

Exploring immuno-genetic resistance in pigs to the food-borne zoonotic pathogen, *Toxoplasma gondii*: A serological study in Central Italy

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Received: 10 May 2024; Accepted: 13 June 2024; Published: 1 July 2024

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SHORT COMMUNICATION

Abstract

Toxoplasmosis is a re-emerging food-borne zoonosis of warm-blooded animals caused by *Toxoplasma gondii*, an obligate intracellular parasite with a complex biological cycle. Until now, the genotypic approach for discovering putative resistance immuno-genetic markers has never been adopted for *T. gondii* infection. Thus, this study aimed, for the first time, to deepen these novel insights. In particular, pigs, reared in different farms in Central Italy, were phenotypically characterized by serological assays performed on diaphragm meat juice. Out of 179 tested animals, 98 resulted seropositive (54.74%), 57 seronegative (31.84%), and 24 doubtful (13.40%), underlining a possible re-emerging diffusion of this protozoan in the investigated areas. Contextually, an RT-PCR assay, followed by Sanger sequencing for *IL-18*, a pro-inflammatory cytokine with a key role upstream of the infection, was developed. At this stage, for this interleukin, significant polymorphic variations were not detected compared to the reference sequence, except in a seropositive animal. However, the starting outcomes of this novel and preliminary study will be investigated in depth with different approaches also on other target genes, with a crucial activity in the immunity pathway towards *T. gondii*, to unveil the possible presence of resistance genetic mechanisms and, finally, to make pork derived food safer.

Keywords: food safety; genetic resistance; *interleukin-18* gene; *One Health*; pig's serological survey; *Toxoplasma gondii*

Introduction

Toxoplasma gondii is a zoonotic, obligate, intracellular protozoan parasite characterized by a complex biological cycle and by the capacity to infect almost all warm-blooded animals, such as humans, livestock, marine mammals, and also birds. The definitive hosts are members of the Felidae family, including domestic cats, in which the extra-intestinal/intestinal sexual cycle of the parasite takes place, characterized by the fecal release

of immature oocysts. In infected warm-blooded animals, the latter is converted to bradyzoites with high tropism for nervous and muscular tissues (Pal *et al.*, 2021; WOAH Terrestrial Manual, 2018). Due to its prevalence and spread, toxoplasmosis is considered an emergent and re-emergent zoonosis and a major public health threat (Pal *et al.*, 2021). In fact, it is estimated that approximately one-third of the world's population has been exposed to the parasite (Dubey *et al.*, 2020; Montoya & Liesenfeld, 2004). Public health issues have been emphasized by

recent studies that have highlighted the possible association of *T. gondii* infection with the onset of neurodegenerative diseases, such as Alzheimer (Bayani *et al.*, 2019) but also brain tumors, particularly glioma in adults (Hodge *et al.*, 2021). While the parasite is sub-clinical in immunocompetent individuals, in pregnant women it may cause abortion or fetal malformations, and immunocompromised individuals like AIDS/HIV seropositive or cancer patients may develop an acute, even lethal infection (Rorman *et al.*, 2006; Wang *et al.*, 2017).

In the majority of the animal species, the infection does not cause clinical illness while in others, such as sheep, goats, and pigs, it causes acute life-threatening forms manifesting itself as a disease of pregnancy (WOAH Terrestrial Manual, 2018). Even if the clinical signs can be more or less marked, toxoplasmosis in animals determines farm management problems and economic issues also for breeders, as well as having a strong impact on animal welfare in indoor or outdoor farms (Dubey, 2009).

Transmission to humans has an environmental or food-borne origin (Dubey, 2010), mainly through the ingestion of food or water contaminated with oocysts excreted by infected cats and through the consumption of raw, undercooked, or cured meat (EFSA, 2024). Pork meat represents the main source of transmission and pig (*Sus scrofa*) is considered highly susceptible to *T. gondii* infection. In particular, pigs of all ages may be infected as a result of an outbreak (Condoleo *et al.*, 2018). In the recent past, the detection of *T. gondii* cysts and DNA in processed or cured pork meats (Costa *et al.*, 2018) has raised concern about the product's food safety. Despite the global resonance of *T. gondii* as a zoonotic agent, currently, the main preventive action is the non-consumption of raw, undercooked, or cured meat. Furthermore, for animals, the only commercial vaccine available is the attenuated tachyzoite S48 strain (Toxovax® – Merck & Co., Inc) used only for sheep and that allows only the development of less infective tissue cysts (Zhang *et al.*, 2022).

