Studies of the microbiota and the prebiotic effects of galacto-oligosaccharides in suckling pigs.

By

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ABSTRACT

Suckling is a unique period in porcine development where the early-life environment affects the composition of the microbiota. Colonic samples were collected from four suckling 22-day old, pigs each, in three separate trials and the microbiome composition assessed by 16s rRNA gene sequencing. Common colonic community indicators were identified from microbiota in each trial. Data could be pooled, where performance, bacterial diversity and abundance were not significantly different between repeated trials, except for a significant difference in Jaccard Similarity. Performance positively correlated with diversity and abundance of protein digesting and short-chain fatty acid producing taxonomic units, suggesting a nutritional role for these organisms. Poorly performing piglets receiving commercial milk replacers in rescue pens do not benefit from naturally occurring prebiotic galacto-oligosaccharides otherwise found in sow milk. In a study investigating effects of complete milk replacer supplemented with galacto-oligosaccharides, milk replacer plus galacto-oligosaccharides improved gut architectural features and villus/crypt ratio throughout the gastrointestinal tract, increased the number of goblet cells and revealed a differential abundance of beneficial probiotic bacteria, particularly *Lactobacillus* and *Bifidobacterium* demonstrating that galacto-oligosaccharides may be a useful addition to animal husbandry. Rotavirus causes significant mortality, morbidity and reduced performance in neonatal pigs. In a study of late gestational sows on a commercial farm with natural endemic rotavirus challenge, supplementation with galacto-oligosaccharides during late gestation significantly increased rotavirus specific antibodies in sow colostrum, reduced the number of infected piglets and suppressed piglet faecal pathogens indicating that galacto-oligosaccharide supplementation during late gestation may have a role in veterinary health settings.

FOREWORD

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LIST OF ABBREVIATIONS

16s rRNA:	Shine-Dalgarno 16 ribosomal ribonucleic acid sequence
α:	Alpha (diversity)
ACE:	Abundance-based coverage estimator
ADG:	Average daily gain
AGP:	Antimicrobial growth promoter
AMOVA:	Analysis of molecular variance
AMR:	Antimicrobial resistance
ANOVA:	Analysis of variance
APHA:	Animal Public Health Authority
ARRIVE:	Animal Research: Reporting of *in vivo* Experiments
ATOH1:	Atonal bHLH transcription factor 1
AWERB	Animal Welfare & Ethical Review Body
β:	Beta (diversity)
bp:	Base pairs
bHLH:	Basic helix-loop-helix transcription factor
BOCM:	British Oil and Cake Mills Ltd.
B&Q:	Block & Quayle
BSC:	Biological safety cabinet
B.Sc.:	Bachelor of Science
Caco-2:	Colorectal adenocarcinoma cells
CAFO:	Concentrated animal feeding operations
cAMP:	Cyclic adenosine monophosphate
CD8+:	Cytotoxic T lymphocyte
cfu:	Colony forming units
CGMP:	Casein glycomacropeptides
cGMP:	Cyclic guanosine monophosphate
CMR:	Complete milk replacer
COVID-19:	Coronavirus disease 2019
csv:	Comma separated values
***czr*C:**	Zinc resistance gene
d:	Day
DEPC:	Diethyl pyrocarbonate

DFI:	Daily feed intake
DNA:	Deoxyribonucleic acid
DNase:	Deoxyribonuclease
dNTPs:	Deoxyribonucleotide triphosphates
dsDNA:	Double stranded deoxyribonucleic acid
DP:	Degree of polymerisation
Dr:	Doctor
dsRNA:	Double stranded ribonucleic acid
EC:	European Commission
***E. Coli*:**	*Escherichia coli*
EDTA:	Ethylenediaminetetraacetic acid
EF:	*Enterococcus faecium*
EFSA:	European Food Safety Authority
ELISA:	Enzyme linked immunosorbent assay
EMA:	European Medicines Agency
EPEC:	Enteropathogenic *Escherichia coli*
eq:	Equivalents
ETEC:	Enterotoxigenic *Escherichia coli*
EU:	European Union
F:	Fimbriae
F4 (K88):	Fimbriae adhesins type 4 Kilodalton 88 enterocyte receptors
F5 (K89):	Fimbriae adhesins type 5 Kilodalton 89 enterocyte receptors
F6 (987P):	Fimbriae adhesins type 6, 987 protein enterocyte receptors
F18:	Fimbriae adhesins type 18 enterocyte receptors
FAM:	6-carboxyfluorescein
FAO:	Food and Agriculture Organization of the United Nations
Fc:	Fragment crystallizable
FDR:	False discovery rates
FEMS:	Federation of European Microbiological Societies
FOS:	Fructo-oligosaccharides
G:	Genotype
Gal:	Galactose
Gal(β1–4)Glc:	Lactose

Gal(β1–4)GlcNAc:	N-acetyl-lactosamine
GAPS:	Goblet cell associated antigen passages
Gb:	Giga bytes
GCs:	Goblet cells
gDNA:	Genomic deoxyribonucleic acid
GIT:	Gastrointestinal tract
Glc:	Glucose
GlcNAc:	N-acetyl-glucosamine
GOS:	Galacto-oligosaccharides
GRAS:	Generally regarded as safe
GSK:	GlaxoSmithKline
G types:	Glycoprotein types
g x:	Gravitational force
HE:	Haematoxylin and eosin
HES1:	Hairy and enhancer of split 1 (gene)
HMOs:	Human milk oligosaccharides
HP:	Hewlett Packard
HPLC:	High performance liquid chromatography
HRP:	Horse radish peroxidase
HS:	High sensitivity
HT:	High throughput
HT-29:	Human colorectal adenocarcinoma cell line 29
i:	Index
IEM:	Illumina Experiment Manager
IFNγ:	Interferon gamma
IgA:	Immunoglobulin A
IgG:	Immunoglobulin G
IgM:	Immunoglobulin M
IL:	Interleukin (1 to 33)
Inc:	Incorporated
KC1:	Kit control one
LAB:	Lactic acid bacteria
Ltd.:	Limited

LEfSe:	Linear discriminant effect size
LPS:	Lipopolysaccharide
LT:	Heat-labile toxin
MA104 cells:	African green monkey foetal kidney cells
MDPI:	Multidisciplinary Digital Publishing Institute
***mec*A:**	Methicillin resistance gene
MOS:	Mannan-oligosaccharides
MRSA:	Methicillin resistant *Staphylococcus Aureus*
Mt:	Million metric tonnes
n:	Number
N:	Normal
NaCl:	Sodium chloride
NaOH:	Sodium hydroxide
NCBI:	National Center for Biotechnology Information
NC3Rs:	National Centre for the Replacement Refinement and Reduction of Animals in Research
Neu5Ac:	N-acetylneuraminic Acid
Neu5Gc:	N-glycolylneuraminic Acid
NF-κB:	Nuclear factor kappa-light-chain-enhancer of activated B cells
NRC:	National Research Council (USA)
NSP:	Non-structural protein
nt:	Nucleotide
OD:	Optical density
OECD-FAO:	Organisation for Economic Co-operation and Development. Food and Agriculture Organisation
ONS:	Office for National Statistics
OTU:	Operational taxonomic unit
***P*:**	Probability
PAS:	Periodic acid Schiff
PBS:	Phosphate buffered saline
PCoA:	Principal coordinate analysis
PCR:	Polymerase chain reaction
pdf:	Portable document format

PEDV:	Porcine epidemic diarrhoea virus
pH:	Potential of hydrogen
PLS:	Pre-lysis solution
pM:	Picomolar
PMO:	Porcine milk oligosaccharides
ppm:	Parts per million
PPS:	Protein precipitate solution
psi:	Pounds per square inch
P types:	Protease types
PWD:	Post weaning diarrhoea
qPCR:	Quantitative polymerase chain reaction
qRT:	Quantitative reverse transcription
RNA:	Ribonucleic acid
RNase:	Ribonuclease
RO:	Reverse osmosis
RT:	Reverse transcription
RV:	Rotavirus
RVA:	Rotavirus A
S.A.:	Société Anonyme (corporation)
SARS-CoV-2:	Severe acute respiratory syndrome coronavirus 2
S-block:	Sample block
SCC*mec*	Staphylococcal cassette chromosome
SCFA:	Short-chain fatty acids
SD:	Standard deviation
SE:	Standard error
SIMPER:	Similarity percentage analysis
SOP:	Standard operating procedure
spp:	Species
SRA:	Sequence read archive
SRP:	Sequence read project
STa:	Heat stable toxin a
STb:	Heat stable toxin b
TAE:	Tris acetate solution

TE:	Tris EDTA buffer
TES:	Tris EDTA solution
tGOS:	Trans-galacto-oligosaccharides
TGFß:	Transforming growth factor beta
Th1:	T helper cell 1
Th2:	T helper cell 2
TIFF:	Tag image file format
TMB:	3,3′,5,5′-Tetramethylbenzidine
TNFα:	Tumour necrosis factor alpha
UDP-Gal:	Uridine diphosphate galactose
UNFAO:	United Nations Food and Agriculture Organisation
UPL:	Universal probe library
UK:	United Kingdom
USA:	United States of America
USB:	Universal serial bus
USDA:	United States Department of Agriculture
USFDA:	United States Food and Drug Administration
UV:	Ultraviolet
v:	Version
V4:	Fourth hypervariable region
VCR:	Villus crypt ratio
VIC:	2′-chloro-7′phenyl-1,4-dichloro-6-carboxy-fluorescein
VLP:	Virus like particle
VP:	Viral protein
v/v:	Volume per volume
WATOK:	Welfare of animals at time of killing
w/v	Weight per volume
w/w:	Weight per weight
x:	Times (multiplication)
XL:	Extra large
YC:	Yue Clayton

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1 Chapter 1. Introduction

The introduction to this thesis is structured to give the reader a current understanding of global meat production, the environmental effects of pig farming, variance in large animal trials and current use of antimicrobial growth promoters in pigs. Loss of production relating to both non-infectious and infectious causes of pre- and post-weaning piglet mortality due to enteric bacteria and rotavirus are introduced, as are implications for human populations whereby animal production may be a reservoir for human pathogens, notably rotavirus and/or a source of bacterial antimicrobial resistance. Further to this, the concepts of pro- and prebiotics are introduced as useful adjuncts to animal husbandry and in particular, naturally occurring prebiotic milk oligosaccharides and synthesised galacto-oligosaccharides used as feed additives. The structure of these diverse molecules are described, as are their effects on adhesion of gut pathogens and capacity for fermentation *in vitro* and *in vivo* with inferences for health benefits to animals, as referenced from published and peer reviewed literature. The physiological effects of galacto-oligosaccharides on gut architecture and immunomodulatory function are also presented demonstrating their biological importance. In these respects, the introduction is constructed to give a global understanding of pig production, current concerns relating to loss of production and the possible intervention with pro- and prebiotics, notably galacto-oligosaccharides which are recognised to modulate the microbiota, immune function, gut architecture and suppress potential pathogens in animals. Results of the studies contained within this thesis are presented in three separate, accepted, peer-reviewed and published papers, the journals being FEMS Microbial Ecology (Chapter 3), Animals (Chapter 4) and Frontiers in Veterinary Science (Chapter 5).

1.1 Meat production Worldwide, in the EU and UK

World meat production was an estimated 339 million metric tonnes (Mt) in 2021 with pork being one of the most widely consumed meats after poultry. Meat production continues to be dominated by Brazil, China, the European Union (EU) and the United States, with total production expected to expand to 377 Mt by 2031 (OECD-FAO, 2021). Pork consumption was an estimated 112.6 Mt in 2021 and is expected to rise to 127.03 Mt worldwide by 2030 (Statista, 2021), with China being the largest producer accounting for 40% of global production (Zira *et al*., 2021). However, there is significant disparity in meat consumption per capita across geographical regions with North America consuming almost 100 kg per capita as opposed to little more than 10 kg per capita in Africa, Figure 1 (OECD-FAO, 2022).

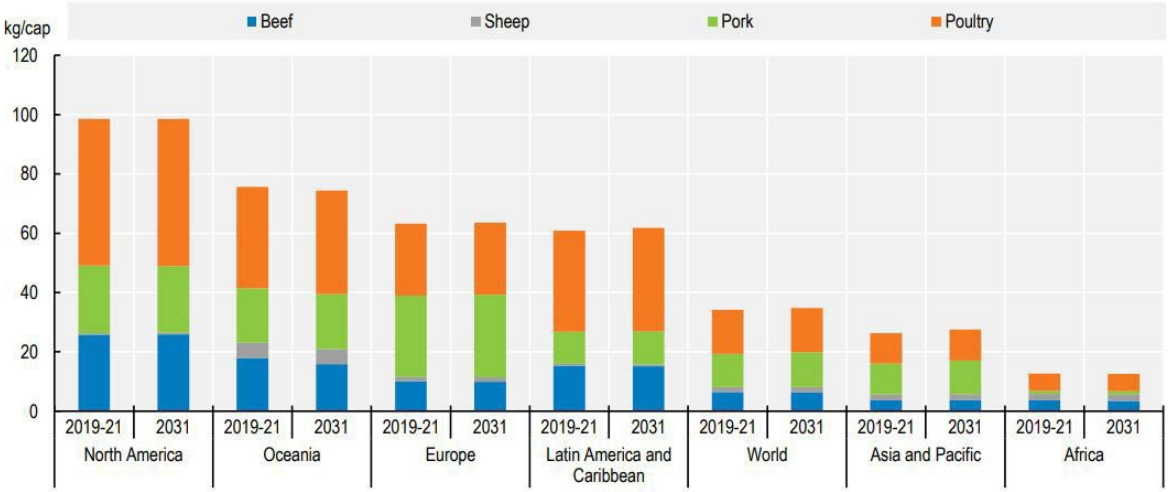

Figure 1. World meat consumption per capita. Actual and projected figures (OECD-FAO, 2022 – direct copy from reference)

Pork is the most consumed meat in the EU, which remains the second and most important producer worldwide, where consumers are attracted by products claimingorganic, free-range, fair trade and/or high welfare status (Proorocu *et al*., 2021). Although global pork consumption is expected to rise by 2030, the forecast in

the EU is different. In the EU, pork consumption is estimated to decrease to 18.5 Mt by 2030 (Statista, 2021) with per capita consumption reducing from 69.8 kg in 2018 to 67 kg by 2031 (EU Commission, 2021). This projected decline in consumption is in part, driven by environmental and animal welfare concerns (OECD-FAO, 2021) with consumers showing attention to the production process and product origin (EU Commission, 2021). In the UK average meat consumption per capita, per day has decreased from 103.7 g (SE 2.3) in 2008 to 86.3 g (SE 2.9) in 2019. For pork, this has decreased by 8.4 g (SE 0.7) in 2008 to 5.8 g in 2019 (SE 0.6) although the trend was not significant (Stewart *et al*., 2021). Nevertheless, pork production and consumption remains highly popular in the EU (Proorocu *et al*., 2021). For example, in England, Scotland and Wales the total number of pig holdings was estimated as 23,722 in 2018/19 ranging from one pig, (organic small-holding pet), to commercial herds in excess of 8000 animals, with a total count of over 4,753,467 animals in Great Britain (APHA, 2022), which is greater than the entire human population of Wales (ONS, 2021). It is interesting to note that UK authorities (APHA, 2022) record data on pig holdings from one animal to plus several thousand, without discriminating between environmental impacts, different farming practices regardless of numbers, or indeed the use of in-feed additives which may enhance health and production. Given the lag between data collection and publishing from government agencies such as the APHA, media sources have recently revealed that there are more than one thousand “USA” style mega-farms in England, Wales and Northern Ireland. These are defined as those that hold more than 125,00 birds, 82,000 egg-laying hens, 2500 pigs, 700 dairy cattle or 1000 beef cattle and defined as “concentrated animal feeding operations" (CAFO). In 2020 there were an estimated 944 mega-farms with at least four poultry farms in the UK registered for one million birds, nineteen dairies holding over a thousand animals and at least three industrial

farms holding more than 20,000 pigs. Most of these animals are kept in intensive units and are “zero grazed”, that is they are permanently housed in agricultural buildings and have no resource to fields and outdoor grazing (The Guardian, 2022; Lymbery, 2022). In these respects, there is some evidence that animal (bovine) faecal microbiotas differ between grazed and non-grazed animals with differences between *Firmicutes* and *Bacteroidetes* composition in cattle suggesting that farming practice between intensively housed and grazed animals may affect the microbiome, animal performance and meat quality (Zhang *et al*., 2021).

1.2 Environmental impacts of pig farming

The environmental impacts of pig farming are considerable and different between producers. For example, the impacts of conventional environmental systems per kg live weight for climate change, acidification, eutrophication, energy use and land occupation were assessed as 2.3 kg CO_2-equivalents (eq), 44.0 g SO_2-eq, 18.5 g PO_4-eq, 16.2 megajoules (MJ) and 4.1 m^2, respectively. In contrast, the impacts for traditional and organic systems were 10% to 60% lower (Dourmad *et al*., 2014). Knowledge of the composition of foods is frequently sought in quantitative studies of human nutrition for the dietary treatment and management of disease. (McCance & Widdowson, 2015). In this respect, the average “bacon sandwich” comprising three rashers, contains at least 1,377 kJ energy, 31.2 g of fat, 11.52 g of protein, 630 mg of sodium, but no carbohydrate (other than the bread) and negligible trace elements (USDA, 2019). Thus, given the nominal portion size of 84 g, one bacon sandwich has an environmental impact of 193.2 g CO_2-eq, 3.7 g SO_2-eq, 1.5 g PO_4-eq, 1.36 megajoules (MJ) and 0.34 m^2, respectively. This simple but crude calculation does not take account of fossil fuel energy costs related to transportation of pigs, heating of pens, processing, packaging, delivery of foods, supply of feed to pigs and cooking,

all of which may add to the total (Tallaksen *et al* 2020; Giraldi-Díaz *et al*., 2021). One mole of any gas has a volume of 24 dm^3 (24,000 cm^3) at standard temperature and pressure, therefore the "average bacon sandwich" contributes at least 105.36 dm^3 CO_2 to the atmosphere based upon data from Dourmad *et al*., 2014 and the USDA. For one finished pig weighing approximately 124 kg the results for the selected impact categories of carbon, water and energy footprints were 538.62 kg CO_2 eq., 21.34 m^3, and 1773.79 MJ, respectively, where the greatest impact was generated in the final stages of pig fattening, mainly due to the large quantity of feed supplied (Giraldi-Díaz *et al*., 2021). Farm size and reproductive efficiency are important factors in the environmental burden of heavy pig production. The largest and most efficient farms (as liveweight produced per sow) have impact potentials per kg liveweight much lower than those generated in the less efficient farms and similar to the ones reported on pigs slaughtered at a lower weight. The wide range of impact values within farms reveals opportunities for environmental improvements in the production of the traditional heavy pig. However, there is a need for further data and models on methane enteric emissions and nitrogen excretions above 100 kg live weight pigs (Bava *et al*., 2017).

1.3 Variance in animal trials

There is a high degree of technical variation between studies of the pig microbiota. Use of different DNA extraction methods, 16s rRNA gene hypervariable regions, 16s rRNA gene library preparation, sequencing platform and protocol introduce study-to-study variation. A wide range of ages for pigs as well as different diets, breeds and treatments also introduce variance between studies (Holman *et al*., 2017). There are multifactorial influences on microbial diversity and composition, these being succession of bacterial populations, the age of the animal, the environment it

inhabits, use of antimicrobial agents, dietary composition, stress and genetics (Pluske, Turpin and Kim 2018), where use of antimicrobials may confound comparisons between studies because of the profound effects upon the microbiota (Holman *et al*., 2017). Eliminating sources of technical variation within a single study can be difficult. For example, sequencing of the human faecal microbiomes has shown high intra-centre reproducibility but significant inter-centre differences of the reported microbiota composition due to variation caused by polymerase chain reaction (PCR) primers for 16S rDNA amplification (Hiergeist *et al*., 2016), thus demonstrating the importance of sequencing all microbiota DNA samples on the same platform and machine. The availability of controlled rearing environments with large animals and the incumbent costs to achieve reproducibility are important study design considerations, which may dictate that, small-scale animal trials are repeated to achieve statistical significance. For example, Megahed *et al*., 2019, analysed the microbiota of piglets managed in several batches over a period of four to ten weeks to achieve study completion. This is in contrast to murine models, which accommodate much larger study populations through ease of animal size and housing. Other advantages of the murine model are that mice have a gestation period of approximately twenty-one days, can be weaned at three to four weeks of age and reach sexual maturity by five to six weeks of age, allowing large numbers of mice to be generated (Bryda, 2013). Nonetheless, not all studies can be accomplished by "murine means" and large animal studies are required that pool data from repeated trials. However, there has been little discussion of the use of data between separate trials that have been repeated over time with the prospect of data pooling as in Thompson, Wang and Holmes 2008; Pajarillo *et al*., 2015; Chen *et al*., 2017. In the case of Thompson, Wang and Holmes 2008, data was collected from thirty-five piglets over three animal trials, but there is no reference to variance between trials to

determine if the data could be truly pooled. In a study of the effects of cohabitation on the faecal microbiome of pigs, two trials were performed two months apart in an attempt to acknowledge that environmental effects such as individual variation, pen and maternal effects might influence experiments (Pajarillo *et al*., 2015). There was no statistical evidence that these trial replicates could be truly compared. Chen *et al*., 2017 extensively studied the maturing–related piglet gut microbiota in two replicated trials. Most of the data has been pooled for single analysis, but there is little evidence of a thorough statistical comparison between both replicates except for Shannon Index and the number of observed OTUs, which are reported as non-significant but do show large variation. Comparison of variation between study replicates is fundamental if they are to be compared. Animal studies often pool data from repeated trials without due consideration of variance between trials and if they can be truly compared, especially for control data (Frommlet & Heinze, 2021), a situation which needs to be improved upon. This is not a major criticism, but rather an observation considering the difficulty in achieving results with large animals as opposed to high "n" murine models, which although informative cannot adequately describe the microbiome of other larger species.

1.4 Use of antimicrobial growth promoters in pigs

Despite technical issues regarding the reproducibility of pig trials, other concerns relate to the use of antibiotics as growth promoters in animal agriculture. Antimicrobial growth promoters (AGPs) have been used for over fifty years leading to improved feed conversion, growth, reduced morbidity and mortality due to clinical and subclinical disease (Gustafson & Bowen, 1997; Butaye *et al*., 2003; Dibner & Richards, 2005). Performance enhancing AGPs such as avoparcin, tylosin, virginiamycin can increase pig feed conversion by 2.5% to 7.0% and growth by 3.0%

to 8.8% depending upon the age of the pig (Viaene & Verbeke, 1999). Moreover, broad spectrum AGPs that promoted growth and feed efficiency at low levels were also shown to control endemic diseases in large groups of animals and their use was promoted with the development of mass confinement rearing from the 1950's onwards (Dibner & Richards, 2005; Gustafson & Bowen, 1997). Globally, the majority (73%) of antimicrobials are used in animals raised for food (Van Boeckel *et al*., 2017). Whilst antimicrobial consumption in animals is threefold that of humans it has enabled large-scale animal protein production for human consumption and in intensive settings. The consequences are development of antimicrobial resistance (AMR) in animals, which has received less attention than in humans. New hotspots of AMR are emerging in countries increasing animal protein production and it is estimated that from 2000 to 2018 the proportion of antimicrobials showing resistance above 50% increased from 0.15 to 0.41 in chickens and 0.13 to 0.34 in pigs (Van Boeckel *et al*., 2019). Based upon the 'Precautionary Principle', consumer, political and scientific concerns over the possibility of animal to human AMR transmission, bans of growth promoting AGPs have been introduced. A total ban was introduced in Sweden in 1986, with bans on avoparcin and virginiamycin in Denmark in 1995 and 1998, and the EU banning the use of avoparcin in 1997 and bacitracin, spiramycin, tylosine and virginiamycin in 1999 (Casewell *et al*., 2003). The EU implemented a full ban on all in-feed AGPs in livestock in diets in 2006 (EC, 2003). However, withdrawal of these AGPs has led to increased morbidity and mortality in pig production herds experiencing an increase in the frequency of diarrhoea and/or reduced weight gain (Casewell *et al*., 2003). Since the withdrawal of AGPs, increased infection has led to a substantial increase in the use of therapeutic antibiotics for food animals in Europe. In the UK, usage of veterinary antimicrobials increased from 383 tonnes in 1999 to 437 tonnes in 2000, post the EU AGP ban.

