Impacts of neonatal iron deficiency anaemia and different iron treatments on growth, haematology, gut microbiota, and systemic lipid profiles using a piglet, a model for human infant

Thesis submitted for the degree of Doctor of Philosophy

Department of Food and Nutritional Sciences

Munawar Abbas

September 2023

Declaration

I confirm that this thesis is my own work and the use of all material from other sources has been properly and fully acknowledged.

Munawar Abbas,

September 2023

Acknowledgement

I would like to thank my first supervisor Dr Marie C. Lewis for her continued support and guidance during my PhD and beyond. I am thankful to my second supervisor Prof. Simon C. Andrews for his continued support and guidance throughout my PhD. I am grateful to the University of Reading for providing full scholarship for my PhD studies.

I would also like to thank Dr Carlos Poveda, Dr Nicola Grey, and Dr Andrea Monteagudo-Mera, and Munsi for their support in laboratory experiments and analysis and running the farm trial. Thanks to my fellows Zeynep, Dan, Chloe for their support.

I can never thank enough to my father who worked very hard to support my education, specially to start my university education, I would have not been able to achieve this without his support in early life. Thanks to my sisters, and friends in and outside the UK for their continued support.

Finally, thanks to my lovely wife Maryam and my cute son Eesa for the sacrifices they had to make during the course of my PhD.

Table of contents

General Abstract

Iron deficiency (ID) is the most prevalent micronutrient deficiency worldwide and is defined by systemic haemoglobin (Hb) levels between 95- and 110 g/L in human infants. If left untreated, ID can progress to iron deficiency anaemia (IDA) when Hb levels are reduced further to <95 g/L. Infants and young children are at particular risk, especially in low- and middle-income countries (LMICs), where IDA can ultimately result in stunted growth, which has life-long health implications. Oral iron supplementation is the most common strategy used worldwide to prevent and treat ID/IDA. However, this has been associated with detrimental side effects including diarrhoea and increased risk of infectious diseases, although there is limited mechanistic understanding in this area. Piglets are valuable models for human infants since they share many physiological characteristics with humans. In addition, their precocial nature permits early separation from maternal interference so their environment and nutrition can be tightly controlled. Furthermore, if left untreated with iron, piglets will reliably develop IDA in the first weeks of life. The aim of this PhD was to explore the effects of IDA, and different forms of iron supplementation on growth and metabolism in human infants using a neonatal piglet model. Twenty-four 1d old piglets were litter-matched into 4 treatment groups (n=6 each). Treatments were goup 1, control (no iron); group 2, intramuscular iron (IM, 200mg Fe); group 3, oral iron (150mg/Kg of iron sulphate); group 4, oral and IM iron. Weight gain was measured twice a week and sequential blood parameters were analysed using a portable haem meter and standard veterinary diagnostic laboratory methods. Host systemic metabolites, microbial-derived short chain fatty acids (SCFAs) and lipid profiles were assessed using liquid chromatography - mass spectrometry (LC-MS), while gut microbiota composition was analysed using 16S Illumina sequencing techniques. The results demonstrated that all types of iron treatments prevented anaemia by sustaining sufficient concentrations of Hb and other iron-dependent blood parameters, and by maintaining host-derived metabolite concentrations. However, oral iron supplementation was associated with significant reductions in weight gain (~ 0.5Kg, $p<0.05$) and abundances of Lachnospiraceae ($p<0.05$) in piglets compared to their IM iron treated siblings, and significant reductions in lactobacilli ($p<0.05$) were observed in all iron treated piglets compared to IDA siblings. In terms of systemic lipids, IDA was associated with reductions in sphingo-

($p<0.05$) and a range of phospholipids ($p<0.05$), which are important for growth and development. Taken together, these results demonstrate that both IDA and iron supplementation have significant impacts on piglet growth and metabolism both directly and via shifts in gut microbiota populations and metabolic outputs. This could have important consequences for oral iron supplementation recommendations, especially in small-for-age infants in LMICs and is especially relevant for iron-replete infant fed iron-rich formula milk.

List of Abbreviations

ATP	adenosine triphosphate
AWERB	Animal Welfare and Ethical Review Body
BCAAs	branch chain fatty acids
BMI	body mass index
Carn	acyl-carnitines
CEDAR	Centre for Dairy Research
Cer	ceramides
CL	cardiolipins
CPT1	carnitine palmitoyltransferase-1
DALYs	disability-adjusted life years
DG	diacylglycerides
DMT1	divalent metal cation transporter
EDTA	ethylenediaminetetraacetic acid
FeDex	iron dextran
Fe-MNPs	iron micronutrient powders
GDP	gross domestic product
GM1	gangliosides
Hb	haemoglobin
HDL	high density lipoprotein
Hex-cer	hexosylceramides
IBD	inflammatory bowel disease
ID	Iron deficiency anaemia
ID	iron deficiency
IDA	Iron deficiency anaemia
IM	intramuscular
LC-MS	liquid chromatography mass spectrometry
LMICs	low-and-middle-income-countries
LPC	lysophoshatidycholines
LPE	lysophosphatidylethanolamines
LPI	lysophosphatidyinositols
LPS	lipopolysaccharides
MCH	mean cell haemoglobin
MCHC	mean cell haemoglobin concentration
MCV	mean cell volume
MG	monoacylglycerides
Mono-lyso_CL	monolyso cardiolipins
NGS	next generation sequencing
PA	phosphtidic acids
PC	phosphatidylcholines
PE	phosphatidylethanolamines
PFPP	pentafluorophenylpropyl
PG	phosphatidylglycerols
PI	phosphatidyinositols

PS	phosphatidylserines
QC	quality control
RBCs	red blood cells
ROS	reactive oxygen species
S	sulfatides
SCFAs	short chain fatty acids
SEM	standard error means
SM	sphingomyelins
SMR	sow milk replacer
TCA	tricarboxylic acid
TF	transcription factors
TG	triacylglycerides
WBCs	white blood cells
WHO	world health organization

List of Publications

1. **Abbas, M**., Hayirli, Z., Drakesmith, H., Andrews, S. C., & Lewis, M. C. (2022). Effects of iron deficiency and iron supplementation at the host-microbiota interface: Could a piglet model unravel complexities of the underlying mechanisms?. *Frontiers in Nutrition*, 1963.
2. Frost, J. N., Tan, T. K., **Abbas, M**., Wideman, S. K., Bonadonna, M., Stoffel, N. U., ... & Drakesmith, H. (2021). Hepcidin-mediated hypoferremia disrupts immune responses to vaccination and infection. *Med*, *2*(2), 164-179.
3. Christoforidou, Z., Mora Ortiz, M., Poveda, C., **Abbas, M**., Walton, G., Bailey, M., & Lewis, M. C. (2019). Sexual dimorphism in immune development and in response to nutritional intervention in neonatal piglets. *Frontiers in Immunology*, *10*, 2705.

List of figures

List of Tables

CHAPTER 1
INTRODUCTION

1.1 Global impact of iron deficiency and iron deficiency anaemia

Inadequate nutrition is recognized as a major factor in abnormal development during early life and has serious implications for long-term health [1, 2, 3]. Iron is an essential micro-nutrient since it is a key prosthetic component (e.g. for haem and iron-sulphur clusters) associated with proteins that have vital roles in a wide range of key biochemical processes including oxygen transport [4], the respiratory chain [5], gut health and metabolic production, and overall growth and development [6, 7]. Iron deficiency (ID), where body iron stores are becoming depleted occurs when iron requirements exceed intake. Exhaustion of these stores leads to iron deficiency anaemia (IDA) which affects around 1.2 billion people worldwide [8, 9]. IDA is clinically defined as serum haemoglobin (Hb) levels of <105 and <100 g/L in 4- and 9-month-old infants respectively [10].

ID and IDA have a significant global economic and societal impact [11]. The global cost of ID and IDA is difficult to estimate as it is highly dependent on the population affected, the severity of the condition and the costs of healthcare in different regions. However, a study conducted in 6–59-month-old Pakistani infants and young children concluded that the medical cost of micronutrient deficiencies including ID and IDA along with zinc, iodine and vitamin A deficiency is in the region of US$ 46 million, with a further US$ 0.3 billion of production-associated loses. This is highly significant and is equivalent to around 1.44% of GDP in Pakistan [12]. Similarly, a study conducted in India estimated that the annual costs of IDA in infants and young children aged 6-59 months includes costs of US$ 8.3 million disability-adjusted life years (DALYs) and production losses of approximately US$ 2.4 billion, which is equivalent to 1.3% of India's GDP [13]. The economic burden of IDA extends to developed countries where, according to a review by Brookes *et al* [14] on the management of IDA in England from 2012 to 2018, emergency hospital admissions cost £42.4 million, while day case admissions cost £46 million, despite treating four times as many patients in the outpatient setting.

It is important to note that these estimates are likely to be conservative, as they do not take into account the long-term consequences of ID and IDA, such as the impact on cognitive function and educational attainment, which are likely to also have significant economic and societal consequences. A study conducted in Nigeria found that the prevalence of IDA in pregnant women was associated with increased maternal mortality [15], linked with higher neonate morbidity and mortality. In addition, these estimates do not take into account the cost of implementing comprehensive strategies to prevent and treat ID and IDA, which may involve a range of interventions, including nutritional education, iron supplementation programmes, and improved access to healthcare.

1.2 Development of iron deficiency and iron deficiency anaemia

In full-term infants born to iron-sufficient mothers, transplacental delivery of iron results in high neonatal reserves, around 75mg/kg, primarily as haemoglobin and in the form of ferritin and hemosiderin [16, 17]. Following birth, excess iron, derived from haemoglobin, is immediately transferred to storage compartments and consequently, under normal conditions, iron supplied through the diet is not required during the first 4-6 months of life [18]. This may partly explain why human breastmilk contains such a low iron content (~0.35 mg/L; bioavailability of 45–100%) [19]. However, after ~6 months, infant iron stores derived from the mother become depleted whilst the developmental demand for iron increases due to higher erythropoietic and brain activity, along with increased tissue accretion as a result of high growth rate [20, 21]. Demand for iron soon exceeds that available from breast milk prompting the need for iron from complementary foods and/or supplementation [22].

Dietary iron requirements during pregnancy are significantly increased. Based on a pre-pregnancy weight of 55kg, it is estimated that an additional 1g of iron is required during pregnancy, equating to around 3.6mg/day on average [23]. This is due to increasingly higher demands from the foetus and placenta, and rapid expansion of the maternal vascular volume during the latter half of gestation especially [24]. However, in both poor and affluent societies, a large proportion of women enter pregnancy with ID or IDA [25] since around 50% of women of childbearing age in low- and middle-income countries (LMICs) are anaemic, although there is significant regional variation [26]. In both ID and IDA, the iron endowment that neonates receive from their mothers is often reduced such that iron reserves are depleted well before

4-6 months (when weaning generally commences) leading to the rapid onset of IDA. Infants born to anaemic or iron-deficient mothers, and those with low birth weight, begin life with reduced iron stores and are at higher risk of developing ID before 4-6 months [27, 28]. Preterm infants are also at increased risk of developing ID as maternal iron transfer to the foetus mostly occurs during the final trimester [29].

Rapid erythropoiesis, inadequate dietary iron consumption and limited iron bioavailability (linked to reduced absorption following enteric infection and/or dietary inhibitors) all contribute to increased risk for ID throughout infancy and early childhood [30]. School-age children primarily consuming unfortified cereal-based diets are at greater risk of ID owing to low dietary iron intake [31]. In addition, non-haem iron, the form derived from plant sources, has lower bioavailability and is more sensitive to enhancers (e.g., ascorbic acid) and inhibitors (e.g. phytate) of iron absorption compared to haem iron, the form derived from animal sources.

Non-nutritional factors, such as infection and inflammation, can also lead to conditional anaemia, or ‘anaemia of inflammation’. This response is instigated by the host to limit systemic iron availability and thus combat ongoing infection [32] and is predominantly mediated through the upregulation of hepcidin [33]. This peptide hormone acts as a major regulator of iron homeostasis. Hepcidin is secreted primarily by hepatocytes in response to various factors including body iron stores and plasma iron levels. It controls iron absorption, recycling, and release from stores by binding to the cellular iron exporter, ferroportin, causing it to internalise, leading to iron retention within cells. This consequently limits iron availability to extra-cellular pathogens [34, 35]. Hepcidin expression is also responsive to elevated inflammatory cytokines including IL-6 and IL-22 [36, 37]. Therefore, anaemia of inflammation is characterised by adequate or high iron stores but low serum iron (bound to the serum iron chaperone, transferrin) [37]. In contrast to IDA, anaemia of inflammation cannot be prevented or resolved by iron supplementation and may even be exacerbated by increased dietary iron [34].

1.3 Iron supplementation to prevent and treat ID and IDA

Iron supplementation is a widely used strategy to prevent and treat ID and IDA in infants and young children [38, 39]. While food-based approaches are important for improving iron status, iron supplements are often needed to meet daily iron

requirements. The WHO report “Daily iron supplementation” endorses iron supplementation as a public-health intervention for infants and children aged 6-23 months across the globe, where the prevalence of IDA in this age group is high (>40%). Recommendations are for daily doses of 10-12.5 mg elemental iron for 3 consecutive months per year for infants and children aged 6-23 months, which increases to 30 mg for preschool-aged children [40].

The efficacy of different types of iron supplementation in preventing and treating ID and IDA in infants and young children has been extensively studied. The most commonly used iron supplements are ferrous salts, such as ferrous sulphate ($FeSO_4$), ferrous fumarate, and ferrous gluconate [41]. $FeSO_4$ has been widely used for decades in the pig industry as well as in human supplements for a number of reasons. For example, it is inexpensive, and available in several forms including tablets, powder and paste [42]. However, oral supplements are associated with gastrointestinal side effects, such as constipation, diarrhoea, and enteric infection, which can limit their effectiveness [43, 44]. To address these issues, alternative forms of iron supplementation are being developed, including haem iron. Haem iron originates from animal products such as red meat and poultry and is well-absorbed by the body, making it an attractive option for infants and young children [45]. It is well-tolerated and helps to prevent the gastrointestinal side effects associated with oral iron treatments.

To mitigate the increased risk of enteric infection and reduce other related side effects associated of oral iron supplementation, perhaps an alternative route could be parenteral iron [46]. Intravenous (IV) iron supplementations have been used as alternative methods to oral iron supplementation, especially in children who have failed to respond to oral iron supplementation. For example, those who suffer from iron malabsorption due to short bowel syndrome or anaemia of inflammation [47]. As IV iron supplementation involves the infusion of iron sucrose, ferric carboxymaltose or iron dextran (FeDex) directly into the bloodstream, it bypasses the hepcidin-ferroportin pathway that controls iron absorption in the gut (**see Figure 1.1**) [48]. Although IV iron can be effective in rapidly correcting IDA, they it is associated with several limitations. Primarily, that it requires close monitoring for adverse effects, which can increase the cost of treatments. Another limitation of IV iron is the need for trained healthcare professionals to administer the supplements and is more

expensive compared to oral iron which can limit access to treatment in low-resource settings [49]. Therefore, IV iron may only be used when oral iron supplementation is not feasible or effective, and the benefits of treatment outweigh the risks and costs.

1.4 Chemical kinetics and absorption of iron from the gut

Dietary iron mostly occurs in the oxidized, ferric (Fe^{3+}) form with the limited bioavailability. For iron absorption to occur, it must either undergo conversion to the ferrous (Fe^{2+}) ionic state, or be present as haem or in nanoparticulate form (e.g. ferritin cores) [50, 51]. A combination of stomach acidity and reducing agents, such as ascorbic acid and ferric reductase enzymes (including duodenal cytochrome B; DcytB) results in reduction of Fe^{3+} to Fe^{2+}. Consequently, dietary non-haem iron is mainly absorbed across the duodenum and proximal jejunum in the highly soluble ferrous form via the divalent metal cation transporter 1 (DMT1, also known as NRAMP2) [52, 53]. Partial absorption of dietary iron contained in food and supplements is a persistent and considerable obstacle in the treatment of ID and IDA [54].

1.5 The role of iron in the development of the gut microbiota

The gut microbiota refers to the diverse community of microbes residing in the gastrointestinal tract of animals, including humans [55]. These microbes include bacteria, archaea, viruses, and fungi, which play important roles in digestion, metabolism, immunity, and overall health [56]. Bacterial numbers and diversity increase in relation to distance from the stomach with the largest, most diverse population residing in the colon due to relatively more favourable conditions [57]. Due to the influence of several factors, including pH and the chemical form of iron present, predicting the bioavailability of iron to the microbiota for each of the various sections of the intestinal tract remains challenging. However, it is estimated that on average ~85% of dietary iron remains unabsorbed and colonic iron concentrations are in the region of 25 mM, of which approximately 0.4 mM is in the form of readily absorbable Fe^{2+} [58]. The majority of gut bacteria have essential requirements for iron and thus require its availability in the gut. However, the Lactobacillaceae are considered to be iron-independent members of the gut microbiota and preferentially utilize manganese instead [59]. It is likely that iron availability influences microbial succession and the developmental stability of the longer-term microbiota.

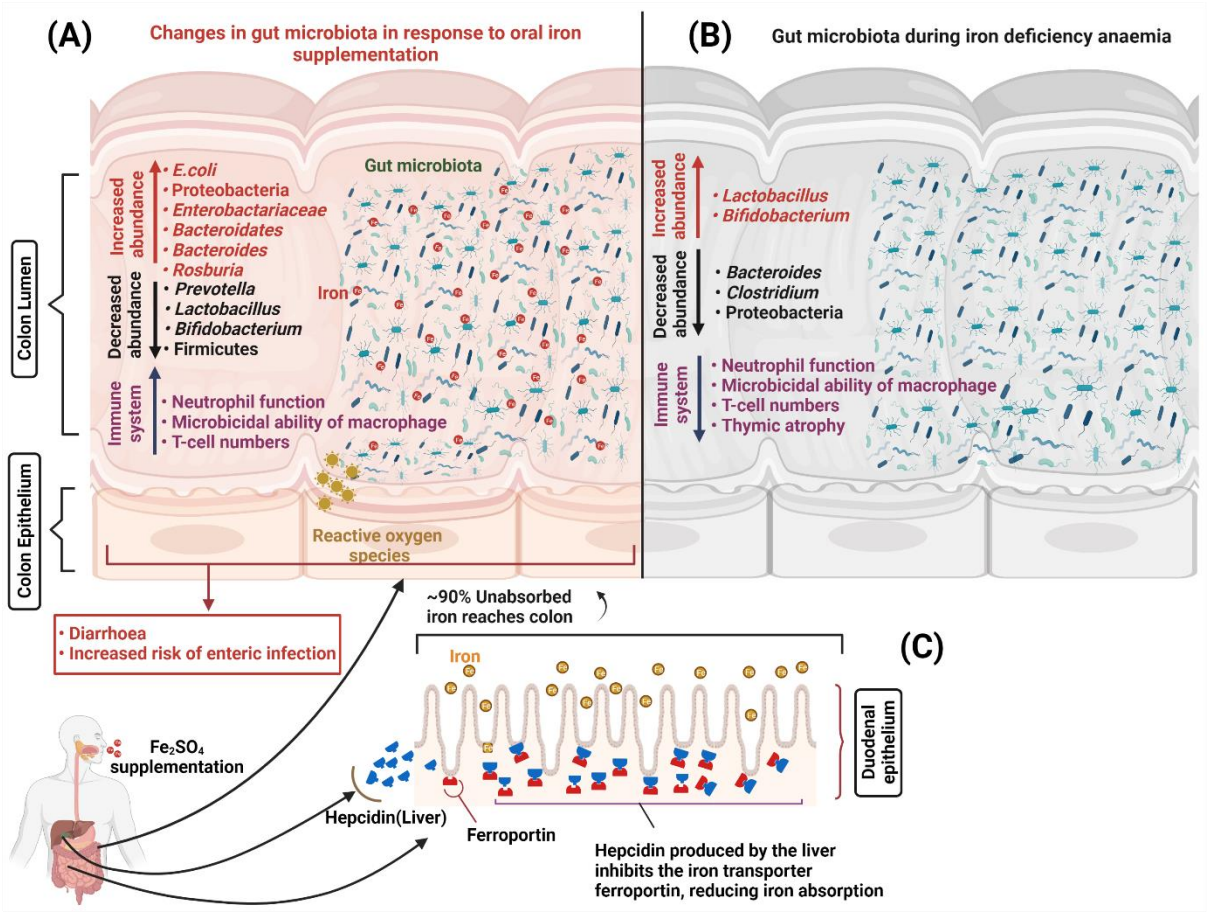

Figure 1. 1 (adapted from Abbas et al [60]). Changes in the gut microbiota in response to oral iron supplementation and anaemia. Iron supplementation prevents iron-deficiency anaemia and aids the generation of appropriate immune responses to invading pathogens. However, approximately 90% of unabsorbed iron reaches the colon where most bacteria have substantial iron requirements for growth. This excess iron results in decreased abundance of beneficial bacteria including lactobacilli and bifidobacteria and increased abundance of potential pathogens including *E.coli* which increase the risk of diarrhoea and enteric infection. In addition, oral iron supplementation may result in the generation of reactive oxygen species (ROS) causing oxidative stress and epithelial damage (A). Abundance of potential pathogens including Bacteroides and *Clostridium* decrease in response to iron deficiency anaemia and promote beneficial bacteria population growth (B). Hepatic cells release hepcidin in response to oral iron supplementation which internalizes the ferroportin (iron transport protein), reducing iron absorption in the duodenum (C).

Increased iron availability has also been associated with a raised risk of the presence, or virulence potential, of enteric pathogens including *Salmonella typhimurium*, *E. coli* and Enterobacteriaceae, along with reductions in more beneficial

bacteria such as bifidobacteria [61, 62, 63]. However, conditionally, ID can protect against infection [64] in both humans and animal models. Since iron is crucial for bacterial growth, in particular for pathogens such as *E. coli* [65, 66], it follows that insufficient iron availability, achieved by nutritional immunity mechanisms in the host, is an effective strategy to limit pathogenic growth and thus reduce infection risk. Also, during chronic inflammation and systemic infection by triggering hepcidin production [67]. Consistent with this, various *in vitro* studies have reported reduced growth of potential enteropathogens, including *Campylobacter jejuni* and *E. coli*, within iron-deprived environments [68, 69]. For example, limiting iron availability resulted in reductions in *E. coli* abundance to 0.8% compared to 10.7% in iron-sufficient controls. Gut fermentation models inoculated with faecal matter from a child and propagated under iron limited conditions (1.56 ± 0.1 mg Fe L^{-1}) showed a relative reduction in *Roseburia* spp., *Eubacterium rectale*, *Clostridium* cluster IV members and *Bacteroides* spp. along with relative increases in *Lactobacillus* spp. and Enterobacteriaceae compared to iron sufficient controls [70]. Similarly, a pilot study assessed the composition of the microbiota in IDA infants and young children concluding that ID is associated with distinct microbial signatures with increased abundance of Enterobacteriacea and Veillonellaceae, and decreased Coriobacteriaceae relative to healthy non-ID controls [71]. Consistent with this, several large-scale human trials using various doses for different durations (2-18 months) have, in general, reported that oral iron supplementation is associated with higher risk (1% to 23%) of developing diarrhoea in infants [72, 73, 74]. However, the outcomes of such studies have been inconsistent [75, 76], which suggests the link is complex and that perhaps other factors are at play.

One theory is that the effects of iron supplementation on the risks of enteric infections are highly dependent on the composition and metabolic activity of the underlying microbiota [77], which in turn is influenced by iron availability during the earlier developmental stages. These studies largely reported compositional changes to the microbiota during ID and IDA. However, an important consideration is how sustained these effects may be once sufficient iron levels have been attained. A recent early-life piglet study showed that ID (rapidly progressing to IDA during the trial) reported 27 bacterial genera differences in faeces following 32 days of iron restriction (from 2 day of age) compared to iron-sufficient controls. It went on to demonstrate that when

both groups received oral iron supplementation (standard 180–300 mg Fe/kg of diet) in weaner mix for a further 30 days, no differences in either bacterial populations or bacterial products of fermentation (volatile fatty acids) were detected.

Although normal microbial communities appeared to have been restored following this period of ID and IDA, bacterial colonization and succession in the gut is the primary driver of metabolic and immune development which occur in a programmed and sequential manner and is largely complete by 49 days following birth [78], well before the completion of the trial. Therefore, different patterns of microbial colonization driven by iron limiting conditions in the gut is highly likely to have had considerable impact on metabolic and immune function in later life and could affect overall growth and susceptibility to infection. In addition, this study reported findings from limited pigs (~n=10/treatment group). Being outbred there are considerable inter-litter and inter-individual variations and in the absence of litter-matching to accommodate this, treatment differences in gut microbiotas at 61 days resulting from earlier ID/IDA would have to have been larger than physiological differences between piglets to have been observed. In addition, the trial was completed using 2 replicate groups and it has previously been demonstrated that minor environmental variations during the first day of life exerted sustained influences on both the microbiota and metabolic phenotype which were of a higher magnitude then differences linked to divergent nutrition [79]. Taken together, this suggests that important questions of longer-term impacts of early-life ID and IDA on the composition and metabolic activity of gut microbiota, and wider development, remain unanswered. The majority of studies have tended to focus directly on the response of the gut microbiota to iron supplementation in ID and IDA infants, rather than underlying mechanisms.

1.6 Impact of ID and iron supplementation on metabolism

Iron plays a critical role in various metabolic pathways, including the tricarboxylic acid (TCA) cycle, haem synthesis, and oxidative phosphorylation [80]. Therefore, ID can significantly impact systemic metabolite production, leading to various adverse health outcomes [81]. The TCA cycle is a crucial metabolic pathway that generates energy by oxidizing acetyl-CoA to CO_2 [82]. ID conditions can significantly impact the TCA cycle by decreasing the levels of TCA cycle intermediates. A study conducted in 6- and 12-month-old Peruvian children (n=200) reported the impaired energy metabolism

indicated by reduced levels of TCA cycle intermediates in 6-month-old IDA children [83]. Iron supplementation has been shown to enhance the activity of TCA cycle enzymes. For example, a study in human cell lines (K-562) showed increased activity of succinate dehydrogenase and aconitase, and isocitrate dehydrogenase in response to iron supplementation [84]. The urea cycle is another critical metabolic pathway that removes toxic ammonia from the body by converting it to urea [85]. ID can also impact the urea cycle by decreasing the activity of urea cycle enzymes. A study conducted in neonatal piglets (n=16) showed that ID was associated with upregulation of urea cycle enzymes in the liver including ornithinetranscarbamoylase and argininosuccinate synthetase compared to iron treated counterparts (150mg FeDex) [86]. IDA can also impact amino acid metabolism by reducing the levels of branched chain amino acids (BCAA). A study in Austrian individuals (n=430) indicated that IDA caused significant reduction in systemic BCAA concentrations including leucin, isoleucine and valine compared to non-IDA subjects, suggesting the important role of iron in amino acid metabolism [87].

In lipid metabolism, iron is involved in the breakdown of fatty acids and in the production of cholesterol and other lipids [88]. Iron is also an integral part of some transporters and enzymes which play important roles in lipid metabolism, as well as their interaction with other lipids in the body [89]. Iron is known to impact one of the key enzymes involved in lipid metabolism, carnitine palmitoyltransferase-1 (CPT1), which is responsible for the transport of long-chain fatty acids into the mitochondria for energy production. A study in male mice (n=12) by Xiong *et al* [90] reported reduced concentrations of CPT1A in mice on normal diets (45mg Fe/Kg food) compared to their counterparts receiving high iron diets (1250mg Fe/Kg food).

Iron also plays a key role in ferroptosis, a type of regulated cell death that plays a significant role in various diseases such as neurodegeneration and organ injury [91]. Phospholipid peroxidation driven by iron is the main event of ferroptosis [92]. Lipid metabolism controls ferroptosis by regulating phospholipid peroxidation and various cellular processes. Multiple signal transduction pathways communicate with ferroptosis by modulating lipid metabolism [93]. This crosstalk between ferroptosis and lipid metabolism, and related signalling pathways, could be exploited to inform the development of therapeutic strategies.

1.7 Dietary iron negatively impacts host-pathogen competition

The universal iron supplementation policy in areas with high prevenance of ID and IDA results in the delivery of additional oral iron to significant numbers of children who are not iron deficient [94]. This is also the case in more affluent societies where infant formula milk is consistently fortified with iron. This 'additional' oral iron may ultimately result in higher concentrations of luminal iron being made available to the developing gut microbiota [33]. Importantly, an *ex vivo* study reported considerably raised growth of bacteria (including *E. coli*, *Salmonella* and *Staphylococcus epidermis*) in the serum of subjects receiving dietary iron supplementation (2 mg of iron /kg body weight). A strong correlation was observed between transferrin saturation and bacterial growth rates [95]. This suggests that even modest levels of oral iron supplementation may contribute to bacteraemia and may have implications for IV iron delivery. Furthermore**,** a randomized controlled study in iron deficient and/or anaemic Kenyan infants demonstrated that iron supplementation caused deleterious shifts in the gut microbial profile which included an increase in pathogenic bacterial population levels and decreases in beneficial *Lactobacillus* and bifidobacterial numbers [77, 96]. Consistent with this, a study in Swedish infants (non-anaemic) demonstrated that the intake of infant formula high in iron (6.6mg/day) was linked with a relatively lower population of bifidobacteria than counterparts consuming low iron formula (1.2mg/day). Similarly, a reduction in the abundance of lactobacilli was observed in response to the administration of oral iron drops (6.6mg/day) along with an increased prevalence of bacterial infection [97]. Even modest levels of iron supplementation, equivalent to that received by infants in fortified formula milk (150 mg Fe/day) and substantially below levels considered toxic, was associated with significant changes in the gut microbiota in young rats. This excess iron was also associated with increased 3-hydroxybutyrate and decreased amino acids, urea and *myo*-inositol. These parameters were linked with adverse cognitive development quantified by memory and learning scoring using the passive avoidance test [98]. Taking the above together, the results suggest that although oral iron supplementation is adequate in preventing IDA, it may also cause a detrimental shift in bacterial populations which is likely to have long-term effects on microbiota and overall growth and development, and possibly on cognitive function. Thus, there appears to be a health trade-off whereby treatment with oral iron supplements can have deleterious as well as beneficial outcomes.

1.8 The piglet model for iron deficiency in human infants

A suitable animal model would be invaluable in generating mechanistic understanding of the multilateral interactions which occur between the gut microbiota, metabolism and iron status. Such a model may also be instrumental in unravelling the reasons why oral iron supplementation may increase the risk of infection and thus aid the development of novel ID treatment strategies to limit such side effects. Rodent models have generated important biomedical information in this field and have several advantages over other animal species including transgenicity, rapid generation time, and accessibility of targeted reagents [99, 100]. However, there are important disadvantages to using rodent models for ID which require iron-deficient diets over long periods of time, which is problematic for modelling early-life ID and IDA [101]. Furthermore, mice have not adapted to adsorb haem which could limit the translational potential of some studies [101].

In contrast, in the absence of iron supplementation neonatal piglets start to become iron deficient within the first week of life. Since early-life environmental and dietary factors can have sustained impact on physiological development [102, 103], it is highly desirable that maternal influence is limited from a very young age and environmental factors are tightly controlled which is far less challenging in precocial species such as the pig; rodents are born relatively underdeveloped and are therefore highly reliant on their mothers during infancy. Consequently, the litter is often the unit which doesn't conform to the 3Rs (replacement, reduction, and refinement) required by UK legislation, to reduce the number of animals required to generate power. In addition, an outbred, rather than inbred, model better reflects the human population, and the piglet model fulfils these criteria.

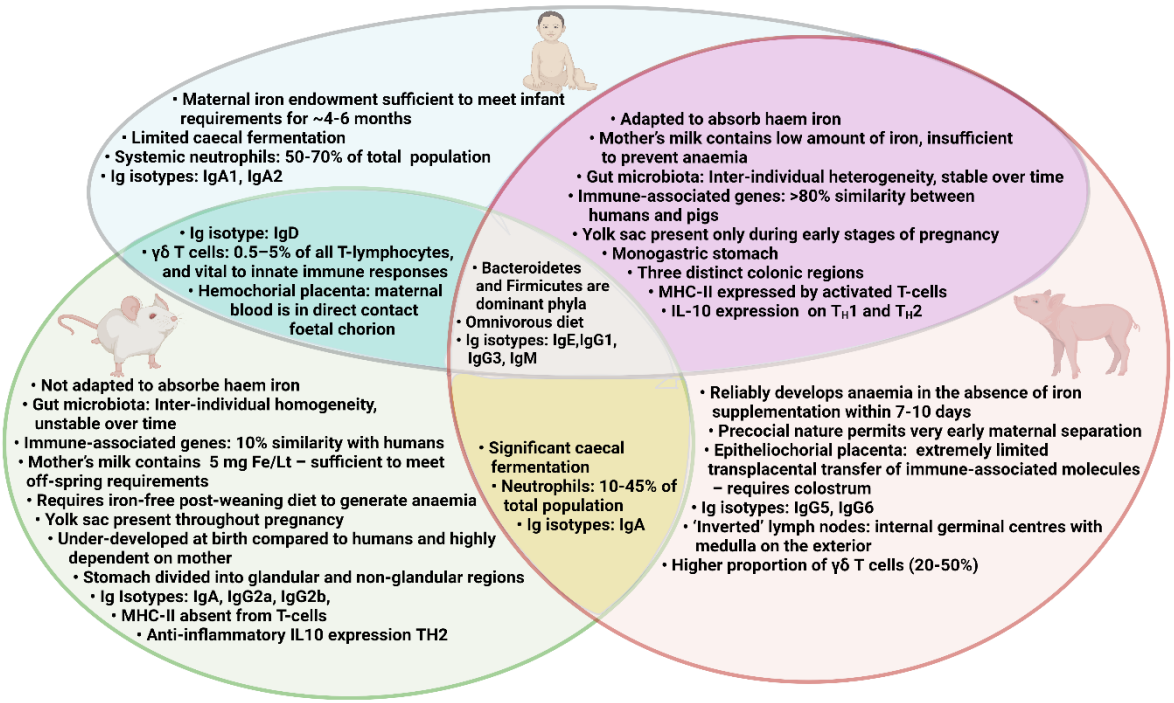

Figure 1. 2 (adapted from Abbas *et al* [60]). Comparison of human, pig and mouse characteristics relevant to assessing the effect of iron deficiency anaemia and iron supplementation

It is well established that omnivorous pigs are a valuable and tractable model for humans [104] as they share several key features including gastrointestinal immunology, physiology, microbiology, pathologies and dietary requirements [105, 106, 107, 108, 109]. Full genome studies show that there are fewer differences between pigs and humans, than rodents and humans [110, 111]. These factors suggest that pigs are valuable intermediates between highly reductionist, mechanistic studies in rodents, and epidemiological studies and clinical trials in humans. Pup-in-a-cup trials, where rats can be individually accommodated from 5 days of age, have been useful for assessing the impact of early nutrition on physiological development. However, precocial piglets are especially valuable models for early life since their self-sufficiency permits very early separation from their mothers and individual housing within a few hours of birth, thus limiting the maternal influence at this critical period of developmental plasticity. Comparative assessment of pig, mouse and human genomes demonstrated that structural and functional analyses of murine genes

involved in immunity an inflammation shared only 10% similarity with humans for measured parameters, whereas in pigs this figure is >80% [112]. In pigs and humans, gut microbiotas are considerably more stable over the passage of time than in rodent models. Additionally, the intra-individual variability is reduced in mice compared to that of humans and pigs [113]. Generally, the microbiota of pigs and humans also share similar diversities and dominant phyla, including Firmicutes and Bacteroides [114, 115]. For these reasons, there is increased potential for determining the mechanisms underlying early microbiota-host interactions in human infants using piglet rather than rodent pup models (**See Figure 1.2**).

As food animals, there is wide public acceptance of piglet use in research, which can be problematic for other non-rodent species such as primates, dogs, and horses. Piglets are particularly prone to ID and will consistently and rapidly develop IDA in the absence of iron supplements [116, 117]. Indeed, iron deficiency has been an established issue in the pig industry since the early 20th century [118] when oral administration of iron salts, as a preventive measure, was first proposed. However, today an early intra-muscular injection of 200mg of iron is standard husbandry practice throughout the pig industry to prevent the early onset of ID/IDA [119]. Piglets are born with very low iron reserves (35-50 mg) which are only sufficient for 3-4 days since daily iron requirements range from 7 to 16 mg [120]. Serum iron at day 4 after birth reduces by 5-fold in non-supplemented piglets and is barely detectable after 6 days [121]. The situation is exacerbated by the rapid increase in litter size over recent years from 12 to 16 piglets [122] thus placing further iron demands on sows. Therefore, the sow-piglet dyad provides a highly useful potential model in the exploration of how manipulation of maternal feed practices and other interventions may improve iron status in offspring.

Poor efficiency of iron transfer through the placenta is an important contributing factor for the relatively low maternal iron endowment received prior to birth in both humans [123] and pigs [124]. In the study by Colomer *et al* [125], 156 infants were closely monitored during their first postnatal year. The risk of developing anaemia was increased by 6.57-fold in infants born to mothers with anaemia (<12 ng/ml) at the time of delivery. The ‘perfect parasite’ is a phrase often used due to the misconception that the foetus is capable of procuring enough iron irrespective of the mother’s iron status. Although

iron is transferred to foetal piglets during gestation, iron supplementation in sows during pregnancy leads to only limited improvements in iron status in offspring and is insufficient to combat the development of IDA in piglets [126]. Similarly in anaemic humans, while iron supplementation during pregnancy improves maternal iron status and may improve pregnancy outcome, including birth weight and reductions in pre-term births, brief periods of iron supplementation are unlikely to counter anaemia in off-spring. Increasing the iron endowment received by infants probably requires improved maternal iron status before the pregnancy begins [127].

A further factor contributing to the development of ID in piglets is the relatively low iron content in sow milk (0.2-0.4 mg per Litre) [128]. From this, piglets can absorb ~60-90% resulting in around 1mg of iron per day which is insufficient to prevent ID in suckling piglets [126]. This is similar to humans where breast milk contains around 0.4mg/L [129]. However, there is a remarkable capacity for transfer of serum iron to milk in rodents resulting in concentrations of ~5mg/litre, sufficient to sustain off-spring iron status before weaning [130]. Moreover, piglets have the highest growth rates of livestock animals typically increasing their plasma volume by 30% as well as doubling their weight in the first week of life [131] followed by a 10-fold increase from birth weight over the following 5 weeks [132]. Most of the functionally active iron (60%) resides in the form of haemoglobin and the majority of the remainder is required for adequate enzymic function and the generation of myoglobin [120]. Liver iron stores and sow milk together cannot meet such high iron requirement in piglets.

The gut of suckling piglets develops very widely with a number of changes happening at the same time including increases in crypt depth, reductions in villus height and reductions in vacuole size [124]. The rate of vacuole disappearance is very much relevant to maturation of the gut. However, due to early weaning practices these days, piglet gut cannot develop naturally, and this is why, perhaps, pigs lack the capacity to cope with the environmental stress and early weaning leads to diarrhoea and intestinal damage [133]. Besides this, the molecular machinery of iron uptake in piglets is relatively immature compared to humans, especially villi length and crypt depth, and reduced expression of iron transporters [134].

1.9 Hypotheses

Since piglets have natural potential to become anaemic in the absence of iron treatment, here we test the following hypotheses to explore how the different systems of this model respond to IDA and different iron treatments.

Hypothesis 1

As iron is one of the key factors for growth and development, IDA will result in reduced growth and impaired metabolism. Different iron treatments will prevent IDA and will have differential impacts on both metabolism and growth.

Hypothesis 2

Components of the gut bacterial population have differential iron requirements. Thus, iron availability in the gut during the neonatal period will impact the development of the gut microbiota in terms of both composition and metabolic output.

Hypothesis 3

Since iron has a key role in overall growth and development and lipids play a key role in early development. IDA will affect the lipid profile and different iron treatments will have distinct impacts on these profiles.

1.10 Aims

- To assess the impact of IDA and different types of iron treatments on infant growth rate.
- To evaluate the effect of IDA and iron treatments on haematological parameters and systemic metabolite production
- To analyze the impact of IDA, and oral and IM iron treatments on both the composition and metabolic activity of the gut microbiota
- To assess the impact of IDA, and oral and IM iron treatments on systemic lipid profiles

1.11 Objectives

- To determine how IDA and different iron treatments affected growth and markers of iron status in infants, a neonatal piglet model was used.
- Haematological parameters were quantified in non-supplemented (IDA) and iron supplemented piglets using portable haem meter and standard veterinary laboratory methods.

- Lipidomic profiles and systemic metabolite production in IDA and iron treated piglets were measured using targeted analytical technique, liquid chromatography / mass spectroscopy (LC-MS).

- Gut microbial composition in terminal rectal samples of IDA and iron treated piglets were analysed using a next generation Illumina sequencing technique.

1.12 References

1. Symonds, M.E. *et al.* Early life nutritional programming of obesity: mother-child cohort studies. *Annals of Nutrition & Metabolism* **62**, 137-145 (2013).

2. Vaiserman, A.M. Early-Life Nutritional Programming of Type 2 Diabetes: Experimental and Quasi-Experimental Evidence. *Nutrients* **9**, 236 (2017).

3. Carolan-Olah, M., Duarte-Gardea, M. & Lechuga, J. A critical review: early life nutrition and prenatal programming for adult disease. *Journal of Clinical Nursing* **24**, 3716-3729 (2015).

4. Boldt, D.H. New perspectives on iron: an introduction. *The American Journal of the Medical Sciences* **318**, 207-212 (1999).

5. Read, A.D., Bentley, R.E., Archer, S.L. & Dunham-Snary, K.J. Mitochondrial iron–sulfur clusters: Structure, function, and an emerging role in vascular biology. *Redox Biology* **47**, 102164 (2021).

6. Anderson, G.J., Frazer, D.M. & McLaren, G.D. Iron absorption and metabolism. *Current Opinion in Gastroenterology* **25**, 129-135 (2009).

7. Pajarillo, E.A.B., Lee, E. & Kang, D.-K. Trace metals and animal health: Interplay of the gut microbiota with iron, manganese, zinc, and copper. *Animal Nutrition* **7**, 750-761 (2021).

8. Camaschella, C. Iron deficiency. *Blood* **133**, 30 (2019).

9. Li, M. *et al.* Prevalence of Anemia among Chinese Rural Residents. *Nutrients* **9**, 192 (2017).

10. Domellöf, M., Dewey, K.G., Lönnerdal, B., Cohen, R.J. & Hernell, O. The Diagnostic Criteria for Iron Deficiency in Infants Should Be Reevaluated. *The Journal of Nutrition* **132**, 3680-3686 (2002).

11. Akhtar, S. *et al.* Iron status of the Pakistani population-current issues and strategies. *Asia Pacific Journal of Clinical Nutrition* **22**, 340-347 (2013).

12. Wieser, S. *et al.* Societal Costs of Micronutrient Deficiencies in 6- to 59-month-old Children in Pakistan. *Food Nutrition Bulleton* **38**, 485-500 (2017).

13. Plessow, R. *et al.* Social Costs of Iron Deficiency Anemia in 6-59-Month-Old Children in India. *PLoS One* **10**, e0136581 (2015).

14. Brookes, M.J., Farr, A., Phillips, C.J. & Trudgill, N.J. Management of iron deficiency anaemia in secondary care across England between 2012 and 2018: a real-world analysis of hospital episode statistics. *Frontline Gastroenterology* **12**, 363-369 (2021).

15. Brabin, B.J., Hakimi, M. & Pelletier, D. An analysis of anemia and pregnancy-related maternal mortality. *The Journal of Nutrition* **131**, S604-S615 (2001).

16. Dallman, P.R., Siimes, M.A. & Stekel, A. Iron deficiency in infancy and childhood. *The American Journal of Clinical Nutrition* **33**, 86-118 (1980).

