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Influence of body lesion severity on oxidative status and gut microbiota of weaned pigs

F. Correa [a], D. Luise [a], G. Palladino [b], D. Scicchitano [b], P. Brigidi [c], P.L. Martelli [b], G. Babbi [b], S. Turroni [b], G. Litta [d], M. Candela [b], S. Rampelli [b], P. Trevisi [a,*]

[a] Department of Agro-Food Sciences and Technologies, University of Bologna, Bologna, Italy
[b] Department of Pharmacy and Biotechnology, University of Bologna, Bologna, Italy
[c] Department of Medical and Surgical Sciences, University of Bologna, Bologna, Italy
[d] DSM Nutritional Products, Animal Nutrition and Health, Wurmisweg 576, 4303 Kaiseraugst, Switzerland

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ABSTRACT

Body lesions in pigs are a common welfare concern, particularly during the weaning period. These lesions can lead to pain, infection, and impaired mobility, resulting in reduced growth performance and increased mortality. Moreover, weaning stress can affect gut microbiota, immune response and increase the oxidative stress of piglets during this transition period. It has been hypothesised that social stress and body lesions could contribute to affect the gut microbiota, physiological and immune response of piglets. The study aims to evaluate the impact of the body lesions due to social stress on microbial profile, immune response, and oxidative status of weaned piglets. Lesion score (**LS**) on skin, tail, ear, neck, middle trunk, and hind quarters was measured 1 week (28 days of age, **T1**) and 7 weeks postweaning (**T2**) on 45 tail-docked pigs according to the method suggested from the Walfer Quality® (2009) on a scale from 0 to 2. Based on the LS, at T1, piglets were classified as High LS (n = 16), when LS was >1 in at least two of the areas considered, or Low LS (n = 29). At T2, based on the same scoring system and to the LS observed at T1, piglets were divided into four groups: High to Low LS (**H-L**, n = 11), High to High LS (**H-H**, n = 5), Low to Low LS (**L-L**, n = 21) and Low to High LS (**L-H**, n = 8). Blood and faecal samples were collected at T1 and T2. At T1, pigs with a high LS had a lower biological antioxidant potential compared with the L group ($P < 0.02$). At T2, the L-H group had a lower Reactive Oxygen Metabolites concentration compared with the H-H group ($P = 0.03$) while the L-L group had a lower concentration of Immunoglobulin A compared with H-H and L-H groups ($P = 0.02$ and $P = 0.04$, respectively). At T1, piglets with high LS had a different microbiota compared to piglets with low LS ($R^2 = 0.04$, $P < 0.01$). Low LS pigs were characterised by a higher abundance of Firmicutes, *Blautia*, *Eubacterium coprostanoligenes*, *Faecalibacterium*, *Megasphaera*, *Subdoligranulum* (*P*.adj < 0.05), while pigs with high LS were characterised by higher abundance of Bacteroidota, Rikenellaceae RC9, Prevotellaceae UCG-003, uncultured-Lachnospiraceae and uncultured-Oscillospiraceae (*P*.adj < 0.05). At T2, the H-H group were characterised by Oscillospirales-UCG-010, H-L by *Agatobachter* and L-L by *Alloprevotella* (*P*.adj < 0.05). Overall, this study provides valuable insights into the relationship between body lesions, oxidative stress, and gut microbiota in weaned pigs.

Implications

Our findings indicate how social stress due to body lesions can alter the physiological homeostasis and affect the intestinal microbiota of weaned pigs. This confirms the urgency of implementing management strategies to reduce lesion incidence, in order to avoid an increase in antibiotic use, which is linked to a higher susceptibility to infections.

Introduction

Conventional intensive pig production can represent a source of a variety of stressors for pigs, including limits in their ability to display natural behaviours such as socializing, exploration, and rooting (Studnitz et al., 2007). The limited ability to express their natural behaviours and the resulted psychological stress may lead to aggressive behaviours (Godyń et al., 2019). The occurrence of aggressive behaviours has been mostly linked to external factors, including housing and management, feeding management, feed contaminants (mycotoxins), poor environmental conditions such as suboptimal

* Corresponding author.
E-mail address: paolo.trevisi@unibo.it (P. Trevisi).

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temperature, poor air quality, lack or suboptimal enrichment materials, diet composition and poor health status (Prunier et al., 2020). This social stressor can affect the physiological status of the piglets. Indeed, chronic stress due to isolation or reduced space allocation has been linked to immune function suppression and induction of free radicals' production at different ages, resulting in an alteration of the blood antioxidation–oxidation balance (Hao et al., 2021).

In the last years, more attention was given to the possible involvement of gut microbiota in aggressive behaviours in pigs (Rabhi et al., 2020; Verbeek et al., 2021; Kobek-Kjeldager et al., 2022). Indeed, microbiota is involved in the regulation of several homeostatic systems of the host, such as the immune system, cardiovascular system, digestive system, and metabolic processes (Jin et al., 2019; Yoo et al., 2020). In addition, microbiota has been shown to affect behaviour via the microbiota-gut-brain axis. This axis can modulate behaviour and stress sensitivity through a complex bi-directional pathway, predominantly mediated by neuroimmune and neuroendocrine mechanisms, involving vagus nerve afferent fibres, hypothalamic–pituitaryadrenal axis, enteric nervous system, or the mucosal immune system. This communication is mediated by microbial metabolites (i.e. branched-chain amino acids and short-chain fatty acids) which can spread signals by interacting with enteroendocrine cells and the mucosal immune system. In addition, microbial metabolites can directly act on the hypothalamic-pituitary-adreno cortical axis (Cryan et al., 2019) by passing through the intestinal epithelium and reaching systemic circulation and eventually passing the blood–brain barrier. Psychological stress, on the other hand, can alter intestinal microbiota via the hypothalamic–pituitaryadrenal axis, increasing gut permeability and bacterial adherence, and increasing the passage of pro-inflammatory factors into the bloodstream (Wiley et al., 2017), eventually leading to microbial dysbiosis. The majority of studies on the microbiota-gut-brain axis have been conducted on rodents and humans. Recent studies, on the other hand, have discovered a link between gut microbiome and tail biting in pigs (Rabhi et al., 2020; Verbeek et al., 2021). Another study conducted by Wen et al. (2021) shown how a lack of environmental stimulation might alter the establishment of gut microbiota. Starting from these assumptions, we hypothesised that timing and intensity of the body lesions can affect the physiological homeostasis of the piglets and in turn affect their gut microbiota. Given the lack of studies specifically designed to investigate this aspect and following our preliminary result (Correa et al., 2022), we aim to evaluate whether lesion severity can affect microbiota profile, oxidative status, and immune status of weaned pigs.

