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The pig in translational medicine: visual and gastro-intestinal systems applications

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The pig in translational medicine

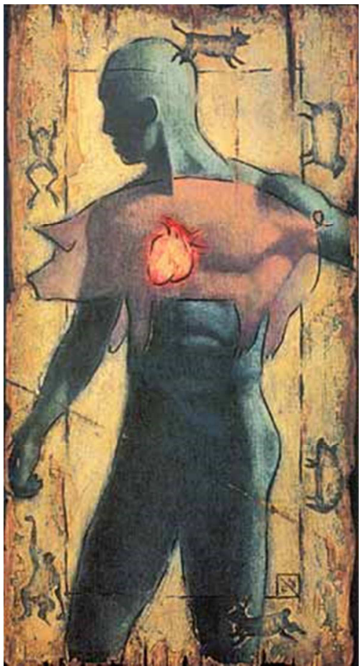
Visual and gastro-intestinal systems applications

Summary

The European Society for Translational Medicine defined Translational Medicine as "*interdisciplinary branch of the biomedical field supported by three main pillars*: *benchside*, *bedside and community. The goal of TM is to combine disciplines, resources*, *expertise*, *and techniques within these pillars to promote enhancements in prevention*, *diagnosis*, *and therapies"* (Cohrs et al., 2015).

"*If [something] works in the pig, then it has a high possibility of working in the human*." - Michael Swindle, author of "Swine in the Laboratory"

1 *Translational medicine*



("Die Abwehr besser verstehen," n.d.)

In the 1950s, clinical systems were structured such that clinical studies, patient care and theoretical research were all done in the same place and members of research teams worked in all these areas. Developments in the decades that followed and the specialization of research areas led to a separation of clinical research (e.g. internal medicine and surgery) and theoretical studies (e.g. biology and molecular biology in particular) ("History of translational medicine | CENTRE FOR TRANSLATIONAL MEDICINE," n.d.). It was this separation of types of research that brought about one of the greatest

challenges in medicine in the late 20th century and introduced the concept of *translational medicine* for the first time.

There is not a clear and univocal definition for *translational medicine* as it assumes different meanings for different people.

The concept of translational medicine as the science to test, in humans, novel therapeutic strategies developed through experimentation was so popular that Bench to Bedside (BtB) Awards was developed within the NIH in 1998 to encourage collaboration between clinicians and basic scientists across institutes ("NIH Clinical Center," n.d.).

Some confusion may occur when we consider the term translational research.

For many, the term refers to the "bench-to-bedside" concept that considers the knowledge coming from the basic sciences a way to assess new drugs, devices, and treatment options for patients. For this area of research the end point is the production of a promising new treatment, and has been characterized as follows: "effective translation of the new knowledge, mechanisms, and techniques generated by advances in basic science research into new approaches for prevention, diagnosis, and treatment of disease is essential for improving health."(Fontanarosa and DeAngelis, 2002)

For others translational research refers to translating research into practice; i.e., ensuring that new treatments and research knowledge actually reach the patients or populations for whom they are intended and are implemented correctly. The production of a new drug, an end point for "bench-

to-bedside" translational research, is only the starting point for this second area of research which works to improve the quality of treatments helping clinicians and patients to change behaviors and make more informed choices, providing reminders and point of- care decision support tools, and strengthening the patient clinician relationship.

In 2003 the Institute of Medicine's Clinical Research Roundtable described 2 "translational blocks" to explain the differences between the two definitions of translational research (Sung et al., 2003), which some now label as T1 and T2. The first roadblock (T1) was described by the roundtable as "the transfer of new understandings of disease mechanisms gained in the laboratory into the development of new methods for diagnosis, therapy, and prevention and their first testing in humans." The roundtable described the second roadblock (T2) as "the translation of results from clinical studies into everyday clinical practice and health decision making."

Referring to T1 and T2 both as translational medicine has become a source of confusion. (Kerner, 2006)

Their goals, settings, study designs, and investigators differ. T1 research requires mastery of molecular biology, genetics, and other basic sciences; appropriately trained clinical scientists and a supportive infrastructure within the institution.

In contrast, the "laboratory" for T2 research is the community and ambulatory care settings, where population-based interventions and practice-

based research networks (Mold and Peterson, 2005) bring the results of T1 research to the public.

In 2009, the American Association for the Advancement of Science announced the publication of a new journal, *Science Translational Medicine*; to try to fill the deep gaps in our fundamental understanding of biology that often have been highlighted in clinical human studies. The Founding Chief Scientific Advisor Elias A. Zerhouni declared: "Never before have scientists had access to the remarkable tools that are available today and that allow rigorous translational investigations to be conducted. However, the creation of a redefined discipline of translational medicine will require the emergence of a new and vibrant community of dedicated scientists, collaborating to fill knowledge gaps and dissolve or circumvent barriers to improved clinical medicine."

In parallel with the WHO's call for translational research (TR) to be applied to much more than just clinical medicine ("WHO | World Report on Knowledge for Better Health," n.d.), it has been recognized in the US that the need for TR extends beyond the areas covered by types T1 and T2.

A variety of four-type models have been proposed, including the one proposed by Khoury and colleagues. In 2010 indeed, highlighting the role of epidemiology in translational medicine, Muin J. Khoury presented the application of translational epidemiology (TE) through four phases designated T1–T4.

The phases and the role of epidemiology are synthetized in table 1. In T1 epidemiology explores the role of a basic scientific discovery (e.g., a disease risk factor or biomarker) in developing a "candidate application" for use in practice (e.g., a test used to guide interventions). In T2, epidemiology can help to evaluate the efficacy of a candidate application by using observational studies and randomized controlled trials. In T3, epidemiology can help to assess facilitators and barriers for uptake and implementation of candidate applications in practice. In T4, epidemiology can help to assess the impact of using candidate applications on population health outcomes. (Khoury et al., 2010)

Table 1. ***T0–T4, designated phases of translational research and the role of epidemiology.*** *From Khoury et al., 2010*

Phase	Details	Role of Epidemiology	Examples From Genomics
T0	Description and discovery	Describing patterns of health outcomes by place, time, and person; finding determinants of health outcomes by use of observational studies	Describing patterns of health outcomes in relation to inbreeding, migration, and family history to generate hypotheses about genetic factors; genome-wide association studies as a tool for gene discovery
T1	From discovery to health applications (tests, interventions)	Characterizing discovery and assessing potential health applications by using clinical and population studies	Assessing prevalence, associations, interactions, sensitivity, specificity, and predictive value of testing for genetic risk factors
T2	From health application to evidence guidelines	Assessing the efficacy of interventions to improve health and prevent disease by using observational and experimental studies	Assessing the clinical utility of genetic risk factors in improving health outcomes
T3	From guidelines to health practice	Assessing the implementation and dissemination of guidelines into practice	Assessing the factors associated with implementation of *BRCA* testing in practice
T4	From health practice to population health outcomes	Assessing the effectiveness of interventions on health outcomes	Assessing the effectiveness of newborn screening programs

The typology suggested by the Harvard Catalyst, a consortium headed by Harvard University, is very similar, but its impact at a population level is clearly stated as relating to public health rather than the application of clinical practice to maximize its impact at a population level ("Pathfinder | Harvard Catalyst," n.d.). The Harvard typology acknowledges the importance of the 'social determinants of health' in considering health at a population level, but the extent to which these and the other determinants of health are addressed in their T4 TR it is not clear.

In 2009 Ogilvie and colleagues noted public and population health TR required more fundamental and wide-ranging societal response than those that were offered through the established systems of delivering health care, so they develop a framework for translational health research at a population level. (Ogilvie et al., 2009)

The complexity of interventions implied by the Ogilvie framework revealed the need for TR to go beyond the impact of specific public health interventions on population-level health outcomes. The addition of T5, as shown in table 2, was proposed by Thomson n in 2012 ("Translational research « What we do « Our work « About « Australian Indigenous HealthInfoNet," n.d.). Unlike the other types of TR, attention to the social and other determinants of health is an essential aspect of T5 TR.

Table 2. ***Translational research, by type, area of translation, attention to social and other determinants of health, and complexity of synthesis****. Source:* **Khoury et al., 2010**,**"Pathfinder | Harvard Catalyst," n.d.** *and* **Ogilvie et al., 2009**

Type	Area of translation	Attention to social and other determinants of health
T1	Basic research to clinical effect	No
T2	Clinical effect to clinical intervention	No
T3	Clinical intervention to clinical practice	Some
T4	Specific public health intervention at a population level	Some
T5	Clinical, public health and determinants-related interventions applied to the population or specific sub-populations	Yes

Analyzing the 4-phase in translational research the T0 phase has given rise to claims that research is translational when, in fact, it is translatable and often quite a distance away from translational medicine.

In 2014 Frank Gannon proposed a classification scheme that aimed to help to define the steps in pre-clinical research from discovery to translation (T0). As the four phases of translational research, Gannon distinguished four phases of discovery D1 to D4. (Gannon, 2014)

D1 is pure basic research to gain knowledge. It is essential and the basis for the following stages, but does not itself focus on clinical use or any other application. D2 is disease-related research or oriented/strategic research: the context is defined by a disease, and the work is designed to obtain new insight or a starting point for diagnosis, treatment or prevention. D3 is the phase when the initial outcome of D2 research, such as a target molecule or a metabolic pathway, has been identified and when its link to the disease is being

investigated. D4 describes the phase when the target from D3 is manipulated in some way to confirm its disease association.

After successfully completing D4, the path to "real" translation or T1 is evident and the product, treatment or diagnostic test can be moved into clinical research. The 4 phases of discovery (D1-D4), T0 and T1 phases are often characterize by the use of animals. The animals are used to model a diseases o to model how a new device/therapy will work on humans. The use of animals is therefore fundamental as the complexity of the human physiology cannot be explained without.

1.1 Historical considerations

Figure 1. ***AVICENNA Liber canonis de medicina - Pavia, Giacomo Pocatela, 1510-1512*****:** *detail of the front page*.

Animal experimentation has played a central role in biomedical research throughout history and for centuries it has also been an issue of heated public and philosophical discussion. Animal use in the life sciences and its moral and social implications in a historical point of view are important to understand the key issues and to evaluate present principles and practices in animal research. (Franco, 2013)

Humans have been using animals as models of their anatomy and physiology since the dawn of medicine, physicians in ancient Greece dissected animals for anatomical studies (Von Staden, 1989).

Galen of Pergamon (2nd–3rd century CE) developed the techniques for dissection and vivisection of animals and on which he based his many treatises of medicine (Guerrini, 2003). These remained canonical, authoritative, and undisputed until the Renaissance (Fig. 1).

Beginning with the decline of the Roman Empire and continuing throughout the Middle Ages, physiological experiments and scientific activity in general would fall almost entirely into disuse and medical knowledge would become dogmatic.

The use of animals for scientific activity restart when the Flemish anatomist Vesalius (1514–1564) and Francis Bacon (1561–1626), examined the similarities and differences between the internal structure of humans and other animals, thus setting the foundations of modern comparative anatomy.

Physiological experiments on animals carried on throughout the seventeenth century, in the period favorable to scientific progress now known as the Age of Enlightenment.

In this period the controversy on animal rights began to take hold, some philosophers, like Baruch Spinoza (1632–1677), though that animals had the ability to feel but that humans could "use them as we please, treating them in a way which best suits us; for their nature is not like ours" (Spinoza, 1677).

John Locke (1632–1704), on the other hand, recognized that animals could feel and stated that "children should be brought up to abhor the killing or torturing of any living thing in order to prevent them from later becoming capable of cruel actions to fellow humans" (Franco, 2013).

Immanuel Kant (1724–1804) regarding animal use in research, stated that "Vivisectionists can justify their cruelty, since animals must be regarded as man's instruments; but any such cruelty for sport cannot be justified"(Kant, 1997).

In 1628 William Harvey (1578–1657), physician to kings James I and Charles I and one of the founders of modern science, published *Exercitatio Anatomica de Motu Cordis et Sanguinis in Animalibus* ("An Anatomical Exercise on the Motion of the Heart and Blood in Living Beings") in which he provided the most accurate description of blood circulation and heart function of his time (Fig. 2).

Figure 2. ***William Harvey, using a live deer, explains to Charles I the circulatory system of blood.*** *From El Museo Popular published Madrid, 1889*

In the eighteen century philosophers like Voltaire, Jean-Jacques Rousseau, Jeremy Bentham and Arthur Schopenhauer began to challenge the anthropocentric view on human duties to animals. Bentham famously state: "The question is not, Can they reason? Nor, Can they talk? But, Can they suffer?" (Bentham, 1907). While knowledge of bodily functions and pathology was still incipient at that time, eighteen-century physiologists differed from their seventeenth-century predecessors, as they believed that medical improvements could one day be achieved through advancing knowledge by the means of animal experimentation.

The idea of inducing disease in model animals was first suggested by Claude Bernard in 1865, when he published the *Introduction to Experimental Medicine* (Bernard, 1957). It was the first time animal models were used for experimental research, rather than for visualization and understanding of biological systems.

Pasteur began hypothesizing that microbes could be responsible for many diseases affecting humans and other animals. Together with his disciples, most notably Emile Roux (1853–1933), he would go on to identify *Staphylococcus*, *Streptococcus*, the "septic vibrio" (now *Clostridium septicum*), the causative agents of anthrax (*Bacillus anthracis*) and chicken cholera (*Pasteurella multocida*), being the first to develop vaccines for these zoonotic diseases, as well as for Swine Erypselas, thus setting the foundations of modern immunology (Bazin, 2011). In his studies Pasteur used dogs, chickens, rabbits, rodents, pigs, cows, sheep, and non-human primates and was the first to introduce the concept of Humane Endpoint by stating that: "The

rabbit should begin to show symptoms on the sixth or seventh day, and die on the ninth or tenth. Usually the rabbit is not allowed to die, but is chloroformed on the last day in order to avoid terminal infections and unnecessary suffering".

Emil von Behring (1854–1917) and Paul Ehrlich (1854–1915) developed the first antitoxin for treatment of diphtheria from horse serum, for which von Behring received the Nobel Prize in 1901.

The twentieth century would witness astonishing advances in medical knowledge and the treatment of disease. The advances of biomedical research to human health since the dawn of the past century are countless, with animal research playing a role in many important discoveries. Of the 103 Nobel Prizes in physiology or medicine given since 1901, in more than 80 the results were obtained from animals.

Consideration towards animals began to change with Regan and Singer works on animal ethics (Regan, 1987) and with the development of the "Three Rs"—*Replacement, Reduction, Refinement*—principles by Russell and Burch in 1959.

Animals are part of the translational process and the importance of animal welfare is emerging thanks to awareness of its fundamental importance in producing data able to establish translation.

1.2 The need for animal model and translational value

Undoubtedly, the greatest achievements in medicine in the 19th and 20th centuries were possible due to the use of animals. There is a strong relationship between a rapid progress in experiments on animals and evident progress in clinical medicine.

The role of the animal model is neatly explained in "The Animal Research War", by Conn and Parker (Conn and Parker, 2008) :

> *"If you are going to study a human disease you can't, for ethical reasons, perform the initial work in humans; you have to develop a model. Some models may be in vitro – literally, in glass tubes – but as you learn more and more, you must eventually test ideas in vivo– in living animals. That means you have to have a way of producing the disease that allows you to study it.*
>
> *Let's consider AIDS, one of Podell's interests. You could take its causative agent, the Human Immunodeficiency Virus (HIV), grow it in a test tube, and kill it by pouring bleach on it. Do you now have a way to kill HIV? Yes, you do. Do you have a treatment that can be used in humans? Absolutely not: bleach is toxic. Killing HIV in a test tube and killing it in a living animal are two very different accomplishments.*
>
> *To complicate things further, viruses grow differently in test tubes than in humans. Humans have an immune system: test tubes do not. A virus growing in a test tube is not a good model for the human disease, but drugs*

that don't kill the test tube virus probably won't work in humans either – and these might be eliminated from further consideration.

Animal models allow closer approximation to a human response. They are not perfect, of course; animals host different diseases and different responses. While the fundamentals of life are the same – there is a 67 percent similarity between the DNA of humans and earthworms – there are differences in species and even in individual animals. Some animals are good human-like models for one thing and some for another; some have a cardiovascular system that is similar to humans while others have similar skin."

We can distinguish three main groups when we talk about types of animal models: (I) normal animals, used in the past and are still used today to investigate physiological situation and to assess the safety/toxicology risk of a putative therapeutic; (II) animals with naturally occurring deficits, i.e. spontaneous and endogenous occurring conditions or mutations, selection and genetic lines; (III) animals with experimentally induced deficits like transgenic and knockout animals, chromosomal substitution strains and chemical/physical induced models.

Another way to defined animal models is by nine categories based on the use of the animals in science and research (Greek and Menache, 2013):

1. Animals are used as predictive models of humans for research into such diseases as cancer and AIDS.

2. Animals are used as predictive models of humans for testing drugs or other chemicals.

3. Animals are used as "spare parts", such as when a person receives an aortic valve from a pig.

4. Animals are used as bioreactors or factories, such as for the production of insulin or monoclonal antibodies, or to maintain the supply of a virus.

5. Animals and animal tissues are used to study basic physiological principles.

6. Animals are used in education to educate and train medical students and to teach basic principles of anatomy in high school biology classes.

7. Animals are used as a modality for ideas or as a heuristic device, which is a component of basic science research.

8. Animals are used in research designed to benefit other animals of the same species or breed.

9. Animals are used in research in order to gain knowledge for knowledge sake.

In both the categorizations, the assumption that animal models are *predictive* of human outcomes is fundamental for much of their use in biomedical research.

The ideal animal model should replicate, to a major extend, both a human disease phenotype and its underlying causality (McGonigle and Ruggeri, 2014) but one ideal animal model for all human condition does not exist. Some animal models are however capable of predicting a particular human condition or define a specific question.

Most medical therapies in use today are initially developed and tested in animals, yet animal experiments often fail to replicate when tested in rigorous human trials (Hackam and Redelmeier, 2006).

To fight this trend is important to validate and improve the translational value of animal models.

It can be done by proper design, conduction and reporting of experiment, as the best validated model is not able to yield conclusive data when the experimental design is flawed or the execution of the study is not well controlled (Denayer et al., 2014).

There are different criteria to validate a model, a more general criteria is the definition of face validity, the similarity in biology and symptoms between the animal model and the human disease; predictive validity, demonstration that clinically effective interventions demonstrate a similar effect in the model and target validity, the target under investigation should have a similar role in the disease model as in the clinical situation.

Another criteria is the one proposed by Sams-Dodd (Sams-Dodd, 2006), a model validity scoring system upon five criteria (Tab. 3).

Table 3. ***Proposed validity scoring system.*** *From Denayer et al.*

Criterion	Value	Score
Species	Human	4
	Non-human primate	3
	Non-human mammal	2
	Non-mammal	1
Disease simulation	True	4
	Complex	3
	Pharmacological	2
	No	1
Face validity	>1 core symptom	4

	1 core symptom	3
	1 symptom	2
	No	1
Complexity	*In vivo*	4
	Tissue	3
	Cellular	2
	Sub-cellular/molecular	1
Predictivity	Graded for all pharmacology principles	4
	Graded for certain pharmacology principles	3
	All or none for certain pharmacology principles	2
	No or not shown	1

This scoring system can help to assemble a screening cascade/combination of models which altogether has maximal validity.

Improving the quality of animal models involves three things: improving the performance quality of existing models (e.g. proper design and execution of experiments), improving the way in which animal models are used in the decision making process and investing in the development of more sophisticated and clinical relevant and predictive models (Denayer et al., 2014).

2 *The pig model*

The pig was first used in biomedical research in ancient Greece and over the past few decades has quickly grown into an important biomedical research tool, table 4 shows the major landmark of the use of pig in biomedical science from Erasitratus to the current day (Schook et al., 2015).

Table 4. ***Timeline of the use of pigs in biomedical science.*** *Table from Schook et al. 2016.*

Date	Event
302–258 BC	Erasistratus: studied the circulatory system of pigs
129–200 AD	Galen: studied the respiratory and nervous system of pigs
1578–1657	William Harvey: established principles of circulation
1865	Claude Bernard: conducted first experimental research on animals
1915–1949	Porcine thyroid extract used to treat hyperthyroidism
1921–1982	Porcine insulin used to treat diabetes
1930–Present	Porcine heparin only FDA-approved source
1960s	Thyrotropin releasing factor and luteinizing hormone releasing factor isolated from porcine hypothalami (Nobel Prize 1978)
1969	First porcine aortic valve implanted
1981	First transgenic mouse (microinjection)
1982	Genentech introduced recombinant human insulin
1986	First transgenic pig (microinjection)
1989	Pig generated by transfer of embryonic cell (blastomere) nucleus into enucleated oocyte
1991	Tao injected cultured fetal fibroblast cells into enucleated oocytes
1996	Dolly born; first clone from adult somatic cell nuclear transfer
1997	Retinitis pigmentosa (Rhodopsin, mutant P347L)
2000	Pigs cloned by nuclear transfer from adult somatic cells
2001	Huntington's disease porcine model
2002	Porcine sequencing initiative white paper submitted
2003	Pig Project Sequencing initiated The Encyclopedia of DNA Elements (ENCODE)
2004	Pilot human epigenome project published
2008	Cystic fibrosis porcine model
2009	Porcine 60k single nucleotide polymorphism chip
2010	Huntington's disease porcine model 2
2012	Pig genome sequence published Neonatal pig brain MRI atlas online (http://pigmri.illinois.edu/) Retinitis pigmentosa porcine model (Rhodopsin, mutant P23H) A porcine model of familial adenomatous polyposis
2013	Genome-edited pigs born (TALEN and ZNF injected into zygote)
2014	Inducible P53/Kras porcine cancer model

The great spread of pig farming made a large quantity of tissues available as source of many therapeutic biomedical products. One great example is the production of insulin. Insulin was discovered in 1924 and porcine insulin

became the treatment of choice (Harington, 1926) until 1982 when Genentech produced the first recombinant human insulin (Evens, 2016).

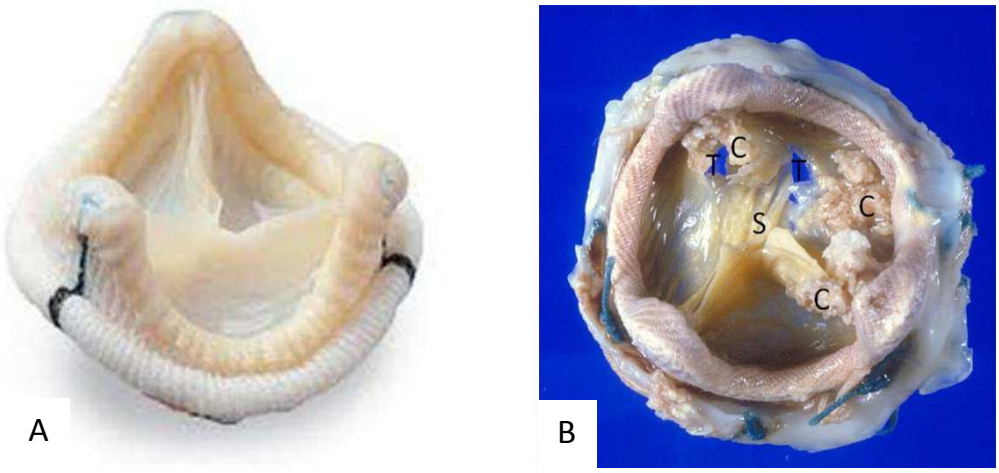
Another example is the porcine thyroxin that was isolated in 1915 (Kendall, 1983) and is still available today (Slater, 2011) .

Heparin, which acts as an anticoagulant, blocking the blood clotting cascade was first isolated from porcine intestines in the 1930s (Pritchard, 1964) and today is still the only Federal Drug Administration–approved source of heparin. Currently, the global use of this product is 1.5 billion doses yearly which production consumes tissue from 700 million pigs (Oduah et al., 2016).

In the 1960s, porcine hypothalami were used for Nobel Prize–winning work that identified, isolated, and determined the structure of the first brain hormones (Schook et al., 2015).

In addition to the extracted biological, porcine tissues are used routinely for replacement heart valves and patches. The bioprosthetic heart valves, first introduced in the 1960s, are made of porcine or bovine pericardium preserved with glutaraldehyde (Manji et al., 2012). Patients receiving these valves do not require anticoagulation therapy. However, these glutaraldehyde-treated implants do pose some risk of deterioration in young patients, which can necessitate additional replacements (Fig. 3).

Figure 3. Comparison of an unused glutaraldehyde-fixed bioprosthetic heart valve (GBHV; "off the shelf") with a GBHV removed from a patient for structural valve deterioration. *From Manji et al., 2012*
A. Porcine bioprosthetic heart valve prior to implantation in patient. There is no calcification and the valve leaflets are thin and clean. B. Porcine bioprosthetic heart valve removed for structural valve deterioration, showing calcification (C), stenosis of the valve orifice (S), and cusp tears (T).



The authors suggested that the production of genetically engineered pigs, such as α1,3-galactosyltransferase gene-knockout pigs, that do not express this protein would provide a potential source of non- or less-immunogenic valves to meet worldwide demand.

Pigs are used in the field of pharmacology and toxicology for their similarities with humans in terms of anatomy and physiology.

Many authors deeply analyzed the role of pig and in particular minipig in toxicology, Swindle et al. provided an overview of the common use of these in research and toxicology testing providing a review of the anatomic and histopathologic characteristic (Swindle et al., 2012).

Thanks to the RETHINK project the use of minipig in this field was validated and the minipig model was assumed to be a better non-rodent model than the dog (Forster et al., 2010) bringing benefits in terms of 3Rs (life-cycle analysis) compared to dogs and non-human primates. Finally the minipig (unlike the dog) is well positioned to take advantage of genomics and gene manipulation technologies (Yum et al., 2016).

In general it can be stated that toxicology studies performed in minipigs are acceptable to regulatory authorities (Bode et al., 2010).

In 2002 a group of scientist submitted the porcine sequencing initiative paper to the National Human Genome Research Institute to provide scientific justification for sequencing the porcine genome to identify new genes and novel regulatory elements in the pig and in humans, mice and rats ("Porcine Genomic Sequencing Initiative," n.d.). The proposal set the stage for the worldwide collaborative effort to develop and publish a high-quality draft genome sequence for a female domestic Duroc pig (Groenen et al., 2012). The initiative had a big impact on scientific world (Fig. 4) leading to the identification of natural mutations, which increase the potential for additional pig biomedical models. In addition, the pig mitochondrial genome was sequenced (Lin et al., 1999) providing support for pig models of mitochondrial diseases.

One hundred and twelve positions have been observed where the porcine protein has the same amino acid that is implicated in a human disease. These include genes implicated in multifactorial diseases, such as obesity, diabetes, Parkinson's, and Alzheimer's disease (Groenen et al., 2012). In addition, the sequencing of the genomes of 48 individual pigs has revealed

32,548 no synonymous SNPs, 6 known to be associated with human disease and 11 that have been linked to human disease phenotypes. Identification of these porcine variants allowed further study of these diseases in a suitable pig model.

Figure 4. ***A high-quality draft genome sequence for a female domestic Duroc pig is published in this issue of Nature, under the auspices of the Swine Genome Sequencing Consortium.*** *Cover: Mike Kemp, Rubberball,Getty Images/ Pompeii: René Mattes, Hemis, Corbis.*

In the post-genomic era the interesting on the pig model increased exponentially. The pig was developed to be a model for multigenetic and monogenic disease, but also to model cancer and lifestyle diseases.

The availability of the pig genome sequence provided an important resource for improving strategies to generate genetically engineered biomedical

models and transgenic animals are now commonly used worldwide as models of human disease.

For example pig proved excellent models for human atherosclerosis (Skold et al., 1966), so that in 2010 Jensen TW et al. demonstrated that cloned, genetically defined ApoE4 (apolipoprotein associated with increased risk of atherosclerosis in humans) pigs fed high-fat, high-cholesterol diets had increased total and low-density lipoprotein cholesterol plasma levels within 60 days, thus providing a model in which the earliest stage in atherosclerosis can be induced and mitigated (Jensen et al., 2010).

Due to recent advances in the sequencing of domestic animal genomes and the development of new organism cloning technologies, it is now very feasible to utilize pigs as a malleable species, with similar anatomic and physiological features with humans, in which to develop cancer models. Recent advancements in porcine tumor modeling and genome editing will bring porcine models to the forefront of translational cancer research (Schook et al., 2016).

Pigs are also very useful as model in surgery new techniques development and training field due to their similar anatomy and human comparable dimensions (Gessaroli et al., 2012).

The pig is an extremely useful biomedical model for improving our understanding of the pathophysiology and for investigating potential treatment/prevention strategies under rigorously controlled conditions for many human diseases and ailments. The optimum porcine model to use is often

dependent on the specific disease being studied. In a review of 2010 Little-Brown and collaborators defined the minipig the best breed for investigation on the metabolic syndrome. The author provide an overview of the use of pig as biomedical model (Litten-Brown et al., 2010).

Due to the considerations above the swine species provide a valuable translational model to bridge the gap between classical rodent models and humans in developing new therapies to aid human health.

These features of the pig combined with an increasing availability of biological tools and reagents for use to study porcine tissue make the pig arguably the best model available for translational biomedical research (Ziegler et al., 2016).

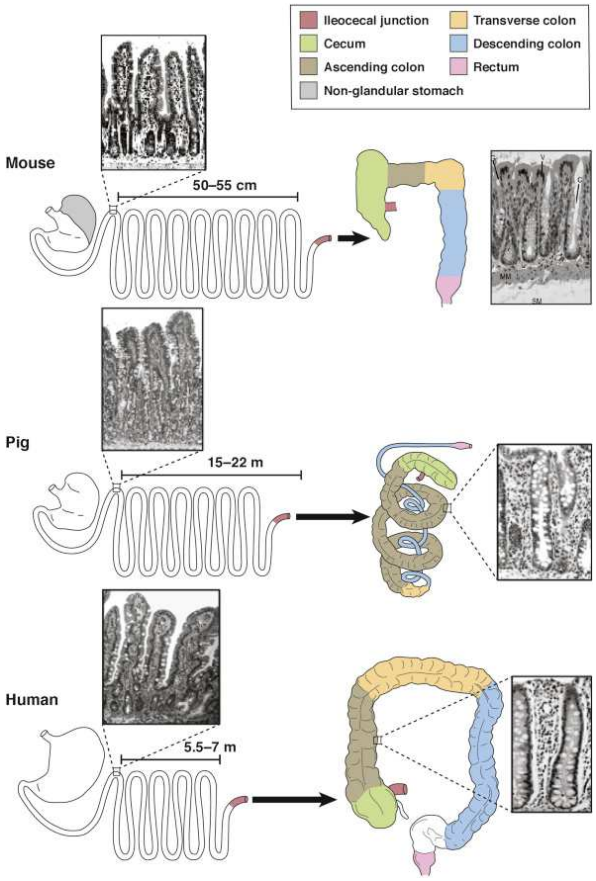
2.1 Gastro-intestinal and metabolic applications

Because of the limitations of data on human gastrointestinal disease in a clinical environment, the use of animal models to clarify the mechanisms of gastrointestinal disease is of great importance.

Rodent models remain the most commonly used animal model for the study of human disease because of their relatively low cost and maintenance requirements, rapid reproduction rates, and availability of research tools such as murine antibodies (Low, 2012). However, rodent models frequently do not mimic clinical signs and significant pathologic hallmarks of human diseases.

Pigs have significant anatomic and physiological similarity with humans, for example, when investigating gastric disease, the pig has a glandular-type stomach similar to that of human beings, whereas the murine stomach has both glandular and non-glandular regions (Fig. 5) (T. T. Kararli, 1995).

Figure 5. ***Schematic diagrams for comparison of murine, porcine, and human gastrointestinal tract anatomy and histology.*** *Derived from* **(Ziegler et al., 2016)**



Humans and pigs shares similarities in the structure of intestine that contribute to the comparable transit time and analogous digestive and absorptive processes reported for these species (Graham and Aman, 1987).

Shared microscopic features also exist, including the structure of the villi and the types of cells that constitute the intestinal epithelium (Fig 6). The villous projection of the small intestine are finger shaped for both humans and pigs and the intestinal epithelium cells, their phenotypic appearance, and their expression of unique protein biomarkers used for cellular identification are similar (Tugrul T. Kararli, 1995). Moreover the morphologic appearance of crypt base columnar stem cells is consistent with the ones described for humans

(Gonzalez et al., 2013). Taken together, these features contribute to fundamental digestive processes such as mucosal transport and motility that are similar between pigs and humans.

The pig serves as a valuable model for studies of human intestinal injury, repair, and efficacy of novel therapeutics, some direct clinical applications are summarized in table 5 (Gonzalez et al., 2015).

Table 5. ***Direct clinical application of porcine models.*** *Modify version from Gonzalez et al. 2015.*
Abbreviations: CF, cystic fibrosis; CRF, corticotropin-releasing factor; FDA, Food and Drug Administration; GLP-2, glucagon-like peptide 2; NEC, neonatal necrotizing enterocolitis; SBS, short bowel syndrome.

Clinical impact	Research support using porcine models
Understanding role of early life stress on the onset and exacerbation of clinical symptoms of human gastrointestinal diseases	-Early life stress induces a marked increase in serum and intestinal CRF that plays a critical role in intestinal permeability and diarrhea. -Administration of CRF receptor antagonists and mast cell stabilizers to pigs prevented stress-induced increases in intestinal permeability
Treatment of reperfusion injury	-Activation of xanthine oxidase plays less important role than initially postulated in inciting injury -Injury is mediated by neutrophil release of oxygen metabolites and transmigration through epithelial tight junctions creating physical damage
Treatment of NEC patients	-Oral probiotics reduce the incidence and severity of NEC in very low birth weight infants[148]
Dietary considerations after massive bowel resection	-GLP-2 hastens intestinal adaptation; FDA drug approval
Successful intestinal transplantation	-Simultaneous liver transplant does not decrease the risk of bowel graft rejection and is no longer recommended in patients with SBS with normal liver function
Cause and treatment of gastrointestinal dysfunction associated with CF	-Pig model of CF accurately displays clinical manifestations of human disease permitting study of pathogenesis of intestinal obstruction
Diagnosis of familial adenomatous polyposis	-Porcine transgenic model accurately represents disease manifestation, including location and histologic appearance of lesions

The pig model has also been used extensively to study esophageal diseases due to the characteristic of the mucosa (Abdulnour-Nakhoul et al., 2007). In pigs, the esophageal and gastroesophageal junction anatomy and physiology are comparable with that of humans, both sharing the existence of large, dissectible sub mucosal glands, making the pig a preferred model for analogous clinical applications (Ziegler et al., 2016). In particular, for what concern the esophagus, pigs are used commonly to test new surgical procedures but also as model for regenerative and protective treatments (Di Simone et al., 2012).

Additionally, pigs, like humans, are true omnivores, whereas other potential mammalian models such as dogs, cats, ruminants, rabbits, and rodents have evolutionarily developed alternative digestive strategies, moreover both pigs and humans are colon fermenters and have similar colonic microbiota composition.

The gut microbiota has been the subject of study for many decades because of its importance on the health and wellbeing of animals. Two types of microbial populations have been described in mammals: autochthonous and allochthonous (Dubos et al., 1965). Autochthonous bacteria are the resident stable microbes that were described by DuBos as co-evolving within a mammalian habitat. The allochthonous bacteria are considered to be non-resident microbes that are 'passing through' a habitat or that represent microbes that are opportunistic colonizers of a habitat and may be associated with disease states or other perturbations.

The gut microbiota is then closely associated with the health of the host and there is a strong interaction between host physiology, nutrition, microbiota and health. The role of gut microbiota is today well recognized from the scientific world, but also from the community and animals breeders as shown in figure 6. The role of microbiota on zootechnical parameters as growth rate and food conversion rate is indeed well correlated.