17. Alleyne, M., Horne, M.K. & Miller, J.L. Individualized treatment for iron-deficiency anemia in adults. *American Journal of Medicine* **121**, 943-948 (2008).

18. Ziegler, E.E., Nelson, S.E. & Jeter, J.M. Iron stores of breastfed infants during the first year of life. *Nutrients* **6**, 2023-2034 (2014).

19. Qasem, W.A. & Friel, J.K. An Overview of Iron in Term Breast-Fed Infants. *Clinical Medicinal Insights of Pediatrics* **9**, 79-84 (2015).

20. Armitage, A.E. *et al.* Rapid growth is a dominant predictor of hepcidin suppression and declining ferritin in Gambian infants. *Haematologica* **104**, 1542-1553 (2019).

21. Andrew, E.A. *et al.* Rapid growth is a dominant predictor of hepcidin suppression and declining ferritin in Gambian infants. *Haematologica* **104**, 1542-1553 (2019).

22. Gilley, S.P. & Krebs, N.F. Chapter 1 - Infant nutrition. In: Marriott, B.P., Birt, D.F., Stallings, V.A. & Yates, A.A. (eds). *Present Knowledge in Nutrition (Eleventh Edition)*. Academic Press, 2020, pp 3-22.

23. Fisher, A.L. & Nemeth, E. Iron homeostasis during pregnancy. *The American Journal of Clinical Nutrition* **106**, 1567S-1574S (2017).

24. Bothwell, T.H. Iron requirements in pregnancy and strategies to meet them. *The American Journal of Clinical Nutrition* **72**, 257S-264S (2000).

25. McMahon, L.P. Iron deficiency in pregnancy. *Obstetrics Medicine* **3**, 17-24 (2010).

26. Kinyoki, D. *et al.* Anemia prevalence in women of reproductive age in low- and middle-income countries between 2000 and 2018. *Nature Medicine* **27**, 1761-1782 (2021).

27. Lönnerdal, B., Georgieff, M.K. & Hernell, O. Developmental physiology of iron absorption, homeostasis, and metabolism in the healthy term infant. *The Journal of Pediatrics* **167**, S8-S14 (2015).

28. Organization, W.H. Nutritional anaemias: tools for effective prevention and control [Internet]. Geneva, Switzerland: World Health Organization; 2017.

29. Moreno-Fernandez, J., Ochoa, J.J., Latunde-Dada, G.O. & Diaz-Castro, J. Iron Deficiency and Iron Homeostasis in Low Birth Weight Preterm Infants: A Systematic Review. *Nutrients* **11**, 1090 (2019).

30. Organization, W.H. *Guideline: daily iron supplementation in infants and children*. World Health Organization, 2016.

31. Organization, W.H.O.F.a.A. Vitamin and Mineral Requirements in Human Nutrition. Geneva, Switzerland; 2004.

32. Hadley, C. & DeCaro, J.A. Does moderate iron deficiency protect against childhood illness? A test of the optimal iron hypothesis in Tanzania. *American Journal of Physical Anthropology* **157**, 675-679 (2015).

33. Drakesmith, H. & Prentice, A.M. Hepcidin and the iron-infection axis. *Science* **338**, 768-772 (2012).

34. Camaschella, C. New insights into iron deficiency and iron deficiency anemia. *Blood Reviews* **31**, 225-233 (2017).

35. Piovanetti, Y. Breastfeeding Beyond 12 Months: An Historical Perspective. *Pediatric Clinics of North America* **48**, 199-206 (2001).

36. Spottiswoode, N., Duffy, P.E. & Drakesmith, H. Iron, anemia and hepcidin in malaria. *Frontiers in Pharmacology* **5** (2014).

37. Nemeth, E. *et al.* Hepcidin, a putative mediator of anemia of inflammation, is a type II acute-phase protein. *Blood* **101**, 2461-2463 (2003).

38. Berglund, S., Westrup, B. & Domellöf, M. Iron supplements reduce the risk of iron deficiency anemia in marginally low birth weight infants. *Pediatrics* **126**, e874-e883 (2010).

39. Domellöf, M. *et al.* Iron requirements of infants and toddlers. *Journal of Pediatric Gastroenterology and Nutrition* **58**, 119-129 (2014).

40. WHO. Daily iron supplementation in infants and children Guideline; 2016.

41. Swan, H. & Jowett, G. Treatment of iron deficiency with ferrous fumarate. *British Medical Journal* **2**, 782 (1959).

42. McDiarmid, T. & Johnson, E.D. Are any oral iron formulations better tolerated than ferrous sulfate? (2002).

43. Santiago, P. Ferrous versus ferric oral iron formulations for the treatment of iron deficiency: a clinical overview. *The Scientific World Journal* **2012** (2012).

44. Finlayson-Trick, E.C., Fischer, J.A., Goldfarb, D.M. & Karakochuk, C.D. The effects of iron supplementation and fortification on the gut microbiota: a review. *Gastrointestinal Disorders* **2**, 327-340 (2020).

45. Schümann, K., Elsenhans, B. & Mäurer, A. Iron supplementation. *Journal of trace elements in medicine and biology* **12**, 129-140 (1998).

46. Silverstein, S.B. & Rodgers, G.M. Parenteral iron therapy options. *American journal of hematology* **76**, 74-78 (2004).

47. Crary, S.E., Hall, K. & Buchanan, G.R. Intravenous iron sucrose for children with iron deficiency failing to respond to oral iron therapy. *Pediatric Blood & Cancer* **56**, 615-619 (2011).

48. Means Jr, R.T. & Weber, K.T. Hepcidin and iron regulation in health and disease. *The American Journal of the Medical Sciences* **345**, 57-60 (2013).

49. Powers, J.M., Shamoun, M., McCavit, T.L., Adix, L. & Buchanan, G.R. Intravenous Ferric Carboxymaltose in Children with Iron Deficiency Anemia Who Respond Poorly to Oral Iron. *The Journal of Pediatrics* **180**, 212-216 (2017).

50. Dan, W. *et al.* Treatments for Iron Deficiency (ID): Prospective Organic Iron Fortification. *Current Pharmaceutical Design* **25**, 325-332 (2019).

51. Powell, J.J. *et al.* A nano-disperse ferritin-core mimetic that efficiently corrects anemia without luminal iron redox activity. *Nanomedicine* **10**, 1529-1538 (2014).

52. Ovesen, L. *et al.* Intraluminal pH in the stomach, duodenum, and proximal jejunum in normal subjects and patients with exocrine pancreatic insufficiency. *Gastroenterology* **90**, 958-962 (1986).

53. Jacobs, A. & Miles, P. Intraluminal transport of iron from stomach to small-intestinal mucosa. *British Medical Journal* **4**, 778-781 (1969).

54. Cook, J.D. Diagnosis and management of iron-deficiency anaemia. *Best Practice & Research Clinical Haematology* **18**, 319-332 (2005).

55. Thursby, E. & Juge, N. Introduction to the human gut microbiota. *Biochemical Journal* **474**, 1823-1836 (2017).

56. Sekirov, I., Russell, S.L., Antunes, L.C.M. & Finlay, B.B. Gut microbiota in health and disease. *Physiological Reviews* (2010).

57. Thursby, E. & Juge, N. Introduction to the human gut microbiota. *Biochemical Journal* **474**, 1823-1836 (2017).

58. Lund, E.K., Wharf, S.G., Fairweather-Tait, S.J. & Johnson, I.T. Increases in the concentrations of available iron in response to dietary iron supplementation are associated with changes in crypt cell proliferation in rat large intestine. *The Journal of Nutrition* **128**, 175-179 (1998).

59. Wooldridge, K.G. & Williams, P.H. Iron uptake mechanisms of pathogenic bacteria. *FEMS Microbiology Reviews* **12**, 325-348 (1993).

60. Abbas, M., Hayirli, Z., Drakesmith, H., Andrews, S.C. & Lewis, M.C. Effects of iron deficiency and iron supplementation at the host-microbiota interface: Could a piglet model unravel complexities of the underlying mechanisms? *Frontiers in Nutrition*, 1963 (2022).

61. Kortman, G.A.M., Boleij, A., Swinkels, D.W. & Tjalsma, H. Iron availability increases the pathogenic potential of Salmonella typhimurium and other enteric pathogens at the intestinal epithelial interface. *PloS one* **7**, e29968-e29968 (2012).

62. Mevissen-Verhage, E.A., Marcelis, J.H., Harmsen-Van Amerongen, W.C., de Vos, N.M. & Verhoef, J. Effect of iron on neonatal gut flora during the first three months of life. *European Journal of Clinical Microbiology* **4**, 273-278 (1985).

63. Zimmermann, M.B. *et al.* The effects of iron fortification on the gut microbiota in African children: a randomized controlled trial in Cote d'Ivoire. *American Journal of Clinical Nutrition* **92**, 1406-1415 (2010).

64. Spottiswoode, N., Duffy, P.E. & Drakesmith, H. Iron, anemia and hepcidin in malaria. *Frontiers in Pharmacology* **5**, 125 (2014).

65. Yu, X. *et al.* Increased Iron Availability Aggravates the Infection of Escherichia coli O157: H7 in Mice. *Biological Trace Element Research* **190**, 457-465 (2019).

66. Nandal, A. *et al.* Induction of the ferritin gene (ftnA) of Escherichia coli by Fe2+–Fur is mediated by reversal of H-NS silencing and is RyhB independent. *Molecular Microbiology* **75**, 637-657 (2010).

67. Cassat, J.E. & Skaar, E.P. Iron in infection and immunity. *Cell Host & Microbe* **13**, 509-519 (2013).

68. Parmanand, B.A. *et al.* A decrease in iron availability to human gut microbiome reduces the growth of potentially pathogenic gut bacteria; an in vitro colonic fermentation study. *The Journal of Nutritional Biochemistry* **67**, 20-27 (2019).

69. Naikare, H., Palyada, K., Panciera, R., Marlow, D. & Stintzi, A. Major Role for FeoB in Campylobacter jejuni Ferrous Iron Acquisition, Gut Colonization, and Intracellular Survival. *Infection and Immunity* **74**, 5433 (2006).

70. Dostal, A., Fehlbaum, S., Chassard, C., Zimmermann, M.B. & Lacroix, C. Low iron availability in continuous in vitro colonic fermentations induces strong dysbiosis of the child gut microbial consortium and a decrease in main metabolites. *FEMS Microbiology Ecology* **83**, 161-175 (2013).

71. Muleviciene, A., D'Amico, F., Turroni, S., Candela, M. & Jankauskiene, A. Iron deficiency anemia-related gut microbiota dysbiosis in infants and young children: A pilot study. *Acta Microbiologica et Immunologica Hungarica* **65**, 551-564 (2018).

72. Gera, T. & Sachdev, H.P.S. Effect of iron supplementation on incidence of infectious illness in children: systematic review. *BMJ* **325**, 1142 (2002).

73. Richard, S.A. *et al.* Zinc and iron supplementation and malaria, diarrhea, and respiratory infections in children in the Peruvian Amazon. *The American Journal of Tropical Medicine and Hygiene* **75**, 126-132 (2006).

74. Chang, S. *et al.* Supplementing iron and zinc: double blind, randomized evaluation of separate or combined delivery. *European Journal of Clinical Nutrition* **64**, 153-160 (2010).

75. Moretti, D. *et al.* Extruded rice fortified with micronized ground ferric pyrophosphate reduces iron deficiency in Indian schoolchildren: a double-blind randomized controlled trial. *The American Journal of Clinical Nutrition* **84**, 822-829 (2006).

76. Manger, M.S. *et al.* A micronutrient-fortified seasoning powder reduces morbidity and improves short-term cognitive function, but has no effect on anthropometric measures in primary school children in northeast Thailand: a randomized controlled trial. *The American Journal of Clinical Nutrition* **87**, 1715-1722 (2008).

77. Jaeggi, T. *et al.* Iron fortification adversely affects the gut microbiome, increases pathogen abundance and induces intestinal inflammation in Kenyan infants. *Gut* **64**, 731 (2015).

78. Stokes, C.R. *et al.* Postnatal development of intestinal immune system in piglets: implications for the process of weaning. *Animal Research* **53**, 325-334 (2004).

79. Merrifield, C.A. *et al.* Neonatal environment exerts a sustained influence on the development of the intestinal microbiota and metabolic phenotype. *Isme Journal* (2015).

80. Eisenstein, R.S. & Ross, K.L. Novel roles for iron regulatory proteins in the adaptive response to iron deficiency. *The Journal of Nutrition* **133**, 1510S-1516S (2003).

81. McClorry, S. *et al.* Anemia in infancy is associated with alterations in systemic metabolism and microbial structure and function in a sex-specific manner: an

observational study. *The American Journal of Clinical Nutrition* **108**, 1238-1248 (2018).

82. Kang, W., Suzuki, M., Saito, T. & Miyado, K. Emerging role of TCA cycle-related enzymes in human diseases. *International Journal of Molecular Sciences* **22**, 13057 (2021).

83. McClorry, S. *et al.* Anemia During Infancy Affects the Fecal Microbiome and Fecal and Serum Metabolomes. *The FASEB Journal* **31**, 958.916-958.916 (2017).

84. Oexle, H., Gnaiger, E. & Weiss, G. Iron-dependent changes in cellular energy metabolism: influence on citric acid cycle and oxidative phosphorylation. *Biochimica et Biophysica Acta (BBA)-Bioenergetics* **1413**, 99-107 (1999).

85. Brosnan, J.T. & Brosnan, M.E. Creatine metabolism and the urea cycle. *Molecular Genetics and Metabolism* **100**, S49-S52 (2010).

86. Dong, Z. *et al.* Effects of Iron Deficiency on Serum Metabolome, Hepatic Histology, and Function in Neonatal Piglets. *Animals* **10**, 1353 (2020).

87. Enko, D. *et al.* Branched-chain amino acids are linked with iron metabolism. *Annals of Translational Medicine* **8**, 1569 (2020).

88. Rockfield, S. *et al.* Links Between Iron and Lipids: Implications in Some Major Human Diseases. *Pharmaceuticals (Basel)* **11** (2018).

89. Ahmed, U., Latham, P.S. & Oates, P.S. Interactions between hepatic iron and lipid metabolism with possible relevance to steatohepatitis. *World Journal of Gastroenterology: WJG* **18**, 4651 (2012).

90. Xiong, Q. *et al.* Regulation of a High-Iron Diet on Lipid Metabolism and Gut Microbiota in Mice. *Animals* **12**, 2063 (2022).

91. Tang, M., Chen, Z., Wu, D. & Chen, L. Ferritinophagy/ferroptosis: Iron-related newcomers in human diseases. *Journal of Cellular Physiology* **233**, 9179-9190 (2018).

92. Imai, H., Matsuoka, M., Kumagai, T., Sakamoto, T. & Koumura, T. Lipid peroxidation-dependent cell death regulated by GPx4 and ferroptosis. *Apoptotic and Non-apoptotic Cell Death*, 143-170 (2017).

93. Jiang, X., Stockwell, B.R. & Conrad, M. Ferroptosis: mechanisms, biology and role in disease. *Nature Reviews Molecular Cell Biology* **22**, 266-282 (2021).

94. Jonker, F.A., Te Poel, E., Bates, I. & Boele van Hensbroek, M. Anaemia, iron deficiency and susceptibility to infection in children in sub-Saharan Africa, guideline dilemmas. *British Journal of Haematology* **177**, 878-883 (2017).

95. Cross, J.H. *et al.* Oral iron acutely elevates bacterial growth in human serum. *Scientific Reports* **5**, 16670 (2015).

96. Paganini, D. *et al.* Prebiotic galacto-oligosaccharides mitigate the adverse effects of iron fortification on the gut microbiome: a randomised controlled study in Kenyan infants. *Gut* **66**, 1956 (2017).

97. Simonyté Sjödin, K. *et al.* Administration of ferrous sulfate drops has significant effects on the gut microbiota of iron-sufficient infants: a randomised controlled study. *Gut* **68**, 2095-2097 (2019).

98. Alexeev, E.E., He, X., Slupsky, C.M. & Lönnerdal, B. Effects of iron supplementation on growth, gut microbiota, metabolomics and cognitive development of rat pups. *Plos One* **12**, e0179713 (2017).

99. Lubs, H.A., Stevenson, R.E. & Schwartz, C.E. Fragile X and X-linked intellectual disability: four decades of discovery. *The American Journal of Human Genetics* **90**, 579-590 (2012).

100. Bryda, E.C. The Mighty Mouse: the impact of rodents on advances in biomedical research. *Molecular Medicine* **110**, 207-211 (2013).

101. Fillebeen, C. *et al.* Mice are poor heme absorbers and do not require intestinal Hmox1 for dietary heme iron assimilation. *Haematologica* **100**, e334-e337 (2015).

102. Merrifield, C.A. *et al.* Weaning diet induces sustained metabolic phenotype shift in the pig and influences host response to Bifidobacterium lactis NCC2818. *Gut* **62**, 842-851 (2013).

103. Christoforidou, Z. *et al.* Development of Immune Cells in the Intestinal Mucosa Can Be Affected by Intensive and Extensive Farm Environments, and Antibiotic Use. *Frontiers in Immunology* **9**, 1061 (2018).

104. Groenen, M.A.M. *et al.* Analyses of pig genomes provide insight into porcine demography and evolution. *Nature* **491**, 393-398 (2012).

105. Meurens, F., Summerfield, A., Nauwynck, H., Saif, L. & Gerdts, V. The pig: a model for human infectious diseases. *Trends in Microbiology* **20**, 50-57 (2012).

106. Sangild, P.T. *et al.* Invited review: the preterm pig as a model in pediatric gastroenterology. *Journal of Animal Science* **91**, 4713-4729 (2013).

107. Bassols, A. *et al.* The pig as an animal model for human pathologies: A proteomics perspective. *Proteomics. Clinical applications* **8**, 715-731 (2014).

108. Pacha, J. Development of intestinal transport function in mammals. *Physiological Reviews* **80**, 1633-1667 (2000).

109. Pabst, R. The pig as a model for immunology research. *Cell and Tissue Research* **380**, 287-304 (2020).

110. Wernersson, R. *et al.* Pigs in sequence space: A 0.66X coverage pig genome survey based on shotgun sequencing. *BMC Genomics* **6**, - (2005).

111. Jorgensen, F.G. *et al.* Comparative analysis of protein coding sequences from human, mouse and the domesticated pig. *BMC Biology* **3**, - (2005).

112. Dawson, H. The Minipig in Biomedical Research. Boca Raton: CRC Press; 2011.

113. Thompson, C.L., Hofer, M.J., Campbell, I.L. & Holmes, A.J. Community dynamics in the mouse gut microbiota: a possible role for IRF9-regulated genes in community homeostasis. *PLoS One* **5**, - (2010).

114. Rinninella, E. *et al.* What is the Healthy Gut Microbiota Composition? A Changing Ecosystem across Age, Environment, Diet, and Diseases. *Microorganisms* **7**, 14 (2019).

115. Hatcher, H.C., Singh, R.N., Torti, F.M. & Torti, S.V. Synthetic and natural iron chelators: therapeutic potential and clinical use. *Future Medicince Chemical* **1**, 1643-1670 (2009).

116. Antonides, A., van Laarhoven, S., van der Staay, F.J. & Nordquist, R.E. Non-anemic Iron Deficiency from Birth to Weaning Does Not Impair Growth or Memory in Piglets. *Frontiers in Behavioural Neuroscience* **10**, 112-112 (2016).

117. Schubert, R. *et al.* Neuroimaging of a minipig model of Huntington's disease: Feasibility of volumetric, diffusion-weighted and spectroscopic assessments. *Journal of Neuroscience Methods* **265**, 46-55 (2016).

118. McGowan, J.P. & Crichton, A. Iron deficiency in pigs. *Biochemical Journal* **18**, 265 (1924).

119. Bhattarai, S. & Nielsen, J.P. Association between hematological status at weaning and weight gain post-weaning in piglets. *Livestock Science* **182**, 64-68 (2015).

120. Jolliff, J. & Mahan, D. Effect of injected and dietary iron in young pigs on blood hematology and postnatal pig growth performance. *Journal of Animal Science* **89**, 4068-4080 (2011).

121. Lipinski, P. *et al.* Benefits and risks of iron supplementation in anemic neonatal pigs. *The American Journal of Pathology* **177**, 1233-1243 (2010).

122. Ward, S.A., Kirkwood, R.N. & Plush, K.J. Are Larger Litters a Concern for Piglet Survival or an Effectively Manageable Trait? *Animals* **10**, 309 (2020).

123. Radlowski, E.C. & Johnson, R.W. Perinatal iron deficiency and neurocognitive development. *Frontiers in Human Neuroscience* **7**, 585 (2013).

124. Szudzik, M. *et al.* Iron supplementation in suckling piglets: An ostensibly easy therapy of neonatal iron deficiency anemia. *Pharmaceuticals* **11**, 128 (2018).

125. Colomer, J. *et al.* Anaemia during pregnancy as a risk factor for infant iron deficiency: report from the Valencia Infant Anaemia Cohort (VIAC) study. *Paediatric and Perinatal Epidemiology* **4**, 196-204 (1990).

126. Svoboda, M. & Drabek, J. Iron deficiency in suckling piglets: etiology, clinical aspects and diagnosis. *Folia Vet* **49**, 104-111 (2005).

127. Scholl, T.O. Maternal iron status: relation to fetal growth, length of gestation, and iron endowment of the neonate. *Nutrition Reviews* **69 Suppl 1**, S23-S29 (2011).

128. Li, Y. *et al.* Effect of different sources and levels of iron in the diet of sows on iron status in neonatal pigs. *Animal Nutrition* **4**, 197-202 (2018).

129. C, C., SV, H. & JK, F. Breast milk iron concentrations may be lower than previously reported: implications for exclusively breastfed infants. *Maternal and Pediatric Nutrition Journal* **2** (2015).

130. Zhang, P. *et al.* The effect of serum iron concentration on iron secretion into mouse milk. *The Journal of physiology* **522 Pt 3**, 479-491 (2000).

131. Svoboda, M. & Píšťková, K. Oral iron administration in suckling piglets–a review. *Acta Veterinaria Brno* **87**, 77-83 (2018).

132. Kim, J.C., Wilcock, P. & Bedford, M.R. Iron status of piglets and impact of phytase superdosing on iron physiology: A review. *Animal Feed Science and Technology* **235**, 8-14 (2018).

133. Zhou, J., Dong, Z., Wan, D., Wang, Q., Haung, J., Huang, P., Li, Y., Ding, X., Li, J., Yang, H. and Yin, Y. Effects of iron on intestinal development and epithelial maturation of suckling piglets. *Journal of Animal Science*, **98(8)**, 213 (2020).

134. Pu, Yutian, Shuhui Li, Haitao Xiong, Xiaofeng Zhang, Yizhen Wang, and Huahua Du. Iron promotes intestinal development in neonatal piglets. *Nutrients*, **10(6)**, 726 (2018).

CHAPTER 2

Effects of iron deficiency anaemia and different types of iron supplementation on growth, blood parameters and systemic metabolite production

Abstract

Iron deficiency (ID) is the primary cause of childhood micronutrient malnutrition worldwide and can lead to anaemia, impaired metabolic function, and stunted growth. Oral iron supplements are commonly used to prevent or treat ID and iron deficiency anaemia (IDA). Neonatal piglets are valuable models for ID in human infants since they rapidly develop the condition if they do not receive exogenous iron. This, along with their shared physiological characteristics with humans, result in them being useful tools for exploring the underlying mechanisms in response to different iron treatments. Twenty-four piglets were lotter-matched into 4 sex-balanced treatment groups at 1-day old: 1. Control (no-iron treatment); 2. Intramuscular iron (IM, 200mgs) injection; 3. Iron supplemented sow milk replacer (oral-iron, 150 mg/kg feed); 4. IM & oral iron for 4 weeks. Results showed, in the absence of iron supplementation, all piglets rapidly developed IDA as quantified by systemic Hb levels (p<0.001). Systemic metabolite production was significantly reduced in anaemic piglets. IDA was prevented by all iron treatments with no significant differences observed in iron parameters or metabolite production between the iron treated groups. Importantly, oral iron supplementation, in the presence or absence of IM iron, led to significant reductions in weight gain over the 28-day preweaning period (~0.5kg) compared to IM iron treated siblings (p=0.01). In conclusion, our results confirm the suitability of piglet models for exploring the physiological effects of IDA and early iron treatments. All forms of iron treatment prevented the development of ID and IDA with no significant differences in any of the blood parameters or metabolites we assessed. However, reductions in weight gain as a direct consequence of oral iron supplementation clearly has important implication for both human infants, and throughout pig industries where the use of iron-fortified sow replacer milks is becoming increasingly prevalent as litter sizes continue to grow.

2.1 Introduction

Iron deficiency (ID) is caused by insufficient body-iron levels, and this can progress to iron deficiency anaemia (IDA) which arise when body iron levels are further reduced such that erythropoiesis is compromised [1]. The clinical definition of IDA is blood haemoglobin (Hb) levels of <120g /L for women, <130 g/L for men [2], and <105 and <100 g/L for 4- and 9-month-old infants, respectively [3]. Red blood cell counts (RBC), mean corpuscular volume (MCV) and mean corpuscular haemoglobin (MCH) are considered complimentary readouts for defining IDA [4]. Being a common form of malnutrition in infants and children, IDA can contribute to stunting, a major public health concern particularly in low- and middle-income countries [5] [6], which results from impairment of the ability of infants to use dietary nutrients efficiently [7]. Indeed, during ID the use of iron for essential functions is prioritized, such as for oxygen transport, over other functions that are necessary for growth and development [8]. This can lead to a reduction in muscle mass [9] and bone density [10], which both contribute to stunting. Stunted children are at increased risk of poor physical and cognitive development, as well as poor health outcomes in later life [11]. The strong association between IDA and stunting was confirmed in Tanzanian children (24–59 months and 6–59 months) by Msaki *et al* [12] where 36.6% of 8014 children were anaemic together with being stunted or underweight. Similarly, a study in Burmese children [13] reported a link between IDA and high prevalence of stunting, low weight, and small head circumference. Taken together, these findings show that appropriate iron levels are necessary for growth potential to be achieved.

Under normal circumstances, iron reserves in neonates are depleted by half by month 4 of life [14]. Infants require at least 0.8 mg of iron per day, of which 75% is used for growth. However, breast milk contains only about 0.3 mg/L of iron with 50% absorption (0.15 mg/L) in infants up to the age of 6-7 months [15]. Considering the period between month 4 and 12 of rapid growth and development, ‘external’ iron is required to meet optimum growth and development demands of infants [16]. During this time, mostly oral, and parenteral iron (in special circumstances) are the two main forms of supplementation for infants [17]. Parenteral iron supplements include iron dextran, iron sucrose and ferric carboxymaltose [18], and are administered through a direct injection into muscle, or intravenous (IV) drip to bypass the gastrointestinal tract. However, these methods of delivery are more likely to cause side effects such as pain or infection at the injection site, iron overload, nausea and dizziness [19].

These supplements are typically used for those who cannot tolerate oral iron supplements, or when oral supplementation has failed to address ID[19]. An alternative mode of delivery is oral iron supplements in the form of liquid drops, syrups or crushed tablets that can be mixed with food or formula milk [20]. The most common oral forms of iron supplementation for infants are ferrous sulphate, ferrous gluconate and ferrous fumarate [21]. It is important to consider that oral supplements can also cause a range of side effects, such as diarrhoea and increased risk of enteric infection [22]. Iron absorption from commercially available oral supplements is limited, at around 10% in infants beyond 6-7 months old [23]. Moreover, universal iron supplementation policies in areas with a high prevenance of ID and IDA result in the delivery of additional oral iron to significant numbers of children who are not iron deficient. This is also true in more affluent societies where infant formula milk is consistently fortified with iron (1-12 mg/L) [24, 25]. The limited absorption of iron supplements results in most of the iron reaching the colon where it could significantly alter the gut microbiota composition and microbial metabolism, which is thought to be linked to increased risks of enteric infection and diarrhoea [26]. This change in the gut ecosystem will be discussed in detail in chapter 3.

During the early postnatal years of life, there is a period of rapid growth and development [27]. During this time, high energy and nutrient demands are met by enhanced metabolic rates [28] and iron plays an essential role in many metabolic processes, including oxygen transport, energy production, and the synthesis of enzymes and hormones [29]. During ID and IDA, production of adenosine triphosphate (ATP), in particular, is reduced via inhibition of metabolic enzymes [30], which is linked with decreased production of specific metabolites that are important for cellular metabolism. This has been confirmed in human infants where IDA subjects were reported to have reduced systemic concentrations of tricarboxylic acid cycle (TCA) intermediates, and some amino acids essential to the TCA cycle [31]. This can lead to reduced energy levels and poor physical endurance, and inhibition of growth and development of muscle and bone tissue [32], resulting in stunted growth. Since IDA can also lead to changes in the composition of the gut microbiota, production of microbial-derived metabolites can also be altered. Some of these metabolites are directly linked to decreases in gut barrier function leading to increased translocation of bacterial-derived products including lipopolysaccharides (LPS) [33]. Immune responses to increased LPS can contribute to the development of systemic

inflammation [34], which is linked with reduced nutrient absorption and further reductions in intestinal barrier function. Thus, IDA can result in both poor absorption of oral iron and stunting, both directly and indirectly.

As previously detailed in chapter 1, piglets are particularly prone to ID and will rapidly develop IDA in the absence of iron supplementation [35, 36]. Indeed, ID has been established as an issue in the pig industry [37] and an early intra-muscular injection of 200mg of iron during first 24 postnatal hours is standard husbandry practice throughout the industry [38]. There are similarities in iron-dependent blood parameters in pigs and humans. Although blood Hb thresholds for IDA in piglets can vary, 80-110 g/L is generally accepted as defining IDA in suckling piglets [39]. In response to ID and IDA, both species have low haemoglobin, MCV, MCH and haematocrit levels [40]. The use of pigs as models for metabolic profiling has shown great potential in advancing the understanding of various diseases and the effects of nutrition on metabolism. In addition, Merrifield *et al* [41] have reported that the metabolic profiles in different pig organs, including kidney and liver, characterized via NMR spectroscopy are qualitatively comparable to those in humans. Furthermore, in a recent study, Fanalli *et al* [42] have reported the impact of nutrition on liver related transcription factors (TF) using pigs as models for human metabolic diseases. They used 36 immuno-castrated and halothane homozygous-negative male pigs to show that TF such as EGFR and RXRA are associated with lipid balance and inflammatory response in pigs administered different amounts of dietary soybean oil. Taken together, these similarities demonstrate that pigs are valuable models for studying the effects of different dietary interventions on metabolic outcomes in humans.

2.2 Hypotheses

The two hypotheses to be tested in this study are indicated below:

Hypothesis 1

Since iron is a key factor for normal growth and development, IDA during infancy will result in stunted growth and impaired metabolism of piglet, which can be reversed by iron treatment.

Hypothesis 2

The two different forms of iron treatment (oral supplements or IM iron) will both prevent the development of IDA but will have distinct metabolic impacts.

2.3 Aims

1. To assess the effects of neonatal IDA on systemic metabolites and growth using a piglet model for human infants.
2. To determine whether the two different forms of iron treatment applied (oral and IM) rescue piglets from developing IDA similarly or whether differential metabolic/physiological effects arise.

2.4 Objectives

1. To establish piglets as a model for human infants in exploration of the effects of neonatal IDA on growth.
2. To use the piglet trial above to determine whether different types of iron supplementation (oral, IM, and oral & IM combined) affect growth to different extents.
3. To determine the impact of IDA and iron treatment on haematological parameters, using a portable haem meter and veterinary diagnostic methodologies.
4. To compare systemic metabolite production in response to IDA and iron treatment, using liquid chromatography mass spectrometry (LC-MS).

2.5 Materials and Methods

2.5.1 Animal Model

Animal housing and experimental procedures were all performed at the Centre for Dairy Research (CEDAR), University of Reading, in accordance with local ethical guidelines. All experiments were approved by the Reading Animal Welfare and Ethical Review Body (AWERB) and were performed under a UK Home Office License. To assess the impact of iron deficiency (ID) and different types of iron supplementation, 24 large white F1 hybrid piglets from 6 litters were allocated into 4 litter- and sex-matched (3 male & 3 female) treatment groups at 1 day old. Treatments were:

Group 1, control (no iron supplementation);

Group 2, intramuscular iron (IM) injection (200mg Fe);

Group 3, iron supplemented ($FeSO_4$) sow-milk replacer (SMR) (oral iron; 150mg/kg body weight/day);

Group 4, IM and oral iron (Figure 2.1).

Prior to their arrival at CEDAR, the piglets did not receive vaccinations, antibiotics or iron injections.

Initially, upon separation from their mothers, piglets were housed in 2 groups (oral iron and no oral iron) and were provided with SMR (in bowls). At 2 days old, piglets were housed in separate pens. Throughout the trial piglets, were housed at 30°C on sawdust and provided with heat lamps under a 12-hour light and dark cycle and had free access to fresh drinking water. Piglets were initially fed with 10% concentration of SMR (**Table 2.1**) every 4 hours (5 feeds/day) and this was increased to 15% and then to 20% (4 feeds/ day) over the following 2 days (standard concentration) in order to reduce the risk of scouring.

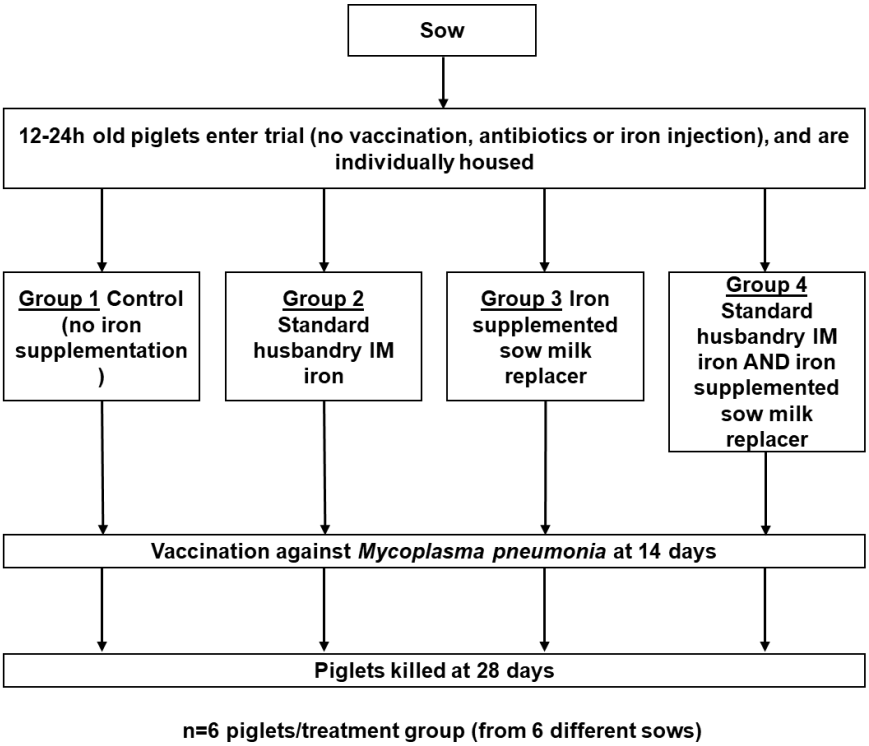

Figure 2. 1. Schematic of experimental design

Following twice-a-week weight measurements, the amount of feed and $FeSO_4$ were increased accordingly. SMR given to all piglets was calculated/measured on the bases of their latest weights to fulfil their overall growth and nutritional demands. All piglets received SMR exclusively throughout the trial (28 days), and the only difference in diet was the iron treatments. Haemoglobin (Hb) was measured weekly to monitor systemic iron using a portable haem meter (HemoCue® 201+, HemoCue AB ©) throughout the trial. Piglets were culled using an overdose of barbiturate (Dolethal®) via the jugular at day 28.

Compounds	**Concentration**
Ash	6%
Betain	114.08 mg/ Kg
Biotin	300 mg/ Kg
Calcium	0.90%
Calcium pantothenate	21 mg/ Kg
Copper	135 mg/ Kg
Crude fibre	0%
Crude oil	14%
Crude protein	22%
Folic acid	1.1 mg/ Kg
Iodine	0.25 mg/ Kg
Lysine	2.10%
Manganese	40 mg/ Kg
Niacin	30 mg/ Kg
Phosphorus	0.70%
Riboflavin	5 mg/ Kg
Selenium	0.4 mg/ Kg
Sodium	0.30%
Vitamin A	240000 IU/ Kg
Vitamin B_1	5 mg/ Kg
Vitamin B_6	5.2 mg/ Kg
Vitamin C	0.15 mg/ Kg
Vitamin D	5000 IU/ Kg
Vitamin E	125 IU/ Kg
Vitamin K	5 mg/ Kg

Zinc	50 mg/ Kg

Table 2. 1. Composition of sow milk replacer (SRM)

2.5.2 Sample collection

Blood samples were collected weekly from day 1 in EDTA vacutainers (BD Vacutainer®) from the jugular vein. On completion of the study, faecal samples were collected directly from rectum and stored temporarily on dry ice. All above samples were then stored at -80˚C.

2.5.3 Analysis

2.5.3.1 Blood parameters

In addition to weekly onsite Haemoglobin (Hb) analysis, parameters including white blood cells (WBCs), red blood cells (RBCs), platelets, mean corpuscular volume (MCV), mean cell haemoglobin (MCH), mean cell haemoglobin concentration (MCHC) and neutrophils were quantified by diagnostic Laboratories, Bristol Veterinary School, Langford, University of Bristol, UK) from baseline (day 1) and terminal (day 28) blood samples.

2.5.3.2 Metabolic profiling

2.5.3.2.1 Sample preparation

In order to assess primary metabolite production, plasma samples were analysed using liquid chromatography mass spectrometry. Plasma from the sequential blood (120) samples were aliquoted into Eppendorf tubes (50µL each) and mixed with 100µL of acetonitrile (Sigma-Aldrich, UK). This mixture was then centrifuged at ~20000 g for 10 minutes at 4°C to facilitate protein precipitation. A pooled sample was used for quality control purposes (QC). Initially, QC and blank (water with 0.1% formic acid) were analysed to evaluate the dilution impact on the results. All samples were then diluted at 1:4 (sample: water) with 0.1% formic acid.

2.5.3.2.2 Liquid chromatography mass spectrometry (LCMS)

The method published by Leung *et al*. [1] was adopted to assess the primary metabolites in plasma samples using pentafluorophenylpropyl (PFPP) as stationary phase. Separation of the target analytes in samples was performed using a HPLC (Shimadzu UK Ltd) coupled with a triple quadrupole mass spectrometer (MS) (Shimadzu Ltd, Milton Keynes). For the analysis of primary metabolites, separation was performed on a Discovery HS F5-3 column (2.1 x 150mm, internal diameter x length; 3µm particle size: Sigma-Aldrich, UK), with an injection volume of 3µl and the column oven temperature set at 40°C.

LabSolutions software (Shimadzu) was used for LCMS data processing including peak assignments. Peak area was used to analyse the metabolite concentrations and compare between the treatments (see **Table 2.2**).

Compound name	Retention time (min)	Polarity	Target ion	Confirmation ion
Cystine	1.908	(+)	241.00>151.95	241.00>73.90
Asparagine	1.953	(+)	133.10>87.15	133.10>28.05
Aspartic acid	1.953	(+)	134.00>74.05	134.00>88.10
Serine	1.960	(+)	105.90>60.10	105.90>60.10
Alanine	1.972	(+)	89.90>44.10	89.90>44.10
4-Hydroxyproline	1.991	(+)	132.10>86.05	132.10>68.05
Cystathionine	2.028	(+)	223.00>88.05	223.00>134.00
Glycine	2.029	(+)	75.90>30.15	75.90>30.15
Citicoline	2.045	(+)	489.10>184.10	489.10>264.05
Glutamine	2.073	(+)	147.10>84.15	147.10>130.10
Threonine	2.133	(+)	120.10>74.15	120.10>56.05
Cysteine	2.148	(+)	122.00>76.05	122.00>59.00
Dimethylglycine	2.189	(+)	104.10>58.05	104.10>44.05
Methionine sulfoxide	2.206	(+)	166.00>74.10	166.00>55.95
Glutamic acid	2.253	(+)	147.90>84.10	147.90>56.10
Cytidine monophosphate	2.260	(+)	324.00>112.05	324.00>95.00
Citrulline	2.321	(+)	176.10>70.05	176.10>159.05
Guanosine monophosphate	2.552	(+)	364.00>152.05	364.00>135.00
Proline	2.609	(+)	116.10>70.15	116.10>28.05
Ornitine	2.679	(+)	133.10>70.10	133.10>116.05
2-Aminobutyric acid	2.831	(+)	104.10>58.05	104.10>41.05
Lysine	2.894	(+)	147.10>84.10	147.10>84.10
Histidine	2.901	(+)	155.90>110.10	155.90>56.10
Adenosine monophosphate	2.969	(+)	348.00>136.05	348.00>97.10
Uracil	2.986	(+)	113.00>70.00	113.00>70.00
Argininosuccinic acid	3.057	(+)	291.00>70.10	291.00>116.05

Thymidine monophosphate	3.070	(+)	322.90>81.10	322.90>207.10
Homocysteine	3.188	(+)	136.00>90.10	136.00>56.10
Arginine	3.365	(+)	175.10>70.10	175.10>60.10
Creatine	3.431	(+)	132.10>44.05	132.10>90.05
4-Aminobutyric acid	3.690	(+)	104.10>87.05	104.10>45.10
Cysteamine	3.980	(+)	78.10>61.05	78.10>61.05
Cytosine	4.044	(+)	112.00>95.10	112.00>95.10
Nicotinic acid	4.080	(+)	124.05>80.05	124.05>78.05
Cytidine 3',5'-cyclic monophosphate	4.093	(+)	306.00>112.10	306.00>112.10
Hypoxanthine	4.251	(+)	137.00>55.05	137.00>110.00
Homocystine	4.321	(+)	269.00>136.05	269.00>88.00
Choline	4.436	(+)	104.10>60.05	104.10>45.10
Uridine	4.444	(+)	245.00>113.05	245.00>113.05
5-Glutamylcysteine	4.449	(+)	251.10>84.10	251.10>122.10
Glutathione	4.543	(+)	308.00>179.10	308.00>179.10
Valine	4.761	(+)	118.10>72.15	118.10>55.05
Creatinine	4.820	(+)	114.10>44.05	114.10>44.05
Norepinephrine	4.988	(+)	170.10>152.15	170.10>152.15
Carnitine	5.284	(+)	162.10>103.05	162.10>60.10
Methionine	5.304	(+)	149.90>56.10	149.90>104.10
Niacinamide	5.344	(+)	123.10>80.05	123.10>53.10
Ophthalmic acid	5.350	(+)	290.10>58.10	290.10>161.10
Carnosine	5.365	(+)	227.10>110.05	227.10>156.05
Guanosine 3',5'-cyclic monophosphate	5.393	(+)	346.00>152.05	346.00>135.05
Thymine	5.448	(+)	127.10>54.05	127.10>110.05
Histamine	5.803	(+)	112.10>95.05	112.10>41.05
Thymidine	6.175	(+)	243.10>127.10	243.10>127.10
Adenosine 3',5'-cyclic monophosphate	6.179	(+)	330.00>136.05	330.00>119.10
Adenylsuccinic acid	6.183	(+)	464.10>252.10	464.10>162.00
Guanosine	6.187	(+)	284.00>152.00	284.00>135.00
Inosine	6.211	(+)	269.10>137.05	269.10>118.95
FAD	6.213	(+)	786.15>136.10	786.15>348.10
Pantothenic acid	6.249	(+)	220.10>90.15	220.10>72.05

Dopa	6.278	(+)	198.10>152.10	198.10>152.10
Cytidine	6.393	(+)	244.10>112.05	244.10>95.00
Adenine	6.460	(+)	136.00>119.05	136.00>65.00
Tyrosine	6.694	(+)	182.10>136.10	182.10>91.10
Adenosine	6.764	(+)	268.10>136.05	268.10>119.00
Symmetric dimethylarginine	6.817	(+)	203.10>70.15	203.10>71.10
S-Adenosylmethionin	6.939	(+)	399.10>250.05	399.10>136.10
Epinephrine	7.164	(+)	184.10>166.10	184.10>77.00
Asymmetric dimethylarginine	7.207	(+)	203.10>70.10	203.10>46.10
Isoleucine	7.241	(+)	132.10>86.20	132.10>69.15
Leucine	7.520	(+)	132.10>86.05	132.10>30.05
Phenylalanine	8.068	(+)	166.10>120.10	166.10>103.10
Dopamine	8.078	(+)	154.10>91.05	154.10>137.05
S-Adenosylhomocystein	8.197	(+)	385.10>134.00	385.10>136.05
Kynurenine	8.340	(+)	209.10>192.05	209.10>94.10
Acetylcarnitine	8.929	(+)	204.10>85.05	204.10>60.10
Acetylcholine	9.165	(+)	147.10>87.05	147.10>88.05
Tryptophan	10.092	(+)	205.10>188.15	205.10>146.10
Serotonin	10.527	(+)	177.10>160.10	177.10>77.05
Allantoin	1.927	(-)	157.00>97.10	157.00>42.05
2-Morpholinoethanesulfonic acid	2.021	(-)	194.00>80.15	194.00>80.15
Methionine sulfone	2.184	(-)	180.00>79.20	180.00>79.20
2-Ketoglutaric acid	2.317	(-)	144.90>101.10	144.90>57.05
Malic acid	2.358	(-)	133.10>114.95	133.10>71.15
Isocitric acid	2.358	(-)	191.20>111.10	191.20>73.00
Pyruvic acid	2.585	(-)	86.90>87.05	86.90>42.95
Orotic acid	2.588	(-)	155.00>111.10	155.00>42.10
Lactic acid	2.795	(-)	89.30>89.05	89.30>89.05
Uric acid	3.159	(-)	167.10>123.95	167.10>96.20
Citric acid	3.209	(-)	191.20>111.10	191.20>87.05
Aconitic acid	3.536	(-)	172.90>85.05	172.90>129.10
NAD	3.882	(-)	663.10>541.05	663.10>540.10
Succinic acid	4.055	(-)	117.30>73.00	117.30>99.05

Xanthine	4.093	(-)	151.00>108.00	151.00>42.00
Fumaric acid	4.571	(-)	115.00>71.10	115.00>26.95
Guanine	5.623	(-)	150.00>133.00	150.00>66.10
FMN	6.193	(-)	455.00>97.00	455.00>78.90
Oxidized glutathione	6.253	(-)	611.10>306.00	611.10>143.05

Table 2. 2. List of target analytes with descriptive information, separated by pentafluorophenylpropyl Column (PFPP) stationary phase.