Thus, alternative prevention and control measures need to be developed (Discontools, 2024).

It is important to state that, mainly in the last decades, an effective and alternative strategy to prevent or control various infectious diseases, based on studies of immunogenetic resistance traits, has been introduced. In order to understand the genetic rules of resistance pathways, different approaches could be adopted, correlating candidate genes, genetic and epigenetic regulation, pedigree status or genome-wide data with phenotypic information as, for instance, those concerning the immuno-competence. The “candidate gene” approach is hypothesis-driven as opposed to the “genome-wide” which is based on the

discovery (Emam *et al.*, 2019). The main method is based on single nucleotide polymorphisms (SNPs) genotyping. In particular, once the haplotypes/genotypes profile related to the disease resistance/susceptibility phenotype have been identified and statistically associated. Marker Assisted Selection (MAS) intervention could be potentially adopted for the breeders, in order to create lines and populations potentially resistant or “resilient” to one or more pathogens. In this regard, the host's genetic attitude towards pathogens of different natures has been evaluated over time, by different research groups (Arcangeli *et al.*, 2021; Fratto *et al.*, 2024; Mazzone *et al.*, 2023; Torricelli *et al.*, 2021, 2023).

That considered, the above-mentioned tools and approaches in the field of genetic resistance have not been investigated or adopted for toxoplasmosis in livestock animals now. In all intermediate hosts, *T. gondii* infection includes different immune pathways, partly to be explored yet (Sasai *et al.*, 2018). Till now, there is no complete clarity on the role of some mediators and pattern recognition receptors (PRRs) as well as how and which toll-like receptors (TLRs) contribute to the parasite recognition and internalization in hosts, such as in pigs (Mukhopadhyay *et al.*, 2020). Thus, it could be of great interest to investigate the presence of possible mutations and polymorphisms (i.e., SNPs) in genes involved upstream of the immunity pathway towards *T. gondii*, prior to the rapid and critical step of the cyst formation and/or diffusion in host tissues in order to make the meat products safer. This is the case of the *interleukin 18 (IL-18)* gene, a member of the IL-1 family of cytokines. *IL-18* was chosen as a target of this study, beyond its immunological role, because it is certainly expressed and present (differently from other markers, such as IFN- γ) in the diaphragmatic muscle, the only currently available matrix (Ensembl; NCBI, 2024). The recognition of *T. gondii* is crucial for IL-12 and IL-18 production by dendritic cells (DCs), which is necessary for a rapid and robust CD4+ T helper 1 (Th1) response leading to the release of IFN- γ . Both IL-18 and IFN- γ cytokines play a pivotal role in host resistance to *T. gondii* when the infection is active (Yarovinsky, 2014). In addition, the study of Lopez-Yglesias *et al.* (2019) demonstrated, in murine models, that in the absence of TLR11, as in the case of pigs, the production of the caspase-1-dependent cytokine IL-18 was sufficient and necessary for CD4+ T cell-derived IFN- γ responses in *T. gondii* infection.

On this basis, the hypothesis and the aim of this preliminary pioneering study are, besides an interesting and updated serological survey in the areas of interest, to start exploring and filling the current gaps on the Toxoplasmosis genetic resistance topic, for the first time, and with a *One Health* future perspective.

Materials and Methods

Pig populations enrolled for the investigation

For this preliminary study, 179 diaphragmatic tissue samples of commercial hybrid pigs were collected, statistically selecting the 20% slaughtered swine per farm with a structure capacity ranging from 70 to 7000 pigs, most of them of fattening type. As detailed in Table 1, the 35 herds were indoors and located in Central Italy regions, mostly in Umbria (31 farms), and also in Toscana (two farms), Marche (one farm), and Emilia-Romagna (one farm).

Samples collection and RNA extraction

Tissue aliquots were taken by authorized veterinarians after slaughter during the mandatory controls planned for the pig breeding sector, therefore no ethical approval was required. Total RNA extraction was performed from the collected diaphragmatic tissues for the host's genetic analysis. Extraction was conducted starting from about 80 mg of each collected tissue homogenized by TissueLyser II (Qiagen®, Hilden, Germany) in 1 mL of Trifast (VWR company®, Avantor, Radnor Township, PA, USA) using 5 mm stainless steel beads, performing two steps at 20.0 Hz for 3' each to allow mechanical lysis. After the homogenization phase, the RNA was purified, precipitated, and washed following manufacturer's instructions for the Trifast reagent (VWR company®, Avantor). The extracted RNA was resuspended in 50 µL of nuclease-free water. RNAs' quantity and quality were estimated photometrically by a Biophotometer (Eppendorf®, Hamburg, Germany).

