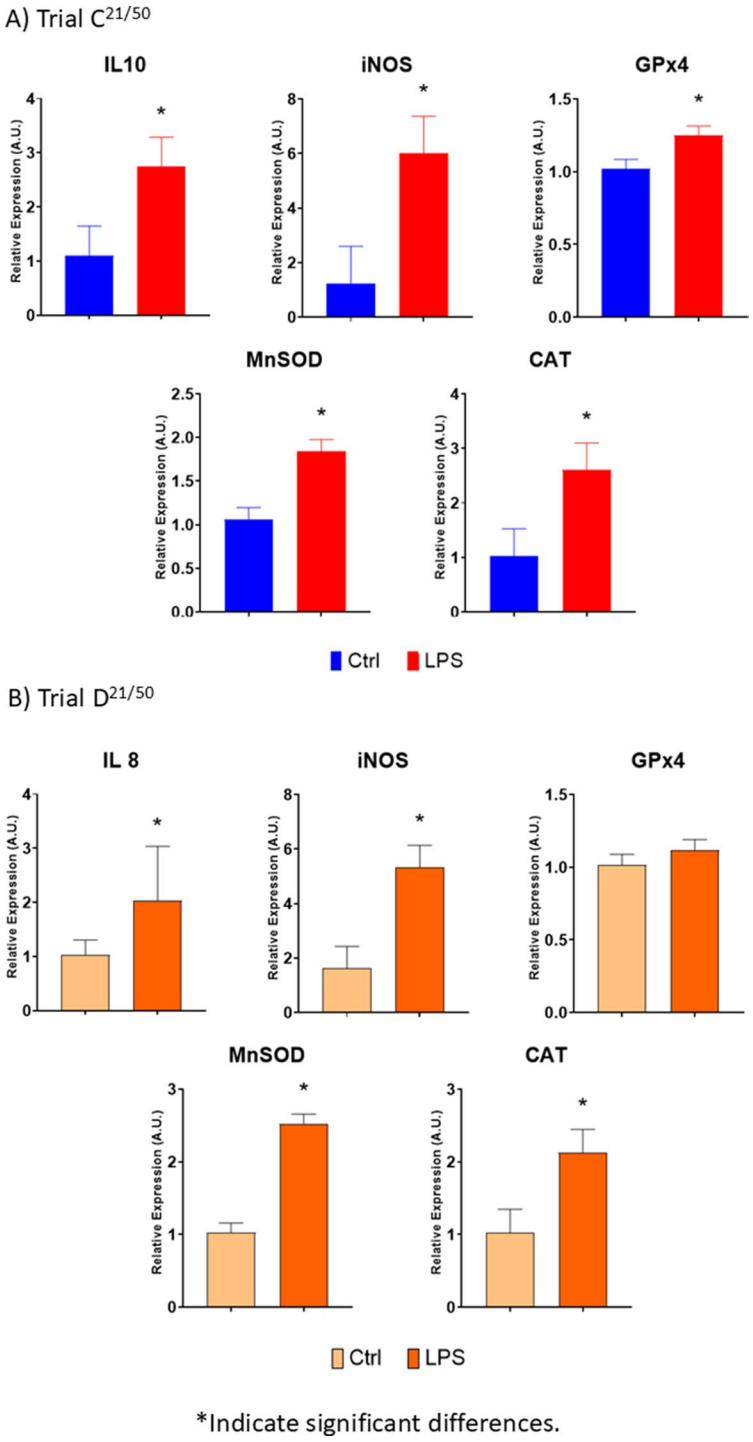

Fig. 1 Gene expression in **A**) trial $C^{21/50}$, and **B**) trial $D^{21/50}$ of antioxidant markers: IL10, iNOS, GPx4, MnSOD, and CAT. *Indicate significant differences ($P < 0.05$)

and LPS IL-10 ($P \geq 0.6489$), IL-1b ($P \geq 0.1353$), TNFα ($P \geq 0.0831$), and GPx1 ($P \geq 0.5917$) did not differ.

Behavioral observations

Figure 2 presents discomfort behaviors' frequency (panting, prostration, trembling, and vomits) in trials $C^{21/50}$ and $D^{21/50}$. In general, Ctrl animals showed less frequency of illness-related behaviors than the LPS treatment group ($P < 0.05$) in both trials. Panting and vomiting behaviors were only displayed in LPS piglets in both trials. LPS pigs displayed more prostration and trembling than Ctrl pigs.