2.5.4 Statistical analysis

Growth rate, haematological parameters and LCMS data (peak area) of primary metabolites were refined using MS Excel (Microsoft ®) and analysed with general linear modelling in SPSS version 26.0 (IBM SPSS, Inc., Chicago, IL, USA) using 'treatment', and 'litter' as factors. Sex was also included as a factor however no significant difference was seen between males and females at any level, probably as a result of low numbers (n=3). A value of $p<0.05$ was considered to be significant.

2.6 Results

2.6.1 Oral iron supplementation, with and without intramuscular iron treatment, caused reduced growth

At 28 days old (end of trial), mean weights in the oral iron supplemented piglets (group 3, 3.40 kg; group 4, 3.54 kg) were significantly lower than their IM iron supplemented siblings (group 2, 4.05 kg, p=0.05). In addition, anaemic (group 1) piglet weights (3.30 kg) were significantly lower than those piglets which received IM iron only (group 2, p=0.01). This represents a significant difference of ~0.5-0.75kg between piglets which received IM iron supplementation only (group 2) compared to the other three treatment groups (**Figure 2.2a**). There were no significant differences in starting weight between any of the treatment groups. This was reflected in piglet total weight gain throughout the trial with group 2 (IM iron only) piglets showing significantly increased weight gain of 4.05kg by day 28 compared to group 1 (anaemic, 3.30kg), group 3 (oral iron only, 3.40kg) and group 4 (oral and IM iron supplementation, 3.54kg) ($p<0.05$, **Figure 2.2b**).

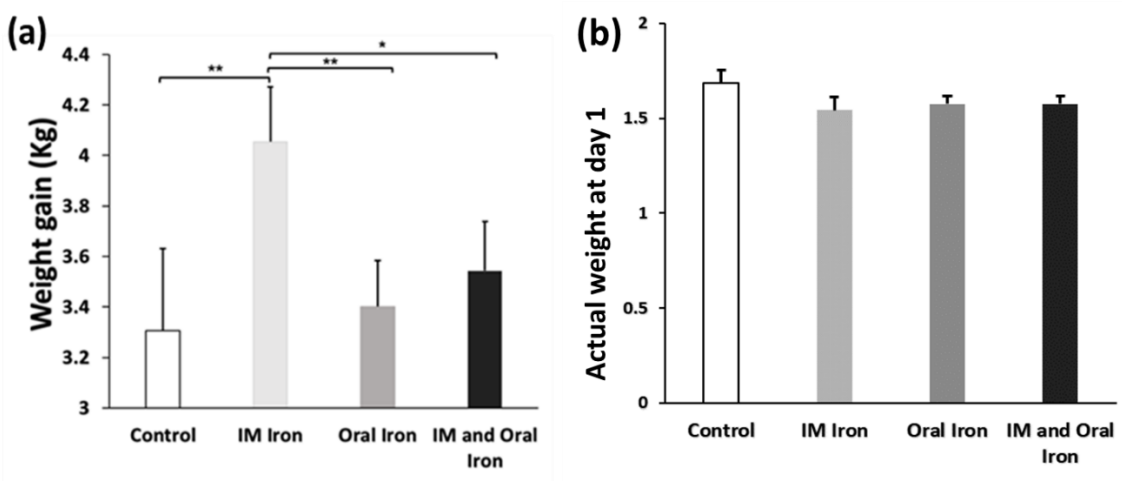

Figure 2. 2. Impact of iron deficiency anaemia (IDA) and different types of iron supplementation on growth (weight gained) in piglets between birth and 28 days old. Growth rate was significantly higher in piglets receiving intramuscular (IM) iron supplementation (200 mg) compared to control pigs which did not receive iron supplementation (p=7x10^{-3}), oral iron (p=0.01), and IM & oral iron (p=0.04) respectively (a). There was no significant difference in the initial weight of piglets between all four groups (b). IM group demonstrated the highest growth rate (4.05 kg). Oral iron supplementation ($FeSO_4$ at 150mg/kg) with and without IM supplementation, and IDA were associated with reduced growth rate. n=6 piglets/treatment group. Error bars = SEM, ** p<0.01, * p<0.05.

2.6.2 Iron supplementation prevented the development of anaemia by correcting systemic haemoglobin and haematocrit concentrations

Since the concentration of Hb in blood is a reliable indicator of iron status in piglets and other species it is often used to define IDA. All piglets that received iron treatment had significantly higher concentrations of systemic Hb (IM iron 95.17 g/L, oral iron 97.5 g/L, and IM and oral iron 104 g/L) than non-iron-treated siblings (52.17 g/L) by day 28 (p<0.001, **Figure 2.3a**). Non-iron-treated piglets developed IDA by day 8 (Hb concentrations <90 g/L). All iron-treated piglets presented with systemic Hb within normal ranges (90 g/L to 110 g/L) (**Figure 2.3b**). Haematocrit (red blood cell volume as a percentage of total blood volume) levels were significantly higher (p<0.001) in iron-treated piglets (IM iron 35.2%, oral iron 35.6%, and IM and oral iron 35.7%) compared to non-iron-treated counterparts (24.1%). No significant differences were observed between the three different iron treatment groups (groups 2-4) (**Figure 2.3c**).

2.6.3 IDA was associated with reduced red blood cell counts, mean cell volume and mean cell haemoglobin concentrations

Red blood cell (RBCs) counts are an iron-dependent blood parameter that was significantly reduced (p<0.001) in non-iron treated piglets ($4.74x10^{12}$/L) compared to iron supplemented siblings (IM iron $5.8x10^{12}$/L, oral iron $5.74x10^{12}$/L, and IM and oral iron $5.86x10^{12}$/L) (**Figure 2.3d**). However, no significant differences were observed between the three different types of iron treatment.

Mean cell volume (MCV) is the measure of the average volume of erythrocyte corpuscle and is obtained by multiplying the blood volume by the cellular portion of blood and dividing that product by the number of RBCs [2]. MCV was significantly lower (p<0.001) in non-iron treated piglets (49.9 fL) against their iron treated siblings (IM iron, 59.9 fL; oral iron only, 62.0 fL; and IM & oral iron, 60.8 fL) (**Figure 2.3e**). Mean cell haemoglobin (MCH) is the average mass of haemoglobin per RBC in a sample [3]. MCH was significantly lower (p<0.001) in non-iron-treated piglets (14.6 pg) compared to iron treated counterparts (IM iron 17.8 pg; oral iron 18.1 pg; and IM & oral iron 18.2 pg) (**Figure 2.3f**). Unlike growth rate, no significant differences were observed between piglets receiving the different types of iron treatment.

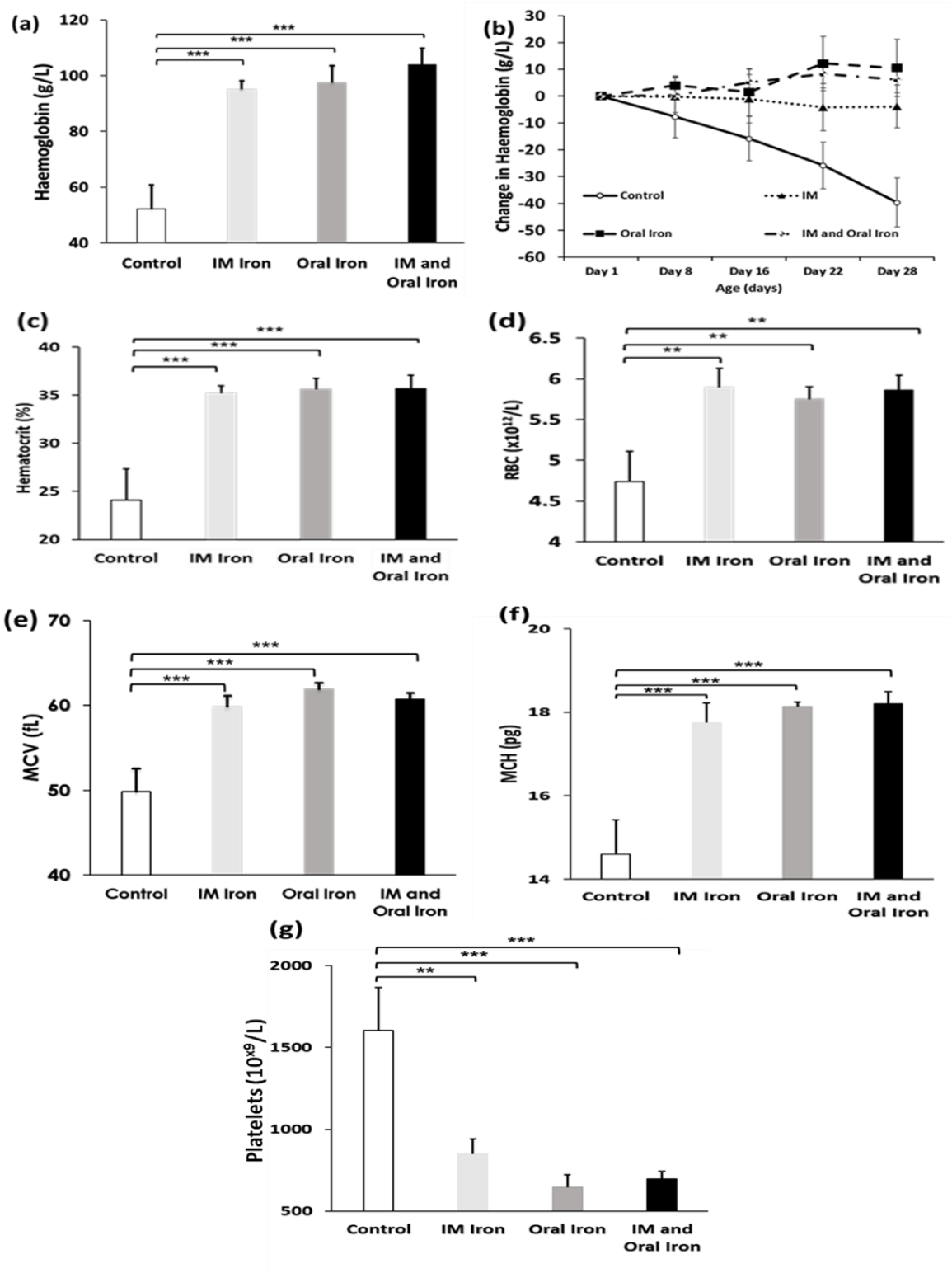

Figure 2. 3. Impact of iron deficiency anaemia (IDA) and different types of iron supplementation on haemoglobin and other iron dependent haematological parameters. Levels of Hb gradually declined in the non-supplemented piglets but were maintained in piglets receiving oral and/or intramuscular iron supplementation (**a**). Sequential changes in systemic haemoglobin (g/L) from 1 to 28 days in iron supplemented (groups 2-4 in Figure

2.1) and non-supplemented (Anaemic, group 1, no iron supplementation), actual Hb values at day 28 (**b**). Haematocrit was found in significantly higher concentrations in all iron supplemented groups (treatment groups 2-4) (p=4x10^{-4}, IM; p=3x10^{-4}, oral; p=3x10^{-4}, IM and oral) compared to control group (**c**). Significantly higher concentrations of red blood cells (RBC) were observed in all iron supplemented groups (groups 2-4) (p=0.003, IM; p=9x10^{-3}, oral; p=4x10^{-3}, IM and oral) were observed compared to non-supplemented siblings (**d**). A significantly higher value of mean cell volume (MCV) was observed in all iron supplemented groups (treatment groups 2-4) (p=2x10^{-5}, IM; p=2x10^{-5}, oral; p=9x10^{-5} respectively, IM and oral) against control group (e). Mean cell haemoglobin (MCH) was significantly higher in IM (p=2x10^{-4}), oral (p=6x10^{-5}) and IM and oral (p=4x10^{-5}) against anaemic group (**f**). Interestingly, significantly lower platelet counts were observed in all iron supplemented groups (treatment groups 2-4) (p=1x10^{-3}, IM; p=1x10^{-4}, oral; p=2x10^{-4}, IM and oral) compared to non-supplemented siblings (**g**). n=6 litter-matched piglets/treatment group. Error bars = SEM, ** p<0.01, *** p<0.001.

2.6.4 Iron supplementation resulted in lower concentrations of platelets

Iron supplementation over the 28 days significantly reduced the systemic platelet count (p<0.001) in all supplemented piglets (IM iron 850x10^9, oral iron 646x10^9, IM and oral iron 696x10^9) compared to their anaemic counterparts (1600x10^9) (**Figure 2.3g**). No significant differences were observed between the piglet groups which received the three different iron treatment regimes.

2.6.5 IDA was associated with reduced production of systemic host metabolites

2.6.5.1 Metabolites of the tricarboxylic acid cycle

The TCA is a series of reactions used by all aerobes to release stored energy via oxidation of acetyl-CoA derived from fat, carbohydrates and protein to generate ATP and reducing equivalents (e.g. NADH) [4]. It also has a critical anabolic function for instance in synthesis of amino acid precursors. Taking all treatments into consideration, three consecutive intermediates of the TCA cycle were observed in significantly lower concentrations in the anaemic (group 1) piglets compared to iron-treated siblings (groups 2-4). Firstly, citric acid concentrations were significantly higher (~ 8 folds) in the IM-iron only, oral-iron only (p<0.001) and IM- & oral-iron iron (p<0.05) treatment groups compared to the anaemic piglets in group 1 (**Figure 2.4a**). Secondly, aconitic acid concentrations were significantly higher (~ 4 folds) in the IM-

iron only, oral-iron only and the IM- and oral-ron (p<0.001) groups compared to non-iron treated siblings (**Figure 2.4b**). The third metabolite of this series, 2-ketoglutaric acid, was also significantly higher (~ 1 fold) in IM-iron only (p<0.05), and IM- & oral-iron (p<0.001) compared to the anaemic counterparts (**Figure 2.4c**). However, no significant differences were observed between the different types of iron supplementation for any of these metabolites.

2.6.5.2 Urea cycle metabolites

In the anaemic piglets (group 1), mean citrulline concentrations were significantly lower (~ 3 folds) compared to the IM, oral and IM & oral (p<0.001) iron treatment groups (**Figure 2.5a**). Arginine, another metabolite resulting from the urea cycle, was also significantly lower (~ 3 folds) in anaemic piglets compared to the IM, oral and IM & oral iron-treatment groups (p<0.001) (**Figure 2.5b**). As observed for the TCA cycle intermediates, there were no significant differences between piglets receiving the threedifferent types of iron treatment.

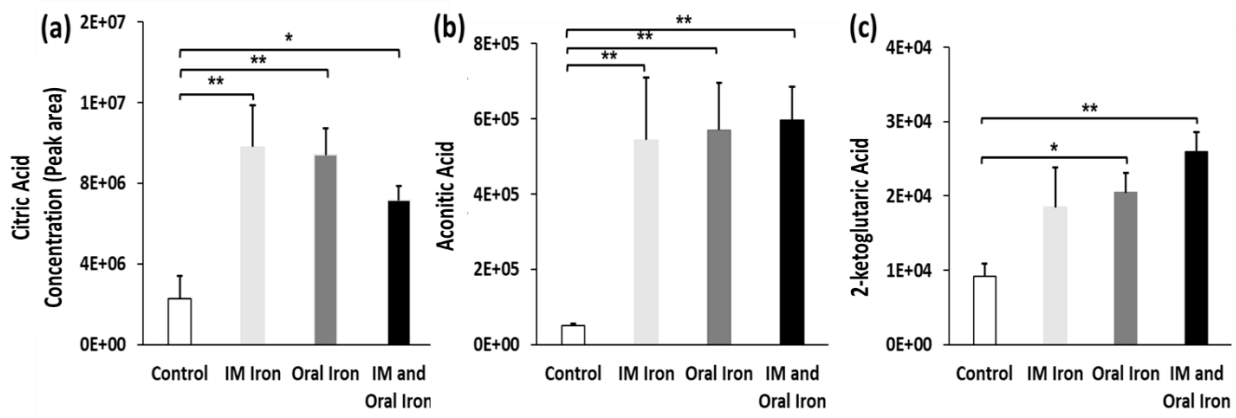

Figure 2. 4. Impact of iron deficiency anaemia IDA and iron supplementation on tricarboxylic acid (TCA) cycle at 28 days. citric acid concentrations were observed significantly higher in all iron supplemented groups (treatment groups 2-4, Figure 2.1) (p=1x10^{-3}, IM; p=1x10^{-3}, oral; p=0.02, IM and oral) compared to non-supplemented siblings (a). Aconitic acid was observed in significantly higher concentrations in all iron supplemented groups (treatment groups 2-4) (p=5x10^{-3}, IM; p=4x10^{-3}, oral; p=2x10^{-3}, IM and oral) compared to non-supplemented anaemic siblings (b). 2-ketoglutaric acid was also found in significantly higher concentrations in oral (p=0.02) and IM+ oral (p=2x10^{-3}) piglets compared to control (c). n=6 litter-matched piglets/treatment group. Error bars = SEM, * p<0.05** p<0.01.

2.6.5.3 Metabolites involved in methylation and phosphorylation, and lactic acid

Methionine and serine are metabolites crucial to the processes of methylation and phosphorylation, respectively. Both were significantly reduced in the anaemic piglets compared to the iron treated piglets. Methionine (**Figure 2.6a**) and serine (**Figure 2.6b**) were significantly higher (~ 7 folds & ~ 4 folds respectively) in IM (p<0.01and p<0.001, respectively), oral (p<0.001) and IM & oral (p<0.001) iron groups, compared to their anaemic siblings (group 1). Lactic acid concentrations were significantly higher (~ 5 folds) in IM (p<0.05), oral (p<0.001) and IM and oral iron (p<0.001) treatment groups, compared to the anaemic piglets (**Figure 2.6c**). However, there were no significant differences between the groups receiving the three different types of iron treatment.

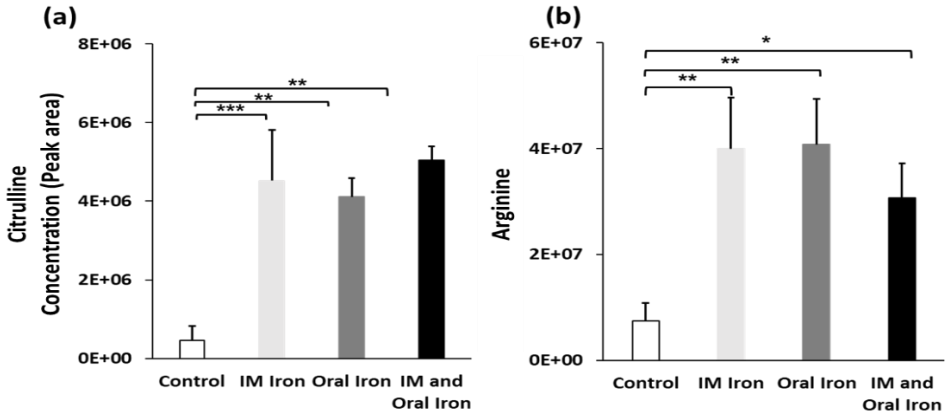

Figure 2. 5. Impact of iron deficiency anaemia (IDA) and iron supplementation on urea cycle metabolites at 28 days. Urea cycle metabolite, citrulline, concentrations were observed significantly higher in all iron supplemented groups (treatment groups 2-4) (p=8x10^{-4}, IM; p=2x10^{-3}, oral; p=2x10^{-3}, IM and oral) compared to non-supplemented siblings (a). Arginine was also found in significantly higher concentration in IM (p=5x10^{-3}) oral (p=4x10^{-3}) and IM and oral (p=0.03) groups compared to control (b). N=6 piglets/treatment group. Error bars = SEM, * p<0.05, ** p<0.01, *** p<0.001.

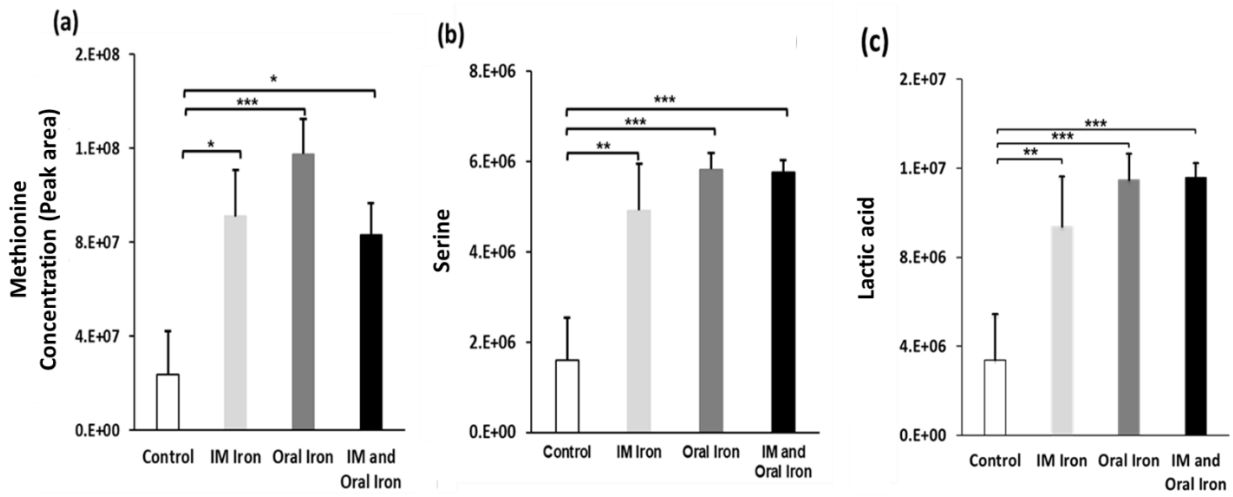

Figure 2. 6. Impact of iron deficiency anaemia (IDA)and iron supplementation on the production of metabolites involved in methylation and phosphorylation, and lactic acid at 28 days. Significantly higher concentrations of methionine were observed in all iron supplemented groups (treatment groups 2-4) (p=0.01, IM; p=7x10^{-4}, oral; p=2x10^{-3}, IM and oral) as compared to non-supplemented siblings (a). Serine was found in significantly higher concentration in IM (p=4x10^{-3}) oral (p=6x10^{-4}) and IM and oral (p=4x10^{-4}) groups compared to control which received no iron supplementation and eventually became anaemic (b). Lactic acid concentration was also significantly higher in IM (p=0.02), oral (p=2x10^{-3}) and IM and oral (p=2x10^{-3}) groups. n=6 piglets/treatment group. Error SEM, * p<0.05, ** p<0.01, *** p<0.001.

2.6.5.4 Non-essential amino acids (asparagine and glycine)

Both asparagine and glycine are non-essential amino acids [5]. Asparagine and glycine were both significantly higher (~ 1.5 folds) in IM (p<0.05), oral (p<0.001) and IM & oral (p<0.05 and p<0.001, respectively) iron-treatment groups compared to their anaemic counterparts (**Figure 2.7**).

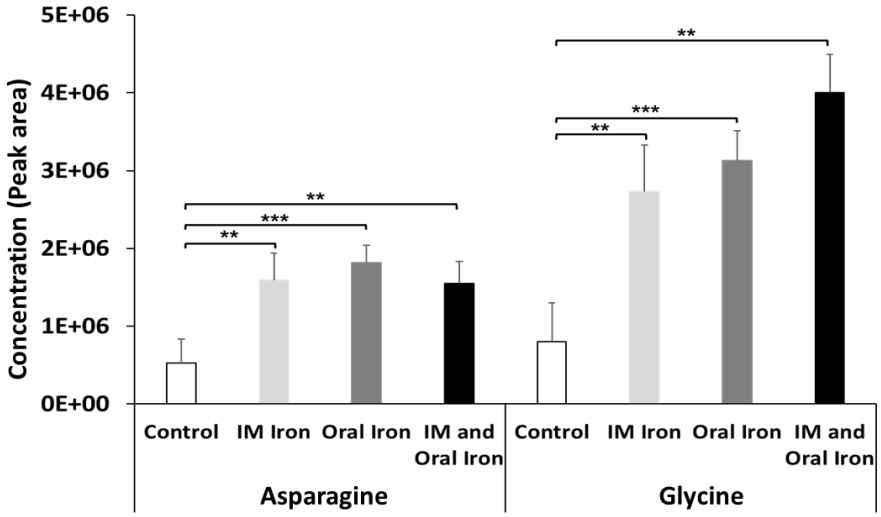

Figure 2. 7. Impact of iron deficiency anaemia (IDA)and iron supplementation on systemic concentrations of asparagine and glycine in 28-day old piglets. Asparagine was observed in significantly higher concentration in all iron supplemented groups (treatment groups 2-4, Figure 2.1) (p=0.01, IM; p=5x10^{-3}, oral; p=0.02, IM and oral respectively) compared to non-supplemented siblings at day 28. Likewise, significantly higher concentrations of glycine were observed in all iron supplemented piglets (treatment groups 2-4) (p=0.01, IM; p=3x10^{-3} oral; p=0.02, IM and oral) compared to non-supplemented siblings. n=6 piglets/treatment group. Error bars = SEM, ** p<0.01, *** p<0.001.

2.6.5.5 Glutamic acid, proline creatine, glutamine and acetylcarnitine

Glutamic acid and proline are amino acids [6], and creatine primarily facilitates the recycling of ATP in brain and muscles [7]. Glutamic acid, proline and creatine concentrations were significantly higher (~ 1, ~ 1.5 and ~ 3 folds respectively) in oral (p<0.05, p<0.05, and p<0.001, respectively) and IM & oral (p<0.001, p<0.05, and p<0.001, respectively) groups, compared to the anaemic piglets (**Figure 2.8**).

Glutamine is a non-essential amino acid [8] and acetylcarnitine is mostly involved in brain metabolism [9]. Both demonstrated similar trends in concentrations in response to IDA and iron treatment. Glutamine and acetylcarnitine were both significantly higher (~ 2) in IM, and oral (p<0.05 and p<0.001, respectively) and IM & oral (p<0.001 and p<0.05, respectively) groups (~ 3 folds) compared to the control group (**Figure 2.9**). No significant differences were observed between anaemic and only IM iron treated piglets for acetylcarnitine nor between the different types of supplementations.

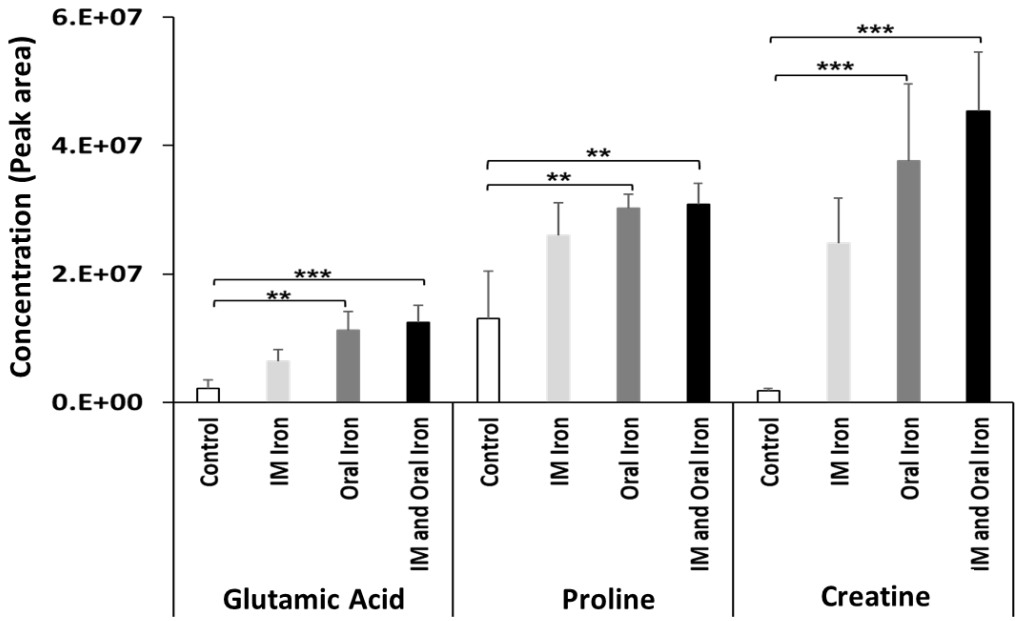

Figure 2. 8. Impact of iron deficiency anaemia (IDA)and iron supplementation on glutamic acid, proline, and creatine concentrations in 28-day old piglets. Significantly higher concentrations of glutamic acid, proline and creatine were observed in oral iron (p=0.01, p=0.02, and p=0.006) and IM and oral (p=5x10^{-3}, p=0.01, and p=1x10^{-3}) group compared to control. n=6 piglets/treatment group. Error bars = SEM, ** p<0.01, *** p<0.001.

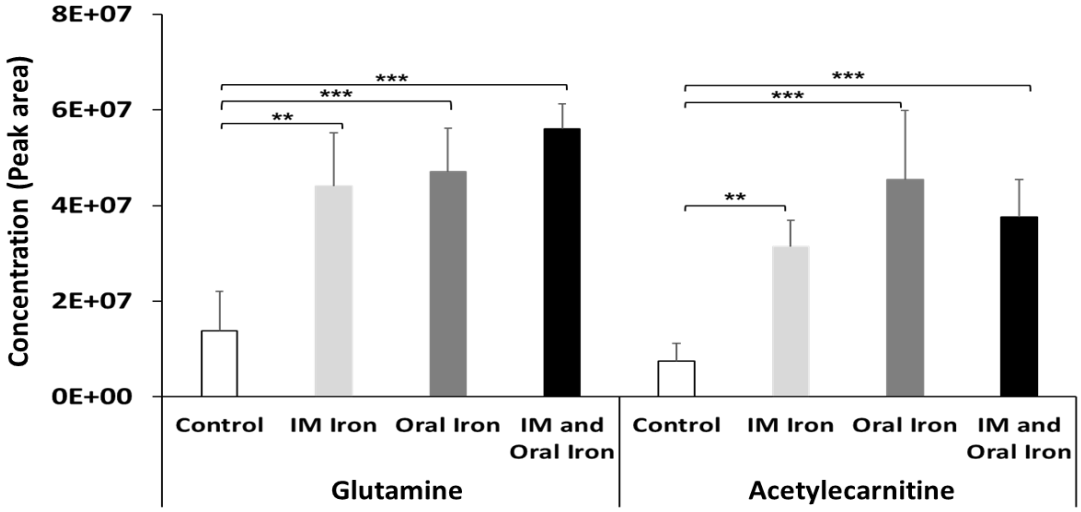

Figure 2. 9. Impact of iron deficiency anaemia (IDA)and different types of iron supplementation on glutamine and acetylcarnitine concentration at 28 days. Glutamine concentration was significantly higher in all iron supplemented groups (treatment groups 2-4) (p=0.02, IM; p=0.01, oral; p=2x10^{-3}, IM and oral) compared to non-supplemented siblings. Also, significantly higher concentrations of acetylcarnitine were observed in all iron supplemented groups except IM (treatment groups 3-4) (p=6x10^{-3} oral; p=0.02, IM and oral)

compared to non-supplemented siblings. n=6 piglets/treatment group. Error bars = SEM, ** p<0.01, ** p<0.001.

2.6.5.6 Leucine, iso-leucine, dimethylglycine and methionine sulfoxide

Leucine is an essential amino acid and iso-leucine is its isomer [10]. Both displayed similar trends in their concentrations in response to IDA and iron treatment. Leucine and iso-leucine were significantly higher (~ 2 folds) in IM (p<0.05), oral (p<0.001) and IM & oral (p<0.05) treatment groups compared to the anaemic siblings (**Figure 2.10**).

Dimethylglycine and methionine sulfoxide were observed in significantly higher (~ 1 & 2 folds respectively) concentrations in the oral (p<0.001and p<0.05, respectively) and IM & oral (p<0.001) treatment groups compared to the anaemic piglets. The IM iron-treated piglets displayed a significantly higher concentration (p=<0.05) of dimethyl glycine with respect to anaemic siblings (**Figure 2.11**). However, no significant difference was observed between the three different types of iron treatment.

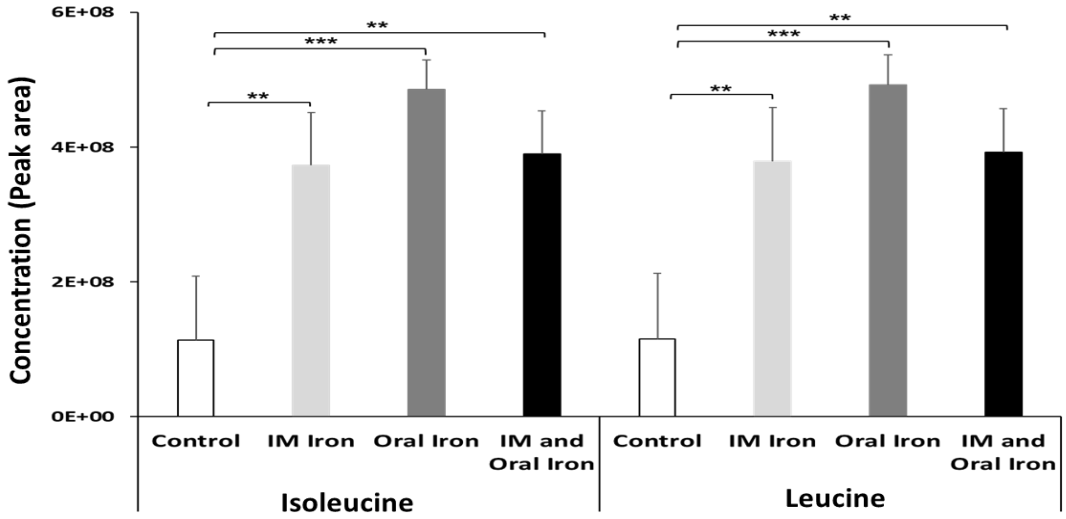

Figure 2. 10. Impact of iron deficiency anaemia (IDA)and iron supplementation on leucine and isoleucine concentrations in 28-day old piglets. Concentrations of leucine and isoleucine were observed significantly higher in all iron supplemented groups (treatment groups 2-4) (IM Iron, p=0.02), (Oral iron, p=0.01), (IM and oral iron, p=2x10^{-3}) for both leucine and isoleucine compared to non-supplemented siblings. n=6 piglets/treatment group Error bars = SEM, ** p<0.01, *** p<0.001.

2.6.5.7 Cystine and Creatinine

Cystine is a non-essential amino acid [11]. Concentrations of cystine were significantly (~ 1-fold) higher in the IM (p<0.05), oral (p<0.001) and IM & oral (p<0.05) treatment groups compared to the anaemic piglets (**Figure 2.12a**).

Creatinine was in significantly higher (~ 7 folds) concentrations in the IM, oral and IM & oral (p<0.001) treatments compared to anaemic piglets. No significant differences in creatinine levels were observed between the three different types of iron treatment (**Figure 2.12b**).

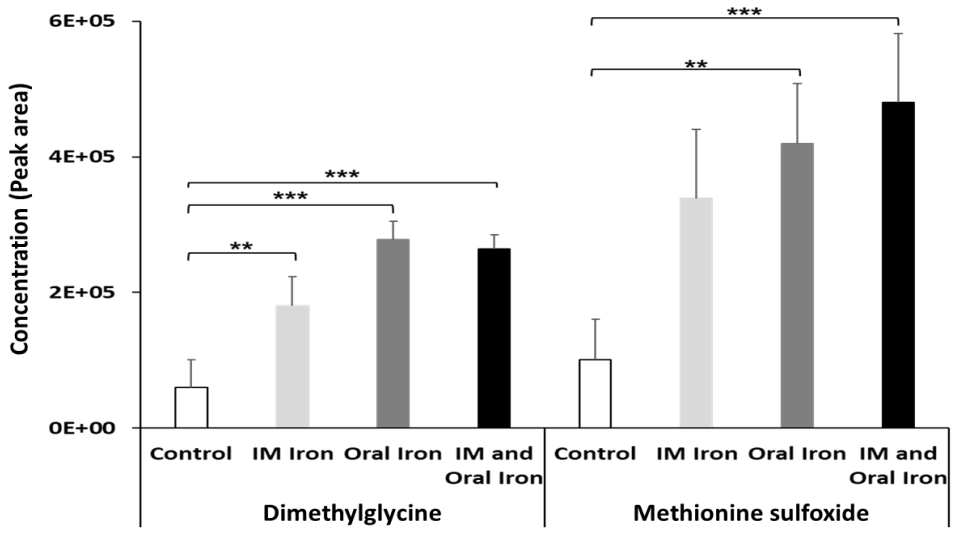

Figure 2. 11. Impact of iron deficiency anaemia (IDA)and different types of iron supplementation on dimethylglycine and methionine sulfoxide concentrations in 28-day old piglets. Significantly higher concentrations of dimethylglycine were observed in all iron supplemented groups (treatment groups 2-4) (p=0.02, IM; p=2x10^{-4}, oral; p=3x10^{-4}, IM+oral) compared to non-supplemented siblings. Also, significantly higher concentrations of methionine sulfoxide were observed in all iron supplemented groups except IM (treatment groups 3-4) (p=0.02 oral; p=7x10^{-3}, IM+oral) compared to non-supplemented counterparts. n=6 piglets/treatment group. Error bars = SEM, * p<0.05, ** p<0.01, ** p<0.001.

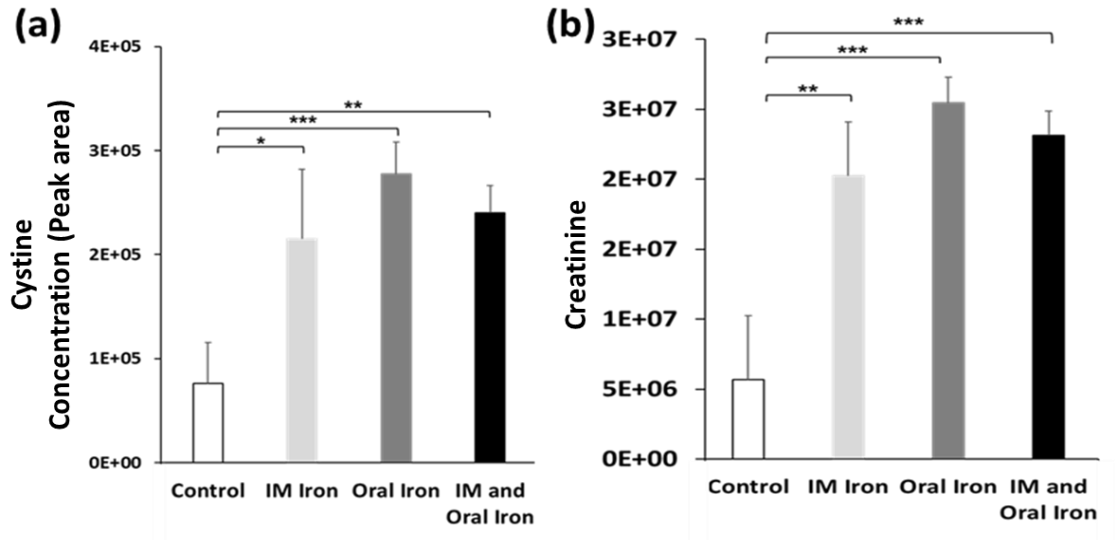

Figure 2. 12. Impact of iron deficiency anaemia (IDA)and different types of iron supplementation on cystine and creatinine concentrations in 28-day old piglets. Significantly higher concentrations of cystine were observed in all iron supplemented groups (treatment groups 2-4) (p=0.03, IM; p=4x10^{-3}, oral; p=1x10^{-3}, IM and oral) compared to non-supplemented siblings at day 28 (a). Significantly higher concentrations of creatinine were observed in all iron supplemented groups (treatment groups 2-4) (p=4x10^{-3}, IM; p=3x10^{-4}, oral; p=1x10^{-4}, IM and oral) compared to non-supplemented siblings at day 28 (b). n=6 piglets/treatment group. Error bars = SEM, * p<0.05, ** p<0.01, ** p<0.001.

2.7 Discussion

Due to its key role in several biological processes, iron is an essential micro-nutrient required by all mammals. However, iron deficiency anaemia (IDA) is a substantial world-wide human health problem, especially for infants, and Iron supplementation is commonly used to address this issue. Here we assessed the impact of IDA on growth and metabolism using piglet models for human infants. We showed that, as expected, lack of iron supplementation was associated with significant reductions in growth rates, and that both oral and intra-muscular iron, and combinations of oral and intramuscular iron supplementation were all sufficient in preventing the development of IDA. We demonstrated that there were no significant differences in any of the measured iron dependent blood parameters, or in systemic metabolite production, resulting from the different forms of iron supplementation assessed. Interestingly, we also showed that administration of oral iron as a single supplement, or in combination

with IM iron supplementation, was associated with significant reductions in weight gain compared to sibling piglets which received only IM iron supplementation. Indeed, the weight of piglets receiving oral iron was comparable to piglets which received no iron supplementation and rapidly developed IDA.