IL-18 RT-PCR and Sanger sequencing

For the amplification of the *IL-18* target gene, primer pair sequences (Table 2) gene were designed by Primer3-BLAST software and the oligonucleotides were purchased from Invitrogen (Thermo Fisher Scientific, Waltham, MA, USA). Reverse Transcriptase amplification (RT-PCR) assay was optimized and performed with the Superscript™ IV One-Step RT-PCR System (Thermo Fisher Scientific). Amplification reactions were set up on a Mastercycler Ep Gradient S (Eppendorf®). The best PCR amplification conditions are described as follows: a final volume of 50 µL containing about 1 µg of target RNA and 400 nM of the forward and reverse primers. PCR protocol was carried out with the following thermal cycling profile: a retro-transcription step at 55°C for 10 min, followed by an initial step of denaturation at 98°C for 2 min, 40 cycles at 98°C for 10 s, 60°C for 10 s, 72°C for 30 s, and a final extension step at 72°C for 5 min. *IL-18* amplicons

Table 1. Information about 35 pig farms from which the samples were derived.

ID Farm	Region	Area (municipality)	Breeding farm type
#1	Tuscany	Cortona (AR)	Fattening – finishing
#2	Tuscany	Cortona (AR)	Fattening
#3	Umbria	Deruta (PG)	Fattening
#4	Umbria	Perugia (PG)	Fattening
#5	Umbria	San Venanzo (PG)	Fattening – semi-wild
#6	Umbria	Fratta Todina (PG)	Closed-cycle reproduction
#7	Umbria	Torgiano (PG)	Fattening complete cycle
#8	Emilia-Romagna	Poggio Torriana (RN)	Fattening complete cycle
#9	Umbria	Torgiano (PG)	Open-cycle reproduction/fattening
#10	Umbria	Assisi (PG)	Fattening – semiwild
#11	Umbria	Perugia (PG)	Fattening – finishing
#12	Umbria	Corciano (PG)	Fattening – weaning
#13	Umbria	Perugia (PG)	Closed-cycle reproduction
#14	Umbria	Perugia (PG)	Open-cycle reproduction
#15	Umbria	Perugia (PG)	Fattening complete cycle
#16	Umbria	Perugia (PG)	Open-cycle reproduction
#17	Marche	Frontone (PU)	Fattening – leaning
#18	Umbria	Gubbio (PG)	Open-cycle reproduction
#19	Umbria	Collazzone (PG)	Closed-cycle reproduction – semiwild
#20	Umbria	Castiglione del Lago (PG)	Fattening – finishing
#21	Umbria	Perugia (PG)	Fattening complete cycle
#22	Umbria	Bettona (PG)	Fattening complete cycle
#23	Umbria	Cannara (PG)	Fattening complete cycle
#24	Umbria	Marsciano (PG)	Fattening complete cycle
#25	Umbria	Castel Ritaldi (PG)	Fattening complete cycle
#26	Umbria	Collazzone (PG)	Fattening – weaning
#27	Umbria	Spello (PG)	Fattening – weaning
#28	Umbria	Bastia (PG)	Fattening – finishing
#29	Umbria	Cortona (AR)	Fattening
#30	Umbria	Deruta (PG)	Fattening
#31	Umbria	Passignano (PG)	None recovered and available information
#32	Umbria	S. Enea/Perugia (PG)	Fattening
#33	Umbria	Perugia (PG)	Fattening
#34	Umbria	S. Enea/Perugia (PG)	Fattening
#35	Umbria	Fratta Todina (PG)	Wild

AR: Arezzo; PG: Perugia; RN: Rimini; PU: Pesaro e Urbino.

Table 2. Primer pair sequences of the *Interleukin-18 (IL-18)* target gene (cDNA) investigated in the study.