Figure 6 ***Figure showing the intestinal tract of a piglet.*** *The picture is the advertising of a pig diet based on Lactobacillus acidophilus fermentation technology. From "SynGenX | Diamond V," n.d.*

Animals develop an intestinal microbiome over time and space. During the growth and development of the pig, the microbiome changes in composition in a process known as the microbial succession, for example the microbiome is an important contributor to the development of the host's immune system (Isaacson and Kim, 2012). There are clear and distinct differences in the composition of the pig intestinal microbiome moving from the proximal end of the intestinal tract to the distal end, the comparative composition of humans

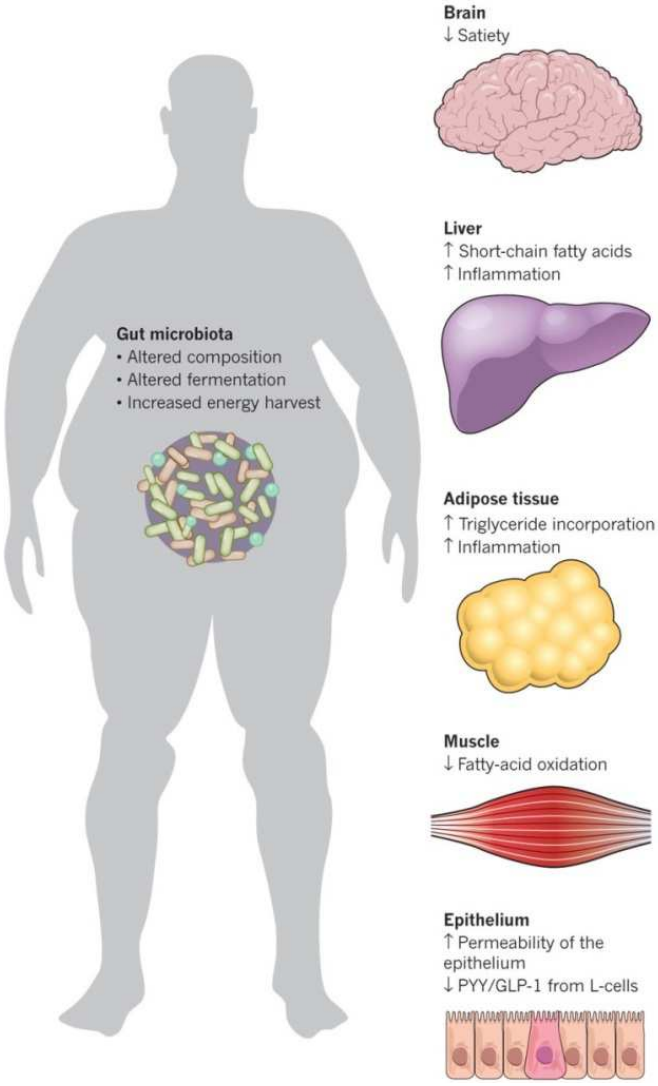
and pigs' microbiota is summarized in table 6. Perturbations to the microbiome occur in response to many factors including stresses, treatment with antibiotics, and diet (Leser et al., 2000; Phillips et al., 2004; Dowd et al., 2007).

Table 6. ***Bacteria Phyla in order of dominance in human and pig gut microbiota.*** *Data from* **(Isaacson and Kim, 2012; Tremaroli and Bäckhed, 2012)**

Human gut microbiota	Pig gut microbiota
Firmicutes	Firmicutes
Bacteroidetes	Bacteroidetes
Actinobacteria	Proteobacteria
Proteobacteria	Cyanobacteria
Verrucomicrobia	Actinobacteria
Euryarchaeota	Spirochaetes
Cyanobacteria	
Fusobacteria	
Lentisphaerae	
Spritochaetes	

The link between the microbes in the human gut and the development of obesity, cardiovascular disease and metabolic syndromes, such as type 2 diabetes, is becoming clearer (Fig 7). However, because of the complexity of the microbial community, the functional connections are less well understood (Tremaroli and Bäckhed, 2012).

Figure 7. ***Alterations to the composition and metabolic capacity of gut microbiota in obesity promote adiposity and influence metabolic processes in peripheral organs, such as the control of satiety in the brain; the release of hormones from the gut (shown as PYY and GLP-1); and the synthesis, storage or metabolism of lipids in the adipose tissue, liver and muscle. Microbial molecules also increase intestinal permeability, leading to systemic inflammation and insulin resistance****.** *From Tremaroli et al. 2010.*



A Human microbiota-associated (HMA) swine have been developed using inocula from infants , children and adult to investigate the role of microbiota on normal development, but also to study microbiota associated disease, like those mentioned above, and microbiota targeted therapies (Wang and Donovan, 2015).

In their review on the pig model for appetite, metabolic syndrome and obese type 2 diabetes, Koopmans and Schurman take in consideration different papers. It emerges that both humans and pigs are prone to the development of obesity and related cardiovascular diseases such as hypertension and atherosclerosis and that bad (LDL) is high and good cholesterol (HDL) is low in pigs, like in humans. Diet-induced obese pigs show a phenotype related to the human metabolic syndrome including high amounts of visceral fat, fatty organs, insulin resistance and high blood pressure. To accelerate the induction of obese type 2 diabetes, obese pigs have been titrated with streptozotocin, a chemical agent which selectively damages the insulin-producing pancreatic beta-cells at such a level that hyperglycaemia was induced but lipolysis was still inhibited due to the fact that inhibition of lipolysis is more sensitive to insulin compared to stimulation of glucose uptake (Koopmans et al., 2006; Larsen et al., 2006).

All the characteristic displayed make the pig one of the most important model in the field of nutrition and microbiota investigation associated with human health (Leulier et al., 2017).

2.2 Visual science applications

As described before in this paper the pig is an excellent model due to its similar anatomy and physiology with humans. For the eyes the situation does not differ, the porcine eye indeed displays many similarities with the human eye.

Anatomic parameters of the porcine eye are summarized in table 7 (Middleton, 2010)

Table 7. ***Measurements of anatomic parameters of the porcine eye. Modified from Middleton, 2010*****.**

Parameter	Measurement	Notes	References
Globe Size (mm) [±SD]	20.1 [0.74] × 23.5 [0.85] × 24.9 [0.87]		(McMenamin and Steptoe, 1991)
Globe Volume (ml) [±SD]	22–24 × 25 × 26–27		(Prince, 1960)
	6.5 [0.3]		(McMenamin and Steptoe, 1991)
Number of Ciliary Processes	95 main processes	Smaller processes between each pair of larger ones	(Prince, 1960)
Intraocular pressure (mm Hg) [±SD]	15.2 [SEM 1.8]	Data from anesthetized animals	(Ruiz-Ederra et al., 2005)
	14.1 [2.2]	Data from anesthetized animals	(Castejon et al., 2010)
	27.3 [3.45] OD 26.3 [3.14] OS	Data from minipigs (not anesthetized)	(Rosolen et al., 2003)
Mean scleral surface area (cm^2) [±SD]	7.78 [0.66], small pigs 9.66 [0.75], medium pigs 11.92 [1.57], large pigs		(Olsen et al., 2002)
Mean corneal surface area (cm^2) [±SD]	1.09 [0.07], small pigs 1.15 [0.09], medium pigs 1.4 [0.19], large pigs		(Olsen et al., 2002)

Corneal dimensions (mm)	17–19 × 14–16.5	(Prince, 1960)
	14–16 × 13–14	(Sisson, 1914)
Corneal thickness (mm)	<1	(Prince, 1960)

Due to the characteristic of the eye the pig is a validated animal model of glaucoma, it has been used in cataract surgery research (Machuk et al., 2016; Nishi et al., 2008; Sugiura et al., 1999), corneal transplant studies (Choi et al., 2011; Kim et al., 2016) and aberrometry studies (Sanchez et al., 2011).

More in detail a pig model of glaucoma based on the occlusion of the episcleral vein method, was produced; the damage was considered to be glaucomatous due to the presence of elevated intraocular pressure, altered eye fundus morphology and retinal ganglion cell loss (Ruiz-Ederra et al., 2005).

To solve the shortage of donor corneas, Choi and colleagues suggested that decellularized porcine cornea could be a promising substitute for human corneal allograft (Choi et al., 2011). Kim proposed Seoul National University (SNU) miniature pig cornea for xenocorneal transplantation using the same preservation protocol as human with respect to biophysical and functional properties; they found that with this protocol the corneas could be stored for up to 7 days for transplantation in human clinical trials (Kim et al., 2016).

When we think of human vision diseases we have to consider retinal degenerations.

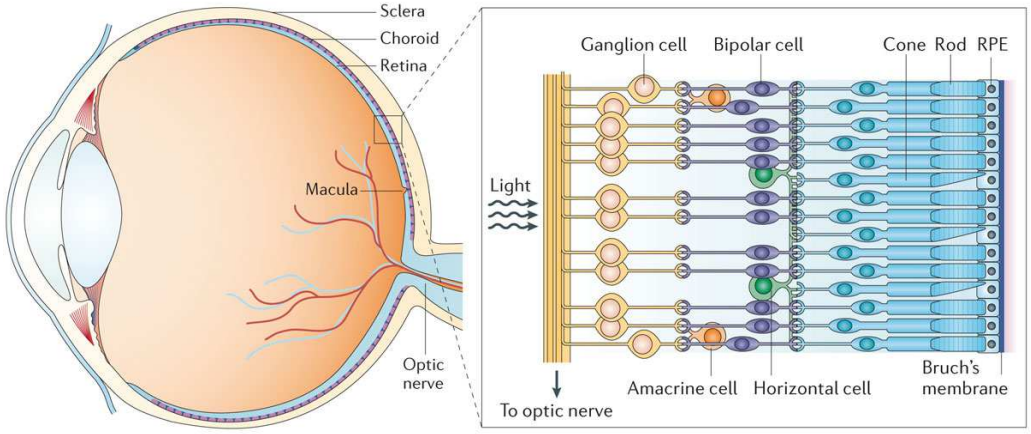
Retinal degenerations (RDs) are a family of inherited retinal degenerative diseases (dystrophies) that lead to vision loss. Although phenotypically very different, the RDs have several characteristic in common.

They are all caused by gene mutations and lead to photoreceptor dysfunction and often to the death of both rod and cones cells. It is estimated that more than fifteen million people around the world have vision loss due to an inherited RD (Chader, 2002).

The principal RDs are dry and wet age-related macular degeneration (AMD), retinitis pigmentosa (RP) and Leber congenital amaurosis.

Human vision is based on three systems of photosensitivity: rod photoreceptors, cone photoreceptors and melanopsin-expressing retinal ganglion cells (Fig. 8) (Kimbrel and Lanza, 2015) .

Figure 8. ***The eye and the cell types of the retina.***
From (Kimbrel and Lanza, 2015)



Rod photoreceptor cells are responsible for the detection of stimuli in dim light, whereas cone photoreceptor cells transmit visual information in bright light conditions. Melanopsin-expressing retinal ganglion cells are also sensitive to bright light, and are implicated in non-image-forming visual functions such as circadian synchronization or pupillary light reflexes (Nasir-

Ahmad et al., 2017). Cones also provide colour vision and high visual acuity, allowing precise tasks such as reading or facial recognition.

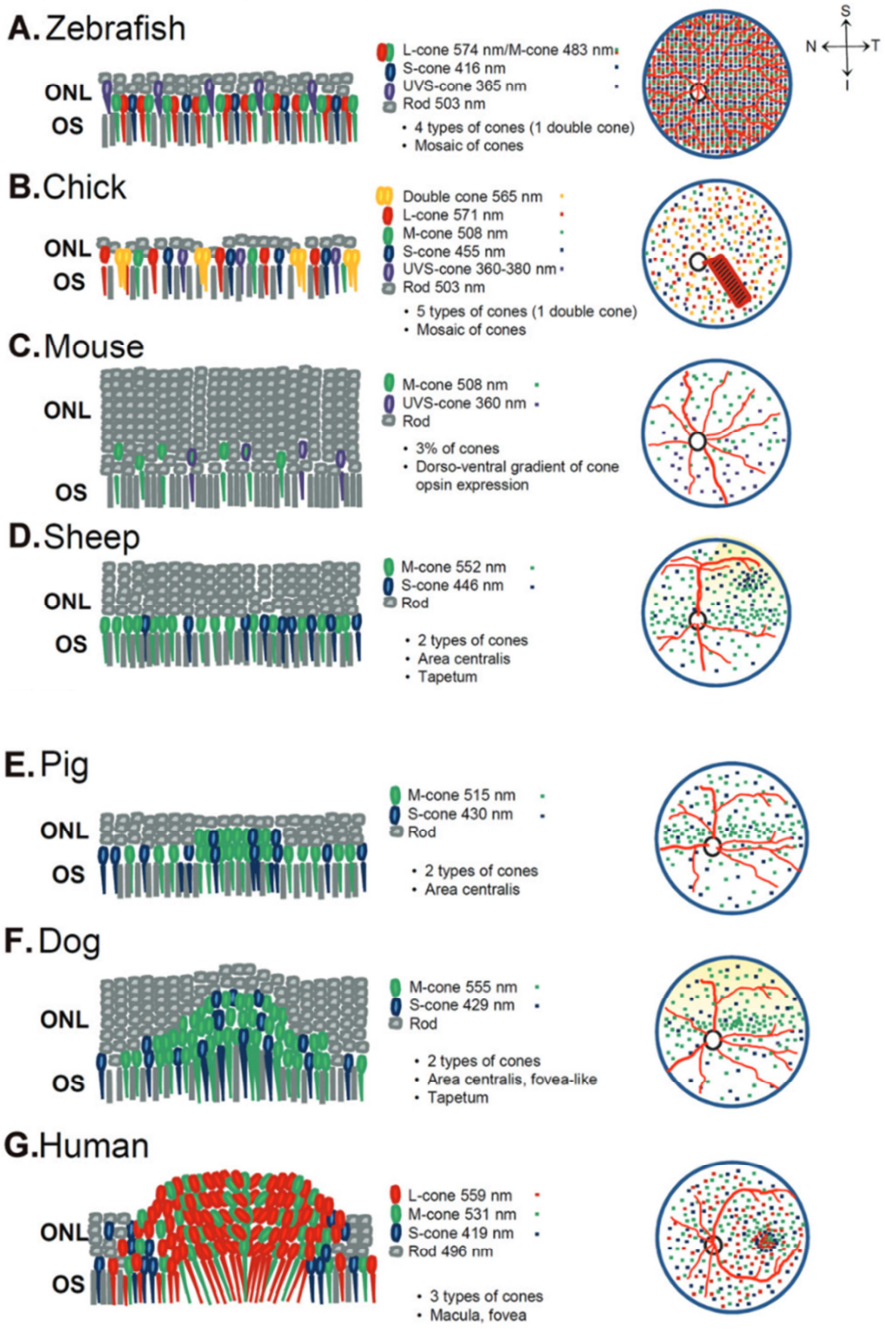
The loss of the cone system is dramatic in our highly technological world, where reading and visual communication are extremely important for social interactions. Moreover,in patients suffering from rod deficiency, a secondary death of cones is observed which definitely worsens their quality of life.

To date, no molecular therapy has been successful in stopping cone degeneration, thus, the study of cone degeneration mechanisms and the examination of innovative therapeutic strategies are a major field of research in ophthalmology (Kostic and Arsenijevic, 2016).

It is very important to find and charactrize animal models to study cone dystrophy, in figure 9 there is a schematic rapresentation of cone distribution in different species.

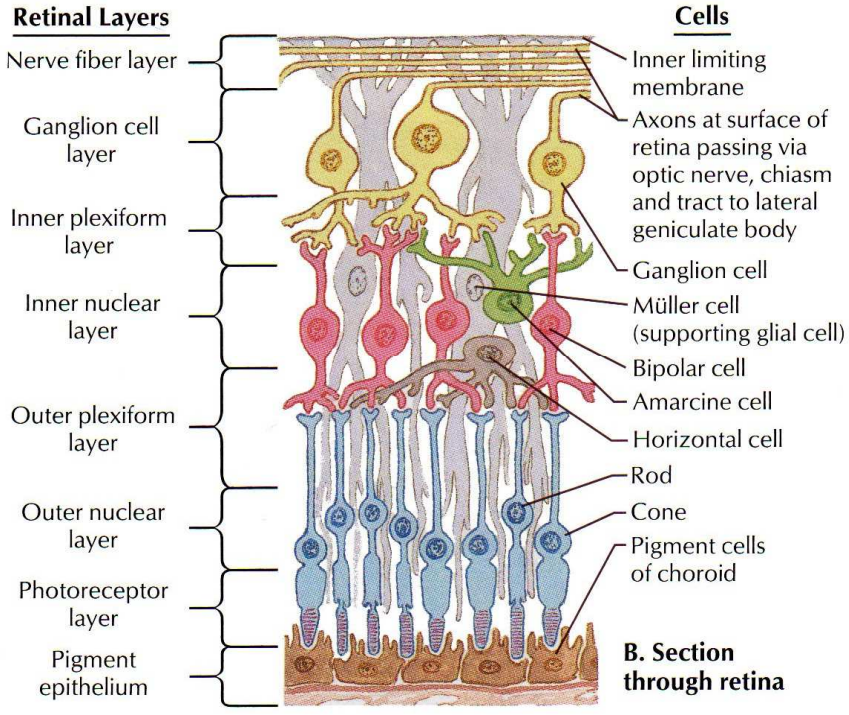
Figure 9. ***Schematic representation of cone distribution in the central retina of different species.*** *Both sections of the outer nuclear layer and visualization of the eye fundus were schematically represented to highlight the specific cone distribution of the retina of zebrafish, chick, mouse, sheep, pig, dog and human. Photoreceptor types are arbitrarily coloured to represent the different categories of cones (L, M, S, and double cones) and rods. The wavelength of each photopigment is also indicated. (A) In zebrafish, in addition to S- and UV-cones, a double cone is observed with fusion at the level of the IS of two OSs containing either L- or M-opsin. The four classes of cones are then laid out in a regular mosaic pattern. (B) The chick retina contains five types of cones comprising a double cone (in yellow) as well. The different types of cones are homogeneously arranged in the retina. A particular vascular extension, the pecten (red rectangle), is apposed at the inner part of the retina. (C) The mouse retina is composed of only 3% cones, distributed throughout the retina. A dorso-ventral gradient of cone expression is observed, with S-opsin mainly expressed in the inferior hemisphere and M-opsin in the superior hemisphere. However, both opsins are observed in single cells in the overlapping gradient. (D) The sheep retina includes two types of cones, with higher densities of cones in the central streak and in the dorso-temporal region. S-cones are enriched in this particular peripheral region. Sheep have a tapetum, a membrane reflecting the light in the superior hemisphere (yellow area) except in the dorso-nasal periphery. (E) The pig retina features two types of cones, with densities higher in the central streak. (F) The dog retina is also characterized by two types of cones and a central streak, but recently a fovea-like region was identified with an increased number of cones and a longer OS. Dogs also have a*

tapetum in the superior hemisphere (yellow area). (G) The human central retina is characterized by a region with exclusively cones named the fovea, containing mainly L- and M-cones. S-cones are distributed in the perifovea region and in the periphery. ONL: outer nuclear layer; OS: outer segment; black circle: optic nerve head; red lines: vessels; S: superior; I: inferior; N: nasal; T: temporal.
Source: (Kostic and Arsenijevic, 2016)



Pig retina maintains the structure of ten layers, the same as in the human retina because embryonic development is similar (Gu et al., 2007; Sanchez et al., 2011). The outer plexiform layer contains the synapses between the photoreceptors and second-order neurons, bipolar and horizontal cells. The inner plexiform layer is the other synaptic zone and connects third-order neurons, like amacrine and ganglion cells, with the bipolar cells. Müller cells are the main retinal glial cells that extend through most of the retina from the outer segments, where their finger-like processes form the outer limiting membrane to their basement membrane, which makes up the inner limiting membrane (Beauchemin, 1974; Beattie et al., 2007). The retina structure is schematically represented in figure 10.

Figure 10. ***Layers of the retina.*** *Light impinging on the retina comes from the top of the figure and passes through all the superficial layers to reach the photoreceptor rods and cones. From* (Koeppen and Stanton, 2017)



Chandler M.J. and Hendrickson Anita studied the distribution and the density of the domestic pig retina finding an overall high density of cones and a large horizontal band running across the retina that contains the highest cone density (Chandler et al., 1999; Hendrickson and Hicks, 2002). This band was found to be free of large blood vessels indicating that the pig has an central area which is similar to the human fovea (De Schaepdrijver et al., 1992).

The cone density in the horizontal band is between 20 and 40 thousand cells/mm^2, moreover the total number of cones in the pig retina is between 17 and 20 million while in the human retina there are around 5 million cones (Curcio et al., 1990). Given that the human and pig retina are about the same size the pig retina can be considered a higher dense cone retina compared with human retina (Hendrickson and Hicks, 2002). The pig retina has also a higher cones/rods ratio, 1:3 to 1:10 from the streak to periphery with a medium of 1:7-8 while the ratio in human is 1:20 and in Macaca monkeys is 1:15 (Chandler et al., 1999; Curcio et al., 1991). Both medium (m) – and short (s) – wavelength sensitive cones were detected in the domestic pig retina, with S cone percentage ranging from 7,4 to 17,5% (Hendrickson and Hicks, 2002).

The color of the optic streak and fundus is orange to pale gray with pigmented epithelial cells, which is very similar to the human eye, and there is no retinal tapetum in the porcine eye (Ollivier et al., 2004; Ng et al., 2008).

Due to the similarities with the human the pig was chosen as model by many scientists and several models of retinal dystrophies have been created (table 8).

Table 8. ***Pig models of cones dystrophies.*** *Modified from (Kostic and Arsenijevic, 2016)*

Model	Gene	Method of generation	Human condition	Reference
Pig TgP347L, Pig-Tg N1Pet	RHO	Pronuclear injection and homologous recombination	RP	(Petters et al., 1997)
Pig TgP23H, line 53-1	RHO	Somatic cell nuclear transfer	RP	(Ross et al., 2012)
Pig ELOVL4-790e794delAACTT	ELOVL4	Pronuclear injection and homologous recombination	STDG3	(Sommer et al., 2011, p. 4)
Pig ELOVL4-Y270terEYFP	ELOVL4	Somatic cell nuclear transfer	STDG3	(Sommer et al., 2011, p. 4)
Pig GUCY2D-E837D;R838S	GUCY2D	Lentiviral-directed transgenesis	CORD6	(Kostic et al., 2013)
IAA Pig	Na	12mg/kg systemic iodoacetc acid (IAA)	PR degeneration	(Scott et al., 2011; Wang et al., 2011; Noel et al., 2012)

The first transgenic (Tg) pig to model a retinal dystrophy was generated by pronuclear microinjection to mimic a severe form of retinitis pigmentosa caused by the dominant rhodopsin (RHO) gene mutation p.P347L (Petters et al., 1997).

This work was then followed by the generation of TgP23Hpigs (Ross et al., 2012) using stable transfected pig fibroblasts, the NIH miniature pig (SLA $^{C/C}$ haplotype) was used. Although these mutations affect specifically rod function and survival, these models are also interesting for cone studies as cone death is observed following rod loss (Kostic and Arsenijevic, 2016). This secondary wave of photoreceptor death is hypothesized to be related to a deficit in a survival factor secreted by rods, the rod-derived cone viability factor (RdCVF) which is an inactive thioredoxin secreted by rod photoreceptors that protects

cones from degeneration (Aït-Ali et al., 2015). RdCVF was found to act through binding to Basigin-1 (BSG1), a trans membrane protein expressed specifically by photoreceptors. BSG1 binds to the glucose transporter GLUT1, resulting in increased glucose entry into cones promoting cone survival by stimulation of aerobic glycolysis. The mechanism that lead to cones death during the late phase of retinitis pigmentosa remain obscure, both TgP347L and TgP23H pigs could help to understand it.

The most severely affected TgP23H animals were investigated at 14 days of age, showing an absence of the scotopic response, the cone response had declined at 2 months and had decreased to 50% at 3 months of age (Fernandez de Castro et al., 2014). The photopic (cones) response then remained at approximately 30% of the wild type WT response until 18–24 months. These two models clearly reveal that cone degeneration rapidly follows rod loss, indicating that cone protection needs to be performed at an early stage of rod dystrophies (Kostic and Arsenijevic, 2016).

A juvenile form of macular degeneration termed Stargardt-like macular dystrophy type 3 (STGD3) is associated with mutations in the elongation of the very long-chain fatty acids-4 gene (ELOVL4), which is mainly expressed in rods and cones (Zhang et al., 2001). The cones in the macula are particularly affected in comparison to rods; the presence of truncated ELOVL4 proteins recapitulates different figures of the STGD3 disease in pigs, thus proposing interesting models to study pathophysiology of this disease.

CORD6 is the most frequent dominant cone dystrophy form in humans. The p.E837D and p.R838S variants of the GUCY2D gene product are associated

with disease appearance (Gregory-Evans et al., 2000). By using a lentiviral vector encoding the mutated GUCY2D cDNA controlled by the cone arrestin promoter and injected in fertilized oocytes, Kostic and colleagues generated three cohorts of transgenic pigs (Kostic et al., 2013).

Although the response variability was high in the transgenic pigs, changes in the behavior tests were observed in several transgenic animals and a significant alteration of transgenic cone activity was noticed by ERG in comparison to WT littermates. Moreover, histology revealed that all transgenic pig eyes had displaced cone nuclei that localized to the OS region, indicating a degenerative process (Kostic et al., 2013).

A rapid inducible swine model of photoreceptors degeneration was generating using the iodoacetic acid IAA by three different scientists. Scott et al. investigated whether IAA produced a damage preferentially for rods or cones and whether IAA induced remodeling of the inner retina using four doses of the chemical (5; 7,5; 10 and 12 mg/kg) (Scott et al., 2011). The author found that systemic IAA administration induces rapid and robust degeneration to photoreceptors and in particular rods. The inner retina was largely spared, exhibiting only subtle changes including dendritic retraction of rod bipolar cells and hypertrophy and swelling of Müller cells.

Wang found the IAA model very similar to the RHO transgenic pig model, they also evaluated ERG signals, finding that the signals from rods were diminished at 2 weeks after IAA injection, and they remained diminished until 12 weeks. By contrast, cone ERG signals dipped at 2 weeks after IAA injection but rebounded significantly by 5 weeks, and this recovery was maintained to 12

weeks. Wang suggested that the dip in response at 2 weeks reflected the initial covalent modification to GAPDH and the resultant block of glycolysis when IAA was injected (Graymore and Tansley, 1959), probably after the injection cones could resynthesize GAPDH and restore glycolysis and that the cone defects was a secondary result of the surrounding rod loss.

Noel and colleagues compared the effectiveness of IAA with Sodium iodate ($NaIO_3$), finding the second one ineffective in swine (Noel et al., 2012). The authors tested the same concentrations of IAA of the previous work published by Scott et al., 5, 7.5, 10 and 12 mg/kg. The data confirm the preferential damage in rods compare to cone due to IAA administration and the damage is concentration-dependent. The mfERG was performed only in 7.5mg/kg IAA treated pigs, but as the light adapted ERG for the same IAA concentration showed an increase of cones responds 5-6- weeks after treatment. This data confirm Wang theory of cones ability to resynthesize GAPDH and restore glycolysis. The trend is not maintained for the higher IAA concentration, thus moderate concentrations of IAA can mimic more initial stages of *retinitis pigmentosa* while the 12 mg/Kg concentration the late state of disease.

This founding make the IAA pig model a rapidly and versatile model of *retinitis pigmentosa* for the study of different therapeutic approaches, from cell transplantation to neuroprosthetic devices (Zhang, 2016).

3 *The role of veterinarians and legislative aspects*

There is an increasing need for veterinarians worldwide. Areas of greatest need include translational medical research, veterinary pathology, laboratory-animal medicine, emerging infectious diseases, public health, academic medicine, and production-animal medicine. Veterinarians have unique skill sets that enable them to serve as leaders or members of interdisciplinary research teams involved in basic science and biomedical research with applications to animal or human health (Rosol et al., 2009).

Translational medicine is connected with basic science and the use of animals and to improve the translational value of animal models is fundamental to "better know" the model itself.

In addition to performing research themselves, veterinarians play crucial roles in the support of biomedical research. Laboratory animal veterinarians support the medical care and health of laboratory animals, promote animal well-being and humane care of laboratory animals, maintain barrier conditions against diseases to improve laboratory research, conduct collaborative research, provide technical instruction to scientists, monitor compliance with state and federal regulations, and serve on institutional animal research review panels.

Veterinary and medical pathologists (that specialize in laboratory animal pathology) are central to the diagnosis of spontaneous diseases, understanding the mechanisms of disease induced by experimental procedures, and analyzing the phenotype of spontaneous or induced genetic modifications in animals. When there is a lack of expert pathology support on a research project

important information may be misinterpreted or go undiscovered, which reduces the impact of animal-based research.

Laboratory animal veterinarians are professionals who by virtue of interest, experience, and training are specialized in working with and caring for laboratory animals. They are integral members of the research team and provide veterinary support for model development and implementation as they work to safeguard animal wellbeing. Although basic veterinary education imparts some of the specialized skills and information necessary to work with laboratory animals, the diversity and complexity of the laboratory environment and the species used within that environment generally require additional experience and training (Turner et al., 2009).

The International Association of Colleges of Laboratory Animal Medicine (IACLAM) and the International Council for Laboratory Animal Science (ICLAS) support the efforts of regional organization and communities of laboratory animal science professionals as well as the development of local associations and professional colleges that promote the training and continuing education of research facility personnel and veterinary specialists (Turner et al., 2015).

3.1 The three R's

In 1959, William Russell and Rex Burch published "The Principles of Humane Experimental Technique". They proposed that if animals were to be used in experiments, every effort should be made to Replace them with non-sentient alternatives, to Reduce to a minimum the number of animals used, and to Refine experiments which used animals so that they caused the minimum pain and distress (Russell and Burch, 1959).

"Good welfare equals good science" has become the mantra ever since Trevor Poole first highlighted the relationship between research animal welfare and the quality of science, and, in the intervening decades, the evidence base for this relationship has been steadily growing (Prescott and Lidster, 2017). Animal welfare means how an animal is coping with the conditions in which it lives. An animal is in a good state of welfare if, as indicated by scientific evidence, it is healthy, comfortable, well-nourished, safe, able to express innate behavior, and if it is not suffering from unpleasant states such as pain, fear and distress. Every effort should be made to minimize unnecessary harm because animals with compromised welfare have disturbed behavior, physiology and immunology. This can lead to unreliable conclusions and/or unwanted variation in scientific output, affecting both the reliability and repeatability of experiments.

Often, appropriately designed experiments that minimize variation, provide standardized optimum conditions of animals care and minimize unnecessary stress or pain yield better more reliable data (Flecknell, 2002)

The definitions of replacement, reduction, and refinement in the *Principles* were crafted with an overriding and clearly expressed aim: the reduction and, whenever possible, the elimination of animal distress consistent with the conduct of sound science. Put another way, it was not the *use* of animals in research that Russell and Burch found problematic, but the infliction on research animals of *unnecessary or avoidable pain, fear, stress, anxiety, bodily discomfort and other significantly unpleasant feelings* (Tannenbaum and Bennett, 2015).

Replacement

Russell and Burch provide a detailed definition of replacement:

"*We shall use the term 'replacement technique' for any scientific method employing non-sentient material which may in the history of experimentation replace methods which use conscious living vertebrates. Among this non-sentient material, we include higher plants, microorganisms, and the more degenerate metazoan endoparasites, in which nervous and sensory systems are almost atrophied*" (Russell and Burch, 1959).

Non-animal research techniques have helped us to reduce the number of situations in which animal procedures are needed. These techniques include the use of statistical data already obtained from animal research, studies of isolated cells and tissues, new scanning methods, computer models that simulate an animal's response to specific experiments, and studies of patients and populations.

Much safety testing can now be undertaken without the use of animals. For example, the Ames test uses bacteria instead of rodents to see whether a chemical is likely to damage DNA or has potential to cause cancer (Rodríguez et al., 2012). Similarly, some tests now use less complex animals than before: scientists can check injected medicines for most pyrogens (fever-causing bacterial contaminants) by using blood cells from the horseshoe crab, replacing tests on rabbits (Wachtel and Tsuji, 1977). More recently, the bovine whole blood assay (bWBA) reproducibly enabled sensitive detection of endotoxin and non-endotoxin pyrogens and may thus become a viable alternative for pyrogen testing (Wunderlich et al., 2014).

Unfortunately, some non-animal research methods only give limited information about what happens in a whole, living animal. Despite the progress made as a result of attention to this principle, several major problems have been identified. When replacing animals with alternative methods, it has often proven difficult to formally validate the alternative. This has proven a particular problem in regulatory toxicology, especially when combined with the labyrinthine processes of the various regulatory authorities (Flecknell, 2002).

Reduction

The *Principles* defines reduction as "reduction in the numbers of animals used to obtain information of a given amount and precision" (Russell and Burch, 1959). Like the other Rs, reduction serves the aim of reducing and when possible removing inhumanity or distress.

This can be achieved in many ways. Good experimental design and statistical analysis ensure that researchers use the optimum number of animals. Using inbred animals means researchers can get reliable results from fewer of them. Animals get fewer secondary infections or diseases, which might interfere with studies, if kept in clean environments. New scanning techniques mean that tumors can be tracked non-invasively, with more data collected from the same animal (Xing et al., 2014). Measures like freezing embryos mean that fewer rodents are needed for genetic modification breeding programs and in general the biobanking system reduce the number of animal and the waste of biological material (Artene et al., 2013).

For ethical and economic reasons, it is important to design animal experiments well, to analyze the data correctly, and to use the minimum number of animals necessary to achieve the scientific objectives, but not so few as to miss biologically important effects or require unnecessary repetition of experiments. Investigators are urged to consult a statistician at the design stage and are reminded that no experiment should ever be started without a clear idea of how the resulting data are to be analyzed (Festing and Altman, 2002).

The principle of Reduction would appear less contentious, but its application has highlighted the difficulties of providing appropriate expert statistical advice, especially in academic research facilities. In some instances, concern to implement Reduction strategies can result in the use of too few animals, which leads to inconclusive results, and wasteful experiments (Flecknell, 2002).

Refinement

The *Principles* defines refinement as “any decrease in the incidence or severity of inhumane procedures applied to those animals which still have to be used” (Russell and Burch, 1959). Russell and Burch subsequently state that the “object [of refinement] is simply to reduce to an absolute minimum the amount of distress imposed on those animals that are still used”

Paul Flecknell, the author of Laboratory Animal Anesthesia, highlighted the problems concerning the Refinement. He noticed that people tend to make assumptions about animals and their feelings that often have little scientific basis. The problem is that these assumptions concerning an animal's likely experience of pain and distress may become incorporated into institutional or national policies, without any attempt to verify them. To give an example - it is reasonable to assure that animals will experience pain after a surgical procedure, so pain-relieving drugs should be given to prevent this. Requiring every animal to have the same dose of the same drug after any surgical procedure is not the best way of dealing with postoperative pain (Roughan and Flecknell, 2003). He suggest to focus on this area, not only to work on new methods of implementing the 3Rs, but also to disseminate current "Best Practice”, and to revise this advice as further progress is made.

A good example of refinement is the new approach developed by Michael Emerson and colleagues for studying pulmonary embolism in mice (Moore and Emerson, 2012). Conventional modeling relies on injection of thrombogenic substances in conscious animals that often results in paralysis and death. In contrast, Emerson's refined model is performed under general anesthesia using

radiolabeled platelets and imaging to measure platelet function in real time during non-fatal thromboembolism. Not only is the new *in vivo* model a significant refinement, it also better mimics the physiology and biochemistry of the condition in man and models a broader spectrum beyond the extreme, fatal stage (Prescott and Lidster, 2017).

The 4th R, Responsibility

The 4th R of Research implies addition of 'responsibility' to the original three R's of Russell and Burch(Banks, 1995). It has grown into a new era of performance-based outcomes, which reflects integrity, honesty, and scientific correctness in appropriate and reasonable use of laboratory animals. This ensures that animal life is required and necessary for biomedical advancement.