Our data shows that the levels of systemic metabolites in anaemic piglets differed significantly from those in piglets which received iron supplementation and thus avoided developing IDA. Metabolites associated with the TCA cycle (citric acid, aconitic acid, and 2-ketoglutaric acid), urea cycle (citrulline and arginine), methylation (methionine) and phosphorylation (serine) processes were significantly reduced in anaemic piglets compared to all iron supplemented counterparts. The systemic concentrations of three consecutive intermediates of TCA cycle were reduced over the period of 28 days in response to IDA. This, in part, is consistent with a study in human cell lines (K-562) by Oxele *et al* [43] who reported decreased activity of TCA cycle enzymes when iron chelator (desferrioxamine) was introduced. This perhaps could be due to the removal (or unavailability in the case of ID) of labile iron atoms from iron-sulphur clusters present in such enzymes. Our data also showed that the systemic concentrations of leucine and isoleucine (energy sources during exercise) and methionine were significantly reduced in IDA piglets. These results are consistent with those reported from an 8-day neonatal piglet trial by Dong *et al* [44] in which non-iron supplemented piglets (n=8) had lower concentrations of leucine, methionine, and tyrosine compared to iron-supplemented counterparts (n=8). Taken together, this is consistent with iron-dependent early-life metabolic shift occurring in anaemic piglets. However, they also reported higher concentrations of urea cycle enzymes which is in contrast with our results where urea cycle metabolites were significantly decreased in anaemic piglets. An explanation for these differences could be a result of the 8-day trial being of insufficient length for the piglets to develop IDA, as evidenced by reported Hb concentrations of (74.3g/L). Although iron deficient, these piglets did not yet develop IDA which is defined by Hb concentrations of <80 g/L. Glucose metabolism, the TCA cycle and oxidative phosphorylation are central biochemical pathways in cellular energy metabolism [45, 46]. There is evidence that the TCA cycle and iron homeostasis can be linked since iron perturbations modulate the expression of the Kreb's cycle intermediates including urea, citrulline, and ornithine[47, 48]. Since decreased production of TCA cycle-associated metabolites is linked with

stunting, this perhaps explains, in part, the reduced growth rate in anaemic piglets in our trial.

All forms of supplementary iron we assessed rescued piglets from developing IDA whilst, as expected, non-supplemented piglets rapidly developed IDA. Our results, in part, are consistent with several findings including Knight *et al*. [49] in which neonatal piglets from day 2 to day 32 consuming an iron deficient diet (equivalent to iron present in sow and human milk (~ 0.3mg/L) developed IDA. However, their counterparts fed on normal iron diets (21.3 mg/L Fe) did not become iron deficient until day 32 after birth. Chen *et al*. [50], and Mazgaj *et al* [51] both reported that piglets which received an IM injection of 100-150mg Fe (iron dextran) at day 3 after birth did not develop IDA for at least the following 25 days. This is consistent with piglets being robust models for assessing the effects of different forms of iron supplementation, and IDA, in human infants. In this study, consistent and appropriate systemic haemoglobin (Hb) levels suggested that having received the IM dose of iron at 1-day old, additional oral iron was unnecessary for at least the following 27 days, when the trial ended. Therefore 'extra' iron from oral supplementation (in addition to IM) was not necessary. However, additional oral iron has been linked with having negative impact on gut and systemic health parameters, including diarrhoea and enteric infection. Iron overload can result in iron deposits occurring in the intestinal mucosa which can result in high levels of reactive oxygen species (ROS) leading to disruption of epithelial tight cell junctions and even ferroptosis [52]. In addition, excess dietary iron triggers hepcidin production by hepatocytes which results in the internalisation of ferroportin, an iron transport protein. Consequently, the capacity for duodenal iron absorption becomes limited[53]. increased concentrations of unabsorbed iron reaching the colon may promote the development of pathogenic microbial populations with high iron requirements. At the same time, high concentrations of luminal iron select against some commercial microbes which do not have such high iron requirement. This could have important implications for increased enteric infection risk in piglets and in infants. The effects of high concentrations of luminal iron on the development of the early gut microbiota is explored in more detail in **chapter 3**.

Growth rate is a useful proxy measure of general health in young animals [54]. Our data shows that the type of iron used to prevent the development of IDA in the pig industry requires careful consideration. Whilst there are negative implications associated with IM iron injection at 1 day old, including iron overload and occasionally inflammation/infection at the site of injection, our results demonstrate that the alternative form of iron supplementation, oral iron, was associated with a significant reduction in growth rate, even in the presence of additional IM iron. This was a substantial reduction over just a 4-week timeframe and represents around a 12.5% reduction in growth rate which may have considerable implications for the pig industry as well as for iron fortified infant formula milk. This difference in weight gain in response to oral iron was not apparent at 2 weeks, at which point ID had not progressed to IDA. This is consistent with an earlier study in pigs by Chwen *et al* [55] which reported that piglets injected with a single dose of 200mg Fe had higher growth rates (weigh gain) over the subsequent 4 weeks compared to piglets given iron supplemented water ($FeSO_4$). One explanation for this is that luminal iron alters the composition of the gut microbiota and correlations have previously been observed between obesity and the gut microbiota based on studies in germ-free mice [56]. These mice were raised in a sterile environment and had no microorganisms in their guts. Conventionally reared mice had a 40% higher body fat content and 47% higher gonadal fat content than germ-free mice even though they consume less food than their germ-free counterparts [57, 58].

Studies in human infants have also demonstrated associations between the intestinal microbiota and weight-gain [59]. In addition, Firmicutes to Bacteroidetes ratios have been shown to have positive correlations with obesity [60]. Aktas *et al* [61] reported weight loss and body mass index (BMI) reductions in 21 iron deficiency anaemic adult women after iron treatment (oral supplementation) over 6 months. Although the cohort size and age of subjects was different from our piglet study, the main outcome (weight loss) in response to oral iron supplementation) is consistent with our observations. We also observed a reduced growth rate in our non-iron supplemented IDA piglets which did not significantly differ in weight from their oral iron supplemented siblings. This is consistent with a piglet study reported by Knight *et al* [62] where IDA was associated with a significant reduction in growth rate compared to their IM iron supplemented counterparts. Overall, our study demonstrates that the

type of iron supplementation (IM, oral or IM & oral) was not important in terms of systemic iron indicators of iron sufficiency and metabolic activity but was a highly significant factor in terms of weight gain.

2.8 Conclusion

In conclusion, our study demonstrated that different types of iron supplementation had the capacity to prevent the development of IDA without differentially affecting either iron dependent haematological parameters including Hb, RBCs, and Haematocrit, or systemic metabolite production. However, oral iron was associated with reduced growth rates, even when used in combination with IM iron injections. Piglets are increasingly being supplemented with sow milk replacers which, until recently, were not fortified with iron. However, iron fortification of these formula-type milks is now standard practice, despite sow milk containing only limited iron. Our results indicate that this practice may have unforeseen consequences and may not address reductions in weight gain, especially if, as is standard practice, piglets have already received an IM injection of iron at 1 day old and so are not deficient in iron (also demonstrated by our results). This also has important implications for human infants. Again, the majority of bovine-based formula milks targeted at infants are also fortified with iron (8-14 mg/L[63]), despite breast milk containing very little iron (~ 0.4mg/L[64]). This could have particularly important implications for low-birth-weight infants as iron status of preterm infants receiving non-fortified breast milk starts to decline within 1 to 4 months which is long before the recommended weaning age of 6 months. Our study confirms that the piglet is a valuable model for exploring ID/IDA and iron supplementation in more detail. There is a clear need to assess the microbiota and its metabolic end products in our trial piglets to explore putative links between weight gain, oral iron supplementation and the gut microbiota which is highly relevant to both the pig industry and human infant health.

2.9 References

1. Bermejo, F. & García-López, S. A guide to diagnosis of iron deficiency and iron deficiency anemia in digestive diseases. *World Journal of Gastroenterol* **15**, 4638-4643 (2009).

2. WHO. (World Health Organization, 2011).

3. Domellöf, M., Dewey, K.G., Lönnerdal, B., Cohen, R.J. & Hernell, O. The Diagnostic Criteria for Iron Deficiency in Infants Should Be Reevaluated. *The Journal of Nutrition* **132**, 3680-3686 (2002).

4. Nivaggioni, V. et al. Use of Sysmex XN-10 red blood cell parameters for screening of hereditary red blood cell diseases and iron deficiency anaemia. *International Journal of Laboratory Hematology* **42**, 697-704 (2020).

5. Seferidi, P., Hone, T., Duran, A.C., Bernabe-Ortiz, A. & Millett, C. Global inequalities in the double burden of malnutrition and associations with globalisation: a multilevel analysis of Demographic and Health Surveys from 55 low-income and middle-income countries, 1992–2018. *The Lancet Global Health* **10**, e482-e490 (2022).

6. Perignon, M. et al. Stunting, poor iron status and parasite infection are significant risk factors for lower cognitive performance in Cambodian school-aged children. *PLoS One* **9**, e112605 (2014).

7. Lawn, J.E., Zupan, J., Begkoyian, G. & Knippenberg, R. in Disease Control Priorities in Developing Countries. (eds. D.T. Jamison et al.) (The International Bank for Reconstruction and Development / The World Bank Oxford University Press Copyright © 2006, The International Bank for Reconstruction and Development/The World Bank Group., Washington (DC) New York; 2006).

8. Georgieff, M.K. Iron assessment to protect the developing brain. *The American Journal of Clinical Nutrition* **106**, 1588s-1593s (2017).

9. Cesari, M. et al. Hemoglobin Levels and Skeletal Muscle: Results From the InCHIANTI Study. *The Journals of Gerontology: Series A* **59**, M249-M254 (2004).

10. Toxqui, L. & Vaquero, M.P. Chronic iron deficiency as an emerging risk factor for osteoporosis: a hypothesis. *Nutrients* **7**, 2324-2344 (2015).

11. Sanou, A.S. et al. Association between stunting and neuro-psychological outcomes among children in Burkina Faso, West Africa. *Child and Adolescent Psychiatry and Mental Health* **12**, 30 (2018).

12. Msaki, R.V. et al. Predictors of iron deficiency anaemia among children aged 6–59 months in Tanzania: Evidence from the 2015–16 TDHS-MIS cross-sectional household survey. *PLOS Global Public Health* **2**, e0001258 (2022).

13. Zhao, A. et al. Prevalence of Anemia and Its Risk Factors Among Children 6–36 Months Old in Burma. *The American Society of Tropical Medicine and Hygiene* **87**, 306-311 (2012).

14. Booth, I.W. & Aukett, M.A. Iron deficiency anaemia in infancy and early childhood. *Archives of Disease in Childhood* **76**, 549 (1997).

15. Lönnerdal, B. Development of iron homeostasis in infants and young children. *The American Journal of Clinical Nutrition* **106**, 1575s-1580s (2017).

16. DALLMAN, P.R. Iron deficiency in the weanling: a nutritional problem on the way to resolution. *Acta Pædiatrica* **75**, 59-67 (1986).

17. Meyer, M.P., Haworth, C., Meyer, J.H. & Commerford, A. A comparison of oral and intravenous iron supplementation in preterm infants receiving recombinant erythropoietin. *Journal of Pediatrics* **129**, 258-263 (1996).

18. Schaefer, B., Meindl, E., Wagner, S., Tilg, H. & Zoller, H. Intravenous iron supplementation therapy. *Molecular Aspects of Medicine* **75**, 100862 (2020).

19. Baird-Gunning, J. & Bromley, J. Correcting iron deficiency. *Aust Prescr* **39**, 193-199 (2016).

20. WHO. Infant and young child feeding: model chapter for textbooks for medical students and allied health professionals. (World Health Organization, 2009).

21. Iannotti, L.L., Tielsch, J.M., Black, M.M. & Black, R.E. Iron supplementation in early childhood: health benefits and risks. *The American journal of clinical nutrition* **84**, 1261-1276 (2006).

22. Tolkien, Z., Stecher, L., Mander, A.P., Pereira, D.I. & Powell, J.J. Ferrous sulfate supplementation causes significant gastrointestinal side-effects in adults: a systematic review and meta-analysis. *PLoS One* **10**, e0117383 (2015).

23. Saarinen, U. & Siimes, M. Iron absorption from infant milk formula and the optimal level of iron supplementation. *Acta Pædiatrica* **66**, 719-722 (1977).

24. Domellöf, M. et al. Iron Requirements of Infants and Toddlers. *Journal of Pediatric Gastroenterology and Nutrition* **58** (2014).

25. Abbas, M., Hayirli, Z., Drakesmith, H., Andrews, S.C. & Lewis, M.C. Effects of iron deficiency and iron supplementation at the host-microbiota interface: Could a piglet model unravel complexities of the underlying mechanisms? *Frontiers in Nutrition* **9** (2022).

26. Seyoum, Y., Baye, K. & Humblot, C. Iron homeostasis in host and gut bacteria - a complex interrelationship. *Gut Microbes* **13**, 1-19 (2021).

27. Sutharsan, R., O'Callaghan, M.J., Williams, G., Najman, J.M. & Mamun, A.A. Rapid growth in early childhood associated with young adult overweight and obesity--evidence from a community based cohort study. *Journal of Health Population and Nutrition* **33**, 13 (2015).

28. Butte, N.F. Fat intake of children in relation to energy requirements. *The American Journal of Clinical Nutrition* **72**, 1246s-1252s (2000).

29. Abbaspour, N., Hurrell, R. & Kelishadi, R. Review on iron and its importance for human health. *Journal of Research Medical Science* **19**, 164-174 (2014).

30. Hoes, M.F. et al. Iron deficiency impairs contractility of human cardiomyocytes through decreased mitochondrial function. *European Journal of Heart Failure* **20**, 910-919 (2018).

31. McClorry, S. et al. Anemia in infancy is associated with alterations in systemic metabolism and microbial structure and function in a sex-specific manner: an

observational study. *The American Journal of Clinical Nutrition* **108**, 1238-1248 (2018).

32. Argilés, J.M., Campos, N., Lopez-Pedrosa, J.M., Rueda, R. & Rodriguez-Mañas, L. Skeletal Muscle Regulates Metabolism via Interorgan Crosstalk: Roles in Health and Disease. *Journal of the American Medical Directors Association* **17**, 789-796 (2016).

33. Yang, W. & Cong, Y. Gut microbiota-derived metabolites in the regulation of host immune responses and immune-related inflammatory diseases. *Cell Molecular Immunology* **18**, 866-877 (2021).

34. Yilmaz, B. & Li, H. Gut Microbiota and Iron: The Crucial Actors in Health and Disease. *Pharmaceuticals (Basel)* **11** (2018).

35. Antonides, A., van Laarhoven, S., van der Staay, F.J. & Nordquist, R.E. Non-anemic Iron Deficiency from Birth to Weaning Does Not Impair Growth or Memory in Piglets. *Frontiers in Behavioural Neuroscience* **10**, 112-112 (2016).

36. Schubert, R. et al. Neuroimaging of a minipig model of Huntington's disease: Feasibility of volumetric, diffusion-weighted and spectroscopic assessments. *Journal of Neuroscience Methods* **265**, 46-55 (2016).

37. McGowan, J.P. & Crichton, A. Iron deficiency in pigs. *Biochemical Journal* **18**, 265 (1924).

38. Loh, T., Jr., Leong, K., Too, H., Mah, C. & Choo, P. The effects of iron supplementation in preweaning piglets. *Malaysian Journal of Nutrition* **7**, 41-49 (2001).

39. Szudzik, M. et al. Iron Supplementation in Suckling Piglets: An Ostensibly Easy Therapy of Neonatal Iron Deficiency Anemia. *Pharmaceuticals (Basel)* **11** (2018).

40. Rydal, M.P., Bhattarai, S. & Nielsen, J.P. An Experimental Model for Iron Deficiency Anemia in Sows and Offspring Induced by Blood Removal during Gestation. *Animals (Basel)* **11** (2021).

41. Merrifield, C.A. et al. A metabolic system-wide characterisation of the pig: a model for human physiology. *Molecular BioSystems* **7**, 2577-2588 (2011).

42. Fanalli, S.L. et al. Effect of dietary soybean oil inclusion on liver-related transcription factors in a pig model for metabolic diseases. *Scientific Reports* **12**, 10318 (2022).

43. Oexle, H., Gnaiger, E. & Weiss, G. Iron-dependent changes in cellular energy metabolism: influence on citric acid cycle and oxidative phosphorylation. *Biochimica et Biophysica Acta (BBA) - Bioenergetics* **1413**, 99-107 (1999).

44. Dong, Z. et al. Effects of Iron Deficiency on Serum Metabolome, Hepatic Histology, and Function in Neonatal Piglets. *Animals* **10**, 1353 (2020).

45. Stryer, L. (Freeman, 1988).

46. Schalinske, K.L., Chen, O.S. & Eisenstein, R.S. Iron differentially stimulates translation of mitochondrial aconitase and ferritin mRNAs in mammalian cells implications for iron regulatory proteins as regulators of mitochondrial citrate utilization. *Journal of Biological Chemistry* **273**, 3740-3746 (1998).

47. Fischer, C. et al. Mitochondrial Respiration in Response to Iron Deficiency Anemia: Comparison of Peripheral Blood Mononuclear Cells and Liver. *Metabolites* **12**, 270 (2022).

48. Volani, C. et al. Metabolic Signature of Dietary Iron Overload in a Mouse Model. *Cells* **7**, 264 (2018).

49. Knight, L.C., Wang, M., Donovan, S.M. & Dilger, R.N. Early-Life Iron Deficiency and Subsequent Repletion Alters Development of the Colonic Microbiota in the Pig. *Frontiers in Nutrition* **6** (2019).

50. Chen, X. et al. Split iron supplementation is beneficial for newborn piglets. *Biomedicine & Pharmacotherapy* **120**, 109479 (2019).

51. Mazgaj, R. et al. Comparative Evaluation of Sucrosomial Iron and Iron Oxide Nanoparticles as Oral Supplements in Iron Deficiency Anemia in Piglets. *International Journal of Molecular Sciences* **22**, 9930 (2021).

52. Huo, C., Li, G., Hu, Y. & Sun, H. The Impacts of Iron Overload and Ferroptosis on Intestinal Mucosal Homeostasis and Inflammation. *International Journal of Molecular Sciences* **23**, 14195 (2022).

53. Li, M. et al. Effect of different iron loads on serum and tissue biochemical parameters and liver hepcidin mRNA abundance of neonatal piglets. *Archives of Animal Nutrition* **65**, 477-485 (2011).

54. Gelander, L. Children's growth: a health indicator and a diagnostic tool. *Acta Paediatrica* **95**, 517-518 (2006).

55. Chwen, L.T., Heng, L.K., Lee, T.H., Kong, M.C. & Yoon, C.P. The effects of iron supplementation in preweaning piglets. *Malaysian Journal of Nutrition* **7**, 41-49 (2001).

56. Bäckhed, F., Manchester, J.K., Semenkovich, C.F. & Gordon, J.I. Mechanisms underlying the resistance to diet-induced obesity in germ-free mice. *Proceedings of the National Academy of Sciences* **104**, 979-984 (2007).

57. Davis, C.D. The Gut Microbiome and Its Role in Obesity. *Nutrition Today* **51**, 167-174 (2016).

58. Backhed, F., Ding, H., Wang, T., Hooper, L. & Koh, G. NagyA., Semenkovich CF & Gordon JI (2004) The gut microbiota as an environmental factor that regulates fat storage. *Proceedings of the National Academy of Sciences of the United States of America101*, 15718-15723.

59. Charbonneau, M.R. et al. Sialylated milk oligosaccharides promote microbiota-dependent growth in models of infant undernutrition. *Cell* **164**, 859-871 (2016).

60. Li, T., Gao, J., Du, M. & Mao, X. Bovine α-lactalbumin hydrolysates ameliorate obesity-associated endotoxemia and inflammation in high-fat diet-fed mice through modulation of gut microbiota. *Food & Function* (2019).

61. Aktas, G. et al. Treatment of iron deficiency anemia induces weight loss and improves metabolic parameters. *Clinical Tertiary* **165**, e87-89 (2014).

62. Knight, L.C. & Dilger, R.N. Longitudinal Effects of Iron Deficiency Anemia and Subsequent Repletion on Blood Parameters and the Rate and Composition of Growth in Pigs. *Nutrients* **10** (2018).

63. Björmsjö, M., Hernell, O., Lönnerdal, B. & Berglund, S.K. Reducing Iron Content in Infant Formula from 8 to 2 mg/L Does Not Increase the Risk of Iron Deficiency at 4 or 6 Months of Age: A Randomized Controlled Trial. *Nutrients* **13** (2020).

64. Cai, C., Harding, S. & Friel, J. Breast milk iron concentrations may be lower than previously reported: implications for exclusively breastfed infants. *Maternal Pediatrics Nutrition* **2**, 2 (2015).

CHAPTER 3

Impact of iron deficiency anaemia, and different iron treatments, on gut microbiota composition and metabolic activity

Abstract

Iron deficiency anaemia (IDA) is prevalent worldwide and can have significant impacts on growth, which may be linked to the gut microbiota. This is because most gut bacteria have essential requirements for iron, and produce metabolic end products, such as short chain fatty acids (SCFAs), which are important sources of energy. Therefore, the aim of this study was to explore the impacts of IDA, and different types of iron treatments, on both the composition and metabolic activity of the gut microbiota using a neonatal piglet model for human infants. Twenty-four piglets were litter-matched into 4 treatment groups (n=6 each) which were either left unsupplemented with iron, or received different iron treatments, as detailed in **section 2.5.1**. During analysis, treatment groups were combined to enable comparisons between anaemic (n=6) and iron replete (n=18) piglets, while comparisons between oral iron (n=12) and intramuscular iron injection (n=6) were also enabled by combining treatment groups. Microbial population assessments were achieved via 16S sequencing using Illumina technology, while metabolite production was quantified using liquid chromatography / mass spectrometry (LC-MS) techniques. The results showed that at 28 days, the abundance of Lachnospiraceae family, which are butyrate producers, was significantly reduced (p<0.05) in response to oral iron supplementation compared to IM iron treatment alone. The relative abundances of Bacilli, Lactobacillales and *Lactobacillus* were significantly reduced (p<0.05) in iron treated piglets compared to their IDA counterparts. In addition, Isobutyric acid was negatively correlated (p=0.02) with weight gain in all piglets, irrespective of iron treatment. Overall, these findings have important implications for formula fed infants, and for pig industries where piglets are increasingly being supplemented with iron-rich sow replacer milked due to increasing litter sizes. Further research is needed to determine the mechanisms underlying the interplay between oral iron, growth, and the developing gut microbiota to inform the development of

regimes to prevent IDA whilst also improving growth and the development of appropriate microbiotas.

3.1 Introduction

Iron deficiency anaemia (IDA), particularly in infants and young children in low- and middle-income countries (LMICs), is a major public health concern [1]. This is partly caused by the high iron demands required for rapid growth and development at these ages [2, 3]. Universal iron supplementation and fortification are the most common strategies used worldwide to prevent and treat IDA [4]. Oral iron supplements are widely available and easy to administer, making them the preferred option for low-resource settings [5]. However, universal iron programmes, which add iron micronutrient powders (Fe-MNPs) directly to home cooked foods (after cooking), are not recommended for infants [6] since they have been demonstrated to have adverse effects on gut bacteria and there is limited evidence for their efficacy and safety in this age group [7]. In addition, providing universal daily iron supplements to all breast-fed infants is unnecessary since not all children, even those in LMIC, are iron deficient. For example, a randomised trial conducted by Dewey *et al* [8] reported that daily universal iron supplementation in breast-fed infants in Sweden (n=101) and Honduras (n=131) improved iron status and growth, but increased the risk of diarrhoea in the children who had initial systemic Hb concentrations > 110 g/L. Similarly, a double-blind randomised trial (n=1958) in Ghanaian infants and pre-school children (age 6-35 months) by Zoltkin *et al* [9], reported an increased rate of hospitalization due to diarrhoea in response to iron fortification. Furthermore, a trial in Zanzibar [10] demonstrated that iron and folic acid supplementation increased the risk of severe illness and death from malaria which led to discontinuation of treatment with Fe-MNPs in areas with high prevalence of malaria [11]. Subsequently, it has been suggested by many researchers that ID may provide protection against malaria [12, 13, 14]. Taken together, these findings suggest that careful consideration is required before making recommendations to commence iron supplementation interventions which should be appropriate for both the target population and environmental conditions.

Humans lack the mechanistic ability to actively remove excess iron [15] and high levels of iron can be toxic [16]. Consequently, absorption of iron from dietary sources is tightly

regulated, and is typically relatively low [17], especially from foods rich in phytic acid, such as cereals, which strongly inhibit iron absorption [18]. In general, less than 20% of iron added to such foods is absorbed [19]. Higher prevalence of enteric inflammation and infection in rural populations in LMICs further limits iron absorption from the lumen due to raised levels of systemic hepcidin [20], as detailed in chapter 1. While fortification and supplementation can deliver additional dietary iron, most remains unabsorbed. Introducing Fe-MNP (12.5mg of iron [21]) can result in significant proportions (~10mg) reaching the colon, perhaps over 30 times more than the amount provided by breast milk which contains only 0.4mg/L of iron which is also absorbed more efficiently [22].

Iron availability is a key requirement for most gut microbes, including enteropathogens such as *E. coli* and *Salmonella* [23]. Excessive, unabsorbed iron in the colon can create an environment that is more favourable to pathogenic bacteria growth compared to normal commensal microbes [24]. For this reason, several studies have reported the effects of oral iron supplementation on gut microbiota compositions. For example, a 6-month trial, in 6–14-year-old Ivorian children (n = 139) by Zimmermann *et al* [25] showed there were significant increases in populations of enterobacteria along with significant decreases in numbers of lactobacilli in response to consumption of iron fortified biscuits. Similar changes in gut microbiota populations in response to iron treatments have been linked with alterations in the production of short chain fatty acids (SCFAs), which are important bacterial end-point metabolites that play crucial roles in both gut and overall health [26]. Dostal *et al* [27] suggested that iron is a strong regulator of butyrate production using *in vitro* fermentation experiments which showed that low iron containing media caused significant reductions in butyrate production and that iron supplementation ameliorated these effects. These findings suggest that ‘excessive’ iron supplementation could have negative impacts on both the composition and metabolic activity of residential gut microbes, potentially promoting the growth of gut pathogens and reducing the abundance of beneficial bacterial populations. This further highlights the necessity for careful consideration of the risks and benefits of iron supplementation, particularly in at risk populations, and the importance of monitoring the effects of iron supplementation on the gut microbiota.

Iron is also an essential nutrient for piglets, and ID is problematic for the pig industry. Piglets have emerged as an important model for studying the effects of IDA, and iron supplementation, as detailed in chapter 1. In addition, the gut microbiota of humans and pigs are considerably more stable over time than in rodent models [28]. Moreover, pigs and humans share similar diversities and dominant phyla, including Firmicutes and Bacteroides [29]. These similarities make the piglet a more suitable model for investigating the mechanisms underlying early microbiota-host interactions in human infants than rodent models do. Piglets have also been shown to exhibit responses to dietary interventions that are more similar to humans than rodents [30]. Furthermore, piglets can be fed a range of diets, including human breast milk, and can be readily colonized with human-derived microbiota [31]. Reflecting the value of the pig model in this area, the impacts of IDA and iron supplementation on gut microbiotas in piglets have been explored in the past. For example, a study by Ding *et al* [32] found that the upper limit (3000mg/Kg feed) of iron supplement ($FeSO_4$) caused significant reductions in *Clostridium* spp. and *Faecalibacterium* in 28 days old weaned piglets. Overall, the piglet model provides a unique opportunity to study the gut microbiota and its interactions with the host in a manner that more closely resembles human physiology than rodent models do.

Since the gut microbiota is largely unstable during the first 3 years in humans[33], early life is a critical period for the establishment of a healthy gut microbiota, and any disruptions at this stage can have lasting impacts [34]. Iron availability could be a key factor for gut microbiota development since it is an essential nutrient for most bacterial growth [35]. Therefore, early exposure to iron or IDA could disrupt the normal pattern of microbiota development, potentially leading to long-term alterations in gut microbial composition and functionality [36]. Thus, understanding the development of the gut microbiota and the factors that influence it is crucial for promoting long-term health and preventing disease.

3.2 Hypotheses

The two hypotheses to be tested in this study were:

Hypothesis 1: Since different components of the gut microbiota have differential iron requirements for growth, iron availability in the lumen will impact gut microbiota composition during infancy.

Hypothesis 2: Bioavailability of iron in the gut will alter the production of bacterial-derived metabolic end products by either altering the populations of bacteria present, or by altering metabolic activity of the residential microbes without altering composition.

3.3 Aim

To assess the effects of IDA and different forms of iron treatments on both the composition and metabolic activity of the gut microbiota in piglet models for human infants.

3.4 Objectives

1. To determine the effects of IDA on the composition of the gut microbiota using faecal samples collected from piglets used in the trial detailed in chapter 2.

2. To determine the effects of IDA, along with oral and intramuscular iron supplementation, on gut bacterial population in early life, using next generation sequencing techniques.

3. To assess the impacts of IDA, and different forms of iron supplementation, on bacterial metabolic end-product concentrations using gas chromatography / mass spectroscopy techniques.

3.5 Materials and Methods

3.5.1 Animal trial

To assess the impact of iron deficiency anaemia (IDA) and different types of iron treatments on gut microbial profiles and systemic concentrations of microbial-derived short chain fatty acids (SCFA), 24 large white F1 hybrid piglets from 6 litters were allocated into 4 litter- and sex-matched (3 male and 3 female) treatment groups at 1 day old. Treatments were group 1, control (no iron treatment); group 2, intramuscular iron (IM) injection (200mg Fe); group 3, iron supplemented ($FeSO_4$) sow milk replacer (SMR) (oral iron; 150mg/kg body weight/day); and group 4, IM and oral iron, as detailed in **Figure 2.1**.

The original treatments were allocated into two different analytic groupings to determine whether overall iron treatment and/or different forms of iron treatment influenced both the composition of the gut microbiota, and subsequent microbial metabolic activity. Specifically, the piglets from the four original treatment groups (**Figure 2.1)** were reassigned into two groups for gut microbiota and SCFA analysis. Thus, groups 2, 3 and 4 (n=18) were combined to generate one iron treatment group (Iron treated) which received IM iron (200mg, one dose) and/or oral iron ($FeSO_4$ 150mg/day). The control (no-iron treatment, which received no iron treatment, n=6) was used as the comparator (**Figure 3.1**). A further analytical grouping was formed to compare the impact of IM iron and oral iron treatments: the IM-only group, IM iron (n=6); and the group, oral iron (with and without IM iron, n=12) (**Figure 3.2)**.

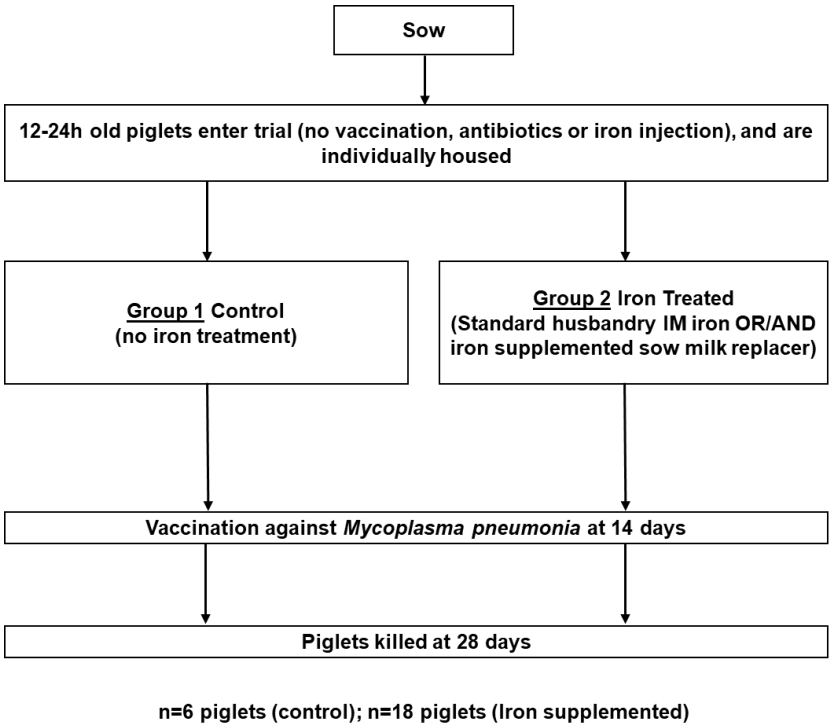

Figure 3. 1. Schematic of experimental design of combined treatments (anaemic vs overall iron supplementation). Schematic of experimental design of combined treatments (anaemic vs overall iron supplementation). For analytic purposes, treatments groups were reallocated to all iron treated piglets (n=18) against their non-iron treated siblings (n=6).

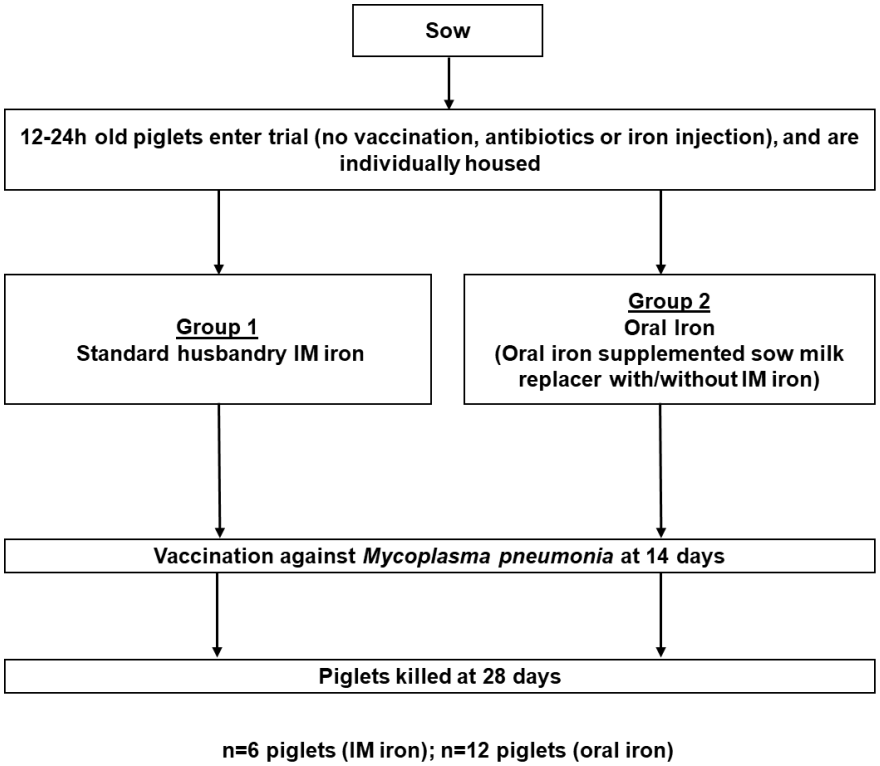

Figure 3. 2. Schematic of experimental design combined treatments (IM iron vs oral iron). For analytic purposes, treatments groups were reallocated to all oral iron treated piglets (n=12) against their IM iron treated siblings (n=6).

3.5.2 Sample collection

Blood samples were collected weekly from day 1 into EDTA vacutainers (BD Vacutainer®). These samples were centrifuged at ~ 1900 g for 10 min at 4^{o}C, and plasma from all samples was separated. Content was directly collected from the rectum of each euthanised piglet. All samples were stored at -80^{o}C.

3.5.2.1 DNA extraction method 'M' adopted from Tang et al [37]

3.5.2.1.1 Pre-treatment

In order to disrupt bacterial cells, this pre-treatment was applied as described by Thiel and Blaut (2005), with some modifications. Briefly, frozen samples (1.6 g) were transferred to sterile tubes (50 ml), diluted in 10 ml of ice-cold 0.05 M PBS buffer (150 mM NaCl, 10 mM Na_2HPO_4, 20 mM NaH_2PO_4, pH 7.4) and homogenized using ten sterile glass beads (3 mm in diameter; Sigma-Aldrich, Cat no. 1040150500) and vortexed for 3 min. Subsequently, the samples were centrifuged at 400 g for 2 min to remove glass beads and cell debris. All the suspension was transferred to a new 50-ml sterile tube and mixed with 3 vol of 4% formaldehyde and incubated on ice for 1 h.

Following that, centrifugation was done at 8000 ×g for 3 min and the cell pellet was resuspended in 4 ml PBS buffer, mixed with 4 ml absolute ethanol, and stored at −20 °C for 20 min. Subsequently, the cell pellet was harvested by centrifugation at 8000 g for 3 min and resuspended in 4 ml of TE buffer (pH 8.0) for use.

3.5.2.1.2 Microbial DNA extraction from pre-treated rectal samples

The chemical-enzymatic method of Niemi et al. (2001) was used, however, it was optimised with the following modifications (particularly varying phenol-chloroform concentration and ratios). A 500 µl volume of pre-treated sample was transferred into new 2.0 ml tubes. Bacterial cells were disrupted using 25 µl lysozyme (20 mg/ml) at 37 °C for 30 min. Subsequently, 50 µl 10% sodium dodecyl sulfate (SDS), and 15 µl proteinase K (10 mg/ml) were added in alkaline (pH 8.0) TE buffer and incubated at 55 °C for 2 h. Microbial DNA was extracted using phenol:chloroform (1:1, v/v), Phenol-choloroform layer was separated and isopropanol was added to precipitate theDNA. Finally, the DNA pellet was suspended in 50 µl of sterile ultra-filtered water.

3.5.2.1.3 PCR Amplification V4 and V5 regions and DNA Sequencing

In order to perform PCR amplifications, extracted microbial DNA was amplified with universal primers for the V4 and V5 regions of the 16S rRNA gene. The primers U515F (5'-GTGYCAGCMGCCGCGGTA) and U927R (5'-CCCGYCAATTCMTTTRAGT) [38] were used to amplify both bacterial and archaeal ribosomal RNA encoding gene regions, whilst providing optimal taxonomic resolution based on published information [39, 40]. 16S rRNA gene amplicon-based sequencing was performed by Novogene (Uk) company ltd. using an Illumina MiSeq instrument to generate paired-end reads. The data output was demuliplexed using the in-built WBI software on the instrument. Forward and reverse fusion primers consisted of the Illumina overhang forward (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG) and reverse adapter (5'-GTCTCGTGGGCTCGGAGATGTGTAATAAGAGACAG) respectively. Amplification was achieved using FastStart HiFi Polymerase (Roche Diagnostics Ltd, UK) under the following cycling conditions: 95°C for 3 min; 25 cycles of 95°C for 30 s, 55 °C for 35 s, 72 °C for 1 min; followed by a final step 72 °C for 8 min. Amplicons were purified using 0.8 volumes of Ampure XP magnetic beads (Beckman Coulter). Each sample was then tagged with a unique pair of indices and the sequencing primer, using Nextera XT v2 Index kits, and 2x KAPA HiFi HotStart

ReadyMix using the following cycling conditions: 95 °C for 3 min; 10 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s; followed by 72 °C for 5 min. Index-tagged amplicons were purified using 0.8 volumes of Ampure XP magnetic beads (Beckman Coulter). The concentration of each sample was measured using the fluorescence based Quantifluor assay (Promega). Concentrations were normalized before pooling all samples, each of which would be subsequently identified by its unique index combination. Sequencing was performed on an Illumina MiSeq with 2 × 300 base reads using V3 chemistry according to the manufacturer's instructions (Illumina Cambridge UK). The data output was demuliplexed using the in-built RTA software on the instrument.

3.5.3 Short chain fatty acids (SCFAs) in plasma

SCFAs are the metabolic end products of gut bacteria which provide energy, and quantifying these in plasma would reflect the amount of gut bacterial metabolites in the system, instead of just produced in the gut. To quantify microbial-derived end-product metabolites including SCFAs, Richardson's [41] method of derivatization was used which uses gas chromatography (GC) which was originally optimized for bacterial cultures. However, this method failed to detect any SCFAs in plasma samples. This could be due to the high protein content in plasma which potentially caused signal suppression in GC. To resolve this issue, an acidification method was used which again failed to detect any SCFA other than standards. Samples were also sent to a commercial lab based in UK (SOCOTEC, Trent, UK). However, this method failed to detect any SCFA due to high detection limits. Finally, another commercial analytical service (Percion Labs, NC, US) was able to detect and quantify the SCFA in plasma samples.

3.5.3.1 SCFA detection and quantification (as provided by Percion Labs, US)

Plasma/serum samples were analysed quantitatively by LC-MS/MS using Precion's Short-Chain Fatty Acid Panel Method for Plasma/Serum (Analytical Test Method ATM004).Individual analytes and their respective calibration ranges are listed in **Table 3.1.**A solution of stable labelled internal standards (sodium acetate-d3, sodium propionate-d5, Isobutyric acid-d3, sodium butyrate-d3, 2-methylbutyric acid-d3, isovaleric acid-d7, valeric acid-d3, hexanoic acid-d3) was added to 50 ul of plasma sample followed by protein precipitation using methanol. After centrifugation, a

portion of the supernatant was removed and derivatized with a substituted hydrazine to form the corresponding acid hydrazides. An aliquot of the resulting mixture was analysed on a Sciex Exion LC/Sciex 5500+ Triple Quadrupole Mass Spectrometer LC-MS/MS system in ESI and multiple reaction monitoring mode (MRM) using C18 reversed phase chromatography.

The peak areas of the respective parent to product ion transitions were measured against the peak areas of the parent to product ion transitions of the corresponding labelled internal standards. Stable labelled versions of each of the analytes were used as internal standards. Quantitation was performed using a weighted linear least squares regression analysis generated from fortified calibration standards (8 to 10 concentration levels, depending on analyte) prepared concurrently with study samples and quality control samples in each analytical run.

Plasma/Serum		
Component Name	**LLOQ (ug/mL)**	**ULOQ (ug/mL)**
2-methylbutyrate	0.0250	2.00
acetate	1.00	160
butyrate	0.0200	1.60
caproate	0.100	8.00
isobutyrate	0.0250	2.00
isovalerate	0.0250	2.00
propionate	0.0800	6.40
valerate	0.0200	1.60

Table 3. 1. Analytes and Calibration Ranges for Plasma/Serum

3.5.4 Data and statistical analysis

Data from 16S rRNA gene sequencing was analysed using the Microbiology module (PERMANOVA performing 99,999 permutations) of QIAGEN CLC Workbench CLC Genomic Workbench was used to further analyse the sequence data including taxonomic assignments and operational taxonomic unit (OTU) clustering. The metadata was merged with the OTU tables for proper aggregation and differentiation. The final taxonomic level of 16S rRNA amplicon sequencing was to show the important changes in the microbiome, the data was presented at different taxonomic levels. Also, data was retrieved and analysed using general linear model, SPSS 26.0 (IBM SPSS, Inc., Chicago, IL, USA). SCFA data were refined using MS Excel (Microsoft®) and analysed with general linear modelling in SPSS version 26.0 using 'treatment' and 'litter' as factors. A value of $p<0.05$ was considered to be significant.

3.6 Results

3.6.1 DNA extraction from rectal contents of neonatal piglets from all treatment groups

The optimized method 'M' resulted in good quality gDNA with distinct bands at ≥10 kb with limited degradation. In addition, there was a higher PCR product yield. This confirmed the suitability of this pre-treatment - extraction method 'M' for isolation of gDNA from piglet gut microbiota samples (**Figure 3.4 a & b**). The gDNA quality and quantity were also analysed by UV-spectroscopy using a NanoDrop and/or DeNovex system.