	Primer Sequence 5'---->3'	Amplicon Length	Reference
IL-18	(Forward) ATGGCTGCTGAACCGGAA	609 bp	This study
	(Reverse) CAGAAAGTTCAAACCATGCAGT		

were controlled on 2% agarose gel electrophoresis containing Midori Green Advanced DNA Stain (Nippon Genetics Europe GmbH, Düren, Germany). A negative control (DEPC water instead of RNA) was introduced in each working session. Then, PCR products were purified with the QIAquick® PCR Purification Kit (Qiagen), following manufacturer's instructions. PCR products' concentration and purity were assessed photometrically with a Biophotometer (Eppendorf®). The sequencing reactions were performed in both directions with the same primer pair used for PCR amplification, using the BrilliantDye™ Terminator Cycle Sequencing Kit v3.1 (NimaGen BV, Nijmegen, The Netherlands), following the instructions of the manufacturer. Sequencing reactions were run in a 3500 Genetic Analyzer (Thermo Fisher Scientific).

Genetic data elaboration

All sequences, in FASTA format, were firstly analyzed and aligned to *Sus scrofa IL18* mRNA complete CDS (GenBank Accession Number: NM_213997.1) with BioEdit v7.2.5 software (Hall, 1999), using the ClustalW algorithm. In order to unveil putative and significant genetic mutations (SNP; insertions/deletions known as *indel...*), each sample sequence, for both primers, was subjected to a multiple-alignment by means of Sequencher® 5.4.6 software (Sequencher® analysis software, www.genecodes.com) using again the deposited sequence NM_213997.1 as reference.

Serological analyses

Serological analyses were carried out to detect specific antibodies for the parasite using ID Screen® Toxoplasmosis Indirect Multi-species kit (ID.vet, rue Louis Pasteur, Grabels, France) from meat juice matrix of the 179 samples object of the study, following manufacturer's instructions. For the interpretation of results, value ranges and relative cut-off, indicated by the kit for meat juice matrix, were applied, in particular: seronegative = SP% ≤ 40%; doubtful = 40% < s/P % < 70%; and seropositive = SP% ≥ 70%, where SP points to "sample positive". Samples of meat juice were also confirmed by Indirect Immunofluorescence (I.F.I) by National Reference Center

for Toxoplasmosis (Ce.Tox), using *Toxoplasma gondii* IFA Feline IgG Antibody KIT – Fuller Laboratories (Fullerton, California 92831 USA), modified with conjugate FITC-labeled anti-pig IgG antibody (Sigma, F1638).

Results

The RT-PCR assay for the target gene *IL-18*, here developed and optimized, proved to be specific as demonstrated by subsequent sequencing analysis and the complete alignment of the consensus sequence with *Sus scrofa IL-18* reference sequence (mRNA, NM_213997.1).

For the SNPs and mutations analysis, the sequences were visualized as contig(s) within Sequencher® 5.4.6 software. Each sequence was aligned to the *IL-18* mRNA reference sequence (NM_213997.1). Initial and terminal ends were manually trimmed to exclude low-quality regions. After a quality control check of chromatograms and sequencing repetition for ambiguous picks and traces, the final consensus sequence was generated (Figure 1).

At this stage, for this interleukin, a heterozygous polymorphism (A>G) at nucleotide position 90 of the consensus was detected (Figure 1) only in a pig with a seropositive phenotype. The putative mutation is a synonymous and conservative one, with no effect on encoded amino acid variation.

Regarding serology, as demonstrated by ELISA and I.F.I tests, out of 179 tested animals, 98 pigs were seropositive, 57 pigs were seronegative, and 24 pigs were doubtful with an inter-farm prevalence of 54.74%, 31.84%, and 13.40%, respectively. These findings are interesting if not partly "alarming", since they indicate a probable re-emergence of *T. gondii* in the examined areas.

Discussion

From the genetic analysis carried out on the investigated pig population, no significant polymorphism was detected in the *IL-18* gene sequence, except for one animal. For this reason, to date, it was not possible to make a statistical association of *IL-18* allelic variants and the SNP genotypes with the phenotype of pigs categorized in positive and negative, based on serological tests.