The concept of responsibility is being mobilized at both the individual level and with relation to good scientific practice more broadly at the organizational level. Institutions and industry that use animals in their research often make reference to the 3Rs, and it appears this is 'used as evidence of the research community's commitment to meet high ethical standards in the care and use of laboratory animals' (Franco and Olsson, 2014). For example, the University of Oxford state on their website that they are 'committed to pursue a policy of reduction, replacement, and refinement (3Rs) in all animal based research and to promote knowledge of the moral and legal responsibilities and a culture of care in all aspects of research' ("University Policy on the Use of Animals in Scientific Research | University of Oxford," n.d.).

At the broader governance and legislative level, the original embedding of the 3Rs principles within the 1986 EU legislation was clearly tied to expectations of 'responsible animal research', and more attention to ethical dimensions within animal research (Matthiessen et al. 2003). More recently, the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs), in collaboration with six other major UK funding bodies, has produced a set of guidelines entitled *Responsibility in the Use of Animals in Bioscience Research* ("Responsibility in the use of animals in bioscience research | NC3Rs," n.d.). Within the guidelines, various responsibilities are set out for researchers, ethics committees, and peer reviewers to ensure implementation of the 3Rs. These responsibilities are mainly tied to legislative obligations, and it is notable that there is no mention of responsibility to the general public or wider society.

4 *Aims*

Scientists are becoming every day more aware that a deep knowledge of an animal used in biomedical science can lead to more reliable data. The role of the 3Rs in promoting health and welfare of laboratory animal models has improved in the last 50 years becoming a guide to the entire scientific and non-scientific community. The translational value of animal models is becoming an important topic of debate due to the high frequency of fail when animal experiments are replicate on humans.

The pig is a largely used and quite validated animal model for many human diseases. The role of the gut microbiota is well known and its impact on the host health is enormous; the pig has already been identified as a strong reliable model to study gut microbiota modulation.

For this reasons the first aim of this work was to better characterize the role of nutrition on the gut microbiota in the pig model. More in detail we wanted to investigate the impact of einkorn flour, sourdough fermentation and iron fortification on the gut microbiota and inflammatory status using the pig as translational model.

Vision is the most precious among the five senses. Unfortunately degenerative retinal diseases that lead to vision decrease and blindness are extremely frequent with enormous social and economic costs. Many strategies have been applied worldwide to find a treatment for retinal degeneration, from gene therapy to bio-prosthetic devices. But new therapeutic approaches need to be tested on animal models.

The pig due to anatomical and physiological similarities is often the gold standard model to test those strategies. The pig retina is indeed rich in cones, the cells responsible for daytime vision and visual acuity. Electrophysiological investigation is the tool to characterize and follow the progression of retinal diseases. It is very useful in clinic but also as preclinical investigation on animal model, for this reasons it is important to standardize and divulgate electrophysiological retinal responses of most commonly used animal models.

The second aim of my work was to investigate a chemically inducible retinal degeneration pig model. This model is a rapidly inducible cost-effective model and the characterization of its visual pathways could help to spread its use in biomedical science.

5 *The pig model to evaluate new bakery functional foods: the European project Bake4Fun*

Nutritional iron deficiency (ID) is estimated to affect 1.5–2 billion people worldwide. In the Western society, consumers are increasingly adopting a preventive dietary strategy to reduce food intake or the consumption of specific foods, thus leading to the decrease of micronutrient intake correlated to ID. BAKE4FUN responds to the needs of food SMEs of innovating food formulations and technologies to overcome the negative effect of iron fortification of bakery products on their sensorial characteristics in order to produce bakery foods with an improved nutritional profile and health-promoting effects.

Figure 11. ***The logo of the European project Bake4Fun***

In particular, BAKE4FUN (Fig. 11) aimed to provide, validate and develop innovative health-promoting bakery products based on a new iron microencapsulation technology and on the use of non-conventional high-added value whole grain flours, able to obtain healthy, sustainable and ethically correct products, to increase antioxidant intake and to improve the gut microbiota.

Research activities carried out within BAKE4FUN were focused on i) the effect of bread obtained by the sourdough fermentation of non-conventional grains on the gut microbiota, the glycemic load, and the inflammatory and oxidative status; ii) the bioavailability of microencapsulated iron in fortified

breads, their effects on the iron status, on biomarkers of oxidation and inflammation.

To respond to the need of developing safe, effective and validated functional bakery products three in vivo experiment were carried out on animal model. The pig was chose for its similarities with human gastro intestinal tract and microbiota.

Ethical Statement

The use of animals in the experiments 1, 2 and 3 was regulated by two protocols approved by the Italian Ministry of Health with D.Lgs 116/92.

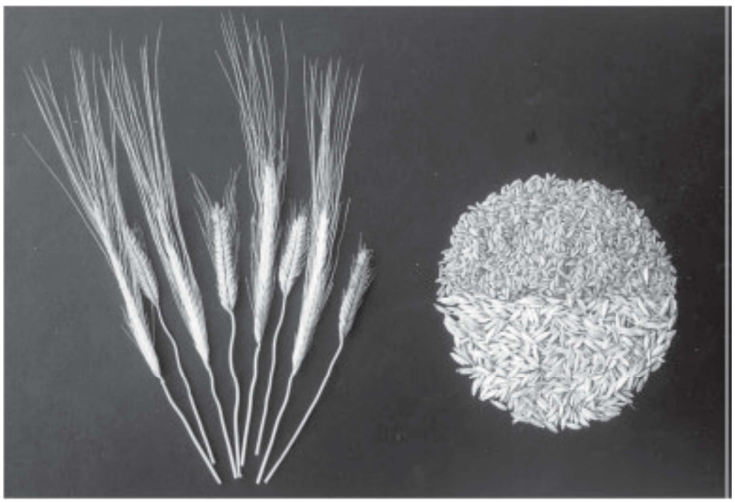
The experimental protocol was presented at the first section of Ethical Committee on Animal Experimentation available after the definition of the research plan. The experimental protocol was numbered 10-75-2013, discussed by the Committee in the session of 15 April 2013 and obtained the approval.

Subsequently the protocol was sent to the Ministry of Health in communication regimen on the 7 June 2013, obtaining authorization by means of a tacit consent 30 days after the receipt.

5.1 Experiment 1. Evaluation of the impact of novel ancient flour bakery products on the intestinal microbiota, oxidative and inflammatory status

Einkorn (*Triticum monococcum* L. ssp. *monococcum*) is a diploid (2*n*=2*x* =14) hulled wheat and a close relative of durum (*Triticum turgidum* ssp. *durum*) and bread (*Triticum aestivum* ssp. *aestivum*) wheats (Fig. 12). It was domesticated about 10 000 years ago in the Fertile Crescent, most probably in the Karacadağ mountains of Turkey, and spread to Europe during the Agricultural Revolution (Hidalgo and Brandolini, 2014).

Figure 12. ***Spikes, hulled seeds and threshed seeds of Triticum monococcum ssp. monococcum.*** *From*(Hidalgo and Brandolini, 2014)



The new interest in einkorn is based on its thrifty nature, particularly appropriate for low-input and organic managements, and on the perceived high nutritional value of its flour. Numerous recent studies confirm a consensus positive evaluation of the dietary merit of *T. monococcum*. Compared with the widespread durum and bread wheats, einkorn kernels are significantly richer in proteins, lipids (mainly PUFA and MUFA) and fructans; some trace elements,

including the ever-important zinc and iron, are also found in higher concentrations (37.2–62.6 mg/kg iron; 42.7–71.1 mg/Kg zinc) . Carotenoids, tocols, conjugated phenolics, alkylresorcinols and phytosterols, antioxidants with relevant healthful properties, are present in significant quantities; the low *β*-amylase and lipoxygenase activities of einkorn whole meal flours limit their degradation during food processing, largely maintaining the positive nutritional properties of this species. On the other hand, compared with bread wheat, einkorn has lower dietary fiber and insoluble bound polyphenol contents, along with higher PPO activity. For the above mentioned characteristic einkorn is a promising candidate for the development of new or special foods such as bakery products, baby foods or products with high contents of dietary fiber, carotenoids and tocols.

The use of sourdough in baked and steamed bread production worldwide is increasing due to the improved sensory and nutritional quality of sourdough bread when compared to bread produced by straight dough processes (Gobbetti et al., 2014). Traditionally, sourdough has been used as leavening agent. The use of sourdough as baking improver in combination with baker's yeast also allows replacement of additives with “clean label” ingredients in industrial baking. Moreover, the fermentation of sourdough in the bakery is an alternative to the use of additives (Gänzle and Ripari, 2016).

The species richness in a single batch of sourdough (α-diversity) is limited; typically, less than 6 different species or strains account for more than 99% of microbial cells.

In contrast, the γ-diversity of sourdoughs, the number of species isolated from sourdoughs globally, encompasses more than 80 bacterial species, including mainly species of the *Lactobacillaceae* and *Leuconostocaceae* but also lactococci, enterococci, and streptococci (Gänzle and Ripari, 2016).

The human intestinal microbial ecosystem plays an important role in maintaining health. A multitude of diseases including diarrhoea, gastrointestinal inflammatory disorders, such as necrotising enterocolitis (NEC) of neonates, and obesity are linked to microbial composition and metabolic activity. Therefore, research on possible dietary strategies influencing microbial composition and activity, both preventive and curative, is being accomplished.

Heinritz reviewed the use of pig as a model to study the dietary modulation of the human gut microbiota (Heinritz et al., 2013), finding the pig a validated model due to its similarities in terms of gastro-intestinal tract and body constitution and microbial fermentation and composition with humans.

The aim of the present experiment was therefore to study the impact of novel ancient flour bakery products on the intestinal microbiota, oxidative and inflammatory status in a pig model.

More specifically we wanted to investigate the role of einkorn flour and sourdough fermentation used to bake breads produced to improve the quality of human health.

5.1.1 Materials & Methods

Animals

36 commercial hybrid pigs (LW x D) of 30 Kg, 18 female and 18 males, all coming from the same piggery were used in this study. The experiment was divided in two blocks in order to guarantee a better management of the animals. The animals were delivered to the ANFI-ASA Unit (Dipartment of Veterinary Medicine, University of Bologna) in two groups, five days before the beginning of the trial (acclimation period) and randomly divided in six groups. The animals were weighed at 0, 7, 14 and 30 days to control the growth performances.

Diet

The pigs were all fed with Swine Standard Diet (SSD) during the acclimation period. The standard diet was particularly formulated for pigs between 30 and 80Kg by CESAC s.c.a. Conselice (RA), and the diet composition is reported in table 9. At the beginning of the trial, each box (three animals per box) was randomly assigned to an experimental diet for 30 days: A, B, C, D, E and F (Tab. 10). From this point all animals and samples were indicated with the alphanumeric code keeping blind all the operators. The experimental bread was prepared and delivered (dehydrated and grinded) to the ASA Unit by Piekarnia W.W.K. Vini in Rogoźnik. The ancient flour was einkorn (*Triticum monococcum* L. ssp. *monococcum*) grown in Italy. The experimental diets were prepared by mixing, for at least 5 minutes, the SSD and one of the experimental bread in 1:1 ratio. A horizontal mixer, single reel of 200 liters capacity with double

concentric coil was used for the mixing. Before and after each mixing session a sample of bread and a sample of the experimental diet were collected and analyzed to quantify total humidity.

Table 9. ***Standard Swine Diet composition (Big 30-80 by CESAC s.c.a).***

Components	
Corn, soy flour, wheat bran, barley, white sorghum, calcium carbonate, vegetable oils and fats, sodium chloride	
Analytical components %	
Crude protein 16.50%, crude oils and fats 3.50%, crude cellulose 4.70%, crude ash 4.10%, calcium 0.50%, phosphorus 0.40%, sodium 0.10%, lysine 0.90%, methionine 0.30%	
Additives /Kg	
Vitamins and pro-vitamins	Vit H 0.25mg
Vit A E627 12500UI	Compounds of trace elements
Vit D3 E671 2000UI	E 4 cupric sulfate, pentahydrate 79mg
Vit E 3°700 50mg	E 8 sodium selenite 0.55mg
Vit B1 2.0mg	E 6 zinc oxide 155mg
Vit B2 4.0 mg	E1 ferrous sulfate monohydrate 456mg
Vit B6 3.0 mg	E5 manganous oxide 129mg
Vit B12 0.025mg	E2 potassium iodide 2.0mg
Vit K 2.5 mg	Antioxidants
Vit PP 25mg	Ethoxyquin E324 34mg
Pantothenic acid 12.5mg	Butylated (BHA) E320 30mg

Table 10. ***Experimental diets.***

Experimental Bread	**Experimental diet**
SFCF = Bread made with Standard Flour + Conventional Fermentation	A
AFCF = Bread made with Ancient Flour + Conventional Fermentation	B
SFSSF = Bread made with Standard Flour + Sourdough Standard Fermentation	C
AFSSF = Bread made with Ancient Flour + Sourdough Standard Fermentation	D
AFSAF = Bread made with Ancient Flour + Sourdough Ancient Fermentation	E
SFSAF = Bread made with Standard Flour + Sourdough Ancient Fermentation	F

Sampling

Fecal samples were collected at 0, 15 and 30 days for gut microbiota evaluation and to investigate differences among digestive process of tested breads by NMR technique. Blood samples (serum, Li-heparin and EDTA) were collected at the beginning and at the end of the trial for clinical evaluation (complete blood count) and to evaluate the impact of tested breads on the oxidative and inflammatory status

For fecal sampling an operator was standing in the box with the animals. The samples were collected directly when spontaneous defecation occurred and immediately stored at -20°C. Samples were then stored at -80°C until the moment of analysis. Blood samples at the beginning and at the end of the trial were collected from jugular vein under general anesthesia with the following protocol: intramuscular (IM) administration of Azaperone (2mg/Kg), after 10 minutes in dark and quite condition an IM bolus of Ketamine (40mg/Kg). Venosafe® Plastic Tubes for Serum Gel, K3EDTA and Li-heparin (Terumo, Tokyo, Japan) were used for blood sampling. Blood samples (K3EDTA) were analyzed at the Veterinary Clinical Pathology Service (SEPAC VET) for complete blood count (CBC). Serum Gel and Li-heparin tubes were centrifuged (100g x 10min), then serum and plasma where stored at -80°C.

Gut microbiota evaluation

DNA extraction

Total bacterial DNA was extracted from fecal material using QIAamp DN Stool Mini Kit (QIAGEN, Hilden, Germany) with a modified protocol (Salonen et al., 2010). Briefly, 250 mg of feces were suspended in 1 ml of lysis buffer (500

mM NaCl, 50 mM Tris-HCl pH 8, 50 mM EDTA, 4% SDS) and 3 times bead-beaten with four 3-mm glass beads and 0.5 g of 0.1-mm zirconia beads (BioSpec Products, Bartlesville, OK) in a FastPrep-24 Instrument (MP Biomedicals, Irvine, CA) at 5.5 m/s for 1 min. After incubation at 95°C for 15 min and centrifugation at full speed for 5 min to pellet stool particles, 260 µl of 10 M ammonium acetate were added to the supernatant, followed by incubation in ice for 5 min and centrifugation for 10 min. One volume of isopropanol was added to the supernatant and incubated in ice for 30 min. Precipitated nucleic acids were washed with 70% ethanol, resuspended in 100 µl of TE buffer and treated with 2 µl of DNase-free RNase (10 mg/ml) at 37°C for 15 min. Each DNA sample was further treated with Proteinase K and purified with QIAamp Mini Spin columns (QIAGEN) following the manufacturer's instructions. DNA concentration and quality was evaluated using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

16S rRNA gene sequencing and processing

For each sample, the V3-V4 region of the 16S rRNA gene was PCR amplified, and the resulting single amplicons of approximately 460 bp were cleaned up and sequenced on Illumina MiSeq platform using a 2×300 bp paired end protocol, according to the manufacturer's instructions (Illumina, San Diego, CA). In detail, PCR reactions were carried out in 25 µl volumes containing 12.5 ng of microbial DNA, 2x KAPA HiFi HotStart ReadyMix (KAPA Biosystems, Resnova, Rome, Italy), and 200 nM of S-D-Bact-0341-b-S-17/S-D-Bact-0785-a-A-21 primers (Klindworth et al., 2013) carrying Illumina overhang adapter sequences. Reaction conditions were as follows: initial denaturation at 95°C for

3 min, followed by 25 cycles of denaturation at 95°C for 30 sec annealing at 55°C for 30 sec, and extension at 72°C for 30 sec, with a final extension step at 72°C for 5 min. Amplicons were purified with a magnetic bead-based clean-up system (Agencourt AMPure XP; Beckman Coulter, Brea, CA). Indexed libraries were prepared by limited-cycle PCR using Nextera technology and further cleaned up with Agencourt magnetic beads. Final libraries were pooled at equimolar concentrations, denatured and diluted to 6 pM before loading onto the MiSeq flow cell.

Raw sequences were processed using a pipeline combining PANDAseq (Masella et al., 2012) and QIIME (Caporaso et al., 2010). High-quality reads were binned into Operational Taxonomic Units (OTUs) at a 0.97 similarity threshold using UCLUST (Edgar, 2010). Taxonomy was assigned using the RDP classifier against Greengenes database. To filter out PCR errors and chimeras, all singleton OTUs were discarded. Alpha rarefaction was performed using the Faith's phylogenetic diversity, Chao1, observed species, and Shannon index metrics. Beta diversity was estimated by computing weighted and unweighted UniFrac distances, which were used as input for principal coordinates analysis (PCoA). All statistical analyses were performed in R 3.1.3. P values were corrected for multiple comparisons using the Benjamini-Hochberg method when appropriate. A corrected $P<0.05$ was considered as statistically significant.

Oxidative and inflammatory status evaluation

Total antioxidant capacity (TAC)

TAC was measured in plasma as previously reported (Amaretti et al., 2013). The method is based on the ability of the antioxidant molecules in the sample to reduce the radical cation of 2,2′-azinobis- (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS·+). TAC was determined in 10µl plasma by evaluating the decoloration of ABTS·+, measured as the quenching of the absorbance at 734 nm. Values obtained were compared to the concentration–response curve of the standard Trolox solution and expressed as micromoles of Trolox equivalents (TE)/mL of plasma.

Glutathione (GSH) content

Plasma reduced GSH concentration was determined as previously described (Amaretti et al., 2013). One hundred µl of plasma were incubated for 30 min in 100 µl reagent buffer (80 mM sodium phosphate, pH 8.0; 2 mM EDTA; 2 % SDS; 250 µM DTNB), and GSH was measured spectrophotometrically by reading at 415 nm the absorbance of TNB. Results were compared to the concentration–response curve of standard GSH solutions and expressed as nanomoles of GSH/mL plasma.

Thiobarbituric acid reactive substances (TBARS) level

TBARS, the end-products of lipid peroxidation, were assayed as previously reported (Valli et al., 2012) with slight modification. Briefly, 100 µl of plasma diluted 1:10 in DPBS were added to a mixture containing 100 µl TCA (30% in 0.25N HCl), 100 µl TBA (0.75% in 0.25N HCl) and 3 µl BHT (1% in

ethanol). The mixture was heated for 10 min in a boiling water bath, allowed to cool and the TBA adducts were detected fluorimetrically (λex 535 nm; λem 595 nm). TBARS level was expressed as relative fluorescence units (RFU).

Cytokines production

Cytokines (IFNα, IFNγ, IL-1β, IL-4, IL-6, IL-8, IL-10, and TNFα) production was evaluated by the Ciraplex® immunoassay kit following manufacturer's instructions. Briefly, the assay is a multiplex sandwich ELISA where each well of the 96-well microplate is pre-spotted with protein-specific antibodies and these antibodies capture specific proteins from the standards and samples of interest. After unbound proteins are washed away, biotinylated detecting antibodies are added and bind to a secondary site on the target proteins. After the removal of excess detection antibody, streptavidinhorseradish peroxidase (SA-HRP) is added. HRP is an enzyme that reacts with a substrate to produce a luminescent signal that is detected by the Cirascan™ Imaging System. The intensity of the signal produced is directly proportional to the quantity of each protein in the standard or sample of interest.

Digestive process investigation

NMR spectra acquisition

Fecal samples were prepared for proton nuclear magnetic resonance spectroscopy (1H-NMR) analysis by vortex mixing 40 mg of stool for 5 minutes with 800 µl of deionized water, followed by centrifugation for 15 minutes at 15,000 rpm at 4°C. 700 µl of supernatant were added to 160 µl of a D2O 1M phosphate buffer at pH 7.00 solution of 3-(trimethylsilyl)-propionic-2,2,3,3-d4

acid sodium salt (TSP) 6.25 mM. The samples were stored at -20°C prior to analysis. Immediately before analysis the samples were thawed and centrifuged again. 1H-NMR spectra were recorded at 298 K with an AVANCE III spectrometer (Bruker, Milan, Italy) operating at 600.13 MHz.

The HOD residual signal was suppressed by applying the first increment of the NOESY pulse sequence and a spoil gradient (Laghi et al., 2014). This was done by employing the NOESYGPPR1D sequence, part of the standard pulse sequence library. Each spectrum was acquired by summing up 256 transients using 32 K data points over a 7211.54 Hz spectral (for an acquisition time of 2.27s). In order to apply NMR as a quantitative technique (Pauli, 2001), the recycle delay was set to 5s, keeping into consideration the longitudinal relaxation time of the protons under investigation. The signals were assigned by comparing their chemical shift and multiplicity with the Human Metabolome Database (Wishart et al., 2007) and Chenomx software data bank (Chenomx Inc., Canada, ver 8.1).

Data analysis

1H-NMR spectra baseline was adjusted by means of the simultaneous peak detection (Coombes et al., 2003) and baseline correction algorithm (SPDBC) implemented in the baseline R package (Liland et al., 2010). Briefly, the algorithm tries to iteratively locate peaks as consistent deviations from noise and interpolate over the spectra after peak removal. To each spectrum a linear correction was then applied to make the points pertaining to the baseline randomly spread around zero. The spectra were overall corrected for errors in chemical shift misalignments using an in-house modified version of Correlation

Optimized Shifting (i-Coshift) (Savorani 2010) able to perform the Co-shift in localized regions of the spectrum. As pigs ileal, duodenal and fecal metabolome have been rarely studied by NMR through a metabolomic approach, literature lacks a detailed description of the NMR spectra pre-processing pipeline. This applies in particular to signals alignment procedure by icoshift, with the following *i*Coshift NMR signals alignment procedure command string:

[Allin, intervals,indexes]=icoshift('median', b4f7.y,[9.14 9.118, 8.278 8.259, 8.211 8.203, 8.112 8.087, 7.962 7.893, 7.879 7.861, 7.553 7.546, 7.54 7.532, 7.125 7.1, 6.984 6.943, 6.426 6.375, 5.993 5.973, 5.641 5.606, 0.7055 0.6133], 'b', [2,0,0,10,1], b4f7.x).

Differences in soluble solids content among samples was taken into consideration by probabilistic quotient normalization (Dieterle et al., 2006). The signals were assigned by comparing their chemical shift and multiplicity with the Human Metabolome Database (Wishart et al., 2007) and Chenomx software data bank (Chenomx Inc., Canada, ver 8). Signals which significantly varied ($P<0.05$) among the samples groups under investigation were looked for by means of a Type II ANOVA test followed by TukeyHSD post hoc test. The assumptions of Anova normal distribution and equality of variance were tested by Shapiro and Bartlett tests respectively.

5.1.2 Results and Discussion

Growth performance and clinical evaluation

The daily observation of the animals showed that pigs housed in box A, B and D had diarrhea throughout the first two weeks and completely recovered within the second part of the trial. The pigs housed in the other three boxes preserved their normal gastrointestinal functions during the entire experiment.

The humidity in the different samples of dried /grinded breads was very similar (9.37±0.53), so it was considered as negligible in the trials.

The body weight of the animals for each group is presented in figure 13. No statistically significant differences were detected between the groups. We must consider that, due to experimental design, the pigs received fixed daily amount of experimental diet (2Kg) that was undersized for energy requirements. We than decide to analyze the data after normalization as body increment/initial weight ratio as shown in figure 14. A two way ANOVA for Flour*Fermentation was performed with a $p<0.027$ for Fermentation, post-hoc test HSD

1 Sourdough Ancient Fermentation (groups E, F) 1.0341243 a

2 Sourdough Standard Fermetation (groups C, D) 0.9932986 ab

3 Conventional Fermentation (groups A, B) 0.8772724 b

The diet E and F (SFSAF, AFSAF) did sustain better the growth of animals in comparison with diet A and B formulated with conventional fermentation.

Complete Blood Count (CBC) is shown in table 11. All CBC values fell within the physiological reference intervals, the differences between day 0 (T0) and day 30 (T30) were mainly related with the animal normal development stages. White Blood cells (WBC) count was decreasing from T0 to T30 in all groups; the decreasing was due to a cleaner environment of the ASA Unit compared to the standard commercial piggery where the animals were bred. The other differences between groups, T0 and T30 were not statistically or biologically significant and fell within the physiological reference intervals.

Figure 13. ***Body weight (kg) expressed as mean ±SD in the groups n= 6***

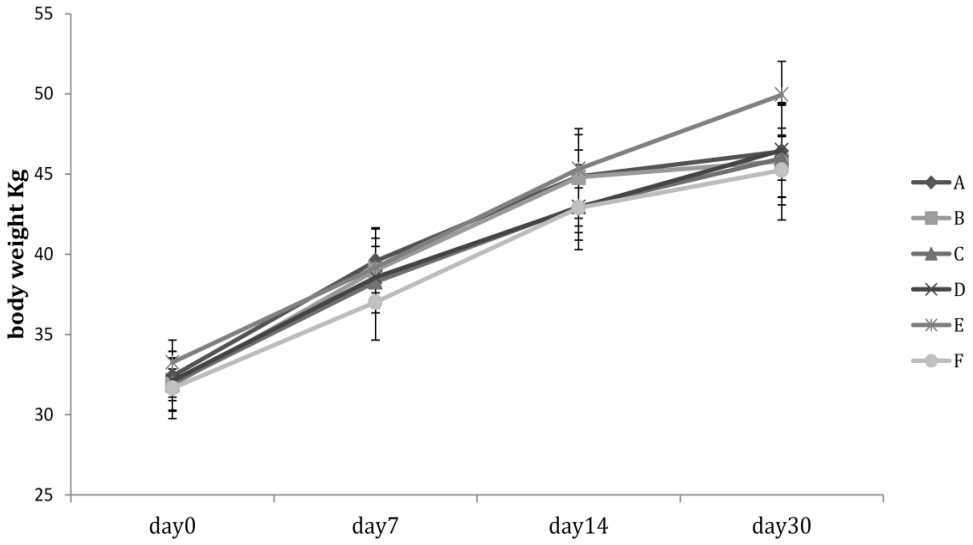


Figure 14. ***Body increment/initial weight ratio after normalization (mean ± SD) in the groups n=6***

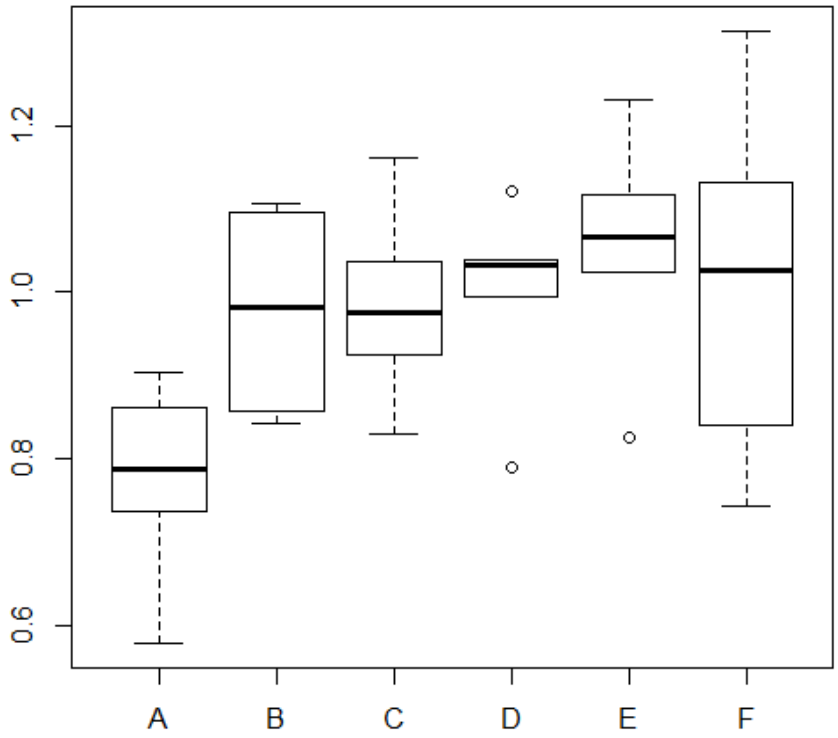


Table 11. ***Complete Blood Count (CBC) expressed as mean ± SD in the groups n=6***

		A		B		C		D		E		F	
Target	Variable (Reference interval)	T_0	T_{30}	T_0	T_{30}	T_0	T_{30}	T_0	T_{30}	T_0	T_{30}	T_0	T_{30}
Anemia	Haematocrit **(32-50 %)**	32.0 ± 0.9	35.4 ± 1.1	32.5 ± 0.7	36.2 ± 0.8	32.4 ± 1.0	35.0 ± 0.4	31.5 ± 1.5	36.0 ± 1.0	32.2 ± 0.8	36.5 ± 0.5	33.0 ± 1.1	36.4 ± 1.0
	Red Blood cell (RBC) **(5-8 x 10^6/mm^3)**	6.0 ± 0.2	6.8 ± 0.2	6.5 ± 0.2	7.1 ± 0.5	6.3 ± 0.2	6.7 ± 0.2	6.2 ± 0.2	6.7 ± 0.2	6.2 ± 0.2	6.9 ± 0.2	6.5 ± 0.1	7.0 ± 0.2
	Hemoglobin **(10-16 gr%)**	10.4 ± 0.5	11.7 ± 0.3	10.6 ± 0.2	11.7 ± 0.2	10.5 ± 0.2	11.4 ± 0.1	10.4 ± 0.4	11.5 ± 0.3	10.7 ± 0.3	11.8 ± 0.3	11.0 ± 0.3	11.5 ± 0.4
	Reticolocytes **(<1%)**	1.1 ± 0.0	2.3 ± 0.4	1.0 ± 0.1	2.7 ± 0.1	1.0 ± 0.2	2.4 ± 0.1	1.2 ± 0.0	2.0 ± 0.0	1.1 ± 0.1	2.8 ± 0.1	1.2 ± 0.1	2.7 ± 0.0
Inflammation Immuno-deficiency	White Blood cells (WBC) **(1.0-2.2 x 10^4 /mm^3)**	2.0 ± 0.1	1.7 ± 0.2	2.4 ± 0.3	2.1 ± 0.2	2.5 ± 0.2	2.0 ± 0.2	2.2 ± 0.2	1.6 ± 1.0	2.2 ± 0.2	2.1 ± 0.2	2.2 ± 0.1	1.7 ± 0.1
	Monocytes **(2-10 %)**	2.5 ± 0.2	2.7 ± 0.1	3.0 ± 0.2	3.1 ± 0.1	3.2 ± 0.2	3.5 ± 0.2	3.2 ± 0.1	2.1 ± 0.0	4.0 ± 0.1	2.0 ± 0.1	3.4 ± 0.1	1.7 ± 0.1
	Neutrophils **(28-47 %)**	43.8 ± 3.2	40.5 ± 3.7	47.0 ± 2.0	42.0 ± 5.0	41.0 ± 4.4	33.0 ± 1.1	34.2 ± 3.8	45.0 ± 1.7	33.0 ± 2.6	42.0 ± 3.9	39.2 ± 3.8	41.7 ± 2.1
	Eosinophil **(0-11 %)**	1.5 ± 0.5	2.1 ± 0.4	2.7 ± 0.4	3.0 ± 0.4	4.0 ± 0.9	2.3 ± 0.4	7.3 ± 2.3	2.3 ± 0.6	3.4 ± 1.4	3.0 ± 0.5	3.4 ± 1.3	3.5 ± 0.5
	Lymphocytes **(39-60 %)**	51.2 ± 3.1	53.0 ± 4.0	46.2 ± 1.8	51.0 ± 4.5	50.5 ± 3.5	60.0 ± 0.7	54.1 ± 2.6	49.7 ± 2.4	58.5 ± 2.1	52.0 ± 3.3	53.0 ± 3.7	52.1 ± 1.5

Gut microbiota evaluation

A total of 8,493,063 sequence reads passing quality filtering (mean per sample, 81,664; range, 23,852-200,038) were obtained and analyzed. Reads were clustered into 5,516 non-singleton OTUs at 97% similarity. Alpha-diversity was almost saturated for all samples, as revealed by the asymptotic shape of the rarefaction curves obtained with Shannon, Chao1, PD whole tree and observed species phylogenetic metrics (Fig. 15).

Principal Coordinates Analysis of weighted UniFrac distances resulted in no significant segregation between the swine gut microbiota profiles at T0 and T30 for any diet group (Fig. 16). However, Procrustes similarity analysis of weighted UniFrac distances revealed that T30 profiles for each diet group partially converged, suggesting a diet-specific effect on the gut microbiota structure (Fig. 17).