Material and methods

Animals and study design

Forty-five weaned pigs from 23 litters (Landrace × Large White × Duroc) were selected for this study. At three days of age, the piglets underwent tail docking using cauterizing pliers without analgesia. Pigs were weaned at 28 days of age (9.65 ± 1.22 Kg BW) and were located in the same pen on a commercial farm. The pen had a completely slatted floor, and the environmental conditions were automatically controlled. The building had a natural ventilation system. On the day of weaning, the starting temperature in the weaning unit was 29 °C and was reduced of 1 °C each week until it reached 26 °C. The pigs had ad libitum access to a mash flour diet (Supplementary Table S1) and water from nipples. The animal density respected the UE directive 120/2008, and the pen was equipped with suboptimal enrichment materials (iron chain coupled with wood blocks). Lesion incidence was assessed at two time points: one-week postweaning (35 days of age) (**T1**) and 7 weeks postweaning (end of the weaning phase) (**T2**). Lesion evaluation was performed within the pen at a distance of 0.5 m from the pig, using a headlight when needed. The lesions were scored on a scale from 0 to 2 according to the Welfare Quality® (2009) guidelines and were measured on the skin, tail, ear, neck, middle trunk, and hindquarters of each pig. At each timepoint, based on the lesion score (**LS**), pigs were classified as high LS when LS was >1 in at least two of the areas considered, otherwise, they were classified as low LS. At T1, 16 piglets were classified as high LS and 29 as low LS. At T2, relying also on the observation at T1, pigs were classified into four categories: High to Low LS (**H-L**, n = 11), High to High LS (**H-H**, n = 5), Low to Low LS (**L-L**, n = 21) and Low to High LS (**L-H**, n = 8). Lesion score and additional phenotic data on the pigs involved in studies are reported in Supplementary Table S2.

Oxidative stress and acute phase proteins

Parameters of oxidative stress and acute phase proteins were measured on blood serum that was obtained by collecting blood samples at T1 and T2 from vena cava using a collection tube with clot activator (Vacutest, Kima). At T1, blood was drawn by placing the animals in dorsal recumbent and by securing their head, hinds, and forelimbs. At T2, blood was drawn by holding the pigs with a snout rope. To obtain serum, blood was kept at room temperature for 2 h and then centrifuged at 3 for 10 min. Then, serum was stored at −20 until further analysis. Parameters of oxidative stress included: Biological Antioxidant Potential (**BAP**), Reactive Oxygen Metabolites (**d-ROMs**) and advanced oxidative protein products. As acute phase proteins, ceruloplasmin, serum amyloid and haptoglobin were analysed. d-ROMs concentration was assessed using d-ROMs test, based on the fact that d-ROMs (mainly hydroperoxides), contained in the biological sample to be analysed, in the presence of iron (released from plasma proteins by an acid buffer), generate alkoxy and peroxyl which, reacting with a substituted aromatic amine oxidise the latter, transforming it into a pink-coloured derivative. The intensity of the developed colour is directly proportional to the concentration of the d-ROMs. BAP was assessed using the BAP test, based on the ability of a coloured solution of ferric ions (Fe3+) complexed to a particular chromogen to discolour when the ferric ions (Fe3+) are reduced to ferrous ions (Fe2+). The determination of advanced oxidative protein products was based on a spectrophotometric detection according to Witko-Sarsat et al. (1996) with some modifications. Forty µl of plasma diluted 1:5 with phosphate saline buffer, pH 7.4, 200 µl of chloramine T (0–100 µmol/L) for calibration and 200 µl of phosphate saline buffer as blank was applied on a microtiter plate. Ten µl of 1.16 M KI and 20 µl of acetic acid were added, and absorbance at 340 nm was measured immediately.

ELISA Kits specific for pig ceruloplasmin, serum amyloid and haptoglobin were used to determine the acute phase protein levels in pig serum. All procedures were performed according to the manufacturer's instructions (MyBioSource, San Diego, USA).

Ceruloplasmin concentration was measured using a double-sandwich ELISA commercial kit (MyBioSource, San Diego, USA) following the manufacturer's instructions. A standard curve ranging from 0.312 to 20 ng/ml and a sample dilution factor of 1:100 was used. Serum amyloid was quantified using the ELISA kit (MyBioSource, San Diego, USA) employing the technique of competitive inhibition enzyme immunoassay. The microtiter plate provided in this kit was precoated with pig serum amyloid A. Standards and samples (diluted 10-fold with phosphate saline buffer and 1/100 with kit sample diluted, total dilution 1/1 000) were pipetted into the wells with a Horseradish Peroxidase conjugated antibody specific. Haptoglobin was quantified using the ELISA kit (MyBioSource, San Diego, USA). Microtiter plate (provided in the kit) was precoated with an antibody specific to haptoglobin. A standard curve ranging from 15.6 to 1 000 ng/ml and a sample dilution factor of 1/1 000 were

used. Standards or samples were then added to the appropriate microtiter plate wells with a biotin-conjugated polyclonal antibody preparation specific for haptoglobin and avidin conjugated to horseradish peroxidase and incubated. Then, a tetramethylbenzidine substrate solution was added to each well. The enzyme-substrate reaction was terminated by the addition of a sulphuric acid solution and the colour change was measured spectrophotometrically at a wavelength of 450 ± 2 nm.