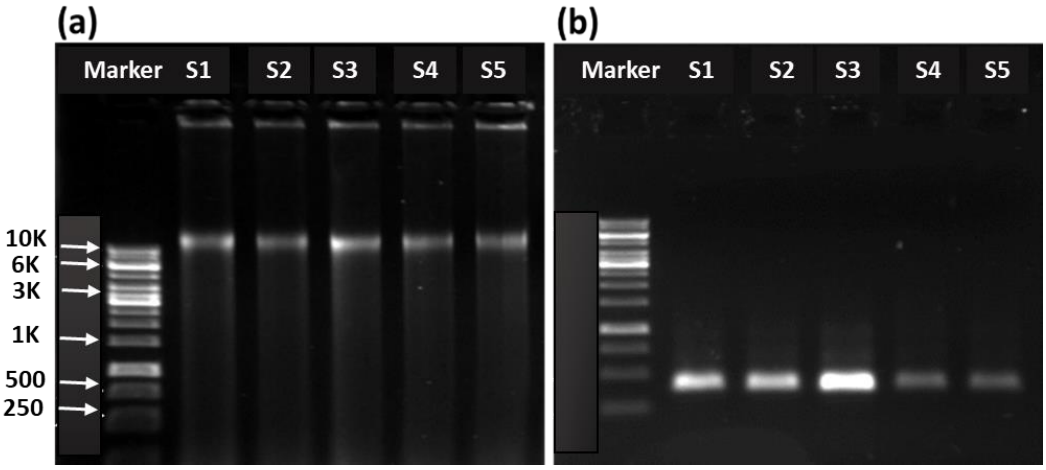

Figure 3. 3. Agarose gel (1%) of microbial gDNA extracted from rectal content of piglets using method 'M' at day 28. Lane 1 represents a 10Kbp DNA ladder.Each lane from S1-S5 represents pooled a sample (homogenized mix of 5 sample of rectal content from 5 different

piglets used for both methods of extraction to minimize the chances of any variable other than extraction method). The microbial gDNA samples extracted using standard QIamp kit method designed for human stool samples (a). A standard RT-PCR was performed to validate the quality of PCR product (regions V4-V5) from gDNA samples (b). Expected and observed gDNA band at ~ 10Kbp, and PCR product at 500bp.

3.6.2 Impact of iron regime on piglet growth and gut microbiota

As a result of 16S sequencing, the total number of reads in OTUs were 742422 and average number of reads per sample was 30934, and in total, 13 phyla, 22 families, 52 genera were detected. Overall treatments were compared using PERMANOVA in Qiagen CLC-workbench to determine the impact on gut microbiota composition using Bonferroni corrections for *p*-values. Principal coordinates of Weighted Unifrac distances (β-diversity) are presented in **Figure 3.5** between non-iron treated and iron treated, between all four treatments, and between IM iron and oral iron treatments. Alpha diversity, the diversity within the treatments (Shannon) is presented in **Figure 3.6.** There were no significant differences between all four treatments at phylum and class levels (**Figure 3.7**).

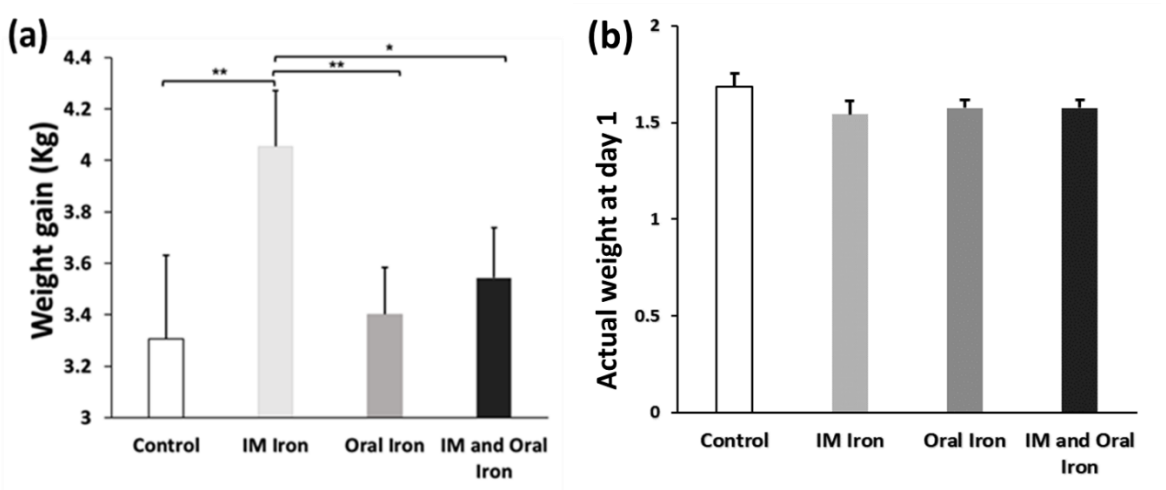

Figure 2.2 (from chapter 2): Impact of iron deficiency anaemia (IDA) and different types of iron treatment on growth (weight gained) in piglets between birth and 28 days old. Growth rate was significantly higher in piglets receiving intramuscular (IM) iron treatment (200 mg) compared to control pigs which did not receive iron treated (p=7x10^{-3}), oral iron (p=0.01), and IM & oral iron (p=0.04) respectively (a). Sequential growth chart of piglets (b). n=6 piglets/treatment group. Error bars = SEM, ** p<0.01, * p<0.05.

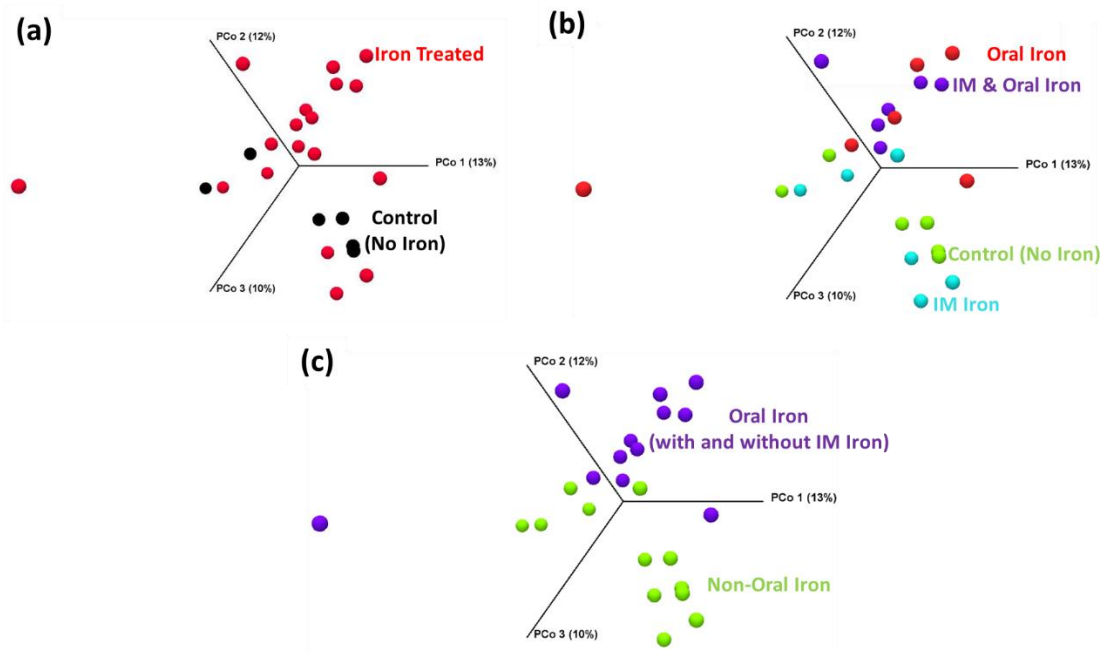

Figure 3. 4. Effect of iron deficiency anaemia (IDA, no iron) and different iron treatments (with different combinations) on beta diversity. Microbiota composition was determined by NGS of 16S rRNA gene amplicons. Principal coordinates of Weighted Unifrac distances (β-diversity) for the corresponding microbiota samples (at the 97% sequence similarly level). Comparing the impact of no-iron and all iron treated (a), all four treatments (b), and oral iron and non-oral iron treated (c) on rectal content samples from piglets.

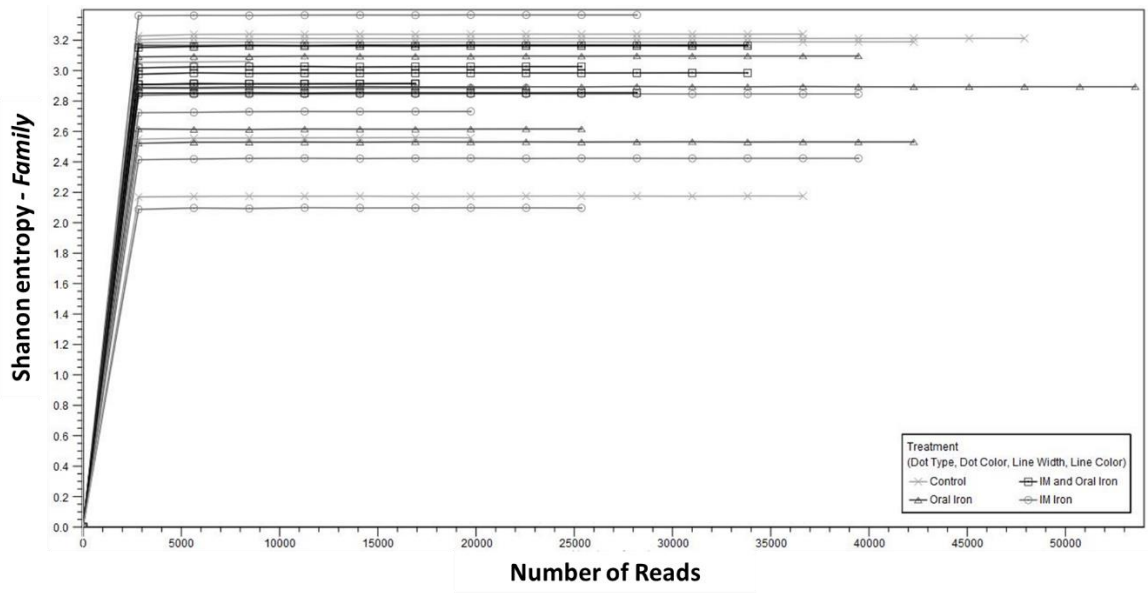

Figure 3. 5. Alpha diversity showing diversity within the treatments using Shannon index. No statistical difference was observed between the samples (Kruskal-Wallis p-value>0.05).

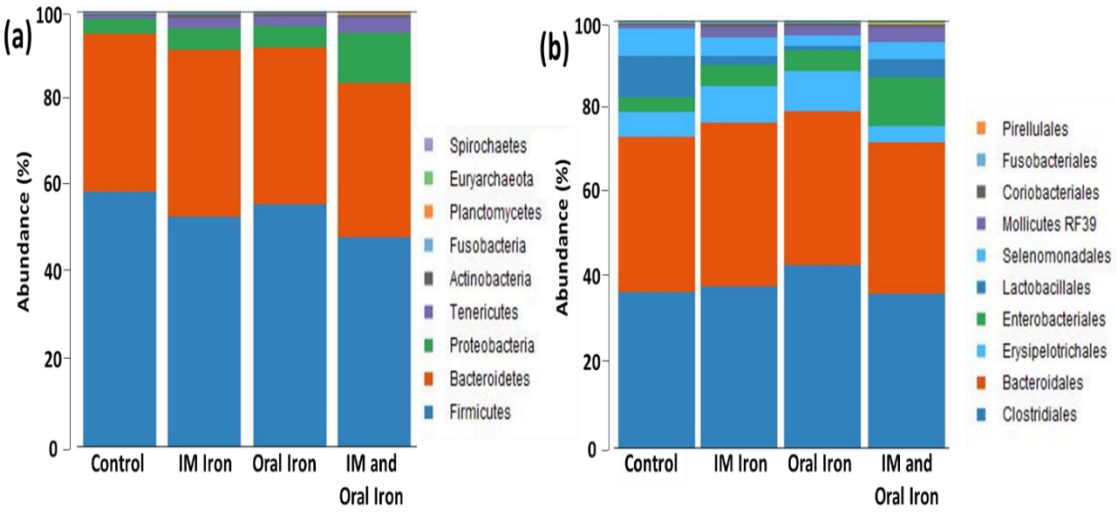

Figure 3. 6. Impact all four treatments on bacterial abundance in rectal content of piglets at different taxa levels at day 28. Control group piglets were not treated with iron and developed IDA (section 2.6.2) however iron-treated litter-matched piglets were given intramuscular iron injection (200mg, one dose) and/or oral iron supplement ($FeSO_4$, 150mg/day). The stacked bar charts display the average relative microbiota abundance compositions in rectal content from control (no-iron) and all iron treated piglets, at phylum (**a**), and class (**b**) taxonomic level.

3.6.2.1 Iron treatments resulted in reductions in the abundance of Bacilli, Lactobacillales and Lactobacillus compared to IDA

Abundance of Bacilli was significantly reduced (p<0.01) in iron treated piglets (0.022) compared to their IDA siblings (0.083) at day 28 (**Figure 3.8a & d**). Lactobacillales abundance was reduced (p<0.05) in piglets which received any form of iron treatment (0.02) compared to their anaemic counterparts (0.071) (**Figure 3.8b & e**). Abundances of *Lactobacillus* were significantly reduced in piglets which received any form of iron treatment (0.02) compared to their anaemic siblings (0.071) at day 28 (**Figure 3.8c & f**).

3.6.2.2 Oral iron supplementation with and without intramuscular iron supplementation was linked with reduced abundance of Lachnospiraceae

Abundance of Lachnospiraceae was significantly reduced (p<0.05) in response to oral iron treatment with or without IM iron compared to only IM iron treatment. IM iron treated piglets had significantly higher abundance of Lachnospiraceae (0.2)

compared to oral (0.14) and oral with IM iron treated counterparts (0.12) at day 28 (**Figure 3.9**). Oral iron treated groups (when combined according to **Figure 3.2**) showed significantly reduced abundance (0.13) of Lachnospiraceae against IM iron treated group (0.21) at day 28.

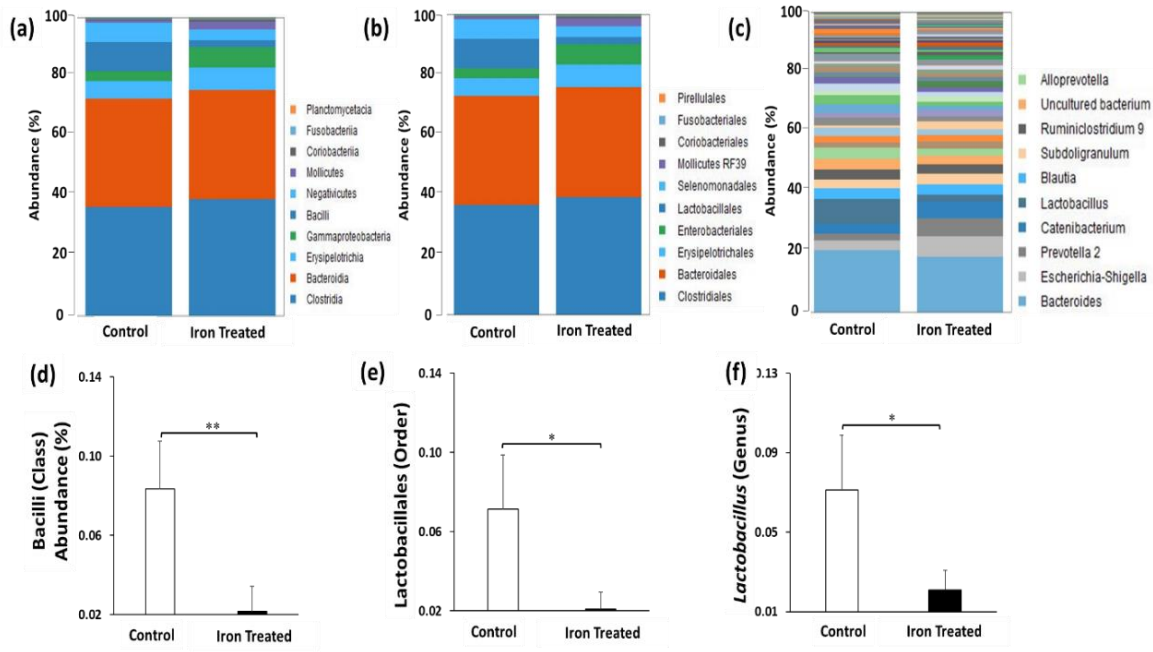

Figure 3. 7. Impact of iron deficiency anaemia (IDA, Control) and iron treatment on bacterial abundance in rectal content in piglets at different taxa levels at day 28. Control group piglets were not treated with iron and developed IDA (section 2.6.2) however iron-treated litter-matched piglets were given intramuscular iron injection (200mg, one dose) and/or oral iron supplement ($FeSO_4$, 150mg/day). The stacked bar charts display the average relative microbiota abundance compositions in rectal content from control (no-iron) and all iron treated piglets, at class (**a**), order (**b**), and genus (**c**) taxonomic level. Bar graphs showing the specific average abundance of Bacilli (d); Lactobacillales (e); *Lactobacillus* (f). Control group, n=6 litter-matched piglets receiving no iron treatment; iron supplemented group, n=18 litter-matched piglets receiving IV-Fe, oral Fe or both oral- and IV-Fe. Error bars = SEM, * $p<0.05$; ** $p<0.01$.

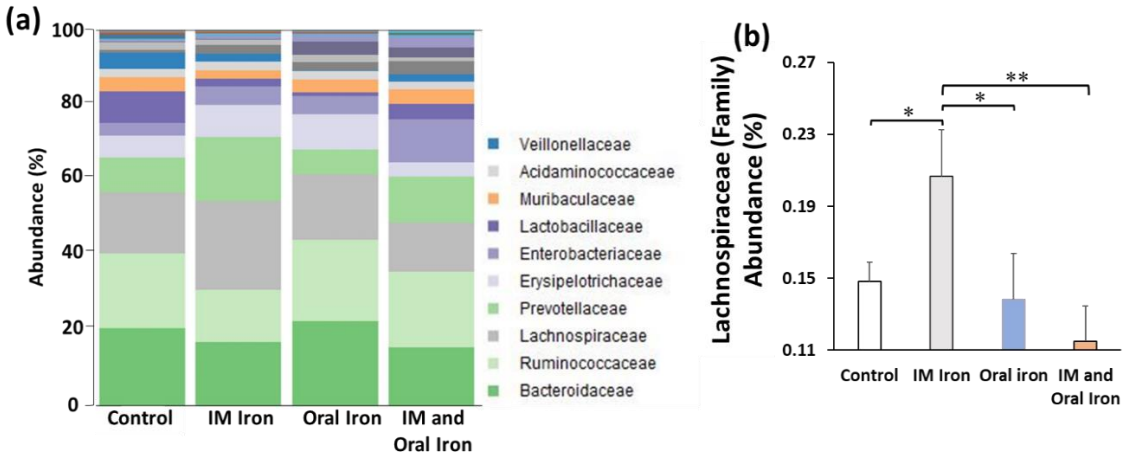

Figure 3. 8. Impact of iron deficiency anaemia (IDA, control) and different types of iron treatments on the abundance of Lachnospiraceae in rectal content in piglets at day 28. Control group piglets were not iron treated and developed IDA however treated litter-matched piglets were given intramuscular iron injection (200mg, one dose) and/or oral iron supplement ($FeSO_4$, 150mg/day). The stacked bar charts display the average relative microbiota abundance compositions for all four treatments, at family taxonomic level (**a**). (**b**) Specifically comparing the impact of different types of iron treatment and the no iron treatment on Lachnospiracea abundance; n=6 litter-matched piglets in each group Error bars = SEM, * p<0.05; ** p<0.01.

3.6.2.3 Iron treatment affects the abundance of Clostridium, Ruminococcus, and Coprococcus

Abundance of *Clostridium* was significantly reduced (p<0.05) in iron treated piglets (0.00005) compared to their IDA siblings (0.0002) at day 28 (**Figure 3.10a**). *Ruminococcus* abundance was reduced (p<0.05) in piglets received oral iron treatment with or without IM iron (0.01) compared to IM treated counterparts (0.03) (**Figure 3.10b**). Abundance of *Coprococcus* was reduced in oral iron treated piglets (0.001) compared to their IM treated siblings (0.004) at day 28 (**Figure 3.10b**).

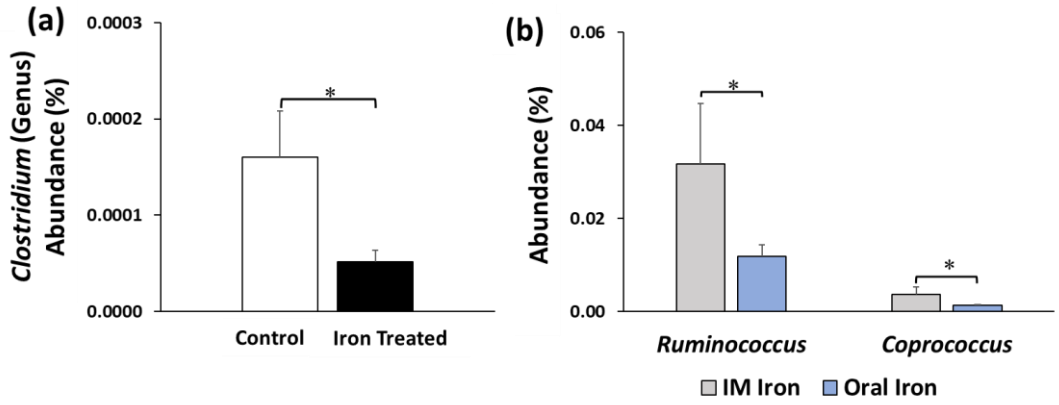

Figure 3. 9. Impact of iron treatments on bacterial abundance in rectal content in piglets at different taxa levels at day 28. (a) abundance of *Clostridium*, control group piglets were not iron treated and developed IDA however iron treated litter-matched piglets were given intramuscular iron injection (200mg, one dose) and/or oral iron supplement ($FeSO_4$, 150mg/day). n=6 litter-matched piglets in control group; n=18 litter-matched piglets in iron treated groups. (b) abundance of *Ruminococcus* and *Coprococcus,* IM iron treated piglets were given intramuscular iron injection (200mg, one dose), and oral iron treated group received $FeSO_4$, 150mg/day with or without IM iron. n=6 litter-matched piglets in IM treated group, and n=12 litter-matched piglets in oral iron treated group. Error bars = SEM, * p<0.05.

3.6.3 Oral and IM iron treatment caused reduction in the systemic concentrations of SCFAs such as propionic, valeric, and caproic acid

Plasma concentration of propionic acid was significantly reduced (p<0.05) in oral iron (-0.05 ug/mL), IM iron (-0.12 ug/mL), and anaemic piglets (-0.0675 ug/mL) compared to IM with oral iron treated siblings (0.12 ug/mL), over the period of 28 days (**Figure 3.11a**). Systemic concentration of valeric acid was significantly reduced (p<0.05) in oral iron (-0.009 ug/mL), IM iron (-0.02 ug/mL), and anaemic piglets (-0.02 ug/mL) compared to IM with oral iron treated siblings (0.014 ug/mL), over the period of 28 days (**Figure 3.11b**). Systemic concentration of caproic acid was significantly reduced (p<0.05) in IM iron (-0.38 ug/mL), and anaemic piglets (-0.35 ug/mL) compared to IM and oral iron treated siblings (0.043 ug/mL), over the period of 28 days (**Figure 3.11c**). Moreover, change in isobutyric concentration over the period of 28 days had significant (p<0.05) negative correlation (R^2=0.199) with weight gain of all piglets (**Figure 3.12**).

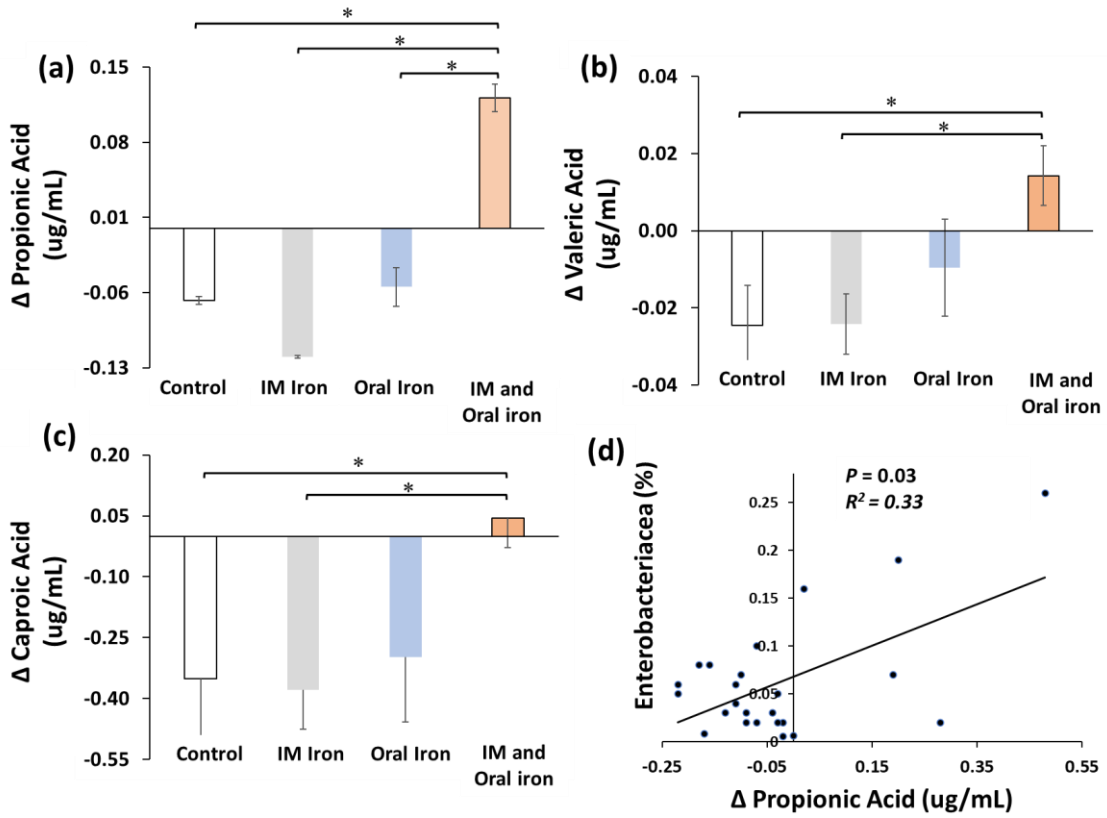

Figure 3. 10. Impact of IDA and different types of iron treatments on systemic concentrations of short chain fatty acids (SCFA) in piglets between day 1 and day 28. Control group piglets were not iron treated and developed IDA however iron treated litter-matched piglets were given intramuscular iron injection (200mg, one dose) and/or oral iron supplement ($FeSO_4$, 150mg/day). (a) change in propionic acid concentration in plasma samples, (b) change in valeric acid concentration in plasma samples, (c) change in valeric acid concentration in plasma samples. n=6 litter-matched piglets in each group. (d) correlation between Enterobacteriaceae and change in propionate over the period of 28 days, X-axis represents the change in propionate concentration between day 1 and 28, and Y-axis represents the abundance of Enterobacteriaceae in rectal microbiota at day 28. Error bars = SEM, * p<0.05.

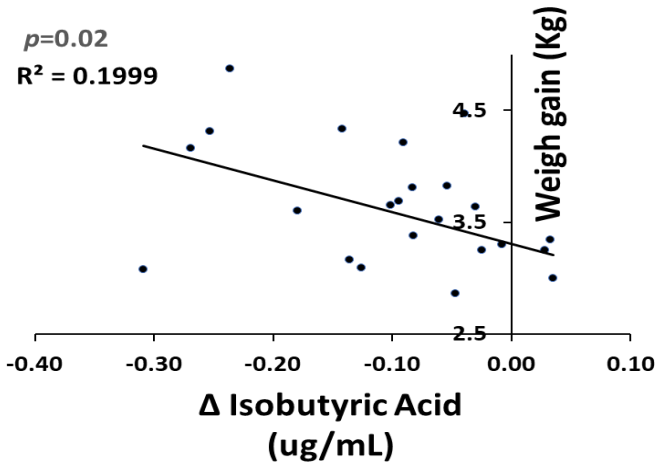

Figure 3. 11. Correlation between weight gain and change in systemic concentration of isobutyric acid between day 1 and day 28. Y-axis represents the change in weight (weight gain/growth) in piglets over the period of 28 days. X-axis represents the change in plasma concentration (increase/decrease) of isobutyric acid between day 1 and 28. Weight gain had significant negative correlation with change in isobutyric acid concentration in plasma over the period of 28 days.

3.7 Discussion

Since iron deficiency anaemia (IDA) is a significant worldwide challenge affecting high proportions of infants and children in low- and middle-income countries (LMIC), [42], a universal iron supplementation approach is the most common and cost-effective method used to combat this debilitating condition [43]. In this chapter, the effects of IDA, and different types of iron treatments, on gut microbiota composition and subsequent metabolic activity (systemic levels of SCFAs) were assessed using piglet models for human infants. Results showed significant impact of IDA and iron treatments on gut microbiota composition; however, this impact was observed only on few, yet important, bacterial taxa. As expected, results showed significant reductions in the abundance of *Lactobacillus,* and *Clostridium* in all iron treated piglets compared to their anaemic siblings. The results also show that oral iron as a sole iron treatment, and in combination with IM iron, caused significant reductions in the abundance of Lachnospiraceae compared to the levels recorded in their solely IM iron treated counterparts. Indeed, abundance of Lachnospiraceae in oral iron treated piglets were comparable to those which received no iron treatment and naturally became anaemic. When comparisons were made between oral iron and IM iron

treated piglets, oral iron caused reductions in abundances of *Ruminococcus* and *Coprcoccus*. In terms of SCFA production, oral iron, and IM iron caused significant reductions in systemic levels of propionic, valeric, and caproic acids in piglets compared to their counterparts who received oral iron in combination with IM iron treatment (group 4). Indeed, the levels of these SCFAs in IM iron and oral iron treated (group 2 & 3) piglets were comparable to their anaemic siblings. Furthermore, isobutyric acid was found to be negatively correlated with weight gain in all piglets, regardless of iron status. Taken together, our results demonstrate that there are significant effects of IDA, and different forms of iron treatments, on gut microbiota composition and their subsequent metabolic activity.

The data indicate that the abundances of the beneficial bacteria genus *Lactobacillus*, order lactobacillales and class Bacilli, were significantly reduced in response to all iron treatments (IM and oral). Our results are consistent with the outcomes of a randomised, placebo-controlled intervention trial by Dostal *et al* [44], in 6–11-year-old ID children who received either oral iron tablets (50 mg Fe) as $FeSO_4$ (n=22) or placebos (n=27) for 4 d/week (for 38 weeks), which reported significant overall decreases in lactobacilli populations ($p<0.05$) in response to the iron treatment. In another double-blind trial in 6-month-old Kenyan children (n=115), Tanja *et al* [45] reported significant increases in pathogenic *E. coli* ($p=0.029$) and faecal calprotectin (a gut inflammatory marker) ($p=0.002$), and also a border significant increase ($p=0.055$) in enterobacteria to lactobacilli ratio in response to administration of 12.5mg Fe-MNP. Knight *et al* [46] have demonstrated similar outcomes in a piglet trial (n=42) where those receiving iron (21.3 mg Fe/L) in sow milk replacer had significantly lower abundances of lactobacilli ($p=0.012$) compared to counterparts which received only 2.72 mg Fe/L) over 32 postnatal days. This, perhaps, is because lactobacilli are known to have a limited ability to utilize iron, which is an important nutrient for the majority of gut bacteria (including potential pathogens) [47], as they utilize manganese instead [48]. This is advantageous for lactobacilli under normal circumstances since breastfed infants have very low levels of iron in their gut [49]. By not competing for iron, lactobacilli in breastfed infants can outcompete other bacteria that require iron to grow, thereby helping to establish a healthy gut microbiome [50]. Since lactobacilli have evolved to use manganese as an alternative to iron, perhaps, it suggests that using manganese instead of iron (in the absence of IDA) may provide

certain advantages for lactobacilli in the gut. Manganese has also been shown to reduce oxidative stress and inflammation, which can be beneficial for gut health [51]. Manganese deficiency can occur in LMIC where children often have limited access to balanced diets [52], and across more developed populations where certain disorders can affect nutrient absorption, such as celiac disease and inflammatory bowel disease (IBD) [53, 54]. It is therefore possible that, manganese deficiency may further exacerbate the detrimental effects of oral iron treatments on lactobacilli populations. Lactobacilli are known to aid in the maintenance of gut barrier integrity by enhancing mucin production [55] which contributes to preventing harmful substances permeating through to the host [56], including translocation of enteric pathogens into host tissues [57]. This could be a mechanism by which oral iron treatments are associated with increased risk of infection [58]. Furthermore, lactobacilli are lactic acid producers and help to maintain a slightly acidic environment (pH 5.4-6.6) in the gut lumen [59]. This acidic environment disrupts the cell wall of Gram-negative bacteria, such as *E. coli* O157:H7, and increases their susceptibility to host-derived antimicrobial peptides including defensins and cathelicidins [60, 61]. In this way, Gram-positive lactobacilli populations may help to maintain a healthy gut microbiota by generating environments inhospitable to enteropathogens and limiting pathogenic colonization of the gut [62]. Taken together, there appear to be several mechanisms by which increased luminal iron availability impacts on gut health by reducing lactobacilli populations, either directly or indirectly.

We show that there were significant reductions in Lachnospiraceae in response to oral iron, with and without IM iron treatment. When comparing oral iron against IM iron treatment in our piglets, abundances of *Ruminococcus* and *Coprcoccus* were significantly reduced in response to oral iron treatment. It is important to note that *Coprcoccus* are part of the Lachnospiraceae, and this family is known to play important roles in the gut microbiome, including the production of certain SCFAs [63]. Systemic concentration of propionic, valeric, and caproic acids were reduced in response to oral iron with- and without IM iron treatment when compared against IM iron treatment alone. Reductions in Lachnospiraceae in our *in vivo* study is consistent with the findings of Dostal *et al* [27] in an *in vitro* colon model inoculated with immobilized gut microbiota from a 2.5-year-old child with diversified gut microbiota (and no antibiotics 3 months before sample collection). Here, a shift towards lower

abundance of Lachnospiraceae and Ruminococcae were observed under high iron (217mg/L) conditions compared to low iron conditions mimicked by feeding a medium containing 200 μM 2,2′-dipyridyl (chelator). They also reported significant increases in propionate and decreases in butyrate production. These specific shifts in SCFA production could be due to reductions in the abundance of Lachnospiraceae which are major butyrate producers [64], and Enterobacteriaceae are major producers of propionate [65]. Interestingly, results of our study show significant positive correlation between the abundance of Enterobacteriaceae and propionate concentrations over 28 days. However, there were no significant differences observed in Enterobacteriaceae abundances between any of the different forms of iron treatments we used. This means, perhaps, iron availability does not affect Enterobacteriaceae populations within gut microbiotas. However, it does suggest that metabolic activity of this population is influenced by iron availability. Including production of propionate as a metabolic end product, Knight *et al* [46] also reported significantly higher concentration of propionate along with lower concentrations of acetate in dry colonic matter from iron supplemented pigs compared to ID pigs. Taken together, our results are consistent with luminal iron availability affecting levels of certain components of the gut microbiota, whilst impacting on metabolic activity in others. Since the neonatal period is a critical time for establishment of health gut microbiotas [66], iron-induced disruptions to normal patterns of development could have sustained effects on its composition and functionality in later life, even after ID and IDA has been resolved.

Microbial-derived SCFAs are known to play important roles in overall growth and development in mammals [67, 68]. For example, a study conducted in young Indonesian children (aged 3-5 years) by Surono *et al* [69] reported higher concentrations of faecal SCFAs (including, butyrate, propionate, acetate and valerate) in stunted children (n=78) compared to counterpart children with normal nutritional status (n=53). This trial also reported that the abundance of Lachnospiraceae was higher in children undergoing normal growth compared to stunted counterparts, and that fibre intake was positively correlated with Lachnospiraceae abundance [70]. These links have been reported in a prospective study, conducted in 1-4 years old Pakistani children (n=60 stunted; 4 with normal growth), by Aziz *et al* who reported positive associations between weight and Lachnospiraceae abundance [71]. This is consistent with our study

where IM iron treated piglets (with the highest weight gain) were observed with the highest abundance of Lachnspiraceae compared to their oral iron supplemented siblings which, perhaps, highlights the important role of Lacnospiraceae in growth. This may be the mechanism by which oral iron consumption significantly inhibited weight gain in our piglets.

3.8 Conclusion

In conclusion, outcomes from our study are consistent with piglets being valuable models for human infants in regard to assessing the impacts of IDA and iron treatments on both gut microbiota composition and production of metabolic end products. The results in this chapter highlight the links between the developing gut microbiota and host growth during the neonatal period. Specifically, it further promotes the existence of positive associations between weight and Lachnospiraceae abundance, possibly as a consequence of butyrate production. This could be the mechanism by which increasing iron availability in the gut leads to reductions in weight gain. This is likely to have important implications for formula-fed infants, compared to those who are exclusively breastfed, since they have very high levels of unabsorbed iron in their gut lumens because the majority of bovine-based formula milks are highly fortified with iron. This iron is available to gut bacteria and could not only skew microbiota development, but also lead to reductions in weight gain. As a direct result of increased iron availability, formula fed infants are also highly likely to have significant reductions in lactobacilli, which are highly abundant in breast-fed infants. These populations provide competitive inhibition of bacterial populations which are detrimental to infant health including, potentially, enteropathogens. Indirectly, lactobacilli-induced reductions in luminal pH can also be reversed in the presence of high iron concentrations which also promotes pathogen growth, whilst lactobacilli-induced promotion of intestinal barrier function is also reduced, increasing bacterial translocation into the underlying tissue which may promote inflammatory immune responses. This study also has important implications for universal iron programmes in LMICs where over half of infant and child populations are not iron deficient and therefore do not benefit from iron treatments. Indeed, such unnecessarily increasing luminal iron may have overall negative impacts on these populations, especially in terms of reductions in weight gain and increased risks of enteric infections. Our results suggest that careful consideration

should be taken when developing iron treatment interventions to both iron deficient and iron replete populations.

3.9 References

1. Ruiz-Argüelles, G.J. Iron deficiency anemia in low- and middle-income countries. *Blood* **122**, 2289-2289 (2013).

2. Iron needs of babies and children. *Paediatr Child Health* **12**, 333-336 (2007).

3. Lönnerdal, B., Georgieff, M.K. & Hernell, O. Developmental Physiology of Iron Absorption, Homeostasis, and Metabolism in the Healthy Term Infant. *Journal of Paediatrics* **167**, S8-14 (2015).

4. Brannon, P.M. & Taylor, C.L. Iron Supplementation during Pregnancy and Infancy: Uncertainties and Implications for Research and Policy. *Nutrients* **9**, 1327 (2017).

5. Iannotti, L.L., Tielsch, J.M., Black, M.M. & Black, R.E. Iron supplementation in early childhood: health benefits and risks. *The American Journal of Clinical Nutrition* **84**, 1261-1276 (2006).

6. Akpan, E. *et al.* Cost-effectiveness of universal iron supplementation and iron-containing micronutrient powders for anemia among young children in rural Bangladesh: analysis of a randomized, placebo-controlled trial. *The American Journal of Clinical Nutrition* **116**, 1303-1313 (2022).

7. Mwangi, M.N., Prentice, A.M. & Verhoef, H. Safety and benefits of antenatal oral iron supplementation in low-income countries: a review. *British Journal of Haematology* **177**, 884-895 (2017)

8. Dewey, K.G. *et al.* Iron Supplementation Affects Growth and Morbidity of Breast-Fed Infants: Results of a Randomized Trial in Sweden and Honduras. *The Journal of Nutrition* **132**, 3249-3255 (2002).

9. Zlotkin, S. *et al.* Effect of iron fortification on malaria incidence in infants and young children in Ghana: a randomized trial. *Jama* **310**, 938-947 (2013).

10. Sazawal, S. *et al.* Effects of routine prophylactic supplementation with iron and folic acid on admission to hospital and mortality in preschool children in a high malaria transmission setting: community-based, randomised, placebo-controlled trial. *The Lancet* **367**, 133-143 (2006)

11. Adam, I. Anemia, Iron Supplementation and Susceptibility to Plasmodium falciparum Malaria. *EBioMedicine* **14**, 13-14 (2016).

12. Gwamaka, M. *et al.* Iron deficiency protects against severe Plasmodium falciparum malaria and death in young children. *Clinical Infectious Diseases* **54**, 1137-1144 (2012).

13. Kabyemela, E.R., Fried, M., Kurtis, J.D., Mutabingwa, T.K. & Duffy, P.E. Decreased susceptibility to Plasmodium falciparum infection in pregnant women with iron deficiency. *The Journal of Infectious Diseases* **198**, 163-166 (2008).

14. Jonker, F.A. *et al.* Iron status predicts malaria risk in Malawian preschool children. (2012).

15. Wallace, D.F. The Regulation of Iron Absorption and Homeostasis. *Clinical Biochemistry Reviews* **37**, 51-62 (2016).

16. Kang, J.O. Chronic iron overload and toxicity: clinical chemistry perspective. *Clinical Lab Science* **14**, 209-219; quiz 222 (2001).

17. Hare, D.J. *et al.* Health outcomes of iron supplementation and/or food fortification in iron-replete children aged 4–24 months: protocol for a systematic review and meta-analysis. *Systematic Reviews* **8**, 253 (2019).

18. Paganini, D., Uyoga, M.A. & Zimmermann, M.B. Iron Fortification of Foods for Infants and Children in Low-Income Countries: Effects on the Gut Microbiome, Gut Inflammation, and Diarrhea. *Nutrients* **8** (2016).

19. Piskin, E., Cianciosi, D., Gulec, S., Tomas, M. & Capanoglu, E. Iron Absorption: Factors, Limitations, and Improvement Methods. *ACS Omega* **7**, 20441-20456 (2022).

20. Armitage, A.E. *et al.* Rapid growth is a dominant predictor of hepcidin suppression and declining ferritin in Gambian infants. *Haematologica* **104**, 1542-1553 (2019).

21. Rahman, S. *et al.* Effect of low-iron micronutrient powder (MNP) on the composition of gut microbiota of Bangladeshi children in a high-iron groundwater setting: a randomized controlled trial. *European Journal of Nutrition* **60**, 3423-3436 (2021).

22. Friel, J., Qasem, W. & Cai, C. Iron and the Breastfed Infant. *Antioxidants (Basel)* **7** (2018).

23. Andrews, S.C., Robinson, A.K. & Rodríguez-Quiñones, F. Bacterial iron homeostasis. *FEMS Microbiology Reviews* **27**, 215-237 (2003).

24. Rusu, I.G. *et al.* Iron Supplementation Influence on the Gut Microbiota and Probiotic Intake Effect in Iron Deficiency-A Literature-Based Review. *Nutrients* **12** (2020).

25. Zimmermann, M.B. *et al.* The effects of iron fortification on the gut microbiota in African children: a randomized controlled trial in Cote d'Ivoire. *American Journal of Clinical Nutrition* **92**, 1406-1415 (2010).

26. Silva, Y.P., Bernardi, A. & Frozza, R.L. The Role of Short-Chain Fatty Acids From Gut Microbiota in Gut-Brain Communication. *Frontiers in Endocrinology* **11** (2020).

27. Dostal, A. *et al.* Iron Modulates Butyrate Production by a Child Gut Microbiota In Vitro. *mBio* **6**, e01453-01415 (2015).

28. Rose, E.C., Blikslager, A.T. & Ziegler, A.L. Porcine Models of the Intestinal Microbiota: The Translational Key to Understanding How Gut Commensals Contribute to Gastrointestinal Disease. *Frontier in Veterinary Sciences* **9**, 834598 (2022).

29. Ma, J. *et al.* Gut Microbiota Composition and Diversity in Different Commercial Swine Breeds in Early and Finishing Growth Stages. *Animals (Basel)* **12** (2022).

30. Chalvon-Demersay, T., Blachier, F., Tomé, D. & Blais, A. Animal Models for the Study of the Relationships between Diet and Obesity: A Focus on Dietary Protein and Estrogen Deficiency. *Frontiers in Nutrition* **4**, 5 (2017).