Analysis of the relative taxon abundance identified many notable differences before and after the different diet interventions at phylum, family and genus level. In particular, at the phylum level the relative abundance of Bacteroidetes significantly increased after B and C intervention, while it decreased after A intervention. Correspondingly, B and C diets were associated with significantly lower amounts of Firmicutes. D and F diets were specifically associated with an increase in Spirochaetes and a decrease in Proteobacteria, respectively

Figure 15. ***Rarefaction curves with different alpha diversity metrics: the Faith's phylogenetic diversity (PD whole tree), the chao1 measure of microbial richness, number of observed species and the Shannon index of biodiversity.***

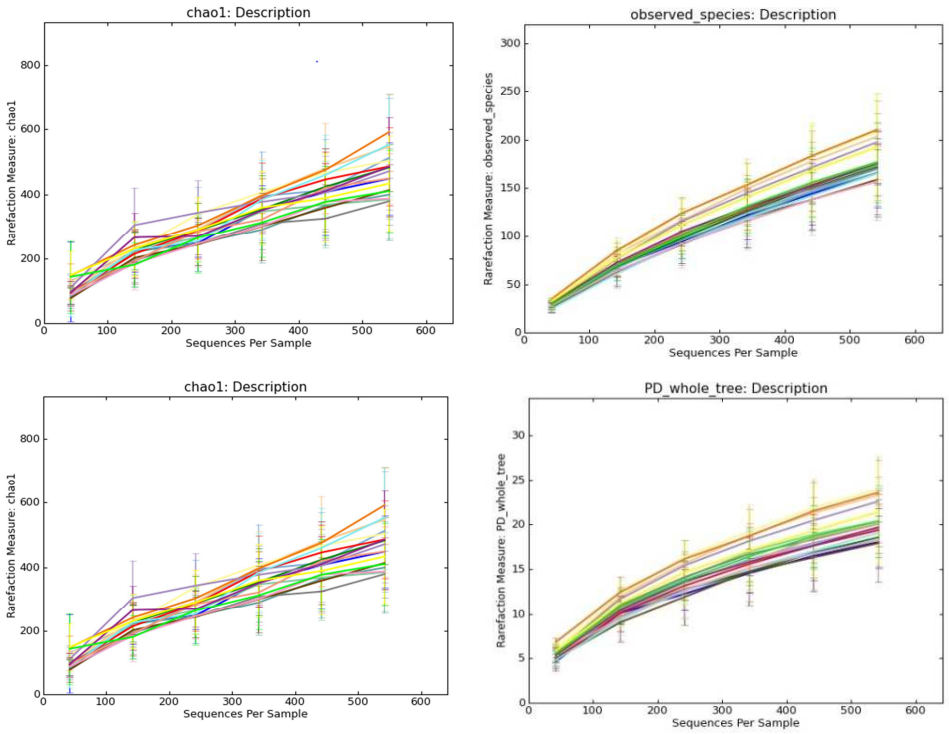


Figure 16. ***PCoA based on weighted UniFrac distances.***

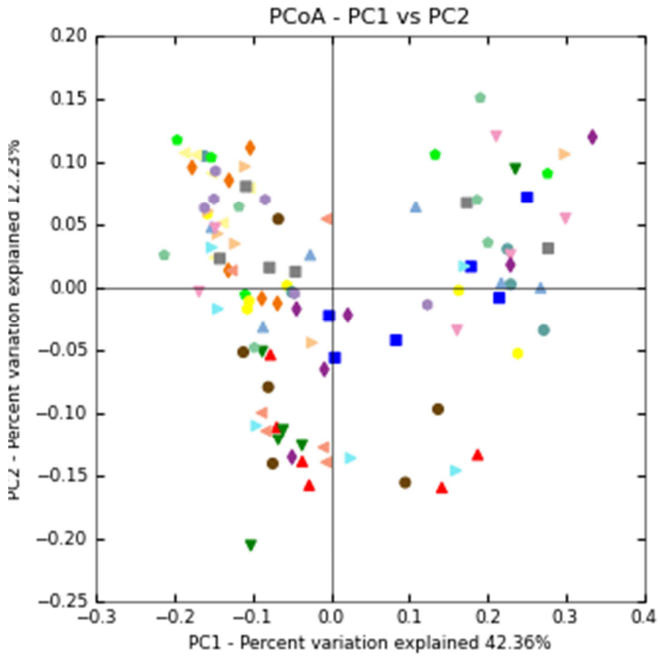
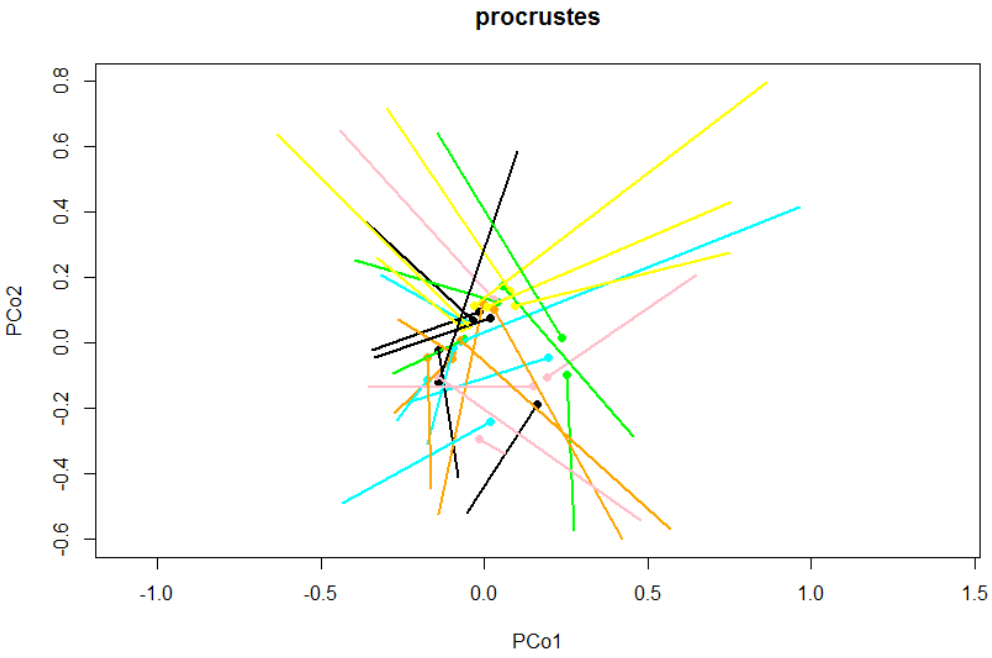


Figure 17. ***Procrustes similarity analysis combining weighted UniFrac distance PCoA of T0 (noncircle end of lines) and T30 profiles (circle end of lines) of the swine gut microbiota. Colors were assigned to each diet group.***



At the family level, the majority of diet groups were characterized by a significant enrichment in *Bifidobacteriaceae* (A, B, C, D), as well as unclassified members of Bacteroidales order (A, B, C, D, E). Most of the groups also showed a significant decrease in *Streptococcaceae* (A, D, E), *Veillonellaceae* (A, D, E, F), and *Enterobacteriaceae* (A, B, D, F). Despite these common features, some intervention-specific changes were identified. In particular, A diet was significantly associated with an increase in *Clostridiaceae*. C diet was associated with an increase of S24-7 and *Desulfovibrionaceae*, and a decrease in *Lactobacillaceae*. D diet was specifically characterized by an increase in *Spirochetaceae* and the Bacteroidetes families BS11, RF16 and p-2534-18B5, as well as by a decrease in *Erysipelotrichaceae* and *Succinivibrionaceae*. A

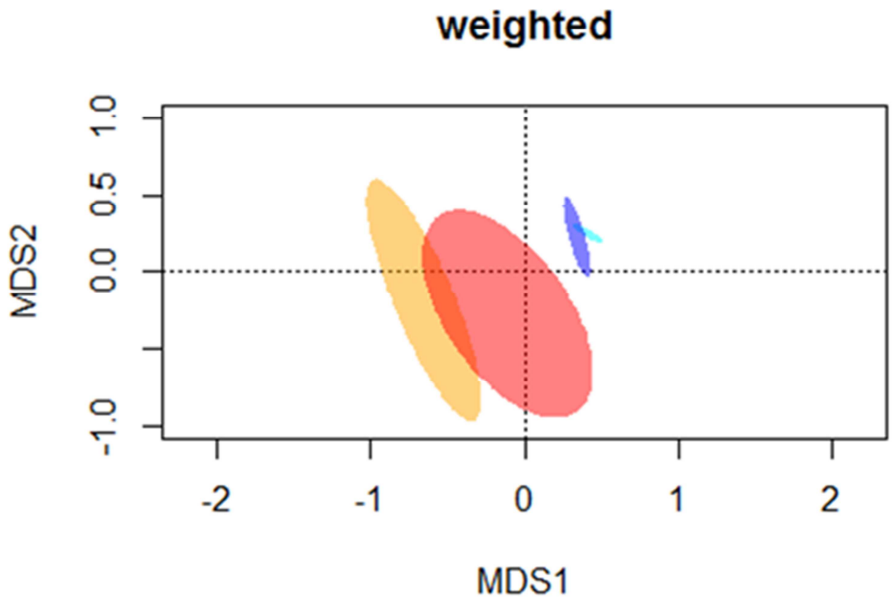
significant increase in *Paraprevotellaceae* and *Lachnospiraceae* was observed in E and F diet group, respectively.

These differences were reflected at the genus level, where both common and unique patterns of variation were identified. In particular, the vast majority of diet groups were characterized by a significant decrease in *Acidaminococcus* (A, B, C, D) and *Megasphaera* (A, B, D, E, F). Moreover, A and F diets were similarly characterized by an increase in *Oscillospira* and *Ruminococcus*, whereas B and D diet groups were both enriched in *Anaerovibrio*. Among the specific effects of the different diets, it is noteworthy that D diet was associated with a significant increase in *Treponema* and a significant decrease in *Phascolarctobacterium*, *Dialister*, and *Catenibacterium*. B diet was specifically characterized by a decrease in *Blautia*, whereas C diet by an increase in *Desulfovibrio*. As anticipated by the family-level comparison, E diet was significantly associated with an increase in the *Prevotella* genus of the *Paraprevotellaceae* family, whereas F diet was associated with enrichment in *Coprococcus*, *Dorea* and *Roseburia*, which are all members of the *Lachnospiraceae* family.

To better understand the gut microbiota modulation due to the different diets in terms of standard and ancient flour (diet A and B) the ileum and cecum microbiota was investigated.

In the ileum statistical differences were observed (Fig. 18), more in detail the diet B lead to a decrease of *Firmicutes* and *Sreprococcus* concentration compared to diet A.

Figure 18. ***Beta diversity of ileum and cecum mucosae microbiota. Group A_ileum is orange; group B_ileum is red; group A_cecum is cyan; group B_cecum is blue***



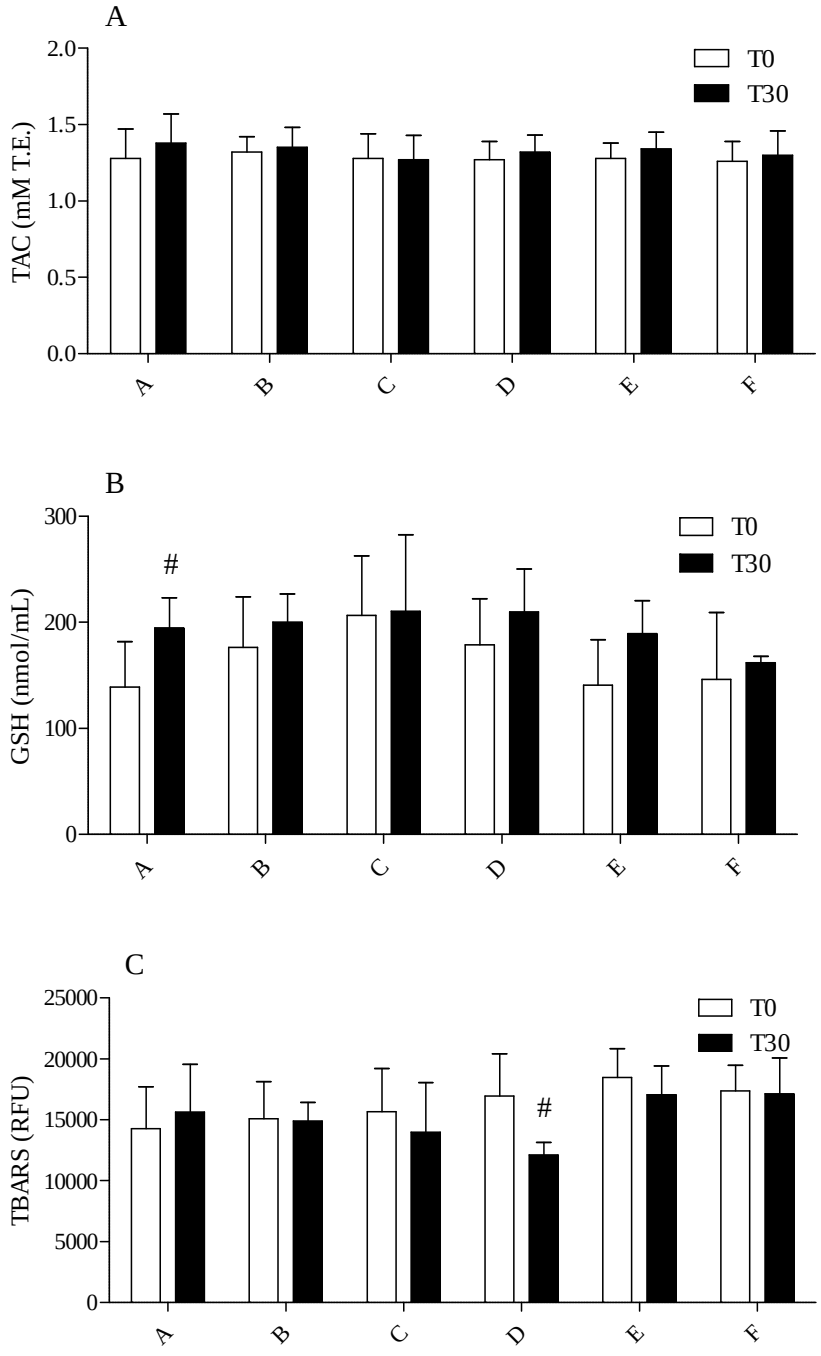
In conclusion, the different diets were able to modulate the swine gut microbiota structure, resulting in both common and unique patterns of variations. Such variations may be related to functional differences in the microbiome with a different impact on host physiology.

Oxidative and inflammatory status evaluation

In figure 19 the TAC (Fig. 19A), GSH content (Fig. 19B) and TBARS level (Fig. 19C) in the plasma of pigs fed the different diets are reported. Among the different dietary groups no significant differences in plasma TAC were detected either at the beginning (T0) or in the end (T30) of the trial. As well, within each group no significant differences between T0 and T30 were evidenced.

At T0, plasma GSH content was similar in all groups (Fig. 19B). In pig fed the SFCF diet (A), plasma GSH concentration increased upon the dietary treatment, while no differences were detected in other groups. As well, plasma TBARS concentration was similar in all groups at T0 (Fig. 19C), and no significant modifications were observed upon the dietary treatment in all groups but group D (fed the AFSSF diet), which evidenced a significant decrease in TBARS level at T30. Overall, the herein reported results indicate that the dietary treatment did not modify the level of antioxidant defenses in pigs, apart from the increase in GSH level observed in animals fed the SFCF diet. The different dietary treatments did not modify the level of oxidative stress, measured as TBARS concentration in plasma, a part from the AFSSF diet. In fact, animals fed this diet showed a lower plasma TBARS concentration, which is suggestive for a reduced lipid peroxidation.

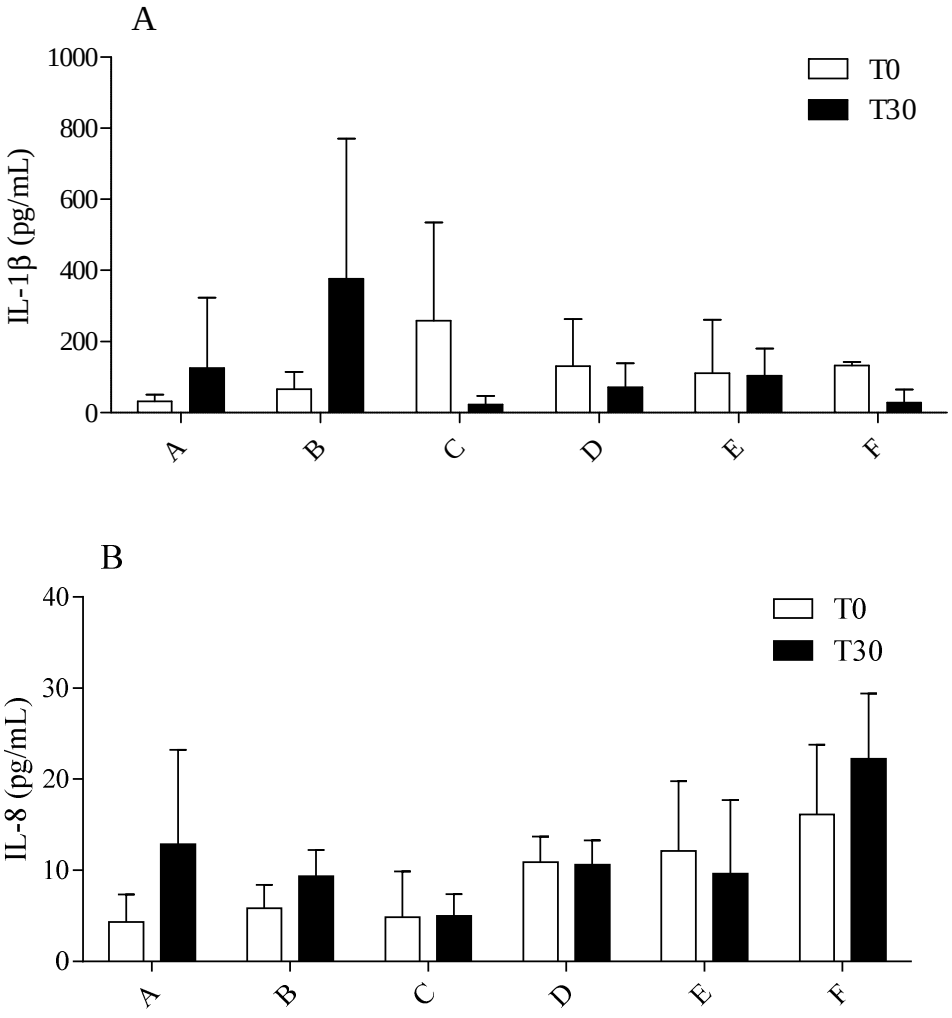
Figure 19. ***TAC (panel A), GSH content (panel B), and TBARS level (panel C) in plasma of pigs fed the different diets.*** *Data are mean +/- SD of six samples, and are expressed as micromoles TE/mL (panel A), nanomoles/mL (panel B), and RFU (panel C). TAC, GSH content and TBARS level were detected at T0 (white bars) and T30 (black bars). Differences among groups at T0 and T30 were tested for statistical significance by the one way ANOVA (n.s.); in the same dietary group differences between T0 and T30 were tested for statistical significance by unpaired Student's t tests (# p<0.05).*



In this study, using a multi-parametric immunoassay kit specific for porcine cytokines, IFNα, IFNγ, IL-4, IL-6, IL-10, and TNFα plasma concentration were under the limit of detection of the assay at both T0 and T30 in almost all animals. The limit of detection were 0.0878 pg/mL, 2.017 pg/mL, 1.435 pg/mL , 0.458 pg/mL, 0.547 pg/mL, and 7.277 pg/mL for IFNα, IFNγ, IL-4, IL-6, IL-10, and TNFα, respectively. Before performing the assay, the calibration curve was run twice and fit with the concentrations used ($0.99<r2<1$ for all cytokines); in addition, in basal, not stressed condition the normal level of interleukins in pigs is reported to be 0-2 pg/mL (Byrami et al., 2013; Ciepielewski et al., 2013; Wyns et al., 2013),which is very close to the detection limits of the multiplex sandwich ELISA test used in the present study.

Therefore it seems that the undetectability of cytokines but IL-1β and IL- 8 was not due to technical problems but to a low inflammatory status of animals. At T0, both IL-1β and IL-8 were detected in plasma (Fig. 20A and 20B, respectively), and a huge variability was evidenced among animals within and among groups. A very high intra and inter groups variability was detected at T30, too. Due to this high variability among animals, no significant differences in IL-1β and IL-8 plasma concentration either comparing all groups at T0 or at T30, or comparing the same group before and after the dietary treatment were observed.

Figure 20. ***IL-1 β (panel A) and IL-8 (panel B)concentration in plasma pigs at T0 (white bar) and T30 (black bar) of treatment.*** *Data are mean +/- SD of six samples, and are expressed as pg/mL of plasma. Differences among groups at T0 and T30 were tested for statistical significance by the one way ANOVA (n.s.); in the same dietary group differences between T0 and T30 were tested for statistical significance by unpaired Student's t tests (n.s.).*



Digestive process investigation

We identified by NMR 83 signals. Among them, 68 were assigned to (and employed to quantify) molecules pertaining to the classes of organic acids and alcohols (Tab. 12 and 13), amino acids (Tab. 14), amines (Tab. 15), nitrogenous bases, mono- and di-saccharides (table 16), while 15 were identified but unassigned (Tab. 17).

Table 12. ***Position in the NMR spectra of the signals employed for the quantification of organic acids and alcohols***

Molecule (Chemical shift)	Spectral portion		Signal protons
Formate-8.460	8.444	- 8.476	1.00
Oxypurinol-8.206	8.202	- 8.209	1.00
Fumarate-6.524	6.517	- 6.531	2.00
Lactate-4.120	4.096	- 4.143	1.00
Glycerol-3.583	3.581	- 3.586	4.00
Methanol-3.365	3.361	- 3.369	3.00
2-oxoglutarate-2.999	2.996	- 3.002	0.25
2-oxoisocaproate-2.623	2.617	- 2.628	1.00
Citrate-2.560	2.556	- 2.564	1.00
Succinate-2.410	2.406	- 2.413	4.00
Butyrate-2.392	2.388	- 2.396	0.31
Pyruvate-2.377	2.375	- 2.378	2.50
Propionate-2.206	2.201	- 2.211	0.25
Isovalerate-2.066	2.063	- 2.070	1.00
Acetate-1.922	1.905	- 1.938	3.00
Valerate-1.517	1.512	- 1.522	0.50
Ethanol-1.188	1.184	- 1.192	1.50

Table 13. ***Position in the NMR spectra of the signals employed for the quantification of nitrogenous compounds and bases***

Molecule (Chemical shift)	Spectral portion		Signal protons
Nicotinate-8.944	8.927	- 8.961	1.00
Adenine-8.218	8.211	- 8.225	1.00
Xanthine-7.926	7.918	- 7.933	1.00
Uracil-7.536	7.534	- 7.539	0.50
Cytosine-7.501	7.496	- 7.506	0.50

Table 14. ***Position in the NMR spectra of the signals employed for the quantification of aminoacids***

Molecule (Chemical shift)	Spectral portion	Signal protons
tyrosine-7.198	7.185 - 7.211	2.00
Tryptophan-7.734	7.729 - 7.739	0.50
Phenylalanine-7.434	7.416 - 7.452	2.00
Histidine-7.891	7.889 - 7.893	1.00
Glycine-3.566	3.565 - 3.568	2.00
aspartate-2.804	2.795 - 2.813	3.00
Methionine-2.648	2.642 - 2.654	1.00
Glutamate-2.357	2.350 - 2.364	1.33
proline-2.015	2.009 - 2.020	1.00
lysine-1.727	1.699 - 1.755	1.00
alanine-1.477	1.473 - 1.482	1.50
isoleucine-1.013	1.004 - 1.022	1.00
valine-0.993	0.983 - 1.003	3.00
leucine-0.964	0.960 - 0.968	1.00

Table 15. ***Position in the NMR spectra of the signals employed for the quantification of amines***

Molecule (Chemical shift)	Spectral portion	Signal protons
Tyramine-7.230	7.224 - 7.235	2.00
Histamine-7.117	7.112 - 7.121	1.00
Ornitine-Putrescine-3.060	3.041 - 3.079	2.00
Cadaverine-3.021	3.015 - 3.027	2.00
TMA-2.883	2.877 - 2.890	9.00
DMA-2.703	2.699 - 2.707	6.00
Methylamine-2.608	2.600 - 2.617	3.00

Table 16. ***Position in the NMR spectra of the signals employed for the quantification of saccharides***

Molecule (Chemical shift)	Spectral portion	Signal protons
Glucose-1-Phosphate-5.462	5.446 - 5.478	1.00
Maltose-5.416	5.406 - 5.426	1.00
Galactose-5.270	5.266 - 5.273	0.50
Glucose-5.270	5.266 - 5.273	1.00
Ribose-5.259	5.256 - 5.262	1.00
Trehalose-5.215	5.211 - 5.218	0.50
xylose-5.200	5.197 - 5.203	0.50
Galactose-4.598	4.595 - 4.601	0.50
Fucose-4.554	4.551 - 4.558	1.00
Arabinose-4.529	4.533 - 4.525	0.50
Sucrose-4.230	4.225 - 4.235	0.50

Table 17. ***Position in the NMR spectra of the signals employed for the quantification of other molecules***

Molecule (Chemical shift)	Spectral portion	Signal protons
Phenylacetate-7.305	7.292 - 7.319	2.00
3-phenylpropionate-7.363	7.357 - 7.369	0.80
Indole-3-lactate-7.174	7.172 - 7.177	0.50
p-cresol-7.150	7.137 - 7.163	2.00
1,3-Dihydroxyacetone-4.425	4.422 - 4.428	4.00
Choline-3.204	3.199 - 3.209	9.00
5-aminolevulinate-2.787	2.780 - 2.793	1.00
2-aminoadipate-2.249	2.244 - 2.255	1.00
Acetone-2-aminoadipate-2.237	2.233 - 2.242	6.00
N-acetilglycine-2.044	2.041 - 2.048	1.00
N-acetilglutamate-2.038	2.035 - 2.040	1.00
2-hydroxy-3-methylvalerate-0.860	0.857 - 0.863	1.00
Cholate-0.738	0.730 - 0.747	1.00
Glycocholate-0.714	0.707 - 0.721	1.00

To gain an insight of the effect of both the flour employed and the kind of leavening agent, we set up, and explored by ANOVA, a two-way model built on each of the 83 signals identified by NMR on the samples obtained at the final timepoint of the experiment.

The leavening agent gave rise to statistically significant differences among the molecules quantified in table 18 A and summarized in table 19, while the flour employed to prepare the bread gave rise to significant differences among the molecules described in table 18 B. To be sure that the found differences could be ascribed to the tested conditions, they were looked for, without success, on samples collected from the same animals at the beginning of the experiment.

Table 18. ***A Molecules influenced by leavening agent. B Molecules influenced by employed flour***

A

	Ancient flour			Standard flour		
	Conv. Leav. agent	Sourd. Ancient	Sourd. Standard	Conv. Leav. agent	Sourd. Ancient	Sourd. Standard
Cytosine	3.7E-5 ± 2.0E-5	5.7E-5 ± 2.0E-5	3.6E-5 ± 3.7E-5	7.7E-5 ± 2.0E-5	6.7E-5 ± 2.8E-5	3.5E-5 ± 1.8E-5
Phenylalanine	6.2E-4 ± 1.1E-4	7.6E-4 ± 1.1E-4	8.2E-4 ± 1.8E-4	6.0E-4 ± 1.4E-4	6.4E-4 ± 1.4E-4	7.0E-4 ± 1.2E-4
Fumarate	1.7E-4 ± 2.2E-5	9.9E-5 ± 5.4E-5	1.4E-4 ± 3.8E-5	1.1E-4 ± 4.6E-5	1.0E-4 ± 3.3E-5	1.2E-4 ± 3.7E-5
X-1.819	1.9E-3 ± 3.3E-4	2.2E-3 ± 3.1E-4	2.0E-3 ± 2.0E-4	2.2E-3 ± 4.0E-4	2.4E-3 ± 1.5E-4	1.8E-3 ± 3.0E-4
alanine	3.5E-3 ± 8.2E-4	3.7E-3 ± 5.1E-4	4.4E-3 ± 1.3E-3	2.8E-3 ± 4.5E-4	3.7E-3 ± 1.4E-3	4.0E-3 ± 1.2E-3
valine	1.9E-3 ± 4.4E-4	2.1E-3 ± 3.7E-4	2.5E-3 ± 6.7E-4	1.7E-3 ± 2.3E-4	1.9E-3 ± 5.0E-4	2.2E-3 ± 4.3E-4
isoleucine	1.4E-3 ± 3.6E-4	1.5E-3 ± 2.6E-4	1.7E-3 ± 4.0E-4	1.3E-3 ± 2.7E-4	1.4E-3 ± 3.5E-4	1.6E-3 ± 2.0E-4
leucine	2.0E-3 ± 3.2E-4	2.3E-3 ± 3.2E-4	2.4E-3 ± 4.6E-4	1.9E-3 ± 2.4E-4	2.0E-3 ± 3.5E-4	2.3E-3 ± 2.8E-4
Cholate	3.6E-4 ± 6.9E-5	2.6E-4 ± 5.2E-5	2.9E-4 ± 5.8E-5	3.0E-4 ± 4.7E-5	2.9E-4 ± 5.1E-5	3.1E-4 ± 1.2E-5

B

	Ancient flour			Standard flour		
	Conv. Leav. agent	Sourd. Ancient	Sourd. Standard	Conv. Leav. agent	Sourd. Ancient	Sourd. Standard
Phenylacetate	5.1E-3 ± 1.7E-3	5.8E-3 ± 1.5E-3	4.9E-3 ± 1.9E-3	7.3E-3 ± 3.3E-3	7.8E-3 ± 2.5E-3	6.1E-3 ± 1.1E-3
Fumarate	1.7E-4 ± 2.2E-5	9.9E-5 ± 5.4E-5	1.4E-4 ± 3.8E-5	1.1E-4 ± 4.6E-5	1.0E-4 ± 3.3E-5	1.2E-4 ± 3.7E-5
X-4.309	6.7E-4 ± 1.4E-4	4.5E-4 ± 1.7E-4	5.9E-4 ± 1.6E-4	4.2E-4 ± 1.1E-4	4.9E-4 ± 1.7E-4	4.3E-4 ± 1.6E-4
Butyrate	1.4E-3 ± 3.7E-4	1.4E-3 ± 3.7E-4	1.2E-3 ± 4.7E-4	1.7E-3 ± 6.5E-4	1.8E-3 ± 5.9E-4	1.5E-3 ± 3.1E-4
Propionate	1.0E-2 ± 2.5E-3	9.8E-3 ± 1.7E-3	9.1E-3 ± 1.2E-3	1.2E-2 ± 1.9E-3	1.2E-2 ± 3.0E-3	9.9E-3 ± 2.9E-3
Isovalerate	3.9E-3 ± 9.8E-4	4.6E-3 ± 1.3E-3	4.0E-3 ± 1.3E-3	5.2E-3 ± 2.0E-3	5.9E-3 ± 1.5E-3	4.8E-3 ± 4.5E-4
Acetate	2.7E-1 ± 5.9E-2	2.9E-1 ± 4.0E-2	2.7E-1 ± 2.8E-2	3.3E-1 ± 6.3E-2	3.5E-1 ± 6.1E-2	2.7E-1 ± 6.9E-2
2-hydroxy-3-methylv.	3.9E-3 ± 1.4E-3	4.3E-3 ± 1.1E-3	3.6E-3 ± 1.5E-3	5.7E-3 ± 2.6E-3	5.9E-3 ± 2.1E-3	4.9E-3 ± 1.2E-3

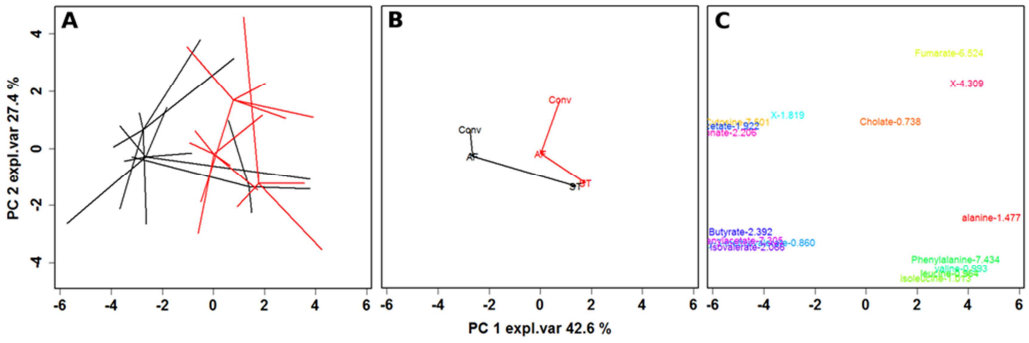
Table 19. ***Statistically significant differences among the molecules of table 9***

	Conv. Leav. agent	Sourd. Ancient	Sourd. Standard
Cytosine	ab	b	A
Phenylalanine	b	a	Ab
Fumarate	a	ab	B
X-1.819	ab	b	A
alanine	b	a	Ab
valine	b	a	Ab
isoleucine	b	a	Ab
leucine	b	a	Ab
Cholate	a	ab	B

To obtain an overview of the changes of molecules influenced by leavening agent or employed flour, a principal component analysis (PCA) model was calculated on their concentration, as shown in figure 21.

PC1, accounting for the 42.6% of the total variance, was mainly influenced by the flour employ, with short chain fatty acids showing higher concentrations in the faeces of pigs fed with standard flour based bred. PC2 was, at the contrary, mainly influenced by the leavening agent, with sourdough based on standard flour leading to the greatest differences from conventional leavening agent, and sourdough based on ancient flour showing intermediate characteristics. The samples pattern was strongly influenced by the amino acids leucine, isoleucine, valine, phenylalanine and alanine, each of them characterized by the highest values as a consequence of conventional leavening agent.

Figure 21. ***Overview by PCA of pigs faecal metabolome influenced by flour and leavening agent of the bread. A) Dispersion scoreplot of the pigs fed with standard (black) and ancient (red) flour, divided in subgroups according to the leavening agent; B) Median of such subgroups; C) PCA loadingplot***



5.1.3 Conclusions

The pigs enrolled in the trial were feed with the experimental breads for 50% of their daily food requirements; the percentage was chose to obtain significant impact on the animals without causing nutritional deficiency. T the end of trial all animals were in good clinical condition and did not displayed nutritional deficiency. The diet A (SFCF) was the less effective to sustain the growth of pigs while sourdough ancient fermentation (groups E, F) breads were the most effective.

No significant segregation between the swine gut microbiota profiles at T0 and T30 for any diet group was recorded. However, Procrustes similarity analysis of weighted UniFrac distances revealed that T30 profiles for each diet group partially converged, suggesting a diet-specific effect on the gut microbiota structure. Some intervention-specific changes were identified; in particular, A diet was significantly associated with an increase in *Clostridiaceae*. C diet was associated with an increase of S24-7 and *Desulfovibrionaceae*, and a decrease in *Lactobacillaceae*. D diet was specifically characterized by an increase in *Spirochetaceae* and the Bacteroidetes families BS11, RF16 and p-2534-18B5, as well as by a decrease in *Erysipelotrichaceae* and *Succinivibrionaceae*. A significant increase in *Paraprevotellaceae* and *Lachnospiraceae* was observed in E and F diet group, respectively.

At the genus level, both common and unique patterns of variation were identified. In particular, the vast majority of diet groups were characterized by a significant decrease in *Acidaminococcus* (A, B, C, D) and *Megasphaera* (A, B, D, E,

F). Moreover, A and F diets were similarly characterized by an increase in *Oscillospira* and *Ruminococcus*, whereas B and D diet groups were both enriched in *Anaerovibrio*. Among the specific effects of the different diets, it is noteworthy that D diet was associated with a significant increase in *Treponema* and a significant decrease in *Phascolarctobacterium*, *Dialister*, and *Catenibacterium*. B diet was specifically characterized by a decrease in *Blautia*, whereas C diet by an increase in *Desulfovibrio*. As anticipated by the family level comparison, E diet was significantly associated with an increase in the *Prevotella* genus of the *Paraprevotellaceae* family, whereas F diet was associated with enrichment in *Coprococcus*, *Dorea* and *Roseburia*, which are all members of the *Lachnospiraceae* family.

Anyway, the different diet did not modify the level of antioxidant defenses in pigs, apart from plasma GSH concentration that increased upon the dietary treatment in pig fed the SFCF diet (A), and TBARS level which evidenced a significant decrease in group D (fed the AFSSF diet), which is suggestive for a reduced lipid peroxidation.

All animals showed a low inflammatory status with IFNα, IFNγ, IL-4, IL-6, IL-10, and TNFα plasma concentration under the limit of detection of the multiplex sandwich ELISA test used in the study. At T0, both IL-1β and IL-8 were detected in plasma with a huge variability among animals. A very high intra and inter groups variability was detected at T30, too. Due to this high variability among animals, no significant differences in IL-1β and IL-8 plasma concentration either comparing all groups at T0 or at T30, or comparing the same group before and after the dietary treatment were observed.

The metabolome approach by NMR identified 68 molecules on T30 fecal samples. The evolution of the animal metabolome as a consequence of the diet was best followed by focusing on the fecal metabolome. The choice to observe the consequences of the diets under investigation after 30 days of administration was therefore considered as appropriate. The concentration of 8 molecules was found to be influenced by the flour, 5 of which being a short chain fatty acid. The univariate statistical analysis allowed to observe 9 molecules with a concentration in the fecal metabolome influenced by the leavening agent. It is interesting to notice that 5 of them were amino acids, suggesting that the efficiency in the proteins dietary content could be finely influenced by the modulation of their source.

Overall our know-how on the healthy and nutritional properties of einkorn flour and einkorn sourdough bread have been significantly increased and, even if a low number of statistically significant differences were found, many trends were detected (better ability to sustain the growth, modulation of the gut microbiota and the fecal metabolome). Results on potential anti-inflammatory role of ancient flour were not univocal, further studies will be necessary to better identify the effects of such as modifications in a challenging condition (e.g. after an inflammatory stimulus) to detect a different level of competence in the response of the host after a different alimentary regime.

The results indicate that the dietary treatment were able to modulate the swine gut microbiota structure, resulting in both common and unique patterns of variations. Such variations may be related to functional differences in the microbiome with a different impact on host physiology.

5.2 Experiment 2. Evaluation of the impact of novel ancient flour bakery products on the glycemic index

Glucose is considered to be the primary trigger for postprandial changes in metabolic and endocrine functioning. As a consequence, defining the postprandial potential glucose response after a meal is of great interest (Giuberti et al., 2012).

Elevated glucose and insulin excursions may induce oxidative stress and subclinical inflammation as well as insulin resistance. By counteracting these phenomena the risk of cardiovascular diseases (CVD) and type 2 diabete (T2D) can be lowered. Indeed, foods with low glycemic index (GI) and low insulin index (II) have been demonstrated to protect against T2D and CVD in observational studies (Rosén et al., 2011).