Immunoglobulins serum concentration

Prior to Immunoglobulin (**Ig**) analysis, serum samples were incubated at 56 °C for 30 min. Serum concentration of IgA, IgM and IgG was analysed using an Ig ELISA assay (Bethyl Laboratories, Montgomery, USA) following the protocol described by Bosi et al. (2007). Briefly, serum samples collected at T1 were diluted in carbonate–bicarbonate buffer, 50 mM, pH 9.4, at 1: 16 000 for IgA, 1: 300 000 for IgG and 1:8 000 for IgM. Serum samples collected at T2 were diluted at 1: 16 000 for IgA, 1: 600 000 for IgG and 1: 25 000 for IgM. For total IgA, IgM and IgG detection, 96-well microtiter plates were coated with goat anti-pig sIgA, goat anti-Pig IgM heavy chain and goat anti-pig IgG heavy and light chain, affinity purified (Bethyl Laboratories, Montgomery, USA), respectively. Subsequently, phosphate saline buffer supplemented with 2 ml/l Tween 20 was added to the wells to block the aspecific binding sites. Pig Immunoglobulin Reference Serum (Bethyl Laboratories, Montgomery, USA) was used as a specific antibody for the standard curve, goat anti-pig sIgA, goat anti-pig IgM heavy chain and goat anti-pig IgG heavy and light chain horseradish peroxidase conjugates (Bethyl Laboratories, Montgomery, USA) were used as a secondary antibody, respectively, and **2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)** (ROCHE Diagnostics, Basel, Switzerland) as an enzyme substrate. The reaction was quantified spectrophotometrically at an absorbance of 405 nm using a microplate reader (Multiskan FC Microplate PhotometerThermo, Fisher Scientific). Concentration values were calculated using a four-point parametric curve and were expressed in mg/mL.

Microbial analysis

For the microbiota analysis, bacterial DNA extraction from the faecal samples was carried out using FastDNA SPIN Kit for Soil (MP Biomedicals, Santa Ana, Ca, USA) following the manufacturer's instruction, as indicated by Correa (2022). DNA concentration and purity (absorbance ratio 260/280 and 260/230) of the isolated DNA were checked by spectrophotometry using a NanoDrop (Fisher Scientific, 13 Schwerte, Germany). The V3-V4 region of the 16S rRNA gene (~460 bp) was amplified, and amplicons were produced using the universal primers: TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG and GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC and using the KAPA HiFi Hotstart Taq DNA Polymerase (KAPA Biosystems, Wilmington, MA, USA) and sequenced using the Illumina NextSeq instruments. The libraries were prepared using the standard protocol for MiSeq Reagent Kit v3 (Illumina Inc., San Diego, Ca, USA). For the bioinformatics analysis, the DADA2 pipeline was used considering the Silva database (version 138) as reference for the taxonomic assignment.

Statistical analysis

The statistical analysis on oxidative stress parameters and Ig concentrations was carried out on R (v4.1.1) software using *car* (v3.0-12) and *emmeans* (v1.7.3) packages, fitting a linear model including the LS class and litter of origin as fixed factors. For the predefined contrasts (H-H vs H-L, H-H vs L-L, H-H vs L-H and L-L vs L-H), H-H was considered as chronic stress group, L-H the acute stress group and L-L and H-L as the reference baseline groups. Contrasts were performed using a Tukey test as posthoc test. Bioinformatic analysis on microbiota data was carried out on R (v4.1.1), the relative abundance of the taxa was normalised using the centred log-ratio transformation. Bacterial richness was estimated using the R package *breakaway* v4.7.5. For the alpha diversity, an ecological network regression model to estimate Shannon and Simpson diversity indices was carried out using the R package *DivNet* v0.4. Differences in bacterial richness, Shannon, and InvSimpson diversity indices between groups were tested using the hierarchical model Betta, which accounts for incomplete community sampling. For the Beta diversity, a dissimilarity matrix using a Euclidian distance on centred log-ratio transformed values was constructed. Results were plotted using Principal coordinate analysis, and then, a permutational ANOVA test was performed to test for any correlation between community composition and LS classification, with 10 000 permutations. LefSe analysis at phylum and genus levels was applied to identify taxa differentially expressed (Effect size > 3 and *P*.adj < 0.05) between experimental group for T1 and T2.

P-values <0.05 were considered statistically significant while *P*-values <0.1 were considered a trend of significance.

The R script used to carry out all the statistical analysis can be found in the Supplementary Material S1.

Results

Oxidative status and immunoglobulins serum concentration

Table 1 shows the effect of LS on the oxidative parameters of the piglets at T1. The H group had a lower BAP compared with the L group (P = 0.02); no differences were observed for d-ROMs, haptoglobin, ceruloplasmin, serum amyloid, advanced oxidative protein products, IgA, IgG and IgM concentration. Table 2 shows the effect of class of LS on oxidative parameters and Ig concentrations of pigs at T2. The H-H group had a higher d-ROMs concentration compared with L-H group (P = 0.03), a higher concentration of IgA compared with the L-L group (P = 0.02) and tended to have a lower IgG concentration compared with the L-L group (P = 0.09). In addition, the L-H group had a higher IgA concentration compared with the L-L group (P = 0.04). No differences were observed for BAP, aptoglobin, ceruloplasmin, serum amyloid, advanced oxidative protein products and IgM concentration between groups.

Microbial profile

A total of 2 287 699 quality checked reads were recovered after the bioinformatic analysis, that were assigned to 4 476 different

Table 1
Effect of lesion incidence on oxidative status and serum immunoglobulin concentration of piglets one-week postweaning.