Increases were mainly in tetracyclines, trimethoprim/sulphonamides and macrolides, with at least seven tonnes attributable to pigs (Casewell *et al*., 2003). Similar trends in the therapeutic use of antimicrobial compounds have been seen in Denmark, a major pig producer, from 1994 to 2002, with large increases in penicillin, cephalosporin and macrolide use (Dibner & Richards, 2005). Thus, the increase in infection, morbidity and mortality across, commercial and conventionally farmed pig herds is due to the withdrawal of AGPs whilst retaining practices of mass confinement rearing for commercial benefit, allowing pathogen transmission that has required an increase in the use of therapeutic antimicrobials.

1.5 Use of pharmaceutical zinc oxide in pig production as alternatives to antimicrobial growth promoters

As an alternative to AGPs and the prevention of post-weaning diarrhoea caused by *Escherichia coli F4* (K88) infections, high doses of pharmaceutical zinc oxide (ZnO), (2500 to 3000 mg ZnO/kg feed) have been included in weaning pig diets (Vondruskova *et al*., 2010; Bonetti *et al*., 2021). Zinc is an integral component of many metalloenzymes (metal cofactors) involved in transcription, intra- and intercellular signaling to cell transcription machinery, cellular respiration and nucleic acid metabolism (Hill & Shannon, 2019). At normal dietary levels, sometimes referred to as “nutritional levels” approximately 100 mg/kg zinc is required in weaned pigs to maintain health (NRC, 2012). Pre-ban AGP studies indicated that there were no negative effects of 2500 ppm dietary ZnO in weaned pigs and that dietary supplementation for two weeks post-weaning suppressed post-weaning diarrhoea and improved performance (Jensen-Waern *et al*., 1998). Other effects of ZnO have been reported. Supplementation of 2500 ppm dietary ZnO to weaned pigs was considered to stabilise the intestinal flora and maintain a high

diversity of coliforms, which may compete for colonising GIT enterocyte receptor sites of diarrhoeagenic strains (Katouli *et al*., 1999). There is also some evidence that 3000 mg/kg ZnO per day alters the mucosal morphology of the small intestine of newly weaned pigs with significant increases in villus height and width (Li *et al*., 2001). ZnO has been shown to reduce enterotoxigenic *Escherichia coli* (ETEC), increase villus height, goblet cell numbers in the small intestine and improve growth and performance in weaned pigs (Slade *et al*., 2011). However, there has been concern that use of pharmaceutical ZnO in pig production may select for methicillin-resistant *Staphylococcus aureus* (MRSA) due to the co-location of the methicillin resistance gene (*mec*A) and zinc resistance gene (*czr*C) within the staphylococcal chromosome *mec* (SCC*mec*), (Cavaco *et al*., 2010). This has been confirmed in randomised controlled pig trials where the prevalence and persistence of MRSA was significantly higher in animals receiving a therapeutic dose of in-feed ZnO (3000 mg/kg) when compared with those receiving only recommended dietary levels (100 mg/kg) (Slifierz *et al*., 2014). Major concerns relate to the effects of high pharmaceutical ZnO administration in pig farming to the environment. Zinc is poorly absorbed in the gastrointestinal tract (GIT) of animals and manure from intensive pig production represents a major source of zinc and also copper salts in Danish agricultural soils and comparable countries, with concentrations of both metals increasing significantly in recent years (Jensen *et al*., 2018). Given the environmental and AMR concerns, in 2017 the European Commission adopted a decision to withdraw marketing authorisation for veterinary medicines containing high doses of pharmaceutical ZnO administered orally to food producing species. Member states were given up to five years from the date of adoption of the decision to withdraw existing authorisations for these products. Moreover, the use of ZnO in pig production must be limited to a maximum level of 150 ppm after 2022 (EMA, 2017).

Thus, growth promoting, microbiome suppressing, AGPs and pharmaceutical ZnOs have been banned leaving the pig production industry wanting for viable alternatives, if current production values are to be preserved. One alternative is a return to less intensive, traditional farming practices and organic farming, but obviously with less economic return than intensive, conventional methods (Dourmad *et al*., 2014). Other alternatives are the inclusion of in-feed prebiotics and/or probiotics to manipulate the microbiome and GIT health with the prospect of beneficial outcomes on pig production (Liao & Nyachoti, 2017; Gresse *et al*., 2017), although the outcomes remain to be researched. Non-infectious and infectious factors affecting pig production will be considered before the effects of alternative in-feed additives such as prebiotics and/or probiotics are introduced here.

1.6 Non-infectious causes of pre-weaning piglet mortality

Non-infectious causes of pre-weaning piglet mortality represent significant economic losses (Panzardi *et al*., 2013; Muns *et al*., 2016) with an average of 50-80% of deaths occurring in the first week *post-partum* with the most critical period being the first 72 hours of life (Koketsu *et al*., 2006). Mortality was estimated at 12.6% in commercial pig herds (Nuntapaitoon *et al*., 2018) with cumulative mortality assessed as 3.3%, 5.4% and 8.7% at 3, 7 and 21 days respectively (Panzardi *et al*., 2013) with most causes being physiological rather than infection. Risk factors associated with pre-weaning mortality in piglets are (sow) colostrum production, parity and litter size; (piglet) birth weight, vitality and sex; (environment) season, herd size and management (Muns *et al*., 2016). Colostrum intake is the main determinant of piglet survival through energy provision and immune protection with long-term effects on growth and immunity (Devillers *et al*., 2011). Daily weight gain and survival until weaning of piglets has been shown to positively correlate with

birth weight, colostrum intake per kg birth weight and negatively to time between birth and first suckle (Decaluwé *et al*., 2014). Colostrum intake and birth weight are important for the growth and survival of piglets with low-birth-weight piglets more dependent on colostrum intake than high-body-weight piglets to ensure their survival (Ferrari *et al*., 2014). Moreover, piglets are born agammaglobulinemic and survival depends on early acquisition of maternal immunity through colostrum (Salmon, 2002) before gut closure within 24 to 48 hours *post-partum* and reduced intestinal enterocyte ability to sequester immunoglobulins from protein rich colostrum (Weström *et al*., 1985). In terms of survival, small piglets have a competitive disadvantage compared with heavier littermates. This is exacerbated in large litters and piglets from older sows. Thus, selection for increased litter size resulting in more low-birth-weight piglets per litter may not be beneficial unless measures are taken to improve the survival of low-birth weight piglets (Milligan *et al*., 2002). The focus on larger litter sizes has increased the number of piglets with decreased viability, lighter birth weights and a reduced ability to thrive in early life (Tucker *et al*., 2021), notwithstanding gut dysbiosis and economic loss due to known bacterial pathogens and/or scour from rotavirus (Monteagudoo *et al*., 2022). The reduction in weaning age may also affect the health of pigs and it is now normal commercial practice that pigs are weaned at a much earlier age than in the wild (Lallès *et al*., 2007). Without human intervention natural weaning in the pig occurs at approximately 70 days. However, advances in housing and nutrition have led to a rapid reduction in weaning age to typically 28 to 35 days in the EU (Whittemore & Green, 2001). Nonetheless, poor pre-weaning performance and failure to thrive without obvious signs of clinical disease during lactation is of concern in piglet production. It is industry standard to remove animals receiving sub-optimal nutrition from the sow from farrowing pens and feed with a commercial milk-replacer (CMR) in controlled pen environments

with no access to the sow. In contrast to natural sow colostrum and milk, which contains naturally occurring prebiotic porcine milk oligosaccharides (PMOs), CMR have not traditionally contained GOS, although studies have demonstrated that formulas supplemented with GOS are safe and well tolerated in neonatal piglets (Kruger *et al*., 2017).

1.7 Infectious causes of pre- and post-weaning pig mortality

1.7.1 Enteric bacterial infections in pigs

Although non-infectious causes of pre-weaning piglet mortality account for the majority of deaths, scours (diarrhoea) and infection account for an estimated 8.1% to 12.2% mortality (Vaillancourt *et al*., 1990). In litters with pre-weaning diarrhoea mortality rate was assessed as 19% compared with 13% in non-diarrhoeic litters. Litters with pre-weaning diarrhoea had reduced weight gain and a significantly increased risk of post-weaning diarrhoea (Svensmark *et al*., 1989a). Enteric bacterial infections are common and cause economically significant diseases affecting pig production at all stages globally. Clinical signs include reduced growth rate, weight loss, diarrhoea and death of pre-weaned, weaned, grower-finisher, young and adult age breeding animals. Common pathogens playing an etiological role in infection are *Escherichia coli*, *Clostridium perfringens*, *Lawsonia intracellularis*, *Salmonella enterica* and *Brachyspira* (Serpulina) spp. (Moxley & Duhamel 1999). Although the focus of this work is pre-weaning animals, some introduction of the concept of "post weaning diarrhoea" (PWD) in weaned pigs is useful. This is mainly because the prebiotics and probiotics discussed in this work, may have a prophylactic role either in the prevention and/or treatment of infection throughout the life of the pig and that pre-weaning health may be a major predictor of post-weaning health in animals (Lu *et al*., 2018). In addition, GIT microbiota diversity pre-weaning, may be predictive of

the susceptibility of animals to enteric disease post-weaning (Dou *et al*. 2017). In these respects, it is just not important to consider the early pre-weaning life of the pig but also the post-weaning life of the pig irrespective of the trial designs and limitations of this work in young animals. There is a "continuum" whereby the early life of pre-weaning animals heavily influences the development and composition of the adult microbiota and intestinal innate immune functions (Bauer *et al*. 2006; Mulder *et al*. 2009; Merrifield *et al*. 2016) and affords immune and microbiological protection to the adult. Weaning is a complex process inducing stress in animals. This is mainly due to change in nutrition from a protein rich liquid colostrum/milk to carbohydrate rich solid diet; separation from mother and litter mates; introduction to a new environment and litter mates; low and variable feed intake (Pluske *et al*., 1997). Withdrawal of sow's milk and the protective immune factors that it contains coupled with a susceptibility to enteric disease due to an immature mucosal immune system at early age weaning induce stress and elevate risk of infection (Lallès *et al*., 2007; Stokes, 2017). Due to stress, dietary change and reduced feeding a state of gut dysbiosis may be induced during weaning where disruption of the gut microbiota composition and intestinal inflammation lead to the expansion and domination of enteric pathogens and the occurrence of PWD (Gresse *et al*., 2017). The post weaning growth check and/or the occurrence of PWD are a major source of economic loss, which can result in young pigs achieving less than 50% of the expected performance (Pluske *et al*., 1997; Pluske *et al*., 2013; Fairbrother *et al*., 2005). ETEC is the main pathotype in PWD in European pig farms post AGP bans (Luppi *et al*., 2016) with total losses due to infection amounting to an estimated 17% in the EU (Lallès *et al*., 2007). PWD caused by ETEC is characterised by mild to severe diarrhoea, typically a few days to one-week post-weaning, dehydration, loss of condition and increased mortality. Death is usually due to dehydration. ETEC

attachment to porcine enterocytes is mediated by the presence of bacterial fimbria. Bacteria colonise the intestine by attachment to glycolipid/glycoprotein receptors on the epithelium by F4 (K88) and F18 fimbrial adhesins. Porcine ETEC also produce enterotoxins such as heat-stable toxin a (STa), heat-stable toxin b (STb) and heat-labile toxin (LT) which induce diarrhoea through accumulation of cellular cAMP and cGMP leading to increased chloride secretion, reduced sodium absorption in pig enterocytes and the critical loss of water into the intestinal lumen (Fairbrother *et al*., 2005; Fairbrother *et al*., 2012). The pathogenesis of ETEC is shown in Figure 2.

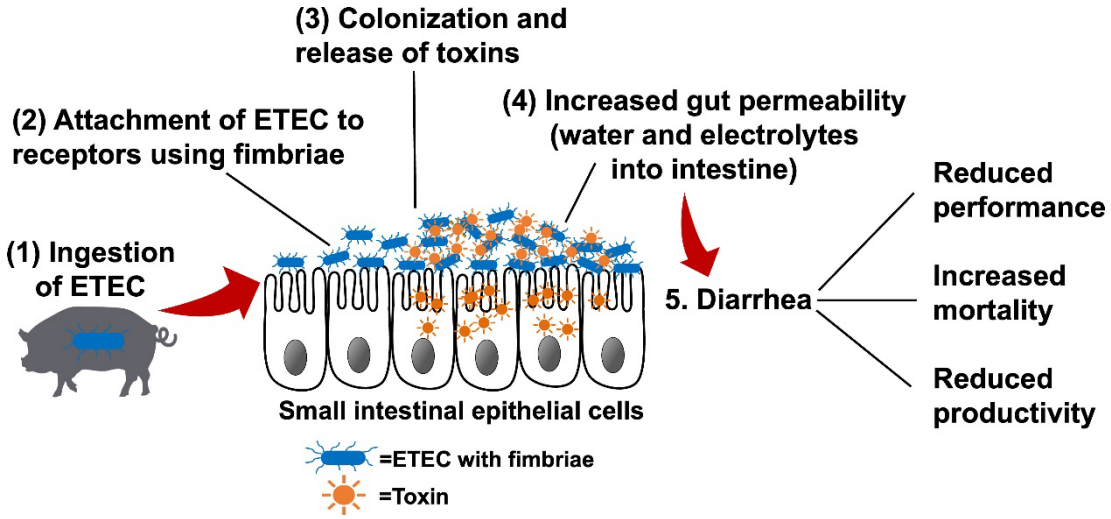

Figure 2. Pathogenesis of ETEC in pigs (Kim *et al*., 2022 – direct copy from reference)

E. coli is also an important cause of pre-weaning diarrhoea in piglets, including neonatal diarrhoea in the first four to five days of life and scours at two to three weeks of age (Fairbrother *et al*., 2012). Studies have shown that in pre-weaning piglets with diarrhoea, the occurrence of *E. coli* fimbrial antigens F4 (K88), F5 (K89) and F were significantly higher than those in piglets without diarrhoea and may be related to poor nutrition in sows (Hong *et al*., 2006). In developing countries with commercial operations of at least one hundred sows each, diarrhoea affected 71.5% of litters born over six months with 31% of faecal samples collected testing positive for at least one of eight porcine ETEC virulence genes. ETEC was

identified as the causative agent of neonatal piglet diarrhoea during the first four days of life in 43% of cases and 23.9% of remaining cases up until the age of weaning (Do *et al*., 2006).

1.7.2 Rotavirus infection in pigs

Other important infectious causative agents of neonatal and pre-weaning diarrhoea in animals including pigs and mortality are the rotaviruses (RVs) (Kapikian *et al*., 2001). These viruses belong to the *Reoviridae* family and are classified into at least ten serogroups (Estes & Kapikian, 2007; Vlasova *et al*., 2017) of which groups A, B and C affect humans (Matthijnssens *et al*., 2011), whilst groups A to H affect pigs (Vlasova *et al*., 2017). Classification is based upon the antigenic and genetic characteristics of the inner capsid viral protein 6 (VP6) (Matthijnssens *et al*., 2012). The most prevalent groups are A, B and C with rotavirus A (RVA) being the most widespread group causing acute dehydrating diarrhoea in veterinary and public health settings (Vlasova *et al*., 2017). RVA reportedly accounts for 53% of pre-weaning and 44% of post-weaning diarrhoea in pigs (Fitzgerald *et al*., 1988) the effects being significant mortality and morbidity in neonates, reduced performance in surviving growers and significant economic loss (Estes *et al*., 2007; Vlasova *et al*., 2017; Svensmark *et al*., 1989b). RVA is the most predominant group in suckling pigs with infections occurring in combination with other pathogens such as *E. coli*, *Clostridium difficile*, *Clostridium perfringens* and porcine epidemic diarrhea virus (PEDV) (Ferrari *et al*., 2022). RVA faecal-oral infection results in destruction of small intestinal enterocytes, the development of malabsorptive diarrhoea (Estes *et al*., 2001) and promotes gut dysbiosis through alteration of the microbiota (Azagra-Boronat *et al*., 2018). Viral replication in epithelial cells at the tip of intestinal villi destroys the enterocytes causing villus atrophy whereby mature columnar epithelial

villus cells are replaced by immature cuboidal enterocytes lacking digestive and absorptive capacities thus increasing malabsorptive diarrhoea, dehydration and death (Shaw *et al*., 1989; Svensmark *et al*., 1989b). Infections with RVs increase the concentration of intracellular calcium, disrupt the cellular cytoskeleton and tight epithelium cellular junctions increasing enterocyte permeability in a similar fashion to infection by ETEC thereby leading to water and electrolyte loss, resulting in mortality through dehydration. Furthermore, RVs produce enterotoxic non-structural protein 4 (NSP4) that also increases intracellular calcium concentrations further contributing to electrolyte imbalance and diarrhoea (Vlasova *et al*., 2017). The clinical symptoms of RVs last up to three days but result in lower weaning weights and average daily gain (ADG) in affected animals that survive (Svensmark *et al*., 1989b). RVA is endemic in UK pig farms with a wide range of genotypes identified in UK pigs these being six G types (VP7); G2, G3, G4, G5, G9 and G11 and six P types (VP4); P6, P7, P8, P13, P23 and P32. The common human genotype P8 can infect pigs highlighting the need for surveillance of porcine rotavirus genotypes to safeguard human and porcine health (Chandler-Bostock *et al*., 2014). RVA are non-enveloped viruses (lacking a lipid membrane derived from the host cell) with a genome composed of eleven double stranded RNA (dsRNA) segments that code for six structural viral proteins VP1, VP2, VP3, VP4, VP6, VP7 and six non-structural proteins NSP1 to NSP6 and where genome segment 11 encodes for NSP5 and NSP6. VP2 forms the inner layer of the virus and encapsulates the genome and two minor structural proteins, VP1 and VP3. The middle layer consisting of VP6 surrounds the core, forming a double layered particle, whilst an outer layer consisting of VP7 and spike-like projections of VP4, enwraps the double layered particle to form the triple-layered RVA virion, Figure 3 (Ciarlet & Estes, 2003; Estes & Kapikian, 2007; Matthijnssens *et al*., 2008).

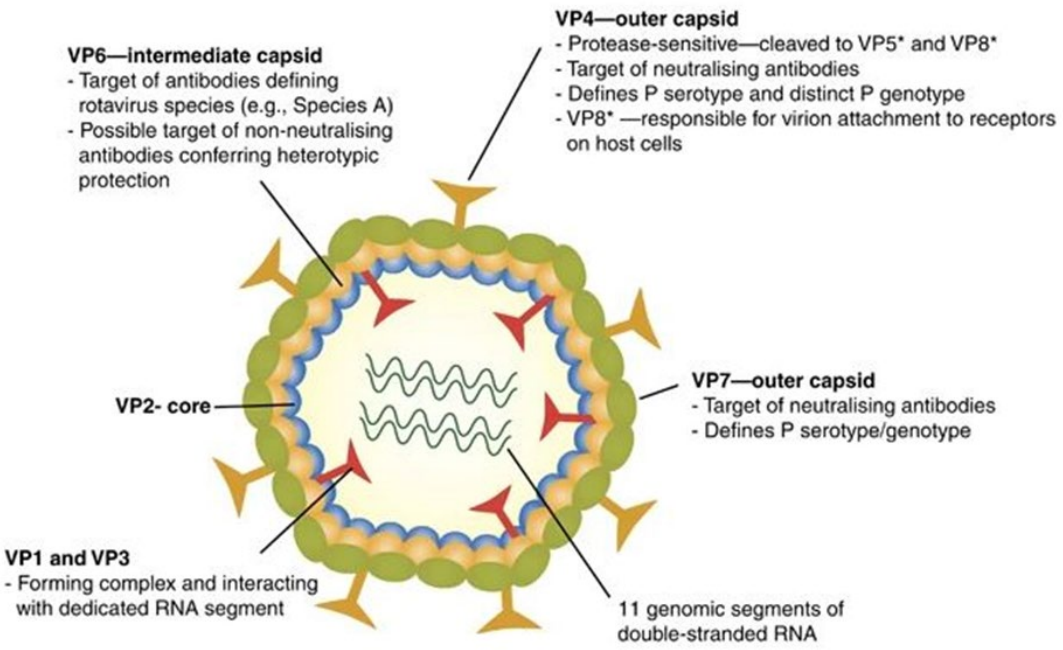

Figure 3. Structure of rotavirus triple layered particle (Clarke & Desselberger, 2014 – direct copy from reference)

Vaccination against RV in livestock is an option, but the wide variety of genotypes complicates effective vaccine production (Vlasova *et al*., 2017). Most animal vaccination strategies have focussed on the induction of active/passive immunity by oral administration of attenuated RV vaccines but these have lacked efficacy in the field due to RV specific IgG and IgA colostral antibodies neutralising and/or inhibiting the vaccines (Saif & Fernandez, 1996). Engineered virus-like particles (VLP) designed as vaccines to boost antibodies in bovine and porcine mammary secretions have shown promise when administered with live-attenuated vaccines (Azevedo *et al*., 2013). However, attenuated replicating porcine RVA vaccines may contribute to the diversity of porcine RVs, through reassortment of vaccine strains with wild type strains and the emergence of novel genetic variants that can evade herd immunity (Vlasova *et al*., 2017; Chandler-Bostock *et al*., 2014). Considering the resilience and longevity of RVA in the environment focus on cleaning with efficacious detergents that not only limit the spread and infectivity of RV but also

other microbial pathogens may be useful (Hancox *et al*., 2013; Chandler-Bostock *et al*., 2014). Nevertheless, endemic porcine RV infection still needs alternative strategies to boost lactogenic immunity in sows, thus providing RV antibodies to the neonate with colostrum and milk (Vlasova *et al*., 2017).

1.7.3 Implications for rotavirus in human populations

Despite the effects of RVs, particularly RVA in commercial pig herds there are major implications for infection in human populations. Rotaviruses, particularly RVA, have a broad host range that include not just humans but other mammalian and avian species (Ciarlet & Estes, 2003; Estes & Kapikian, 2007; Matthijnssens *et al*., 2008). RVs, mainly RVA have been a major cause of morbidity and mortality in children under five years of age with an estimated 440,000 deaths annually pre-vaccine introduction in 2006 (Parashar *et al*., 2003). Two RV vaccines were licenced in 2006 (RotaTeq, Merck & Company and Rotarix, GSK Biologicals) (Vesikari *et al*., 2006; Ruiz-Palacious *et al*., 2006), but despite major vaccination programmes RV is still a major causative agent of lethal gastroenteritis in young children globally (Tate *et al*., 2012; Tate *et al*., 2016; Burnett *et al*., 2017). However, and more recently, cost effective Rotasiil, Rotavac, and Rotavin-M1 vaccines have been licenced demonstrating safety and efficacy against RV with promising results in vulnerable children (Skansberg *et al*., 2021). Nevertheless, there were an estimated 528,000 RV deaths in children under five years of age in 2000, 453,000 in 2008 and 215,000 deaths attributable to this disease in 2013 post-vaccine introduction (Tate *et al*., 2012; Tate *et al*., 2016). Moreover, the majority of countries implementing successful RV vaccination were low mortality areas, whereas many countries including sub-Saharan Africa remained disproportionately affected (Tate *et al*., 2016; Burnett *et al*., 2020; Damtie *et al*., 2020). Whilst vaccination programmes have

undoubtedly been successful, with significant reductions in hospitalisation and mortality (Burnett *et al*., 2017; Burnett *et al*., 2020; Lai *et al*., 2020), problems still relate to health care provision, sanitation, hygiene, nutrition, adequate vaccination program delivery, cost, health education and national vaccine uptake (Abou-Nader *et al*., 2018; Apte *et al*., 2018; Damtie *et al*., 2020). It is also noted that where RV is endemic, other pathogens such as *Cryptosporidium* in humans are prevalent, indicating the need for collaborative strategies to combat RV and other enteric diseases in susceptible communities (Msolo *et al*., 2020). Furthermore, the successful spread of RV post-vaccination and genetic drift of RV strains over time may undermine the effectiveness of human vaccines (Harastani *et al*., 2020). There is also some evidence that the close relationship amongst porcine RVA strains and some unique porcine-like genotypes detected sporadically in human children may share those with pig farms indicating that pigs might serve as a reservoir for potential RVA zoonotic transmission to humans and novel genotype evolution in insular environments. This indicates the continued need for RVA strain surveillance in animals and vaccine effectiveness in children (Wu *et al*., 2022). However, the addition of prebiotics to animal diets appears promising in attempts to improve lactogenic immunity in sows with benefits conferred to the offspring (Vlasova *et al*., 2017; Azagra-Boronat *et al*., 2018; Azagra-Boronat *et al*., 2019).