A higher dietary glycemic index has been proposed as a risk factor for weight gain and obesity. This notion builds on findings from experimental studies that a high-glycemic-index meal may induce reactive hypoglycemia in the late postprandial phase, which may in turn lead to hunger and higher voluntary energy intakes. These adverse effects of a high-glycemic-index meal may only become relevant if long time intervals elapse between meals, allowing enough time for reactive hypoglycemia to develop (Cheng et al., 2009)

The reason for differences in glycemic response appears to relate to the rate at which the foods are digested and the many factors influencing this. The glycemic index (GI) is a system of classification in which the glycemic responses of foods are indexed against a standard (white bread or glucose). Low GI foods

may influence amino acid metabolism although the implications of these are unknown. In addition, low GI foods increase colonic fermentation. The physiologic and metabolic implications of this relate to increased bacterial urea utilization, and to the production and absorption of short chain fatty acids in the colon.

The aim of this trial was therefore to evaluate how glycemic index change between breads produced with different type of flour (ancient vs conventional) and different type of fermentation (yeast vs sourdough). In addition we evaluate the insulin level to better characterize the effects of einkorn and sourdough fermentation on the carbohydrates metabolism.

5.2.1 Materials & Methods

Animals

6 hybrid (LW x D) female pigs of 50Kg all coming from the same piggery were used in this study. The animals were delivered to the ASA Unit two at a time five days before the beginning of the experimental procedure and stabled in multiple boxes.

The animals were cannulated in the jugular vein with a central permanent silicon catheter (BBraun, Bethlehem, USA) under general anesthesia and stabled in single overhead boxes for a week to recover before the beginning of the trial. For the surgery pigs were premedicated with an intramuscular (IM) bolus of ketamine (20 mg/kg) and medetomidine (0.03 mg/kg). Ten minutes after, when recumbent, a venous access was achieved through an auricular vein and animals were inducted with a bolus of tiopenthal (0,25 gr/animal). Orotracheal intubation was performed using a 8 mm ID endotracheal tube. Anesthesia was maintained with isoflurane (2-3%) in a mixture 1:1 of oxygen and air.

Diet

Although the experimental breads have excellent organoleptic characteristic, the pigs bred in standard commercial conditions were not use to their flavor. Therefore the animals have been trained to eat the experimental bread. During the training period the animals were fed with a Swine Standard Diet (SSD) and experimental bread mix, in a 1:1 ratio. After a week of training pigs were spontaneously eating the experimental breads by itself. In the morning, after 18 hours of fasting, pigs were feed with 50gr of one of the six

experimental breads, then the blood was sampled every 15 min for two hours. At the end of the experimental session the animals were fed with SSD to meet the daily nutritional requirements. The bread was changed with a Latin Square design (6 x 6). After 2 days of standard diet the challenge was repeated twice.

Sampling

Blood samples were collected from jugular vein 15 minute before the administration of the bread and every 15 minute for 2 hours to measure glucose and insulin level by standard clinical chemistry analysis (Fig. 22). For the blood glucose detection a rapid test glucometer (Contour Glucometer Xt, Bayer S.p.a., Leverkusen, Germany) was used while the blood for the insulin quantification was collected in a K3EDTA tube and stored at +4°C until the end of the post prandial sampling period. The K3EDTA tubes were then centrifuged (1000g x 10min) and the plasma was stocked at -20°C. The insulin level was detected with a Ria Kit.

Figure 22. ***A pig with a jugular cannula that allowed recurring blood samples and the rapid test glucometer used in the trial***

5.2.2 Results and discussions

All animals recovered completely from the surgery in about seven days, the food training was successful and the pigs ate the fixed experimental bread quantity within five minutes. The jugular catheters were constantly washed with saline during the trial and left with heparin to maintain patency and prevent thrombus formation at the catheter tip. Despite that, the catheters from two animals did not enable to standardize the blood sampling so we decide to exclude the subjects from the study.

The data were analyzed dividing the initial groups in 4 groups as indicated in table 20. The division was necessary to highlight the effect of different fermentation strategy and flours on the bread characteristics.

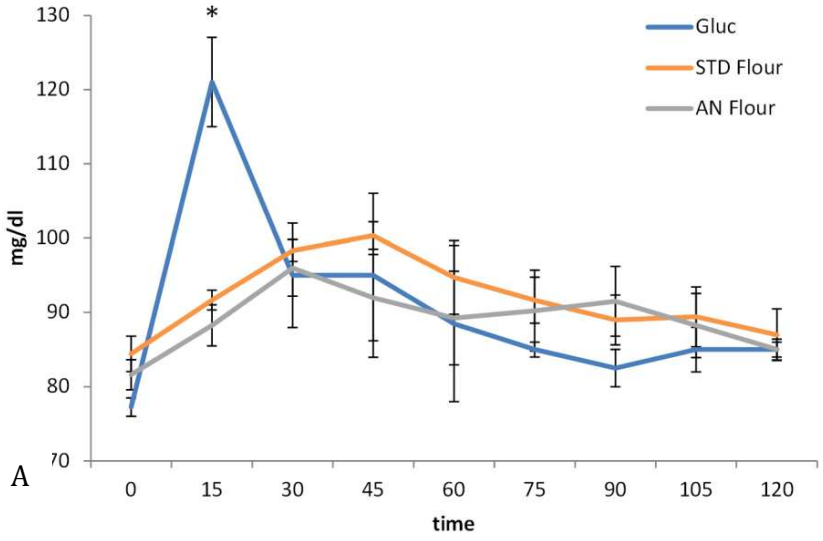
Table 20. ***Groups Yeast, Sourdough, Standard (STD) four and Ancient (AN) flour characteristic.***

Experimental Bread	**Group**	**Group on fermentation**	**Group on flour**
SFCF = Bread made with Standard Flour + Conventional Fermentation	A	yeast	STD flour
AFCF = Bread made with Ancient Flour + Conventional Fermentation	B		AN flour
SFSSF = Bread made with Standard Flour + Sourdough Standard Fermentation	C	sourdough	STD flour
AFSAF = Bread made with Ancient Flour + Sourdough Ancient Fermentation	E		AN flour

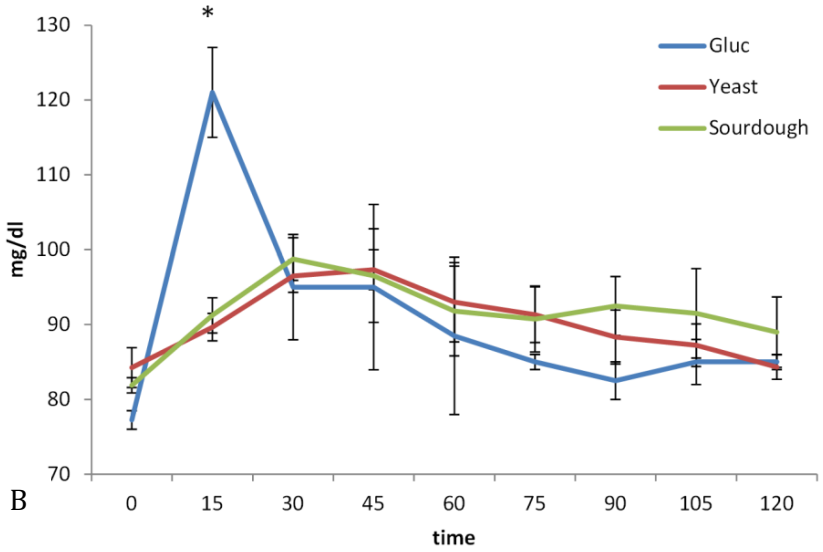
Blood glucose and insulin curves are represented in figure 23 and 24.For Blood glucose no statistical differences were found between ST and AN flour and between Yeast and Sourdough fermentation, while all four differ from glucose after 15 minute from the feed.

The shape of blood glucose curve of einkorn flour and sourdough fermented breads suggest that the glucose level was more stable, but more subjects are needed to confirm it due to the extremely high variability of the carbohydrate metabolism within the same subject.

Figure *23. A. Glycaemia of yeast and sourdough fermentation breads compared to glucose. B. Glycaemia of standard (STD) and ancient (AN) flour breads compared to glucose.* *The data are presented as mean ± SD. Time is reported as minutes. At 15 minutes the glycemic level of glucose is statistically different ($p < 0.001$) from yeast and sourdough breads which are not different among each other (fig 23 A). At 15 minutes the glycemic level of glucose is statistically different ($p < 0.001$) from STD and AN flour breads which are not different among each other (fig 23 B).*

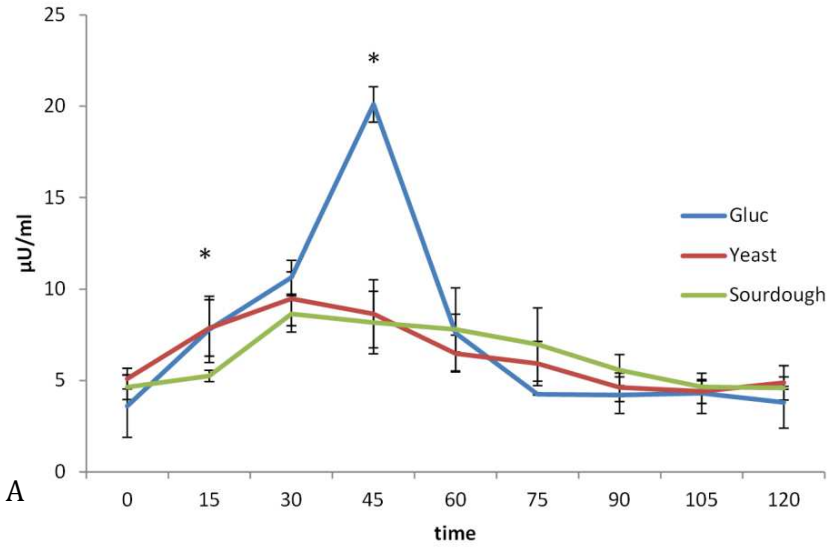


A

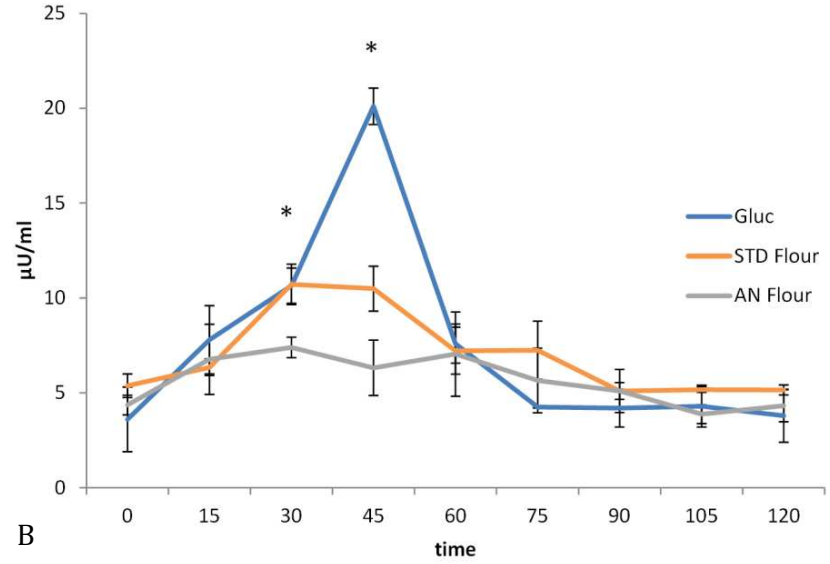


B

Figure ***24. A. Insulin levels of yeast and sourdough fermentation breads compared to glucose. B. Insulin levels of standard (STD) flour and ancient (AN) flour compared to glucose.*** *The data are presented as mean ± SD. Time is reported as minutes. In figure 24 A at 15 min insulin level of sourdough breads is statistically different ($p < 0.05$) from glucose and yeast breads which are not different among each other; at 45 min Glucose insulin differ ($p<0.001$) from all the other. In figure 24 B at 30 min insulin level of ST flour breads and glucose are statistically different ($p < 0.05$) from AN flour breads; at 45 min all insulin levels differ ($p<0.001$) with the glucose level highest, the AN flour lowest and the ST flour in the middle.*



A



B

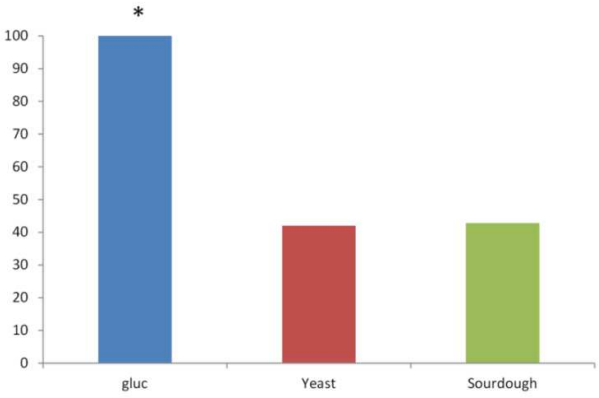
The insulin plasma levels of yeast and sourdough fermented breads does not biologically differ, while there is an interesting differences between einkorn and standard flour. The insulin level of AN flour breads was stable for the entire

post prandial sampling period (120 min) suggesting that the einkorn does less stimulate the insulin release.

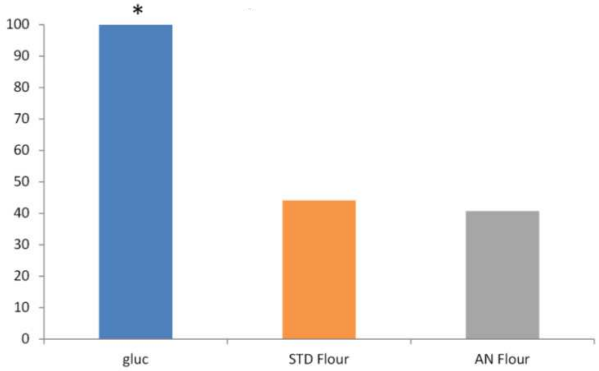
Glycemic index (GI) for each diet was calculate as area under the curve (AUC) normalized on the glucose AUC. The GI of AN was lower compared to ST flour while GI of sourdough fermented bread was higher compared to yeast fermented bread.

The glycemic load (GL) of the different breads was also calculated to take in account the amount of carbohydrates intake, but did not show any difference between diets (Fig. 25).

Figure 25. ***A Glycemic loads (GL) of yeast and sourdough fermented breads compared to glucose. B. Glycemic loads of standard (STD) flour and ancient (AN) flour breads compared to glucose*.**



A



B

5.2.3 Conclusions

The trial was conducted on jugular cannulated pigs, after a period of training all animals eat the experimental diet within 5 minutes leading to a standardized post prandial blood samples collection. The blood glucose and insulin spikes of glucose were coherent as the insulin spike followed the glucose spike after 30 minutes. Despite that some animals were excluded from the trial due to technical problem with the jugular cannula.

Glycemic index (GI) and glycemic load (GL) were calculated.

Einkorn (AN flour) breads and sourdough fermented breads GI and GL did not lead to a statistical differences compared with standard flour and standard fermented breads, but the trend of the blood glucose curves suggest that AN flour bread made with einkorn has a lower GI and GL that might be highlighted by increasing the subjects enrolled in the study.

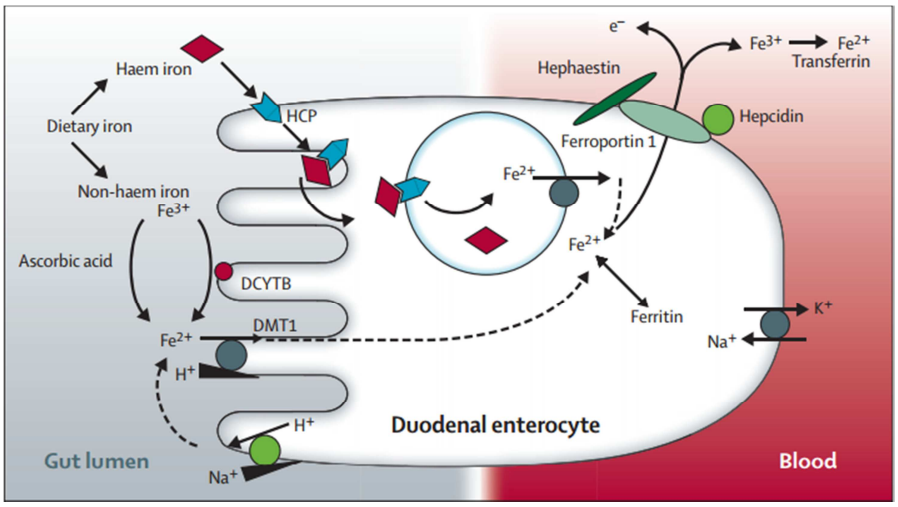
This study confirms that whole flour bread compared to standard flour bread might protect against insulin spikes and subsequent hypoglycemia with the reactivation of the hunger and higher voluntary energy intakes in a pig model.

Moreover the role of sourdough microbioma on the gut pig micriobiota and its role on the modulation of carbohydrate metabolism were studied. The results suggest that the sourdough fermentation can lead to a better exploitation of food carbohydrates. This finding make the einkorn sourdough fermented bread a high nutritional value food.

5.3 Experiment 3. Evaluation of iron bioavailability and impact of novel bakery products enriched by microencapsulated iron on the intestinal microbiota, oxidative and inflammatory status

Human beings are unable to excrete iron actively, so its concentration in the body must be regulated at the site of iron absorption in the proximal small intestine. Diets contain both haem and non-haem (inorganic) iron and each form has specific transporters (Zimmermann and Hurrell, 2007). In figure 26 regulation of intestinal iron uptake is represented, haem iron is taken up by the haem iron transporter (HCP), undergoes endocytosis, and Fe^{2+} (ferrous iron) is liberated within the endosome or lysosome. Non-haem iron includes Fe^{2+} and Fe^{3+} (ferric iron) salts. Fe^{3+} is reduced to Fe^{2+} by ascorbic acid in the lumen or by membrane ferrireductases that include duodenal cytochrome B (DCYTB). At the apical membrane, the acid microclimate provides an H^{+} electrochemical gradient that drives Fe^{2+} transport into the enterocyte via the divalent metal-ion transporter (DMT1). At the basolateral membrane, iron transport to transferrin in the circulation is mediated by ferroportin 1, in association with hephaestin. Hepcidin, produced by the liver, binds to ferroportin 1, causing its internalisation and degradation and decreasing iron transfer into the blood.

Figure 26. ***Regulation of intestinal iron uptake.*** *From Zimmermann and Hurrell, 2007*



Nutritional iron deficiency arises when physiological requirements cannot be met by iron absorption from diet. Dietary iron bioavailability is low in populations consuming monotonous plant-based diets with little meat (Zimmermann et al., 2005). In meat, 30–70% of iron is haem iron, of which 15–35% is absorbed. However, in plant-based diets in developing countries most dietary iron is non-haem iron, and its absorption is often less than 10% (Hurrell, 2002). The absorption of non-haem iron is increased by meat and ascorbic acid, but inhibited by phytates, polyphenols, and calcium. Iron deficiency affects predominantly infants, young children, adolescents, menstruating and pregnant woman when iron requirements are greater than energy needs; this condition has substantial health and economics costs.

The main strategies to correct iron deficiency are supplementation and fortification. Although in industrialized countries, oral iron therapy is commonly associated with gastrointestinal side effects, it is not known to increase the burden of infection in this setting (Kortman et al., 2014) . In contrast, in an African region with high infection pressure (e.g. malaria) and low hygiene

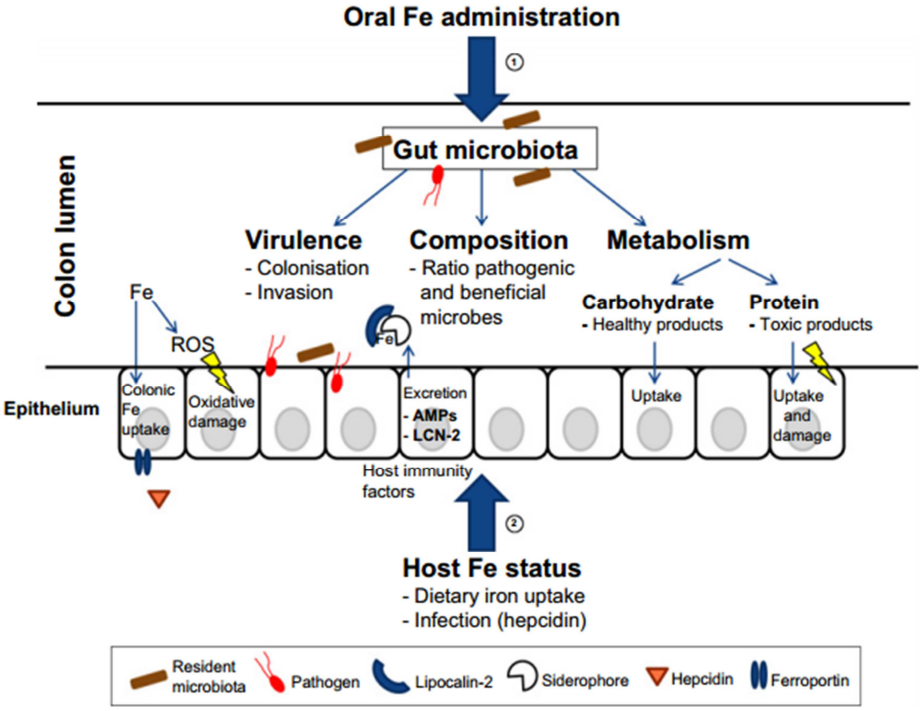
standards, oral iron supplementation has been associated with increased morbidity and mortality of children (Goheen et al., 2016). Furthermore, oral administration can increase the incidence of diarrhea, which may partly be caused by the outgrowth of enteric pathogens.

Fortification is probably the most practical and sustainable long-term solution to control iron deficiency, but often the iron compound react with other food components causing off-flavors, color changes and or fat oxidation (Spieldenner, 2016).

Moreover accumulating evidence indicates that excess of unabsorbed iron that enters the colonic lumen causes unwanted side effects at the intestinal host–microbiota interface, a schematic overview of this process is exposed in figure 27 (Kortman et al., 2014). In their article review Kortman et al. described the dynamic effects and impact of oral administration on intestinal host-microbiota interactions pointing out that oral iron administration changes the gut microbiota profile on several fronts and a shift towards a potentially pathogenic profile has been confirmed.

The clinical relevance of these effects is not fully clear yet, but it is highly recommended that potentially pathogenic effects on the gut microbiota need to be avoided as much as possible.

Figure 27. ***Schematic overview of the main gut microbial factors that are influenced by oral iron administration and host iron status.*** *This figure summarizes the main two iron-related components that influence the gut microbiota composition, virulence and metabolism. (1) Orally administered iron can have direct impact on the gut microbiota composition (and the ratio of pathogenic bacteria to beneficial bacteria) and metabolism and potentially on the virulence of enteric pathogens. (2) Host iron status is influenced by dietary iron uptake and infection; this in turn influences host immunity factors which can affect the gut microbiota. From* **(Kortman et al., 2014)**



To study the role of iron supplementation on gut microbiota and intestinal mucosa in vitro gut models and animal models that closely resemble the relevant in vivo conditions are needed.

The suckling piglet is an optimal model for iron deficiency as this condition is particularly frequent and severe in pigs, regardless of the breed and the system of piglet rearing (Starzyński et al., 2013).

The common reason for iron deficiency in newborn piglets is their rapid growth, particularly the increase in blood volume and the number of red blood cells (RBCs). Indeed, erythroid precursors in the bone marrow use most of the

iron found in the plasma for the synthesis of hemoglobin. As sow's milk largely provides piglets with iron below their daily requirements, an exogenous source of iron is essential to prevent the reduction in RBC hemoglobin level. Intramuscular administration of large amounts of iron dextran (FeDex) on days 3 to 6 postpartum is current practice in the swine industry and has been proven to rectify the hematological status of piglets.

The newborn piglet develop a para-physiological iron deficiency anemia that is comparable with human nutritional iron deficiency making the piglet without iron parenteral supplementation a feasible, rapidly inducible and cost-effective model.

The need for different approaches to iron supplementation or fortification able to protect the gut microbiota leads to the development of iron microencapsulation. This technology was used by Bake4Fun partners to produce an iron microencapsulate fortified bread that had similar organoleptic properties to the conventional breads.

One of the aims of this study was to evaluate the effect of this novel bakery product on the gut microbiota and inflammatory status in a piglet model. Moreover we wanted to validate the iron microencapsulated fortification as possible strategy to contrast iron deficiency in a piglet model.

In order to be able to compare iron fortification efficiency on the model, valuable references data for piglets at precise ages were needed. Moreover the bibliography lacks of hematology and clinical chemistry references to evaluate

iron profile in newborn piglets with and without iron exogenous supplementation.

We therefore decide to create a dataset to improve current knowledge and to improve the Reduction in future animal experiments as suggested by the 3Rs principle.

5.3.1 The biomedical piglet: establishing reference intervals for haematology and clinical chemistry parameters of two age groups with and without iron supplementation

The similarities between swine and humans in physiological and genomic patterns, and the great correlation in size and anatomy, make pigs extremely useful in preclinical studies. New-born piglets can represent a model for congenital and genetic diseases in new-born children. It is known that piglets may have significant differences in clinicopathological results compared to adult pigs. Therefore, adult laboratory reference intervals cannot be applied to piglets. The aim of this study was to compare hematological and chemical variables in piglets of two ages and determinate age-related reference intervals for commercial hybrid young pigs.

Blood samples were collected under general anesthesia from 130 animals divided into five- (P5) and 30- (P30) day-old piglets. Only P30 animals were treated with parenteral iron after birth. Samples were analyzed using automated hematology (ADVIA 2120) and chemistry analyzers, and age-related reference intervals were calculated.

Significant higher values of RBC, Hb and HCT were observed in P30 animals when compared to P5, with an opposite trend for MCV. These results were associated with a reduction of the RBC regeneration process and the thrombopoietic response. The TSAT and TIBC were significantly higher in P30 compared to P5; however, piglets remained iron deficient compared to adult reference intervals reported previously.

In conclusion, this paper emphasizes the high variability occurring in clinicopathological variables between new-born and 30-day-old pigs, and between piglets and adult pigs. This study provides valuable reference data for piglets at precise ages and could be used in the future as historical control improving the Reduction in animal experiments, as suggested by the 3Rs principle.

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RESEARCH ARTICLE Open Access

The biomedical piglet: establishing reference intervals for haematology and clinical chemistry parameters of two age groups with and without iron supplementation

Domenico Ventrella, Francesco Dondi*, Francesca Barone, Federica Serafini, Alberto Elmi, Massimo Giunti, Noemi Romagnoli, Monica Forni and Maria L. Bacci

Abstract

Background: The similarities between swine and humans in physiological and genomic patterns, and the great correlation in size and anatomy, make pigs extremely useful in preclinical studies. New-born piglets can represent a model for congenital and genetic diseases in new-born children. It is known that piglets may have significant differences in clinicopathological results compared to adult pigs. Therefore, adult laboratory reference intervals cannot be applied to piglets. The aim of this study was to compare haematological and chemical variables in piglets of two ages and determinate age-related reference intervals for commercial hybrid young pigs.
Blood samples were collected under general anaesthesia from 130 animals divided into five- (P5) and 30- (P30) day-old piglets. Only P30 animals were treated with parenteral iron after birth. Samples were analysed using automated haematology (ADVIA 2120) and chemistry analysers, and age-related reference intervals were calculated.

Results: Significant higher values of RBC, Hb and HCT were observed in P30 animals when compared to P5, with an opposite trend for MCV. These results were associated with a reduction of the RBC regeneration process and the thrombopoietic response. The TSAT and TIBC were significantly higher in P30 compared to P5; however, piglets remained iron deficient compared to adult reference intervals reported previously.

Conclusions: In conclusion, this paper emphasises the high variability occurring in clinicopathological variables between new-born and 30-day-old pigs, and between piglets and adult pigs. This study provides valuable reference data for piglets at precise ages and could be used in the future as historical control improving the Reduction in animal experiments, as suggested by the 3Rs principle.

Keywords: ADVIA 2120, Clinical chemistry, Haematology, Reference intervals, Swine

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Background

The interest in the pig as an animal model for experimental medicine can be traced back to Galen, in 1586 [1]. One reason for this interest is the strong similarities between the pig and the human in both physiological [2] and genomic [3] patterns. In addition, size and anatomy can be easily related to the development stages of people, making the pig the perfect preclinical model for human diseases [4, 5], surgical techniques and, more recently, for transplantation research [6–8]. When compared to other models such as mice or rats, the pig has a longer lifespan of 10–15 years [9], so disease progression is more similar to that seen in humans [1]. Furthermore, in the neonatal period, pigs represent an accurate model for studying congenital and genetic diseases in humans [10]. Piglets can even represent a good model for the preterm neonate, as they show similar anthropometric and physiological characteristics [11].

However, age differences, even within the same species, significantly affect the comparison of some developmental patterns, especially in extremely young subjects. Therefore, these processes need to be thoroughly investigated in order to create an accurate and standardised preclinical model and to help reduce and refine experimental protocols. As an important example, iron deficiency, which is one of the most common nutritional defects during the neonatal period in mammals [12, 13], is extremely common in swine, due to the high reproductive performance required of these animals. Unless given iron supplements, piglets may develop iron-deficiency a few days after birth [14, 15]. This condition occurs regardless of the breed and management system, and is the result of interactions of several factors including low levels of iron stores, increased requirements, poor exogenous supply and immaturity of absorption mechanisms [15, 16]. Similarly, iron requirements cannot be completely fulfilled by hepatic reserves and milk consumption due to the constant request for larger litter sizes, higher birth weights and faster growth that result in a greater blood volume and red blood cell (RBC) count [17]. It is therefore mandatory to supplement piglets with exogenous iron to prevent dangerous deficiency [18]. This procedure may interfere with several clinical chemistry parameters [19] and is the reason why it is very inaccurate to evaluate piglets based on the clinicopathological findings of older pigs. Therefore, it is extremely important to have specific age-related reference intervals for both haematological and chemical variables for piglets. Some values have been described in a single litter of Duroc x Jersey piglets [20], but the small number of animals and the lack of information about iron supplementation make them hard to rely on.

The aim of this study was to evaluate haematological and chemical variables in two groups of healthy hybrid piglets of different ages. Secondary objectives were to establish age-related reference intervals (RI) for both haematology and clinical chemistry variables and to evaluate the iron profile in new-born piglets without exogenous supplementation (5 days old) and young pigs administered with exogenous iron within 3 days after birth (30 days old).

Methods

Animals

All of the animals were Italian Large White x Duroc x Landrace commercial hybrids used in our facility. We only selected control and/or pre-treated animals previously enrolled in other experimental protocols and approved by the local ethical committee to be part of this study. The above mentioned protocols included blood tests to evaluate the animals, and we decided to work on the obtained data set of blood values.

We analysed piglets at two different time points: P5 were five-day-old piglets born in our facility that had not received any iron supplementation before blood sampling and were not neutered; P30 were 30-day-old pigs that were transferred to our piggery on the day of weaning (28th day of life) and were administered a single iron injection (100 mg IM; Endofer, FATRO, Italy) within the first 72 h after birth, and males were neutered. None of the animals was included in both age groups. In order to rule out any possible variation in genetic line and management, both pregnant sows and pigs were born and raised in the same farm. All of the animals were housed in multiple stalls and fed with a standard swine diet; P5 were housed in the farrowing crate with the sow until weaning. Body weight (kg) was measured in P5 and P30 and recorded.

A total of one hundred-thirty animals were included in the study; 74/130 (57%) were females, while 56/130 (43%) were males. Body weight was 2.3 (1.2–3.8) kg in P5 and 8.0 (5.3–11.2) kg in P30. For haematologicalal analyses, samples from 130 animals were available: 66 P5 and 64 P30. For chemistry evaluations, samples from 119 animals were available: 56 P5 and 63 P30.

Blood sample collection and analyses

Blood samplings were performed on day 5 (P5) or 30 (P30) under general anaesthesia, using an advanced anaesthesia delivery unit (Datex-Ohmeda ADU S/5, GE Healthcare, USA), achieved by inhalation induction with sevoflurane (Sevoflo, Abbott Laboratories, Chicago, USA). No premedication was performed in order to avoid blood alterations due to injected drug adsorption. After oro-tracheal intubation, anaesthesia was maintained with $4 \pm 0.5\%$ sevoflurane in a 1:1 oxygen/air mixture. Samples were obtained from the femoral artery using a 21 G butterfly needle and a vacuum system;

tubes with K_3EDTA anticoagulant, citrate and clot activator were used. The total volume of withdrawn blood was approximately 10 ml, which was considered completely safe and negligible for these animals.

Blood samples (K_3EDTA tubes) were analysed within 30 min from collection; serum (clot activator tubes) and citrate plasma (citrate tubes) were obtained by centrifugation (10 min at 3000 × g) within 1 h and analysed or stored at −80 °C until analysis.

Complete blood count (CBC) was performed with a new automated haematology analyser (ADVIA 2120, Siemens Healthcare Diagnostics, Tarrytown NY, USA) that combines classic haematological variables with individual cell indices. The variables evaluated in our study were haematocrit value (HCT), haemoglobin concentration (Hb), cellular haemoglobin content (CH), cellular haemoglobin content of mature red blood cells (CHm), red blood cells count (RBC), mean corpuscular volume (MCV), mean corpuscular volume of mature RBCs (MCVm), mean corpuscular haemoglobin concentration (MCHC), mean corpuscular haemoglobin (MCH), corpuscular haemoglobin concentration mean (CHCM), corpuscular haemoglobin concentration mean of mature RBCs (CHCMm), haemoglobin concentration distribution width (HDW), haemoglobin concentration distribution width of mature RBC (HDWm), RBC distribution width (RDW) and mature RBC distribution width (RDWm). Total white blood cell (WBC) count and differential WBC count were also performed. Platelet indices were analysed and included platelet count (PLT), mean platelet volume (MPV), PLT volume distribution width (PDW), plateletcrit (PCT), mean PLT component (MPC), platelet component distribution width (PCDW), mean PLT mass (MPM) and platelet mass distribution width (PMDW).

In addition to the above mentioned variables, we evaluated the following reticulocyte indices: absolute reticulocyte count (Retic), percentage of reticulocytes (%Retic), average size of reticulocytes (MCVr), average cell haemoglobin concentration (CHCMr), average haemoglobin content (CHr), distribution width of reticulocyte cell size (RDWr), distribution width of CHCMr (HDWr), percentage of microcytic reticulocytes (%Micro-r), percentage of macrocytic reticulocytes (%Macro-r), percentage of hypochromic reticulocytes (%Hypo-r), percentage of hyperchromic reticulocytes (%Hyper-r), percentage of reticulocytes with a low CH (%LowCHr), percentage of reticulocytes with a high CH (%HighCHr), CHr-CHm (CH delta), CHCMr-CHCMm (CHCM delta), CHDWr-CHDWm (CHDW delta), HDWr-HDWm (HDW delta), MCVr-MCVm (MCV delta), and RDWr-RDWm (RDW delta). The haematologicalal evaluation was completed by a blood smear microscopic examination using Romanovsky staining.

All chemistry analyses were carried out on an automated chemistry analyser (Olympus AU 400, Beckman Coulter/Olympus) and included aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), creatinine, urea, glucose, total proteins (TP), albumin, albumin to globulin ratio (A/G), sodium, potassium, total iron (TI), unsaturated iron binding capacity (UIBC), total iron binding capacity (TIBC) and TIBC saturation (TSAT). Total iron and UIBC were measured using colorimetric methods (Iron Ferene, KAL 002, Olympus/Sentinel Diagnostics, Milan, Italy; UIBC OSR61205, Olympus/Beckman Coulter, O'Callaghan's Mills, Ireland). Total iron binding capacity and TSAT were calculated as follows: TIBC = TI + UIBC; TSAT = (TI × 100)/TIBC.