31. Brink, L.R. *et al.* Neonatal Diet Impacts Bioregional Microbiota Composition in Piglets Fed Human Breast Milk or Infant Formula. *The Journal of Nutrition* **149**, 2236-2246 (2019).

32. Ding, H. *et al.* Tolerable upper intake level of iron damages the intestine and alters the intestinal flora in weaned piglets. *Metallomics* **12**, 1356-1369 (2020).

33. Wernroth, M.-L. *et al.* Development of gut microbiota during the first 2 years of life. *Scientific Reports* **12**, 9080 (2022).

34. Lv, H., Zhang, L., Han, Y., Wu, L. & Wang, B. The Development of Early Life Microbiota in Human Health and Disease. *Engineering* **12**, 101-114 (2022).

35. Yilmaz, B. & Li, H. Gut Microbiota and Iron: The Crucial Actors in Health and Disease. *Pharmaceuticals (Basel)* **11** (2018).

36. Abbas, M., Hayirli, Z., Drakesmith, H., Andrews, S.C. & Lewis, M.C. Effects of iron deficiency and iron supplementation at the host-microbiota interface: Could a piglet model unravel complexities of the underlying mechanisms? *Frontiers in Nutrition* **9**, 927754 (2022).

37. Tang, J.N. *et al.* An effective method for isolation of DNA from pig faeces and comparison of five different methods. *Journal of Microbiolgy Methods* **75**, 432-436 (2008).

38. Ellis, R.J. *et al.* Comparison of the distal gut microbiota from people and animals in Africa. *PLoS One* **8**, e54783 (2013).

39. Wang, Q., Garrity, G.M., Tiedje, J.M. & Cole, J.R. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied Environmental Microbiology* **73**, 5261-5267 (2007).

40. Wang, Y. & Qian, P.Y. Conservative fragments in bacterial 16S rRNA genes and primer design for 16S ribosomal DNA amplicons in metagenomic studies. *PLoS One* **4**, e7401 (2009).

41. Brotz, P.G. & Schaefer, D.M. Simultaneous determination of lactic and volatile fatty acids in microbial fermentation extract by gas-liquid chromatography. *Journal of Microbiological Methods* **6**, 139-144 (1987).

42. Joo, E.Y., Kim, K.Y., Kim, D.H., Lee, J.E. & Kim, S.K. Iron deficiency anemia in infants and toddlers. *Blood Research* **51**, 268-273 (2016).

43. Akpan, E. *et al.* Cost-effectiveness of universal iron supplementation and iron-containing micronutrient powders for anemia among young children in rural Bangladesh: analysis of a randomized, placebo-controlled trial. *The American Journal of Clinical Nutrition* **116**, 1303-1313 (2022).

44. Dostal, A. *et al.* Effects of iron supplementation on dominant bacterial groups in the gut, faecal SCFA and gut inflammation: a randomised, placebo-controlled intervention trial in South African children. *British Journal of Nutrition* **112**, 547-556 (2014).

45. Tanja, J. *et al.* Iron fortification adversely affects the gut microbiome, increases pathogen abundance and induces intestinal inflammation in Kenyan infants. *Gut* **64**, 731 (2015).

46. Knight, L.C., Wang, M., Donovan, S.M. & Dilger, R.N. Early-Life Iron Deficiency and Subsequent Repletion Alters Development of the Colonic Microbiota in the Pig. *Frontiers in Nutrition* **6** (2019).

47. Huynh, U. *et al.* Differential Effects of Transition Metals on Growth and Metal Uptake for Two Distinct Lactobacillus Species. *Microbiology Spectrum* **10**, e01006-01021 (2022).

48. Poddar, D. *et al.* Manganese accumulation in probiotic Lactobacillus paracasei ATCC 55544 analyzed by synchrotron X-ray fluorescence microscopy and impact of accumulation on the bacterial viability following encapsulation. *Food Research International* **147**, 110528 (2021).

49. van den Elsen, L.W.J., Garssen, J., Burcelin, R. & Verhasselt, V. Shaping the Gut Microbiota by Breastfeeding: The Gateway to Allergy Prevention? *Frontiers in Pediatrics* **7** (2019).

50. Sousa Gerós, A., Simmons, A., Drakesmith, H., Aulicino, A. & Frost, J.N. The battle for iron in enteric infections. *Immunology* **161**, 186-199 (2020).

51. Carroll, I.M. *et al.* Anti-inflammatory properties of Lactobacillus gasseri expressing manganese superoxide dismutase using the interleukin 10-deficient mouse model of colitis. *American Journal of Physiology-Gastrointestinal and Liver Physiology* **293**, G729-G738 (2007).

52. Khayat, S., Fanaei, H. & Ghanbarzehi, A. Minerals in pregnancy and lactation: a review article. *Journal of Clinical and Diagnostic Research: JCDR* **11**, QE01 (2017).

53. Johnson, M.W., Ellis, H.J., Asante, M.A. & Ciclitira, P.J. Celiac disease in the elderly. *Nature Clinical Practice Gastroenterology & Hepatology* **5**, 697-706 (2008).

54. Goldschmid, S. & Graham, M. Trace element deficiencies in inflammatory bowel disease. *Gastroenterology Clinics of North America* **18**, 579-587 (1989).

55. Caballero-Franco, C., Keller, K., De Simone, C. & Chadee, K. The VSL# 3 probiotic formula induces mucin gene expression and secretion in colonic epithelial cells. *American Journal of Physiology-Gastrointestinal and Liver Physiology* **292**, G315-G322 (2007).

56. Alp, D. & KuleaŞan, H. Determination of competition and adhesion abilities of lactic acid bacteria against gut pathogens in a whole-tissue model. *Bioscience Microbiota Food Health* **39**, 250-258 (2020).

57. Dempsey, E. & Corr, S.C. Lactobacillus spp. for Gastrointestinal Health: Current and Future Perspectives. *Frontiers in Immunology* **13** (2022).

58. Reid, G. & Burton, J. Use of Lactobacillus to prevent infection by pathogenic bacteria. *Microbes and Infection* **4**, 319-324 (2002).

59. Ilhan, Z.E., Marcus, A.K., Kang, D.-W., Rittmann, B.E. & Krajmalnik-Brown, R. pH-mediated microbial and metabolic interactions in fecal enrichment cultures. *Msphere* **2**, e00047-00017 (2017).

60. Chen, X. *et al.* Synergistic effect of antibacterial agents human beta-defensins, cathelicidin LL-37 and lysozyme against Staphylococcus aureus and Escherichia coli. *J Dermatol Sci* **40**, 123-132 (2005).

61. Dempsey, E. & Corr, S.C. Lactobacillus spp. for Gastrointestinal Health: Current and Future Perspectives. *Frontiers in Immunology* **13**, 840245 (2022).

62. Liévin-Le Moal, V. & Servin, A.L. Anti-infective activities of lactobacillus strains in the human intestinal microbiota: from probiotics to gastrointestinal anti-infectious biotherapeutic agents. *Clin Microbiol Rev* **27**, 167-199 (2014).

63. Vacca, M. *et al.* The Controversial Role of Human Gut Lachnospiraceae. *Microorganisms* **8** (2020).

64. Louis, P. & Flint, H.J. Diversity, metabolism and microbial ecology of butyrate-producing bacteria from the human large intestine. *FEMS Microbiology Letters* **294**, 1-8 (2009).

65. Hosseini, E., Grootaert, C., Verstraete, W. & Van de Wiele, T. Propionate as a health-promoting microbial metabolite in the human gut. *Nutrition Reviews* **69**, 245-258 (2011).

66. Merrifield, C.A. *et al.* Neonatal environment exerts a sustained influence on the development of the intestinal microbiota and metabolic phenotype. *The ISME Journal* **10**, 145-157 (2016).

67. den Besten, G. *et al.* The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. *Journal of Lipid Research* **54**, 2325-2340 (2013).

68. Cong, J., Zhou, P. & Zhang, R. Intestinal Microbiota-Derived Short Chain Fatty Acids in Host Health and Disease. *Nutrients* **14** (2022).

69. Surono, I.S. *et al.* Differences in immune status and fecal SCFA between Indonesian stunted children and children with normal nutritional status. *Plos One* **16**, e0254300 (2021).

70. Surono, I.S., Widiyanti, D., Kusumo, P.D. & Venema, K. Gut microbiota profile of Indonesian stunted children and children with normal nutritional status. *Plos One* **16**, e0245399 (2021).

71. Aziz, I. *et al.* A prospective study on linking diarrheagenic E. coli with stunted childhood growth in relation to gut microbiome. *Scientific Reports* **13**, 6802 (2023).

CHAPTER 4

Impact of iron deficiency anaemia and different iron treatments on systemic lipidomic profiles

Abstract

The effects of iron deficiency anaemia (IDA) extend beyond well-established detrimental impacts on blood oxygenation and emerging evidence suggests that IDA has the potential to affect systemic lipids, although there is a paucity of knowledge in this field. However, differences have recently been reported between systemic lipidomic profiles in formula-fed and breast-fed infants, and iron content is an important difference between these two forms of infant nutrition. This chapter aimed to directly explore the impact of IDA on plasma lipidomic profiles, and to determine whether different forms of iron treatments influenced these profiles in differential manners. Twenty-four, 1 day old, piglets were assigned to 4 litter-matched treatment groups as detailed in **section 2.5.1** Treatment groups were then combined to assess the effects of IDA (n=6) on systemic lipids profiles compared to those in iron replete siblings (n=18). Treatment groups were also combined to compare the effects of oral iron (n=12) compared to intramuscular iron treatments (n=6) on systemic lipids using liquid chromatography / mass spectrometry (LC-MS). Results showed that IDA was associated with significant reductions in sphingolipids gangliosides (GM1), phospholipids especially lysophoshatidycholines (LPC), sphingomyelins SM, and sulfatides (p<0.05). When comparisons between IM iron and oral iron treatments were made, oral iron caused reductions in ceramides (Cer) (p<0.05), and IM iron caused reduction in phosphatidylglycerols (PG) (p<0.05). These findings suggest that IDA can significantly impact the metabolism of certain lipid subclasses (GM1, SM, sulfatides), which may directly, or indirectly, impact overall growth and neurodevelopment. Type of iron treatment can have differential impact on lipids especially involved in growth (Cer, PG), therefore selection of iron treatment should be given careful consideration. This is the first study, to the best of our knowledge, to explore the iron-lipidomics axis in piglets. Further research is needed to fully understand underlying mechanisms involved and to determine the optimal iron treatment strategies for restoring normal lipidomic profiles in infant populations.

4.1 Introduction

Lipidomic profiling is a comprehensive analysis of the types and concentrations of lipids present in a biological sample, such as plasma [1]. This includes triglycerides, cholesterol and its derivatives, phospholipids, sphingolipids, glycolipids, and steroids [2]. These lipids play critical roles in various biological processes including energy storage [3], cell membrane formation [4], regulation of signalling pathways [5], and overall growth and development [6]. In general, healthy systemic lipid profiles in humans are characterized by normal levels of cholesterol, triglycerides and other lipids [7] whereas in disease states these lipid profiles can be disrupted. In addition to cardiovascular disease, changes in lipid profiles have been linked to diseases such as inflammatory conditions, impaired brain development and diabetes. High levels of triglycerides and low levels of high-density lipoprotein (HDL) are often observed in diabetic patients [8]. This has been confirmed in an 8-year retrospective cohort study in Chinese adults by Zhao *et al* [9] where they concluded that there were significant associations between high levels of triglycerides and type-2 diabetes which suggests the importance and diverse role of lipid metabolism in health and disease.

Lipid profiles in early life are crucial as adequate amounts and balances of lipids are necessary for normal growth and development. Lipid profiles in breast-fed and formula-fed infants have been shown to differ significantly. For example, Acharjee *et al* [10] have reported specific types of sphingo- and phospholipids as robust biomarkers to differentiate between formula- and breastfed infants. Iron, in part, makes an important difference between breast milk and formula milk, as breast milk contains very low concentrations of iron (~ 0.3mg/ L) compared to formula (1-12mg/ L) [11]. In addition, iron and lipid metabolism are closely related since changes in systemic iron levels can affect systemic lipid profiles [12]. An ^{1}H NMR spectroscopy-based report by Rao *et al* [13] has shown significant differences in compositions of cerebrospinal fluid between anaemic and iron sufficient 7-month-old rhesus monkeys. Since lipids are a major part of brain structure, Vlasova *et al* [14] have observed the negative impact of IDA on brain development in non-human primate infants. More importantly they reported significant differences in anaemic subjects following iron repletion, compared to those having sustained normal iron status. This emphasises the importance of early diagnosis of iron deficiency, and subsequent iron

supplementation, in early life to limit the negative consequence of IDA on lipid profiles, and indirectly on brain development, as infants age.

Iron supplements, including oral iron and intravenous iron therapy, are commonly used to treat IDA [15]. These supplements can have a direct, as well as indirect, impact on lipid profiles. Directly, iron supplements can increase levels of iron storage markers in the body such as transferrin saturation and ferritin [16]. High levels of these markers are associated with increased peroxidation of lipids leading to increased risks of cardiac diseases [17]. A study in Iranian men (age 50-58) by Pourmoghaddas *et al* [18] has shown a strong association between high serum ferritin levels and coronary heart disease. Indirectly, Iron supplements can alter the composition and diversity of the gut microbiota [19] which plays a critical role in lipid metabolism [20]. One way in which the gut microbiome can affect lipid metabolism is through the production of short-chain fatty acids (SCFAs) [21]. These SCFAs, particularly acetate, propionate, and butyrate, can affect the expression of genes involved in lipid metabolism and reduce the absorption of lipids from the gut [22]. Additionally, the gut microbiome can affect the absorption and metabolism of bile acids, which are essential for the digestion and absorption of lipids [23]. In mice, Besten *et al* [24] have observed that propionate, butyrate and acetate can affect the expression of genes associated with hepatic metabolic pathways. For example, they concluded that caecal acetate and butyrate were crucial SCFA for mammalian lipid metabolism, while propionate is mainly involved in glucogenesis.

As previously detailed in **chapter 1**, pigs have been widely used as models for humans in a considerable range of nutritional studies [25, 26]. In relation to our study, lipid metabolism in pigs is largely comparable to that in humans, with similar fatty acid compositions in cell membranes between the two species [27], This is consistent with pigs being suitable models for exploring lipid-related bioprocesses such as the role of different types of lipids in overall growth performance [28]. In addition to being a natural IDA model [29], pigs have similar responses to diet [30] and genetic manipulation as humans do [31]. Consequently, pigs are valuable models for determining the effects of iron deficiency and iron supplementation on lipid metabolism, thus providing valuable insights into human lipid metabolism and potential therapies for lipid-related disorders. However, there is currently a paucity of knowledge of lipid profiling in infant

pigs, especially in in relation to the impact of iron status on these profiles. However, Shao *et al* [32] have reported the impact of abrupt weaning on lipid composition in jejunal crypt cells, where they established that certain types of phospho- and sphingolipids were reliable biomarkers for weaning stress. To the best of our knowledge, lipidomic profiles have not been explored in relation to IDA and iron supplementation in humans, or other species.

4.2 Hypotheses

Hypothesis 1

Since iron has both direct and indirect roles in overall growth, and lipids are basic building blocks for these processes, IDA will result in disruptions in systemic lipidomic profiles.

Hypothesis 2

Depending on microbial requirements, iron availability will generate selective advantages and disadvantages for different bacterial taxa present in gut microbiotas. Different types of iron supplementation will result in differential lipidomic profiles.

4.3 Aim

The aim of this study was to identify disruptions in lipidomic profiles in response to IDA, and how different types of iron supplementations could correct or/and change these profiles using piglet models for human infants.

4.4 Objectives

4. To determine whether IDA disrupts systemic lipid profiles in infants using piglet models.
5. To quantify the impact of different types of iron supplementation on lipidomic profiles, use liquid chromatography mass spectrometry (LC-MS).

4.5 Materials and Methods

4.5.1 Animal Model

To assess the impact of iron deficiency anaemia (IDA) and different types of iron treatments on systemic lipid profiles, 24 large white F1 hybrid piglets from 6 litters were allocated into 4 litter- and sex-matched (3 male + 3 female) treatment groups at 1 day

old. Treatments were group 1. Control (no iron treatment): 2. Intramuscular iron (IM) injection (200mg Fe): 3. Iron supplemented ($FeSO_4$) sow milk replacer (SMR) (oral iron; 150mg/kg body weight/day): 4. IM and oral iron, as detailed in **Figure 2.1.**

Additionally, we distributed the treatments in two different manners for analysis purposes to see whether overall supplementation or different type of iron supplementation influence the lipidomic profiles. The second set of treatments was group 1. Control (no iron supplementation, n=6): group 2. Iron supplementation (IM iron or/and oral iron, n=18) (**figure 3.1**). The third set of treatments was group 1. IM iron (n=6): groupv2. Oral iron (with and without IM iron, n=12) (**figure 3.2)**.

4.5.2 Sample collection

Blood samples were collected weekly from day 1 in EDTA vacutainers (BD Vacutainer®). These samples were centrifuged at centrifuged at 15000 g for 10 minutes at 4^{o}C. All above samples were then stored at -80^{o}C.

4.5.3 Analysis

4.5.3.1 Systemic lipid profile

We quantified 360 lipids (of 20 different types) in plasma samples using LC-MS. Those twenty types are given below.

Carn = acyl-carnitines

Cer = ceramides

CL = cardiolipins

GM1 = gangliosides

Hex-cer = hexosylceramides

LPC = lysophoshatidycholines

LPE = lysophosphatidylethanolamines

LPI = lysophosphatidyinositols

PI = phosphatidyinositols

PS = phosphatidylserines

Mono-lyso_CL = monolyso cardiolipins

DG = diacylglycerides

MG = monoacylglycerides

PA = phosphtidic acids

PC = phosphatidylcholines

PE = phosphatidylethanolamines

PG = phosphatidylglycerols

SM - sphingomyelins

S - sulfatides

TG – triacylglycerides

4.5.3.1.1 Sample preparation

The protein-precipitation liquid extraction protocol has previously been described. (Jenkins et al., 2020)[33]. Briefly, 50 µL of plasma was transferred into 2 mL screw cap Eppendorf plastic tube (Eppendorf, Stevenage, UK). Immediately, 650 µL of chloroform was added to each sample, followed by thorough mixing. Then, 100 µL of the LIPID-IS (5 µM in methanol), 100 µL of the CARNITINE-IS (5 µM in methanol) and 150 µL of methanol was added to each sample, followed by thorough mixing. Then, 400 µL of acetone was added to each sample. The samples were vortexed and centrifuged for 10 minutes at ~20,000 g to pellet any insoluble material. The supernatant was pipetted into separate 2 mL screw cap amber-glass auto-sampler vials (Agilent Technologies, Cheadle, United Kingdom). The organic extracts were dried down to dryness using a Concentrator Plus system (Eppendorf, Stevenage, UK) run for 60 minutes at 60 degrees Celsius. The samples were reconstituted in 100 µL of 2: 1: 1 (propan-2-ol, acetonitrile and water, respectively) then thoroughly vortex. The reconstituted sample was transferred into a 250 µL low-volume vial insert inside a 2 mL amber glass auto-sample vial ready for liquid chromatography with mass spectrometry detection (LC-MS) analysis.

4.5.3.1.2 Liquid chromatography mass spectrometry

Full chromatographic separation of intact lipids was achieved using Shimadzu HPLC System (Shimadzu UK Limited, Milton Keynes, UK) with the injection of 10 µL onto a Waters Acquity UPLC® CSH C18 column (Waters, Hertfordshire, UK); 1.7 µm, I.D. 2.1 mm X 50 mm, maintained at 55 degrees Celsius. Mobile phase A was 6:4, acetonitrile and water with 10 mM ammonium formate. Mobile phase B was 9:1, propan-2-ol and acetonitrile with 10 mM ammonium formate. The flow was maintained at 500 µL per minute through the following gradient: 0.00 minutes_40% mobile phase B; 0.40 minutes_43% mobile phase B; 0.45

minutes_50% mobile phase B; 2.40 minutes_54% mobile phase B; 2.45 minutes_70% mobile phase B; 7.00 minutes_99% mobile phase B; 8.00 minutes_99% mobile phase B; 8.3 minutes_40% mobile phase B; 10 minutes_40% mobile phase B. The sample injection needle was washed using 9:1, 2-propan-2-ol and acetonitrile. The mass spectrometer used was the Thermo Scientific Exactive Orbitrap with a heated electrospray ionisation source (Thermo Fisher Scientific, Hemel Hempstead, UK). The mass spectrometer was calibrated immediately before sample analysis using positive and negative ionisation calibration solution (recommended by Thermo Scientific). Additionally, the mass spectrometer scan rate was set at 4 *Hz*, giving a resolution of 25,000 (at 200 *m/z*) with a full-scan range of *m/z* 100 to 1,800 with continuous switching between positive and negative mode.

4.6 Statistical analysis

LCMS data (absolute values in uM) were refined using MS Excel (Microsoft ®) and analysed with general linear modelling in SPSS version 26.0 (IBM SPSS, Inc., Chicago, IL, USA) using 'treatment', and 'litter' as factors. A value of $p<0.05$ was considered to be significant.

4.7 Results

4.7.1 Impact of iron deficiency anaemia on cellular lipid molecular species

4.7.1.1 Iron deficiency anaemia was associated with reduced systemic ganglioside (GM1), lysophosphatidylcholines (PC), and phosphatidylcholine (LPC) concentrations

Systemic concentrations of four GM1 (34:1, 34:1-OH, 34:0, 36:0) were significantly reduced in anaemic piglets ($p<0.05$) compared to their siblings which received iron supplementation (IM or/and oral iron), between the day 7 and day 28 (**Figure 4.1**).

Systemic concentrations of different isoforms of lysophosphatidylcholines (LPC) (16:0, 17:0, 18:0, 19:0, 20:3) in piglets not receiving iron were significantly reduced ($p<0.05$) compared to their siblings which received iron treatment (IM or/and oral iron), between the day 7 and day 28 (**Figure 4.2a-e**). Similarly, PC (38:0) concentration was significantly reduced ($p<0.05$) in non-supplemented piglets compared to their iron supplemented counter parts over the period of 21 days (**Figure 4.2f**).

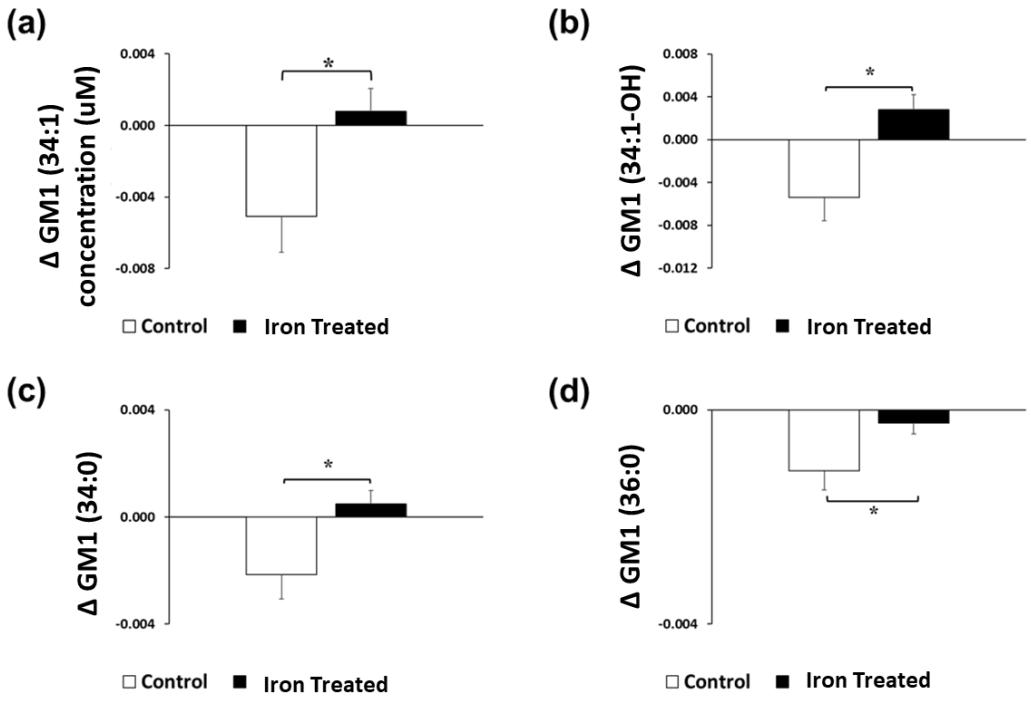

Figure 4. 1. Impact of iron deficiency anaemia (IDA) and iron treatment on systemic concentrations of ganglioside (GM1) between day 7 and 28. Control group piglets were not iron treated and developed IDA however iron-treated litter-matched piglets were given intramuscular iron injection (200mg, one dose) and/or oral iron supplement ($FeSO_4$, 150mg/day). (**a**), GM1 (34:1); (**b**), GM1 (34:1-OH); (**c**), GM1 (34:0), and (**d**), GM1 (36:0). n=6 litter-matched piglets in control group; n=18 litter-matched piglets in iron treated groups. Error bars = SEM, * p<0.05.

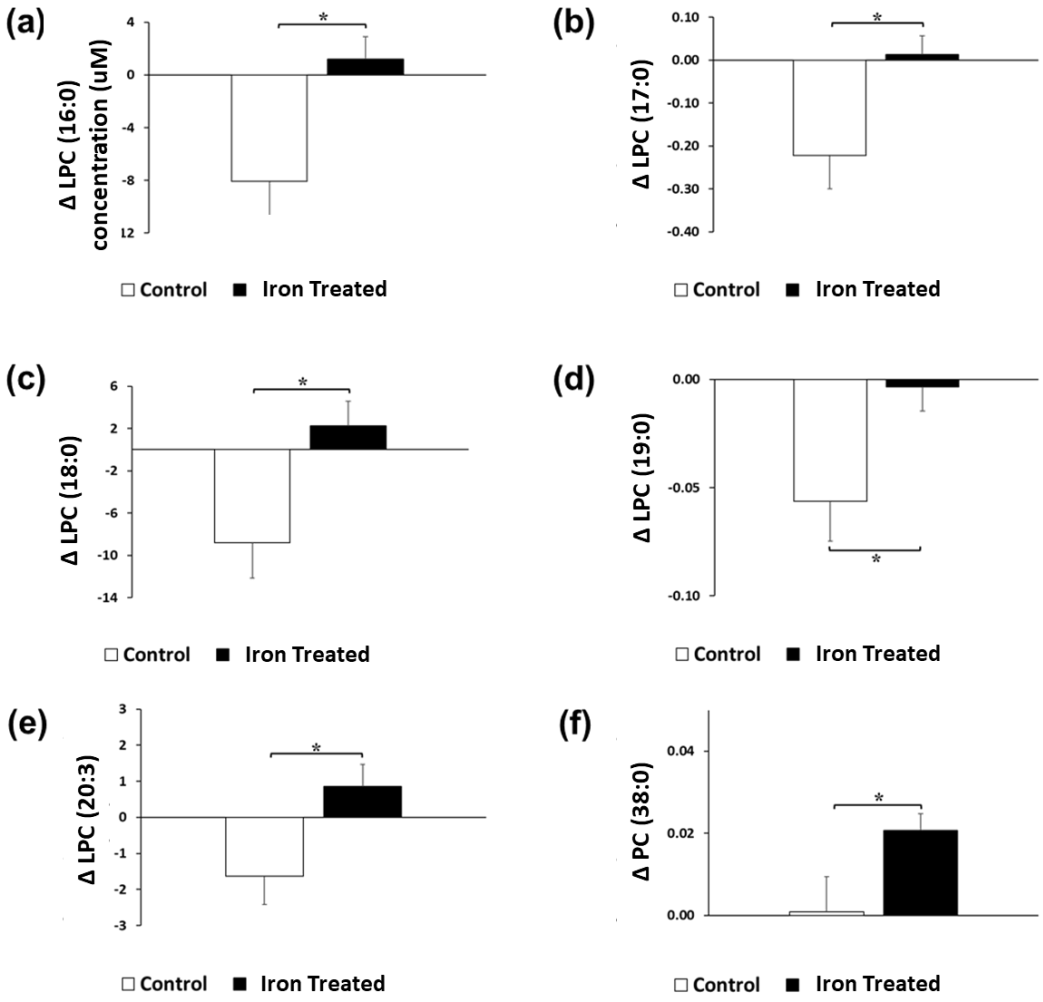

Figure 4. 2. Impact of iron deficiency anaemia (IDA) and iron treatments on systemic concentrations of lysophoshatidycholines (LPC) and phoshatidycholine (PC) by 21 days old. Control group piglets were left non-iron treated and IDA, however iron treated pigs were given intramuscular iron injection (200mg, one dose) or/and oral iron supplement ($FeSO_4$, 150mg/day) (**Figure 3.1**). (**a**), LPC (16:0); (**b**), LPC (17:0); (**c**), LPC (18:0); (**d**), LPC (19:0); (**e**), LPC (20:3); (**f**), PC (38:0). n=6 litter-matched piglets in control group; n=18 litter-matched piglets in iron treated groups. Error bars = SEM, * p<0.05.

4.7.1.2 Cognitive maturation may be influenced by the iron status

Non-supplemented piglets were observed with significantly reduced systemic concentrations (p<0.05) of two different isoforms of SM (42:2, 40:1) compared to their iron supplemented siblings (**figure 4.3**). Similarly, Systemic concentrations of different isoforms of S (33:0-OH, 34:0, 34:1-OH, 36:1-OH, 38:1-OH) in piglets receiving no-iron treatment were significantly reduced (p<0.05) compared to their

siblings which received iron treatment (IM or/and oral iron), between the day 7 and day 28 (**Figure 4.4**).

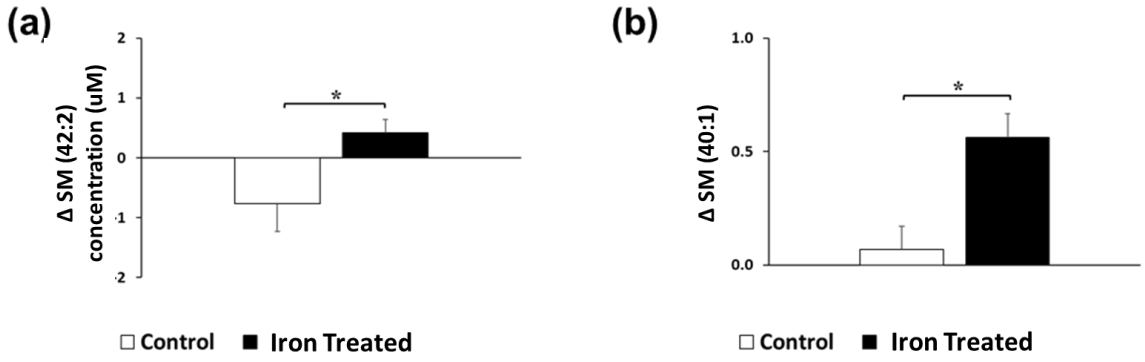

Figure 4. 3. Impact of iron deficiency anaemia (IDA) and iron treatments on concentrations of sphingomyelins (SM) between day 7 and day 28. Control group piglets remained non-iron treated and developed iron deficiency (IDA), however iron treated pigs were given intramuscular iron injection (200mg, one dose at 24h) or/and oral iron supplement ($FeSO_4$, 150mg/day) (**Figure 3.1**). (**a**), SM (42:2); (**b**), SM (40:1). n=6 litter-matched piglets in control group; n=18 litter-matched piglets in iron treated group. Error bars = SEM, * $p<0.05$.

4.7.1.3 Iron deficiency anaemia and iron supplementation, both were linked with reduction in certain isoforms of hexosylceramides

Systemic concentration of Hex-cer (34:2) in piglets not receiving iron was significantly reduced ($p<0.05$) compared to their siblings which receiving iron treatment (IM or/and oral iron), between the day 7 and day 28. However, Hex-cer (35:0) concentration was significantly reduced in response to iron treatment against their non-supplemented siblings (**Figure 4.5**).

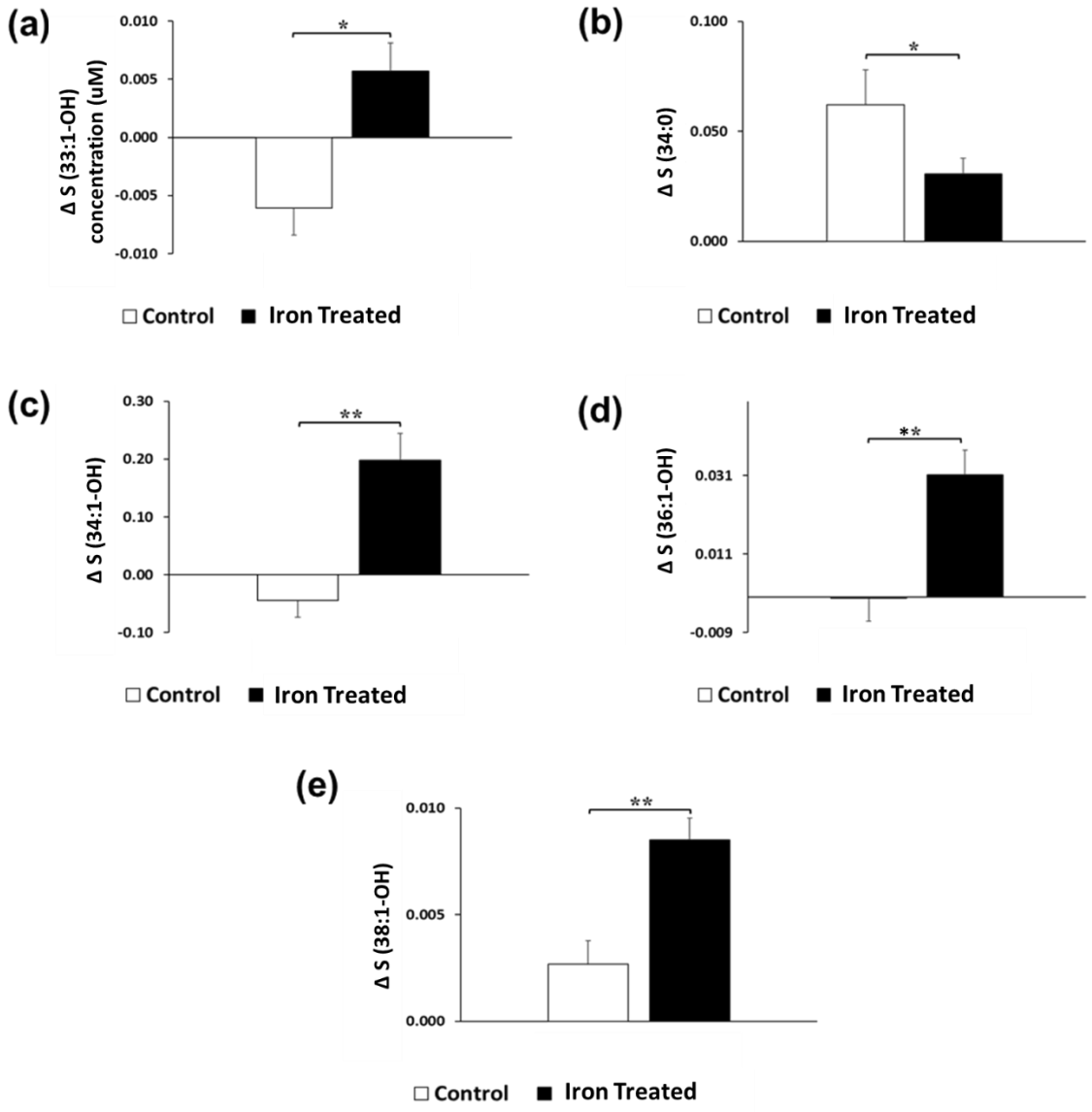

Figure 4. 4. Impact of iron deficiency anaemia (IDA) and iron treatments on systemic concentrations of sulfatides (S) between day 7 and 28. Control group piglets were left non-iron treated and developed iron deficiency (IDA), however iron treated pigs were given intramuscular iron injection (200mg, one dose) or/and oral iron supplement ($FeSO_4$, 150mg/day) (**Figure 4.1**). (**a**), S (33:0-OH); (**b**), S (34:0); (**c**), S (34:1-OH); (**d**), S (36:1-OH); (**e**), S (38:1-OH). n=6 litter-matched piglets in control group; n=18 litter-matched piglets in iron treated groups. Error bars = SEM, * $p<0.05$, ** $p<0.01$.

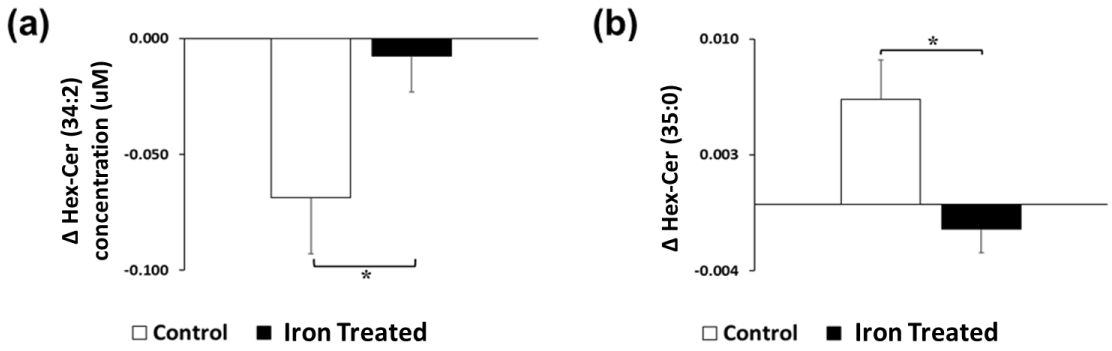

Figure 4. 5. Impact of iron deficiency anaemia (IDA) and iron treatments on systemic concentrations of different hexosylceramides (Hex-cer) between day 7 and 28. Control group piglets were left non-iron treated and developed iron deficiency (IDA), however iron treated pigs were given intramuscular iron injection (200mg, one dose) or/and oral iron supplement ($FeSO_4$, 150mg/day) (**Figure 4.1**). (**a**), Hex-cer (34:2); (**b**), Hex-cer (35:0). n=6 litter-matched piglets in control group; n=18 litter-matched piglets in iron treated groups. Error bars = SEM, * p<0.05.

4.7.2 Type of iron supplementation may influence the lipidomic profiles

4.7.2.1 Oral iron supplementation is associated with reduction in ceramide, phoshatidycholine and lysophoshatidycholines levels

Systemic concentrations of two isoforms of Cer (40:0, 41:0) in piglets receiving oral iron treatment (with or without IM iron treatment) were significantly reduced (p<0.05) compared to IM iron treated siblings, over the period of three weeks (**Figure 4.6**).

Systemic concentration of PC (38:0) in piglets receiving oral iron treatment (with or without IM iron treatment) were significantly reduced (p<0.05) compared to IM treated counterparts, between the day 7 and day 28 (**Figure 3.2**). Similarly, LPC (20:3) concentration was significantly reduced in response to oral iron treatment compared to IM iron treatment (**Figure 4.7**).

4.7.2.2 Intramuscular iron caused reduction in phosphatidylglycerol (PG), cardiolipins (CL), and phosphatidylserine (PS) concentrations

Systemic concentrations of two isoforms of PG (40:4, 32:0) in piglets receiving IM iron were significantly reduced (p<0.05) compared to oral iron treated siblings (with or without IM iron treatment) siblings, over the period of 21 days (**Figure 4.10**).

CL (66:7) concentration in plasma was significantly reduced (p<0.05) in IM iron treated piglets compared to oral iron treatment, between the day 7 and day 28

(Figure 4.2). Similarly, Mono-lyso-CL (53:5) concentration was significantly reduced (p<0.05) in response to IM iron treatment compared to oral iron treatment (**Figure 4.11**).

IM iron treated piglets were observed with significantly reduced systemic concentrations (p<0.05) of PS (40:2) compared to their oral iron treated siblings (**figure 4.3**). On the contrary, GM1 (34:0) concentration was significantly reduced (p<0.05) in response to oral iron treatment compared to IM iron treated piglets (**figure 4.12**).

Analysing the lipidomic data as per treatment plan shown in figure 2.1 did not result in any significant results between all four treatments. Additionally, the data was also analysed using 'sex' as a factor which did not show significant differences between male and female piglets.

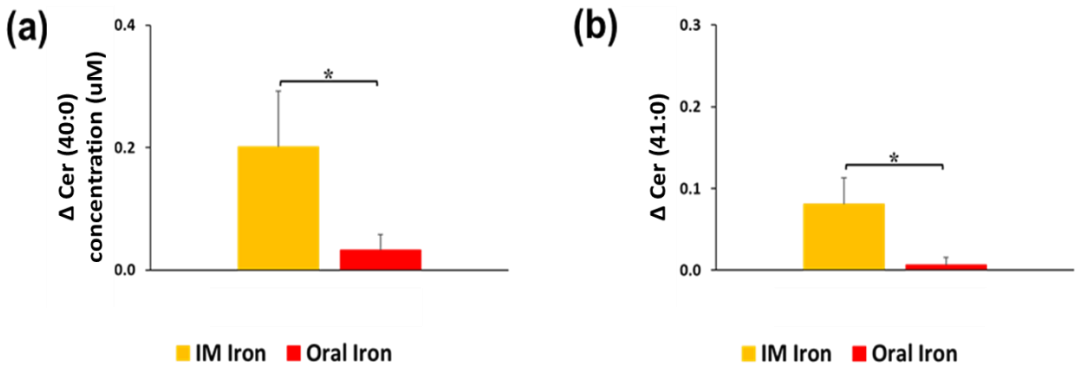

Figure 4. 6. Impact of IM iron and oral iron treatments on systemic concentrations of ceramides (Cer) between day 7 and 28. IM iron group piglets were administered 200mg iron injection and oral iron group was given $FeSO_4$ (150mg/day) in sow milk replacer (**Figure 3.2**) with or with IM iron injection. (**a**), Cer (40:0); (**b**), Cer (41:0). n=6 litter-matched piglets in IM iron group; n=12 litter-matched piglets in oral iron supplemented group. Error bars = SEM, * p<0.05.

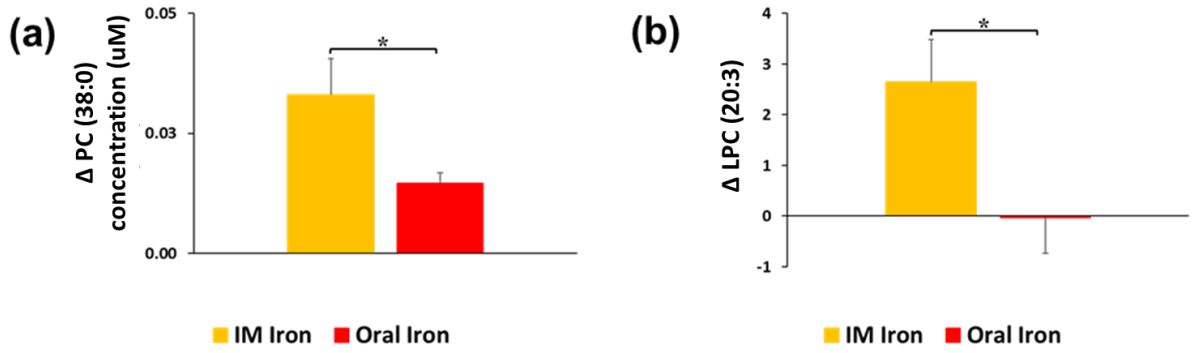

Figure 4. 7. Impact of IM and oral iron treatments on Systemic concentrations of phoshatidycholine (PC) and lysophoshatidycholines (LPC) over the period of 21 days. IM iron group piglets were administered 200mg iron injection and oral iron group was given $FeSO_4$ (150mg/day) in sow milk replacer (**Figure 3.2**) with or with IM iron injection. (**a**), PC (38:0); (**b**), LPC (20:3). n=6 litter-matched piglets in IM iron group; n=12 litter-matched piglets in oral iron supplemented group. Error bars = SEM, * p<0.05.