1.8 The concept of probiotics

Probiotics are living microorganisms comprising mainly bacteria, some yeasts and fungi that are considered, when ingested in sufficient quantity, to have health benefits upon the host (Angelakis, 2017). As early as the 1860's Metchnikoff made the association between human longevity and lactobacilli consumption in edible fermented milk products. Metchnikoff demonstrated such products could be made

from pure cultures of *Lactobacillus bulgaricus* and suggested these organisms were able to contribute to colonic health by eliminating pathogens (Metchnikoff, 1908). Further advances were made in the 1920's and 1930's with the discovery that *Lactobacillus acidophilus* could be used for the successful treatment of chronic constipation, diarrhoea and eczema (Rettger & Cheplin, 1922; Kopeloff *et al*., 1932). The term probiotic is introduced by Lilly & Stillwell in 1965 and is defined as "substances secreted by one microbe that stimulate the growth of another". However, this work was based on protozoa and not beneficial GIT bacteria (Lilly & Stillwell, 1965). More recent definitions include "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance (Fuller, 1989); "a live microbial feed supplement that is beneficial to health (Salminen, 1998); "live microorganisms, that when administered in adequate amounts, confer a health benefit on the host" (UNFAO, 2002). Moreover, Fuller, 1989, considers that probiotic treatments may re-establish natural conditions, which exist in wild animals, but that which have been disrupted by modern trends in the conditions used for rearing young animals, and thereby early recognition that intensive farming affects the establishment of a natural microbiome. In animals, probiotic microorganisms can be classified into different groups according to various criteria (Bajagai *et al*., 2016):

1) Single versus multi strain probiotics composed of a single species or strain to multi species/strain microorganisms, examples of single species probiotics being *Saccharomyces cerrevisiae* (Bro-bio-fair) and *Enterococcus faecium* (Anta Pro EF). Multi species probiotics contain microorganisms such as *Lactobacillus* spp., *Enterococcus faecium* and *Bifidobacterium thermophilum* (PrimaLac).
2) Bacterial versus non-bacterial probiotics. Most probiotics are bacterial and include *Lactobacillus*, *Bifidobacterium*, *Bacillus* and *Enterococcus*. Fungal and

yeast probiotics include *Aspergillus oryzae*, *Candida pintolopesii*, *Saccharomyces cerevisiae* and *Saccharomyces boulardii*.

3) Spore forming versus non-spore forming probiotics. Spore forming bacteria such as *Bacillus subtilis* and *Bacillus amyloliquefaciens* are increasing in use because of their spore longevity and perceived advantages in storage, shelf life and incorporation into feedstuffs.

4) Allochthonous versus autochthonous probiotics, where allochthonous microorganisms describe those not normally present in the GIT of animals, for example, some yeasts and fungi versus autochthonous microorganisms, indigenous to the GIT such as *Lactobacillus* and *Bifidobacterium*. A diverse range of microorganisms are used as probiotics in animal production, including *Lactobacillus*, *Enterococcus*, *Streptococcus*, *Leuconostoc*, *Lactococcus*, *Pedicoccus*, *Bifidobacterium*, *Bacillus*, *Saccharomyce*s and *Aspergillus* (Anee *et al*., 2021; Angelakis, 2017; Liao & Nyachoti, 2017), the first six of those listed belonging to the lactic acid bacteria (LAB) and that are most frequently used in animal production (Yang *et al*., 2015).

In animals the benefits of probiotic consumption mainly relate to increased weight gain and sometimes improved feed efficiency, modulation of the GIT microbiome, the immune system and suppression of potential pathogens (Anee *et al*., 2021; Angelakis, 2017; Liao & Nyachoti, 2017). There are several proposed and cross-correlating mechanisms for probiotic modes of action. These include:

1) Inhibition of pathogen adhesion by out competing pathogens for adhesion sites on the GIT surface.

2) Direct inhibition of pathogens through the secretion of molecular bacteriocins and/or defensins.

3) Modulation of host immune responses through enhancement of intestinal barrier function, for example, increased mucin production by secretory goblet cells and presentation of GIT luminal antigens to the immune system.

4) Reduction of luminal pH through fermentative activity producing short chain fatty acids (SCFAs) which suppress pathogens, but which are beneficial to the host.

5) Reduction in diarrhoea and pathogen enterotoxin effects by inhibition of toxin expression and neutralisation of toxins.

6) Modulation of the immune system via T and B-cell activation through mucosal dendritic cells which present probiotics to mesenteric lymph nodes (Anee *et al*., 2021).

However, there is controversy surrounding the efficacy of probiotics and their ability to successfully populate the GIT over the long term, with some studies showing that viability may only be transient (Zmora *et al*., 2018). In contrast, the prebiotic concept states that non-viable dietary components fortify autochthonous probiotic residents of the GIT flora without the need for survival of a live ingested probiotic microorganism (Gibson, 1999).

1.9 The concept of prebiotics

The concept of "prebiotics" was introduced by Gibson and Roberfroid in 1995 and defined as "A prebiotic is a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number

of bacteria in the colon, and thus improves host health." (Gibson & Roberfroid, 1995). The definition includes how food ingredients can be classified as prebiotics. "In order for a food to be classified as a prebiotic, it must:

1) Be neither hydrolysed nor absorbed in the upper part of the gastrointestinal tract.

2) Be a selective substrate for one or a limited number of beneficial bacteria. commensal to the colon, which are stimulated to grow and/or are metabolically activated.

3) Consequently be able to alter the colonic flora in favour of a healthier composition.

4) Induce luminal or systemic effects that are beneficial to the host health." (Gibson & Roberfroid, 1995).

In this case, the focus is upon human colonic health and not necessarily animal monogastric species, although the definition may be generally applied at this time. Although several non-digestible and low-digestible dietary carbohydrates are identified as candidate prebiotics only the fructo-oligosaccharides and questionably the galacto-oligosaccharides are considered prebiotic at this time (Gibson & Roberfroid, 1995). However, the concept of prebiotics and the criteria to define them has evolved through time with Gibson *et al*., 2004 refining the concept as "A prebiotic is a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host wellbeing and health." Review of the concept was based on potential prebiotics showing resistance to upper intestinal acid and enzymatic hydrolysis and absorption, capacity for GIT bacteria fermentation and the selective stimulation of growth of GIT bacteria associated with health and wellbeing, notably lactobacilli

and bifidobacteria. Thus, inulin, transgalacto-oligosaccharides (tGOS) and lactulose were awarded prebiotic status, with isomalto-oligosaccharides, xylo-oligosaccharides and lactosucrose showing some promise (Gibson *et al*., 2004). Having recognised the beneficial effects of prebiotic foods, the expansion of foods considered prebiotic and the increased knowledge of GIT bacterial populations through metagenomics studies, the UNFAO evaluated the functional and health properties of prebiotics and defined them as "a nonviable food component that confers a health benefit on the host associated with modulation of the microbiota" (Pineiro *et al*., 2008). Considering prebiotics may modulate other non-GIT mixed bacterial ecosystems, a niche for "dietary prebiotics" was defined as "a selectively fermented ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health'' (Gibson *et al*., 2010). Another definition challenged previous definitions as prebiotic effects were too selective and/or specific and proposed prebiotics as "a non-digestible compound that, through its metabolism by microorganisms in the gut, modulates composition and/or activity of the gut microbiota, thus conferring a beneficial physiological effect on the host" (Bindels *et al*., 2015). However, the definition of the concept of prebiotics was further expanded in 2016 to include non-carbohydrate molecules such as polyphenols and polyunsaturated fatty acids and applied for use in animals where "The beneficial effect(s) of a prebiotic on health must be confirmed in the target animal for its intended use and mediated through the microbiota" with the updated definition of a prebiotic being "a substrate that is selectively utilized by host microorganisms conferring a health benefit." (Gibson *et al*., 2017). The family of prebiotics now extends to inulin, fructo-oligosaccharides (FOS), lactulose, tGOS, human milk oligosaccharides (HMOs) and GOS with candidate molecules including lactosucrose, soya oligosaccharides, xylo-

oligosaccharides, resistant starches, mannan-oligosaccharides (MOS) and arabinoxylans (Bedu-Ferrari *et al*., 2022; Gibson *et al*., 2017). The majority of prebiotics conferring a host health benefit in key human studies are GOS, FOS and inulin in comparison to animals where short-chain FOS, MOS, GOS, inulin, lactulose, fructans and galactans have been used (Gibson *et al*., 2017). Health benefits considered targets for prebiotic effects are summarised in Table 1. However, to satisfy the criterion of conferring a health benefit, a wide variety of controlled studies establishing direct links between the prebiotic and health are required in the target host.

Table 1. Summary of the main physiological and patho-physiological targets for prebiotic effects (Roberfroid *et al*., 2010)

1	Improvement and/or stabilisation of gut microbiota composition.
2	Improvement of intestinal functions (stool bulking, stool regularity, stool consistency).
3	Increase in mineral absorption and improvement of bone health (bone calcium content, bone mineral density).
4	Modulation of gastro-intestinal peptides production, energy metabolism and satiety.
5	Initiation (after birth) and regulation/modulation of immune functions.
6	Improvement of intestinal barrier functions, reduction of metabolic endotoxemia.
7	Reduction of risk of intestinal infections.
8	Tentatively: Reduction of risk of obesity, type 2 diabetes, metabolic syndrome, etc.
9	Tentatively: Reduction of risk and/or improvement in the management of intestinal inflammation.
10	Tentatively: Reduction of risk of colon cancer

With randomised controlled trials being the "gold standard" in humans, the strength of evidence for prebiotic status must be commensurate with the strength of the health benefit claim (Gibson *et al*., 2010; Gibson *et al*., 2017). Early relevant studies with oligosaccharides attempting to confirm a prebiotic effect focussed on human adults ingesting mainly short-chain FOS, inulin, tGOS and lactulose with studies commonly reporting an increase in *Lactobacillus* and *Bifidobacterium* as reviewed by Gibson, 1999 and Gibson *et al*., 2010. Thus, the focus of the prebiotic effect was considered as an increase in the relative abundance of beneficial members of the microbial community, mainly *Lactobacillus* and *Bifidobacterium*, which are considered as probiotic organisms. However, in contrast to commonly tested prebiotics such as inulin, FOS and GOS other candidate molecules may be slow to gain prebiotic status because there are fewer consistent studies confirming health benefits as described above (Gibson *et al*., 2017).

1.10 Galacto-oligosaccharides

1.10.1 Human and animal milk oligosaccharides

Prebiotic oligosaccharides are a major constituent of human and animal milk (Niñonuevo *et al*., 2006; Albrecht *et al*., 2014). However, human milk oligosaccharides (HMOs) have been the focus of research for some time in contrast to animal milk oligosaccharides which have been more recently investigated (Urashima *et al*., 2001; Urashima *et al*., 2013; Albrecht *et al*., 2014). Human milk is rich in oligosaccharides and contains 20 mg/L in colostrum and 12 to 13 mg/L in mature milk, with over two hundred different oligosaccharide components identified showing the complexity and diversity of these molecules (Niñonuevo *et al*., 2006; Urashima *et al*., 2007). Although mammalian milk oligosaccharides have no direct nutritive value to the neonate through limited upper intestinal hydrolysis and

absorption, their effects relate to stimulating the development of the microbiota in neonates and conferring a variety of health benefits including innate and adaptive immune development (Bode, 2012; Donovan & Comstock, 2017). In terms of shaping the neonatal GIT microbiome, HMOs are digested by *Bifidobacterium longum* and *Bifidobacterium infantis* (Underwood *et al*., 2015) with evidence of a strong bifidogenic effect, reduction in faecal pathogens and an improved intestinal immune response in neonates receiving oligosaccharide supplemented formula milk compared to non-supplemented milk (Estorninos *et al*., 2022). HMOs are mainly fermented by *Bifidobacterium longum*, *Bifidobacterium breve*, and *Bacteroides* spp. from infant inocula producing beneficial SCFAs such as acetate, propionate and butyrate as well as lactate and succinate, butyrate and propionate being produced by *Bacteroides* spp., not *Bifidobacterium* spp. (Xu *et al*., 2022). To be clear, *Bifidobacterium* spp., produce acetate and lactate where lactate and succinate are not SCFAs, but rather important intermediaries in their bacterial metabolism and formation (Markowiak-Kopeć & Śliżewska, 2020). A major protective effect of HMOs is to inhibit bacterial and viral infection by either binding to pathogens in the GIT lumen and/or inhibiting binding to cell surface glycan receptors, presumably acting as soluble cell surface decoys (Newburg *et al*., 2005; Bode, 2012; Li *et al*., 2014). As immune modulators, specific HMOs promote maturation of epithelial cells of the small intestine and enhance barrier function mainly through upregulation of tight junction proteins, claudin-8 and claudin-5 (Holscher *et al*., 2014, Šuligoj *et al*., 2020). Goblet cells (GCs) that line the entirety of the GIT are essential to maintaining barrier function, intestinal homeostasis, epithelial integrity and physical lubrication of luminal contents (Forder *et al*., 2007; McCauley & Guasch 2015). It is now recognised that GC intrinsic sensing of the GIT microbiota plays a critical role in regulating the exposure of the immune system to microbial challenges (McDole *et*

al., 2012; Knoop *et al*., 2015; Knoop *et al*., 2018). There is some evidence that HMOs enhance mucosal barrier function through direct modulation of GC function and upregulation of GC secretory product genes (Bhatia *et al*., 2015; Cheng *et al*., 2020).

1.10.2 Structure of galacto-oligosaccharides

Animal mammalian colostrum and milk contains, in addition to lactose, a variety of neutral and acidic oligosaccharides. These are typically composed of three to ten monosaccharide units, including glucose (Glc), galactose (Gal) and N-acetyl-glucosamine (GlcNAc) as well as fucose and sialic acids, HMOs being a good example, demonstrating how component molecules can be arranged across core polymerised structures leading to complex and diverse sialylated and fucosylated structures (Figure 4).

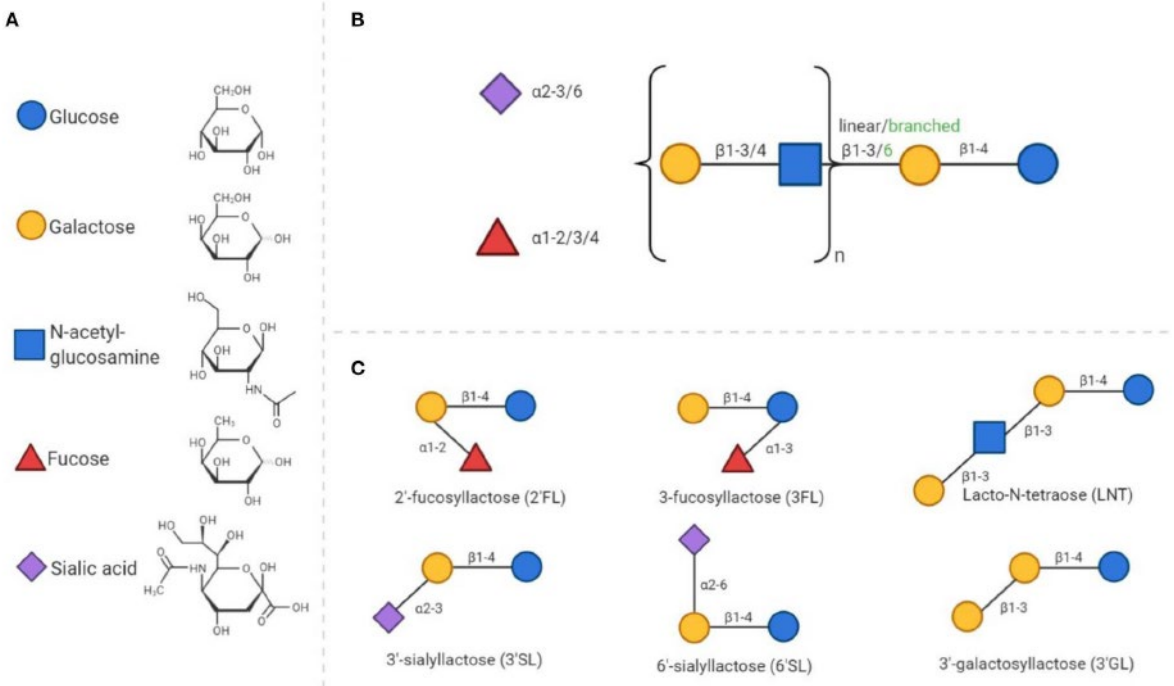

Figure 4. General composition of human milk oligosaccharides and synthetic analogues. A) All HMOS consist of five different monosaccharides. B) The

composition of HMOs follows a distinct structure. Elongation of the core structure and decoration with fucose and/or sialic acid residues leads to a large number of structures discovered. C) As examples, six simple oligosaccharide structures shown (Zuurveld *et al*., 2020 – direct copy from reference)

The core moiety present at the reducing end of milk oligosaccharides is either lactose (Gal(β1–4)Glc) or N-acetyl-lactosamine (Gal(β1–4)GlcNAc) (Urashima *et al*., 2001). The dominant saccharide in mammalian milk or colostrum is lactose, which is synthesised from Glc and uridine diphosphate galactose (UDP-Gal) by β4galactosyltransferase *in vivo* in the mammary gland (Urashima *et al*., 2001). Most animal milk oligosaccharides are sialylated (sialic acid at glycoprotein terminal ends), containing N-acetylneuraminic acid (Neu5Ac) and/or N-glycolylneuraminic acid (Neu5Gc) (Urashima *et al*., 2001; Urashima *et al* 2013). Sialic acids are nine carbon atom sugars also present at terminal ends of glycolipids and glycoproteins involved in cellular communication and survival (or non-survival) of pathogens (Cavalcante *et al*., 2021). Compared with other domestic animals, porcine milk contains the highest percentage of neutral oligosaccharides (20%), the most abundant variety of mono-sialylated and di-sialylated large oligosaccharides and are the closest to human milk oligosaccharide composition (Albrecht *et al*., 2014). Between twenty- nine and sixty PMOs have been identified by a variety of high-performance liquid chromatography (HPLC) and mass spectrometry techniques (Tao *et al*., 2010; Albrecht *et al*., 2014; Cheng *et al*., 2016; Difilippo *et al*., 2016a; Mudd *et al*., 2016; Salcedo *et al*., 2016; Wei *et al*., 2018). However, the abundance and composition of PMOs changes throughout lactation. There is a lactation-stage related decrease in the total number of PMOs by 36% and 24% in sow and gilt milk respectively over the course of lactation from colostrum to mature milk (Wei *et al*., 2018). Even in the first week of lactation, the majority of PMOs decrease in

abundance by 43% with the concentration of acidic PMOs decreasing and that of neutral-fucosylated and neutral PMOs increasing (Difilippo *et al*., 2016a; Mudd *et al*., 2016). Furthermore, significant decreases in sialylated PMOs correlates with significant increases in fucosylated PMOs at day fourteen *post-partum* (Salcedo *et al*., 2016) with an estimated decrease in sialylated PMOs from 80% content at farrowing to 60% in early lactation (days four to seven), to 40% in late lactation (day 24) (Tao *et al*., 2010) indicating a change in functionality during lactation. Commercially available GOS are typically composed of galactose units bound by various β(1-2), β(1-3), β(1-4) and β(1-6) linkages with a terminal glucose, where the degree of polymerisation, "n" is two to eight molecules (Figure 5). These are generally synthesised by the transgalactosylation of lactose by βgalactosidases (Intanon *et al*., 2014).

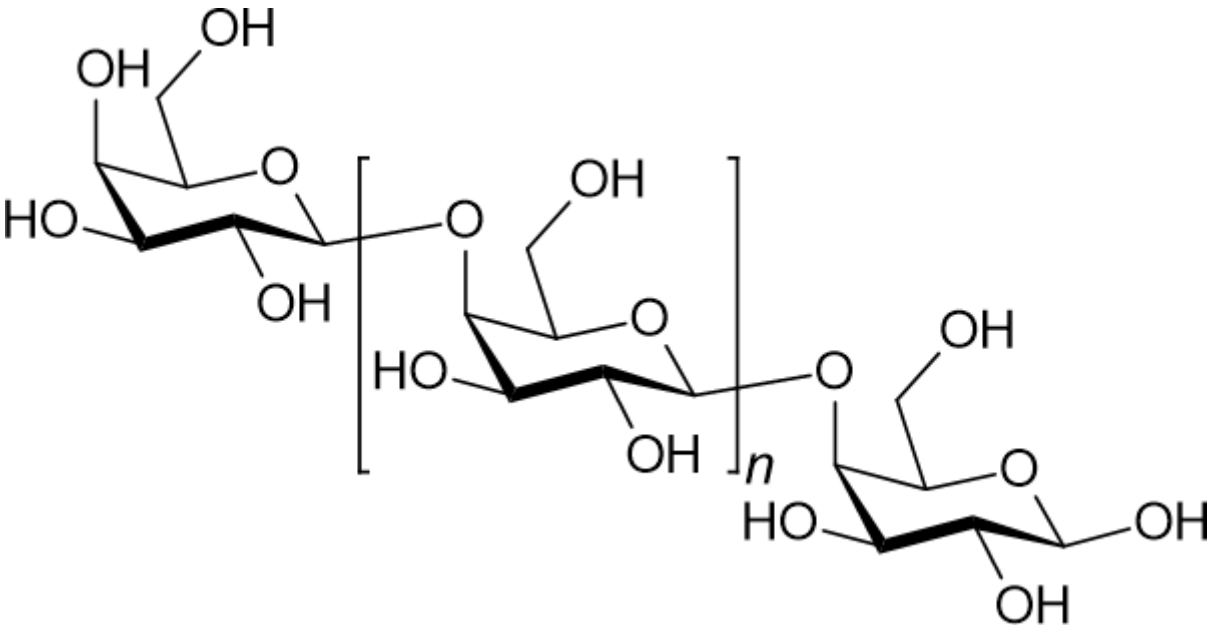

Figure 5. General structure of commercially available galacto-oligosaccharides where "n" is two to eight repeating molecules and synthesised by transgalactosylation of lactose by βgalactosidases (Intanon *et al*., 2014 – direct copy from reference)

1.11 Effects of galacto-oligosaccharides

1.11.1 Adhesion of pathogens

As introduced, attachment of pathogens, particularly ETEC to GIT epithelial cells by fimbrial adhesins is important in the development of infection and disease in pigs (Fairbrother *et al*., 2005; Fairbrother *et al*., 2012). Studies have shown that GOS strongly inhibits the attachment of enterohepatic *E. coli* and *Salmonella enterica*, serotype Typhimurium to human colon HT29 cells and adhesion of enteropathogenic *E. coli* (EPEC) to human GIT epithelial cells Hep-2 and Caco-2 lines by 65% and 75% respectively (Tzortzis *et al*., 2005; Shoaf *et al*., 2006). Moreover, the adherence inhibition of GOS outperforms that of other probiotics such as lactulose, inulin, raffinose and FOS (Shoaf *et al*., 2006) suggesting that expression of complex mammalian milk oligosaccharides has evolved over time, not just to prime the microbiome but also to prevent infection through pathogen adherence inhibition (Bode, 2012). Furthermore, galactosylated oligosaccharides derived from chitin have been shown to strongly reduce adhesion of enterotoxigenic ETEC K88 to porcine erythrocytes by 60% (Yan *et al*., 2017). Recent studies have shown that GOS significantly reduces adhesion of *E. coli* to HT-29 cells but also significantly reduced *E. coli* growth *in vitro* demonstrating the capacity for GOS to react directly with pathogenic cells (Asadpoor *et al*., 2021). Clinically relevant inhibition of RVA by GOS has been demonstrated. Up to 50% inhibition of RVA binding to MA104 cells at concentrations of 4 mg/ml for 3′- sialylated, 2 mg/ml for 6′-sialylated and 12 mg/ml HMOs has been shown, with constant exposure of cells to oligosaccharides being most effective in reducing RVA infectivity (Hester *et al*., 2013). When compared with RVA infections in the absence of GOS, oligosaccharides and GOS

significantly reduced the infectivity of RVA in MA104 cells, the effect being considered as one mediated by the virus and not the cells (Laucirica *et al*., 2017).