Parameter	Mean		SEM	*P*-value	
	L (n = 29)	H (n = 16)		LS class	Litter
d-Roms, CARR U	247.41	250.71	5.20	0.71	0.03
BAP, µmol/L	2 227.82	1 977.48	58.71	0.02	0.02
HP, ug/ml	42.55	43.21	0.66	0.56	0.03
CER, ng/ml	67.35	64.88	7.07	0.84	0.21
SAA, ng/ml	38.31	31.72	2.52	0.13	0.01
AOPP, nmol/mg	3.08	1.91	0.56	0.22	0.69
IgM, ng/mL	0.70	0.78	0.47	0.73	0.36
IgA, ng/mL	1.57	1.41	0.85	0.76	0.95
IgG, ng/mL	9.41	8.71	6.56	0.73	0.01

Abbreviations: d-ROMs = Reactive Oxygen Metabolites; CARR U = Carratelli Units; BAP = Biological Antioxidant Potential; HP = Haptoglobin; CER = Ceruloplasmin; SAA = serum amyloid A; AOPP = Advanced Oxidative Protein Products; Ig = immunoglobulins, LS = Lesion score; L = Low Lesion Score; H = High Lesion Score.

Amplicon sequence variants. One sample (Low LS, T1) was excluded from analysis for low DNA yield. From a taxonomic perspective, 19 phyla were identified, the most abundant belonged to Firmicutes 74.78 ± 1.38%, Bacteroidota 20.66 ± 0.29%, Actinobacteriota 1.30 ± 0.19% and Spirochaetota 1.19 ± 0.15%. At family level, 93 families were identified, with Lactobacillaceae 24.19 ± 6.00%, Lachnospiraceae 14.81 ± 0.37%, Prevotellaceae 13.77 ± 0.31% and Ruminococcaceae 6.36 ± 0.36% being the most abundant. At genus level, 212 genera were identified, the majority belonged to *Lactobacillus* 24.19 ± 6.00%, *Prevotella* 8.28 ± 0.39%, *Megasphaera* 5.04 ± 2.94% and *Agathobacter* 3.14 ± 0.55% genera. The effect of LS class on the estimated richness, Shannon and InvSimpson diversity of faecal samples is showed in Fig. 1. At T1, no differences were observed between H and L for Estimated richness, Shannon and InvSimpson (Fig. 1A). At T2, the L-L group had a significantly lower Richness and Shannon diversity compared to the H-H and L-H groups ($P < 0.001$) (Fig. 1B). In addition, bacterial richness tended to be lower in the L-H group compared to H-H group ($P = 0.057$)

Table 2
Effect of lesion incidence on oxidative status and serum immunoglobulin concentration of piglets 7-week postweaning.

Parameter	Mean				SEM	*P*-value		Pair-wise contrasts, *P*-value			
	L-L (n = 21)	L-H (n = 8)	H-L (n = 11)	H-H (n = 5)		LS class	Litter	H-H vs H-L	H-H vs L-L	H-H vs L-H	L-L vs L-H
d-Roms, CARR U	259.85	228.90	256.13	266.74	7.85	0.02	0.03	0.65	0.45	0.03	0.69
BAP, µmol/L	2 061.01	2 127.07	1 985.92	2 250.05	94.81	0.41	0.32	0.16	0.27	0.53	0.51
HP, ug/ml	48.65	47.03	46.57	49.38	1.85	0.73	0.44	0.43	0.83	0.54	0.35
CER, ng/ml	95.98	90.37	92.73	106.32	13.00	0.94	0.71	0.59	0.66	0.55	0.83
SAA, ng/ml	111.59	92.28	88.84	68.91	16.26	0.36	0.02	0.52	0.15	0.49	0.25
AOPP, nmol/mg	1.59	1.53	1.64	0.52	0.39	0.46	0.51	0.14	0.13	0.22	0.91
IgM, ng/mL	1.74	1.83	1.25	1.48	0.27	0.39	0.16	0.67	0.59	0.53	0.14
IgA, ng/mL	1.65	2.57	3.71	5.26	0.79	0.05	0.62	0.31	0.02	0.11	0.04
IgG, ng/mL	17.75	16.97	14.36	7.65	3.23	0.35	0.28	0.29	0.09	0.17	0.38

Abbreviations: d-ROMs = Reactive Oxygen Metabolites; CARR U = Carratelli Units; BAP = Biological Antioxidant Potential; HP = Haptoglobin; CER = Ceruloplasmin; SAA = serum amyloid A; AOPP = Advanced Oxidative Protein Products; Ig = immunoglobulin; LS = Lesion score; H-L = High to Low Lesion score, H-H = High to High Lesion score, L-L = Low to Low Lesion score, L-H = Low to High Lesion score.