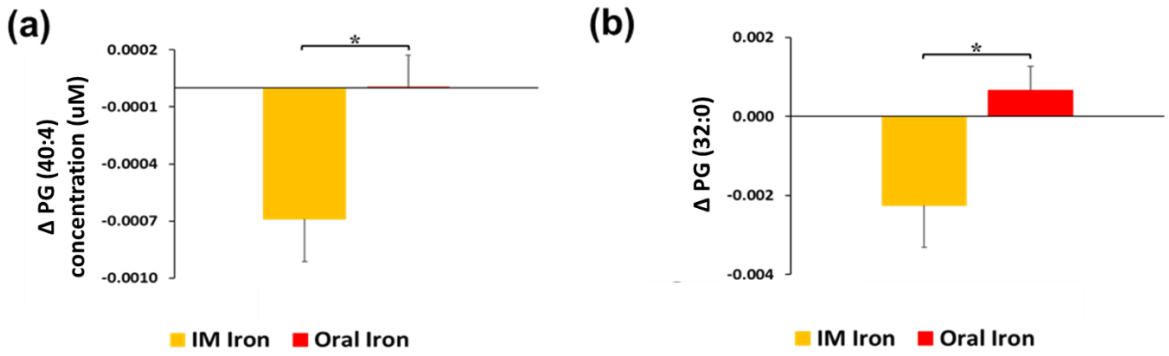

Figure 4.8: Impact of IM and oral iron treatments on systemic concentrations of two different types of phosphatidylglycerols (PG) between day 7 and 28. IM iron group piglets were administered 200mg iron injection and oral iron group was given $FeSO_4$ (150mg/Kg) in sow milk replacer (**Figure 3.2**) with or with IM iron injection. (**a**), PG (40:4); (**b**), PG (32:0). n=6 litter-matched piglets in IM iron group; n=12 litter-matched piglets in oral iron supplemented group. Error bars = SEM, * p<0.05.

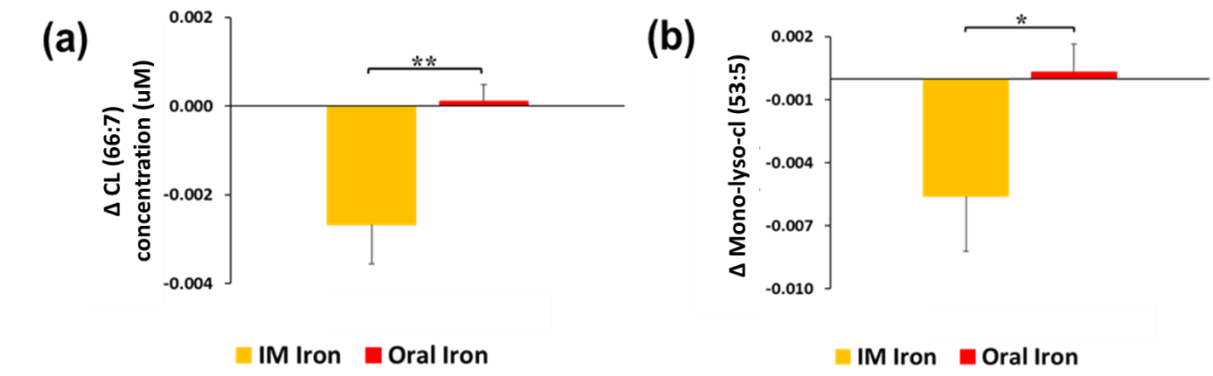

Figure 4.9: Impact of IM and oral iron treatments on systemic concentrations of cardiolipin (CL) and monolyso-cardiolipins (Mono-lys-cl) in 3-week time. IM iron group piglets were administered 200mg iron injection and oral iron group was given $FeSO_4$ (150mg/Kg) in sow milk replacer (**Figure 4.2**) with or with IM iron injection. (**a**), CL (66:7); (**b**), Mono-lys-cl (53:5). n=6 litter-matched piglets in IM iron group; n=12 litter-matched piglets in oral iron supplemented group. Error bars = SEM, * p<0.05.

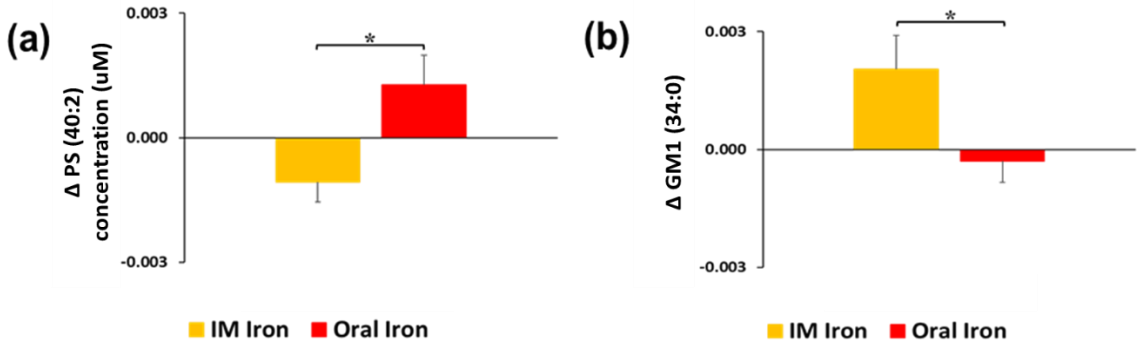

Figure 4.10: Impact of IM and oral iron treatments on systemic concentrations of phosphatidylserine (PS) and gangliosides (GM1) between day 7 and 28. IM iron group piglets were administered 200mg iron injection and oral iron group was given $FeSO_4$ (150mg/Kg) in sow milk replacer (**Figure 4.2**) with or with IM iron injection. (**a**), PS (40:2); (**b**), GM1 (34:0). n=6 litter-matched piglets in IM iron group; n=12 litter-matched piglets in oral iron supplemented group. Error bars = SEM, * p<0.05.

4.8 Discussion

Systemic lipids not only help to address high energy demands in mammals, but also perform several metabolic and physiological functions which contribute to normal

growth and development in infants and young children [34, 35]. This includes brain development [36] and changes in systemic lipid profiles have been shown to affect cognitive function [37, 38]. Here, the impact of IDA and different types of iron treatments on lipidomic profiles during infancy were assessed. This study demonstrated that IDA was associated with significantly reduced levels of gangliosides (GM1), lysophosphatidylcholines (LPC), phosphatidylcholine (PC), and certain isoforms of hexosylceramides (Hex-cer) in plasma in 28-day old piglets compared to their iron replete siblings. We also demonstrated that the type of iron treatment appears to play an important role in modulating lipidomic profiles in neonatal piglets; oral iron supplementation was associated with significant reductions in concentrations of specific ceramides (Cer), PC, LPC levels, while intramuscular iron treatment caused a reduction in phosphatidylglycerol (PG) and phosphatidylserine (PS) concentrations. To the best of our knowledge, this is the first time different types of iron supplementation have been linked to changes in systemic lipid profiles in neonates.

Sialic acid residue containing gangliosides (GM) are complex glycol-sphingolipids [39] found in high concentrations in the nervous system in mammals, particularly in the brain [40]. Breast milk is a rich source of GMs, and nutrition has been shown to influence systemic levels of GM [41]. This was demonstrated in a study conducted by Wang *et al* [42] in infants who died of sudden infant death syndrome. These infants were observed to have had higher levels (32%) of sialic acid bound GMs present in brain (frontal cortex grey matter) of breastfed infants (n=12) compared to their formula-fed counterparts (n=10). In addition, a recent observational study conducted in Chinese pre-term infants (approximately 32-week gestation) by Zhang *et al* [43] where those fed breastmilk (n=34) had greater regional grey matter development and subsequent function compared to formula-fed counterparts (n=22). Grey matter is responsible for movement control, memory and emotions [44]. Although this is not direct evidence confirming the impact of iron on GMs, iron concentration is an important difference between breast milk (~0.4mg/L) and formula milk (8-14mg/L). This means, perhaps, higher dietary iron concentrations may, in parts, play a negative role in GM metabolism. Another explanation for this, perhaps, could be that GM metabolism is independent of iron, and IDA or iron supplementation may not influence the GM metabolism as breastmilk contains very low amount of iron. An important consideration is that all piglets in our study were fed sow milk replacer

(SMR) in the absence of sow milk. Consequently, in relation to iron concentration, our findings are inconsistent with the finding of Wang *et al* who showed that breastmilk fed infants (low iron concentration) had higher concentrations of sialic acid bound GMs, while 66% of piglets with higher levels of GM1s in our study were iron treated (fed on oral iron supplement at 150mg/day), if compared to iron concentrations of human formula milk. However, they did not report the iron status of subjects, which is a limitation.

A study in 630 first-time mothers by Henly *et al* [45] showed that IDA during lactation can result in a decreased milk production and quality. Although there is currently no direct evidence regarding the role of iron in GM metabolism, this may suggest that, perhaps, GM concentration may be indirectly influenced by iron status as GM are significant proportion of human milk lipid profile. Some studies have suggested that overall dietary supplementation with gangliosides may have benefits for brain development and cognitive function. For example, not specifically GM1, but impacts of overall GM consumption on brain and cognition development has been confirmed in piglets where Liu *et al* [46] reported the improved spatial learning and brain growth in response to formula feeding supplemented with gangliosides and phospholipids. Clearly, further research is required to fully understand the relationship between IDA and GM1 metabolism, as well as the potential benefits of iron treatment on systemic GM1 concentrations.

Phospholipids such as LPC have been shown to play a significant role in the modulation of inflammation. LPC is formed by the hydrolysis of PC, which is a major component of cell membranes. Although we did not assess markers of inflammation in our piglets in response to IDA and iron supplementation, we have previously shown that systemic hepcidin, an inflammatory marker released by hepatocytes, is significantly increased in oral iron supplemented piglets [47]. Systemic levels of hepcidin are raised during inflammation and is part of a key protective mechanism by internalizing ferroportin to limit further influx of iron into the system. Trovato *et al* [48] reported that LPC (18:0) and LPC (16:1) were significantly high in adult patients with acute liver failure (n=96) compared to healthy controls (n=71). This may, in part, be consistent with our study as we have demonstrated increased levels of these LPC in iron supplemented piglets and reported higher levels of hepcidin in our published

work [47], especially in oral iron supplemented piglets at day 28. However, the overall role of LPC in inflammation is complex and context dependent; while it can act as both a pro-inflammatory and an anti-inflammatory mediator, the balance between these effects may depend on the specific cell types and signalling pathways involved. Understanding the mechanisms underlying the role of LPC in inflammation may provide new opportunities for the development of therapeutic interventions for inflammatory diseases.

Around 36% of the complex lipids present in human milk are sphingomyelins (SM) [49], a sphingolipid crucial for the formation of myelin sheaths, the fatty layer which insulates nerve fibres resulting in increased efficiency of impulse transmission [50,51]. Myelin is around 70% lipid [52], of which 7% are sulfatides (S) [53]. Iron is required for the synthesis of myelin as it is a critical component of the enzymes involved in myelin assembly [54]. Specifically, iron is involved in the maturation and differentiation of oligodendrocytes, the cells responsible for myelin production in the central nervous system [55]. ID can impair myelin synthesis and result in abnormal myelin composition, including decreased levels of associated lipids such as S and SM [56]. Here we report significant reductions in concentrations of systemic SM and S in anaemic piglets compared to their iron treated siblings. Studies have shown that ID can lead to impaired myelination [57], which can result in delayed cognitive development and reduced cognitive function [57]. Additionally, iron is required for oxidative metabolism, which occurs at a higher rate in oligodendrocytes than other cells in the brain [58]. However, certain factors such as cytokines and conditions like ID may decrease iron acquisition by oligodendrocytes. These cells have high levels of iron in their cytoplasm, making them more susceptible to oxidative injury which can have negative effects on myelin production and the survival of oligodendrocytes [59]. Overall, this is consistent with iron being essential for myelination and cognitive development, and that maintaining adequate iron levels is crucial for optimal neurodevelopmental outcomes.

4.9 Conclusion

In conclusion, to the best of our knowledge, we present the first study to explore the direct impact of IDA and iron treatments on systemic lipidomic profiles. IDA may, directly or indirectly, affect phospho- and sphingolipid metabolism which contribute to

brain growth and cognitive development. There is currently a paucity of knowledge in this area, despite the recognised detrimental effects IDA has on neurodevelopment. Iron modulates lipid metabolism through its interaction with several key enzymes and regulatory proteins involved in lipid synthesis and transport. These findings have implications for understanding the role of iron in cellular function and metabolism and may inform future research on the effects of ID and iron supplementation on health outcomes. Lipids play key roles in neurodevelopment and lack of iron during ID and IDA may disrupt lipid metabolism via downregulation of the specific enzymes involved in this process. Further research is required to identify potential links between lipid concentrations and inflammation to determine the mechanisms involved.

4.10 References

1. Quehenberger, O. *et al.* Lipidomics reveals a remarkable diversity of lipids in human plasma. *Journal of Lipid Research* **51**, 3299-3305 (2010).

2. Gille, B., Galuska, C.E., Fuchs, B. & Peleg, S. Recent Advances in Studying Age-Associated Lipids Alterations and Dietary Interventions in Mammals. *Frontiers in Aging* **2**, 773795 (2021).

3. Welte, M.A. & Gould, A.P. Lipid droplet functions beyond energy storage. *Biochim Biophys Acta Molecular and Cell Biology of Lipids* **1862**, 1260-1272 (2017).

4. Horn, A. & Jaiswal, J.K. Structural and signaling role of lipids in plasma membrane repair. *Current Topics in Membranes* **84**, 67-98 (2019).

5. Sunshine, H. & Iruela-Arispe, M.L. Membrane lipids and cell signaling. *Current Opinion in Lipidology* **28**, 408-413 (2017).

6. Rodrigo Valenzuela, B. & Alfonso Valenzuela, B. Overview About Lipid Structure. In: Rodrigo Valenzuela, B. (ed). *Lipid Metabolism*. IntechOpen: Rijeka, 2013, p Ch. 1.

7. O'Neal, D.N. *et al.* Australian Aboriginal people and Torres Strait Islanders have an atherogenic lipid profile that is characterised by low HDL-cholesterol level and small LDL particles. *Atherosclerosis* **201**, 368-377 (2008).

8. Farbstein, D. & Levy, A.P. HDL dysfunction in diabetes: causes and possible treatments. *Expert Review of Cardiovascular Therapy* **10**, 353-361 (2012).

9. Zhao, J. *et al.* Triglyceride is an independent predictor of type 2 diabetes among middle-aged and older adults: a prospective study with 8-year follow-ups in two cohorts. *Journal of Translational Medicine* **17**, 403 (2019).

10. Acharjee, A. *et al.* The translation of lipid profiles to nutritional biomarkers in the study of infant metabolism. *Metabolomics* **13**, 25 (2017).

11. Lönnerdal, B. Development of iron homeostasis in infants and young children. *The American Journal of Clinical Nutrition* **106**, 1575s-1580s (2017).

12. Verma, U. *et al.* Relationship between iron deficiency anaemia and serum lipid levels in Indian adults. *Journal of Indian Medical Association* **108**, 555-558, 562 (2010).

13. Rao, R. *et al.* Metabolomic Analysis of Cerebrospinal Fluid Indicates Iron Deficiency Compromises Cerebral Energy Metabolism in the Infant Monkey. *Neurochemical Research* **38**, 573-580 (2013).

14. Vlasova, R.M. *et al.* Infantile Iron Deficiency Affects Brain Development in Monkeys Even After Treatment of Anemia. *Frontiers in Human Neuroscience* **15** (2021).

15. Macdougall, I.C. Strategies for iron supplementation: oral versus intravenous. *Kidney International Supplements* **69**, S61-66 (1999).

16. Vaucher, P., Druais, P.L., Waldvogel, S. & Favrat, B. Effect of iron supplementation on fatigue in nonanemic menstruating women with low ferritin: a randomized controlled trial. *Canadian Medical Association Journal* **184**, 1247-1254 (2012).

17. Kadoglou, N.P.E. *et al.* The association of ferritin with cardiovascular and all-cause mortality in community-dwellers: The English longitudinal study of ageing. *Plos One* **12**, e0178994 (2017).

18. Pourmoghaddas, A., Sanei, H., Garakyaraghi, M., Esteki-Ghashghaei, F. & Gharaati, M. The relation between body iron store and ferritin, and coronary artery disease. *ARYA Atherosclerosis* **10**, 32-36 (2014).

19. Puga, A.M. *et al.* Iron Supplementation at the Crossroads of Nutrition and Gut Microbiota: The State of the Art. *Nutrients* **14**, 1926 (2022).

20. Schoeler, M. & Caesar, R. Dietary lipids, gut microbiota and lipid metabolism. *Reviews in Endocrine and Metabolic Disorders* **20**, 461-472 (2019).

21. Deleu, S., Machiels, K., Raes, J., Verbeke, K. & Vermeire, S. Short chain fatty acids and its producing organisms: An overlooked therapy for IBD? *EBioMedicine* **66**, 103293 (2021).

22. He, J. *et al.* Short-Chain Fatty Acids and Their Association with Signalling Pathways in Inflammation, Glucose and Lipid Metabolism. *International Journal of Molecular Sciences* **21** (2020).

23. Staley, C., Weingarden, A.R., Khoruts, A. & Sadowsky, M.J. Interaction of gut microbiota with bile acid metabolism and its influence on disease states. *Applied Microbiology and Biotechnology* **101**, 47-64 (2017).

24. Besten, G.d. *et al.* Gut-derived short-chain fatty acids are vividly assimilated into host carbohydrates and lipids. *American Journal of Physiology-Gastrointestinal and Liver Physiology* **305**, G900-G910 (2013).

25. Houpt, K.A., Houpt, T.R. & Pond, W.G. The pig as a model for the study of obesity and of control of food intake: a review. *Yale Journal of Biology and Medicine* **52**, 307-329 (1979).

26. Rodríguez, R.R. *et al.* The Iberian pig fed with high-fat diet: a model of renal disease in obesity and metabolic syndrome. *International Journal of Obesity* **44**, 457-465 (2020).

27. Liu, Y. Fatty acids, inflammation and intestinal health in pigs. *Journal of Animal Science and Biotechnology* **6**, 41 (2015).

28. Rincker, M., Hill, G., Link, J. & Rowntree, J. Effects of dietary iron supplementation on growth performance, hematological status, and whole-body mineral concentrations of nursery pigs. *Journal of Animal Science* **82**, 3189-3197 (2004).

29. Antonides, A., van Laarhoven, S., van der Staay, F.J. & Nordquist, R.E. Non-anemic Iron Deficiency from Birth to Weaning Does Not Impair Growth or Memory in Piglets. *Frontiers in Behavioural Neuroscience* **10** (2016).

30. Nielsen, K.L. *et al.* Similar metabolic responses in pigs and humans to breads with different contents and compositions of dietary fibers: a metabolomics study. *The American Journal of Clinical Nutrition* **99**, 941-949 (2014).

31. Perleberg, C., Kind, A. & Schnieke, A. Genetically engineered pigs as models for human disease. *Disease Models and Mechanisms* **11** (2018).

32. Shao, Y. *et al.* Early weaning leads to the remodeling of lipid profile in piglet jejunal crypt cells during post-weaning days. *Animal Nutrition* **11**, 102-111 (2022).

33. Jenkins, B., Ronis, M. & Koulman, A. LC–MS Lipidomics: Exploiting a Simple High-Throughput Method for the Comprehensive Extraction of Lipids in a Ruminant Fat Dose-Response Study. *Metabolites* **10**, 296 (2020).

34. Mazzocchi, A. *et al.* The role of lipids in human milk and infant formulae. *Nutrients* **10**, 567 (2018).

35. Delplanque, B., Gibson, R., Koletzko, B., Lapillonne, A. & Strandvik, B. Lipid quality in infant nutrition: current knowledge and future opportunities. *Journal of Pediatric Gastroenterology and Nutrition* **61**, 8 (2015).

36. Salvati, S., Attorri, L., Avellino, C., Di Biase, A. & Sanchez, M. Diet, lipids and brain development. *Developmental Neuroscience* **22**, 481-487 (2000).

37. Lozoff, B. Early iron deficiency has brain and behavior effects consistent with dopaminergic dysfunction. *The Journal of Nutrition* **141**, 740S-746S (2011).

38. Beard, J. Iron deficiency alters brain development and functioning. *The Journal of nutrition* **133**, 1468S-1472S (2003).

39. Aureli, M. *et al.* GM1 ganglioside: past studies and future potential. *Molecular Neurobiology* **53**, 1824-1842 (2016).

40. Matsuda, J. *et al.* Chemical chaperone therapy for brain pathology in GM1-gangliosidosis. *Proceedings of the National Academy of Sciences* **100**, 15912-15917 (2003).

41. McJarrow, P., Rowan, A. & Norris, C. Diet-Induced Changes in Serum Ganglioside Spectrum Patterns in 6-Month-Old Infants.

42. Wang, B., McVeagh, P., Petocz, P. & Brand-Miller, J. Brain ganglioside and glycoprotein sialic acid in breastfed compared with formula-fed infants. *The American Journal of Clinical Nutrition* **78**, 1024-1029 (2003).

43. Zhang, Y. *et al.* The impact of breast milk feeding on early brain development in preterm infants in China: An observational study. *Plos One* **17**, e0272125 (2022).

44. Zhou, Y. *et al.* Gray matter abnormalities in Internet addiction: a voxel-based morphometry study. *European Journal of Radiology* **79**, 92-95 (2011).

45. Henly, S.J. *et al.* Anemia and Insufficient Milk in First-Time Mothers. *Birth* **22**, 87-92 (1995).

46. Liu, H. *et al.* Early Supplementation of Phospholipids and Gangliosides Affects Brain and Cognitive Development in Neonatal Piglets. *The Journal of Nutrition* **144**, 1903-1909 (2014).

47. Frost, J.N. *et al.* Hepcidin-mediated hypoferremia disrupts immune responses to vaccination and infection. *Med* **2**, 164-179. e112 (2021).

48. Trovato, F.M. *et al.* Lysophosphatidylcholines modulate immunoregulatory checkpoints in peripheral monocytes and are associated with mortality in people with acute liver failure. *Journal of Hepatology* **78**, 558-573 (2023).

49. Ramiro-Cortijo, D. *et al.* Breast Milk Lipids and Fatty Acids in Regulating Neonatal Intestinal Development and Protecting against Intestinal Injury. *Nutrients* **12** (2020).

50. Hartline, D.K. What is myelin? *Neuron Glia Biology* **4**, 153-163 (2008).

51. Barkovich, A.J. Concepts of myelin and myelination in neuroradiology. *American Journal of Neuroradiology* **21**, 1099-1109 (2000).

52. Ho, P.P. *et al.* Identification of naturally occurring fatty acids of the myelin sheath that resolve neuroinflammation. *Science Translational Medicine* **4**, 137ra173-137ra173 (2012).

53. Chrast, R., Saher, G., Nave, K.-A. & Verheijen, M.H.G. Lipid metabolism in myelinating glial cells: lessons from human inherited disorders and mouse models. *Journal of Lipid Research* **52**, 419-434 (2011).

54. Todorich, B., Pasquini, J.M., Garcia, C.I., Paez, P.M. & Connor, J.R. Oligodendrocytes and myelination: the role of iron. *Glia* **57**, 467-478 (2009).

55. Badaracco, M.E., Siri, M.V.R. & Pasquini, J.M. Oligodendrogenesis: the role of iron. *Biofactors* **36**, 98-102 (2010).

56. Olsen, A.S. & Færgeman, N.J. Sphingolipids: membrane microdomains in brain development, function and neurological diseases. *Open biology* **7**, 170069 (2017).

57. Wu, L.-l. *et al.* Effect of perinatal iron deficiency on myelination and associated behaviors in rat pups. *Behavioural Brain Research* **188**, 263-270 (2008).

58. Connor, J.R. & Menzies, S.L. Relationship of iron to oligondendrocytes and myelination. *Glia* **17**, 83-93 (1996).

59. Piñero, D.J. & Connor, J.R. Iron in the brain: an important contributor in normal and diseased states. *The Neuroscientist* **6**, 435-453 (2000).

CHAPTER 5

General Discussion

Iron is an essential micronutrient required for many physiological processes in the body, including the formation of red blood cells, transport of oxygen, and overall growth and development [1]. Iron deficiency (ID) is a common problem worldwide, particularly among infants and young children [2]. Globally, an estimated 273 million infants and young children (<5 years) suffer from iron deficiency anaemia (IDA) [3], which can lead to impaired physical and cognitive development, and increased morbidity and mortality [4]. To address this major public health concern, universal iron treatment programmes for infants and young children in low- and middle-income countries (LMICs) are widespread. However, the success of these programmes has been questioned because of several associated challenges [5]. These programmes are usually untargeted and therefore include high percentages of non-ID individuals. In addition, universal iron-associated challenges include limited iron absorption across the intestinal tract [6] and whether iron status of the subject is in relation to specific inflammatory gut conditions which are known to interfere with iron absorption pathways [7]. Oral iron supplementation is also commonly linked with negative side effects, including gastrointestinal distress, nausea, constipation, and diarrhoea [8]. Oral iron supplementations have also been linked to increased risks of enteric infections [9] and even increased morbidity and mortality from malaria [10].

There is growing evidence that dietary iron may impact on the gut microbiota, resulting in dysbiosis [11]. Since normal patterns of microbial colonisation of the gut during early-life are essential for healthy physiological development, deviations from this can have longer-term health implications, especially for later immune and metabolic functionality. In practice, implementing successful iron supplementation programmes, where appropriate levels of iron are administered at the appropriate time to appropriate individuals can be problematic, especially in LMICs. This is due to limited coverage and uptake, infrastructure and high prevalence of additional health conditions. Clearly, better understanding of IDA in early life would inform the development of improved strategies to reduce the health and economic burden of this global issue without the current debilitating side effects. This body of work explored the impact of IDA on the gut microbiota, metabolism and lipid profiles in

neonatal piglet models for human infants. It then assessed the efficacy of different iron treatments in reducing IDA whilst focusing on how they effected early microbial colonisation in the gut, metabolic development and their impact on systemic lipid profiles.

Partial absorption of dietary iron contained in food and supplements is a persistent and considerable obstacle in the treatment of ID and IDA. Typically, oral iron salt absorption is highly inefficient and varies between 2% and 13% under normal conditions but can be improved, to some extent, to between 5% and 28% if administered following fasting [12]. However, a recent randomized, single-blind, crossover study in ID women demonstrated that coadministration of 15g of galacto-oligosaccharides (GOS) or fructo-oligosaccharides (FOS) and a single 100 mg Fe tablet, labelled with 4 mg ^{57}Fe or ^{58}Fe, resulted in 45% and 51% increases in absorption respectively compared to counterparts who received carbohydrate-based placebos [13]. Similarly, a 4 week study in iron-sufficient adult Sprague-Dawley rats demonstrated an 8% increase in iron adsorption from standard feed when fortified (5g/100g feed) with GOS [14]. The disparities in efficacy between these human and rodent trials could be linked with iron status, or species differences. Some probiotic strains have also been demonstrated to improve iron uptake, as recently reviewed by Rusu *et al* [15]. For example, meta-analysis of 15 studies found significant improvements in iron uptake in response to co-administration with *Lactobacillus plantarum 299v (Lp299v)*, but not for other probiotic lactobacilli strains [16]. Consistent with these findings, a clinical trial conducted in pre-school children (n=190) by Silva *et al* [17] concluded that children who received iron-fortified fermented milk containing *Lactobacillus acidophilus* presented with higher increases in systemic haemoglobin (Hb) compared to counterparts who received iron-fortified, non-fermented milk. Similarly, a study in 66 Indonesian children (5-12 years) by Manoppo *et al* [18] indicated that the children administered *Lactobacillus reuteri* DSM 17938 alongside oral iron supplementation were observed with higher reticulocyte haemoglobin equivalent compared to those receiving iron supplement alone. The mechanism appears to be largely *via* probiotic-induced reduction of luminal Fe^{3+} to Fe^{2+} and thus promotion of iron uptake by enterocytes [15]. Although further work in this area is required, taken together the findings above suggest that co-administration of specific pre- or probiotics with oral iron treatments offers promise in reducing the amount of

oral iron required to address ID and IDA, which could limit the associated side-effects whilst also reducing the amount of non-absrbed iron available to the gut microbiota.

Since both GOS and FOS are established prebiotics known to selectively stimulate growth and activity of beneficial microbial strains residing in the gut [19, 20], there is potential for both supplements to reduce the impact of increased luminal iron availability on microbial population skewing to a less beneficial phenotype. This could be especially relevant for Lactobacillaceae which are abundant in healthy infant guts [21]. This is because, as previously mentioned, Lactobacillaceae have very limited requirements for iron and so are disadvantaged under iron-rich conditions. Along with specific pre- and probiotics appearing to increase iron absorption in infants and young children, there is also potential for these supplements to further promote gut health during oral iron supplementation at critical early stages of gut microbiota development. For example, *Lp299v* has been shown to reduce the incidence and/or severity of a range of enteric infections, and to prevent *E. coli* attachment to enterocytes and the associated tight cell junction disruption in Caco-2 monolayers [22, 23, 24, 25], which occur under high iron availability conditions. Consistent with this, a randomized, double blind, controlled trial conducted in 155 Kenyan infants (age 6.5-9.5 months) by Paganini *et al* [26] determined that GOS helped to improve a number of negative side-effects associated with oral iron treatments. Specifically, the infants who received GOS in combination with iron containing micronutrient powder (Fe-MNP) had significantly higher abundances of *Lactobacillus* and *Bifidobacterium* along with decreased expression of toxin-encoding and other virulence-factor associated genes, and reductions in biomarkers of enterocyte damage compared to those children who received Fe-MNP in the absence of GOS. Given that oral iron availability is also associated with increased enteric infection, such probiotics and prebiotics may be beneficial in limiting some of the side-effects associated with oral iron treatments in addition to their roles in increasing iron adsorption.

Nano encapsulation involves the packaging of target nutritional or pharmacological elements, such as iron, within a protective coating or matrix [27]. This technique has recently been explored as a means of reducing the negative effects of oral iron supplementation on gut microbiotas. For example, the research published by Pereira *et al* [28], which involved a randomized, double-blind, placebo-controlled trial in mild-

moderately ID, pre-menopausal females, along with a pilot study in rats, investigated the use of nano-iron (nano-Fe) compared to conventional soluble iron supplements ($FeSO_4$). They reported that the nano-Fe supplement was well-tolerated as it did not accumulate in intestinal mucosa and resulted in significantly higher iron bioavailability (~ 80%) than $FeSO_4$ in humans and was observed to be 14-fold less toxic than $FeSO_4$. In their rat study, they reported higher abundances of *Lactobacillus* and lower abundances of *Escherichia* in subjects receiving nano-Fe, compared to $FeSO_4$ supplemented counterparts. This suggests that nano techniques may help to protect the active element within oral iron supplements whilst reducing the negative effects on gut microbiotas. Application of such technologies may have important implications for the quality of iron formulations and treatment of ID and IDA, particularly in vulnerable populations such as infants and young children.

The program of work in this PhD has focused on ID and IDA, along with different forms of iron supplementation, in piglets as models for human infants. However, as outlined in the introduction (**chapter 1**), ID and IDA are significant problems for pig industries worldwide. The pork exports from the UK were valued at £339M in 2021 and thus make significant contributions to the British Agrifood industry [29]. Commercially, piglets are weaned at 24-26 day old [30] which involves separation from sows, mixing in large unfamiliar groups and exposure to novel feed, environments and pathogens. This imposes abrupt and simultaneous stressors leading to various negative physiological changes in the gastro-intestinal tract. Consequently, piglets are extremely vulnerable to enteric infection leading to diarrhoea and increased morbidity and mortality at this time. In addition, piglets that fail to gain weight (or indeed lose weight) during the first 10 days post-weaning require an additional 10 days to reach market weight [31]. Post-weaning diarrhoea (PWD) is the most frequent cause of economic loss in the industry and novel approaches are urgently required to improve both productivity and welfare around weaning.

At weaning, piglet diets are supplemented with high levels of zinc oxide (ZnO) since it stimulates weight gain[32]; this effect is largely achieved through its antimicrobial properties resulting in reductions in PWD caused by enteropathogens. However, the mechanisms of ZnO action are unknown. ZnO use in pigs increased following the ban on growth promoter antibiotics in 2006. However, it is now thought that since

genes encoding antibiotic resistance and genes encoding zinc resistance often occur on the same plasmids, ZnO could actively promote the generation of antibiotic resistant microbes[32, 33]. Zinc from pig farms is an established environmental pollutant and the prophylactic use of ZnO was banned in the EU in 2022, although use of current stocks is still permitted [34]. It is highly likely that the UK will comply with this due to the desire for continued trade following Brexit. According to the National Pig Association, this is the most urgent issue facing the pig industry as there are currently no reliable alternatives to ZnO administration in place. Thus, there is a pressing need to find alternative approaches to using ZnO at weaning.

This PhD has focused on the neonatal stage of development as changes at this critical time can have sustained effects on later health and physiological systems functionality. However, weaning is also a fundamental phase of development for the gut microbiota, and the microbiota is the primary driver of immune development[35]. Deviation from normal microbial colonisation may have a major impact on later immune functionality[36] and is thus associated with a reduced ability to combat infection, as discussed in **chapter 3**. Under normal conditions, the pre-weaned pig gut is largely iron-free since sow-milk contains limited iron (0.2-4mg/L) [37], as described in **chapter 1**. However, the introduction of iron at weaning (375mg/Kg) generates an abrupt iron-rich environment. As in humans, this promotes the growth of enteropathogenic populations with high-iron requirements whilst disadvantaging beneficial bacteria that have low-iron requirements. Following birth, piglets rapidly develop iron-deficiency anaemia, which we have exploited to generate valuable models of ID and IDA for human infants. In the pig industry, this is extremely problematic, and it is standard husbandry practice for piglets to receive an IM injection of 200mg of iron sulphate to alleviate this issue. Following this initial dose of IM iron, our piglets were not anaemic at weaning, while non-anaemic iron deficiency does not impair growth in pre-weaned piglets [38]. Given this, and the other findings reported from this PhD, it is questionable whether piglets should be exposed to high dietary iron levels at a time when their guts are already undergoing considerable disruption due to weaning and are at higher risk of enteric infections. Indeed, dietary iron within tolerable upper intake limits is associated with considerable physiological GI tract damage and diarrhoea [39] and could result from non-ID piglets receiving high dietary iron at weaning. In addition, our study showed that oral iron, with and without

IM iron, drove significant decreases in weight gain in pre-weaned piglets. It is not yet clear if this is also the case in weaner piglets. If it is, then this clearly has implications for pig industries where profit margins are already small and reduced further if piglets take longer to reach market weight. Since ZnO reduces enteric infection at weaning partly by inhibiting microbial iron uptake, thus reducing pathogenic expansion, then initially weaning piglets onto iron-free diets could reduce enteropathogen growth and thus negate the need for ZnO. This could also reduce microbiota skewing during this important phase of development whilst reducing the prevalence of antibiotic resistance in pig industries. As there are no current strategies to replace ZnO once UK reserves are depleted, it follows that limiting iron at weaning could provide an effective and cost-efficient method to limit PWD whist also increasing weight gain.

Malnutrition, the primary cause of death and disease worldwide, is a complex issue and fuelled by worsening dysfunctional global food systems. Malnutrition contributes to the development of environmental enteric dysfunction (EED) which has devastating consequences for development and effects 200 million children LMICs each year. The syndrome is defined by intestinal inflammation, reduced barrier function, increased susceptibility to enteric infection and reduced absorptive capacity. Development of the gut microbiota appears to be impaired in EED, and the inflammatory nature of this condition is highly likely to impair iron uptake from the gut, as described in **chapter 1**. There is currently no satisfactory animal model of human EED and biopsies from children are scarce, while early ID is difficult to model in very young animals due to their reliance on their mothers. Consequently, there is little understanding of the underlying cause, or the development of pathology in EED, nor how this common condition could reduce iron absorption which is especially relevant to LMICs. As detailed in **chapter 1**, pigs share many features of immunology, gastrointestinal physiology, metabolism, microbiology, and dietary requirements with humans [40, 41, 42], and are therefore valuable translatable models [43]. Additionally, precocial piglets are important models for studies of the impact of early nutrition on physiological development since their self-sufficiency permits early separation from their mothers, thus limiting the maternal influence at this critical period of developmental plasticity. Importantly, many of the characteristic features of EED are routinely observed in piglets subjected to early, abrupt weaning. This causes

disruption of gut function with induced morphology similar to EED, and results in a ‘growth check’ and increased susceptibility to a range of infectious diseases. Under normal conditions, the gut recovers within 2 weeks and piglets ‘catch up’ with gradually weaned counterparts in terms of weight gain. However, affected piglets later show distinct physiological ‘clustering’ compared to gradually weaned littermates. It could be that abrupt, early weaning triggers symptoms of EED and subsequent malnutrition, as occurs in some LMIC population, could prevent recovery, and thus generate a novel model for EED in human infants. Since piglets are also prone to developing dietary iron deficiency, there is potential to generate a combined model of both EED and IDA. Our results showed that oral iron drives reductions in weight gain in otherwise healthy neonatal piglets. This could have huge implications for infants with EED who already under-absorbing nutrients are. Therefore, this putative model of EED and IDA could be used not only to further understanding of ID and EED commodities, but also informing the development of strategies and intervention to improve iron absorption and decrease the negative side effects in extremely vulnerable children with EED.

Conclusion

In conclusion, this PhD has demonstrated that IDA has significant impacts on the developing microbiota, and both microbial- and host-derived metabolism using a piglet model for human infants. It also demonstrates that oral and IM iron treatments prevent the development of IDA with no discernible differences on parameters of anaemia or the production of host-derived systemic metabolic end products. However, oral iron, in comparison to IM iron, is linked with significant reductions in growth rates, disruptions to normal patterns of microbial colonisation in the gut and altered systemic lipid profiles, all of which have important consequences for human health and agrifood industries.

Future Work

Since oral iron supplementation is associated with side effects which includes gut microbial dysbiosis, diarrhoea, eneteric infection and even causing reduction in growth rate. In future, using certain pre- and probiotics with oral iron supplementation may help to mitigate the side effects of oral iron along with increased bioavailability of iron.

References

1. Zhang, D.L., Ghosh, M.C. & Rouault, T.A. The physiological functions of iron regulatory proteins in iron homeostasis - an update. *Frontiers in Pharmacology* **5**, 124 (2014).

2. Joo, E.Y., Kim, K.Y., Kim, D.H., Lee, J.E. & Kim, S.K. Iron deficiency anemia in infants and toddlers. *Blood Research* **51**, 268-273 (2016).

3. Stevens, G.A. *et al.* Global, regional, and national trends in haemoglobin concentration and prevalence of total and severe anaemia in children and pregnant and non-pregnant women for 1995–2011: a systematic analysis of population-representative data. *The Lancet Global Health* **1**, e16-e25 (2013).

4. Aditi, K., Esha, S., Alexandra, M., Mark, A.S. & Matthew James, B. Iron deficiency anaemia: pathophysiology, assessment, practical management. *BMJ Open Gastroenterology* **9**, e000759 (2022).

5. Viteri, F.E. Iron supplementation for the control of iron deficiency in populations at risk. *Nutrition Reviews* **55**, 195-209 (1997).

6. Gesquiere, I. *et al.* Iron deficiency after Roux-en-Y gastric bypass: insufficient iron absorption from oral iron supplements. *Obesity Surgery* **24**, 56-61 (2014).

7. Schröder, O. *et al.* Intravenous iron sucrose versus oral iron supplementation for the treatment of iron deficiency anemia in patients with inflammatory bowel disease—a randomized, controlled, open-label, multicenter study. *Official journal of the American College of Gastroenterology| ACG* **100**, 2503-2509 (2005).

8. Bloor, S.R., Schutte, R. & Hobson, A.R. Oral iron supplementation—gastrointestinal side effects and the impact on the gut microbiota. *Microbiology Research* **12**, 491-502 (2021).

9. Mahalhal, A. *et al.* Oral iron exacerbates colitis and influences the intestinal microbiome. *Plos One* **13**, e0202460 (2018).

10. Prentice, A.M., Ghattas, H., Doherty, C. & Cox, S.E. Iron metabolism and malaria. *Food and Nutrition Bulletin* **28**, S524-S539 (2007).

11. Puga, A.M. *et al.* Iron Supplementation at the Crossroads of Nutrition and Gut Microbiota: The State of the Art. *Nutrients* **14** (2022).

12. Cook, J.D. Diagnosis and management of iron-deficiency anaemia. *Best Practice & Research Clinical Haematology* **18**, 319-332 (2005).

13. Giorgetti, A., Husmann, F.M.D., Zeder, C., Herter-Aeberli, I. & Zimmermann, M.B. Prebiotic Galacto-Oligosaccharides and Fructo-Oligosaccharides, but Not Acacia Gum, Increase Iron Absorption from a Single High-Dose Ferrous Fumarate Supplement in Iron-Depleted Women. *The Journal of Nutrition* **152**, 1015-1021 (2022).

14. Maawia, K. *et al.* Production of impure prebiotic galacto-oligosaccharides and their effect on calcium, magnesium, iron and zinc absorption in Sprague-Dawley rats. *PharmaNutrition* **4**, 154-160 (2016).

15. Rusu, I.G. *et al.* Iron Supplementation Influence on the Gut Microbiota and Probiotic Intake Effect in Iron Deficiency—A Literature-Based Review. *Nutrients* **12**, 1993 (2020).

16. Vonderheid, S.C. *et al.* A Systematic Review and Meta-Analysis on the Effects of Probiotic Species on Iron Absorption and Iron Status. *Nutrients* **11** (2019).

17. Silva, M.R., Dias, G., Ferreira, C.L., Franceschini, S.C. & Costa, N.M. Growth of preschool children was improved when fed an iron-fortified fermented milk beverage supplemented with Lactobacillus acidophilus. *Nutrition Research* **28**, 226-232 (2008).

18. Manoppo, J., Tasiringan, H., Wahani, A., Umboh, A. & Mantik, M. The role of Lactobacillus reuteri DSM 17938 for the absorption of iron preparations in children with iron deficiency anemia. *Korean journal of pediatrics* **62**, 173 (2019).

19. Walton, G.E. *et al.* A randomised crossover study investigating the effects of galacto-oligosaccharides on the faecal microbiota in men and women over 50 years of age. *The British Journal of Nutrition* **107**, 1466-1475 (2012).

20. Wang, S. *et al.* Rational use of prebiotics for gut microbiota alterations: Specific bacterial phylotypes and related mechanisms. *Journal of Functional Foods* **66**, 103838 (2020).

21. Tandon, D. *et al.* A prospective randomized, double-blind, placebo-controlled, dose-response relationship study to investigate efficacy of fructo-oligosaccharides (FOS) on human gut microflora. *Scientific Reports* **9**, 5473 (2019).

22. Liévin-Le Moal, V. & Servin, A.L. Anti-infective activities of lactobacillus strains in the human intestinal microbiota: from probiotics to gastrointestinal anti-infectious biotherapeutic agents. *Clinical Microbiology Reviews* **27**, 167-199 (2014).

23. Kaźmierczak-Siedlecka, K. *et al.* The role of Lactobacillus plantarum 299v in supporting treatment of selected diseases. *Central-European Journal of Immunology* **45**, 488-493 (2020).

24. Potočnjak, M. *et al.* Three New Lactobacillus plantarum Strains in the Probiotic Toolbox against Gut Pathogen Salmonella enterica Serotype Typhimurium. *Food Technology and Biotechnology* **55**, 48-54 (2017).

25. Qin, H., Zhang, Z., Hang, X. & Jiang, Y. L. plantarum prevents Enteroinvasive Escherichia coli-induced tight junction proteins changes in intestinal epithelial cells. *BMC Microbiology* **9**, 63 (2009).