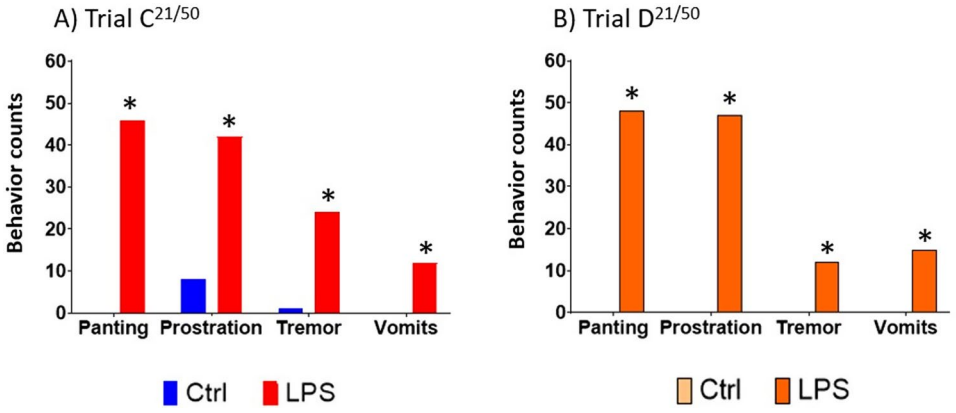

Fig. 2 Frequency of discomfort behaviors after the oxidative challenge, including (panting, prostration, trembling, and vomits frequency presented is the sum of each behavior category. **A**) Trial $C^{21/50}$; **B**) Trial $D^{21/50}$. *Indicate significant differences ($P < 0.05$). The absence of mean bars means the value is zero or close to zero

Discussion

There is a need for simple, accurate, and less aggressive experimental setups to evaluate nutritional or husbandry strategies to mitigate oxidative stress in pigs under commercial production conditions. The ultimate goal of the current research project was to develop an experimental oxidative stress model for pigs minimizing the negative impact on the welfare of the animals and without compromising the accuracy of the results obtained. The trials performed in this study attempted to detect oxidative stress indicator changes triggered by a mild LPS injection. Each independent trial attempted to refine the oxidative stress model based on the results of the previous trial. In trials $A^{41/25}$ and $B^{28/25}$, the goal was to trigger a measurable antioxidant response using an LPS dose of 25 µg/KgBW. Different age groups (28- and 41-days post-weaning) of pigs were tested to see when they would be most responsive to the LPS challenge. However, since the antioxidant response couldn't be detected through the proposed methods (assessment of the antioxidant activity in plasma and intestinal mucosa), trials $C^{21/50}$ and $D^{21/50}$ were adjusted. These trials used a higher LPS dose compared to trials $A^{41/25}$ and $B^{28/25}$ and were conducted on younger pigs (21 days post-weaning). In addition, we conducted gene expression analyses and behavioral assessments to improve the likelihood of detecting changes resulting from the LPS challenge. However, we did not measure antioxidant activity in the intestinal mucosa in Trials $C^{21/50}$ and $D^{21/50}$ due to its lack of sensitivity.

Overall, the trials in the current study, using different LPS doses and in different piglet ages, were not able to detect any physiological responses toward the mild oxidative challenge. Antioxidant responses such as changes in the activity of the antioxidant enzymes were expected as Hou et al. [24], and Hu et al. [34] reported with mild LPS doses. Piglets' variability (e.g. individual immune system maturity) or the mild severity of the proposed challenges might be some of the reasons for the inconsistent oxidative stress responses detected among the different trials. When assessing the physiological response of the piglets in trial $A^{41/25}$, no significant differences were detected in plasmatic markers between treatment groups with a mild LPS dose. Whereas in trial $B^{28/25}$, only a significant increase in the GST plasmatic activity was detected when pigs received the LPS challenge. The maturity of the animals is an important factor to consider in the lack of detected responses on trial $A^{41/25}$. Immune system resilience increases when the animal grows, becoming more robust or primed towards immune challenges [35]. In trials, $C^{21/50}$ and $D^{21/50}$, LPS dose and age were modified (higher dose in younger animals) aiming to detect the acute oxidative response, but again the oxidative response obtained was not consistent. No GPx, SOD, and GST activity changes were observed in trial $C^{21/50}$, but in trial $D^{21/50}$, only a significant increase in SOD plasmatic activity in the LPS group was detected.