1.11.2 *In vitro* fermentation of galacto-oligosaccharides and porcine milk oligosaccharides

GOS is readily and completely fermented by pig faecal flora *in vitro* (Martinez *et al*., 2013; Difilippo *et al*., 2016b). However, there are differences in the extent and duration of fermentation of GOS depending upon structure of oligosaccharide molecules be they inherently expressed PMOs or additive GOS. Acidic PMOs and GOS with degrees of polymerisation between four and seven monomers were rapidly depleted within twelve hours of *in vitro* fermentation in contrast to more complicated molecules being fucosylated and phosphorylated PMOs, which were partially resistant to fermentation. GOS structures containing ß1-2 and β1-3 linkages were fermented in preference to GOS containing β1-4 and β1-6 linkages. This suggests that there are different physiological roles for different structures, with some readily lower molecular weight PMOs and GOS being preferentially fermented to SCFAs and others being less fermented but retaining sialylated, di-sialylated, fucosylated and large oligosaccharide structures implicated in preventing pathogen binding to piglet intestinal cells (Albrecht *et al*., 2014; Difilippo *et al*., 2016b). Using an *in vitro* model of the large intestine, inoculated with human or pig faeces, GOS fermentation and degradation was more pronounced with pig faecal inocula rather than human inocula. GOS significantly stimulated the growth of *Lactobacillus* and *Bifidobacterium* in both human and pig inocula, but with more complex communities from pig faecal material and higher SCFA production in contrast to human inocula indicating differences between these species (Martinez *et al*., 2012). GOS significantly increases SCFA production *in vitro*, mainly producing acetate,

propionate, butyrate, succinate and lactate (Martinez *et al*., 2013; Tanner *et al*., 2014; Difilippo *et al*., 2016b). These are trophic to GIT epithelial cells, stimulate GIT cell proliferation, reduce pH of luminal contents, are antineoplastic, particularly butyrate and favour beneficial bacteria such as LABS (Scheppach *et al*., 2001; Macfarlane *et al*., 2008). There is also evidence that acetate and propionate production from GOS is responsible for inhibition of *Salmonella enterica* colonisation in a pig *in vitro* fermentation model through reduction of pH and modulation of bacterial communities (Tanner *et al*., 2014).

1.11.3 Beneficial effects of galacto-oligosaccharides *in vivo*

GOS and PMOs are readily fermented in the GIT of pigs (Tzortzis *et al*., 2005; Difilippo *et al*., 2015; Difilippo *et al*., 2016a; Alizadeh *et al*., 2016). The majority of ingested GOS reaches the colon where it is fermented by resident bacteria with very little absorbed systemically. At three and twenty-six days after feeding GOS to animals, only trace amounts could be found in faecal samples, indicating almost complete fermentation of GOS *in vivo* (Difilippo *et al*., 2015). Even in nursing piglets receiving PMO rich colostrum, no intact PMO structures from faeces could be found at one to two days of nursing indicating intestinal fermentation of GOS at a very early age (Difilippo *et al*., 2016a). Similarly, no intact original molecular GOS structures could be found in piglet faeces at day three and day twenty-six following GOS feeding in milk replacer (Alizadeh *et al*., 2016). GOS decreases digesta pH in pigs with pigs fed 4% w/w GOS having a lower proximal colonic pH than pigs fed control diets (Tzortzis *et al*., 2005) and significantly lower pH values in caecum digesta of pigs fed GOS in milk replacer (Alizadeh *et al*., 2016). The lowering of pH is indicative of SCFA production *in vivo* with increases in SCFA in the proximal colon almost entirely due to acetic acid, which is a major fermentation product of

Bifidobacterium (Tzortzis *et al*., 2005). In contrast, GOS was only found to increase caecal butyrate digesta concentrations in pigs (Alizadeh *et al*., 2016), whereas the pH of ileal digesta decreased in GOS fed piglets with significantly increased concentrations of propionate, butyrate, and valerate compared with controls (Tian *et al*., 2019). The effects of GOS on pH and SCFA concentrations are not just limited to healthy pigs. In suckling piglets challenged with lipopolysaccharide (LPS) endotoxin derived from *E. coli*, the pH value of colonic digesta increased and the concentrations of acetate, butyrate and lactate significantly decreased. In contrast, animals fed GOS and challenged with lipopolysaccharide showed a significant decrease in the pH of colonic digesta and a significant increase in acetate, butyrate, lactate and total SCFA demonstrating the ability of GOS to relieve colonic inflammation (Gao *et al*., 2021). In 30 kg pigs receiving 2.5 g/kg GOS in their basal diet, *Lactobacillus* spp. were significantly increased in caecal and colonic digest despite challenge with 1 x 10^8 cfu *Salmonella typhimurium* (Bouwhuis *et al*., 2017) showing the ability of GOS to modulate the microbiota during infection. GOS also modulates the GIT microbiota in young pigs with addition of GOS to diets significantly increasing *Lactobacillus* and *Bifidobacterium* in caecal contents and faeces (Tzortzis *et al*., 2005; Alizadeh *et al*., 2016; Xing *et al*., 2020). After twenty-six days feeding with GOS, a significant reduction in *E. coli* and clostridial counts were observed (Alizadeh *et al*., 2016). Similar results were obtained by Xing *et al*., 2020, who noted a significant decrease in the number of *E. coli* in a linear and dose dependent manner from 500 mg/kg up to 2000 mg/kg GOS fed daily. The modulatory effects of GOS do not extend to the lower GIT or just faeces alone. The ileal microbiota composition of the upper GIT was significantly enriched with *Lactobacillus* and reduced in *Clostridium sensu stricto* in twenty-one-day old pigs

following an initial ten-day GOS feeding period demonstrating the ability of GOS to affect the microbiome over time (Tian *et al*., 2019). GOS also affects infection in animals by viruses. In an RVA infection model in rats, GOS significantly reduced the incidence, duration and severity of diarrhoea, with a second RVA challenge failing to provoke significant symptomatology in GOS treated groups (Massot-Cladera *et al*., 2022). Similar studies have shown that GOS/FOS mixtures prevent infection and gut dysbiosis caused by RV (Azagra-Boronat *et al*., 2019). Prebiotic oligosaccharides are included in human infant formulas and mixtures of short-chain GOS and long-chain FOS at a 9:1 ratio in infant formulas has significantly reduced the incidence of numerous infections in infants (Arslanoglu *et al*., 2007). GOS/FOS mixtures also have modulatory effects in pigs infected with RV. These oligosaccharides increased luminal pH, lowered dry matter content of the colon, enhanced numbers of butyrate producing bacteria and reduced the duration of RV induced diarrhoea in piglets (Li *et al*., 2014).

1.11.4 Galacto-oligosaccharides and gut architecture

A healthy well-differentiated intestinal mucosa has long, regular villi and high villus-to-crypt ratios (Jeurissen *et al*., 2002), with the villus epithelium lining consisting mainly of absorptive enterocytes and specialised secretory GCs (Epple *et al*., 1977). Moreover, the healthy maturation of the jejunum is beneficial for maintaining high growth rates in neonates (Buddington & Sanglid, 2011) and is considered the main organ for nutrient absorption in pigs (Tian *et al*., 2018). Although some studies have shown little effect of GOS on gut architecture (Tian *et al*., 2018) some have shown histomorphological differences, reported as increased villus height after only three days of GOS feeding (Alizadeh *et al*., 2016). At twenty-six days, the jejunal villi were thicker and larger with a significant increase in villus height, villus breadth top

and villus breadth base in GOS fed pigs compared with controls (Alizadeh *et al*., 2016). In LPS challenged pigs GOS has a protective effect upon the GIT mucosa and alleviates inflammation. Histomorphological differences were observed in the jejunum of LPS plus GOS pigs who had significantly higher villus height and VCR compared with LPS control pigs who were not fed GOS. Higher villus heights were also seen in the duodenum of LPS GOS fed pigs. (Gao *et al*., 2021; Tian *et al*., 2021). The physiological effect of GOS on GIT architecture and immunomodulatory GC expression has also been reported in chickens. Villus length and crypt depth were significantly greater in GOS fed juvenile birds compared with control birds, but these differences did not result in a difference in the VCR between treatments. However, greater villus tissue densities of GCs were observed in GOS fed birds throughout trials (Richards *et al*., 2020). In early-stage infection, *Campylobacter jejuni* challenged broiler chickens, GOS significantly increased villus height and crypt depth at eight and fifteen days old. The effect was also seen in late-stage *Campylobacter jejuni* challenged birds with increased villus height and crypt depth at twenty-two days and twenty-eight days old respectively, again demonstrating the capacity of GOS to protect the GIT epithelium when under pathogen challenge (Lafontaine *et al*., 2020). It is known that food-chain contaminants such as aflatoxins disrupt GIT intestinal barrier function by reducing tight junction expression and promote cellular apoptosis in pigs and mice (Zhang *et al*., 2022). GOS can mitigate the intestinal tight junction disruption associated with the mycotoxin treatment of Caco-2 cells by stimulating tight junction assembly (Akbari *et al*., 2015). This demonstrates the immunomodulatory and cellular physiological effects of GOS at molecular levels despite mycotoxin challenge. This may suggest that GOS is not just protective against pathogens but may have a role to play in aflatoxin and deoxynivalenol mycotoxin contamination in populations, particularly pregnant

mothers exposed to these contaminants in African populations (Piekkola *et al*., 2012).

1.11.5 Immunomodulatory effects of galacto-oligosaccharides

The immunomodulatory effects of GOS on the immune responses and expression of cytokines and chemokines in intestinal tissue has been well studied. GOS directly upregulates tight junction protein genes (claudin 1, zona occludens 1 and 2, occludin) which regulate epithelial permeability and tight junction scaffolding proteins linking claudins and occludins to the cell cytoskeleton (Alizadeh *et al*., 2016; Akbari *et al*., 2015). GOS also directly upregulates secretory product genes linked with high molecular weight glycoprotein mucins and intestinal factors which stabilise the integrity of the mucus layer (mucin 2, mucin 4 and trefoil factor 3) as well as antimicrobial proteins and peptides such as resistin like molecule beta and poricine beta defensin 2 (Alizadeh *et al*., 2016; Bhatia *et al*., 2016). The secretory goblet cell lineage gene Atonal bHLH transcription factor 1 (ATOH1) is upregulated by prebiotics whilst the gene acting as a suppressive ATOH1 transcription factor HES1 is downregulated by probiotics (Zhang *et al*., 2017). A number of pro-inflammatory cytokines such as TNFα, IL1ß, IL6, IL8, IL12A, Il17A, IL17F, IL22 and IL33 are down regulated by GOS as well as immune-regulatory and pro-inflammatory cytokines IFNγ and NF- κB (Bouwhuis *et al*., 2017; McDonnell *et al*., 2016; Tian *et al*., 2018, Richards *et al*., 2020, Dai *et al*., 2018; Verheijden *et al*., 2015). IL10 which is a cytokine synthesis inhibitory factor, with major anti-inflammatory effects is upregulated by GOS as is TGFß, an anti-inflammatory cytokine which regulates cell proliferation and growth (McDonnel *et al*., 2016; Tian *et al*., 2018). Generally, prebiotics including GOS have both direct and indirect immunomodulatory effects. Prebiotics can directly act on GIT epithelial cells through toll like receptors, which

leads to cytokine production through NF- κB activation that eventually leads to IL-2, IL-4 and IL-10 production. Indirect effects are mediated by GIT bacteria which ferment GOS to SCFA. These can bind to G-protein- coupled receptors on GIT epithelial cells and induce production of IL10 and tissue growth factor-β (Pujari & Banerjee, 2021). In suckling piglets it has been demonstrated that GOS increases abundance of SCFA producing bacteria such as *Prevotella*, *Barnesiella* and *Parabacteroides* and increases SCFA concentrations in the colon. Furthermore, the higher colonic SCFA concentration of GOS piglets altered gene expression of inflammatory factors through regulation of NF- κB and protein-kinase signalling pathways demonstrating microbial mediation of immune function (Wang *et al*., 2019).

1.12 Summary to the Introduction

Global pork production continues to increase but not without concerns relating to environmental impacts and use of AGPs in animal agriculture. Despite bans of AGPs in territories such as the EU, there has been an increase in the therapeutic use of antibiotics in animals and emergence of AMR in non-EU countries intensifying animal protein production who still continue to use AGPs as growth promoters. Therefore, there is a need for viable alternatives to AGPs in animal production. Nevertheless, pigs are large animals and there is a statutory requirement to study any feed additive in the host of choice in the EU. There is also a high degree of technical variation between studies of the pig microbiota with many pooling data from repeated trials. This may be necessary to achieve statistical significance, but the variance between trials needs to be truly compared. Despite technical issues in trial design and comparison, increased pig production is not without problems. Non-infectious causes of pre-weaning piglet mortality represent significant economic losses with mortality estimated as high as 12% in some commercial pig herds. Risk factors associated with pre-weaning mortality in piglets are mainly sow colostrum production,

parity and litter size and piglet birth weight, vitality and sex. However, it is considered that selection for increased litter size resulting in more low-birth-weight piglets is not beneficial unless measures are taken to improve their survival. In this respect, removal of poorly performing piglets who do not receive adequate nutrition from sows to environmentally controlled pens with CMR feeding is seen as useful, particularly with pro- and prebiotic intervention. Infectious enteric diseases are also a significant cause of pre- and post-weaning mortality in pigs with losses approaching 20% in diarrhoeagenic litters. Common bacterial pathogens are *Escherichia coli*, *Clostridium perfringens*, *Lawsonia intracellularis*, *Salmonella enterica* and *Brachyspira* (Serpulina) spp., with ETEC being the main pathotype in pre- and post-weaning diarrhoea. Attachment of ETEC to porcine enterocytes is mediated by fimbrial adhesins with bacteria producing toxins responsible for increased GIT permeability leading to dehydration, reduced performance and possible mortality. Notwithstanding pathogenesis due to enteric bacterial infections, other important infectious causative agents of neonatal and pre-weaning diarrhoea and mortality in pigs are the rotaviruses. RVA is the most prevalent viral group in suckling pigs, infections often occurring in tandem with other enteric bacteria as described. RVA faecal-oral infection results in the destruction of small intestinal enterocytes thus promoting malabsorptive diarrhoea, dehydration and death. Vaccination of animals is an option, but the wide variety of RVA genotypes and the ability of colostral antibodies to neutralise vaccines complicates their effective production. Moreover, RVA is still a major causative agent of lethal gastroenteritis in young children globally. The effectiveness of human vaccines may be undermined, post vaccination, by genetic drift of RVA strains over time, as in animals. There is also evidence that pigs may serve as a reservoir of RVA zoonotic transmission to humans. Thus, collaborative adjunct strategies, such as use of prebiotics in endemic RVA infection remains to be investigated. Overall, there is an absolute need for alternative strategies to improve pre- and post-weaning pig health in relation to non-infectious

physiological failure to thrive and infectious causes of failure to thrive. In these respects, feeding with pro- and prebiotics may be useful. Probiotics are living microorganisms, mainly bacteria, but include some yeasts and fungi, that when ingested are considered to confer health benefits upon the host. In animals probiotics can be classed as single or multi strain, bacterial or non-bacterial, spore forming or non-spore forming and allochthonous or autochthonous. The benefits of probiotic consumption relate to increased weight gain, improved feed efficiency, modulation of the GIT microbiome, the immune system and suppression of potential pathogens. There are various mechanisms for these effects, which include inhibition of pathogen adhesion and toxin expression, enhanced barrier function and T and B-cell activation. However, the efficacy of probiotics and their ability to successfully populate the GIT in animals is questioned. Alternatively, the prebiotic concept states that non-viable dietary components fortify autochthonous probiotic residents of the GIT flora without the need for survival of a live ingested probiotic microorganism. The original definition of a prebiotic was "A prebiotic is a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon improving host health." (Gibson & Roberfroid, 1995) In order for a food to be classed as a prebiotic, it must not be digested in the upper GIT, be a selective substrate for beneficial bacteria, alter the colonic flora and induce effects beneficial to host health. Only FOS and questionably GOS are considered prebiotic at the first original definition. However, the definition of prebiotics has been revised and updated several times and is now recognised as "a substrate that is selectively utilized by host microorganisms conferring a health benefit." (Gibson *et al*., 2017). The family of prebiotics now includes inulin, FOS, lactulose and GOS. The main prebiotic effects are considered as improvement of gut microbiota composition, intestinal and barrier function, immune regulation and pathogen suppression. Prebiotic oligosaccharides that are a major constituent of human and animal milk have no direct nutritive value to the neonate through limited

upper intestinal hydrolysis and absorption. Instead, their effects relate to stimulating the development of the microbiota in neonates and conferring a variety of health benefits including innate and adaptive immune development. Human milk oligosaccharides are digested by *Bifidobacterium longum* and *Bifidobacterium infantis* with evidence of a strong bifidogenic effect, reduction in faecal pathogens and an improved intestinal immune response in neonates. Other species such as *Bacteroides* produce beneficial SCFAs from human milk oligosaccharides. Protective effects include inhibition of viral and bacterial enteric pathogens by inhibiting binding to cell surface glycan receptors, thereby acting as soluble cell surface decoys and enhanced barrier function through direct modulation of intestinal goblet cells. Animal milk oligosaccharides are complex, diverse molecules and are typically composed of three to ten monosaccharide units, including glucose, galactose and N-acetyl- glucosamine as well as fucose and sialic acids. Commercially available GOS is typically composed of galactose units bound by various β(1-2), β(1-3), β(1-4) and β(1-6) linkages with a terminal glucose, where the degree of polymerisation is two to eight molecules and are generally synthesised by the transgalactosylation of lactose by βgalactosidases. *In vitro*, studies have demonstrated that GOS strongly inhibits the adhesion of *E. coli*, *salmonella* and RVA to human cell lines. GOS is also completely fermented by pig faecal flora *in vitro*, producing SCFA and increasing populations of *Lactobacillus* and *Bifidobacterium*. Both GOS and porcine milk oligosaccharides are readily fermented in the GIT of pigs, lowering pH, producing SCFA, increasing populations of *Lactobacillus* and *Bifidobacterium* and decreasing *E. coli* demonstrating beneficial health effects. In addition, GOS and FOS mixtures have been shown to attenuate RV induced diarrhoea in piglets. Other effects of GOS are increased villus height, goblet cell expression and protection of the GIT epithelium when under pathogen or toxin challenge. Immunomodulatory effects of GOS are upregulation of tight junction protein, glycoprotein mucin and goblet cell expression genes with downregulation of many pro-inflammatory cytokines.

1.13 Hypotheses

The overall hypothesis for this work is that data from small-scale pig trials can identify common community indicators allowing data pooling and GOS when fed to pigs, modulates the microbiome, improves gut architecture and suppresses GIT pathogens.

1.13.1 Hypothesis Chapter 3

The hypothesis for Chapter 3 was that microbiota data from three separate suckling pig studies could be used to identify common colonic community indicators, the data pooled and correlated with animal performance. The objectives of this study were to investigate if common colonic community indicators could be identified from the microbiota of suckling pigs in repeated small-scale trials and if pooled data in terms of microbial diversity and abundance of the colonic microbiota related to animal performance in three controlled reproducible trials.

1.13.2 Hypothesis Chapter 4

The hypothesis for Chapter 4 was that GOS when added to CMR would benefit poorly performing piglets by increasing the abundance of beneficial probiotic bacteria, improve gut architecture and immunomodulatopry GC expression. The objectives of this study were to investigate the effects of GOS on the microbiome, gut architecture and GC expression in poorly performing (non-thriving) piglets with below average birthweight who may benefit from milk replacement feeding alone or supplemented with GOS in four separate and repeated trials.

1.13.3 Hypothesis Chapter 5

The hypothesis for Chapter 5 was that late gestational feeding of GOS to sows could confer immunity to RVA in neonates and reduce infection. The aims of this study were to determine if GOS supplementation in late gestational sows conferred immunity, reduced

infectivity and modulated the microbiome in neonatal piglets in a commercial pig farm setting where RV challenge was naturally endemic and as confirmed by previous veterinary reports.

2 Chapter 2. Methodological approaches

This chapter provides supplementary information for the methods and materials sections described within three separate studies that are derived from publications 1 to 3 presented in Chapters 3, 4 and 5 respectively. Unless stated, all reagents and consumables were part of the commercial kits provided. Common consumables, reagents and apparatus for all studies/trials are referenced in the first case. A full list of laboratory reagents, consumables, apparatus, animal trial consumables and reagents are provided as follows.

Table 2. **Reagents**

Reagent	**Supplier**	**Catalogue/Part Number**
Agarose, molecular biology grade	Merck, Gillingham, UK	A9539-500G
Diethyl pyrocarbonate 0.1% v/v	Merck, Gillingham, UK	D5758-25ML
DNA polymerase & dNTPs, Accuprime Pfx Supermix	Thermo, Fisher Scientific, Loughborough, UK	12344040
Ethanol 96% v/v	Fisher Scientific, Loughborough, UK	15518181
Ethidium bromide	Merck, Gillingham, UK	E1510
Fluorometer reagents, Qubit dsDNA High Sensitivity (HS)	Thermo Fisher Scientific, Loughborough, UK	Q32854
HT1 buffer	Illumina, San Diego, USA	20015892
Hydrochloric acid 0.5 M	Thermo Fisher Scientific, Loughborough UK	J/4330/15
Isopropanol 99.5% v/v	Thermo Fisher Scientific, Loughborough UK	184130010
Ladder, DNA 100 bp	Promega Corporation, Madison, Wisconsin, USA	G2101
Loading dye	Promega Corporation, Madison, Wisconsin, USA	G210A
Microbial Community 10 ng	ZymoBIOMICS, Cambridge Bioscience, Cambridge, UK	D6300

Reagent	Supplier	Catalogue/Part Number
PCR grade water	Thermo Fisher Scientific, Loughborough, UK	AM9935
Peroxidase-labelled goat anti-porcine IgA	Thermo Fisher Scientific, Bonn, Germany	PA1-84624
PhiX	Illumina, San Diego, USA	FC-110-3001
Phosphate buffered saline (PBS) pH 7.4	Thermo Fisher Scientific, Loughborough UK	J62036.K2
Primers A701 – A712	Merck, Gillingham, UK	8816450674-170/0 to 8816450674-280/0
Primers B501 – B508	Merck, Gillingham, UK	8816450674-90/0 to 8816450674-160/0
Primers B701 – B712	Merck, Gillingham, UK	8816450674-290/0 to 8816450674-400/0
Primers A501 – A508	Merck, Gillingham, UK	8816450674-10/0 to 8816450674-80/0
Primer Read 1 V4	Merck, Gillingham, UK	8816450674-410/0
Primer Read 2 V4	Merck, Gillingham, UK	8816450674-420/0
Primer Index V4	Merck, Gillingham, UK	8816450674-430/0
Reverse osmosis water	Milli-Q, Merck, Gillingham, UK	ZOOQSVC01
RNase free water	Fisher Scientific, Loughborough, UK	BP561-1
RNAse Zap	Thermo Fisher Scientific, Loughborough, UK	AM9780
Sodium chloride NaCl	Merck, Gillingham, UK	S9625-1KG
Sodium hydroxide NaOH 1.0 N	Merck, Gillingham, UK	S2770-100ML
TapeStation reagents, D1000	Agilent Technologies, Inc., Santa Clara, California, USA	5067-5583
TE buffer (10 mM Tris/Cl; 1 mM EDTA, pH 8)	Thermo Fisher Scientific, Loughborough, UK	12090015
Tris acetate solution	Thermo Fisher Scientific, Loughborough, UK	B49

Table 3. Consumables

Consumable	Supplier	Catalogue/Part Number
Absorbent paper towel, Rolled Blue Paper Towel, 198 x 200mm, 7200 Sheets	Kimberley-Clark, Reigate, UK	6668
Aluminium foil, Kirkland Signature	Costco Wholesale UK Ltd., Watford, Hertfordshire, UK	RK611
Bijou bottle 7 mL	Fisher Scientific, Loughborough, UK	14803562
Cling film, Clingorap	Terinex, Bedford, Bedfordshire, UK	NA
Conical flask 250 mL	Schott, Duran, Mainz, Germany	212163605
Disposable loop 10 µL sterile	Sarstedt, Leicester, UK	86.1562.050
DNA Purification kit, NucleoSpin Tissue Genomic	Macherey-Nagel, Düren, Germany	740952.50.
DNA Purification kit, FastDNA SPIN Kit for Feces	MP Biomedicals, Solon, USA	657020017
DNA purification kit robotic, QIAmp 96 PowerFecal QIA Cube HT Kit	Qiagen, Hilden, Germany	51531
ELISA kit total IgA, IgA Pig ELISA Kit	Abcam plc, Cambridge, UK	ab190536
ELISA kit total IgG, IgG Pig ELISA Kit	Abcam plc, Cambridge, UK	ab291065
ELISA kit RVA, Ingezim rotavirus porcine	Immunologia Y Genetica Aplicada S.A. Madrid, Spain	11.RTP.K.1
Ethanol wipes, Azowipes	Synergy Health, Chorley, Lancashire, UK	81103
Graduated filter tip sterile 1000 µL XL	Starlab (UK) Ltd., Milton Keynes, Buckinghamshire, UK	S1122-1830
Graduated filter tip sterile 200 µL	Starlab (UK) Ltd., Milton Keynes, Buckinghamshire, UK	S1120-8810
Graduated filter tip sterile 20 µL	Starlab (UK) Ltd., Milton Keynes, Buckinghamshire, UK	S1122-1810