26. Daniela, P. *et al.* Prebiotic galacto-oligosaccharides mitigate the adverse effects of iron fortification on the gut microbiome: a randomised controlled study in Kenyan infants. *Gut* **66**, 1956 (2017).

27. Gharibzahedi, S.M.T. & Jafari, S.M. The importance of minerals in human nutrition: Bioavailability, food fortification, processing effects and nanoencapsulation. *Trends in Food Science & Technology* **62**, 119-132 (2017).

28. Pereira, D.I.A. *et al.* Nanoparticulate iron(III) oxo-hydroxide delivers safe iron that is well absorbed and utilised in humans. *Nanomedicine: Nanotechnology, Biology and Medicine* **10**, 1877-1886 (2014).

29. Bedford, E. https://www.statista.com/statistics/316374/pork-export-value-in-the-united-kingdom-uk/. *statista.com* (2022).

30. Report. UK pigs - Facts and Figures Annual Report for 2018: Agriculture and Horticulture Development Board; 2019.

31. Collins, C.L. *et al.* Post-weaning and whole-of-life performance of pigs is determined by live weight at weaning and the complexity of the diet fed after weaning. *Animal Nutrition* **3**, 372-379 (2017).

32. Dębski, B. Supplementation of pigs diet with zinc and copper as alternative to conventional antimicrobials. *Polish Journal of Veterinary Sciences* **19**, 917-924 (2016).

33. Ciesinski, L. *et al.* High dietary zinc feeding promotes persistence of multi-resistant E. coli in the swine gut. *Plos One* **13**, e0191660-e0191660 (2018).

34. Veterinary, Medicine & Division. Questions and answers on veterinary medicinal products containing zinc oxide to be administered orally tofood-producing species (Outcome of a referral procedure under Article 35 of Directive 2001/82/EC (EMEA/V/A/118). In: Agency, E.M., editor. EMA/394961/2017; 2017.

35. Okada, H., Kuhn, C., Feillet, H. & Bach, J.F. The 'hygiene hypothesis' for autoimmune and allergic diseases: an update. *Clinical Experminatal Immunolgy* **160**, 1-9 (2010).

36. Bjorksten, B., Sepp, E., Julge, K., Voor, T. & Mikelsaar, M. Allergy development and the intestinal microflora during the first year of life. *Journal Allergy Clinical Immunology* **108**, 516-520 (2001).

37. Li, Y. *et al.* Effect of different sources and levels of iron in the diet of sows on iron status in neonatal pigs. *Animal Nutrition* **4**, 197-202 (2018).

38. Antonides, A., van Laarhoven, S., van der Staay, F.J. & Nordquist, R.E. Non-anemic Iron Deficiency from Birth to Weaning Does Not Impair Growth or Memory in Piglets. *Frontiers in Behavioural Neuroscience* **10**, 112-112 (2016).

39. Ding, H. *et al.* Tolerable upper intake level of iron damages the intestine and alters the intestinal flora in weaned piglets. *Metallomics* **12**, 1356-1369 (2020).

40. Meurens, F., Summerfield, A., Nauwynck, H., Saif, L. & Gerdts, V. The pig: a model for human infectious diseases. *Trends in Microbiology* **20**, 50-57 (2012).

41. Roura, E. *et al.* Critical review evaluating the pig as a model for human nutritional physiology. *Nutrition Research Reviews* **29**, 60-90 (2016).

42. Stachowiak, M., Szczerbal, I. & Switonski, M. Genetics of Adiposity in Large Animal Models for Human Obesity-Studies on Pigs and Dogs. *Progress in Molecular Biology and Translational Science* **140**, 233-270 (2016).

43. Groenen, M.A.M. *et al.* Analyses of pig genomes provide insight into porcine demography and evolution. *Nature* **491**, 393-398 (2012).

Appendices

Phylum	Groups	Mean ± SE	*p*-value
Proteobacteria	Control	0.0321±0.002	0.38
	IM Iron	0.0517±0.0125	
	Oral iron	0.0698±0.0083	
	IM and Oral Iron	0.0933±0.0226	
Actinobacteria	Control	0.0054±0.0033	0.4
	IM Iron	0.0106±0.002	
	Oral iron	0.0062±0.0049	
	IM and Oral Iron	0.0061±0.0032	
Tenericutes	Control	0.0063±0	0.46
	IM Iron	0.0191±0.0032	
	Oral iron	0.0242±0.012	
	IM and Oral Iron	0.0299±0.0114	
Fusobacteria	Control	0.0026±0.0468	0.48
	IM Iron	0.0031±0.0013	
	Oral iron	0.0019±0.0016	
	IM and Oral Iron	0.0003±0.0016	
Firmicutes	Control	0.58±0	0.51
	IM Iron	0.5483±0.0334	
	Oral iron	0.4917±0.0505	
	IM and Oral Iron	0.51±0.0645	
Spirochaetes	Control	0±0.0427	0.56
	IM Iron	0±0	
	Oral iron	0.0001±0	
	IM and Oral Iron	0.0001±0.0001	
Bacteroidetes	Control	0.3733±0.0031	0.83
	IM Iron	0.3617±0.0358	
	Oral iron	0.4067±0.0502	
	IM and Oral Iron	0.3533±0.0478	

Table 3.1: Impact of iron deficiency anaemia and different types of iron supplementation on gut microbial profiles in rectal samples from 28 days old piglet, at phylum level. This table shows the microbial relative abundances (%) assessed through 16S rRNA sequencing. No significant differences were found between the different types of iron supplementation. Ordinary one-way ANOVA test was used for the statistical analysis among the different groups. Data presented as mean ± SEM, n=6 piglets/treatment group.

Phylum	Groups	Mean ± SE	*p*-value
Tenericutes	Control	0.00626±0.0032	0.143
	Iron Supplemented	0.0244±0.00671	
Proteobacteria	Control	0.03212±0.01251	0.184
	Iron Supplemented	0.0716±0.0159	
Firmicutes	Control	0.58±0.03337	0.27
	Iron Supplemented	0.51667±0.03008	
Spirochaetes	Control	0±0	0.354
	Iron Supplemented	0.00006±0.00004	
Actinobacteria	Control	0.00544±0.00203	0.575
	Iron Supplemented	0.00766±0.00212	
Fusobacteria	Control	0.0026±0.00131	0.597
	Iron Supplemented	0.00177±0.00077	
Bacteroidetes	Control	0.37333±0.03584	0.991
	Iron Supplemented	0.37389±0.02557	

Table 3.2: Impact of iron deficiency anaemia and iron supplementation on gut microbial profiles in rectal samples from 28 days old piglet, at phylum level. This table shows the microbial relative abundances (%) assessed through 16S rRNA sequencing. No significant differences were found between anaemic (control) and iron supplemented piglet siblings. Ordinary one-way ANOVA test was used for the statistical analysis among the different groups. Data presented as mean ± SEM, n=6 (control); n= 18 (iron supplemented) piglets/treatment group.

Phylum	Groups	Mean ± SE	*p*-value
Fusobacteria	IM Iron	0.00311±0.00163	0.232
	Oral Iron	0.0011±0.00081	
Actinobacteria	IM Iron	0.01064±0.00486	0.336
	Oral Iron	0.00617±0.00212	
Proteobacteria	IM Iron	0.05167±0.00833	0.392
	Oral Iron	0.08157±0.02331	
Firmicutes	IM Iron	0.54833±0.05049	0.473
	Oral Iron	0.50083±0.03807	
Spirochaetes	IM Iron	0.00002±0.00002	0.476
	Oral Iron	0.00008±0.00006	
Tenericutes	IM Iron	0.01909±0.01201	0.592
	Oral Iron	0.02705±0.00835	
Bacteroidetes	IM Iron	0.36167±0.05016	0.746
	Oral Iron	0.38±0.03055	

Table 3.3: Impact of intramuscular iron (IM iron) and oral iron supplementation on gut microbial profiles in rectal samples from 28 days old piglet, at phylum level. This table shows the microbial relative abundances (%) assessed through 16S rRNA sequencing. No significant differences were found between IM, and iron supplemented piglet siblings. Ordinary one-way ANOVA test was used for the statistical analysis among the different groups. Data presented as mean ± SEM, n=6 (IM iron); n= 12 (oral iron) piglets/treatment group.

Class	Groups	Mean ± SE	*p*-value
Bacilli	Control	0.08333±0.02431	0.07
	IM Iron	0.02351±0.00792	
	Oral iron	0.00828±0.00454	
	IM and Oral Iron	0.03347±0.02933	
Gammaproteobacteria	Control	0.03198±0.01256	0.386
	IM Iron	0.05167±0.00833	
	Oral Iron	0.06978±0.02263	
	IM and Oral Iron	0.09333±0.04271	
Actinobacteria	Control	0.00038±0.00028	0.403
	IM Iron	0.00011±0.00003	
	Oral Iron	0.00007±0.00007	
	IM and Oral Iron	0.00002±0.00002	
Mollicutes	Control	0.00626±0.0032	0.464
	IM Iron	0.01909±0.01201	
	Oral Iron	0.02419±0.01142	
	IM and Oral Iron	0.02992±0.01317	
Fusobacteriia	Control	0.0026±0.00131	0.482
	IM Iron	0.00311±0.00163	
	Oral Iron	0.00191±0.00162	
	IM and Oral Iron	0.0003±0.00015	
Erysipelotrichi	Control	0.065±0.01765	0.541
	IM Iron	0.09±0.02745	
	Oral Iron	0.075±0.03274	
	IM and Oral Iron	0.04167±0.00946	
Betaproteobacteria	Control	0.00011±0.00006	0.605
	IM Iron	0.00011±0.00007	
	Oral Iron	0.00003±0.00002	
	IM and Oral Iron	0.00007±0.00003	
Deltaproteobacteria	Control	0.0003±0.00009	0.683
	IM Iron	0.00048±0.00019	
	Oral Iron	0.00051±0.00015	
	IM and Oral Iron	0.00046±0.00005	
Coriobacteriia	Control	0.00535±0.00206	0.703
	IM Iron	0.01058±0.00488	
	Oral Iron	0.00614±0.00316	
	IM and Oral Iron	0.00614±0.00312	
Bacteroidia	Control	0.37333±0.03584	0.834
	IM Iron	0.36167±0.05016	
	Oral Iron	0.40667±0.0478	
	IM and Oral Iron	0.35333±0.03921	
Clostridia	Control	0.43333±0.02848	0.975
	IM Iron	0.43333±0.0349	
	Oral Iron	0.40833±0.05885	
	IM and Oral Iron	0.435±0.06233	

Table 3.4: Impact of iron deficiency anaemia and different types of iron supplementation on gut microbial profiles in rectal samples from 28 days old piglet, at class level. This table shows the microbial relative abundances (%) assessed through 16S rRNA sequencing. No significant differences were found between the different types of iron supplementation. Ordinary one-way ANOVA test was used for the statistical analysis among the different groups. Data presented as mean ± SEM, n=6 piglets/treatment group.

Class	Groups	Mean ± SEM	*p*-value
Bacilli	Control	0.08333±0.02431	0.011*
	Iron Supplemented	0.02176±0.00994	
Actinobacteria	Control	0.00038±0.00028	0.066
	Iron Supplemented	0.00007±0.00003	
Mollicutes	Control	0.00626±0.0032	0.143
	Iron Supplemented	0.0244±0.00671	
Gammaproteobacteria	Control	0.03198±0.01591	0.183
	Iron Supplemented	0.07159±0	
Deltaproteobacteria	Control	0.0003±0.00009	0.225
	Iron Supplemented	0.00048±0.00008	
Betaproteobacteria	Control	0.00011±0.00006	0.469
	Iron Supplemented	0.00007±0.00003	
Coriobacteriia	Control	0.00535±0.00206	0.568
	Iron Supplemented	0.00762±0.00213	
Fusobacteriia	Control	0.0026±0.00131	0.597
	Iron Supplemented	0.00177±0.00077	
Clostridia	Control	0.43333±0.02848	0.886
	Iron Supplemented	0.42556±0.02913	
Erysipelotrichi	Control	0.065±0.01765	0.888
	Iron Supplemented	0.06889±0.01455	
Bacteroidia	Control	0.37333±0.03584	0.991
	Iron Supplemented	0.37389±0.02557	

Table 3.5: Impact of iron deficiency anaemia and iron supplementation on gut microbial profiles in rectal samples from 28 days old piglet, at class level. This table shows the microbial relative abundances (%) assessed through 16S rRNA sequencing. At class level, iron supplementation was associated with reduced abundance of Bacilli (0.02176; *p*=0.011), compared to anaemic counterparts (0.0833). Ordinary one-way ANOVA test was used for the statistical analysis among the different groups. Data presented as mean ± SEM, n=6 (control); n= 18 (iron supplemented) piglets/treatment group.

Order	Groups	Mean ± SEM	*p*-value
Lactobacillales	Control	0.08333±0.02431	0.07
	IM Iron	0.02351±0.00792	
	Oral iron	0.00828±0.00454	
	IM and Oral Iron	0.03337±0.02935	
Bifidobacteriales	Control	0.0003±0.00029	0.387
	IM Iron	0±0	
	Oral iron	0±0	
	IM and Oral Iron	0±0	
Turicibacterales	Control	0.00004±0.00004	0.632
	IM Iron	0.00002±0.00002	
	Oral iron	0±0	
	IM and Oral Iron	0.0001±0.0001	
Coriobacteriales	Control	0.00535±0.00206	0.703
	IM Iron	0.01058±0.00488	
	Oral iron	0.00614±0.00316	
	IM and Oral Iron	0.00614±0.00312	
Actinomycetales	Control	0.00008±0.00008	0.779
	IM Iron	0.00011±0.00003	
	Oral iron	0.00007±0.00007	
	IM and Oral Iron	0.00002±0.00002	
Bacteroidales	Control	0.37333±0.03584	0.834
	IM Iron	0.36167±0.05016	
	Oral iron	0.40667±0.0478	
	IM and Oral Iron	0.35333±0.03921	

Table 3.6: Impact of intramuscular iron (IM iron) and oral iron supplementation on gut microbial profiles in rectal samples from 28 days old piglet, at class level. This table shows the microbial relative abundances (%) assessed through 16S rRNA sequencing. No significant differences were found between IM, and iron supplemented piglet siblings. Ordinary one-way ANOVA test was used for the statistical analysis among the different groups. Data presented as mean ± SEM, n=6 (IM iron); n= 12 (oral iron) piglets/treatment group.

Order	Groups	Mean ± SEM	*p*-value
Lactobacillales	Control	0.08333±0.02431	0.07
	IM Iron	0.02351±0.00792	
	Oral iron	0.00828±0.00454	
	IM and Oral Iron	0.03337±0.02935	
Enterobacteriales	Control	0.03198±0.01256	0.386
	IM Iron	0.05167±0.00833	
	Oral iron	0.06978±0.02263	
	IM and Oral Iron	0.09333±0.04271	
Bifidobacteriales	Control	0.0003±0.00029	0.387
	IM Iron	0±0	
	Oral iron	0±0	
	IM and Oral Iron	0±0	
Fusobacteriales	Control	0.0026±0.00131	0.482
	IM Iron	0.00311±0.00163	
	Oral iron	0.00191±0.00162	
	IM and Oral Iron	0.0003±0.00015	
Erysipelotrichales	Control	0.065±0.01765	0.541
	IM Iron	0.09±0.02745	
	Oral iron	0.075±0.03274	
	IM and Oral Iron	0.04167±0.00946	
Burkholderiales	Control	0.00011±0.00006	0.605
	IM Iron	0.00011±0.00007	
	Oral iron	0.00003±0.00002	
	IM and Oral Iron	0.00007±0.00003	

Order	Groups	Mean ± SEM	*p*-value
Turicibacterales	Control	0.00004±0.00004	0.632
	IM Iron	0.00002±0.00002	
	Oral iron	0±0	
	IM and Oral Iron	0.0001±0.0001	
Desulfovibrionales	Control	0.0003±0.00009	0.683
	IM Iron	0.00048±0.00019	
	Oral iron	0.00051±0.00015	
	IM and Oral Iron	0.00046±0.00005	
Coriobacteriales	Control	0.00535±0.00206	0.703
	IM Iron	0.01058±0.00488	
	Oral iron	0.00614±0.00316	
	IM and Oral Iron	0.00614±0.00312	
Actinomycetales	Control	0.00008±0.00008	0.779
	IM Iron	0.00011±0.00003	
	Oral iron	0.00007±0.00007	
	IM and Oral Iron	0.00002±0.00002	
Bacteroidales	Control	0.37333±0.03584	0.834
	IM Iron	0.36167±0.05016	
	Oral iron	0.40667±0.0478	
	IM and Oral Iron	0.35333±0.03921	
Clostridiales	Control	0.43333±0.02848	0.975
	IM Iron	0.43333±0.0349	
	Oral iron	0.40833±0.05885	
	IM and Oral Iron	0.435±0.06233	

Table 3.7: Impact of iron deficiency anaemia and different types of iron supplementation on gut microbial profiles in rectal samples from 28 days old piglet, at order level. This table shows the microbial relative abundances (%) assessed through 16S rRNA sequencing. No significant differences were found between the different types of iron supplementation. Ordinary one-way ANOVA test was used for the statistical analysis among the different groups. Data presented as mean ± SEM, n=6 piglets/treatment group.

Order	Groups	Mean ± SEM	*p*-value
Lactobacillales	Control	0.08333±0.02431	0.01*
	Iron Supplemented	0.02172±0.00995	
Bifidobacteriales	Control	0.0003±0.00029	0.075
	Iron Supplemented	0±0	
Enterobacteriales	Control	0.03198±0.01256	0.183
	Iron Supplemented	0.07159±0.01591	
Desulfovibrionales	Control	0.0003±0.00009	0.225
	Iron Supplemented	0.00048±0.00008	
Burkholderiales	Control	0.00011±0.00006	0.469
	Iron Supplemented	0.00007±0.00003	
Coriobacteriales	Control	0.00535±0.00206	0.568
	Iron Supplemented	0.00762±0.00213	
Fusobacteriales	Control	0.0026±0.00131	0.597
	Iron Supplemented	0.00177±0.00077	
Actinomycetales	Control	0.00008±0.00008	0.798
	Iron Supplemented	0.00007±0.00003	
Clostridiales	Control	0.43333±0.02848	0.886
	Iron Supplemented	0.42556±0.02913	
Erysipelotrichales	Control	0.065±0.01765	0.888
	Iron Supplemented	0.06889±0.01455	
Turicibacterales	Control	0.00004±0.00004	0.947
	Iron Supplemented	0.00004±0.00003	
Bacteroidales	Control	0.37333±0.03584	0.991
	Iron Supplemented	0.37389±0.02557	

Table 3.8: Impact of iron deficiency anaemia and iron supplementation on gut microbial profiles in rectal samples from 28 days old piglet, at order level. This table shows the microbial relative abundances (%) assessed through 16S rRNA sequencing. Iron supplementation was associated with reduced abundance of Lactobacillales (0.02172; *p*=0.010), compared to anaemic siblings (0.0833). Ordinary one-way ANOVA test was used for the statistical analysis among the different groups. Data presented as mean ± SEM, n=6 (control); n= 18 (iron supplemented) piglets/treatment group.

Order	Groups	Mean ± SEM	*p*-value
Fusobacteriales	IM Iron	0.00311±0.00163	0.232
	Oral Iron	0.0011±0.00081	
Actinomycetales	IM Iron	0.00011±0.00003	0.29
	Oral Iron	0.00005±0.00004	
Burkholderiales	IM Iron	0.00011±0.00007	0.301
	Oral Iron	0.00005±0.00002	
Erysipelotrichales	IM Iron	0.09±0.02745	0.319
	Oral Iron	0.05833±0.017	
Coriobacteriales	IM Iron	0.01058±0.00488	0.34
	Oral Iron	0.00614±0.00212	
Enterobacteriales	IM Iron	0.05167±0.00833	0.392
	Oral Iron	0.08156±0.02332	
Lactobacillales	IM Iron	0.02351±0.00792	0.446
	Oral Iron	0.02082±0.01466	
Methanobacteriales	IM Iron	0.00008±0.00008	0.51
	Oral Iron	0.00169±0.00166	
Turicibacterales	IM Iron	0.00002±0.00002	0.704
	Oral Iron	0.00005±0.00005	
Bacteroidales	IM Iron	0.36167±0.05016	0.746
	Oral Iron	0.38±0.03055	
Clostridiales	IM Iron	0.43333±0.0349	0.857
	Oral Iron	0.42167±0.04106	
Desulfovibrionales	IM Iron	0.00048±0.00019	0.963
	Oral Iron	0.00049±0.00008	

Table 3.9: Impact of intramuscular iron (IM iron) and oral iron supplementation on gut microbial profiles in rectal samples from 28 days old piglet, at order level. This table shows the microbial relative abundances (%) assessed through 16S rRNA sequencing. No significant differences were found between IM, and iron supplemented piglet siblings. Ordinary one-way ANOVA test was used for the statistical analysis among the different groups. Data presented as mean ± SEM, n=6 (IM iron); n= 12 (oral iron) piglets/treatment group.

Family	Groups	Mean ± SEM	*p*-value
Lachnospiraceae	Control	0.14833±0.01078	0.039*
	IM Iron	0.20667±0.02591	
	Oral iron	0.13833±0.02535	
	IM and Oral Iron	0.115±0.01945	
Lactobacillaceae	Control	0.07126±0.02744	0.189
	IM Iron	0.02181±0.00695	
	Oral iron	0.0079±0.00459	
	IM and Oral Iron	0.03283±0.02946	
Porphyromonadaceae	Control	0.02179±0.00592	0.239
	IM Iron	0.01502±0.00708	
	Oral iron	0.02905±0.01162	
	IM and Oral Iron	0.00758±0.00123	
Paraprevotellaceae	Control	0.03761±0.01308	0.372
	IM Iron	0.03335±0.01382	
	Oral iron	0.01265±0.00506	
	IM and Oral Iron	0.025±0.00719	
Bifidobacteriaceae	Control	0.0003±0.00029	0.387
	IM Iron	0±0	
	Oral iron	0±0	
	IM and Oral Iron	0±0	
Streptococcaceae	Control	0.01201±0.0116	0.439
	IM Iron	0.00103±0.0008	
	Oral iron	0.00033±0.00012	
	IM and Oral Iron	0.00084±0.00037	
Veillonellaceae	Control	0.065±0.01765	0.465
	IM Iron	0.04133±0.02032	
	Oral iron	0.0281±0.00668	
	IM and Oral Iron	0.04076±0.01737	
Eubacteriaceae	Control	0.00011±0.00011	0.481
	IM Iron	0.00001±0.00001	
	Oral iron	0.00001±0.00001	
	IM and Oral Iron	0.00002±0.00001	
Fusobacteriaceae	Control	0.0026±0.00131	0.482
	IM Iron	0.00311±0.00163	
	Oral iron	0.00191±0.00162	
	IM and Oral Iron	0.0003±0.00015	
Peptococcaceae	Control	0.00036±0.00015	0.517
	IM Iron	0.00014±0.00007	
	Oral iron	0.00044±0.00021	
	IM and Oral Iron	0.00057±0.0003	
Enterococcaceae	Control	0.00005±0.00004	0.538
	IM Iron	0.00003±0.00001	
	Oral iron	0.00005±0.00002	
	IM and Oral Iron	0.00001±0.00001	
Erysipelotrichaceae	Control	0.065±0.01765	0.541
	IM Iron	0.09±0.02745	
	Oral iron	0.075±0.03274	
	IM and Oral Iron	0.04167±0.00946	
Bacteroidaceae	Control	0.17721±0.07029	0.621
	IM Iron	0.17756±0.0703	
	Oral iron	0.26167±0.06019	
	IM and Oral Iron	0.15167±0.04222	
Turicibacteraceae	Control	0.00004±0.00004	0.632
	IM Iron	0.00002±0.00002	
	Oral iron	0±0	
	IM and Oral Iron	0.0001±0.0001	
Ruminococcaceae	Control	0.18333±0.01909	0.66
	IM Iron	0.15±0.03098	
	Oral iron	0.17833±0.02442	
	IM and Oral Iron	0.19333±0.02512	
Corynebacteriaceae	Control	0.00008±0.00008	0.779
	IM Iron	0.00011±0.00003	
	Oral iron	0.00007±0.00007	
	IM and Oral Iron	0.00002±0.00002	
Prevotellaceae	Control	0.09785±0.05354	0.824
	IM Iron	0.11174±0.06821	
	Oral iron	0.04888±0.02204	
	IM and Oral Iron	0.09037±0.04132	
Clostridiaceae	Control	0.00136±0.00053	0.9
	IM Iron	0.0012±0.00077	
	Oral iron	0.00149±0.00062	
	IM and Oral Iron	0.00184±0.00053	
Rikenellaceae	Control	0.00024±0.00013	0.999
	IM Iron	0.00028±0.00027	
	Oral iron	0.00026±0.00022	
	IM and Oral Iron	0.00025±0.00015	

Table 3.10: Impact of iron deficiency anaemia and different types of iron supplementation on gut microbial profiles in rectal samples from 28 days old piglet, at family level. This table shows the microbial relative abundances (%) assessed through 16S rRNA sequencing. Lachnospiraceae abundance was significantly higher (p=0.039) in IM supplemented (0.20667) piglets compared to anaemic (0.14833) and oral supplemented without- and with IM iron injected siblings (0.13833 and 0.115 respectively). Ordinary one-way ANOVA test was used for the statistical analysis among the different groups. Data presented as mean ± SEM, n=6 piglets/treatment group.

Family	Groups	Mean ± SEM	*p*-value
Lactobacillaceae	Control	0.07126±0.02744	0.04*
	Iron Supplemented	0.02085±0.0099	
Bifidobacteriaceae	Control	0.0003±0.00029	0.075
	Iron Supplemented	0±0	
Streptococcaceae	Control	0.01201±0.0116	0.092
	Iron Supplemented	0.00073±0.00029	
Eubacteriaceae	Control	0.00011±0.00011	0.111
	Iron Supplemented	0.00001±0	
Ruminococcaceae	Control	0.18333±0.01909	0.135
	Iron Supplemented	0.17389±0.01528	
Enterobacteriaceae	Control	0.03198±0.01256	0.183
	Iron Supplemented	0.07159±0.01591	
Desulfovibrionaceae	Control	0.0003±0.00009	0.225
	Iron Supplemented	0.00048±0.00008	
Enterococcaceae	Control	0.00005±0.00004	0.347
	Iron Supplemented	0.00003±0.00001	
Alcaligenaceae	Control	0.00011±0.00006	0.406
	Iron Supplemented	0.00007±0.00003	
Elusimicrobiaceae	Control	0±0	0.467
	Iron Supplemented	0.00001±0.00001	
Erysipelotrichaceae	Control	0.065±0.01765	0.597
	Iron Supplemented	0.06889±0.01455	
Fusobacteriaceae	Control	0.0026±0.00131	0.597
	Iron Supplemented	0.00177±0.00077	
Porphyromonadaceae	Control	0.02179±0.00592	0.618
	Iron Supplemented	0.01722±0.00479	
Helicobacteraceae	Control	0.00002±0.00002	0.691
	Iron Supplemented	0.00001±0.00001	
Bacteroidaceae	Control	0.17721±0.07029	0.783
	Iron Supplemented	0.19697±0.03383	
Corynebacteriaceae	Control	0.00008±0.00008	0.798
	Iron Supplemented	0.00007±0.00003	
Prevotellaceae	Control	0.09785±0.05354	0.8
	Iron Supplemented	0.08366±0.02667	
Clostridiaceae	Control	0.00136±0.00053	0.825
	Iron Supplemented	0.00151±0.00036	
Lachnospiraceae	Control	0.14833±0.01078	0.863
	Iron Supplemented	0.15333±0.01597	
Veillonellaceae	Control	0.065±0.01765	0.888
	Iron Supplemented	0.03673±0.00875	
Rikenellaceae	Control	0.00024±0.00013	0.905
	Iron Supplemented	0.00026±0.00012	
Peptococcaceae	Control	0.00036±0.00015	0.923
	Iron Supplemented	0.00039±0.00013	
Turicibacteraceae	Control	0.00004±0.00004	0.947
	Iron Supplemented	0.00004±0.00003	

Table 3.11: Impact of iron deficiency anaemia and iron supplementation on gut microbial profiles in rectal samples from 28 days old piglet, at family level. This table shows the microbial relative abundances (%) assessed through 16S rRNA sequencing. Iron supplementation was associated with reduced abundance of Lactobacillaceae family (0.02085; *p*=0.040), compared to anaemic siblings (0.07126). Ordinary one-way ANOVA test was used for the statistical analysis among the different groups. Data presented as mean ± SEM, n=6 (control); n= 18 (iron supplemented) piglets/treatment group.

Family	Groups	Mean ± SEM	*p*-value
Lachnospiraceae	IM Iron	0.20667±0.02591	0.013*
	Oral Iron	0.12667±0.01563	
Helicobacteraceae	IM Iron	0.00004±0.00003	0.073
	Oral Iron	0±0	
Peptococcaceae	IM Iron	0.00014±0.00007	0.181
	Oral Iron	0.00051±0.00018	
Fusobacteriaceae	IM Iron	0.00311±0.00163	0.232
	Oral Iron	0.0011±0.00081	
Ruminococcaceae	IM Iron	0.15±0.03098	0.282
	Oral Iron	0.18583±0.01685	
Corynebacteriaceae	IM Iron	0.00011±0.00003	0.29
	Oral Iron	0.00005±0.00004	
Alcaligenaceae	IM Iron	0.0001±0.00007	0.3
	Oral Iron	0.00005±0.00002	
Erysipelotrichaceae	IM Iron	0.09±0.02745	0.319
	Oral Iron	0.05833±0.017	
Enterobacteriaceae	IM Iron	0.05167±0.00833	0.392
	Oral Iron	0.08156±0.02332	
Eubacteriaceae	IM Iron	0.00001±0.00001	0.469
	Oral Iron	0.00002±0.00001	
Prevotellaceae	IM Iron	0.11174±0.06821	0.473
	Oral Iron	0.06962±0.02318	
Clostridiaceae	IM Iron	0.0012±0.00077	0.556
	Oral Iron	0.00167±0.00039	
Elusimicrobiaceae	IM Iron	0.00001±0.00001	0.634
	Oral Iron	0.00002±0.00001	
Bacteroidaceae	IM Iron	0.17756±0.0703	0.698
	Oral Iron	0.20667±0.03878	
Turicibacteraceae	IM Iron	0.00002±0.00002	0.704
	Oral Iron	0.00005±0.00005	
Veillonellaceae	IM Iron	0.04133±0.02032	0.722
	Oral Iron	0.03443±0.00907	
Porphyromonadaceae	IM Iron	0.01502±0.00708	0.757
	Oral Iron	0.01831±0.00644	
Rikenellaceae	IM Iron	0.00028±0.00027	0.917
	Oral Iron	0.00025±0.00013	
Lactobacillaceae	IM Iron	0.02181±0.00695	0.948
	Oral Iron	0.02037±0.0147	
Desulfovibrionaceae	IM Iron	0.00048±0.00019	0.963
	Oral Iron	0.00049±0.00008	
Enterococcaceae	IM Iron	0.00003±0.00001	0.974
	Oral Iron	0.00003±0.00001	

Table 3.12: Impact of intramuscular iron (IM iron) and oral iron supplementation on gut microbial profiles in rectal samples from 28 days old piglet, at Genus level. This table shows the microbial relative abundances (%) assessed through 16S rRNA sequencing. Lachnospiraceae abundance was significantly reduced (p=0.013) in iron supplemented (0.12667) piglets compared to IM injected siblings (0.20667). Ordinary one-way ANOVA test was used for the statistical analysis among the different groups. Data presented as mean ± SEM, n=6 (IM iron); n= 12 (oral iron) piglets/treatment group.

Genus	Groups	Mean ± SEM	*p*-value
Mogibacterium	Control	0.00019±0.00013	0.013*
	IM Iron	0.00002±0.00004	
	Oral iron	0.00007±0.00012	
	IM and Oral Iron	0.00002±0.00003	
Escherichia	Control	0.00024±0.00028	0.041*
	IM Iron	0.0006±0.00053	
	Oral iron	0.00014±0.00016	
	IM and Oral Iron	0.00078±0.00053	
Dehalobacterium	Control	0.00001±0.00002	0.155
	IM Iron	0±0	
	Oral iron	0±0	
	IM and Oral Iron	0±0	
Clostridium	Control	0.00016±0.00012	0.16
	IM Iron	0.00007±0.00013	
	Oral iron	0.00005±0.00007	
	IM and Oral Iron	0.00004±0.00004	
Coprococcus	Control	0.00191±0.00121	0.161
	IM Iron	0.00375±0.00393	
	Oral iron	0.0016±0.0009	
	IM and Oral Iron	0.00097±0.00045	
Lactobacillus	Control	0.07126±0.06722	0.189
	IM Iron	0.02181±0.01701	
	Oral iron	0.0079±0.01125	
	IM and Oral Iron	0.03283±0.07215	
Parabacteroides	Control	0.02179±0.01451	0.239
	IM Iron	0.01502±0.01734	
	Oral iron	0.02905±0.02846	
	IM and Oral Iron	0.00758±0.00301	
Christensenella	Control	0.00005±0.00007	0.369
	IM Iron	0.00005±0.00012	
	Oral iron	0.00011±0.0002	
	IM and Oral Iron	0.00039±0.0007	
Bifidobacterium	Control	0.0003±0.00071	0.387
	IM Iron	0±0	
	Oral iron	0±0	
	IM and Oral Iron	0±0	
Faecalibacterium	Control	0.02874±0.03675	0.392
	IM Iron	0.01084±0.01179	
	Oral iron	0.00964±0.01244	
	IM and Oral Iron	0.0243±0.02137	
Ruminococcus	Control	0.01887±0.01071	0.392
	IM Iron	0.00942±0.01079	
	Oral iron	0.00782±0.01141	
	IM and Oral Iron	0.01902±0.02144	
Fusobacterium	Control	0.00187±0.00224	0.428
	IM Iron	0.0031±0.00399	
	Oral iron	0.00175±0.00359	
	IM and Oral Iron	0.00025±0.00034	

Genus	Groups	Mean ± SEM	*p*-value
Streptococcus	Control	0.01201±0.02842	0.439
	IM Iron	0.00103±0.00197	
	Oral iron	0.00033±0.00029	
	IM and Oral Iron	0.00084±0.00092	
Catenibacterium	Control	0.03518±0.03309	0.441
	IM Iron	0.07035±0.0665	
	Oral iron	0.0566±0.07067	
	IM and Oral Iron	0.02455±0.02018	
Dorea	Control	0.01247±0.00617	0.524
	IM Iron	0.01022±0.00779	
	Oral iron	0.02149±0.02054	
	IM and Oral Iron	0.0184±0.01796	
Enterococcus	Control	0.00005±0.00009	0.538
	IM Iron	0.00003±0.00003	
	Oral iron	0.00005±0.00006	
	IM and Oral Iron	0.00001±0.00002	
Citrobacter	Control	0.00002±0.00002	0.555
	IM Iron	0.00001±0.00002	
	Oral iron	0.00001±0.00001	
	IM and Oral Iron	0.00004±0.00007	
Collinsella	Control	0.00513±0.00506	0.559
	IM Iron	0.01035±0.01215	
	Oral iron	0.00416±0.00452	
	IM and Oral Iron	0.00594±0.00774	
Bacteroides	Control	0.17721±0.17216	0.621
	IM Iron	0.17756±0.1722	
	Oral iron	0.26167±0.14743	
	IM and Oral Iron	0.15167±0.10342	
Turicibacter	Control	0.00004±0.0001	0.632
	IM Iron	0.00002±0.00006	
	Oral iron	0±0.00001	
	IM and Oral Iron	0.0001±0.00025	
Shigella	Control	0.00002±0.00003	0.648
	IM Iron	0.00003±0.00003	
	Oral iron	0.00001±0.00001	
	IM and Oral Iron	0.00002±0.00004	
Corynebacterium	Control	0.00008±0.00021	0.779
	IM Iron	0.00011±0.00008	
	Oral iron	0.00007±0.00017	
	IM and Oral Iron	0.00002±0.00006	
Prevotella	Control	0.09785±0.13115	0.824
	IM Iron	0.11174±0.16708	
	Oral iron	0.04888±0.05399	
	IM and Oral Iron	0.09035±0.10122	
Butyricimonas	Control	0.0006±0.00031	0.857
	IM Iron	0.00058±0.00047	
	Oral iron	0.00051±0.00046	
	IM and Oral Iron	0.00042±0.00031	

Table 3.13: Impact of iron deficiency anaemia and different types of iron supplementation on gut microbial profiles in rectal samples from 28 days old piglet, at Genus level. This table shows the microbial relative abundances (%) assessed through 16S rRNA sequencing. *Mogibacterium* abundance was significantly higher (p=0.013) in anaemic piglets (0.00019) compared to IM supplemented (0.00002) and oral supplemented without- and with IM iron injected siblings (0.00007 and 0.00002 respectively). *Escherichia* was also found in significantly higher abundance (p=0.041) in

IM injected piglets (0.0006) and oral & IM supplemented group (0.00078) compared to anaemic (0.00024) and oral supplemented siblings (0.00014). Ordinary one-way ANOVA test was used for the statistical analysis among the different groups. Data presented as mean ± SEM, n=6 piglets/treatment group.

Genus	Groups	Mean ± SEM	*p*-value	Genus	Groups	Mean ± SEM	*p*-value
Mogibacterium	Control	0.00019±0.00005	0.002**	***Butyricimonas***	Control	0.0006±0.00013	0.611
	Iron Supplemented	0.00004±0.00002			Iron Supplemented	0.0005±0.00009	
Dehalobacterium	Control	0.00001±0.00001	0.019*	***Parabacteroides***	Control	0.02179±0.00592	0.618
	Iron Supplemented	0±0			Iron Supplemented	0.01722±0.00479	
Clostridium	Control	0.00016±0.00005	0.023*	***Collinsella***	Control	0.00513±0.00207	0.656
	Iron Supplemented	0.00005±0.00002			Iron Supplemented	0.00682±0.00203	
Lactobacillus	Control	0.07126±0.02744	0.04*	***Bacteroides***	Control	0.17721±0.07029	0.783
	Iron Supplemented	0.02085±0.0099			Iron Supplemented	0.19697±0.03383	
Bifidobacterium	Control	0.0003±0.00029	0.075	***Corynebacterium***	Control	0.00008±0.00008	0.798
	Iron Supplemented	0±0			Iron Supplemented	0.00007±0.00003	
Streptococcus	Control	0.01201±0.0116	0.092	***Prevotella***	Control	0.09785±0.05354	0.8
	Iron Supplemented	0.00073±0.00029			Iron Supplemented	0.08366±0.02667	
Faecalibacterium	Control	0.02874±0.015	0.21	***Coprococcus***	Control	0.00191±0.00049	0.858
	Iron Supplemented	0.01493±0.00386			Iron Supplemented	0.00211±0.00059	
Escherichia	Control	0.00024±0.00011	0.222	***Fusobacterium***	Control	0.00187±0.00092	0.907
	Iron Supplemented	0.00051±0.00012			Iron Supplemented	0.0017±0.00074	
Ruminococcus	Control	0.01887±0.00437	0.328	***Turicibacter***	Control	0.00004±0.00004	0.947
	Iron Supplemented	0.01209±0.0036			Iron Supplemented	0.00004±0.00003	
Enterococcus	Control	0.00005±0.00004	0.347	***Shigella***	Control	0.00002±0.00001	0.963
	Iron Supplemented	0.00003±0.00001			Iron Supplemented	0.00002±0.00001	
Dorea	Control	0.01247±0.00252	0.542	***Citrobacter***	Control	0.00002±0.00001	0.964
	Iron Supplemented	0.0167±0.00381			Iron Supplemented	0.00002±0.00001	
Catenibacterium	Control	0.03518±0.01351	0.544				
	Iron Supplemented	0.0505±0.0135					

Table 3.14: Impact of iron deficiency anaemia and iron supplementation on gut microbial profiles in rectal samples from 28 days old piglet, at phylum level.

This table shows the microbial relative abundances (%) assessed through 16S rRNA sequencing. Iron supplementation was associated with reduced abundance of *Lactobacillus* Genus (0.02085; *p*=0.040), compared to anaemic siblings (0.07126). Iron supplementation caused highly significant reduction in *Mogibacterium* abundance (0.00004; *p*=0.002) compared to anaemic siblings (0.00019). *Clostridium-1*, and *Clostridium-3* were also significantly reduced (*p*=0.05 and *p*=0.023) in response to iron supplementation. Ordinary one-way ANOVA test was used for the statistical analysis among the different groups. Data presented as mean ± SEM, n=6 (control); n= 18 (iron supplemented) piglets/treatment group.

Genus	Groups	Mean ± SEM	*p*-value	Genus	Groups	Mean ± SEM	*p*-value
Coprococcus	IM Iron	0.00375±0.00161	0.047*	***Enterococcus***	IM Iron	0.00103±0.0008	0.492
	Oral Iron	0.00129±0.00022			Oral Iron	0.00059±0.0002	
Fusobacterium	IM Iron	0.0031±0.00163	0.191	***Bifidobacterium***	IM Iron	0.00058±0.00019	0.582
	Oral Iron	0.001±0.00074			Oral Iron	0.00047±0.00011	
Corynebacterium	IM Iron	0.01035±0.00496	0.229	***Escherichia***	IM Iron	0.0006±0.00022	0.591
	Oral Iron	0.00505±0.00177			Oral Iron	0.00046±0.00014	
Mogibacterium	IM Iron	0.01035±0.00496	0.229	***Citrobacter***	IM Iron	0.00001±0.00001	0.595
	Oral Iron	0.00505±0.00177			Oral Iron	0.00002±0.00001	
Dorea	IM Iron	0.01022±0.00318	0.24	***Clostridium***	IM Iron	0.00007±0.00005	0.596
	Oral Iron	0.01994±0.00533			Oral Iron	0.00004±0.00002	
Turicibacter	IM Iron	0.00003±0.00003	0.256	***Ruminococcus***	IM Iron	0.00942±0.0044	0.616
	Oral Iron	0±0			Oral Iron	0.01342±0.00502	
Catenibacterium	IM Iron	0.07035±0.02715	0.313	***Collinsella***	IM Iron	0.17756±0.0703	0.698
	Oral Iron	0.04057±0.0151			Oral Iron	0.20667±0.03878	
Shigella	IM Iron	0.00003±0.00001	0.355	***Lactobacillus***	IM Iron	0.00002±0.00002	0.704
	Oral Iron	0.00002±0.00001			Oral Iron	0.00005±0.00005	
Streptococcus	IM Iron	0.00002±0.00002	0.442	***Butyricimonas***	IM Iron	0.01502±0.00708	0.757
	Oral Iron	0.00005±0.00003			Oral Iron	0.01831±0.00644	
Faecalibacterium	IM Iron	0.01084±0.00481	0.47	***Prevotella***	IM Iron	0.02181±0.00695	0.948
	Oral Iron	0.01697±0.0053			Oral Iron	0.02037±0.0147	
Bacteroides	IM Iron	0.11174±0.06821	0.473	***Parabacteroides***	IM Iron	0.00003±0.00001	0.974
	Oral Iron	0.06961±0.02319			Oral Iron	0.00003±0.00001	

Table 3.15: Impact of intramuscular iron (IM iron) and oral iron supplementation on gut microbial profiles in rectal samples from 28 days old piglet, at phylum level. This table shows the microbial relative abundances (%) assessed through 16S rRNA sequencing. Oral iron supplementation was associated with reduced abundance of *Coprococcus* Genus (0.00129; *p*=0.047), compared to IM injected siblings (0.00375). Ordinary one-way ANOVA test was used for the statistical analysis among the different groups. Data presented as mean ± SEM, n=6 (IM iron); n= 12 (oral iron) piglets/treatment group.