Contrary to the results of the current study, other authors, such as Hu et al. [34] applied an LPS dose of 10 µg/KgBW concentration, and Hou et al. [24] used an LPS dose of 25 µg/KgBW able to trigger and detect plasmatic antioxidant responses through mild LPS doses. Even though Hu et al. [34] reported Antioxidant Capacity (AOC) differences in the LPS group relative to treatment groups with nutritional additives. The samples for the AOC analyses were collected two hours after the LPS injection, which might increase the chance of detecting a response to the oxidative threat. On the other hand, Hou et al. [24] compared the effect of the LPS injection against a saline solution after six hours of the intraperitoneal injection; the differences are presented as total AOC. Thus, it is worth considering that antioxidant responses are easier to detect when the results are presented as AOC, which is a measurement of the cumulative action of all the antioxidant components in plasma

[36], instead of the individual activity of antioxidant enzymes. Additionally, as reported by Hu et al. [34] an earlier sampling might increase the likelihood to detect antioxidant enzyme activity changes. In the present study, AOC capacity was not estimated because the focus was on observing how the different antioxidant enzymes reacted to the LPS challenge.

On the other hand, antioxidant activity on the intestinal tissues measured in trials $B^{28/25}$, only detected an increase in the SOD activity in the ileum. Other authors have reported changes in the antioxidant activity markers, either in plasma or intestinal tissues, when a higher dose was applied. For instance, Sun et al. [31] reported significant reductions of the GPx activity in different tissues (serum, liver, spleen, thymus, and lymph node) of pigs challenged with an LPS dose of 100 μg/kg BW relative to the control pigs. Chen et al. [21] registered a notorious decline of the plasmatic GPx of the LPS group compared to the saline group, but no differences were detected in the SOD activity in plasma with an LPS dose of 100 μg/kg. Also, the antioxidant capacity of intestinal tissues (jejunum and ileum) was reduced after the oxidative challenge, but no differences were reported in SOD activity at the jejunum, ileum, or colon. Cao et al. [5] noticed a decrease in the GPx and SOD activity in the jejunum mucosa with the 100 μg/kg BW LPS dose. However, not all the markers are sufficiently sensitive to measure oxidative stress at this high LPS concentration. For example, studies from Kang et al. [27], Yi et al. [26], and Cai et al. [30] were not able to detect differences in CAT activity with an LPS dose of 100 μg/kg BW. Hence, a high dose on an LPS challenge does not guarantee a higher expression of the oxidative markers, but it induces acute behavioral responses (i.e. trembling, vomiting, anorexia, among others) in the animal that deteriorates the animal welfare of the subjects challenged, as observed in the studies mentioned as an example.

The absence of differences among the oxidative markers in the present study could be also related to the breed. The piglets in current trials were crossbred (Large White x Landrace) x Pietrain from the same original farm. Other studies used crossbred Duroc × Landrace × Yorkshire, in which they were able to observe differences among the antioxidant markers [5, 21, 24, 25]. Thus, the breed factor might be a possible cause of the oxidative stress sensibility [13, 37]. Nguyen and McPhee [38] have reported Large White phenotypic advantages that might reduce the susceptibility towards oxidative damage, as Large White pigs are more resilient to high environmental temperatures. Also, Camara et al., [39] have reported that the inclusion of Pietran genetics might increase the growth performances of pig genetic lines, which might have an impact on the resilience to immune challenges. However, the mechanism underlying the breed-specific response towards stress is not fully understood and requires further studies [40]. Another point to consider when comparing the antioxidant results from other authors is the differences in the management and the facilities where the trials were performed, as environmental, sampling protocols, or other unmeasurable factors could affect the physiology of the piglets [14].

Literature has reported that higher doses of LPS (>60 μg/Kg BW) do not guarantee a measurable change in physiological parameters [26, 27, 30]. Furthermore, some antioxidant markers such as the CAT might reduce its own activity as Kang et al. [27], Yi et al. [26], and Cai et al. [30] observed when a high LPS dose was used (100 μg/kg BW). Large concentrations of LPS as an oxidative stressor would result in an amount of oxidative damage that constrains the antioxidant enzymes' energy [4] to respond with a higher capacity to control the oxidative menace [4, 41]. The activity of the antioxidant enzymes might be higher at relatively lower levels of the stressor.