Consumable	Supplier	Catalogue/Part Number
Graduated filter tip sterile 10 µL	Starlab (UK) Ltd., Milton Keynes, Buckinghamshire, UK	S1121-3810
Graduated filter tip 200 µL extra-long sterile, Biosphere	Sarstedt, Leicester, UK	70.1189.215
Lint free wipes, Kimtech	Kimberley-Clark, Reigate, UK	05511
Loading tips	Agilent Technologies, Inc., Santa Clara, California, USA	5067-5153
Lysing tubes, Matrix E	MP Biomedicals, Solon, USA	116914100
Microtiter plate 96 well	Thermo Fisher Scientific, Newport, UK	612U96
Micro tube nuclease free 2 mL, SafeSeal	Sarstedt, Leicester, UK	72.695.400
Nano flow cell	MiSeq, Illumina, San Diego, USA	15035217
Normalization plate (96) kit, SequalPrep	Invitrogen, Thermo Fisher Scientific, Loughborough, UK	A1051001
Optical tubes, 8 x strip	Agilent Technologies, Inc., Santa Clara, California, USA	401428
Pathogen lysis tube, Type L	Qiagen, Hilden, Germany	19092
PCR microplate, 384 well	Corning Incorporated, Salt Lake City, USA	PCR-384-LC480-W
PCR plate, 96 well half skirt	Sarstedt, Leicester, UK	72.1981.202
PCR seal adhesive	Sarstedt, Leicester, UK	95.1993
PCR seal non-adhesive, Microseal A	Bio-Rad Laboratories Ltd., Watford, UK	MSA5001
PCR strip tubes and caps 0.2 mL	Roche Diagnostics GmbH, Mannheim, Germany	11 667 009 001
PCR tubes 0.2 mL	Sarstedt, Leicester, UK	72.737.002
Petri dish	Sarstedt, Leicester, UK	82.1472
Pipette 25 mL, Serological	Sarstedt, Leicester, UK	86.1685.001
Plasticware robotic workstation, QIAcube HT	Qiagen, Hilden, Germany	950067
Reagent cartridge and PR2bottle. Nano Kit v2, MiSeq	Illumina, San Diego, USA	MS-103-1001

Consumable	Supplier	Catalogue/Part Number
Reagent reservoir 10 mL	Thermo Fisher Scientific, Loughborough, UK	95128095
Reagent trough with lid 70 mL, QIAcube HT	Qiagen, Hilden, Germany	990554
Reagent trough with lid 170 mL, QIAcube HT	Qiagen, Hilden, Germany	990556
RNase Free DNase Set	Qiagen, Hilden, Germany	79254
RNA purification kit robotic. QIAamp 96 Virus QIAcube HT Kit	Qiagen, Hilden, Germany	57731
Rotavirus A detection kit. Techne qPCR rotavirus A kit	Cole-Parmer, Stone, Staffordshire UK	TKIT11031M
Screen tape. D1000	Agilent Technologies, Inc., Santa Clara, California, USA	5067-5582
Universal bottles, glass 30 mL	Fisher Scientific, Loughborough, UK	14803572
Wash bottle, Nalgene Color-Coded LDPE	Thermo Fisher Scientific, Loughborough, UK	2422-4500

Table 4. Apparatus

Apparatus	Supplier	Catalogue/Part Number
Balance	Kern & Sohn, Balingen, Germany	EMS 300-3
Biological safety cabinet "Advantage"	Thermo Fisher Scientific, Loughborough, UK	NA
Centrifuge Mikro 185	Hettich GmbH & Co. KG, Tuttlingen, Germany	1203
Centrifuge PCR strip tube mini, SciSpin Mini	SciQuip Ltd., Newtown, Shropshire, UK	9011002012
Commercial microwave oven	Sharp Corporation, Osaka, Japan	1000W/R21-ATP
Digital pathology system, NanoZoomer	Hamamatsu, Welwyn Garden City, UK	C13220-04
Digital pathology image program, NDP2.view2	Hamamatsu, Welwyn Garden City, UK	U12388-01
Fluorometer, Qubit 3.0	Thermo Fisher Scientific, Loughborough, UK	Q33216
Fluorospectrometer, NanoDrop 1000	Thermo Fisher Scientific, Loughborough, UK	ND-1000
Gel casting tray 96 well	ABgene, Epsom, Surrey, UK	AB-0708

Apparatus	Supplier	Catalogue/Part Number
Gel documentation, Gel Doc XR+ gel documentation system	Bio-Rad, Watford, Hertfordshire, UK	1708195
Homogeniser/bead beater, FastPrep, 24 5G	MP Biomedicals, Solon, USA	116004500
LightCycler	Roche 480, Hoffman La Roche, Basel, Switzerland	05015243001
Microplate reader	Labtech International Ltd., Ringmer, East Sussex, UK	LT-4000
PCR hood	Labcaire Systems Ltd., Clevedon, Somerset, UK	SC8R(UV)
Pipette 1000 µL, Pipetman G P1000	Gilson UK, Dunstable, Bedfordshire UK	F144059M
Pipette 200 µL, Pipetman G P200	Gilson UK, Dunstable, Bedfordshire UK	F144058M
Pipette 100 µL, Pipetman G P100	Gilson UK, Dunstable, Bedfordshire UK	F144057M
Pipette 20 µL, Pipetman G P20	Gilson UK, Dunstable, Bedfordshire UK	F144056M
Pipette 10 µL, Pipetman G P10	Gilson UK, Dunstable, Bedfordshire UK	F144055M
Pipette 2 µL, Pipetman G P2	Gilson UK, Dunstable, Bedfordshire UK	F144054M
Pipette 0.5 – 10 µL 12 channel, mLINE	Sartorius Biohit Liquid Handling, Helsinki, Finland	725220
Pipette 10 – 50 µL 8 channel, Finnpipette F2	Fisher Scientific, Loughborough, UK	11807381
Pipette controller	Heathrow Scientific, Nottingham, Nottinghamshire, UK	RF 3000
Plate spinner, Axygen Axyspin	Corning, New York, USA	230UK
Robotic workstation, QIAcube HT	Qiagen, Hilden, Germany	9001896
Sequencer, Illumina MiSeq	Illumina, San Diego, USA	SY-410-1003
Rotator	Grant Instruments Ltd., Cambridge, UK	PTR-25
Seal applicator	3M, Bracknell, Berkshire, UK	PA-1
Seal roller	Elkay Laboratory Products UK Ltd., Basingstoke, UK	RRLE303
TapeStation, Agilent 2200	Agilent Technologies, Inc., Santa Clara, California, USA	G2964A

Apparatus	Supplier	Catalogue/Part Number
Thermal cycler, Bio-Rad T-100	Bio-Rad Laboratories Ltd., Watford, UK	18, UK61096
Vortexer, IKA	IKA England Ltd., Oxford, Oxfordshire, UK	0003208002
Vortexer, TopMix	Fisher Scientific, Loughborough, UK	FB150120

Table 5. Animal studies consumables and reagents

Consumable/reagent	Supplier	Catalogue/Part Number
Cable ties 140 mm	B&Q, Eastleigh, UK	03618660
Coccidiostat, Baycox toltrazuril	Bayer, Newbury, UK	NA
Complete milk replacer, Faramate	Volac International Ltd., Royston UK	NA
Disinfectant, animal pens, MS MegaDes Novo	MS Schippers, Hapert, Netherlands	2509899
Disinfectant, foaming, animal pens, Top Foam LC	MS Schippers, Hapert, Netherlands	2509903
Disinfectant animal trials, Virkon,	VWR International Ltd., Lutterworth, UK	DIUKANT0190
Dry ice	Harper Adams University, Newport, UK	NA
E. coli vaccine, Porcilis Porcoli Diluvac Forte	Intervet International BV, Boxmeer, The Netherlands	NA
E. coli/rotavirus vaccine, Rokovac Neo	Bioveta, Ivanovice na Hané, Czech Republic	NA
Galacto-oligosaccharide, Nutrabiotic	Saputo Dairy UK, Weybridge, Surrey, UK	NA
Iron injection, Gleptosil	Alstoe Ltd, York, UK	NA
Iron injection, Ferroferon	Iron4u, Holte, Denmark	NA
Lactation diet wheat based diet	BOCM Pauls ltd, Wherstead, UK	NA
Lactation diet wheat based diet, Gold Lactator	Noble Foods, Stokesley, UK	11648
Pentobarbitone sodium, Dolethal	Vétoquinol, Buckingham, UK	NA
Screw top containers 70 mL plastic	Sarstedt, Leicester, UK	75.9922.683
Sire line semen 900	JSR Genetics, Driffield, UK	NA
Sows, Landrace x Large white	JSR Genetics, Driffield, UK	JSR 9T
Specimen jars 40 mL prefilled	Leica Microsystems UK Ltd., Milton Keynes, UK	3800770C

Consumable/reagent	Supplier	Catalogue/Part Number
Straw and hemp bedding, Aubiose	Datesand Ltd., Stockport, UK	CS1A07
Surgical scalpel blade, No. 22A stainless steel sterile	Swann-Morton, Sheffield, UK	0508
Titan semen	JSR Genetics, Driffield, UK	NA
Universal tubes plastic 30 mL	Thermo Scientific, Loughborough, UK	10096A

2.1 Animal studies

Studies involving work with animals need approval from an institution's Animal Welfare and Ethical Review Body (AWERB). The AWERB considers the potential benefit gained from conducting a study weighed against the cost to the animals involved. The AWERB provides constructive feedback to the applicant along with recommendations they feel appropriate regarding the programme of work. Any comments/recommendations must be addressed by the applicant to the satisfaction of the AWERB before the application is approved. Application for approval to use animals in each of the non-licensed studies carried out at the University of Nottingham and Harper Adams University were made to their respective AWERB. In completing the AWERB cover form, details of the animal species and number, study purpose, scientific background, study objectives, housing and care, potential benefits and what happens to animals at the end of the study were provided. Details of application of the "3Rs" these being "Replacement", "Reduction" and "Refinement" were provided (Section 2.1.2). The applicant confirmed that the procedures to be conducted will not result in any animal experiencing pain, suffering, distress or lasting harm and were therefore non-regulated under the Animals (Scientific Procedures) Act 1986. The AWERB considered the information given and gave their comments and recommendations. For example, in Chapter 5 one of the comments was "Confirmation is required that the animals will be held

according to DEFRA guidelines for stocking density." The applicant then provided responses to the AWERB with the intention of satisfying their comments and recommendations. These were considered by the AWERB committee and approved for each animal trial conducted. For Chapter 3, (paper 1) all three animal trials were conducted at the University of Nottingham, Sutton Bonington, UK in a dedicated, non-commercial, pig research unit. Animals were euthanised by a Schedule 1 method in accordance with the UK animals (Scientific Procedures) Act 1986. For Chapter 4, (paper 2) all four animal trials were conducted at Harper Adams University, Newport, UK in a dedicated commercial facility according to the "Guidelines on Good Clinical Practice for Clinical Trials for Registration of Veterinary Medicinal Products" (EMEA, 2000). Animals were housed in accordance with Red Tractor Assurance for Farms - Pigs Scheme Standards (Version 5.0) (Red Tractor, 2021) and any animals euthanised on farm used the method of captive bolt and pithing before confirmation of euthanasia by exsanguination in compliance with the Welfare of Animals at the Time of Killing guidance, (WATOK, 2015) and Schedule 1 of the Animals (Scientific Procedures) Act 1986. Trials met appropriate current quality standards "Administrative guidance for the preparation of applications on additives for use in animal nutrition" (EFSA, 2021). For Chapter 5, (paper 3) a randomised controlled trial was performed on a commercial farrow-to-finish pig farm at Worsall Manor Farms, Darlington, UK. The trial was approved by the farm veterinary consultant. No animals were euthanised or invasive samples taken during trial procedures for Chapter 5.

2.1.1 Animals

Preparation of manuscripts was, as far as possible, carried out in accordance with the "Animal Research: Reporting of *In Vivo* Experiments" guidelines ARRIVE (Percie du Sert *et al*., 2020). All metadata are available for 16S rRNA gene sequencing of GIT bacterial communities in the National Center for Biotechnology Information NCBI sequence read archive database available at https://www.ncbi.nlm.nih.gov/sra. For Chapter 3 (paper 1) sequence data were deposited in the NCBI database within the Bioproject PRJNA494528 under the SRA study SRP164374. For Chapter 4 (paper 2), sequence data were deposited within the Bioproject PRJNA866473. For Chapter 5 (paper 3), sequence data were deposited within the Bioproject PRJNA884280. The latter two BioProject accession numbers are now provided instead of SRP/SRA references and should be used for publications enabling better searching in NCBI.

2.1.2 The National Centre for the Replacement Refinement and Reduction of Animals in Research principles

The National Centre for the Replacement Refinement and Reduction of Animals in Research NC3Rs, principles were applied to studies as far as possible for Chapters 3, 4 and 5 https://www.nc3rs.org.uk/. No adverse effects were envisaged other than those associated with farming pigs.

2.1.2.1 Replacement: The use of pigs in experiments was unavoidable to meet obligations under Commission Regulation (EC) No 429/2008 (EC, 2008) that requires efficacy studies to allow evaluation of feed additives common to farming practices in the EU. Additionally, other animal species or *in silico* models would not adequately represent the growth performance of pigs under the experimental conditions, as biological data on the interactions of the host/microbiome is not

sufficiently refined. Furthermore, other species require different dietary levels of nutrients that would not be appropriate for pig production. These regulations apply to Chapters, 3, 4 and 5.

2.1.2.2 Reduction: Chapter 3 combined the results of three separate studies where only four pigs were used in each case, giving a total number of twelve animals studied. No treatment effects were investigated in these studies. A minimal number of animals were used allowing data pooling from each study to achieve statistical significance. In Chapter 4, Harper Adams University maintained a small but high-status commercial herd, producing good quality animals with low variation in production parameters. This was achieved, in part, due to the selection of a commercial hybrid genotype. Nevertheless, during the production process there were always some poorly performing piglets that were removed from the sow and cared for in a rescue pen (Figure 6). This is normal commercial practice for Harper Adams University and gave piglets a better chance of surviving to achieve acceptable performance, as more individual care and attention was provided. However, given the commercial nature of production, the number of poorly performing piglets available for trials and their ability to thrive could never be predicted. Only a certain number of trials could be replicated, given the availability of dedicated rescue pens, tissues and intestinal contents from euthanised piglets deemed unsuitable for introduction into the commercial herd. The hypothesis was that an indeterminate number of poorly performing piglets may benefit from GOS dietary supplementation as opposed to any number of piglets failing to thrive and placed in rescue deck pens for intensive care receiving CMR as opposed to CMR supplemented with GOS. In Chapter 5, trials were conducted on a large commercial pig farm and trial size was determined using a power calculation http://clincalc.com/stats/samplesize.aspx where $\alpha = 0.05$, $\beta = 0.2$ and power = 0.8, the extent of commercial operations

allowing a statistically number of sows and piglets to be studied.

Figure 6. Rescue pens used in Chapter 4 for care of poorly performing piglets

2.1.2.3 Refinement: Pork is the most consumed meat in the EU and therefore pigs must be the animal of choice to assess a) Chapter 3, common colonic community indicators b) Chapter 4, the effects of dietary prebiotics on the microbiota and gut architecture and c) Chapter 5, effects of dietary prebiotics on RVA infection and the microbiome. Pigs are social animals and prefer to live in herds rather than in isolation. Grouping animals into pens rather than separating individuals allowed animals to express normal social behaviour. Given the nature of trials, it was not expected that diets or housing conditions would produce any adverse effects. Balancing this with the stress that individual housing may cause, pens of animals were the most appropriate method to meet trial objectives. Minor distress (sub-

threshold) to the animals may be associated with movement and weighing operations but these tasks were conducted in a familiar environment to reduce the stress experienced by the animals. Environmental conditions in trials met Red Tractor standards for commercial practice, which sought to maximise performance by reducing stress and maintaining healthy stock (Red Tractor, 2021). Metal chain toys with plastic balls were provided in weaning pens as environmental enrichment. Poorly performing piglets potentially receiving sub-optimal nutrition from the sow were selected within the first seven days of life by visual assessment and the appearance of "non-thriving" by qualified animal technicians. This was based upon poor weight gain, a high degree of contamination with faecal material, the presence of watery faeces and overall health. Animals displayed no clinical symptoms of underlying disease, for example, scour or lameness, but were considered to benefit from a complete milk replacement feeding program. As introduced earlier, poor performance may be a function of the focus on larger litter sizes which has increased the number of piglets with decreased viability, lighter birth weights and a reduced ability to thrive in early life (Tucker *et al*., 2021).

2.1.3 Test products

No product was tested in Chapter 3. The product under test in Chapters 4 and 5 was Nutrabiotic® which is a GOS based feed material with prebiotic properties. GOS is included in the feed material register (registration number: 03101-EN) https://www.feedmaterialsregister.eu/register and is approved for use in human infant formula/foods in the USA and generally regarded as safe (GRAS) (USFDA, 2019). GOS produced by β-galactosidases is authorised and included in the EU Union list of novel foods (EU, 2015). In the EU, GOS intake has not raised any safety concerns and it was concluded that the proposed use of GOS as a novel food is safe under

proposed conditions of use (EFSA, 2021). The manufacturer was Saputo Dairy UK (formerly Dairy Crest, Weybridge, Surrey UK). Nutrabiotic was stored in a cool, dry place, protected from moisture and light in a well-sealed pack until mixed with the trial diets. An analysis of the product is shown in Table 6. It should be noted that Nutrabiotic is not 100% pure GOS. The product contains galactose, glucose and lactose which are hydrolysed and digested in the upper pig GIT. The remainder is GOS of varying degrees of polymerisation from 3 to 7 as determined by the producers analysis (Dairy Crest) and would be expected to be fermented in the lower pig GIT. Given that non-GOS control piglets received only contemporary CMR, without GOS, it is apparent that test animals are receiving an additional digestible fraction in the test product that may affect production values. This needs to be addressed in future studies by perhaps making diets isocalorific. However, given the low 1% dose of total Nutrabiotic, the effects may be neglible, but nonetheless close examination of product formulation and possible effects of digestible and non-digestible fractions obviously need to be more clearly examined in design of future studies. In Chapter 4, Nutrabiotic was dosed at 1% (w/w) on a dry solids basis into CMR (Faramate, Volac International Ltd., Royston, UK). For control groups, 150 g of CMR was dissolved in 500 mL of warm water (30 – 40 °C) and made up to a total volume of 1 L with warm water. For test groups 150 g of CMR was dissolved in 500 mL of warm water, 3 g GOS Nutrabiotic added and made up to a total volume of 1 L with warm water. In Chapter 5, sows received 30 g per day GOS Nutrabiotic top-dressed into lactation diet.

Table 6. Analysis of Nutrabiotic® % w/w dry solids. (Dairy Crest, 2018).

Galactose	1.61%
Glucose	21.83%
Lactose	31.66%
DP3 GOS	25.18%
DP4 GOS	12.23%
DP5 GOS	5.00%
DP6 GOS	1.77%
DP7 GOS	0.70%

DP = Degree of polymerisation.

2.1.4 Pig gastrointestinal dissection and sampling

After euthanasia, each pig was cut down the ventral line using a sterile surgical scalpel blade No. 22A (Swann-Morton, Sheffield, UK) and the GIT removed from the carcass and laid out in sections as in Figure 7. For Chapter 3, only the colonic contents were sampled. Sampling locations were identified and tied off with 140 mm cable ties (B&Q, Eastleigh, UK) to prevent flow of luminal contents or cross-contamination. These were UV irradiated for 30 minutes in a biological safety cabinet (BSC, Thermo Fisher Scientific, Loughborough, UK) before use. Luminal contents were sampled from the duodenum, jejunum, ileum, caecum, colon and rectum for bacterial DNA extraction. Tissue samples were collected from the jejunum, ileum, caecum and colon for histopathological examination and GC enumeration. Separate dissection kit tools including surgical blades were used for each sampling location to prevent cross-contamination and dissection tables

disinfected with 1% w/v 10 g in 1 L H_2O, Virkon (VWR International Ltd, Lutterworth, UK – see Appendix 4 for composition) between each animal dissection. The GIT was initially examined to identify sampling regions these being the small intestine comprising the duodenum, jejunum and ileum; the caeco- colic junction leading to the caecum and colon; the descending colon and rectum. Sampling sites were representative for each trial according to methodology below.

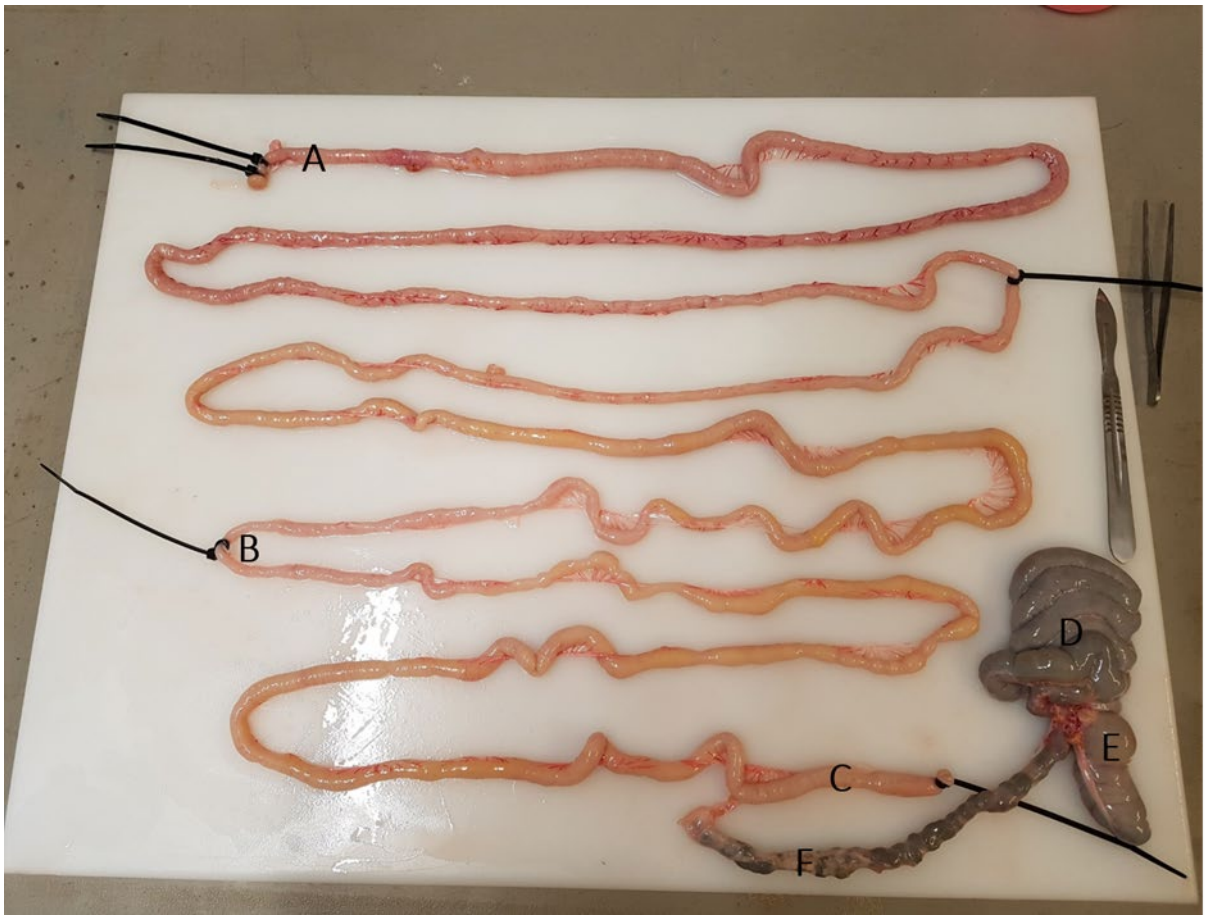

Figure 7. Pig GIT dissection showing A) Duodenum B) Jejunum C) Ileum D) Colon E) Caecum and F) Rectum

2.1.4.1 The duodenum: The stomach was identified, and the descending duodenum found to where it turned through 180 degrees to become the ascending duodenum. The pancreas was identified within the "u-bend" thus formed. The mesentery was dissected away from the duodenum to release a 15-20 cm loop and sampled at the

turning point.