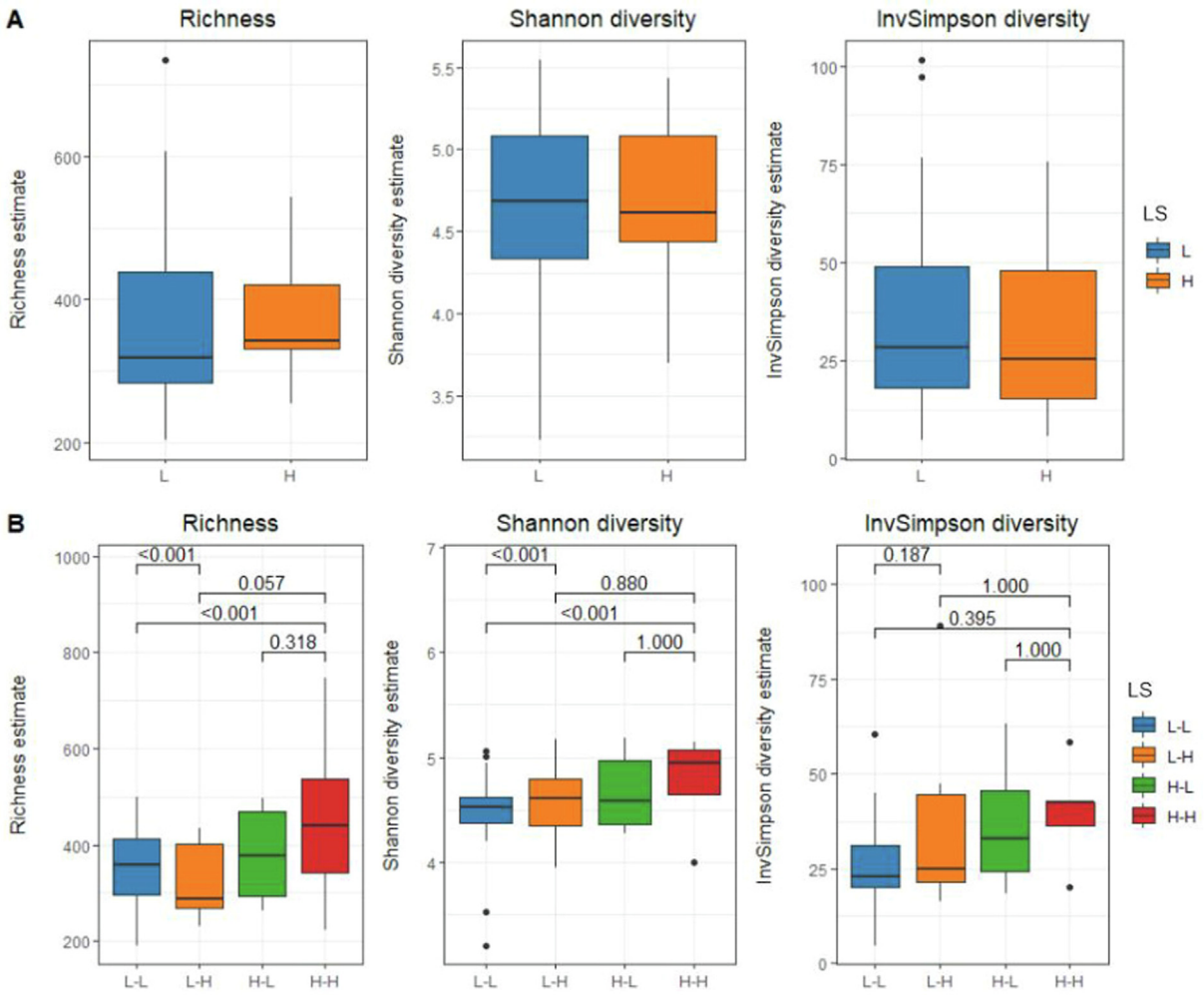

Fig. 1. Boxplots showing estimated richness, Shannon and Inverse Simpson diversity of piglets' faecal samples at T1 (A) and T2 (B). Abbreviations: LS = Lesion score; L = Low Lesion Score; H = High Lesion Score; H-L = High to Low Lesion score, H-H = High to High Lesion score, L-L = Low to Low Lesion score, L-H = Low to High Lesion score, T1 = 28 days of age, T2 = 7 weeks postweaning.

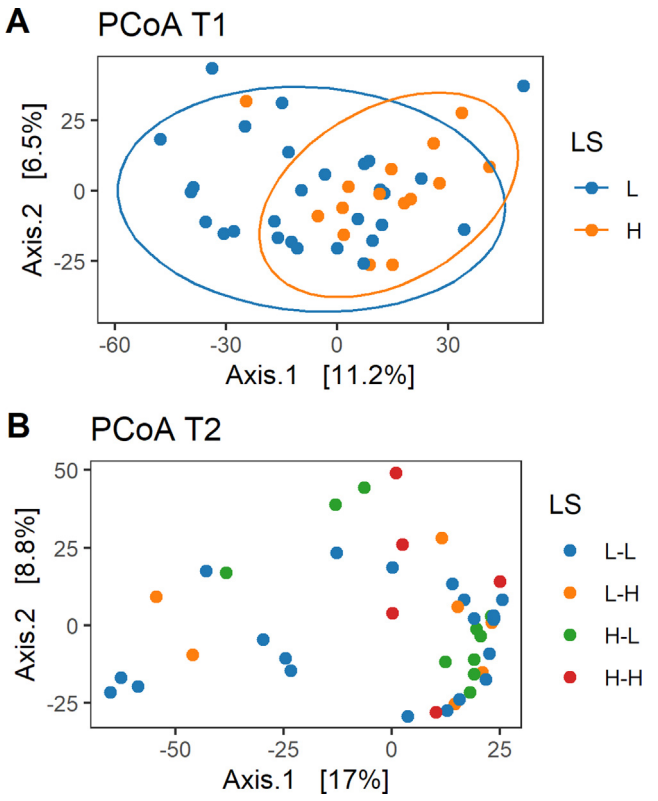

Fig. 2. PCoA plot showing beta diversity of piglets faecal samples at T1 (A) and T2 (B) constructed using a dissimilarity matrix using Euclidian distances on centred log-ratio transformed values. Abbreviations: PCoA = Principal Coordinates Analysis; LS = Lesion score; L = Low Lesion Score; H = High Lesion Score; H-L = High to Low Lesion score, H-H = High to High Lesion score, L-L = Low to Low Lesion score, L-H = Low to High Lesion score, T1 = 28 days of age, T2 = 7 weeks postweaning.

(Fig. 1B). For the beta diversity, the Principal Coordinates Analysis in Fig. 2A shows that samples tend to separate based on their LS score at T1. In addition, the PERMANOVA test confirmed that the beta diversity was significantly affected by LS score class (R^2 = 0.04, P = 0.008) and litter of origin (R^2 = 0.54, P = 0.04). At T2, the beta diversity was not affected by the LS score class (R^2 = 0.07, P = 0.46) and tended to be affected by the litter of origin (R^2 = 0.56, P = 0.074) (Fig. 2B).