Considering these preliminary results, further studies conducted with the same (Sanger) approach or exploiting Next Generation Sequencing (NGS) high-throughput technologies, are necessary to comprehend if other genetic regions or target genes encoding for other immunity regulators act in the initial steps of the infection

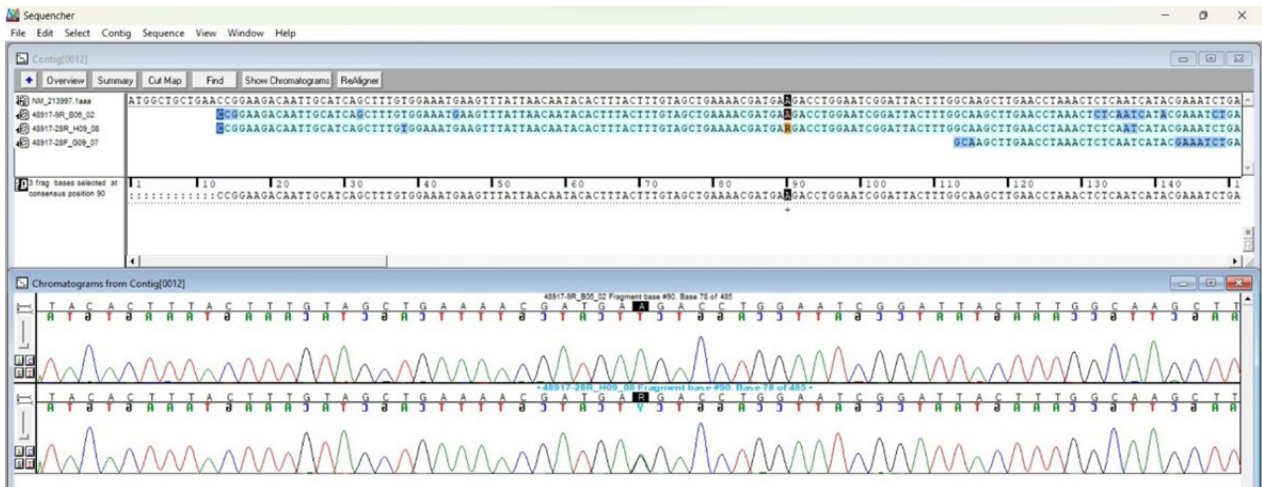


Figure 1. Example of sequence alignment to reference (NM 213997.1) through the Sequencher software. The figure highlights nucleotide position 90 and the heterozygous peak, detected in a sample, during the analysis.

could behave as *hot spot* of mutations (i.e., SNPs), confirming or not this assumption. In fact, in the host, such polymorphic variants could condition the *T. gondii* recognition and entry, the cysts formation/diffusion and in general the immunological response, thus determining a host profile susceptible or resistant to the infection.

Concerning host-pathogen relationship and immunological *cross-talk*, *T. gondii* modulates host pathways through the secretion and delivery of endogenous effectors in order to try to evade host immunity or, in other cases, activate it. This balance is required by the parasite for the conversion into bradyzoites that form tissue cysts, orally transmitted to intermediate hosts. A complete evasion of *T. gondii* from immune defense mechanisms corresponds to an uncontrolled proliferation of the parasite with a negative, occasionally fatal, impact on the host (Mukhopadhyay *et al.*, 2020).

Furthermore, the susceptibility to *Toxoplasma* depends on the host's genetic background, on the immune status and its interplay with the parasite. Scientific evidence suggests a species-dependent susceptibility to toxoplasmosis. For instance, some farm animals, most of all bovines, seem to be more resistant to *T. gondii* and they are characterized by a permanent infection without showing clinical signs (Stelzer *et al.*, 2019).

The in-depth study of the immunity mediators, conducted with the aim of evaluating the genetic markers putatively associated with underlying resistance mechanisms or resistant phenotypes, could be challenging and very impactful in terms of public health. Currently, similar works on immunity targets, particularly on interleukins (Andrade *et al.*, 2020; Wujcicka *et al.*, 2018) involved in toxoplasmosis have been conducted, until now, on pregnant women, mainly for epidemiological

and knowledge purposes. In these studies, multiple SNP analyses were carried out to unveil those genotypes (significantly) associated with toxoplasmosis and with a decreased or increased risk of parasitic infection.

The preliminary findings of our study pertain to a pig population with a specific geographic area. These findings are based solely on a particular marker gene, chosen because it is involved in the up-stream immunity pathway towards *T. gondii* and it is expressed at a rate of 0.7 transcripts per million (TPM) in the diaphragmatic matrix (Ensembl; Martin *et al.*, 2023; NCBI, 2024), which was the only available matrix for the study.