ADVIA 2120 erythrocytes, reticulocytes and platelet indices, and other variables evaluated in the study are reported in the Additional file 1, including their abbreviations.

Statistical analyses

Statistical analyses were performed using MedCalc statistical software (version 15.6; MedCalc Software, Ostend, Belgium). The D'Agostino-Pearson test was used to assess normal distribution of data. Data were reported as mean ± SD or median (minimum-maximum) based on their distribution. Comparisons between the two age groups were performed using the Mann-Whitney *U* test due to the non-Gaussian distribution of the majority of the data. Reference intervals were obtained using the 2.5th − 97.5th percentiles method following the Clinical and Laboratory Standards Institute (CLSI) guidelines for estimating percentiles and their 90% confidence intervals [21]. Outliers were identified with the Tukey test. Differences were considered to be statistically significant with $P < 0.05$.

Results and discussion

For the haematological and chemical analyses, the number of samples available for each analyses, descriptive statistics, differences between groups and estimated RI in P5 and P30 are reported in Tables 1, 2 and 3. A significant increase in the circulating erythrocyte mass was detected in P30 compared to P5 as demonstrated by the higher values of Hb, HCT and RBC count. This finding was associated with a significant reduction in volume (MCV, MCVm) and a significant increase in anisocytosis (RDW, RDWm). Erythrocyte haemoglobin content indices (CH, CHm, MCH) were significantly lower in P30 compared to P5 with the exception of CHCM which was significantly higher in P30, while MCHC and CHCMm were not significantly different between groups (Table 1). Absolute reticulocyte count and percentage of reticulocytes were significantly lower in P30 compared to P5.

Table 1 Descriptive statistics, differences between groups and estimated reference intervals for haematological variables. Data are expressed as mean ± SD or median (minimum-maximum)

Variable	P5	P5	P5	P30	P30	P30	*P*-value
	Descriptive data	Reference Interval	*n*	Descriptive data	Reference Interval	*n*	
Hb (g/dL)	6.03 ± 1.02	3.56-7.74	61	8.84 ± 2.0	4.32-13.31	64	<0.0001
HCT (%)	20 ± 3	13-25	61	29 ± 6	16-41	64	<0.0001
CH (pg)	16.85 (14.10–22.30)	14.30–21.82	66	14.0 (9.0–19.0)	9.3–18.9	64	<0.0001
CHm (pg)	18.3 (16.1–22.4)	16.27–22.01	50	15.4 ± 1.5	12.7–18.8	33	<0.0001
CHDW (pg)	3.72 (2.90–5.84)	3.01–5.71	66	3.99 ± 0.66	2.66–5.33	64	0.3541
CHDWm (pg)	3.36 (2.82–5.47)	2.83–5.34	50	4.78 (2.61–5.55)	2.61–5.50	33	<0.0001
RBC (10^6/μL)	3.26 ± 0.52	1.88–4.11	61	6.08 ± 0.93	4.08–8.17	64	<0.0001
MCV (fL)	61.97 ± 5.39	51.41–73.65	61	48.5 (32.4–61.5)	34.2–61.3	64	<0.0001
MCVm (fL)	65.9 ± 4.5	56.4–74.9	50	52.9 ± 3.9	45.3–60.5	33	<0.0001
MCHC (g/dL)	30.0. ± 1.6	26.1–32.7	61	29.9 ± 1.59	26.5–33.6	63	0.9606
MCH (pg)	18.50 ± 1.36	15.45–21.54	61	14.8 (9.1–20.2)	9.4–19.8	64	<0.0001
CHCM (g/dL)	27.35 ± 1.11	24.84–29.13	66	28.35 ± 1.60	25.13–31.62	64	0.0003
CHCMm (g/dL)	28.2 (26.7–30.8)	26.8–30.8	50	28.6 (26.8–30.9)	26.8–30.9	33	0.5389
RDW (%)	18.8 (15.8–25.7)	15.9–25.7	56	26.5 (13.5–38.5)	13.5–38.0	64	<0.0001
RDWm (%)	15.9 (14.2–25.7)	14.2–25.1	50	25.5 (12.7–32.2)	12.7–32.0	33	<0.0001
HDW (g/dL)	2.89 (2.28–4.13)	2.29–3.93	66	2.63 ± 0.40	2.02–3.50	64	<0.0001
HDWm (g/dL)	2.60 (2.14–3.68)	2.16–3.63	50	2.73 ± 1.99–3.28	1.99–3.28	33	0.1685
WBC (10^3/μL)	7.47 (4.39–12.58)	4.50–12.55	58	11.6 ± 3.2	5.6–18.5	58	<0.0001
neutrophil (%)	44.71 ± 9.66	22.77–61.33	60	35.3 ± 15.0	10.8–70.6	63	0.0001
lymphocyte (%)	49.30 ± 9.49	33.45–70.86	60	57.9 ± 14.5	26.2–82.9	64	0.0005
monocyte (%)	3.11 ± 1.13	1.10–5.85	59	4.4 ± 1.5	1.4–8.3	64	<0.0001
eosinophil (%)	0.5 (0.1–3.1)	0–2.3	57	0.6 (0.1–7.6)	0–1.9	64	0.5699
basophil (%)	0.41 ± 0.16	0–0.79	60	0.4 (0.2–1.2)	0–0.9	63	0.1183
neutrophil (10^3/μL)	3.17 ± 0.98	1.15–5.43	54	3.5 (0.8–9.9)	0.8–9.7	61	0.1472
lymphocyte (10^3/μL)	3.85 ± 1.11	1.91–6.45	58	6.6 ± 2.4	2.7–12.8	60	<0.0001
monocyte (10^3/μL)	0.23 (0.07–0.7)	0.075–0.68	59	0.4 (0.1–1.1)	0.1–1.1	59	<0.0001
eosinophil (10^3/μL)	0.04 (0.01–0.48)	0–0.28	60	0.07 (0.01–0.38)	0.00–0.20	63	0.0014
basophil (10^3/μL)	0.03 (0.01–0.2)	0–0.08	60	0.05 (0.02–0.33)	0.00–0.13	63	<0.0001
PLT (10^3/μL)	594 (219–1142)	253–1286	61	503 ± 141	192–832	63	<0.0001
MPV (fL)	17.84 ± 3.78	9.99–25.32	61	8.5 (6.2–13.0)	6.5–12.7	63	<0.0001
PDW (%)	84.22 ± 8.32	69.17–100.30	61	60.40 (17.90–106.60)	18.59–101.98	64	<0.0001
PCT (%)	1.20 ± 0.50	0.29–2.52	61	0.40 (0.12–0.94)	0.18–0.91	62	<0.0001
MPC (g/dL)	24.5 (17.2–26.3)	17.81–26.30	61	24.09 ± 0.96	21.69–26.50	64	0.0182
MPM (pg)	2.84 ± 0.34	2.10–3.44	61	1.96 ± 0.22	1.58–2.45	63	<0.0001
PCDW (g/dL)	4.6 (4.0–5.7)	4.0–5.6	66	4.6 (3.8–5.9)	3.9–5.9	64	0.4354
PMDW (pg)	1.45 (0.99–1.62)	0.99–1.61	66	0.87 (0.61–1.55)	0.62–1.54	64	<0.0001

Many other reticulocyte indices were significantly different between age groups (Table 2).

Circulating platelet number (PLT, PCT) was significantly decreased in P30 compared to P5; these results were associated with a significant reduction in platelet volume and mass (MPV, MPM) in P30 animals.

In the chemistry analysis, P5 subjects had significantly lower values of creatinine, urea, ALT, albumin and A/G and significantly higher ALP and potassium compared to P30 animals. Total iron concentration and TSAT % were significantly higher in P30 piglets compared to P5 (Table 3).

Table 2 Descriptive statistics, differences between groups and estimated reference intervals for reticulocyte indices. Data are expressed as mean ± SD or median (minimum-maximum)

Variable	P5	P5	P5	P30	P30	P30	*P*-value
	Descriptive data	Reference Interval	*n*	Descriptive data	Reference Interval	*n*	
Retic (10^9cell/L)	369.3 ± 101.1	152.2–547.9	45	148.2 (53.4–554.5)	53.4–554.5	33	<0.000001
%Retic (%)	11.8 ± 3.8	4.6–20.7	49	2.5 (0.8–9.2)	0.8–9.2	33	<0.000001
MCVr (fL)	74.5 ± 7.3	60.7–89.4	48	56.5 ± 6.8	43.0–71.1	33	<0.000001
CHCMr (g/dL)	24.9 ± 0.9	23.4–27.0	48	26.1 ± 1.0	24.2–28.0	33	0.000008
CHr (pg)	18.1 (16.0–24.3)	16.2–23.8	48	14.6 ± 1.4	12.1–18.0	33	<0.000001
CHDWr (pg)	3.7 ± 0.3	3.1–4.4	48	3.5 (2.4–4.6)	2.4–4.6	33	0.166288
RDWr (%)	17.6 ± 2.1	14.2–20.7	48	19.5 (13.0–33.0)	13.0–33.0	33	0.055130
HDWr (g/dL)	3.36 ± 0.36	2.66–3.84	48	3.9 ± 0.62	2.7–5.1	33	0.000003
%Micro-r (%)	0.05 (0–0.8)	0.0–0.45	48	0.7 (0.0–15.5)	0.0–15.5	33	0.000005
%Macro-r (%)	26.5 (4.8–71.9)	6.7–70.7	48	2.6 (1.0–17.4)	1.0–17.4	33	<0.000001
%Hypo-r (%)	83.0 (62.8–94.3)	62.2–92.2	48	76.4 (57.2–85.8)	57.2–85.8	33	0.000036
%Hyper-r (%)	0.1 (0.0–0.3)	0.0–0.3	48	0.3 (0.0–2.7)	0.0–2.7	33	<0.000001
%LowCHr (%)	10.9 (0.5–35.4)	0.5–32.1	48	57.3 (6.0–76.8)	6.0–76.8	33	<0.000001
%HighCHr (%)	22.6 (9.2–76.3)	10.1–74.9	48	7.1 ± 2.5	3.3–13.0	33	<0.000001
CH delta (pg)	−0.1 ± 1.6	−3.3–2.1	48	−0.7 ± 0.7	−2.4–0.7	33	0.012741
CHCM delta (g/dL)	−3.4 ± 0.9	−5.2–(−1.8)	48	−2.6 ± 1.7	−5.3–0.8	33	0.075
CHDW delta (pg)	0.38 (−0.54–0.86)	−0.33–0.67	48	−1.0 ± 0.6	−2.19–(−0.05)	33	<0.000001
HDW delta (g/dL)	0.66 ± 0.27	0.12–1.07	48	1.26 ± 0.46	0.18–2.06	33	<0.000001
MCV delta (fL)	8.9 ± 6.6	−3.4–18.3	48	3.6 ± 4.4	−3.8–12.5	33	0.000255
RDW delta (%)	1.4 (−7.4–5.2)	−6.3–4.2	48	−3.8 ± 4.3	−12.6–3.0	33	<0.000001

Table 3 Descriptive statistics, differences between groups and estimated reference intervals for clinical chemistry. Data are expressed as mean ± SD or median (minimum-maximum)

Variable	P5	P5	P5	P30	P30	P30	*P*-value
	Descriptive data	Reference Interval	*n*	Descriptive data	Reference Interval	*n*	
Glucose (mg/dL)	124 (69–200)	71–196	49	111 ± 26	34–159	62	0.0016
Urea (mg/dL)	12.4 (4.9–31.7)	5.0–30.8	55	17 (4–40)	4–39	59	0.0002
Creatinine (mg/dL)	0.65 (0.30–0.88)	0.38–0.85	56	1.09 (0.31–1.41)	0.51–1.39	62	<0.0001
AST (U/L)	29 ± 7	10–47	56	31 (11–68)	13–65	62	0.9915
ALT (U/L)	23 ± 6	5–38	56	30 (11–58)	14–54	60	<0.0001
ALP (U/L)	3773 ± 1017	1324–6031	56	770 ± 270	110–1292	61	<0.0001
TP (g/dL)	5.0 ± 0.5	3.7–6.2	54	4.8 (1.2–6.7)	2.5–6.6	59	0.4464
Albumin (g/dL)	1.8 ± 0.3	1.0–2.6	55	3.0 (1.8–4.0)	1.9–4.0	59	<0.0001
A/G	0.56 (0.36–0.99)	0.37–0.98	53	1.5 ± 0.3	0.7–2.2	60	<0.0001
TI (μg/dL)	15 (9–31)	9–30	49	34 (7–157)	7–151	60	<0.0001
UIBC (μg/dL)	338 ± 72	218–524	44	326 (40–1058)	63–980	62	0.2031
TIBC (μg/dL)	354 ± 72	230–542	44	397 (128–882)	142–877	60	0.0031
TSAT (%)	4.2 ± 1.2	1.3–6.7	42	8.6 (0.8–55.6)	0.9–54	61	0.0058
Potassium (mEq/L)	4 ± 0.5	2.8–5.1	54	3.7 ± 0.4	2.9–4.6	63	0.0252
Sodium (mEq/L)	135 (126–140)	126–140	53	136 ± 4.7	125–147	63	0.2448

Swine are probably one of the most important models for translational medicine [7], and therefore a complete and accurate knowledge of their physiology should be mandatory. This kind of knowledge would represent a big improvement when it comes to refinement of animal experiments. When using commercial hybrids for scientific purposes, it is important to acknowledge that those animals are the products of strong zootechnical manipulation that constantly requires higher breeding and production performance [18]. Moreover, very young piglets (first month of age) present high variability in many clinical and clinicopathological variables due to rapid growth, nutrition and other metabolic conditions, some which are potentially related to iron status [15, 20]. As in other species, new-born and young piglets are extremely different than adult animals regarding laboratory results [20], thus, the determination of reliable age-related reference intervals for both haematology and chemistry variables in 5- and 30-day-old piglets is warranted. It is important to acknowledge the fact that our results may not perfectly translate to every other pigs' breeds, but still represent a valid and robust general reference especially because obtained by one of the most common hybrid cross in Europe. Another important issue to address, before the discussion of the results, is the feeding protocol: P5 only received milk from the sows, while P30 were weaned at 28 days of life, therefore only ate solid feed for 2 days before blood sampling. This difference in alimentation may have contributed to some of the differences alongside with evolution of the gastro-intestinal system.

The results reported in this study showed high variability in the blood profiles among P5 and P30 animals. Unfortunately, it is hard to compare our data with the ones previously described in the literature mainly because of the poor or absent age distinction and the different types of animals used. However, our P5 and P30 animals had lower Hb, HCT, RBC, MCV and MCH values compared to animals of similar ages (days 6 and 36 from birth, respectively) [20].

In our study, P5 animals had a RBC regenerative response that was significantly reduced in P30, as further demonstrated by the results of reticulocyte indices (significantly higher Retic, %Retic, MCVr, %Macro-r and MCV delta). Similar findings have been reported previously and could represent a physiological response in the new-born pigs [15, 20]. However, a condition of iron deficiency could not be excluded in our piglets. It is well known that piglets can suffer from iron deficiency due to many causes such as immature iron metabolism, decreased iron intake or absorption and rapid growth. Iron deficiency can be associated with reduced weight gain, anaemia and even with increased mortality in these animals [22]. For these reasons, iron supplementation is highly recommended and the benefit of this treatment is well documented [18]. The role of iron deficiency in P5 animals, although suspected based on haematological results, could not be demonstrated with the current study design. In the initial phase, iron deficiency is characterised by enhanced erythropoiesis and even a regenerative anaemia, while microcytosis and hypochromasia (reduction of MCV and MCHC) are late findings associated with iron deficiency in animals and humans [23, 24]. The P30 animals that were supplemented with parenteral iron within the first 72 h after birth had a decreased reticulocyte response compared to the new-born animals. In addition, the results of iron profile parameters, particularly TI and TSAT, supported the potential role of iron deficiency in the piglets included in the present study. Total iron and TSAT values in P5 and even P30 animals were extremely decreased compared to the adult pig values reported in the literature [25]. However, TI and TSAT values were significantly higher at 30 days after birth, compared to new-born piglets. A previous study, using a different schedule of iron treatment, reported different results with particular regard to the iron profile parameters, compared to our study [15]. In our study, the upper limit for TI in P30 animals was five times higher than the P5 upper limit, however the lower limit was similar; the same happened for TSAT. The TIBC showed a slightly different trend, with a wider interval and both lower and higher values in P30 compared to P5. The overall pattern shown by iron-related parameters indicated that circulating iron was very low in these healthy animals. For this reason iron deficiency is hard to investigate in piglets and the determination of other iron-related variables such as ferritin, which is considered the main iron storage protein, may be helpful. Further studies on non-treated animals should be performed in order to investigate and quantify the physiological erythroid response of growing pigs. However, it is possible that an enhanced erythropoiesis accompanied by a relative iron deficiency may be considered a paraphysiological condition for this type of piglet in the first month after birth.

Swine are known to have an elevated platelet count compared to other animals, with frequent platelet clumping upon blood smear examination [20]. Although the total platelet count at both experimental times was comparable to adult animal values available in the literature, PLT, volume and mass were significantly reduced in P30 pigs compared to the new-born piglets. Similar data for young pigs are lacking in veterinary medicine to the best of our knowledge. Analogous to erythropoiesis, an enhanced thrombocytopoiesis that decreases shortly after birth could be explained by the increased need for platelets due to rapid growth or even iron deficiency. Iron deficiency, in fact, is recognised as a stimulus for the bone marrow to produce and release platelets, leading to thrombocytosis in small animals and humans [26].

The P5 and P30 total WBC count results in our study were consistent with the literature: neutrophil percentage decreased from P5 to P30 while the neutrophil total number did not differ significantly. On the contrary, the lymphocyte count and percentage were significantly increased in older animals, as previously reported [20].

Many chemistry variables evaluated were significantly different between the two age groups. These differences may impact the RI definition, however, it is difficult to clarify the exact physiological significance of these changes from an observational study. Interestingly, new-born swine may have a very low concentration of albumin that was significantly higher in 30-day-old animals, while TP concentration did not differ significantly between groups. As supported by the increase in A/G in P30 piglets, albumin concentration increased with the growth of these animals and this process was associated with a progressive reduction in the globulin fraction. Similar findings are lacking in previous studies.

Creatinine and urea values were significantly higher in P30 animals compared to P5, however both values were fully comparable to the adult values available in the veterinary literature [27]. In the authors' opinion, this is the result of the fast growing rate of pigs leading to a rapid increase in muscle mass, helped by the gradual supplementation of enriched solid diet throughout the lactation period.

Another significant difference can be noticed in the ALP concentration, which was extremely higher in younger animals. The literature suggests that this finding is related to higher osteoblast activity in young, growing animals; nevertheless the exact role of ALP in swine in poorly understood, and its clinicopathological usefulness needs to be clarified in further studies [28, 29].

Age-related reference intervals for haematological and biochemical variables in wild boars have been recently published [30]. Although a similar automated haematology system (ADVIA 120) was used in that study, animals from zero to six months were referred to as piglets, and therefore a comparison of their data with our results would not be accurate or reliable. This different stratification of the population in their study design reflects changes in chemical variables as well, and their data seem to be more comparable to our P30 results.

Conclusions

In conclusion, this paper highlights the high variability in haematological and chemistry variables between new-born and 30-day-old pigs, and between these animals and adult pigs. Moreover, our study provides specific age-related reference intervals for healthy commercial hybrid piglets that can be used as physiological standards for both translational and swine medicine. Age-related reference intervals will help in the correct interpretation of experimental results and should be considered an important step towards a more in-depth knowledge and standardisation of the swine animal model.

Additional file

Additional file 1: Haematological and Biochemical variables evaluated in the study. (DOCX 18 kb)

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Availability of data and material
The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

Authors' contributions
DV drafted the manuscript, conceived and designed the study, organized the data and performed statistical analyses. FD drafted the manuscript, helped designing the study, carried out the laboratory analyses and helped organizing and interpreting the data. FB helped handling the animals and collecting blood samples, organized and interpreted the data and review the draft. FS helped acquiring the data and carrying out the laboratory and statistical analyses. AE helped collecting blood samples and organizing the data, performed and reviewed the statistical analyses. MG helped handling the animals and collecting blood samples and critically reviewed the results and the entire draft. NR coordinated the activities performed on the animals and helped drafting the manuscript. MF co-funded the study and helped conceiving and designing the study. MLB funded and conceived the study, coordinated the work team and finally approved the manuscript. All authors read and approved the manuscript.

Competing interests
The authors declare that they have no competing interests.

Consent for publication
Not applicable.

Ethics approval
Animals used in this study were already enrolled in experimental protocols approved by the Italian Ministry of Health.

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5.3.2 Bioavailability of Microencapsulated Iron from Fortified Bread Assessed Using Piglet Model.

The aim of this study was to investigate the influence of oral iron supplementation, in the form of fortified breads, on the growth performance, health, iron status parameters, and fecal metabolome of anemic piglets. A study was conducted on 24 hybrid (Large White × Landrace × Duroc) piglets. From day 44, the post-natal 12 piglets were supplemented with 100 g of one of two experimental breads, each fortified with 21 mg of ferrous sulphate, either encapsulated or not. After one week of oral supplementation, hematological parameters (hematocrit value, hemoglobin, and red blood cells) showed statistically significant differences ($p \leq 0.05$). Piglets fed with the fortified breads had higher iron concentrations in the heart, liver, and intestinal mucosa compared to anemic piglets fed with control bread. Gene expression of hepcidin, iron exporter ferroportin (IREG1), and divalent metal transporter 1 (DMT1), together with concentrations of plasma ferritin, showed no significant statistical differences between groups. Both fortified breads could be used as sources of bioavailable iron. The seven-day intervention trial showed microencapsulation to have only a mild effect on the effectiveness of iron supplementation in the form of fortified bread.

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Bioavailability of Microencapsulated Iron from Fortified Bread Assessed Using Piglet Model

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Abstract: The aim of this study was to investigate the influence of oral iron supplementation, in the form of fortified breads, on the growth performance, health, iron status parameters, and fecal metabolome of anemic piglets. A study was conducted on 24 hybrid (Large White × Landrace × Duroc) piglets. From day 44, the post-natal 12 piglets were supplemented with 100 g of one of two experimental breads, each fortified with 21 mg of ferrous sulphate, either encapsulated or not. After one week of oral supplementation, hematological parameters (hematocrit value, hemoglobin, and red blood cells) showed statistically significant differences ($p \leq 0.05$). Piglets fed with the fortified breads had higher iron concentrations in the heart, liver, and intestinal mucosa compared to anemic piglets fed with control bread. Gene expression of hepcidin, iron exporter ferroportin (IREG1), and divalent metal transporter 1 (DMT1), together with concentrations of plasma ferritin, showed no significant statistical differences between groups. Both fortified breads could be used as sources of bioavailable iron. The seven-day intervention trial showed microencapsulation to have only a mild effect on the effectiveness of iron supplementation in the form of fortified bread.

Keywords: microencapsulated iron; fortified bread; iron bioavailability; piglet model; iron deficiency; anemia; metabolome

1. Introduction

Iron is an essential metalloelement for the physiology and biochemistry of most forms of living organism [1]. Well-nourished people generally have 4 to 5 grams of iron in their bodies, making it the most abundant trace element in the human body [2,3]. The functional iron pool consists of structural components in heme proteins—hemoglobin, myoglobin, and cytochrome-dependent proteins [3]. Approximately 2 grams of iron is stored by adult men in proteins such as ferritin and hemosiderin, and somewhat less by women of childbearing age. Both the functional and stored iron pools are maintained by the balance between intestinal absorption of dietary iron and iron losses via the gastrointestinal tract, loss of blood (e.g., menstruation), sweat, skin, or urine [4,5].

The absorption of dietary iron is regulated by physiological factors such as bioactive hepcidin peptide, iron importer divalent metal transporter 1 (DMT1), and the cellular iron exporter ferroportin (IREG1) [6]. The molecular target of hepcidin is IREG1, which supplies iron to plasma from duodenal

enterocytes engaged in dietary iron adsorption, from macrophages of the spleen and liver which recycle old red blood cells, and from hepatocytes involved in iron storage [7,8]. The interaction of hepcidin and IREG1 results in the internalization and degradation of the ligand-receptor complex, which decreases the delivery of iron to plasma. DMT1 is the most important apical uptake transporter of inorganic iron in enterocytes, which import ferrous iron. DMT1 is essential for normal iron homeostasis [6].

Iron deficiency is the most common nutritional disorder, affecting people of all ages worldwide, and mammals in general [9,10]. According to the WHO, nutritional iron deficiency affects 1.5–2 billion people worldwide [11,12]. Iron deficiency is associated with diminished work productivity, lower immunity, and impaired cognitive development. Iron deficiency can be caused by inadequate iron intake, compromised bioavailability, and increased iron losses, or a combination of these. The predominant dietary form of iron is non-heme iron, which accounts for 70%–90% of dietary iron intake in the world, with higher intake in the developing world than in developed countries [13]. Non-heme iron absorption is in the range of 5%–15%. The two major dietary inhibitors of absorption are phytates and polyphenols [14]. The most significant enhancer of iron bioavailability is ascorbic acid, which both reduces and chelates iron, rendering it soluble and available for absorption in the gut [15].

Iron deficiency can be treated using oral iron supplements, such as ferrous sulfate, ferrous gluconate, and ferrous fumarate [16]. However, inorganic iron supplements have limitations, including poor bioavailability and potential adverse effects in the gastrointestinal tract, primarily due to the oxidative toxicity of ferrous iron [17]. New types of iron supplements with minimal side effects and dietary sources with high absorption efficiency, such as fortified foods, are therefore needed. Food fortification is considered the most cost-effective population-based strategy in the long-term to combat micronutrient malnutrition, effective with no or fewer side effects than supplementation [18].

This study set out to investigate the effects of two experimental breads—each fortified with ferrous sulphate, either encapsulated or not—on iron status indicators, with piglets used as models. Iron encapsulation was chosen because of its potential to help overcome unwanted sensory changes in fortified food, reduce interactions of Fe with food components, and increase Fe bioavailability [19,20]. Swine provide possibly the best preclinical animal model for investigating the gastrointestinal system and its functions [21,22]. New piglet models are constantly emerging [23]. Iron deficiency is extremely common in pigs, and is considered to be a para-physiological condition by zootechnical standards. Indeed, it is mandatory to supplement piglets with exogenous iron within few days of birth, in order to compensate for poor iron storage by the mothers and the immaturity of the piglets' absorption mechanisms [24]. This species is therefore extremely useful for use in preclinical trials concerning iron deficiency and food supplementation.

2. Material and Methods

2.1. Experimental Breads and Diet of Piglets

The control bread (CB) and iron fortified breads (FeMB and FeSB) were made according to a procedure developed and optimized at the University of Bologna (UNIBO). The breadmaking process was based on dried compressed yeast (Lesaffre, San Quirico, Italy). The dough was fermented for 45 min at a controlled temperature (30 °C) and then portioned. The pieces were fermented for another 45 min under the same conditions, then baked at 195 °C for 45 min. All samples were obtained from the same batch.

The fortified bread was made from an iron fortified mix of white wheat flour (type 650) and encapsulated ferrous sulphate supplied by EPSA (Valencia, Spain). The experimental breads were produced in the form of sandwich loafs, sliced, and fresh frozen. Each morning, a small aliquot was thawed and given to the animals after crumbling.

Analysis of the humidity, fat content, and content of soluble sucrose/saccharose in the breads was performed according to Polish standard norm ref. no PN-A-74108:1996, Bakery products—testing

methods. Protein content was calculated based on nitrogen content, determined using the Kjeldahl method (AOAC 950.36-1950, Protein in bread). The ash residue of the flour was measured using AOAC Official method 923.03. Total dietary fiber was quantified using an assay kit (Sigma, St. Louis, MO, USA).

A low iron swine diet (LISD), providing the lowest possible amount of iron, was designed for the purposes of the experiment. The standard commercial swine feed could not be used as the basal diet, due to its iron content equivalent to dietary requirements. The LISD was composed of corn flour (40%), barley flour (10%), barley flakes (20%), soybean flour (20%), and bran (10%). The iron content .in this diet formula was one tenth of that found in standard commercial swine feed.

2.2. Animals

Twenty-four commercial hybrid (Large White × Landrace × Duroc) piglets, born within the ASA Unit (DIMEVET, UNIBO), were enrolled in the study. Twelve of the animals were born of one sow, and the other 12 to two different sows 4 days later. All of the sows were inseminated using artificial insemination doses from the same boar, in an attempt to standardize the piglets as much as possible. The animals were monitored daily to assess their clinical condition. All of the activities performed on the animals were approved by the Local Ethics Committee and the Italian Ministry of Health (Legislative Decree 116/92, protocol number 10-75-2013).

2.3. Trial Design and Feeding Protocol

Five days after birth (P5), the animals were randomly assigned to four groups (six piglets in each group, $n = 6$). Only one of the four groups received regular intramuscular (IM) supplementation with 1 mL iron dextran (Endofer, Fatro IT, 100 mg·iron·mL^{-1}). Groups are represented in Table 1. The experiment was divided into two identical blocks to guarantee better management of the animals and to ensure that they were of the same age at the starting point. During their first 14 days of life, the piglets were suckled on sow milk only. During the last phase of lactation (P14), LISD was placed in the mangers of the farrowing crates to accustom the animals to solid feed. The same diet was then left in quantities sufficient for the piglets to eat ad libitum throughout the rest of the trial.

Table 1. Experimental groups.

Treatments	Piglets Group			
	FeMB-G1	**FeSB-G2**	**CB-G3**	**CB-G4**
Intramuscular iron supplementation	No	No	No	Yes
Administered Bread	Microencapsulated Iron Bread	Non-Microencapsulated Iron Bread	Control Bread	Control Bread

In order to start training the piglets to eat the experimental breads, control bread (CB) was added to the LISD until weaning. After weaning (P28), the piglets were individually fed with 50 g of CB each day. Starting on day 44 (P44), the animals were individually fed 100 g of either the control or fortified bread, depending on the experimental group, for 7 days until the end of the trial (P51).

Two groups were fed with iron fortified experimental breads. One group was fed microencapsulated iron bread (FeMB-G1), the other was fed bread containing the same quantity of iron sulphate, starch and citric acid (FeSB-G2). The remaining two groups were the control groups. One (CB-G3) ate control bread, while the other (CB-G4) ate control bread and was given IM iron supplements from P5.

To guarantee that the piglets ingested the required quantity of bread, it was mixed with water (100 g of bread, 130 mL^{-1} water) and fed to the piglets through a 50 mL syringe connected to a silicon tube, which the animals were given to suck. Vanilla extract was added to improve the flavor.

The weight of individual piglets was monitored periodically (P5, P28, P44, and P51) using a livestock scale (CO.BA di Melloni Giuseppe e C. SNC, Pieve di Cento IT).

2.4. Sampling Protocol

Blood samples were collected at P5, P44, and P51. Fecal samples were collected from the rectum at P44 and P51. Urine and tissue samples were collected only at P51, upon euthanasia.

Blood samples were collected under general anesthesia via the femoral artery, using a 21G butterfly needle and a vacuum system. Tubes with K3EDTA anticoagulant and clot activator were used (Venosafe®, Terumo, Leuven, Belgium). Samples in K3EDTA for a complete blood count (CBC), and serum (obtained by 1000 $g \times 10$ min centrifugation) for biochemical profiling, were analyzed by the Veterinary Clinical Pathology Service of DIMEVET (UNIBO).

At the end of the trial, the animals were sedated by an intramuscular (IM) injection of Tiletamine-Zolazepam (Zoletil, Virbac, Praga) (5 mg·kg^{-1}). General anesthesia was induced 10 min later, using an intravenous (IV) bolus of Thiopental sodium (Pentothal, MSD Animal Health s.r.l.) (10 mg·kg^{-1}). After blood and urine had been collected, the animals were euthanized by an IV bolus of Tanax (0.3 mL·kg^{-1}, Intervet, Milano, Italy) for extensive sampling.

Immediately after euthanasia, the digestive tract was removed and separated into its anatomical parts. Mucosal scraping of the stomach wall (lower body part, near pyloric antrum) was performed using a glass microscope slide, and the mucosa was flash frozen in liquid nitrogen. A 25 cm long segment of the small intestine (beginning ~10 cm distal to the pyloric sphincter) was removed, cut open longitudinally, and rinsed with PBS [25]. Duodenal scrapings from the first 10 cm of exposed mucosa were taken and the tissue was immediately frozen in liquid nitrogen for gene expression analysis. The second 15 cm of mucosa was scraped and frozen for total iron content analysis. Samples of organs (liver, heart, spleen, and kidney) were in part conserved for gene expression analysis using RNAlater (Ambion, ThermoFisher, Waltham, MA, USA) and in part flash frozen in liquid nitrogen for iron determination. Stomach contents, feces, and urine were collected in sterile tubes, frozen immediately, and stored at −20 °C.

2.5. Hematological Tests

CBC was performed using an automated hematology analyzer (ADVIA 2120, Siemens Healthcare Diagnostics, Tarrytown, NY, USA). All of the classic hematological variables were analyzed, along with relatively new platelet and reticulocyte indices.

Biochemical analyses were performed on an automated chemistry analyzer (Olympus AU 400, Beckman Coulter/Olympus, Brea, CA, USA), including analyses of total iron (TI) and unsaturated iron binding capacity (UIBC). TI and UIBC, analyzed using a colorimetric method, were used to further calculate the total iron binding capacity (TIBC) and the TIBC saturation percentage (TSAT).

2.6. Total Iron and Fe(II)

The concentration of iron in wet-digested samples of organs (liver, spleen, heart, and kidney), whole blood, diets, and feces was determined using atomic absorption spectrophotometry. The Standard Berghof (Berghof Products + Instruments GmbH Labor Technik, Eningen, Germany) method for microwave digestion of wheat was applied to the gastric content and whole blood samples. Briefly, 1 g or 0.5 mL (blood) samples were digested in 10 mL of concentrated nitric acid and 2 mL hydrogen peroxide using a three-step digestion program, with the temperature raised to 170 °C and a total digestion time of 25 min. For the intestinal mucosa samples, the volume of acid and hydrogen peroxide was reduced by half. Before digestion, the samples of gastric content were homogenized using a Glas Col GKH Control Stirrer Mixer.

The digested samples were diluted with deionized water and the iron content was determined by AAS using a GBC 932 spectrophotometer (GBC Scientific Equipment Pty Ltd., Braeside, Australia), with a hollow cathode lamp for iron, at 248.3 nm. Acetylene and air flow were fine-tuned daily.

Standard curves were prepared daily by diluting iron standard reference materials (1000 μg·cm^{-3}; JT Baker®Chemicals, Phillipsburg, NJ, USA) with deionized water to concentrations of between 0 and 10 μg·cm^{-3}. Certified reference material, whole blood L-1 (Seronorm™ Trace Elements Serum L-1), was used to test the accuracy of the methods. Iron recovery from whole blood was 97%. During analysis, precautions were taken to avoid contamination of the iron samples. A ceramic cutter was used. The glassware was washed in 10% HCl and rinsed three times in ultrapure water.

The presence of Fe(II) was determined through the reaction of formation blue coordinating compound, formed by chelation of Fe(II) with Ferene S reagent (3-(2-pyridyl)-5,6-difurylsulfonic acid-1,2,4-triazine sodium salt) under acidic conditions [26].