Results of the LefSe analysis performed at T1 are reported in Fig. 3. At phylum level, the pigs of the L group were characterised by a higher abundance of Firmicutes (Effect size = 4.73, *P*.adj = 0.02) and a lower abundance of Bacteroidota (Effect size = 4.70, *P*.adj = 0.03) (Fig. 3A). At genus level, the pigs of the L group were characterised by a higher abundance of *Blautia* (Effect size = 3.93, *P*.adj = 0.01), *Faecalibacterium* (Effect size = 3.77, *P*.adj = 0.01), *Megasphaera* (Effect size = 3.74, *P*.adj = 0.03), *Subdoligranulum* (Effect size = 3.71, *P*.adj = 0.01), *Eubacterium coprostanoligenes* group (Effect size = 3.72, *P*.adj = 0.03) and *Solobacterium* (Effect size = 3.51, *P*.adj = 0.01) (Fig. 3B). The pigs of the H group were characterised by a higher abundance of Oscillospirales-UCG-005 (Effect size = 4.13, *P*.adj = 0.01), Rikenellaceae RC9 gut group (Effect size = 4.04, *P*.adj = 0.01), Bacteroidales-p-251-o5 (Effect size = 3.82, *P*.adj = 0.04), Lachnospiraceae (uncultured genera) (Effect size = 3.82, *P*.adj = 0.04), Bacteroidales-F082 (Effect size = 3.69, *P*.adj < 0.001), Oscillospiraceae (uncultured genera) (Effect size = 3.63, *P*.adj < 0.001), Prevotellaceae UCG-003 (Effect size = 3.59, *P*.adj = 0.02) and Family XIII AD3011 group (Effect size = 3.54, *P*.adj = 0.03). Results of the LefSe analysis from T2 are reported in Fig. 4. No differences among groups were observed at phylum level. At genus level, the pigs of the L-L group were characterised by a higher abundance of *Alloprevotella* (Effect size = 3.98, *P*.adj = 0.02); the pigs of the H-L by a higher abundance of *Agathobacter* (Effect size = 3.79, *P*.adj = 0.02) and the pigs of the H-H group by a higher abundance of Oscillospirales-UCG-010 (Effect size = 3.91, *P*.adj = 0.03). No discriminant genera were observed for the L-H group.

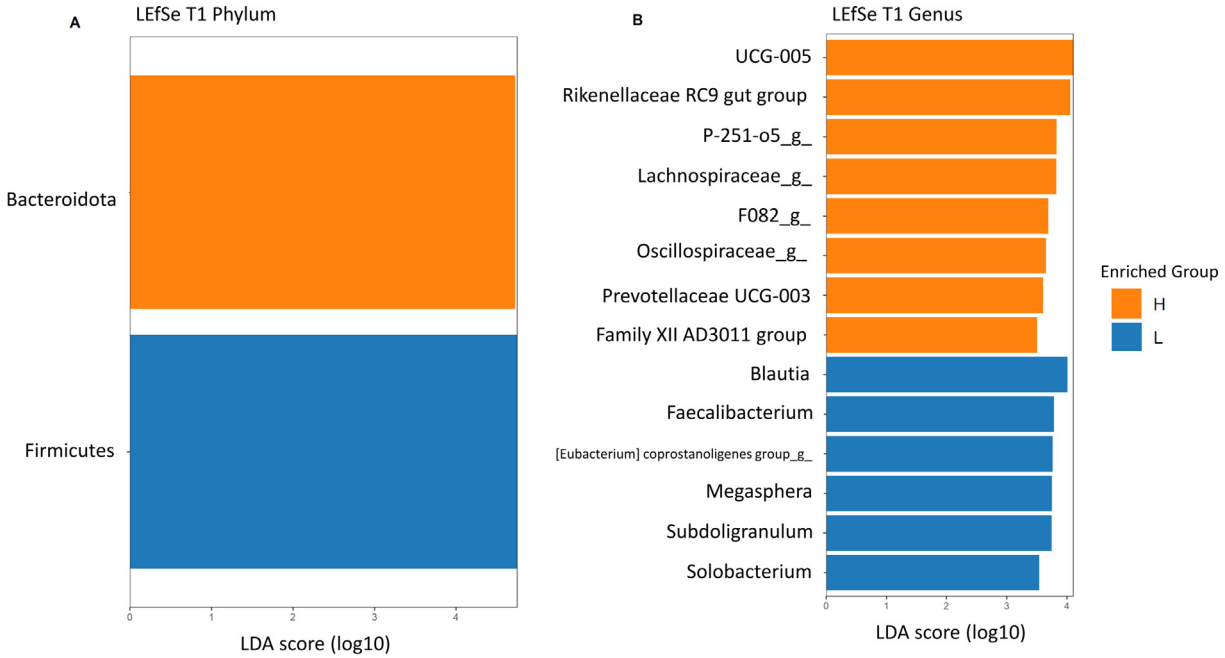

Fig. 3. Linear discriminant analysis effect size (LEfSe) plots of the biomarkers taxa, identified in piglets faecal samples, for the LS category at Phylum (A) and Genus level (B) at T1. Abbreviations: LS = Lesion score; LDA = Linear discriminant analysis; L = Low Lesion Score; H = High Lesion Score, T1 = 28 days of age.