2.1.4.2 The jejunum: The mesentery was dissected away from the jejunum starting at the junction with the ileum and continued to the region where the jejunum became firmly fixed to the root of the mesentery. This was only a short distance from the duodeno-jejunal junction and there was no need to completely dissect the mesentery to reach the junction. Grasping the fixed end of the jejunum enabled it to be matched against the ileo-jejunal junction. The doubled-up jejunum was then pulled (without squeezing to avoid tissue damage) through one hand to reach the middle of the jejunum giving an approximate mid-jejunum point for sampling.

2.1.4.3 The ileum: The ileum was identified as the terminal 10-15 cm of the small intestine before it entered the caeco-colic junction. It was identified by the presence of two mesenteries, one of which was attached to the caecum. Some of the mesentery was dissected away, but the junction between the ileum and jejunum was marked first with cable ties, since apart from the double mesentery there were no visual differences between these two sections of the small intestine. Sampling was half-way along the length of the ileum.

2.1.4.4 The caecum: The caecum, attached to the large colon and ileum was identified as a large blind-ended piece of bowel. Samples were taken from the tip of the caecum.

2.1.4.5 The colon: The colon was identified as large spiral structure with a smaller spiral inside and a “turn-around” at the bottom of the spiral (lowest in the abdomen). Samples were taken from the turning point of the spiral where it formed a U-bend.

2.1.4.6 The rectum: The rectum was identified as from where the large colon narrowed and headed towards the pelvis forming the rectum from which faecal samples were taken.

2.1.4.7 Sampling of gastrointestinal contents and tissues: At least 1 g of luminal contents from each sampling location were collected into 70 mL plastic, screw-top containers (Sarstedt, Leicester, UK) and placed on dry ice for subsequent storage at -80°C. Excised GIT tissue samples were fixed in 10% v/v neutral buffered formalin (see Appendix 4 for composition) in 40 mL prefilled specimen jars (Leica Microsystems UK, Ltd., Milton Keynes, UK) and stored at room temperature prior to histological analyses.

2.2 Nucleic acid extractions from trial samples

For all nucleic acid extractions common reagents, consumables and equipment are referred to in the first case. All buffers and reagents from nucleic acid extraction kits are referenced as far possible, but abbreviations for many kit components cannot be determined since they are not described by the manufacturer for proprietary reasons.

2.2.1 Chapter 3. Manual DNA extraction from gastrointestinal samples

Bacterial DNA from colonic samples of twelve pigs were extracted using the Macherey-Nagel NucleoSpin Tissue Genomic DNA Purification Kit (Macherey-Nagel, Düren, Germany). All consumables were provided in the kit unless otherwise stated. Prior to DNA extraction, 28 mL 96% v/v ethanol (Fisher Scientific, Loughborough, UK) was added to each bottle of wash buffer B5 (80% ethanol, 20 mM NaCl, 2 mM Tris-HCl) and 1.35 mL proteinase buffer (30 mM Tris-HCl) added to 30 mg lyophilised proteinase K. Before DNA elution the elution buffer BE (5 mM Tris-HCL) was pre-heated to 70°C. A 2 mL nuclease-free micro tube (Sarstedt, Leicester, UK) was placed in a sterile 7 mL Bijou bottle (Fisher Scientific, Loughborough, UK) for support and stood upon a sterile Petri dish (Sarstedt, Leicester, UK) for weighing GIT samples (Kern & Sohn, Balingen, Germany). Using aseptic technique, 250 mg of colonic samples were weighed into the tube with a

sterile 10 µL disposable loop (Sarstedt, Leicester, UK) and 1 mL buffer TE (10 mM Tris-HCl, 1 mM EDTA) (Thermo Fisher Scientific, Loughborough, UK) added. Samples were resuspended by vigorous vortexing for 30 seconds (Vortexer TopMix, Fisher Scientific, Loughborough, UK.) Samples were centrifuged at 21°C using a Mikro 185 centrifuge (Hettich GmbH & Co. KG, Tuttlingen, Germany) for 15 minutes at 4,000 x g and the supernatant discarded. One mL lysis buffer T1 (composition not available) was added, and the pellet resuspended. Of this, 200 µL was transferred to a new 2 mL micro tube and 25 µL proteinase K solution added. Samples were vortexed and then incubated at 56°C for 3 hours with occasional vortexing. After incubation, samples were briefly vortexed, 200 µL buffer B3 (50% v/v guanidine hydrochloride, 50% v/v H_2O) added, vortexed vigorously and incubated at 70°C for 10 minutes. Ethanol, 210 µL 96% v/v was added and samples vortexed vigorously. For each sample one NucleoSpin column was placed in a collection tube. Samples were pipetted into columns, centrifuged at 21°C and 11,000 x g for 1 minute and the flow through discarded. Wash buffer BW (50% v/v guanidine hydrochloride, 50% v/v isopropanol), 500 µL was added, samples centrifuged at 21°C and 11,000 x g for 1 minute and the flow through discarded. Wash buffer B5 (80% ethanol v/v, 20 mM NaCl, 2 mM Tris-HCl) , 600 µL was then added, samples centrifuged at 21°C and 11,000 x g for 1 minute and the flow through discarded. Samples were then centrifuged in columns for 1 minute at 21°C and 11,000 x g. Columns were then placed in 2 mL micro tubes, 100 µL pre-warmed BE buffer added, incubated at room temperature for 1 minute and finally centrifuged at 21°C and 11,000 x g to elute DNA. A kit control was prepared that did not contain GIT contents.

2.2.2 Chapter 4. Manual DNA extraction from gastrointestinal samples

Bacterial DNA was extracted from GIT luminal/pig digesta samples using the MP Biomedicals FastDNA SPIN Kit for Feces (MP Biomedicals, Solon, USA). All consumables were provided in the kit unless otherwise stated. The order in which DNA extractions from GIT samples were randomised using the random number generator https://www.random.org to control for possible DNA extraction kit effects. To prepare the wash buffer for DNA extraction, 100 mL of 96% v/v ethanol was added to wash buffer 2 (Composition not available), and the bottle marked to signify the addition. A 2 mL lysing matrix E tube (MP Biomedicals, Solon, USA) was placed in a sterile 7 mL Bijou bottle for support and stood upon a sterile Petri dish for weighing GIT samples. Using aseptic technique, 250 mg of GIT samples were weighed into the lysing matrix E tube with a 10 µL sterile disposable loop and 275 µL of pre-lysis solution (PLS - composition not available) and 825 µL sodium phosphate buffer added (5% sodium phosphate w/v, 95% H_2O v/v). A kit control was prepared that did not contain GIT contents. Lysing tubes were shaken by hand to mix and vortexed for 10 to 15 seconds. Contamination around the neck of the tube was avoided by covering with sterile absorbent tissue during vortexing. Lysing tube caps were secured, but not over-tightened, to prevent sample leakage. Preparation of GIT luminal content samples were performed in a sterile, pre-cleaned BSC until caps on lysing tubes were secured. Lysing tubes were centrifuged at 21°C and 13,300 x g for 5 minutes and the supernatant pipetted out and disposed of. Sodium phosphate buffer 978 µL and 122 µL MT buffer (1% sodium dodecyl sulphate v/v, 99% v/v H_2O) were added and vortexed briefly to mix. Samples were homogenized in the FastPrep 24 5G instrument at setting of 6.0m/s for 40 seconds (MP Biomedicals, Solon, USA). Care was taken to double label tubes and arrange in order of processing since the FastPrep 24 5G instrument could easily destroy labelling through gravitational

acceleration. Homogenized samples were then centrifuged at 21°C and 13,300 x g for 15 minutes to ensure elimination of excessive cell debris from samples. Using aseptic technique, the supernatant was transferred to a clean nuclease-free 2.0 mL micro tube. Protein precipitate solution, 250 µL (PPS - 5% acetic acid v/v, 95% v/v H_2O) was added, shaken vigorously to mix, and incubated at 4°C for 60 minutes. Samples were then centrifuged at 21°C and 13,300 x g for 2 minutes. Binding matrix solution, (87.8% silicon dioxide v/v, 12.2% H_2O v/v) 750 µL was added to a clean 2 mL micro tube. The supernatant was then added to the binding matrix solution in the 2 mL micro tube, shaken gently by hand to mix and placed on a PTR-25 rotator (Grant Instruments Ltd., Cambridge, UK) for 3-5 minutes. Samples were then centrifuged at 21°C and 13,300 x g for 2 minutes. The supernatant was pipetted out down to the pellet and discarded. The binding mixture pellet was then washed by gently re-suspending with 1 mL wash buffer 1 (Guanidine thiocyanate 30% v/v, 70% H_2O). In a two-step process, 600µL of the binding mixture was transferred to a spin filter tube and centrifuged at 21°C and 13,300 x g for 1 minute. The catch tube was emptied, and the remaining binding mixture added to the spin filter tube, pipetted to re-suspend and centrifuged as before, with the catch tube emptied again. Prepared wash buffer 2, 500 µL was added to the spin filter tube and gently resuspended using the force of the liquid from the pipette tip to re- suspend the pellet. Samples were then centrifuged at 21°C and 13,300 x g for 2 minutes. The flow-through from tubes was then discarded. Samples were centrifuged again for 2 minutes to extract residual ethanol from the binding matrix and dry the samples. The spin filter bucket was then transferred to a clean 1.9 mL catch tube. One-hundred µL Tris-EDTA (TES - Composition not available) solution was added and pipetted up and down to re-suspend the pellet. Samples were then centrifuged at 21°C and 13,300 x g for 2 minutes to elute purified DNA into clean catch tubes. The spin filters were then

discarded leaving purified DNA in catch tubes for PCR and other downstream applications and stored at -20°C until use. DNA integrity and quality was checked for each sample using the NanoDrop 1000 Fluorospectrometer (Thermo Fisher Scientific, Loughborough, UK). Both optical surfaces were cleaned using 2 µL RO (reverse osmosis) H_2O (Milli-Q, Merck, Gillingham, UK) before use and wiped with Kimtech wipes (Kimberley- Clark, Reigate, UK) between readings. 0.1% v/v HCL (0.1% v/v HCL, 99.9% H_2O) solution was used as a cleaning agent before measurements (Thermo Fisher Scientific, Loughborough UK). A reference reading using 2 µL TES elution buffer pipetted onto the lower optical surface of the NanoDrop was used as a control reading. 2 µL samples were pipetted onto the lower optical surface of the NanoDrop, avoiding air bubbles, to measure DNA concentration. Samples were then stored at -20°C for downstream processing.

2.2.3 Chapter 5. Automated purification of bacterial DNA from porcine faecal samples

2.2.3.1 Initial preparation

All reagents from the QIAmp 96 PowerFecal QIA cube HT Kit (Qiagen, Hilden, Germany) were equilibrated to room temperature (15 - 25°C) and checked for any precipitates. Any reagents containing precipitates were incubated at 37°C with gentle shaking to dissolve precipitates. Vigorous shaking was avoided to prevent foaming. Buffers were supplied as concentrates that required dilution with solvents before use. Before using for the first time, 484 ml 96% v/v ethanol was added to buffer AW1 (Guanidine hydrochloride 50%, v/v 50% H_2O) and mixed well to obtain a working solution. Three-hundred mL 96% v/v ethanol was added to buffer AW2 (70% ethanol v/v, 30% H_2O v/v) and mixed to obtain a working solution. Buffer PW1 (Guanidine thiocyanate 2.5% v/v, 97.5% H_2O) was warmed to 60°C for 10 minutes

before use. Check boxes on buffer bottle labels were ticked to indicated that solvents had been added. Filter plates, filter tape, elution microtubules, “OnCor C 7” filter tips and sample S-blocks were provided as part of the QIAcube HT Plasticware kit (Qiagen, Hilden, Germany).

2.2.3.2 Pre-treatment of faecal samples

Preparation of faecal samples for DNA extraction was carried out in a BSC and all equipment subjected to 30 minutes UV irradiation before use. Faecal samples were defrosted over ice and 200 mg weighed into a sterile pathogen lysis tube type L (Qiagen, Hilden Germany) placed in a 7 mL Bijou bottle for support on a Petri dish on a balance. PW1 buffer, 650 µL was added and samples homogenized in the FastPrep 24 5G instrument at setting of 6.0m/s for 40 seconds. Samples were then centrifuged at 21°C and 13,300 x g for 15 minutes to pellet faecal debris. Of this, 400 µL supernatant was then pipetted into a clean 2 mL micro tube and 150 µL buffer C3 (composition not available) added with thorough mixing by pipetting up and down. Samples were then incubated for 5 minutes at 4°C and centrifuged at 21°C and 13,300 x g for 1 minute. Of each sample, 300 µL was added to a sample S-Block and 20 µL proteinase K (20 mg/mL in 30 mM Tris-HCL) added, mixed and incubated for 10 minutes at room temperature.

2.2.3.3 Robot worktable preparation

The QIAcube HT (Qiagen, Hilden, Germany) was switched on and the QIAcube HT icon chosen to open the QIAcube HT “Prep Manager” software for extraction of DNA from samples. The “home” tab was chosen on the software followed by the “QIAmp 96 PowerFecal” protocol from the drop-down menu. The experimental information was completed by selecting sample type as “stool” and pre-treatment as “pathogen”. The check box for optional vacuum performance check after the binding

step was ticked. Samples were defined by entering the sample numbers 1 to 48. The input positions of samples were selected and assigned according to the graphic of the 96 well plate on screen. The waste bottle was checked for sufficient capacity prior to the run and the tip chute and tip disposal box loaded. The channeling block holder, channeling block, transfer carriage and Qiagen QIAmp 96 filter plate were loaded (Figure 8). Unused wells were covered with filter tape on the 96 well filter plate. The riser block and elution microtubules were then loaded. A full box of 96 OnCor C filter tips was loaded in the robot worktable tip rack position C1 and all tips set to available in the software. A second box of filter tips was loaded onto the C2 tip rack and selected as available. The instrument calculated the volume of reagents required for each reagent trough.

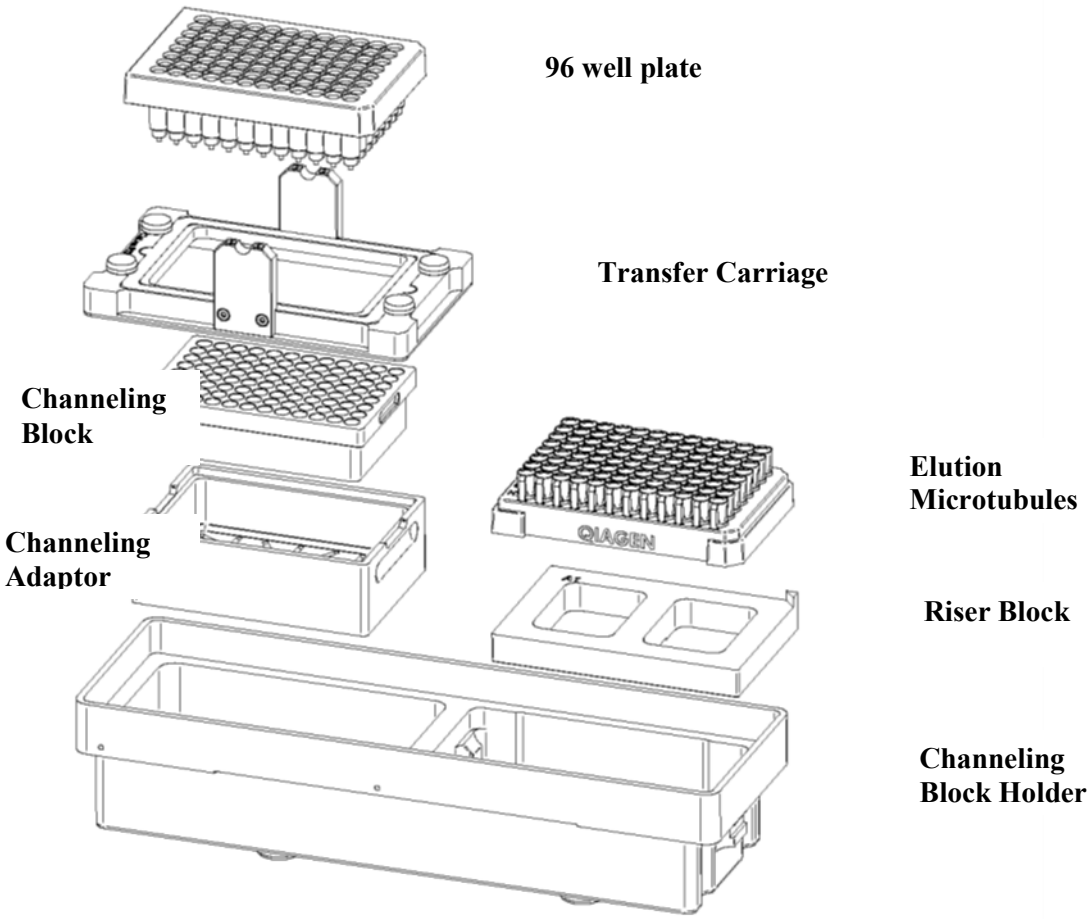

Figure 8. Component parts of the QIAcube HT robot

Worktable positions A1 and B1 took 180 mL reagent troughs (Qiagen, Hilden, Germany) and positions A2, B2 and C1 to C4 70 mL reagent troughs (Qiagen, Hilden, Germany). Reagents were added to troughs using a 25 mL serological pipette (Sarstedt, Leicester, UK) using a pipette controller (Heathrow Scientific, Nottingham, Nottinghamshire, UK). A 27 mL aliquot of solution C4 (50% v/v guanidine hydrochloride, 50% v/v isopropanol) was added to reagent trough A2. Ninety-six % v/v ethanol, 27.7 mL was added to reagent trough B2. Buffer AW2, 32 mL was added to reagent trough C1. Buffer AW1, 53.2 mL was added to reagent trough C2. Top elute fluid, (100% white mineral oil) 2.1 mL was added to reagent trough C3 and 5.2 mL buffer ATE (composition not available) was added to reagent trough C4. The robotic DNA extraction protocol was then launched. Once complete, samples were then stored at -20°C for downstream processing.

2.2.4 Extraction of rotavirus A RNA from porcine faecal samples.

2.2.4.1 Pre-treatment of faecal samples

Eight glass universal bottles (Fisher Scientific, Loughborough, UK) were cleaned with 0.1% v/v diethyl pyrocarbonate (DEPC) (Merck, Gillingham, UK) in RO water, shaken vigorously and allowed to stand overnight at 37°C. These were autoclaved on a liquid cycle at 121°C and 15 psi, for twenty minutes, to eliminate residual DEPC. Five hundred mL sterile 0.9% w/v NaCl solution was prepared by adding 4.5 g NaCl (Merck, Gillingham, UK) to 500 mL RNase free water (Fisher Scientific, Loughborough, UK) and autoclaved. Twenty-five mL of the NaCl solution was then aliquoted into the eight DEPC cleaned universal bottles giving enough pre-prepared isotonic diluent for extracting RNA from at least 140 pig and piglet faecal samples. All further work was carried out in a BSC, wearing double gloves and having thoroughly sprayed and cleaned work surfaces, racks, bijou bottles, pipettes and any

other equipment with RNase Zap (Thermo Fisher Scientific, Loughborough, UK) before commencing work. All equipment was then subjected to 30 minutes UV irradiation in the BSC before use. Animal faecal samples were defrosted over ice and 100 mg weighed into a sterile, RNase-free 2 mL micro tubes, placed in a Bijou bottle for support on a Petri dish on the balance. Nine-hundred µL 0.9% w/v NaCl solution was pipetted into the tube containing 100 mg faecal sample and vortexed for one minute. The suspension was centrifuged at 21°C and 16,000 g for 5 minutes to pellet debris and the clear supernatant with at least 200 µL transferred to a clean micro tube for storage at -80°C.

2.2.4.2 Initial preparation

All reagents from the QIAmp 96 Virus QIA cube HT Kit (Qiagen, Hilden, Germany) were equilibrated to room temperature (15 - 25°C) and checked for any precipitates. Those reagents containing precipitates were incubated at 37°C with gentle shaking to dissolve precipitates. Vigorous shaking was avoided to prevent foaming. Before using for the first time, 40 mL 99.5% v/v isopropanol (Fisher Scientific, Loughborough, UK) was added to buffer ACB (Guanidine thiocyanate 50% v/v, 50% H_2O) and mixed well to obtain a working solution. Ethanol, 250 mL 96% v/v was added to buffer AW1 (Guanidine hydrochloride 50% v/v, 50% H_2O v/v) and mixed to obtain a working solution. Ethanol, 300 mL was added to buffer AW2 (70% ethanol v/v, 30% H_2O v/v) and mixed to obtain a working solution. Check boxes on buffer bottle labels were ticked to indicated that solvents had been added.

2.2.4.3 Preparation of RNA carrier stock solution

Buffer AVE (0.04% v/v sodium azide, 99.96% H_2O v/v), 1550 µL was added to the tube containing 310 µg lyophilized carrier RNA to obtain a stock solution of 0.2

µg/µL. Aliquots of 255 µL carrier RNA stock solution were pipetted into six RNase-free 2 mL micro tubes and frozen at -24°C. Aliquots of carrier RNA were not subjected to more than three freeze-thaw cycles. For a 48-sample run using the QIAcube HT robotic workstation for RNA purification, 250 µL prepared carrier RNA stock solution was required.

2.2.4.4 Preparation of the RNase free DNase set

DNase I stock solution was prepared before using the RNase-free DNase Set (Qiagen, Hilden, Germany) for the first time. RNase-free water, 550 µL was pipetted into the vial containing Lyophilized DNase I (1500 Kunitz units) and gently inverted to mix. For long-term storage of DNase I, stock solution was removed from the glass vial and divided into single-use aliquots in micro tubes and stored at -24°C. Reconstituted DNase I was not vortexed because of sensitivity to physical denaturation, but rather mixing by gentle inversion. To prepare enough DNase-mix for 48 samples 540 µL DNase stock was added to 3.78 mL RDD Buffer (supplied with kit – composition not available), leaving enough for tip and reagent trough wastage.

2.2.4.5 Robot worktable preparation

The instrument was prepared as in section 2.2.3.2 except that the "Custom QIAamp 96 Virus CR 1564" protocol was chosen. The volume of reagents required was 49.3 mL of 96% v/v ethanol added to reagent trough A1, 4.5 mL of DNase mix added to reagent trough A2, 18.5 mL buffer ACB added to reagent trough B2 and 30.5 mL buffer AW1 added to trough C1. Buffer AW2, 38.9 mL was added to trough C2, 2.1 mL top elute was added to trough C3 and 5.2 mL buffer AVE added to trough C4.

2.2.4.6 Sample preparation

The Qiagen sample S-Block for RNA extraction required in addition to samples, the addition of proteinase K, buffer ACL (Guanidine thiocyanate 30% v/v, 70% H_2O v/v), carrier RNA and an RNA internal extraction control from the qPCR test rotavirus A kit (Cole-Parmer, Stone, UK). Two hundred µL of 48 test samples were pipetted into the bottom of the S-Block followed by 20 µL Qiagen proteinase K. The RNA internal extraction control was pulse spun in a centrifuge before opening and reconstituted by the addition of 600 µL RNase/DNase free water from the qPCR kit and vortexed. To prepare the buffer ACL, 250 µL carrier RNA stock solution and 192 µL RNA internal extraction control was added to 8 mL ACL buffer. Buffer ACL, 160 µL mixture was pipetted into each sample in the S-Block and mixed well. The S-Block was covered with adhesive tape and incubated at 56°C for 30 minutes. The adhesive tape was aseptically removed and the S-Block transferred to the robot worktable ready for RNA extraction. Once started, the instrument performed the RNA extraction protocol run and when finished, purified viral RNA was stored at -80°C for downstream applications.