2.7. RNA Isolation and Quantitative Real Time PCR (qPCR) for HAMP, IREG1, and DMT1

Total RNA was isolated from liver and duodenal mucosa samples using a NucleoSpin®RNA Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany). One microgram of total high quality RNA (A260/A280 ratio above 2.0) was reverse-transcribed in cDNA using an iScript cDNA Synthesis Kit (Bio-Rad Laboratories Inc., Hercules, CA, USA), with a final volume of 20 μL. Real-time quantitative PCR was carried out using a CFX 96 Real Time System (Bio-Rad) and iTaq Universal SYBR Green Supermix (Bio-Rad). All samples were analyzed in duplicate (10 μL/well). The amplification reaction (20 μL) contained 10 μL of iTaq Universal SYBR Green Supermix, 0.8 μL of each forward and reverse primer (5 μM), and 2 μL of cDNA. All samples were performed in duplicate. Controls lacking a cDNA template were included to determine the specificity of target amplification. The real-time program included an initial denaturation period of 1 min 30 s at 95 °C, 40 cycles at 95 °C for 15 s, and 60 °C for 30 s, followed by a melting step with ramping from 55 °C to 95 °C at a rate of 0.5 °C/10 s. The specificity of the amplified PCR products was confirmed by melting curve analysis and agarose gel electrophoresis. The expression of the hepcidin gene (HAMP, hepcidin antimicrobial peptide) was evaluated on cDNA derived from liver tissue, while the gene expression of iron transporters (IREG1 and DMT1) was assessed on cDNA derived from duodenum. The swine primer sequences are reported in Table 2.

The relative expressions of the studied genes were normalized based on the geometric mean of two reference genes (GAPDH, glyceraldehyde-3-phosphate dehydrogenase and RPL35 ribosomal protein L35). The relative mRNA expression of the tested genes was evaluated in relation to the control group (CB-G4) using the $2^{-\Delta\Delta Ct}$ method [27].

Table 2. Primer sequences used for quantitative real-time polymerase chain reaction analysis.

Gene	Sequence (5′-3′)	PCR Product Length (bp)	Gene Bank Accession Number	Reference
HAMP	For: TCTCCCATCCCAGACAAGAC Rev: AAGATGCAGATGGGGAAGTG	123	NM_214117	Hansen et al., 2009 [25]
IREG1	For: CTCTATGGAAACAGCCTTCTC Rev: AGGATGACCGAAACATTCTG	158	XM_003359590.1	Present study
DMT1	For: AGGATCTAGGGCATGTGGTG Rev: CCACAGTCCAGGAAGGACAT	124	NM_001128440.1	Present study
GAPDH	For: TGGTGAAGGTCGGAGTGAAC Rev: TGTAGTGGAGGTCAATGAAGGG	120	AF017079	Dall'Aglio et al., 2011 [28]
RPL35	For: AACCAGACCCAGAAAGAGAAC Rev: TTCCGCTGCTGCTTCTTG	145	NM_214326	Alexander et al., 2012 [29]

2.8. Ferritin in Plasma

The specific Pig Ferritin Sandwich ELISA (Cod. LS-F6483, LifeSpan BioScience, Inc., Seattle, WA, USA) was used for the ferritin assay. Plasma ferritin concentrations were extrapolated from a standard curve, as described in the manufacturer's protocol.

2.9. Metabolomics of Feces

Fecal samples were prepared for proton nuclear magnetic resonance spectroscopy (^{1}H-NMR) analysis by vortex mixing 80 mg of stool for 5 min with 1 mL of deionized water, followed by centrifugation, for 15 min at 18,630 g and 4 °C. Seven hundred microliters of supernatant was added to 100 µL of a 10 mM solution of 3-(trimethylsilyl)-propionic-2,2,3,3-d4 acid sodium salt (TSP) in D_2O, buffered at pH 7.00 with 1 M phosphate buffer. Immediately before analysis, the samples were centrifuged again. ^{1}H-NMR spectra were recorded at 298 K using an AVANCE III spectrometer (Bruker, Milan, Italy) operating at 600.13 MHz.

The HOD residual signal was suppressed by applying the first increment of the NOESY pulse sequence and a spoil gradient [30]. The NOESYGPPR1D sequence, part of the standard pulse sequence library, was used. Each spectrum was acquired by summing up 256 transients using 32 K data points over 7211.54 Hz (for an acquisition time of 2.27 s). To apply NMR as a quantitative technique, the recycle delay was set to 5 s, taking into consideration the longitudinal relaxation time of the protons [31]. The signals were assigned by comparing their chemical shift and multiplicity with the Human Metabolome Database and Chenomx software data bank (Chenomx Inc., Edmonton, AB, Canada, ver 8.1) [32].

The ^{1}H-NMR spectra were pre-processed for quantitative analysis following a method already described in literature [30]. Briefly, the spectra were baseline-adjusted by means of simultaneous peak detection and a baseline correction algorithm (SPDBC) implemented in the baseline R package [33]. The spectra were corrected for errors in chemical shift misalignments using an in-house modified version of Correlation Optimized Shifting (i-Coshift) [34].

2.10. Statistical Data Analysis

The data were analyzed using a Student's *t*-test and one-way analysis of variance (ANOVA), followed by a Tukey post hoc comparison test (SPSS program version 13.0; SPSS Inc., Chicago, IL, USA). Differences of at least $p \leq 0.05$ were considered significant. Iron concentration underwent statistical analysis in R computational language. In each group, data were considered as probable outliers when they exceeded by 1.5 times the 0.25–0.75 percentile range. Metabolomics data were analyzed in R, with sparse principal component analysis (sPCA) performed using a mixOmics package, applying the sparsity principle as detailed by Shen and Huang [35].

3. Results and Discussion

3.1. Experimental Bread

Three types of experimental bread were prepared, using conventional yeast fermentation: microencapsulated iron fortified bread (FeMB), ferrous sulphate fortified bread (FeSB), and control bread (CB) without any iron supplementation. The formula of the FeSB was based on that of FeMB, but instead of microencapsulated iron an equivalent amount of modified starch, ascorbic acid, and ferrous sulphate was used, in the form of individual compounds. The content of iron in the fortified breads was 21 mg Fe 100 g^{-1}·WM (Wet Mass), ten times higher than that in the control bread. To meet WHO recommendations for cereal flour fortification, ferrous sulphate was selected as the iron source [36]. The presence of ferrous ions in the final experimental breads was determined, as ferrous ions are easily oxidized to ferric ions, especially under the conditions used in the baking process (high temperature and humidity), which may accelerate the reaction. The presence of Fe(II) in the experimental breads was confirmed in a reaction with Ferene S. During the trial period, the daily iron intake of each piglet was 21 mg. This dose complies with experimental findings and recommendations. According to the National Research Council's current guidelines, neonatal pigs require 7 to 16 mg of iron per day for normal growth [37]. Table 3 presents the composition of the experimental breads.

Table 3. Component analysis of breads administered to the pigs. All values are given in respect to the fresh bread mass. (FeMB: bread with microencapsulated iron; FeSB: bread with free iron; CB: control bread).

Sample/Breads	FeMB	FeSB	CB
Humidity (g 100 g^{-1})	38.90 ± 0.83	38.11 ± 0.19	41.27 ± 0.77
Proteins (g 100 g^{-1})	7.62 ± 0.53	8.10 ± 0.57	7.55 ± 0.53
Fat (g 100 g^{-1})	1.76 ± 0.20	1.43 ± 0.05	1.78 ± 0.42
Carbohydrates			
Total (g 100 g^{-1})	45.59	46.56	44.01
Soluble (Sucrose/Saccharose) (g 100 g^{-1})	1.35	1.00	0.69
Insoluble (fiber) (g 100 g^{-1})	1.61	1.61	1.61
Ash (g 100 g^{-1})	1.71 ± 0.04	1.73 ± 0.04	1.62 ± 0.05
NaCl (g 100 g^{-1})	1.47	1.47	1.47
Total Fe (mg 100 g^{-1})	21.10 ± 0.82	21.20 ± 0.63	2.80 ± 0.20

3.2. Body Weight and Blood Analyses

The body weights of the piglets and their main blood parameters are reported in Table 4.

Table 4. Effect of bread administration on growth performance and blood parameters. Value are means ± SD. Means with different superscripts differ $p < 0.05$. Capital superscripts represent differences within the same group between P44 and P51. Lowercase superscript represent differences between the groups at the same experimental time. (BW: body weight; HCT: hematocrit; Hb: hemoglobin; RBC: red blood cells; WBC: white blood cells; RET: reticulocytes; GGT: gamma glutamyltransferase; TIBC: total iron binding capacity; UIBC: unsaturated iron binding capacity; TSAT: TIBC saturation percentage).

Intramuscular Iron Supplementation		Piglets Group			
		FeMB-G1	FeSB-G2	CB-G3	CB-G4
		no	no	no	yes
Body weight (kg)	P44	6.9 ± 1.33 ^{a}	7.2 ± 1.5 ^{a}	7,6 ± 1.0 ^{a}	10.5 ± 1.5 ^{b}
	P51	6.8 ± 1.1 ^{a}	6.3 ± 1.4 ^{a}	7.0 ± 1.6 ^{a}	10.5 ± 1.9 ^{b}
Hb (g/dL)	P44	6.1 ± 2.0 ^{aA}	6.0 ± 2.5 ^{aA}	5.3 ± 1.5 ^{a}	8.8 ± 1.7 ^{b}
	P51	7.2 ± 2.2 ^{abB}	8.0 ± 2.5 ^{abB}	6.3 ± 2.4 ^{a}	9.3 ± 1.7 ^{b}
HCT (%)	P44	21.7 ± 5.6 ^{a}	21.1 ± 6.5 ^{aA}	20.2 ± 4.8 ^{a}	29.7 ± 3.6 ^{b}
	P51	23.5 ± 5.5 ^{ab}	25.5 ± 6.7 ^{abB}	21.7 ± 5.9 ^{a}	29.1 ± 4.1 ^{b}
RBC (10^6/mm^3)	P44	5.10 ± 0.9 ^{a}	4.59 ± 1.18 ^{aA}	5.26 ± 1.68 ^{a}	7.37 ± 0.86 ^{b}
	P51	5.8 ± 0.9 ^{a}	5.60 ± 1.12 ^{aB}	5.78 ± 1.70 ^{a}	7.27 ± 0.40 ^{b}
WBC (10^3/mm^3)	P44	14.3 ± 2.2	11.5 ± 3.4	11.5 ± 1.6	17.5 ± 4.1
	P51	10.3 ± 3	8.1 ± 3.9	8.2 ± 2.5	14.1 ± 4.6
RET (10^3/mm^3)	P44	415.5 ± 189.2	634.1 ± 171.7	503.3 ± 64.5	299.1 ± 93.7
	P51	86.6 ± 57.9	112.4 ± 48.9	95.8 ± 28.5	58.6 ± 18.3
Glucose (mg/dL)	P44	107.3 ± 17.2	104.5 ± 9	112.5 ± 15.7	104 ± 7.3
	P51	162.2 ± 33.2	136.3 ± 26.9	150.8 ± 52.1	145.7 ± 27.3
GGT (U/L)	P44	32.5 ± 5.2	30.1 ± 3.5	28.3 ± 5.3	36.9 ± 4.2
	P51	38.4 ± 8.1	37.3 ± 8.3	29.3 ± 4.4	36.8 ± 5.5
Iron (serum) (µg/dL)	P44	97.2 ± 81.5	83.8 ± 59.1	41.8 ± 30.6	85 ± 47.2
	P51	66.3 ± 54.1	69.7 ± 47.8	39.8 ± 44	88.5 ± 42.5
TIBC (µg/dL)	P44	507 ± 51	528.5 ± 79.1	532.5 ± 118.9	455.7 ± 38.1
	P51	402.8 ± 53.5	412.5 ± 60.2	388.3 ± 59.6	399 ± 60.4
UIBC (µg/dL)	P44	409.8 ± 75.3	444.7 ± 117	490.8 ± 144.1	370.7 ± 76.2
	P51	336.5 ± 84.9	342.8 ± 99.7	348.5 ± 91.8	310.5 ± 101
Iron (whole blood) (mg/L)	P51	229.7 ± 64.2 ^{a}	253.2 ± 65.8 ^{ab}	215.7 ± 63.1 ^{a}	284.3 ± 49.2 ^{b}

Parameters showing statistical differences and their correlations are reported in the upper part of the table. All piglets were of similar weight on day 5 (data not shown) and analysis showed that all of the animals enrolled in the study were healthy. The group that received IM iron supplements subsequently gained significantly more weight than those which did not receive the experimental diet. This might be explained by the fact that animals that received the IM supplementation at 5 days, had more time (from P5 to P44) to restore and correct anemia and balance all of the physiological gastrointestinal mechanisms. Their weight is indeed significantly higher since the beginning of the trial P44. Over the week of the trial, the differences were maintained between the live body weights of the piglets in the positive control group (CB-G4) and those of the piglets in all other groups.

Episodes of diarrhea were reported from the second day of the experimental diet. However, none of the four groups showed a higher incidence of diarrhea than the others. Thomaz et al. [38] report that animals fed diets supplemented with trace minerals in inorganic form suffer higher rates of diarrhea than animals fed diets without mineral supplements. However, this was not confirmed in the present study.

Levels of variables such as hematocrit (HCT), hemoglobin (Hb), and red blood cells (RBC) seem to have been influenced by the kind of bread administered (Table 4). Groups that received iron fortified breads (FeMB-G1; FeSB-G2) showed significant increases in these parameters ($p \leq 0.05$) as compared to piglets fed with the control bread (CB-G3 and CB-G4). At the beginning of the experiment, the FeSB-G2 group was classified as anemic, whereas after the treatment they showed only borderline anemia [39]. The impact of iron microencapsulation on Hb was not as marked as that of unencapsulated ferrous sulfate, although there was no significant difference between the treatments ($p > 0.05$).

No changes in levels of leukocytes were observed in any of the groups over the course of the experiment. Clinical chemistry analyses did not reveal significant variations at different times or between groups, proving that the animals were healthy. CB-G4 showed the highest concentrations of Gamma-glutamyl transferase (GGT) at P44. By the end of the trial, GGT had increased in the groups fed with iron fortified breads (FeMB-G1 and FeSB-G2), reaching or exceeding the level observed previously for CB-G4, while it remained stable in group CB-G3 (the lowest noted concentration). Therefore, this parameter seems to be directly related to iron supplementation, since it increased after both IM and oral administration. In all of the groups, the glucose concentration in the blood was higher on day 51 than on day 44. This is related to the fact that the animals were fed 1 hour before euthanasia in order to collect gastric content. The iron content in serum varied widely between animals. Despite this large individual variation, it can be noted that the iron levels in orally supplemented groups (FeMB-G1 and FeSB-G2) decreased, while they remained stable in groups fed with control bread (CB-G3 and CB-G4). Our hypothesis is that the increase in nutritional iron availability may stimulate iron storage or tissue-binding mechanisms, therefore lowering the UIBC (Unsaturated Iron Binding Capacity) and TIBC (Total Iron Binding Capacity). TSAT (TIBC Saturation Percentage) appears to have remained stable, with minimal variations.

CB-G4 did not show large differences for the main indices of hematopoiesis and anemia. The probable reason is that the diet used (LISD) was designed to have a low iron content. Whereas, a normal farm practice is IM supplementation and the use of specific iron-rich diets.

Overall, all of the blood parameters fell within the physiological reference intervals available in the literature for piglets. Therefore, despite some differences between the groups at P44 and P51, the trial did not determine any dangerous or important alterations in the animals, which remained healthy throughout the trial [40].

3.3. Relative Expression of HAMP, IREG1, and DMT1

The effects of the different bread types on mRNA of hepcidin (HAMP) in the liver, and on IREG1 and DMT1 at the duodenal level, did not differ in a statistically significant manner (Figure 1). Moreover, the gene expression of HAMP calculated with reference to CB-G4 shows a progressively decreasing trend, starting from the group of anemic piglets fed bread with microencapsulated iron (FeMB-G1),

followed by the group given non-encapsulated iron bread FeSB-G2 and finally the anemic group, CB-G3, which was fed with control bread (Figure 1A). This trend, showing a slight up-regulation in groups with iron fortified bread in respect to the anemic group, can be explained by the fact that iron is the most important proven biological factor for inducing hepcidin expression, while there is also evidence that both iron saturation of plasma and hepatic iron loading stimulate hepcidin synthesis [25,29].

The relative gene expression of IREG1 (Figure 1B) and DMT1 (Figure 1C) in relation to the group with encapsulated iron supplementation (CB-G4) showed a relatively higher value for piglets in FeSB-G2. The increasing trend in intestinal DMT1 mRNA (Figure 1C) for piglets fed with FeMB-G1 and FeSB-G2 is likely due to the animals' anemic status and increased need for dietary Fe to meet erythropoietic demands. The increasing trend in IREG1 expression observed for animals fed with FeMB-G1 and FeSB-G2 as compared with CB-G4 corresponds to the opposite trend observed for HAMP expression. Hepcidin binds to IREG1, causing internalization and degradation of the protein and decreasing cellular Fe export [7,41].

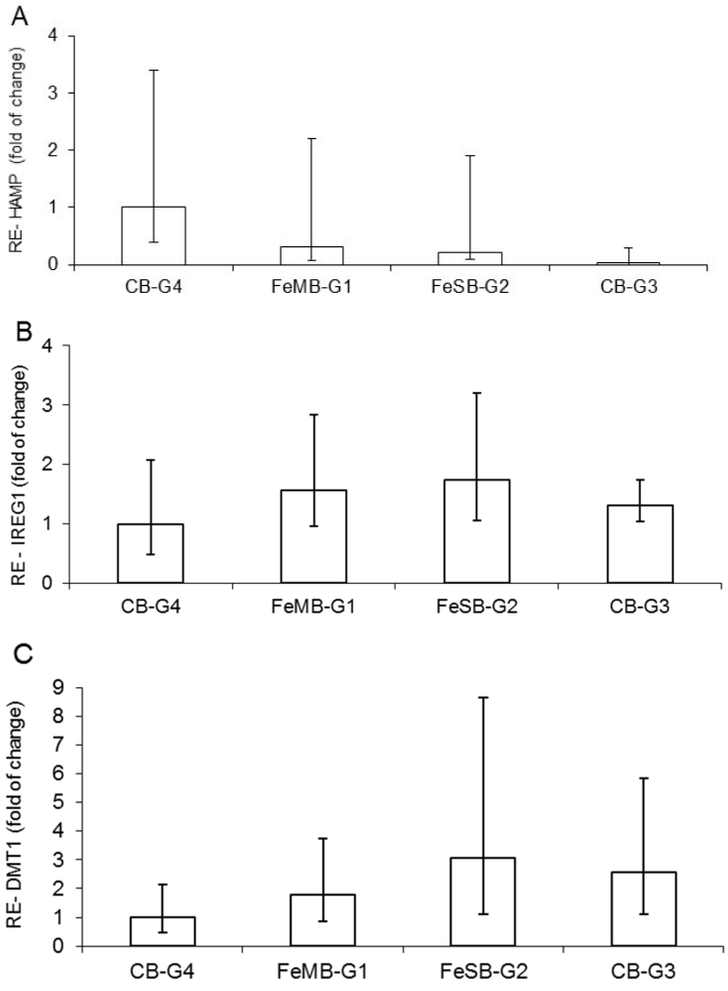

Figure 1. Effect of different breads on relative expression (RE) of hepcidin (HAMP) mRNA in liver (**A**); ferroportin (IREG1) (**B**); and Fe importer divalent metal transporter 1 (DMT1) mRNA in duodenum (**C**) determined by qPCR. Data are expressed as fold changes with respect to CB-G4. FeMB-G1: no iron supplementation/Microencapsulated Fe Fortified bread; FeSB-G2: no iron supplementation/Free Fe Fortified bread; CB-G3: no iron supplementation/control bread; CB-G4: iron supplementation/control bread.

3.4. Ferritin Determination

Although ferritin is mainly found as a cytosolic protein, small amounts of ferritin are secreted into serum, in amounts closely reflecting the size of the body's iron stores [42,43]. Plasmatic ferritin can be used to identify differences within the physiological range, reflecting non-heme iron levels in the liver and spleen more than other blood values [44].

The effects of consuming the different types of bread on the piglets' plasmatic levels of ferritin are presented in Figure 2. There were no statistically significant differences between the groups in terms of the amounts of ferritin detected in the plasma samples using a specific ELISA kit, although the group fed with FeMB-G1 had the highest ferritin levels. This lack of significant differences may be due to the short duration of the experiment.

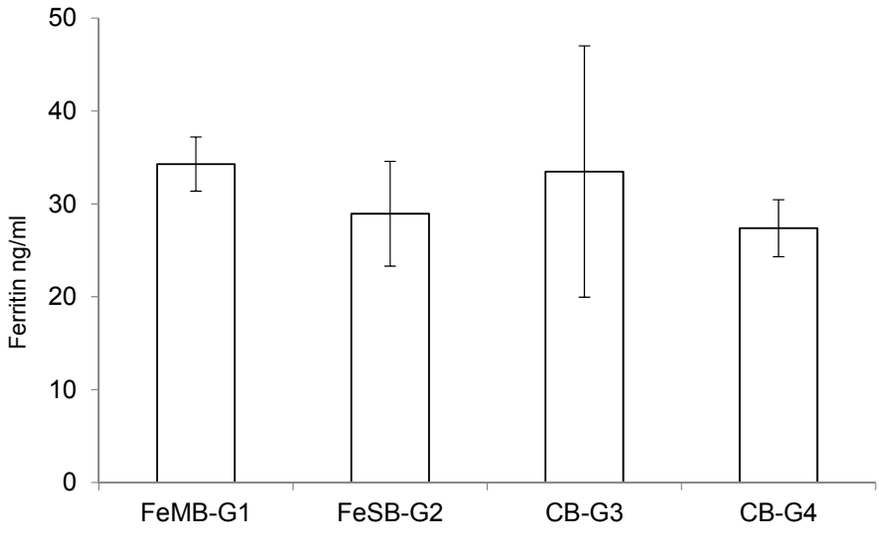

Figure 2. Effect of consuming different breads on plasmatic levels of ferritin in piglets.

3.5. Iron Absorption and Storage

The total iron content in the intestinal mucosa and internal organs after 51 days of life is shown in Table 5. The iron content in the intestines of the animals which did not receive iron supplementation was 18.4 ± 4.6 mg·Fe·kg^{-1}. This was significantly lower ($p < 0.05$) than in CB-G4. In contrast, the iron content in the intestinal mucosa of the animals that received either oral or IM iron supplementation ranged from 25.0 to 28.0 (mg/kg·WM), and did not differ from the CB-G4 group ($p > 0.05$). Iron content in the intestinal mucosa is known to be controlled tightly by double regulation [3]. The first regulatory mechanism controls the passage of iron into intestinal mucosal absorptive cells (enterocytes). Iron transfer from the basal surface into the blood is then regulated according to the body's needs at the time. Excess iron entering the mucosal cells can be incorporated into ferritin, stored in the intestinal cells for 2–3 days, and finally lost by exfoliation and apoptosis of the cell [37]. The iron levels of the piglets fed with iron fortified breads were close to iron adequate status. This suggests that iron absorbed from lumen was not stored in the epithelial cell, but uptaken by transferrin and transported systemically to the body.

Table 5. Effects of dietary iron supplementation in the form of iron fortified bread and intramuscular injection on iron concentration (mg·kg^{-1}·WM) in organs of post-weanling piglets. Values are expressed as means ± SD; Values in the same row with different superscripts differ significantly $p < 0.05$ ($n = 6$).

Samples		Piglets Group			
		FeMB-G1	**FeSB-G2**	**CB-G3**	**CB-G4**
Intestinal mucosa	P51	25.0 ± 8.1 ^{a}	28.0 ± 11.5 ^{a}	18.4 ± 4.6 ^{b}	27.2 ± 4.4 ^{a}
Liver	P51	29.0 ± 7.6 ^{b}	30.3 ± 10.1 ^{b}	20.0 ± 6.9 ^{c}	56.2 ± 24.7 ^{a}
Heart	P51	33.0 ± 9.9 ^{b}	35.4 ± 8.1 ^{b}	26.3 ± 8.4 ^{c}	46.0 ± 12.4 ^{a}
Spleen	P51	9.4 ± 2.0 ^{ab}	10.1 ± 1.2 ^{ab}	8.3 ± 1.6 ^{b}	10.5 ± 2.4 ^{a}
kidney	P51	28.2 ± 8.6 ^{ab}	27.3 ± 8.0 ^{ab}	24.5 ± 7.3 ^{b}	33.0 ± 8.6 ^{a}
Feces	P44	130.4 ± 39.7 ^{a}	84.2 ± 17.2 ^{a}	98.2 ± 31.3 ^{a}	119.9 ± 27.3 ^{a}
	P51	324.4 ± 129.5 ^{b}	168.8 ± 83.7 ^{ab}	58.5 ± 18.5 ^{a}	139.2 ± 92.4 ^{a}

Oral supplementation with iron in FeMB or FeSB forms resulted in a statistically significant increase in the iron content of the liver, by up to 51.6% and 53.9%, respectively, with respect to the CB-G3 group. The concentration of Fe showed a clear tendency to increase in orally supplemented groups compared to CB-G3, confirming the positive effect of this Fe source on absorption and storage in the body.

To understand the extent to which oral supplementation moved iron content away from typical levels for anemic non-supplemented piglets, we calculated the differences shown by each supplemented piglet from those of the piglets in groups CB-G3 and CB-G4. We then set up a paired comparison between the distances, using Welch's *t*-test. [45] The supplemented piglets, independent of the kind of supplementation, showed an intermediate iron content in relation to the CB-G3 and CB-G4 groups in each of the studied body organs with the exception of liver, where the iron content was closer to that of CB-G3.

Piglets given increased daily iron intake in the form of FeMB-1 or FeSB-2 showed significantly higher iron content in their hearts in comparison with CB-G3 ($p < 0.05$), but did not reach the level observed in piglets given IM supplementation from day 5 of life. In both orally supplemented groups, iron accumulation in the heart and liver, the organs most commonly affected by iron overload, remained below the values observed in CB-G4. At the end of the intervention trial, there were no statistical differences in terms of iron content in the spleens of supplemented animals, where iron content reached similar levels of around 10 mg·g^{-1}. The iron content in the spleens of animals not given supplementation was lower, although the increase caused by one week of oral supplementation was not statistically significant.

A comparison of the iron content in feces from individual animals showed a broad range of variation. In the feces of the FeMB-1 group, iron content varied from 94 to 180 mg/kg on 44 day and from 282 to 485 mg/kg on day 51. However, with increased iron intake there was an up to twofold increase in excretion. At the same time, in the CB-G3 iron excretion decreased by half.

Pigs fed with $FeSO_4$ or microencapsulated $FeSO_4$ had higher iron concentrations in the heart, liver, and intestinal mucosa compared with iron-deficient pigs, indicating that iron supplementation was effective at improving the iron status of the tested organs. These results are in agreement with those obtained by Fang et al., [46] who observed that 10 days of supplementation in the form of ferrous sulfate or iron glycine chelate increased iron content in the heart, liver, and lungs of iron-deficient piglets.

3.6. Fecal Metabolome

To provide an overview of the changes that occurred over the course of the experiment in the intestinal environments of piglets in different test groups, the metabolome of feces collected at the beginning and at the end of the experiment was investigated by means of high-resolution proton magnetic resonance spectroscopy (^{1}H-NMR) and multivariate analysis. In several related studies, these methods have been found optimal for following the possible consequences of inflammation

caused by exogenous agents [47–49]. Sixty-six molecules were quantified, mainly pertaining to amino acids, short chain fatty acids and organic acids. Fifteen other signals were detected, pertaining to different unknown molecules. The underlying structure of the data was represented in a multidimensional space using principal component analysis (Figure 3). The score plot highlighted the differences between the samples, while the loading plot provided a visual representation of the most important molecules that determined those differences. Along the first principal component (PC1), accounting for 23.5% of the total variance, the samples collected at P44 are grouped around negative values, while the samples collected at P51 are grouped around positive values. The linear combination of the molecules giving rise to PC1 therefore provides a superbly concise representation of the growth of the animals. Piglets belonging to group CB-G4 appear at P44 with less negative values than the others, which are closer to CB-G4 animals at P51, as a one tail Wilcox test confirmed. This was not unexpected, as such piglets were anticipated to be characterized by a more balanced physiological condition than those in any other group, with positive consequences for the overall development of the animals. The relative position of samples collected at P51 is remarkably different from the position of samples obtained at P44, as revealed by an ANOVA test. In fact, the CB-G4 samples are flanked by FeMB-G1, which has the highest PC1 values, by FeSB-G2 with intermediate values, and by CB-G3 which has the lowest values.

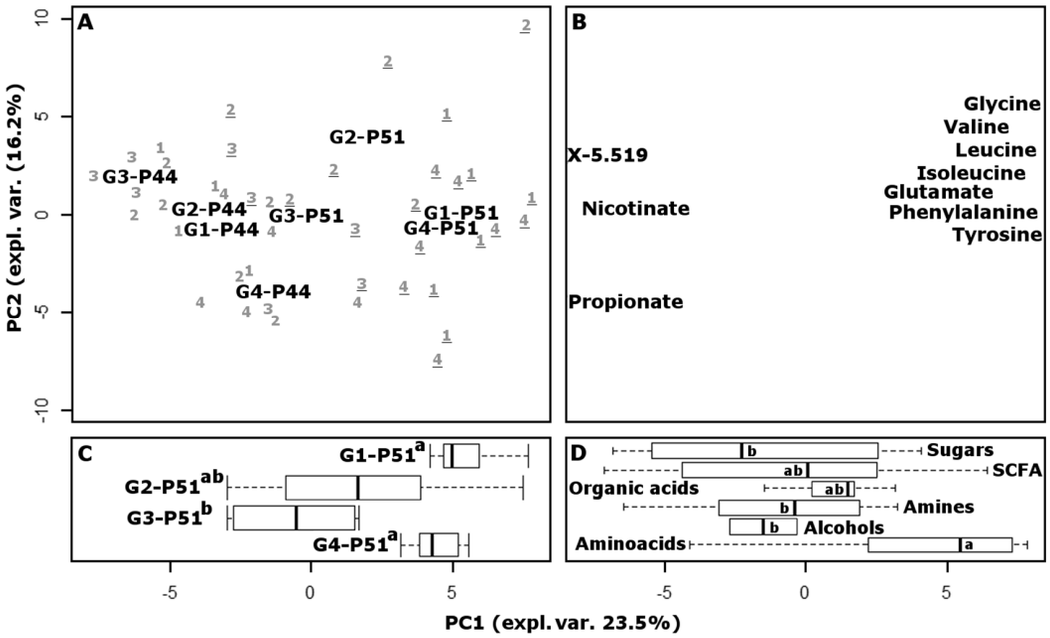

Figure 3. Score plot (**A**) of a PCA analysis of fecal metabolome. To facilitate the visualization of the differences of the samples at P51, their scores along PC1 are summarized by boxplots (**C**); Corresponding loading plot (**B**), in which only the 10 most important variables, as calculated by sPCA, are reported. The 81 loadings are also summarized by the molecular group in panel (**D**). In panels (**C**,**D**), different lowercase letters indicate statistically different groups ($p < 0.05$). (G1, G2, G3, G4 refer respectively to the groups fed with: microencapsulated iron bread (FeMB-G1); bread containing the same quantity of iron sulphate; starch and citric acid (FeSB-G2); control bread (CB-G3), and control bread with IM iron supplements at P5 (CB-G4)).

A recent work on human subjects showed that some iron replacement therapies may cause inflammation accompanied by drastic modification of gut microbiota and metabolome [50]. Prompted by these findings, we investigated the intra-group distances of the piglets at P44 and P51, by considering the Euclidean space constituted by the concentration of all the molecules studied by NMR, scaled to unit variance. FeSB treatment caused a significant ($p < 0.05$) increase in the variability of the metabolome, with the average distance between FeSB-G2 piglets increasing by

175% after iron supplementation. This trend was confirmed when the samples were observed in the principal component space shown in Figure 3, or in the space of intermediate complexity. In contrast, groups FeMB-G1, CB-G3, and CB-G4 showed no statistical differences between P44 and P51, suggesting that iron supplementation through microcapsules had a milder effect on gut microbiota and metabolome than did fortification with iron sulphate.

The loading plot of the PCA model reveals that the metabolic cycles mostly responsible for the highlighted trends involved the production of amino acids and decarboxylation. In fact, amino acids appear significantly more concentrated in samples located around positive PC1 values, while molecules belonging to the chemical group of amines appear more concentrated in samples with negative PC1 values.

4. Conclusions

This study has shown that bread fortified with ferrous sulphate or encapsulated ferrous sulphate provided effective treatment of anemia in piglets. Indeed, after a relatively short treatment time (7 days), animals initially classified as anemic, due to lack of exogenous iron supplementation, showed noticeable signs of improvement regarding hematological indicators of anemia. Fortified bread could therefore also provide a source of bioavailable iron for humans, which should be of interest to the functional foods industry. However, little difference was observed during the seven-day intervention trial between using microencapsulated iron or the same amount of ferrous sulphate. It might be expected that over the course of a longer study iron microencapsulation could obviate the negative effects of free iron supplementation, and this should be the focus of further research.

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Author Contributions: Monica Forni, Andrea Gianotti, and Maria L. Bacci conceived and designed the experiments; Augusta Zannoni and Danielle L. Taneyo Saa prepared the fortified breads; Maria L. Bacci, Francesca Barone, and Domenico Ventrella performed the experiments on the animals and tissue samplings; Monica Forni and Augusta Zannoni performed the gene expression analyses and the ferritin determination; Malgorzata A. Bryszewska performed the total iron and Fe(II) determination analyses; Luca Laghi performed the metabolome analysis and the statistical analyses; Malgorzata A. Bryszewska, Luca Laghi, Augusta Zannoni, and Domenico Ventrella drafted the manuscript which was implemented and approved by all of the co-authors.

Conflicts of Interest: The authors declare no conflict of interest.

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6 *Morpho-functional characterization of the visual pathways in the iodoacetic acid (IAA) pig model*

Photoreceptors degeneration is the major cause of adult blindness in industrialized countries, *retinitis pigmentosa* (RP) and age-related macular degeneration (AMD) are the most common.(Ting et al., 2009; Wright et al., 2010) Typically RP results from a primary defect in rods leading to diminished night vision and progressive narrowing of the visual field as vision becomes totally dependent on cones, but this almost invariably induce a secondary cone loss (rod–cone degeneration) with consequent visual loss up to complete blindness.(Hamel, 2006) AMD is a multifactorial cause of photoreceptor degeneration that is becoming a growing health problem as the segment of the population aged 60 and older will experience a demographic growth.(Smith, 2010; Velez-Montoya et al., 2014) Different animal models have been used to study genotype-phenotype correlation and to evaluate new therapeutic strategies for photoreceptor degeneration. Spontaneous animal model are available for retinal degeneration, but their repertoire is limited and still many models has to be found.(Chader, 2002) Thanks to the great progress in genetic engineering over the last few years an increasing number of transgenic model are available, small animals might be very useful for the earlier phase of investigation, but before embarking on human clinical trials it is imperative to demonstrate both efficacy and safety in a large animal model. The pig is an excellent retinal model as with humans, the swine retina contains a cone-dominant central visual streak with rods enriched in the peripheral retina,(Chandler et al., 1999; Hendrickson and Hicks, 2002; Kostic and Arsenijevic, 2016) and the eye is comparable in size to the humane eye (Prince and Ruskell, 1960) . Different transgenic pig model have been created for studying the cone degeneration,(Petters et al., 1997; Ross et al., 2012) and

macular degeneration.(Sommer et al., 2011; Colella et al., 2014) Despite the clear advantages, those models share slow time course of disease progression and can only features a specific gene defect while the photoreceptor degeneration is a multifactorial disease. This could be a deterrent for their use in many cases when a rapidly inducible swine model of photoreceptor damage would be advantageous. Iodoacetic acid (IAA) was proved to blocks glycolysis and to be toxic to neurons which depend upon this pathway.(Orzalesi et al., 1970; Winkler et al., 2003; Liang et al., 2008) The degeneration of photoreceptors and in particular of the rods in the pig by IAA have been described.(Scott et al., 2011; Wang et al., 2011; Noel et al., 2012) The IAA swine model could be a useful model to study biocompatibility, surgical approach and efficacy of new artificial retinal prosthesis. In the last twenty years indeed many research groups worked on this new rising technology (Zrenner, 2002; Bertschinger et al., 2008; Ghezzi et al., 2013) proving its feasibility in micropigs (Schwahn et al., 2001). The IAA pig model has been described but the lack of information regarding the functionality of the entire visual pathways does not allow its use in this field. Our aim is to characterize the IAA swine model visual pathways to enlarge the use of this low cost and rapidly inducible model.