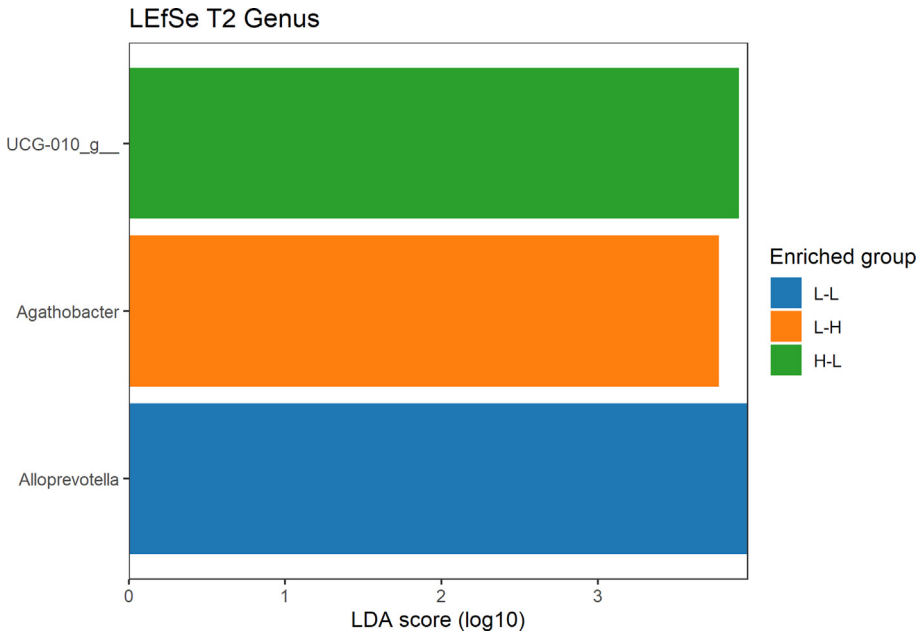

Fig. 4. Linear discriminant analysis effect size (LEfSe) plots of the biomarkers taxa, identified in piglets faecal samples, for the LS category at Genus level at T2. Abbreviations: LS = Lesion score; LDA = Linear discriminant analysis; H-L = High to Low Lesion score, L-L = Low to Low Lesion score, L-H = Low to High Lesion score, T2 = 7 weeks postweaning.

Discussion

Taken together, results indicate that, one-week postweaning, pigs with a high LS had an impaired antioxidant defence system and a different microbial profile, characterised by a higher abundance of Bacteroidota and a reduction of Firmicutes compared with pigs with a low LS. Six weeks later, the pigs that continued to have a high LS showed a higher oxidative stress as well as modifications in their immunological status.

As reported by Sauerwein et al. (2005), d-ROMs and acute phase proteins can provide an objective measure of the oxidative and health status of an animal and they are increasingly used as markers of animal health and welfare. In our study, at one-week postweaning, BAP concentration was lower in high LS pigs, while no difference was observed for d-ROMs between groups. BAP express the combined effects of several antioxidants' molecules including uric and ascorbic acids, proteins, alpha-tocopherol or bilirubin (Benzie and Strain, 1996). A transient reduction of BAP level in the first-week postweaning has been observed by Buchet et al. (2017), and it reflects the use of antioxidant reserves, in particular of vitamins E and A, to cope with the high production of free radicals. The antioxidant enzyme activities start to be restored after the first-week postweaning (Luo et al., 2016). However, in the present study, this recovery phase was impaired in pigs more exposed to aggressive behaviours, and this could be linked to a higher production of free radicals during the first days postweaning that led to a depletion of the antioxidant reserves. In addition, seven weeks postweaning, when the antioxidant capacity is restored, pigs which continued to present a high LS (H-H group) had an impaired antioxidant status, characterised by a higher d-ROMs concentration; however, this was only observed if compared with L-H pigs. Taken together, this finding may suggest that, in the first-week postweaning, stress associated to body lesion can exacerbate the transient physiological reduction in antioxidant capacity and cause oxidative stress. Whereas seven weeks postweaning, when the antioxidant capacity is restored, stress linked to a high LS score, in the same pigs, leads to increased d-ROMs production. However, this aspect needs to be elucidated in further studies.

In addition to oxidative stress, social stress and aggressive behaviours have been shown to affect the immune system. The most widely held opinion is that stress decreases immune system components; however, some authors have found that stress can enhance immunity (Wrona et al., 2001). The precise response of the immune system to stress remains controversial, owing to the complexity of the immune response. Furthermore, the response depends on the types and durations of the stressors, age, genetics, and social status (Salak-Johnson and McGlone, 2007). In our study, seven weeks after weaning, pigs with a high LS at both timepoints (H-H group) or just at the second timepoint (L-H group) had a higher IgA concentration compared to pigs that consistently had a low LS (L-L group). Usually, the IgA concentration in serum or saliva is used as a reliable marker of acute stress in response to various stressors in pigs (snitch restraint, isolation, mixing) (Muneta et al., 2010; Kanitz et al., 2019). The results of the present study agree with what was observed by Kanitz et al. (2019), in which study an increase of the IgA concentration in blood serum related to injuries due to social interaction was observed. The increase in IgA production seems to be linked to its strong anti-inflammatory action, which is mediated by the FcαRI receptor to avoid an excessive immune response (Monteiro, 2014). As a result, pigs with a high LS could be more exposed to inflammation related to open wound, and a high level of serum IgA may be a functionally suitable response to avoid the development of an excessive inflammatory response. A tendency for a lower IgG serum concentration was observed in the H-H pigs compared to L-L pigs. Previous studies have shown how social stress related to social hierarchy establishment that causes a chronic stress is linked to an immune-suppressive activity (Hessing et al., 1994; Tuchscherer et al., 1998; de Groot et al., 2001). In our study, acute stress due to aggressive behaviours may lead to inflammation and overproduction of IgA, while chronic stress observed in pigs who were always subjected to aggressive behaviours could have an immunosuppressive activity.

Since the first study showing how gut microbiota can affect behaviour in mice (Heijtz et al., 2011), research in pigs has started to assess in which extent gut microbiota can be linked to aggressive behaviours and the resulting social stress. Two studies were