2.3 16S rRNA gene sequencing with the Illumina MiSeq: Library generation, quality control and DNA sequencing

The protocol for 16S rRNA gene sequencing was based upon the "Development of a dual- index sequencing strategy and curation pipeline for analysing amplicon sequence data on the MiSeq Illumina sequencing platform" (Kozich *et al*, 2013) and the "MiSeq Wet Lab SOP" as described by Schloss *et al*, 2013. The Illumina MiSeq Sequencer (Illumina, San Diego, USA) can produce 2 x 250 base pair (bp) paired end reads and up to 81.5 giga bytes (Gb) of data in a single run. Dual indexing of library samples allows up to three hundred and eighty-four samples to be run

simultaneously equivalent to four 96 well plates. The instrument is capable of producing twenty-five million reads. For low diversity runs approximately twelve million reads can be expected. A wide range of applications are possible including 16S rRNA gene sequencing, metagenomics, genome sequencing, transcriptomics and RNA sequencing. 16S rRNA gene sequencing typically sequences the V4 region of the 16S rRNA gene, its short length of 250 bp allowing for fully overlapping forward and reverse reads, resulting in low error rates. Steps in preparing samples for sequencing on the MiSeq included 1) Sample plate map and sample sheet generation. 2) Reconstitution of primers. 3) PCR of genomic DNA (gDNA) samples isolated from GIT samples. 4) Gel electrophoresis of all samples from initial PCR of gDNA samples for quality control. 5) gDNA concentration normalisation and pooling of samples by SequalPrep. 6) gDNA library quality control and quantification by the Agilent 2200 TapeStation System to determine the number of gDNA base pairs and Qubit DNA assay to determine gDNA concentration.
7) Sequencing, run quality assessment and data export.

2.3.1 Sample plate map and sample sheet generation

Using the Illumina Experiment Manager https://emea.support.illumina.com/sequencing/sequencing_software/experiment_manager/downloads.html a sample plate map of samples and indexed primers layout for each 96 well plate was created. “Ian Connerton” was selected from the sample preparation kit selection menu and a project name entered. The plate tab was clicked to show the 96 well plate layout and the sample names entered. The appropriate indexes were selected from the pull-down menu for each plate. Indexes were chosen that corresponded to one of the two index pair plates below:

A701 – A712 with B501 – B508

B701 – B712 with A501 – A508

One plate was prepared for each of the four Harper Adams pig trials using A701 – A712 with B501 – B508 as in Chapter 4. Two plates were prepared for Chapter 5 using A701 – A712 with B501 – B508 and B701 – B712 with A501 – A508. An example from Chapter 4 for Harper Adams pig trial 1 and for samples D1A to D8F is shown in Figure 9. The plate included a kit control KC1, the synthetic bacterial MOCK community and reverse osmosis (RO) water.

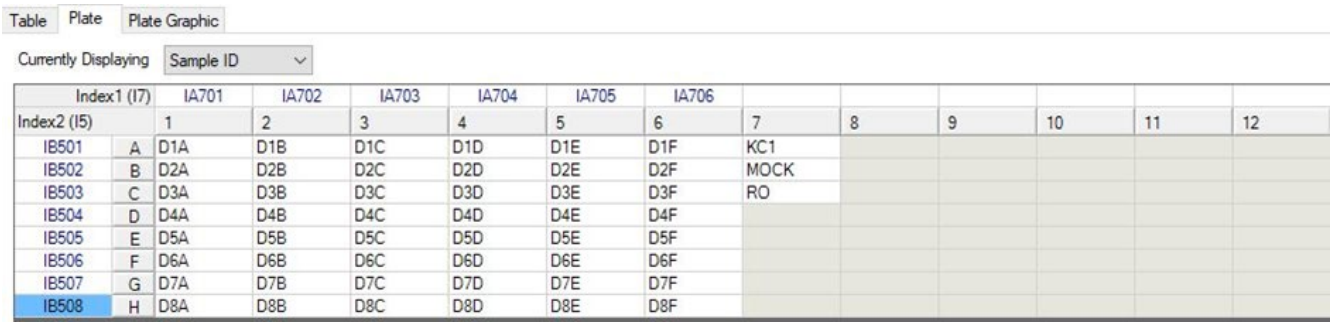

Table | Plate | Plate Graphic

Currently Displaying: Sample ID

Index1 (I7)		IA701	IA702	IA703	IA704	IA705	IA706						
Index2 (I5)		1	2	3	4	5	6	7	8	9	10	11	12
IB501	A	D1A	D1B	D1C	D1D	D1E	D1F	KC1					
IB502	B	D2A	D2B	D2C	D2D	D2E	D2F	MOCK					
IB503	C	D3A	D3B	D3C	D3D	D3E	D3F	RO					
IB504	D	D4A	D4B	D4C	D4D	D4E	D4F						
IB505	E	D5A	D5B	D5C	D5D	D5E	D5F						
IB506	F	D6A	D6B	D6C	D6D	D6E	D6F						
IB507	G	D7A	D7B	D7C	D7D	D7E	D7F						
IB508	H	D8A	D8B	D8C	D8D	D8E	D8F						

Figure 9. Sample plate map generated by Illumina experiment manager for Harper Adams pig trial 1.

A sample sheet was then created using the IEM software by selecting the instrument selection page and selecting MiSeq. “Fastq only” was selected on the MiSeq application selection page. The barcode from the MiSeq reagent kit to be used for the sequencing run was entered. “Ian Connerton” was selected as the sample preparation kit. An experiment name, number and description were entered, and the number of cycles changed to 251 for both read 1 and read 2. The appropriate sample plate file was then selected for plate 1 (as created above). “Select All” was chosen, and all the selected samples added to the sample sheet which, was saved as csv file for transferal to the MiSeq before sequencing commenced.

2.3.2 Reconstitution of primers

Primers were supplied by Merck, Gillingham, UK. One hundred µL, 10 µM aliquots of index primers were prepared. Sequencing primers were not diluted. PCR grade

water (Thermo Fisher Scientific, Loughborough, UK) was added to each primer vial at x µL to reconstitute to100 µM concentration, where x equals the volume of PCR grade water specified in the technical data sheet (Merck, Gillingham, UK). Vials were spun down before reconstitution to ensure the DNA pellet was at the bottom of the vial. Micro tubes, 2 mL were labelled with the primer set name, A701 to A712, B501 to B508, B701 to B712 and A501 to A508. A full list of primers is shown in Appendix 1. Ninety µL of PCR grade water and 10 µL each reconstituted primer was added to each labelled micro tube. Using a multichannel pipette (Sartorius Biohit Liquid Handling, Helsinki, Finland), 5 µL of A701 to A712 were pipetted onto a clear 96 well microtiter plate (Fisher Scientific, Loughborough, UK) followed by 5 µL of B501 to B508 onto the plate to give the correct combination of primers. Primer plates were stored at -20°C for subsequent sequencing runs.

2.3.3 PCR of genomic DNA samples

Using the isolated DNA as a template, the V4 region of the bacterial 16S rRNA genes were PCR amplified using primers 515f (5′ GTGCCAGCMGCCGCGGTAA 3′) and 806r (5′ GGACTACHVGGGTWTCTAAT 3′) (Caporaso *et al*., 2011). All PCR steps were carried out in a PCR hood (Labcaire Systems Ltd., Clevedon, Somerset, UK) under sterile conditions. The 96 well PCR plate (Sarstedt, Leicester, UK) was kept on ice when preparing PCR. For ease of working most pipetting steps were performed using a multichannel pipette (Fisher Scientific, Loughborough, UK). The 96 well PCR plate, pipette filter tip boxes and any other plastic ware were sterilised by UV irradiation for 30 minutes. Two µL of each paired set of index primers were pipetted from the primer plate to the corresponding well on the 96-well PCR plate according to the Sample Plate Map design. Seventeen µL of Accuprime Pfx Supermix (Thermo Fisher Scientific, Loughborough, UK – see Appendix 4 for

composition) was pipetted into each well of the 96-well PCR plate, from a sterile 10 mL reagent reservoir (Thermo Fisher Scientific, Loughborough, UK) to assist with multichannel pipetting. One µL of template gDNA, including DNA extraction kit controls, were pipetted to the corresponding wells on the 96-well PCR plate and mixed by pipetting up and down. One µL of Milli-Q/molecular biology grade H_2O was added to the negative control well and 1 µL of ZymoBIOMICS Microbial Community (10 ng) (Cambridge Bioscience, Cambridge, UK) added to the positive control well on the PCR plate. The plate was sealed using an adhesive PCR seal (Sarstedt, Leicester, UK), seal roller (Elkay Laboratory Products UK Ltd., Basingstoke, UK) and an adhesive seal applicator used (3M, Bracknell, Berkshire, UK) to finally seal the plate, whilst placed on the PCR block, to prevent tipping or warping of the plate. A second non- adhesive seal, (Bio-Rad) was applied to help maintain the seal on the plate and prevent sample loss by evaporation. The plate was vortexed for three seconds on the IKA Vortexer (IKA England Ltd., Oxford, Oxfordshire, UK.) and the contents spun down using the Axygen Axyspin (Corning, New York, USA.), for 20 seconds. The plate was placed in the thermal cycler (Bio-Rad T-100) and the program "16S MISE" selected with a run time of approximately 3 hours and 55 minutes to completion (Table 7).

Table 7. PCR parameter settings.

Temperature °C	Time minutes
95	02:00
30 cycles	
95	00:20
55	00:15
72	05:00
1 cycle	
72	10:00
4	20:00

2.3.4 Gel electrophoresis

To check for DNA quality a 1% w/v agarose gel, using a 96-well, 12 ladder wells casting tray/running tray was prepared. For one gel, 2 g of molecular biology grade agarose (Merck, Gillingham, UK) was weighed into 200 mL tris acetate solution (TAE - Tris 11.31% v/v, EDTA disodium salt 0.87% v/v, acetic acid sodium salt 7.66% v/v, HCl 3.74% v/v) (Thermo Fisher Scientific, Loughborough, UK) in a 250 mL conical flask (Schott, Duran, Mainz, Germany). This was microwaved (Sharp Corporation, Osaka, Japan) for 20 seconds and swirled gently. This step was repeated until all the agarose dissolved. Cling film (Clingorap, Terinex, Bedford, Bedfordshire, UK) was placed over the top of the conical flask and pierced before microwaving. The flask was cooled under running water so as just cool enough to touch. Ten µL ethidium bromide (Merck, Gillingham, UK - 10 mg/mL in H_2O) was added, swirled gently and the liquid agarose poured into the 96 well casting tray (ABgene, Epsom, Surrey, UK). For a 96 well plate, 4 µL of loading dye x 96 equalled 384 µL needed, plus 10% equalled 422.4 µL. Six x Promega loading dye (Promega Corporation, Madison, Wisconsin, USA) was used where 1.5 x was needed, equating to 1.6/6 x 422 µL = 105.5 µL dye plus 316.9 RO H_2O. Two µL of sample and 4 µL of loading dye diluted to 1.5 x were loaded onto the agarose gel and run at 97V for 30 minutes and 20 mA alongside a 100 bp ladder (Promega Corporation, Madison, Wisconsin, USA). The gel was photographed and documented under UV light using the Gel Doc XR+ system (Bio- Rad, Watford, Hertfordshire, UK).

2.3.5 Normalization and pooling

The following steps were performed using the SequalPrep Normalization Plate (96) Kit. (Thermo Fisher Scientific, Loughborough, UK) Reagents were provided by the

kit unless otherwise stated. Depending upon the product volume from PCR, between 10 and 18 µL of PCR product from the 96-well PCR plate were pipetted into the corresponding wells on the normalization plate. An equivalent volume of binding buffer (HCl 10% v/v, 90% H_2O v/v) was added to each well, mixed by pipetting up and down and the plate sealed with adhesive PCR seal, vortexed on the IKA Vortexer for 3 seconds and spun down briefly using the Axygen Axyspin. The plate was incubated at room temperature for 60 minutes. Fifty µL of wash buffer (composition not available) was added to each well, pipetted up and down twice and aspirated immediately. The plate was inverted and gently tapped onto clean absorbent blue paper towel (Kimberley-Clark, Reigate, UK) to ensure there was no residual wash buffer remaining in any well. Twenty µL of elution buffer (10 mM Tris-HCl) was added to each well and mixed by pipetting up and down five times. The plate was sealed, vortexed and spun as previously and incubated at room temperature for 5 minutes. A pool (the DNA library) was created from the entire contents of the plate by pipetting 5 µL of each well into a 2 mL micro tube using an empty 96 well plate and multichannel pipette to aid pooling.

2.3.6 DNA Library quality control and quantification

The number of bp's from the pooled DNA library preparations were determined using the Agilent 2200 TapeStation System Trace using D1000 ScreenTape, Agilent D1000 reagents and Agilent plastic ware (Agilent Technologies, Inc., Santa Clara, California, USA). The Agilent sample buffer (20mM KCl, 60mM PO_4 buffer, 60mM guanidine-HCl, 240mM NaCl, 60mM NaOAc) was allowed to equilibrate at room temperature for 30 minutes and vortexed before use. The Agilent ladder was prepared by mixing 2 µL D1000 sample buffer with 2 µL D1000 ladder. Two µL D1000 sample buffer was mixed with 2 µL DNA library samples in Agilent optical tubes, spun down in a PCR strip tube mini-centrifuge (SciSpin Mini, SciQuip Ltd.,

Newtown, Shropshire, UK) and vortexed using the IKA Vortexer and adaptor at 2000 rpm for 1 minute to position samples at the bottom of tubes. Samples were prepared in triplicate. The Agilent 2200 TapeStation controller software was launched on the HP laptop attached to the Agilent 2200 TapeStation and the D1000 ScreenTape device and loading tips loaded into the instrument. The samples and ladder in the optical tubes were loaded into the instrument and the required number of samples selected with the controller software. The instrument was started, and the results saved as a pdf file to disk. The average number of bp's was taken from three readings.

2.3.7 Qubit DNA assay

DNA concentration was assayed using the Qubit 3.0 Fluorometer, reagents and assay tubes (Thermo Fisher Scientific, Loughborough, UK). The Qubit working solution was prepared by diluting Qubit dsDNA High Sensitivity (HS) Reagent (composition not available) 1:200 dilution in Qubit dsDNA HS Buffer (1 µL reagent in 199 µL buffer – composition not available) in a clean nuclease free Qubit assay tube. For preparation of two DNA standards and one DNA library sample in triplicate, 5 Qubit assay tubes and 5 µL reagent in 995 µL buffer were required. One hundred and ninety µL of Qubit working solution was added to each tube to be used for DNA standards. Ten µL of each Qubit standard was added to the appropriate tube and vortexed for 3 seconds giving a final volume of 200 µL. One hundred and ninety-eight µL of Qubit working solution was added to each of the assay tubes used for samples and vortexed for 3 seconds. Two µL of pooled DNA sample was added to each assay tube giving a final volume of 200 µL. All tubes were incubated at room temperature for 2 minutes. On the home screen of the Qubit 3.0 Fluorometer, "dsDNA High Sensitivity" was selected as the assay type. DNA standard 1 was

inserted in the instrument and read, followed by DNA standard 2. “Run Samples” was then selected on the instrument screen and the “+” or “–“ buttons used to adjust the sample volume added to the assay tube (2 µL). Units for the output sample concentration were selected from the drop-down menu as ng/µL. Sample tubes were inserted in the instrument one-by-one and the DNA concentration in ng/µL read. An average for three readings was taken. The following formula was used to convert DNA library concentration from ng/µL to nM.

DNA concentration nM = (DNA concentration ng/µL) / (660 g/mol x average library size bp) x 1 x 10^6

2.3.8 Sequencing

2.3.8.1 Preparing and diluting the DNA library

The 500 cycle MiSeq Reagent Nano Kit v2 cartridge (Illumina, San Diego, USA) was removed from the -20°C freezer and placed in a water bath at room temperature for one hour. The HT1 buffer tube (Illumina, San Diego, USA - 10 mM Tris-HCl, 0.1% v/v Tween 20) was removed from the cartridge packing and placed in a fridge at 4°C. Fresh 0.2N NaOH was prepared by adding 200 µL 1N NaOH (Merck, Gillingham, UK) to 800 µL PCR grade water. To a 2 mL micro tube, 10 µL of DNA library and 10 µL of 0.2N NaOH were added (library tube). To a separate tube 2 µL PhiX (Illumina, San Diego, USA), 3 µL PCR grade water and 5 µL of 0.2N NaOH were added (PhiX tube). Tubes were pipetted up and down to mix and allowed to incubate at room temperature for 5 minutes. Ice cold HT1 buffer, 980 µL was then added to the library tube, and 990 µL HT1 buffer to the PhiX tube. For a DNA library starting concentration of 5 nm this gave a DNA concentration of 50 pM once diluted. The resultant 20 pM PhiX solution was frozen and used for subsequent runs

once used. HT1 buffer was then used to further dilute the library and PhiX preparations to 5 pM. Nine hundred µL HT1 buffer was added to 100 µL 50 pM library giving a concentration of 5 pM DNA containing 0.0002 N NaOH. PhiX, 250 µL, 20 pM was added to 750 µL HT1 buffer giving a concentration of 5 pM PhiX containing 0.00025 N NaOH. Library, 850 µL was then added to 150 µL PhiX solution in a separate micro tube and vortexed for 3 seconds. This gave an overall concentration of 5 pM with a 4.25 pM library concentration, 0.75 pM PhiX and 0.0002075 N NaOH concentration. The NaOH concentration on the Illumina MiSeq flow cell must remain under 0.001N. Adjusting the concentration of the NaOH used to denature the DNA to 0.1N may be necessary if library concentration is 1 nM or below.

2.3.8.2 Preparing the reagent cartridge for sequencing

Once thawed, the reagent cartridge was gently dried with absorbent towel. The cartridge was gently inverted ten times to mix the reagents and ensure each well was thawed. Using a clean 1000 µL pipette tip, the foil covering wells 12, 13, 14, and 17 of the reagent cartridge were broken. Final Library/PhiX solution 600 µL, was loaded into well 17 of the reagent cartridge. Using a Biosphere extra-long tip (Sarstedt, Leicester, UK) on a P200 Gilson pipette, 10 µL of the cartridge reagent was removed from well 12 and placed into a sterile PCR tube. Three µL of the 100 µM Read 1 sequencing primer for the V4 region (TATGGTAATTGTGTGCCAGCMGCCGCGGTAA) (Merck, Gillingham, UK) was pipetted into the PCR tube containing reagents from well 12 and pipetted up and down to mix. Reagent and primers, (13 µL) were then pipetted into well 12, pipetting up and down to mix. Some of the liquid from the cartridge well was withdrawn to double check the pipette tip had reached the liquid in the cartridge and was then replaced. This process was repeated for well 13 and the index primer for the V4

region (ATTAGAWACCCBDGTAGTCCGGCTGACTGACT) (Merck, Gillingham, UK), well 14 and the read 2 sequencing primer for the V4 region (AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT) (Merck, Gillingham, UK) to ensure that all primers and the reagents in wells were properly mixed. The cartridge was then gently tapped on the bench to remove air bubbles that might have accumulated in the bottom of reagent wells. This prevented the "sippers" on the Illumina MiSeq instrument sucking air rather than reagent into the instrument. The reagent cartridge was set aside on ice and the MiSeq flow cell (Illumina, San Diego, USA) unboxed from storage at 4°C. The flow cell was thoroughly rinsed with Milli-Q water and carefully dried with absorbent free wipes (Kimberley-Clark, Reigate, UK) giving special attention to the edges and points of intersections between the glass and plastic. Using ethanol wipes (Synergy Health, Chorley, Lancashire, UK), the glass of the flow cell was gently cleaned to remove any blemishes, particles and/or fibres and salts residual from the storage buffer, whilst avoiding the rubber intake ports. This cleaning process was repeated until the flow cell was judged finally clean and suitable for sequencing. The flow cell, reagent cartridge and PR2 bottle were then transferred to the Illumina MiSeq ensuring that the waste bottle on the instrument was empty. The Sample Plate Map csv file was then transferred to the MiSeq operating system via a virus protected USB. On screen instructions on the Illumina MiSeq were followed to load each component, these being the Sample Plate Map csv file, reagent cartridge, flow cell and PR2 bottle. It was ensured that the Illumina MiSeq recognised the correct Sample Plate Map csv file and that all pre-run checks were correct before pressing start. Run monitoring was examined by the Illumina Sequence Analysis Viewer (on screen on instrument) and the cluster density and quality scores for clusters passing >85% filters monitored. At the end of sequencing, generated fastq files were then transferred by a virus free and password

protected USB for downstream bioinformatics processing by Mothur (Schloss *et al*., 2009) and R programming, examples of which are shown in Appendices 2 and 3.

2.4 Quantification of rotavirus A from porcine faecal samples using the Techne qPCR kit for rotavirus A.

2.4.1 Reagent reconstitution protocol

To minimise risk of contamination all procedures were carried out in a BSC. All consumables and equipment were irradiated with UV for 30 minutes and treated with RNase Zap. The qPCR kit was stored at -20°C. The kit contained components shown in Table 8. Tubes 1 and 3 were pulse-spun to ensure that lyophilised primers and probe mixes were in the base of each tube. These were reconstituted in 165 µL RNase/DNase free water as supplied. Each tube was vortexed thoroughly to ensure complete resuspension. Tube 4, the RNA internal extraction control had been previously reconstituted and added to samples for RNA extraction. Tube 2, the positive control RVA template was pulse-spun, reconstituted with 500 µL template preparation buffer (composition not available) and vortexed. This was performed in a separate PCR hood away from other equipment and components of the qPCR test to avoid contamination. The Lyophilised OneStep 2X RT-qPCR Master Mix (composition not available) was re-suspended in 525 µL resuspension buffer. Once re-suspended the OneStep 2X RT-qPCR Master Mix was stored at -20°C if needed.

2.4.2 One-step reverse transcription-PCR detection protocol

A one-step approach combining RNA reverse transcription and amplification in a single closed tube was used. All pipetting steps and the PCR plate set-up were performed on ice. For 50 viral RNA samples, the RNA extraction kit control and a negative control of RNAse/DNAse free water, a reaction mix was prepared according to Table 9.

Table 8. Techne qPCR kit for rotavirus A components
1) Rotavirus A specific primer/probe mix (150 reactions BROWN)
2) Rotavirus A positive control template (for Standard curve RED).
3) Internal extraction control primer/probe mix (150 reactions BROWN).
4) Internal extraction control RNA (150 reactions BLUE).
5) Template preparation buffer (YELLOW) for resuspension of positive control template and standard curve preparation.
6) Lyophilised OneStep 2x qRT-PCR MasterMix Containing complete one step qRT-PCR MasterMix and resuspension buffer
7) RNAse/DNAse free water (WHITE) for resuspension of primer/probe mixes and internal extraction control RNA

For 50 samples plus 10% tip wastage a total of 825 µL PCR reaction mix was needed. This was prepared by pipetting each component into a 1 mL micro tube. This was divided into eight aliquots of 103.1 µL by pipetting into each of eight PCR strip tubes (Roche Diagnostics GmbH, Mannheim, Germany) and 15 µL pipetted using a multichannel pipette into the sample and control wells on the 384 well PCR plate (Corning Incorporated, Salt Lake City, USA). Samples and controls 5 µL, were pipetted onto the PCR plate giving a total volume of 20 µL for each well.

Table 9. PCR reaction mix preparation for viral RNA samples

Component	**1 Sample**	**50 Samples**	**50 Samples plus 10%**
OneStep 2x qRT-PCR MasterMix (6)	10 µL	500 µL	550 µL
Rotavirus A primer/probe mix (1)	1 µL	50 µL	55 µL
Internal extraction control primer/probe mix (3)	1 µL	50 µL	55 µL
RNAse/DNAse free water (7)	3 µL	150 µL	165 µL
Final volume	**15 µL**	**750 µL**	**825 µL**

2.4.3 Preparation of the standard curve

In a separate PCR hood, 90 µL of template preparation buffer 5) was pipetted into five 2 mL micro tubes and labelled 2 to 6. Positive control template 10 µL, 2) was pipetted into tube 2 and vortexed thoroughly. The pipette tip was changed and 10 µL from tube 2 pipetted into tube 3 and vortexed thoroughly. This was repeated for tubes 4, 5 and 6 to complete the dilution series from 2 x 10^5 to 2 per µL copy number. A PCR reaction mix for 6 samples plus 10% tip wastage was prepared by pipetting 66 µL OneStep 2x qRT-PCR MasterMix 6), 6.6 µL rotavirus A primer/probe mix 1) and 26.4 µL RNAse/DNAse free water 7) into a 2 mL micro tube. PCR reaction mix, 15 µL was added to each of the six standard curve wells on the PCR plate followed by 5 µL of the standard template dilution series to give a final volume of 20 µL. The plate was sealed with adhesive tape. Plates were wrapped in aluminium foil (Kirkland Signature, Costco Wholesale UK Ltd., Watford, Hertfordshire UK) to avoid daylight interference with fluorescent qPCR chemistry.