6.1 Materials and Methods

Animals and study design

The use of animals in this study was regulated by two protocols approved by the Italian Ministry of Health, one with D.Lgs 116/92 and the other with the new low D.Lgs 26/2014.

Eighteen commercial hybrid pig [(Large White x Landrance) x Duroc] were enrolled in the study and stable in multiple boxes according to their origin dominance group to reduce stress and aggressiveness. The animals (male and female, 32 ± 12 Kg bodyweight) were anesthetized, a group was treated with iodoacetic acid (IAA) (IAA group n=11), the other group was injected with saline (Control group n=7). Before and a month after treatment animals were evaluated with a panel of functional and morphological test, full-field and pattern electro-retinography (ffERGs and pERG), Flash Visual Evoked Potential (fVEP) and behavioral tests. At the end of the trial animals were put under general anesthesia and euthanized with 0.3 ml/kg of a compound containing embutramide 200 mg/ml, mebenzonio iodide 50 mg/ml, tetracaine hydrochloride 5 mg/ml (Tanax, Intervet Italia srl, Milan, Italy) for the anatomical and pathological study of the retina and the visual pathways.

Anesthesia protocol and IAA treatment

Animals were weight and sedated with an intramuscular (IM) injection of Tiletamine-Zolazepam (Zoletil, Virbac, Prague) (5mg/Kg). General anesthesia was induced and maintained with sevoflurane (SevoFlo, Esteve, Barcelona, Spain). During anesthesia hematic and biochemical analysis were performed as

previously described.(Ventrella et al., 2017) The IAA group piglets received an intravenous injection, in the auricular vein, of 12 mg/kg body weight of sterile, pH balanced, iodoacetic acid (ICH_2CO_2H) lot n. BCBQ7348V and BCBS2675V (CAS Number: 64-69-7, Sigma Chemical Corp., St Louis, MO), the administration last five minutes. The IAA was freshly prepared the day of the administration with saline solution (NaCl 0,9%) and use at the concentration of 12 mg/ml.

Electrophysiological (ffERG, pERG and fVEP) analysis

The analysis was performed with the animals under general anesthesia, to maintain the eye in a central and stable position atracurium besylate (Tracrium, GlaxoSmithKline, Bredford, United Kingdom) 0.25mg/kg was injected every 30 min. Two drops of oxybuprocaine hydrochloride (Novesina, Visufarma s.r.l. Rome, Italy) were used as a local anesthetic for the corneal surface and a barraquer blepharostat and the electrodes were positioned. Contact lenses electrodes (ERG-jet®, Universo Plastique, Switzerland) as active electrodes during ffERG and pERG and sub dermal needle electrodes as reference near the ipsilateral and the controlateral ear were used. For fVEP a dermal needle was placed close to the occipital protuberance as active electrode. A drop of 3% carbomel (Dacriogel, Alcon, Fort Worth, Texas, USA) was also applied to the inner surface of the lens electrode to protect the cornea and to ensure good electrical contact. The pERG was performed before pupillary dilatation; maximal pupillary dilation was obtained by applying 2 drops of tropicamide phenylephrine hydrochloride (Visumidriatic fenilefrina 10% + 0.5%, Visufarma s.r.l. Rome, Italy) 15 min prior to the beginning of the ffERGs session. The International Society for Clinical Electrophysiology of Vision

(ISCEV) Standards for ffERGs, pERG and fVEP (Bach et al., 2013; McCulloch et al., 2015; Odom et al., 2016) were used with the settings adjusted for the swine species displayed in table 21 A and B. Both eyes were investigated separately and the contralateral eye was covered to reduce contamination. The ffERG stimuli were produce by a Ganzfeld dome and pattern stimuli by a screen connected to a pattern generator. The data were amplified and acquired by WinAverager Software. The Ganzfeld dome, the pattern screen and generator, the amplifier and the Software used were part of the *BM6000-MAXI Electrophysiology Unit* shown in figure 28 (Biomedica Mangoni, Pisa, Italy). Data were acquire as single waves and filtered with OriginPro 9.1. A FFT filter low pass 50 Hz was used for the ffERGs and fVEP waves while pattern ERG were filtered with a band pass 5-20 Hz FFT filter.

Figure 28 ***BM6000-MAXI Electrophysiology Unit***

Table 21. ***A. Stimulus and recording parameters for swine adapted ISCEV Standard ERGs.*** ** Dark-adapted ERGs are recorded sequentially without further dark adaptation. Thus, only the 0.01 ERG is a fully dark-adapted response*

B. Stimulus and recording parameters for swine adapted ISCEV Standard pERG and fVEP

A

ERG test	Adaptation/ background strength and time	Stimulus strength $cd \bullet s \bullet m^{-2}$	Inter stimulus time	Recording bandpass	Main physiological generator(s)
Dark-adapted 0.01 ERG	DA = 20min	0.01	0.49 Hz	1 - Hz - 500	*b-wave*: rod-initiated on pathways
Dark-adapted 3.0 ERG*	DA = 20 min	3	0.1 Hz	1 - Hz - 500	*a-wave*: combined response photoreceptors & post-receptoral on pathways *b-wave*: on & off bipolar cells
Light-adapted 3.0 ERG	30 cd m^{-2} = 10 min	3	1.1 Hz	2 - Hz - 500	*a-wave*: cones with post-receptoral on & off pathways *b-wave*: on & off bipolar cells

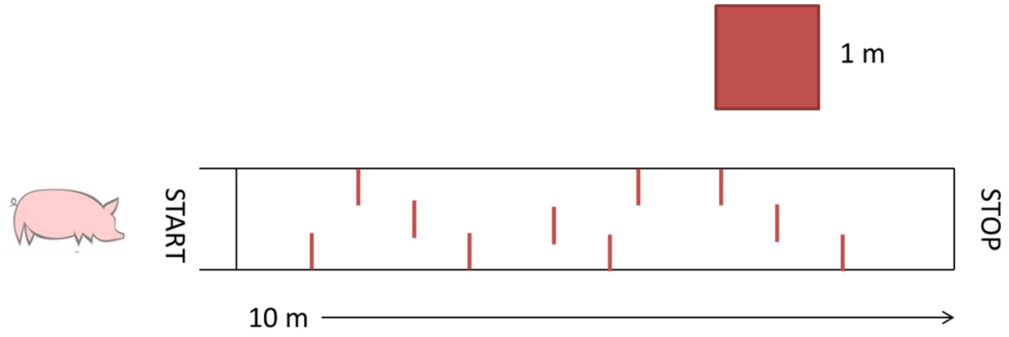
B

Analysis	Stimulus	Luminance	Contrast	Presentation rate	Recording bandpass
Pattern ERG	Vertical square 0.10 C/°	47 cd m^{-2}	96 %	1.98 rps (0.5 Hz)	2 - Hz - 200
Flash VEP	Flash	3 $cd \bullet s \bullet m^{-2}$	-	2 Hz	1 - Hz - 500

Behavioral Vision Assessment

Two week after treatment pigs (IAA group n=11 and C group n=7) were evaluated with a behavior test proposed for cats in 2013 (Nan et al., 2013) with some modifications. The testing course was 10 meter long and 2 meter wide, with 10 obstacles every meter randomly positioned on the right, the left or the center to obtain 4 configurations (Fig. 29). In the morning before food administration the animals were tested with artificial light condition and semidarkness condition. Time to complete the course and number of collision with the obstacles were recorded by two different operators at the same time. The test was performed twice a week for 8 replicates to reduce errors due to smells and noises, to reduce the possibility that the animals where learning the obstacles position, configuration was changed every time the test was performed.

Figure 29. ***Clinical vision evaluation testing course design***



Retinal histology

The eye globes from two C and two IAA animals were enucleated after suppression and immediately put in Ambion™ PBS Phosphate-Buffered Saline (10X) pH 7.4 precooled at 4°C (Fisher Scientific, Waltham, Massachusetts, United States). The eyes were prepared for the shipment to the Department of Biotechnological and Applied Clinical Science, University of L'Aquila, with the following protocol: a cut near to the corneal limbus was performed to expose the posterior chamber, that the eyes were perfixed in 4% paraformaldehyde for 24 hours. At the Department of Biotechnological and Applied Clinical Science the samples were dehydrated in an increasing concentration solution of sucrose (10-20-30%), each step last 24 hours.

The retinas were than orientated cut and stained for the immunohistochemistry analyzes with the protocols of the Department of Biotechnological and Applied Clinical Science of University of L'Aquila.

Visual pathways (anatomical and pathological) evaluation

Five pigs were submitted to necroscopic and histologic investigations, focusing on the study of the optic tracts, IAA n=3 and C n=2.

After opening of the skull the CNS tissues were gently removed; the cerebral ventricles were opened and the tissue exposed to favour the subsequent fixation. (In case of detection grossly), the optic chiasm and the optic nerve were sampled and put in a plastic biocassette (Bio-Optica, Milan). Then, cerebrum cerebellum and pons and the tissues within the biocassettes were *in toto* in formalin fixed (10%).

Brains samples were fixed in 4% paraformaldehyde for at least 72 h. The lateral geniculate bodies and adjacent diencephalon were isolated and postfixed in the same fixative for 4 h. After rinsing in phosphate buffer saline (PBS, pH 7.4), the tissue was cryoprotected in 30% sucrose solution in PBS (pH 7.4) at +4°C, and cut on a sliding freezing microtome in serial coronal sections (50 μm). The sections were stored in PBS (pH 7.4) containing sodium azide (0.01%).

Samples of the optic chiasm, optic nerve, the left and right optic tracts, the left and right lateral geniculate bodies were routinely processed, embedded in paraffin, cut into 4-μm thick sections and stained with Haematoxylin–Eosin (H&E).

Thionin staining

To identify cytoarchitectonic features of the lateral geniculate nucleus, sections were stained with thionin as follows. Sections were taken out of the PBS, mounted on gelatin-coated slides, and dried overnight at 37 ◦C. Sections were defatted 1 h in a mixture of chloroform/ethanol 100% (1:1), and then rehydrated through a graded series of ethanol,2×2 min in 100% ethanol, 2 min in 96% ethanol, 2 min in 70% ethanol, 2 min in50% ethanol, 2 min in dH2O, and stained 30 s in a 0.125% thionin (Fisher Scientific)solution, dehydrated and coverslipped with DPX (BDH Laboratory Suplies Poole,England).

Analysis of sections

Sections were analyzed using a Leica DMRB microscope. Brightfield images were acquired by means of a Polaroid DMCdigital camera (Polaroid Corporation, Cambridge, MA, USA) and DMC 2 software. Contrast and brightness

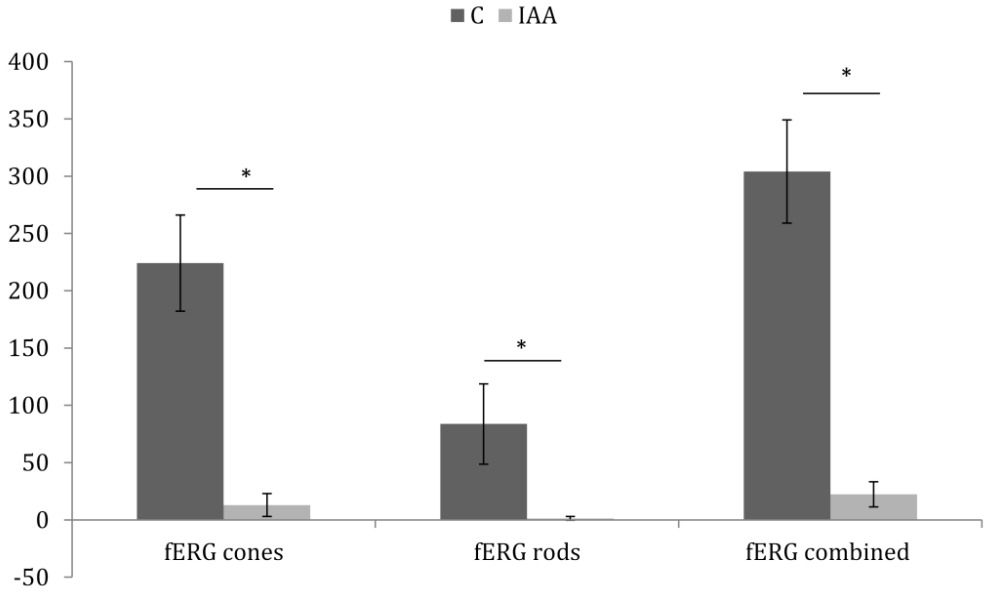
were adjusted to reflect the appearance of the labeling seen through the microscope using Adobe Photoshop CS3 Extended 10.0 software (AdobeSystems, San Jose, CA). Data concerning the percentage of the image covered by neurons and glial cells in lateral geniculate nucleus were obtained using the automatic threshold algorithm of ImageJ (version IJ 1.46r downloaded from http://imagej.nih.gov/ij/download.html). For this analysis images were taken using a Leica DMRB microscope under identical acquisition parameters from control and treated pigs. For each pig, 5 sections were analyzed. All data are given as mean ± standard deviation and differences between controls and treated were evaluated using Student's t-test, with a significance level of $p < 0.05$.

6.2 Results and discussions

Electrophysiological (ffERGs, pERG and fVEP) analysis

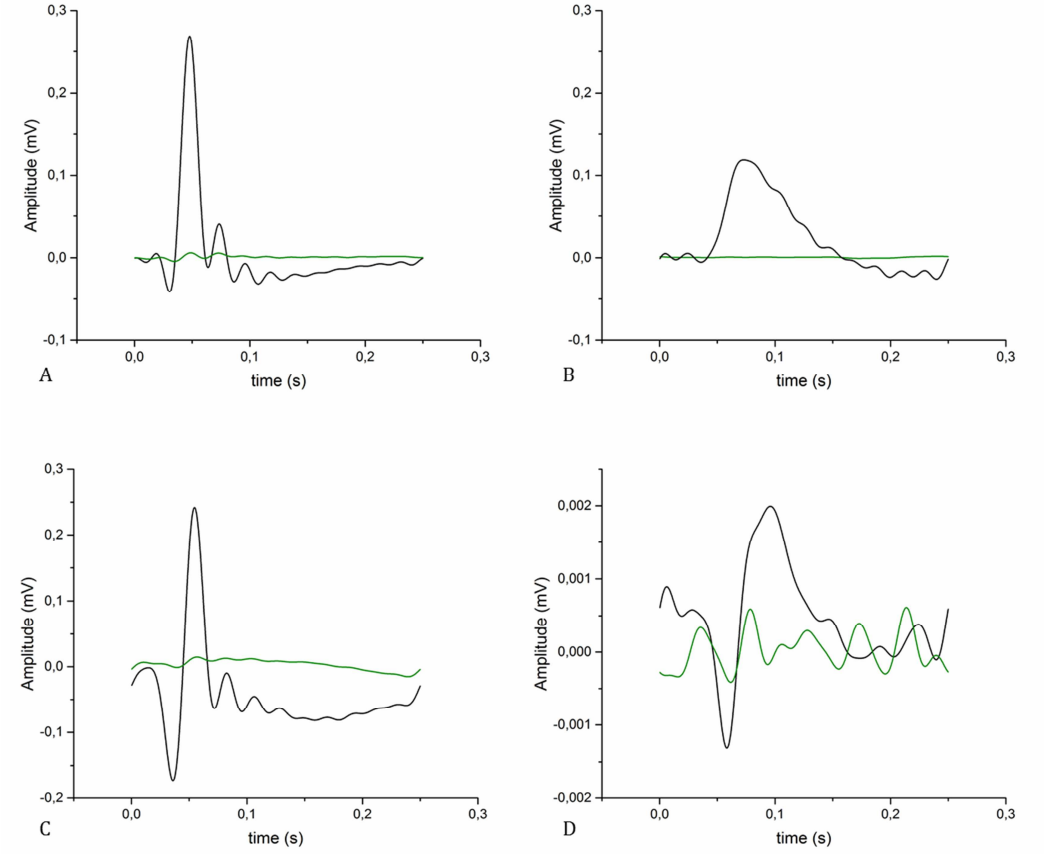
The animals were tested before and four weeks after IAA treatment (12 mg/kg). The recording of ffERG and fVEP stimuli was reproducible amplitudes of *b-wave* are shown in figure 30. The data were analyzed for pair data in animals treated with the IAA and for in comparison with the control group. In both analyzes the data confirmed the complete degeneration of rod photoreceptor while few cones were still able to react to light. As previously describe by Weng, Noel and Scott the 12 mg/kg IAA concentration lead to severe photoreceptor degeneration. The fVEP stimuli are were reduced but not completely extinguished, as we can see in figure 31D the shape of the wave after IAA treatment is maintained suggesting that the visual pathways are still intact and able to conduct the stimuli from photoreceptor to visual cortex.

Figure 30. ***B wave amplitudes of ffERG in C and IAA pigs four week after treatment.***



In figure 31 (A, B, C and D) an example wave for each of the full field flash ERGs stimulus and fVEP is reported. It is important to notice that the waves are well characterized and comparable to the one described for humans (McCulloch et al., 2015; Odom et al., 2016). FVEP wave (fig 31 D) has a different y axis scale to allow a better visualization of the wave; the fVEP amplitude is indeed smaller compared to ffERG waves.

Figure 31. ***ffERGs and fVEP waves shape in IAA (green) and C (black) animals.*** *A light adapted fERG 3.0 expressing the cones response. B dark adapted fERG 10.0 expressing the rods response. C dark adapted fERG 3.0 expressing both cones and rods response. D fVEP expressing the activation of visual cortex in response to flash stimuli.*



The pERG arises largely in the ganglion cells, driven by the photoreceptors and corresponding retinal cells. Since the pERG (in contrast to the flash ERG) is a local response from the area covered by the retinal stimulus

image, it can be used as a sensitive indicator of dysfunction within the macular region and it reflects the integrity of the optics, photoreceptors, bipolar cells and retinal ganglion cells.

To evaluate the ganglion cells functionality pERG was evaluated. We start from the ISCEV protocol parameters for reconding pPERG in human as very little is described in swine (Janknecht et al., 2001) and we end up with the setting displayed in table 21 B.

PERGs are small signals, typically around 2–8 μV in normal humans, making pERG recording more technically demanding than standard flash ERGs (Fig. 32) (Bach et al., 2013).

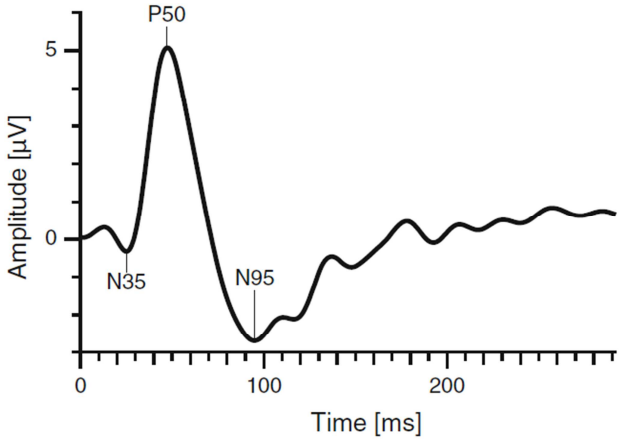


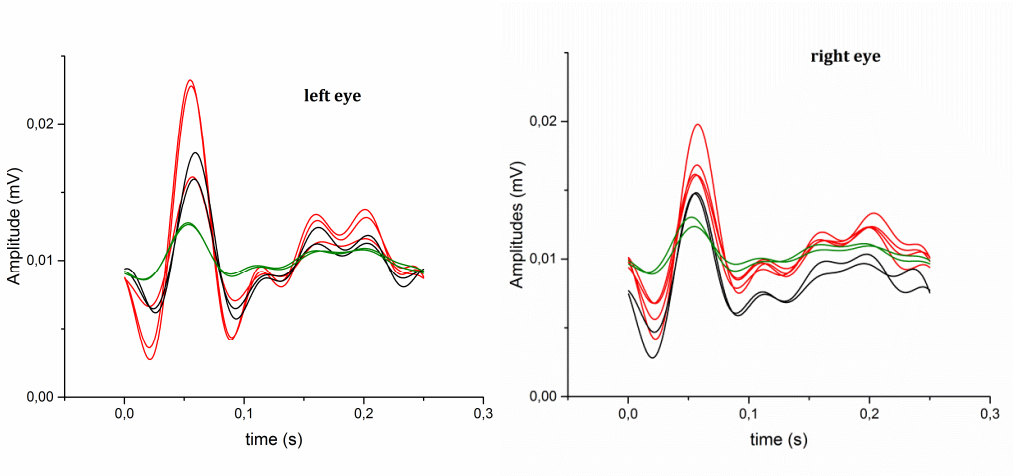
Figure 32. ***A typical standard pERG. The amplitude of the P50 is typically between 2.0 and 8.0 μV.***
From Bach et al., 2013

Therefore, obtaining reliable results requires careful attention to technique, including stimulus and electrode quality as well as to sources of extraneous noise.

We recorded pattern ERGs in normal pigs (Fig. 33), but we were not able to recorder it after IAA treatment. Most probably the very high technical

demanding to record pattern ERG requires a faraday cage to maintai the noises under control and enable investigators to recorder a decrease signal.

Figure 33. ***pERG waves of three pigs without IAA treatment. Data of right and left eye are reported, each animal is assigned to a different color, and at least two replicates were made for each animal.***



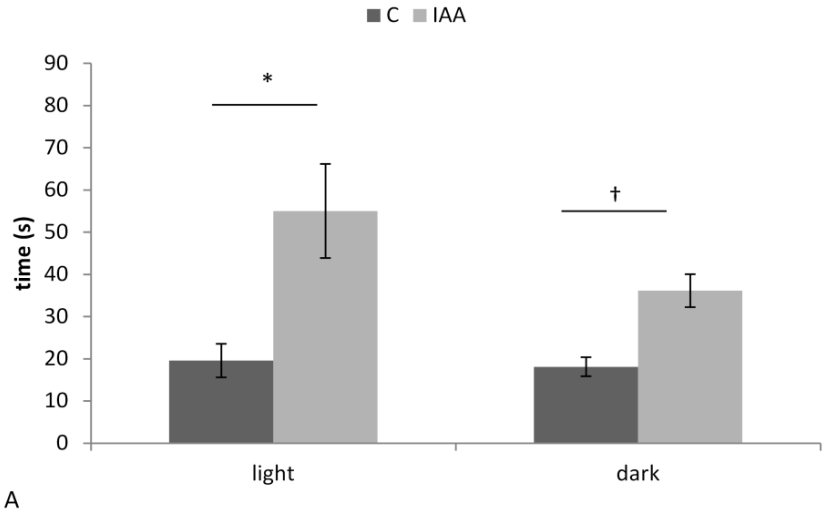
The pERG presented show a high variability between subject (inter variability) but a reduced intra subject variability confirming the reproducibility of the records. The tested animals (Fig. 32) have different age, black and red color was assigned to 80 days old pigs while the green color refear to a six mounth old pig. We can notice that their might be a difference in pERG amplitude due to the animal development, but more studies are necessaty to confirm this hypotesis.

Behavioral vision assessment

The animals were easily trained to complete the trial within 20 ± 5 seconds in four days. All animals were than tested before treatment. After treatment the IAA group showed a statistically significant increase in the time needed to complete the trial and in the number of collision with the obstacles

(Fig. 34) compared with C group in both light and semidarkness conditions. In semidarkness condition IAA treated animals had more collision with obstacle compared with the same group in light condition. The results confirm that vision can be assessed with behavioral test in pigs, endorsing the hypotheses obtained from electrophysiology.

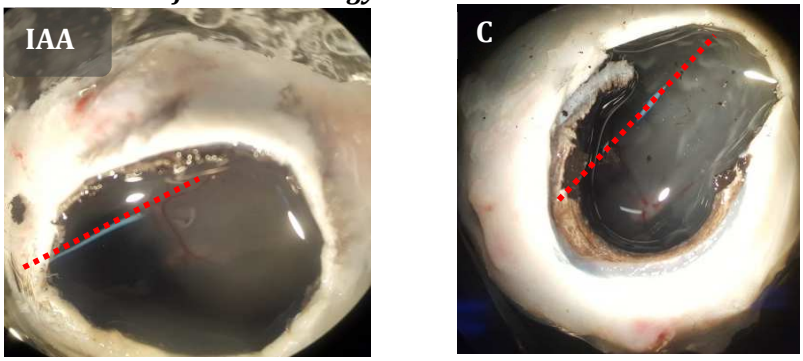
Figure 34. ***Behavioral vision assessment. A. Time (s) in light and semidarkness condition to finish the path in control (C) and iodoacetic acid treated (IAA) groups. B. numbers of hit obstacles in light and semidarkness condition in control (C) and iodoacetic acid treated (IAA) groups.*** ** for p<0.05; † for p<0.001*



Retina histology

The eyes were macroscopically evaluated (Fig. 35), all samples were in good conditions and no inflammation or alterations were observed.

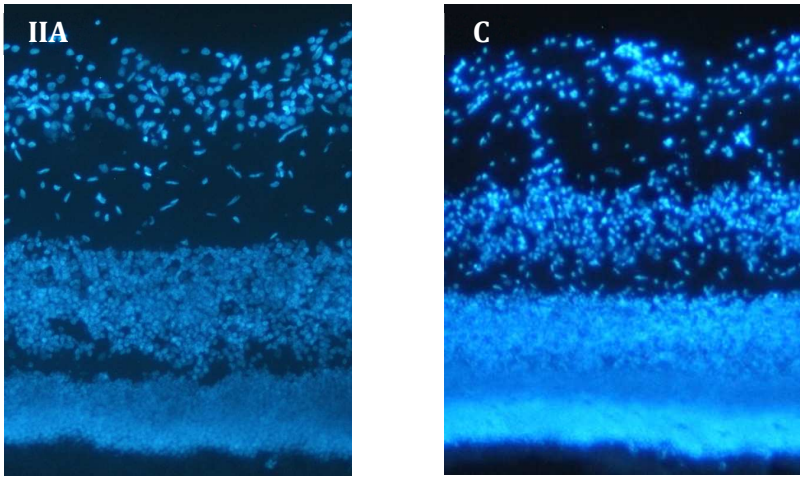
Figure 35. ***Macroscopic views of IAA and C eyes after ablation of the cornea, anterior chamber and the crystalline during the processing. The red line indicates were the retina was cut for the histology*.**



The photoreceptors from the central cone rich horizontal band were completely degenerated in IAA treated retinas as shown in figure 36.

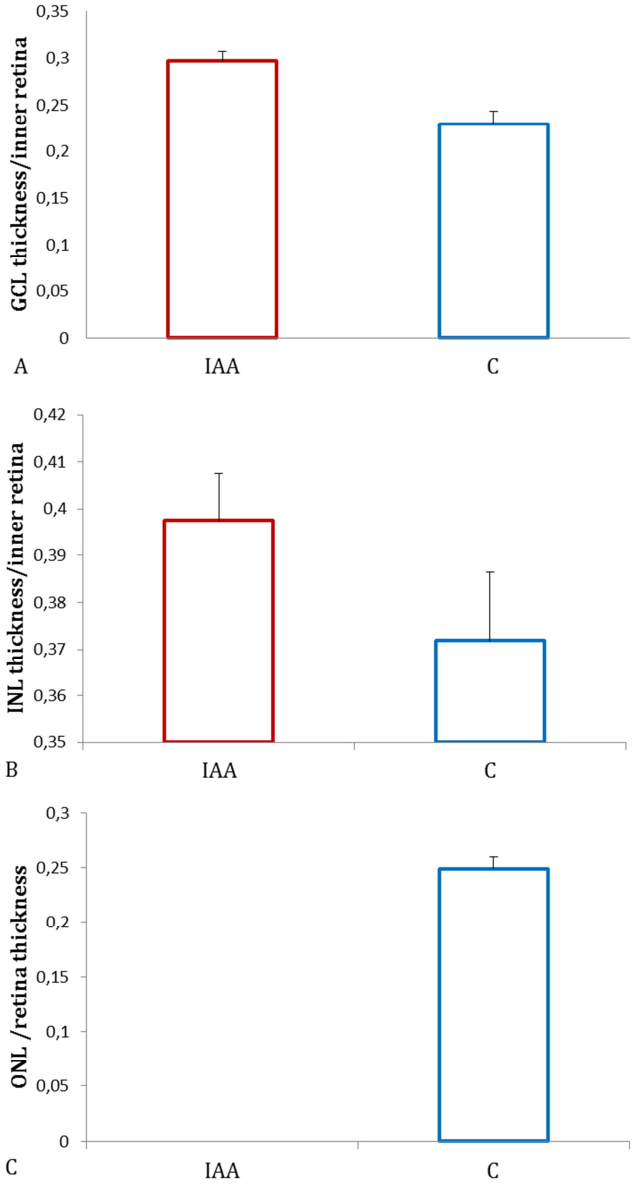
As previously described the 12 mg/kg IAA concentration lead to a severe photoreceptors degeneration affecting both rods and cones (Wang et al., 2011).

Figure 36. ***Immunohistochemistry of the retina showing the differences within the IAA and C retinas.***



The thickness of retinal layers was evaluated (Fig. 37 A, B and C). The ganglion cell layer (GCL) and the inner layer (INL) were found relatively increased in IAA group due to the outer layer (ONL) degeneration. The data are confirming previously findings of hypertrophy and swelling of Müller cells (Scott et al., 2011).

Figure 37. ***Retinal Thickness measures in C and IAA retina samples.*** *A GCL thickess to inner retina thickness ratio. B INL thickness to inner retina thickness ratio. C ONL over total retinal thickness ratio.*



The data suggest a mild anatomical alteration of GCL layer that is however not compromising the optic nerve anatomy.

Visual pathways histo-morphological evaluation

Histology of the optic/visual tracts

A mild satellitosis of the optic chiasm along with a mild to moderate gliosis of the optic nerve were detected. Glial cells immunohistochemistry is ongoing; the results could help to investigate a possible inflammation reaction toward the treatment.

Histology of the lateral geniculate nucleus

In thionin preparations, the neuronal population of the lateral geniculate nucleus was made up in its grater part of polygonal neurons, but also included fusiform and spheroidal neurons. Although the lateral geniculate nucleus had a rather striated appearance, it was difficult to recognize the existence of a clear laminar organization. As reported in table 22, the average area covered by neurons and glial cells was significantly larger in C than in IAA animals.

Table 22. ***Average percentage (± standard deviation) of the image area covered by neurons and glial cells (nuclei) in lateral geniculate nucleus (LGN) of control (C) and treated (IAA) groups***

Group	Right and left LGN	Right LGN	Left LGN
C	25,6 ± 4,7*	27,8 ± 3,4§	21,5 ± 3,9
IAA	23,2 ± 3,3*	23,2 ± 2,8§	23,3 ±3,8

* $P < 0.05$ at the t-test, C versus IAA

§ $P < 0.001$ at the t-test, C versus IAA

Histopathological extra-CNS findings were **e**nzootic pneumonia (one case of necro-suppurative bronchio-alveolar pneumonia), inguinal lymphadenomegaly (chronic follicular hyperplasia, subcapsular histiocytosis, and eosinophilic lymphadenitis), and multifocal chronic hepatitis ("white spot liver disease"). Those findings were compatible with a standard pig farming health condition.

6.3 Conclusions

We evaluate morpho-functional characteristic of the visual pathways in a IAA pig model of photoreceptor degeneration. The project is not concluded, more histological and electrophysiological investigations are indeed necessary to better characterize the visual pathways.

Behavioral visual functional test were assessed for the first time in pigs. The test was easily conduced on animals and was able to discriminate between C and IAA groups. We can therefore state that the proposed pig behavioral vision assessment test is validated and can be applied to any research that involves the pig as animal model. Moreover it can be adopted as clinical evaluation of pig vision acuity as the pig, and in particular minipig, is becoming more frequently a domestic pet.

The full field ERG was recorded and waves were described; the differences between groups were strong as the IAA concentration of 12 mg/kg

lead to a complete rods and severe cones degeneration mimic the late stage of *retinitis pigmentosa.*

The IAA concentration dependent pig model was reproduced and innovative investigations were proposed to better characterize the model. The components of optic tract following the retina up to the visual cortex were indeed never investigated in IAA pig model.

The morphological investigation suggest that a 12 mg/kg of IAA administration can cause reorganization in the structure of lateral geniculate nucleus (LGN) as a possible consequence of decreased incoming inputs due to photoreceptor degeneration. None the less the optic investigated tract did not show any pathological status, a very mild gliosis was observed, but further investigations are needed to understand this phenomenon.

We can therefore state that the IAA pig model is an easy reproducible, costs-effective model. At the concentration of 12mg/Kg the IAA treatment lead to anatomical and functional findings comparable with the late stage of *retinitis pigmentosa*.

The 12mg/kg IAA pig model of photoreceptors degeneration could therefore be a suitable model for the study of bioprosthetic devices that are one of the only treatment choices for end stage photoreceptors degeneration diseases.

7 *General Conclusions*

The role of veterinarians in biomedical science is increasing and becoming fundamental due to the awareness that more efforts are needed to increase translational value of animal models.

Deep knowledge of the physiology of the chosen species combined with the etiopatological knowhow of human diseases could be the right path to take.

The new findings in science concerning the role of microbiota on the host health increased the need for comparable animal models to allow more in deep investigations and to test microbiota associated treatments.

The pig full fields this need, as it is a widespread and validated model of gastro enteric and metabolic system.

The Bake4fun project aimed to investigate the impact of highly nutritional value foods on the gut microbiota and the potential benefits to the human health on a pig model. At the same time the piglet was chosen for its Para physiological iron deficiency to investigate microencapsulated iron fortification as potential treatment for human iron nutritional deficiency.

The results lead to a thorough understanding of pig gut microbiota and its modulation to nutritional changes, these

understandings are fundamental for the interpretation of data when experiments are conducted on animals.

The potential role in reduction and refinement is clear especially considering the pig as the emerging gold standard model for gastro intestinal research.

The microencapsulation of iron was found to be protective against the side effects of iron dietary supplementation maintaining the healing effect, opening a new prospective on iron fortified foods.

The pig model is also very important in ophthalmology.

The human vision is based on three photo sensible cells and the most important are cones. The cones are responsible for day light color vision and visual acuity therefore the loss of cone system is dramatic in our highly technological world.

The rich cone pig retina is therefore an extremely important model for the study of cones degeneration mechanisms and the examination of innovative therapeutic strategies.

A rapidly inducible costs-effective chemical pig model of photoreceptor degeneration was investigated. New tools have been established for the assessment of vision in a pig model. An easy

visual evaluation behavioral test has been validated, giving to researchers and clinicians the opportunity to assess vision in a sentient pig model.

Moreover the pattern electroretinogram (pERG) was recorded for the first time in pigs. The pERG is a sensitive indicator of dysfunction within the macular region and it reflects the integrity of the optics, photoreceptors, bipolar cells and retinal ganglion cells.

The advance in ophthalmology research given by the above described results is enormous and leads to a better use of the pig model in retinal research.

Hopefully this work will contribute to the translational value of the pig model, which in my opinion is the future gold standard model for gastro-intestinal and vision systems and will completely replace the use of dogs and non-humane primates in biomedical science.

8 *Acknowledgments*

This work is the results of two multidisciplinary collaborations.

The European project Bake4Fun was an integrated work between small medium business and research groups. I want to thank Luca Laghi, Andrea Gianotti, Silvia Turroni, Patrizia Brigidi, Alessandra Bordoni, Danielle Laure Taneyo Saa and Mattia di Nunzio for allowing me to expose the data on the gut microbioma, fecal metabolome and inflammation parameters.

I want to thank Fabio Benfenati that gave me the possibility to cooperate in a multidisciplinary and multicultural framework as the IIT.

Thanks to the IIT group I had the possibility to enlarge my knowledge on retinal electrophysiology by using technical instrumentation and dedicated Software.

The ASA Unit experience

From my fourth year as a veterinary medicine student I start to look at the Physiology department with interest and I decide to ask to professor Bacci the opportunity to join her work team.

From that moment I began a new experience becoming aware that veterinary medicine is not only clinic.

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