The jejunum gene expression in the animals challenged with LPS in trials $C^{21/50}$ and $D^{21/50}$ detected the increase of the inflammatory or oxidative activity caused by the oxidative stimuli. Gene expression is a sensible method to detect responses towards oxidative challenges, either with mild oxidative stimuli (10 μg/kg BW on day 14 and 21 p.w.) [42], or severe doses of the oxidative challenge (100 μg/kg BW on day 14 and 21 p.w.) [43]. However, the practical use of this methodology is constrained due to the relatively elevated price of the analyses and the need for euthanasia to obtain the tissues.

On the other hand, behavioral responses were detected in both trials $C^{21/50}$ and $D^{21/50}$, LPS piglets displayed panting, prostration, tremors, and vomiting at a higher frequency than Ctrl piglets. Contrarily, Ctrl piglets in both trials $C^{21/50}$ and $D^{21/50}$ displayed significantly less of these discomfort behaviors. Animal behavior changes have been pointed out as an adaptative strategy to cope with the effects of a stressor and recover homeostasis [11, 44]. Thus, considering behavior as a physiological reflection of the state of an organism, it is plausible to propose the incidence of discomfort behaviors as a non-invasive oxidative stress indicator. Remarkably, the development of remote and continuous measurement methodologies to monitor animal behavior and welfare is urgently required by animal production stakeholders to effectively oversee the health and fitness of the animals [15, 45].

In the present study, the mild challenges resulted in discomfort in the pigs, as evidenced by their behaviors. However, there was no measurable antioxidant response in plasma or the intestinal mucosa. More detailed assessment methods, such as gene expression analysis, would be necessary to detect the changes caused by the mild challenges. Nevertheless, conducting such analyses

would require sacrificing the pigs and collecting tissue samples.

The combination measurement methodologies have the potential to provide an accurate approach to the assessment of oxidative stress [15]. However, behavioral observation methodologies are not able yet to quantify the magnitude of the impact of the oxidative challenge. A sensitivity scale of the behaviors related to stress responses can be a versatile tool for pig production stakeholders to assess the impact of stressors.

Finally, confronting the results of the current study with other results from the literature, severe LPS doses (>60 µg/Kg BW) might be considered physiological challenges as those can affect the physiological homeostasis of the pigs [5, 21–23, 25, 26, 31], reflected in the changes in antioxidant activity (gene expression or protein abundance). Contrarily, mild LPS doses, as the ones tested in the current trials (up to 50 µg/Kg BW), might be studied as physical discomfort that might not alter severely the physiological balance and is mainly reflected in the behavior of the animal. Important to consider is that piglets' resilience toward the LPS challenge was not measured (i.e. time to recover homeostasis after the intraperitoneal LPS injection) as sampling protocols were done aiming to detect the highest point of the physiological response. Also, if such a resilience test was done, more piglets would be exposed to the LPS challenge and handling protocol which would produce unnecessary discomfort for piglets and would increase the cost of the study. However, antioxidant results obtained in the current trials can suggest that the LPS doses did not trigger severe pathophysiological responses for the piglets and support the proposal of a mild oxidative stress model.

Conclusions

Based on the empirical results obtained, it is possible to assume that the magnitude of the physiological response may be associated with the intensity of the oxidative challenge (LPS dose) and the maturity of the animal's immune system (age). The presence of oxidative stress was verified through the measurement of gene expression markers in intestinal tissues but not in the plasma. On the other hand, our study might elucidate the possibility that behavior may be used as an indicator for continuous and non-invasive monitoring of piglets, which is sensitive enough for early detection of physiological responses to stressors able to induce oxidative stress. Nevertheless, behavior sensitivity and specificity have not been defined and require further research.

Animal models for research must guarantee the best possible welfare conditions, as well as researchers and producers' expectations in terms of accuracy, feasibility, and reproducibility of the results obtained. For this reason, the optimization and development of less intense stress experimental models would fulfill the *Refinement* concept from *the three R's* principle (*Replacement, Reduction, and Refinement*).

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Author contributions

RDG, JJP, SLV, XM, GT, and PL contributed to the conception and design of the study. RDG, JJP and SLV performed the study. RDG, JJP and SLV organized the database and performed the statistical analysis. RDG wrote the first draft of the manuscript (including Figures and tables). All authors contributed to the article and approved the submitted version.

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Data availability

Data is provided within the manuscript.

Declarations

Ethics approval and consent to participate

All procedures were approved by the Laboratory Animal Care Advisory Committee of the Universitat Autònoma de Barcelona (CEA/9310/P1).

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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