Future in-depth investigations are required to comprehend the presence and the possible merit or contribution, in *T. gondii* infection as well as in the genetic tolerance mechanisms, of polymorphic variants in those genes with a key role prior to the critical step of cysts formation. These markers could be, for example, *Interleukin 12 – IL12*, *Interferon Regulatory Factor 8 – IRF8*, *Myeloid Differentiation Primary Response 88 – Myd88*, and *NLRPs* inflammasome mediators (Mukhopadhyay *et al.*, 2020).

Regarding the serological survey, the initial focus of the work, the outcomes of a seroprevalence higher than 50% have strengthened the need to explore preventive and alternative “genetic approaches”. To date, there are some reports from Europe concerning *T. gondii* infections in organically raised pigs. Similar to the population examined in this study, a very high serological prevalence of *T. gondii* was detected in 21 pigs from three organic farms in Italy: in particular, antibodies to *T. gondii* were found in 20 out of 21 (95.2%) meat juice samples (Bacci *et al.*, 2015). A survey conducted on pig farms in Umbria, the main region investigated in our survey, showed a seroprevalence of approximately 16.14% (Veronesi *et al.*, 2011)

but using serum as matrices instead of meat juice and in a different and rather distant period from this current survey. In order to have a general overview of this issue, other findings about the presence of the protozoan parasite in tissues from pigs are available, particularly in the review of Dubey *et al.* (2020) that reports outcomes of infection in the period 2009–2020. In particular, in Italy, the frequency of positive cases varies, ranging from 13.6% to 57.1% based on the investigated Italian regions (Northern, Central, and Southern), on sampled tissues and target genes of *T. gondii*, more commonly the *B1* or the *529 bp Repeat Element (529 RE)*. These data pointed out how *T. gondii* infection in pigs represents a significant public health issue, depending on multiple factors such as farm management, the area of interest, and other environmental conditions of risk.

Conclusions

In conclusion, it is important to constantly monitor the *T. gondii* seroprevalence in livestock animals. Furthermore, it is of great interest to investigate and understand the putative presence or role of “host mutations”, underlying the genetic variation in non-coding and coding genomic regions that culminate in the gene’s function impairment, in the organism’s phenotype alteration, and consequently in the molecular divergence of populations.

We believe that as in the field of genetic resistance against other recognized infectious diseases (parasites included) it is worth exploring and establishing whether genetic selection could be a strategic preventive measure for the creation of animal lines genetically resistant to toxoplasmosis or not. From a future and “idealistic” perspective, the expected impacts could be both the valorization of “Toxo-free” or safer food products and, in general, the prevention and the control of toxoplasmosis, in a holistic vision and with a *One Health* approach.

Funding

The authors declare that currently no funds, grants, or other support were received during the investigation, and during the preparation of this manuscript.

Acknowledgments

A special thanks to the scientific committee of the Sisvet (*Italian Society for Veterinary Science*) federation for the “One Health 2023” Award by *Zooprophylactic and Zootechnical Initiatives Foundation* (“*Fondazione Iniziative Zooprofilattiche e Zootecniche*”), conferred to Dr Martina Torricelli for the originality and innovation

of presented Abstract entitled: “*Toxoplasma gondii* 20.23: serological survey in Central Italy pig farms and novel insights for genetic resistance studies”.

Conflicts of interest

The authors have no relevant financial or non-financial interests to disclose.

Author contributions

M.T. conceptualized the study; M.T.; M.B. formulated the study; M.T., A.F., H.L., C.P., C.S., M.C., and D.C., did the formal analysis; M.T., A.F., H.L., C.P., M.C., and D.C., were in charge of investigation; S.C., H.L., A.T., S.Cr., P.M., and M.B. arranged the resources; M.T. prepared the original draft; M.T., H.L., C.S., S.Cr., and M.B. reviewed and edited the manuscript; M.T., A.T., S.C., and M.B. supervised the study; M.T., S.C., A.T., S.Cr., P.M., and M.B. were in charge of project administration; S.C., A.T., and M.B. acquired funding.

Data availability

Not applicable.

Ethics approval

Not applicable. In particular, as reported in the main text in the Materials and Methods section, “*Tissue aliquots were taken by authorized veterinarians after slaughter during the mandatory controls planned for the pig breeding sector, therefore no ethical approval was required.*”

Consent to participate

Not applicable.

Consent to publish

Not applicable.

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