carried out in pigs (Rabhi et al., 2020; Verbeek et al., 2021), however, these studies were only focused on the occurrence of tail biting, while to our knowledge, no studies have been carried out on the effect of social stress related to aggressive behaviours on gut microbiota. Our results show that one week after weaning, gut microbiota is significantly different between pigs who had a higher LS compared with the other with a low LS. These results can be compared with the results of Rabhi et al. (2020), who observed how biter and bitten pigs, who experienced a higher stress compared with negative control pigs, had significantly different gut microbiota compared with the latter. However, these differences were not observed later, this could be related to the fact that most of the aggressive behaviours occur in the first-week postweaning when social hierarchy must be established, and then tend to diminish (Fels et al., 2014). The higher social stress due to aggressive behaviours in the first-week postweaning was associated to a decrease in Firmicutes abundance and an increase in bacteria from the Bacteroidota phylum. These findings agree with several studies involving humans diagnosed with major depressive disorder or in rodents exposed to social stress (Bailey et al., 2011; Jiang et al., 2015). In pigs, a reduction of Firmicutes have been reported in victims of tail biting by Verbeek et al. (2021). However, in our case, the reduction of Firmicutes involved mainly bacteria belonging to *Megasphaera*, *Subdoligranulum* and *Eubacterium coprostanoligenes* genera, while, in the study of Verbeek et al. (2021), this was primarily driven by a reduction of the families Lachnospiraceae, Ruminococcaceae and Clostridiales Family XIII. The differences on microbial profile, especially at genera level, can be ascribed to the different environmental conditions and the diets, which differ among countries. Moreover, pigs with high lesions at the first observation were characterised by a higher abundance of Prevotellaceae-UCG03; this agrees with the results of Jiang et al. (2015), who observed an alteration in the abundance of *Prevotella* linked to major depressive disorder in humans. Similarly, a higher abundance of *Prevotella* was recorded in stress-susceptible mice who were exposed to mild chronic social defeat (McGaughey et al., 2019). The mechanism behind the alteration of this bacterial taxa is still unclear; *Prevotella* is not traditionally considered a pathogenic genus, but recent evidence suggested that it may play a role in causing gut inflammation and the subsequent release of substances which can worsen anxiety and depression symptoms. This is thought to happen by interfering with the regulation of cortisol, a hormone that, when elevated, has been linked to increased symptoms of anxiety and depression (Miller et al., 2013).

In addition, in the study of McGaughey et al. (2019), the defeated mice displayed an increase in the relative abundance of unclassified genera of the Lachnospiraceae family. This result agrees with the result observed in the present study as a reduction of Lachnospiraceae was seen in pigs with high LS.

The differences in microbiota composition observed in the first timepoint between pigs with high or low LS can also help to explain the reduction of BAP observed in pigs with high LS. As mentioned earlier, serum BAP is highly influenced by serum concentration of liposoluble vitamin (vitamins A and E); the intestinal absorption of these vitamins can be influenced by the microbiota through microbial deconjugation of bile salts (Lauridsen et al., 2021). Several bacteria, including *Bacteroides*, *Bifidobacterium*, *Clostridium* and *Lactobacillus*, possess bile salt hydrolase enzymes which can reduce the efficacy of bile acids for the emulsification of dietary lipid and micelles formation. In the present study, no differences in the abundance of these bacteria were observed, however, a general increase in the Bacteroidota phylum, which comprise *Bacteroides* and the mentioned genera, was seen; therefore, a reduction in BAP in pigs with high LS could be due to a modification in the microbial deconjugation of bile salts by the Bacteroidota phylum.

The effect of lesion severity on the gut microbiota structure during the second timepoint seems to be more limited, and this could be related to the fact that stress related to the establishment of social hierarchy is probably lower (Fels et al., 2014). However, subjects who experienced a higher incidence of lesions at both timepoints had a significantly higher bacterial richness and Shannon diversity compared with those who had a lower lesion score in both timepoints. This result contradicts with most of the research carried out in other animals, in which social stress has been linked to a reduction of alpha diversity and a disruption of the gut microbial ecosystem (Bailey et al., 2011). However, the assumption that a high microbial diversity always represents a favourable condition for the bacterial community to respond to external disturbances has been argued (Shade, 2017). In addition, the lower number of pigs within the H-H group did not allow to make solid inferences. Moreover, differences observed in this study can be also explained by other factors which were not recorded. For instance, a reduction in feed intake that may have been occurred in pigs more exposed to aggressive behaviour could help to explain some of the results, this can be especially true for the faecal microbiota composition. However, this study was conducted in a commercial farm and it was not possible to measure individual feed consumption.

Conclusion

In conclusion, our findings indicated that the occurrence of aggressive behaviour which can lead to body lesions in early postweaning negatively affected the physiological homeostasis of the piglets, by altering their oxidative status and affecting their gut microbial profile. The long-term effect of these physiological alterations resulted in a modulation of their immune response until 7 weeks postweaning. Finally, our finding consolidates the knowledge regarding the detrimental effect of aggressive behaviours on piglets' health, confirming the urgency to implement management strategies to reduce them (i.e. increasing the space allowance and group size, mixing the litters during the lactation, selecting less aggressive breeds or using nutritional additives and appeasing pheromones), in order to avoid an increase in the antibiotic use linked to a higher susceptibility to infections.

Supplementary material

Supplementary material to this article can be found online at https://doi.org/10.1016/j.animal.2023.100818.

Ethics approval

The procedures complied with Italian law pertaining to experimental animals and were approved by the Ethic-Scientific Committee for Experiments on Animals of the University of Bologna (Italy) and by the Italian Ministry of Health (Prot. N. 574/2019-PR, released on the 29th July 2019).

Data and model availability statement

The datasets used and/or analysed during the current study are available from the corresponding author upon reasonable request. Raw reads are publicly available at Sequence Read Archive under the accession number PRJNA886053.

Author ORCIDs

DL: https://orcid.org/0000-0001-7864-7822.
FC: https://orcid.org/0000-0001-8558-6503.
GP: https://orcid.org/0000-0002-5432-919X.

DS: https://orcid.org/0000-0002-1583-7239.
PB: https://orcid.org/0000-0001-8375-6150.
PLM: https://orcid.org/0000-0002-0274-5669.
GB: https://orcid.org/0000-0002-9816-4737.
ST: https://orcid.org/0000-0003-2345-9482.
GL: https://orcid.org/0000-0001-8079-1743.
MC: https://orcid.org/0000-0001-7420-790X.
SR: https://orcid.org/0000-0002-5655-6695.
PT: https://orcid.org/0000-0001-7019-6828.

Author contributions

PT, PLM, MC and **PB** designed the experiment. **FC, DL** and **PT** performed the experiment and collected samples. **DS, GP, ST** and **SR** performed the laboratory work, including 16S rRNA gene amplification, and library preparation for sequencing, while **FC, DL** and **GB** analysed the data. **GL** performed the analysis of oxidative and inflammation marker. **FC, DL** and **PT** conceptualised the paper, compiled all the information, and prepared the manuscript. All authors read and approved the final manuscript.

Declaration of interest

The authors declare that there is no conflict of interests regarding the publication of this article.

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