2.4.4 Real-time quantitative PCR

Real-time quantitative PCR data were collected using the Roche LightCycler 480 (Hoffman La Roche, Basel, Switzerland). The amplification protocol was reverse transcription for 10 minutes at 42°C, enzyme activation for 2 minutes at 95°C, then 50 cycles of denaturation for 10 seconds at 95°C and fluorogenic data collection for 60 seconds at 60°C followed by one cycle of cooling. The detection format was dual colour hydrolysis/Universal Probe Library (UPL), with dynamic integration time mode and a filter combination of duplexing TaqMan probes, FAM (6- carboxyfluorescein) and VIC (2′-chloro-7′phenyl-1,4-dichloro-6-carboxy- fluorescein). Amplification curves were initially analysed using the LightCycler 480 Software release 1.5.0.39. as obtained from https://pim-eservices.roche.com/eLD/web/ accessed 20-02-20.

2.5 Enzyme linked immunosorbent assays for rotavirus IgG and IgA in sow colostrum

2.5.1 Determination of rotavirus specific IgG and IgA in sow colostrum

The Ingezim rotavirus porcine ELISA kit (Immunologia Y Genetica Aplicada S.A. Madrid, Spain) was used to determine specific anti-RVA IgG and anti-RVA IgA activity in colostrum samples. For the detection of anti-RVA IgA antibodies, ELISA was performed as with IgG, but the secondary antibody was substituted with peroxidase-labelled goat anti-porcine IgA (Thermo Fisher Scientific, Bonn, Germany) at a dilution of 1/10,000 as according to Kreuzer *et al*., 2012. To determine the optimum dilution for IgG determinations in the colostrum samples, seven test samples were diluted in dilution buffer (composition not available), from 1 in 1000, to 1 in 15,000 and assayed using the kit according to methodology below. Samples were chosen at random using https://www.random.org/ . Four samples were from control (non-GOS) sows and three samples were from GOS fed sows. The optimum dilution that ensured the maximum number of samples would be in range for optical density (OD) determination, was determined to be 1 in 10,000. Similarly, the optimum from IgA was determined to be 1 in 1000. The remaining samples were assayed in duplicate, using these dilutions, with OD measured at 450 nm and the blanks subtracted. The positive control serum supplied with the kit, was assayed on each occasion and the mean value from these measurements was used to obtain a normalised absorbance ratio to reduce assay-to-assay variation as in Ramanakumar *et al*., 2010.

2.5.1.1 Preparation of samples and reagents

Procedures were carried out in a BSC and reagents supplied in the kit unless otherwise stated. Sow colostrum samples were defrosted, and 1 mL aliquots centrifuged at 21°C and 13,000 g for 15 minutes to separate the fat from the

colostrum. Aqueous phase colostrum was pipetted from underneath the fat layer and into sterile 2 mL micro tubes for analysis. These samples were serially diluted to 1 in 10,000 for IgG and 1 in 1000 for IgA by consecutive 1 in 10 dilutions of 50 µL sample plus 450 µL diluent (composition not available) in 2 mL micro tubes. A 1 in 200 dilution of negative and positive control sera were made by diluting 5 µL of serum with 1 mL diluent and stored at -20°C. Conjugates were made immediately before use. IgG conjugate was diluted to 1 in 100 with diluent. IgA conjugate was diluted to 1 in 10,000 with diluent.

2.5.1.2 Assay procedure

One hundred µL of the diluted samples, negative and positive controls were pipetted onto the ELISA plate in duplicate. One hundred µL of diluent was added to two wells as blanks. The plate was sealed and incubated at 37°C for 30 minutes. The contents of the plate were "brusquely" thrown out to avoid mixing one plate well with another. The plates were washed by adding 300 µL diluted washing solution (composition not available) into each well using a wash bottle (Thermo Fisher Scientific, Loughborough UK), the plate shaken gently and then emptied as before. This was repeated three times and the plate inverted on absorbent paper. One hundred µL of diluted conjugate was added to each well and incubated at 37°C for 30 minutes. The plate was washed four times and inverted on absorbent paper. One hundred µL of 3,3′,5,5′- Tetramethylbenzidine (TMB – composition not available) solution was added to each well and the plate incubated in the dark at room temperature for ten minutes. One hundred µL of stop solution (composition not available) was added to each well and the plate transferred to the ELISA LT-4000 microplate reader for determination of absorbance at 450 nm (Labtech International Ltd., Ringmer, East Sussex, UK).

2.5.2 Determination of rotavirus total non-specific IgG and IgA in sow colostrum

2.5.2.1 Preparation of samples and reagents

Total non-specific IgG and IgA in colostrum were assayed using the IgG and IgA Pig ELISA Kits (Abcam plc, Cambridge, UK). An aliquot of 50 mL IgG or IgA pig diluent was diluted to 1 in 5 with 200 mL RO H_2O and stored at 4°C for use. The wash buffer 50 mL, (composition not available) was diluted to 1 in 20 with 950 mL RO H_2O and stored at 4°C. IgG and IgA pig HRP conjugate was prepared by adding 10 µL to 990 µL diluent (composition not available) for each 96 well plate. IgG standards ranging from 0.0 ng/mL to 1000 ng/mL and IgA standards ranging from 0.0 ng/mL to 200 ng/mL were used to prepare standard curves against which samples could be compared and the concentration of IgG and IgA calculated. A blank diluent control containing no IgG or IgA was also prepared. Samples were diluted to 1 in 10,000 by consecutive 1 in 10 dilutions of 50 µL sample plus 450 µL diluent in 2 mL micro tubes for IgG and 1 in 100,000 for IgA.

2.5.2.2 Assay procedure

All standards, controls and samples were assayed in duplicate. One hundred µL of each IgG and IgA standard, zero controls and samples were pipetted into 96 well microtiter plates and incubated at room temperature for 45 minutes. The contents were discarded, and wells completely filled with wash buffer and aspirated after shaking four times. Plates were sharply struck on absorbent paper to remove residual buffer. One hundred µL of diluted IgG or IgA pig HRP conjugate was added to each well and incubated at room temperature for fifteen minutes in the dark. Plates were then washed and blotted as before four times. One hundred µL TMB (composition not available) was pipetted into each plate well and incubated at room temperature for ten minutes in the dark. One hundred µL of stop solution

was added to each well and the absorbance determined at 450 nm using the ELISA LT-4000 microplate reader.

STUDY RESULTS

Study results for Chapters 3, 4 and 5 are presented as three published papers. Contributions were as follows:

Chapter 3. Common colonic community indicators of the suckling pig microbiota: All microbiota diversity, bioinformatics and statistical analyses. All programming, data analyses and validation. Writing the manuscript.

Chapter 4. Effects of prebiotic galacto-oligosaccharides in poorly performing pre-weaning piglets: Conceptualisation, sample collection and DNA extraction. PCR amplification of 16S rRNA gene sequences, sequencing and microbiota diversity analysis. Histological, bioinformatics and statistical analyses. All programming, data analyses and validation. Writing the manuscript.

Chapter 5. Effects of prebiotic galacto-oligosaccharides on RVA antibodies in sow colostrum, reduced infectivity and microbiome modulation in neonatal piglets: Conceptualisation and study design. ELISA for specific anti-RVA IgG and anti-RVA IgA in colostrum. All nucleic acid extractions and qPCR detection of RVA. PCR Amplification of 16S rRNA Gene Sequences, sequencing and microbiota diversity analyses. All bioinformatics and statistical analyses. All programming, data analyses and validation. Writing the manuscript.

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27th February 2023

Dear

Sir/Madam

Ref: Adam Lee

I am the primary supervisor of Adam Lee and this letter is to confirm his contribution to the three manuscripts in his thesis submitted March 2023.

Adam has been the primary driver in ALL aspects of the manuscripts presented including writing of the manuscripts. I have no reservations in stating that the work generated was developed during the course of his research and represents an original portfolio as defined by the University of Nottingham.

Kindest Regards,

Dr KH Mellits
Associate Professor in Virology and Cellular Microbiology
University of Nottingham

3 Chapter 3. Common colonic community indicators of the suckling pig microbiota

The primary objective of this study was to compare variation in the microbiota of suckling pigs from three separate trials conducted at different times of year and to determine if common colonic community indicators could be identified. Pig weights, ADG, bacterial diversity and abundance were not significantly different between repeated trials, except for a significant difference in Jaccard Similarity. Relative abundance of common colonic community indicators were the *Porphyromonadaceae* unclassified (15.81%), *Ruminococcaceae* unclassified, (12.78%), *Prevotella* (7.26%), *Clostridiales* unclassified (6.99%), *Lactobacillus* (6.58%), *Phascolarctobacterium* (6.52%), and *Firmicutes* unclassified (5.69%). Pig weight at day 22 and ADG positively correlated with α-diversity. Abundance of operational taxonomic units ascribed to *Terrisporobacter*, *Ruminococcaceae* unclassified, *Intestinimonas*, and *Dorea* correlated with weight and ADG. Apart from demonstrating that small-scale studies can be pooled, this study is one of the first to correlate animal performance with alpha diversity and the abundance of short-chain fatty acid producers.

The paper was peer reviewed and accepted for publication by "FEMS Microbiology Ecology", an official journal of the Federation of European Microbiological Societies and is accessible at: https://academic.oup.com/femsec/article/98/5/fiac048/6576765 in portable document format.

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Research Article

Common colonic community indicators of the suckling pig microbiota where diversity and abundance correlate with performance

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One sentence summary: Common colonic community indicators can be identified from suckling pigs in repeated trials without major significant differences in diversity, abundance, or microbiota composition.

Editor: Leluo Guan

Abstract

The primary objective of this study was to investigate if common colonic community indicators could be identified from the microbiota of 22-day-old suckling pigs in repeated small-scale trials. A total of three separate trials were conducted at different times in the same year and facility with genetically similar animals. Colonic samples were collected from four pigs in each trial and the micro- biome composition assessed by 16s rRNA gene sequencing. Pig weight, average daily gain (ADG), bacterial diversity, and abundance were not significantly different between repeated trials, except for a significant difference in Jaccard Similarity. At genus level, the most abundant taxa identified were *Porphyromonadaceae* unclassified (15.81%), *Ruminococcaceae* unclassified, (12.78%), *Prevotella* (7.26%), *Clostridiales* unclassified (6.99%), *Lactobacillus* (6.58%), *Phascolarctobacterium* (6.52%), and *Firmicutes* unclassified (5.69%). The secondary objective was to establish if pooled data in terms of microbial diversity and abundance of the colonic microbiota related to weight and ADG. Pig weight at day 22 and ADG positively correlated with α-diversity. Abundance of potential protein digesting and short-chain fatty acid producing operational taxonomic units ascribed to *Terrisporobacter*, *Ruminococcaceae* unclassified, *Intestinimonas*, and *Dorea* correlated with weight and ADG, suggesting a nutritional role for these common colonic community microbiota members in suckling pigs.

Keywords: microbiota, pigs, suckling, colonic, diversity, performance

Introduction

Suckling is a unique period in Porcine development, where the early-life environment significantly affects the development and composition of the adult microbiota. The gastrointestinal tract (GIT) microbiota, contribute to the developmental and metabolic needs of animals through vitamin synthesis, short-chain fatty acid (SCFA) production, complex carbohydrate digestion, and immune system regulation (Brestoff and Artis 2013; Kim and Isaacson 2015). In neonatal pigs, development of the intestinal microbiota is a gradual and sequential process (Inoue *et al*. 2015) in which the GIT is colonized by bacteria from maternal, and environmental sources (Katouli *et al*. 1997; Konstantinov *et al*. 2006; Thompson *et al*. 2008). During suckling, the formation of an increasingly differential, milk-oriented and protective *Lactobacillaceae* rich microbiota is favoured (Mulder *et al*. 2009; Petri *et al*. 2010; Frese *et al*. 2015; Bian *et al*. 2016). This is a unique period in porcine development, where acquisition of maternal immunity (Salmon *et al*. 2009) and the early-life environment heavily influences the development and composition of the adult microbiota and intestinal innate immune functions (Bauer *et al*. 2006; Mulder *et al*. 2009; Merrifield *et al*. 2016). In addition, GIT microbiota diversity may be predictive of the susceptibility of the animals to enteric disease postweaning (Dou *et al*. 2017). Indeed, diversity at weaning might not be an accurate predictor of diversity in later life, but earlier measures preweaning, may be more predictive (Lu *et al*. 2018). The abundance and diversity of the pig GIT microbiota increases with age (Niu *et al*. 2015; Chen *et al*. 2017), with operational taxonomic units (OTUs) ascribed to *Lachnospiraceae*, *Ruminococcaceae*, *Prevotella*, *Treponema*, and *Bacteroides* showing association with fatness in older pigs (He *et al*. 2016). It has also been shown that piglets with above average daily gain (ADG) had significantly higher abundances of *Lactobacillus*, unclassified *Ruminococcaceae*, and unclassified *Prevotella* (Gaukroger *et al*. 2020), and that microbial richness positively correlated with weight gain in preweaning pigs (Ding *et al*. 2019), thus indicating the link between microbiota composition and performance. Considering the profound influence of weaning weight on the lifetime growth and health performance (Collins *et al*. 2017), there is a lack of information on performance and the association between microbial diversity and abundance in the suckling pig. There are multifactorial influences on microbial diversity and composition, these being succession of bacterial populations, the age of the animal, the environment it inhabits, use of antimicrobial agents, dietary composition, stress, and genetics, to name but a few (Pluske *et al*. 2018).

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Given these influences and large variations between studies (Holman *et al*. 2017), there has been little discussion of the use of data between separate trials that have been repeated over time with the prospect of data pooling (Thompson *et al*. 2008; Pajarillo *et al*. 2015; Chen *et al*. 2017). The availability of controlled rearing environments with large animals and the incumbent costs to achieve reproducibility are important design considerations, which may dictate that small-scale trials are repeated to achieve statistical significance, in contrast to Murine models, which accommodate much larger study populations through ease of animal size and housing. Moreover, animal studies often pool data from repeated trials without due consideration of variance between trials and if they can be truly compared, especially for control data (Frommlet and Heinze 2021). In this study, we have attempted to reproduce three suckling pig trials in which multifactorial influences, as described above, on the microbiota were controlled for as far as possible. Not all variables can be controlled, e.g. succession of bacterial populations. However, examining animals of the same age, similar genetic traits, reproducing environmental and housing conditions, diets, and reducing physical contact between animals may allow studies to be compared. Indeed, long-term cohousing increases the similarity of pig faecal microbiota (Pajarillo *et al*. 2015) and there is a significant correlation between the intestinal microbiota of cohoused pigs particularly at 3–4 weeks postpartum (Thompson *et al*. 2008). We have attempted to reduce these pen effects in this study since samples relate to independently housed suckling pigs (n = 12 litters where "n" is the pen) rather than cohoused animals who might influence each other's microbiota as in other studies (Chen *et al*. 2017). In these respects, the objectives of this study were to investigate if common colonic community indicators could be identified from the microbiota of suckling pigs in repeated small-scale trials and if pooled data in terms of microbial diversity and abundance of the colonic microbiota related to animal performance in three controlled reproducible trials.

Materials and methods

Animals and trial design

This study was carried out under license and in accordance with UK Animals, (Scientific Procedures) Act 1986. All procedures were approved by the Local Ethics Committee of the University of Nottingham.

For all trials, A, B, and C, Landrace x Large white sows of parity seven were artificially inseminated with the same batch of Titan semen (JSR Genetics, Driffield, UK). Titan semen was pooled from three sibling boars bred from the same Piétran line and selected for similar breeding traits, as per industry standard by the supplier. On day 113 of gestation, sows were moved to individual 3.8 × 2.4 m farrowing pens with a 2.1 × 0.62 m farrowing crate (Figure S1, Supporting Information). Animals were housed in a single facility in separate pens. Pens were of solid concrete block construction with 1.5 m high walls. There were no apertures through which animals could physically contact one another through pens. Each pen was provided with its own colour coded tools for cleaning so as not to cross-contaminate pens. Pens had two secure lockable metal gates at opposite ends. One for allowing feeding of sows without technicians standing on bedding and one for removing contaminated bedding into a concrete corridor for disposal. This area was 60 cm lower than the base of the pen so that any "run off" could not contaminate other pens. The solid concrete construction of pens allowed no egress of contaminated bedding or fluid "run off" between pens. Technicians wore disposable gloves, facemasks, overshoes, and suits when cleaning pens and attending to animals. These were changed when attending to different pens. Trials were conducted during A; January–February, B; April–May, and C; October–November 2010 with four litters per trial kept under identical housing and environmental conditions. Pens were deep cleaned with Virkon between trials (VWR International Ltd, Lutterworth, UK). Pens were not used for any other experiments in between trials. Farrowing pens contained a 1 × 1 m piglet box heated with an industry standard heat lamp. Animals were bedded on a mixture of dust extracted straw and hemp bedding (Aubiose, Datesand Ltd., Stockport, UK) on concrete in farrowing pens and on straw, on plastic slats, and in weaning pens. Bedding was obtained from the same source for all three trials. Metal chain toys with plastic balls were provided in weaning pens as environmental enrichment. Temperature was kept at range 18–20°C for sows and 23–24°C for piglets with light periods from 7:30 a.m. to 7:00 p.m. Sows received a wheat-based lactation diet (BOCM Pauls ltd, Wherstead, UK) containing 16% protein, 4.5% oil, 5.5% crude fibre, 5.5% ash, 0.75% lysine, 1000 iu.kg^{-1} vitamin A, 2000 iu.kg^{-1} vitamin D3, 100 iu.kg^{-1} vitamin E, 0.40 mg.kg^{-1} selenium, and 25 mg.kg^{-1} copper, plus water *ad libitum*. For prevention of iron deficiency and coccidiosis, new-born pigs received a 1-ml intramuscular iron injection (Gleptosil, Alstoe Ltd, York, UK) 24 hours after birth, 0.7 ml of Baycox toltrazuril coccidiostat (Bayer, Newbury, UK) orally 3 days after birth and were ear tagged at day 5 for identification. Pigs did not receive any creep feed supplementation or any other prophylactic antibiotic treatment during the trials. Pigs were cross-fostered within 24 hours of birth to achieve homogenous litter size for welfare purposes and as per standard industry practice. However, cross-fostered pigs were excluded from euthanasia for collection of colonic samples. Not all pens had cross-fostered piglets. In Trial A, pen 2 had two cross-fostered pigs. Pens 1–4 contained 12, 13, 12, and 13 pigs, respectively. In Trial B, pen 4 had two cross-fostered pigs. Pens 1–4 contained 11, 12, 11, and 12 pigs, respectively. In Trial C, pen 1 had two cross-fostered pigs and pen 2, one cross-fostered pig. Pens 1–4 contained 10, 10, 10, and 12 pigs, respectively. From 24 hours of birth (post cross-fostering), to day 22 of sampling, there was no contact between litters and sows of different pens. Pigs were individually weighed at days 5, 12, 19, and 22 to determine ADG, with one pig per litter randomly selected at day 22 for euthanasia by intraperitoneal injection of Dolethal (1 ml kg^{-1} body weight; 20% w/v Pentobarbitone Sodium, Vétoquinol, Buckingham, UK).

Sample collection and DNA extraction

Samples of digesta from euthanized pigs were aseptically collected from the colonic lumen and held on ice for 5 minutes prior to transfer to the laboratory and storage at −80°C until bacterial DNA isolation. Bacterial DNA was isolated from ~0.2 g colonic contents using the NucleoSpin Tissue Kit (Macherey-Nagel GmbH & Co. KG., GER) according to the manufacturer's instructions.

PCR amplification of 16S rRNA gene sequences

Using the isolated DNA as a template, the V4 region of the bacterial 16S rRNA genes were PCR amplified using primers 515f (5ᵗ GTGCCAGCMGCCGCGGTAA 3ᵗ) and 806r (5ᵗ GGACTACHVGGGTWTCTAAT 3ᵗ; Caporaso *et al*. 2011). Amplicons were sequenced on the Illumina MiSeq platform (Illumina, Inc., USA) using 2 × 250 bp cycles by LGC Genomics GmbH (GER). Sequence data were deposited in the NCBI database within the Bioproject PRJNA494528 under the SRA study SRP164374.

Microbiota diversity analysis

The 16S rRNA sequence analysis was performed using Mothur v. 1.39, using default settings (Schloss *et al*. 2009). Analysis was performed according to the MiSeq SOP (accessed online 09/11/2017; Kozich *et al*. 2013). The 16S rRNA gene sequences were aligned against a reference alignment based on the SILVA rRNA database (Pruesse *et al*. 2007) for use in Mothur (release 128; available at: https://www.mothur.org/wiki/Silva_reference files) and clustered into OTUs using the "opticlust" clustering algorithm (Westcott and Schloss 2017). The similarity cut off for OTUs was 0.03. The consensus taxonomy of the OTUs was generated using the "classify.otu" command in Mothur with reference data from the Ribosomal Database Project (version 14; Wang *et al*. 2007; Cole *et al*. 2014) adapted for use in Mothur (available at: https://www.mothur.org/wiki/RDP_reference_files).

Statistical analyses

Coverage and α-diversity expressed as Inverse Simpson diversity (Magurran 2004) and Chao Richness (Chao 1984) were calculated using the "summary.single" command in Mothur (Schloss *et al*. 2009). Quantile plots and Shapiro–Wilk tests (Shapiro and Wilk 1965) were used to determine normality for pig weights at days 5, 12, 19, and 22, ADG and α-diversity metrics. Significant differences were tested using ANOVA in R Studio (v4.1.1) with repeated measures for weight (R Core Team 2021). Estimates of β-diversity were calculated in Mothur as Yue and Clayton Dissimilarity (θ_{YC}; Yue and Clayton 2005), Bray–Curtis Dissimilarity (Bray and Curtis 1957) and Jaccard Similarity (Jaccard 1901). Homogeneity of variance for all three β-diversity metrics were analyzed by the Levene test (Levene 1960) using the "Car" package (v3.0-11) in R Studio. Analysis of molecular variance executed in Mothur (AMOVA) was used to test for differences in β-diversity between samples (Excoffier *et al*. 1992; Anderson 2001). Similarity Percentage (SIMPER) analysis was used to identify OTUs that most contributed to Bray–Curtis β-diversity measures (Clarke 1993) as performed in the "Vegan" Community Ecology Package (v2.4-3) in R Studio (Oksanen *et al*. 2017). Linear discriminant analysis effect size (LEfSe) was used to examine differential OTU abundances in Mothur (Segata *et al*. 2011). The abundance of phyla and OTUs at the genus level were analyzed by Kruskal–Wallis rank sum tests (Kruskal and Wallis 1952) to determine differences between trials. Correlations between pig weights, ADG, abundance of phyla, and OTUs at the genus level were analyzed by Kendall rank sum correlations with regression analysis performed using linear modelling in R as previously reported (Dill-McFarland *et al*. 2017). Pig weights at day 22, ADG, and α-diversity were correlated using Pearson's Product-Moment Correlation, with regression analysis performed using linear modelling in R Studio. Where appropriate, multiple comparisons (ANOVA and AMOVA) were adjusted for false discovery rates (FDR) by the Benjamini and Hochberg procedure (Benjamini and Hochberg 1995).

Results

Weight and ADG distribution

Pig weights at days 5, 12, 19, and 22 and ADG were normally distributed according to quantile plots, R^2 = 0.96, 0.96, 0.95, 0.91, and 0.94, respectively and Shapiro–Wilk normality tests, P = .64, .78, .51, .27, and 0.42, respectively. A total of four pigs each from separate litters in their own pens were analyzed for each trial, A, B, and C. Weights at days 5, 12, 19, and 22 and ADG were not significantly different between trials A, B, and C; P = .92, .92, .78, .84, and .79, respectively (ANOVA; Table 1). Weight significantly increased with time where D5–D12 P = .002, D12–D19 P < .001, and D19–D22 P < .001 (adjusted).

Colonic microbiota diversity

A total of 357 133 high quality V4 16S rRNA sequence reads were obtained from twelve suckling pig colonic microbiota samples, from which 8718 sequences per sample were subsampled to achieve a coverage of 97%–99%. Inverse Simpson diversity and Chao Richness were normally distributed according to quantile plots, R^2 = 0.90 and 0.97, respectively and Shapiro–Wilk normality tests, P = .21 and P = .88, respectively. Inverse Simpson diversity and Chao Richness were not significantly different between trials A, B, and C, P = .70 and P = .10, respectively (ANOVA; Table 1). Calculated β-diversity θ_{YC} and Bray–Curtis distances between trials A, B, and C were not significantly different, as determined by AMOVA, P = .586 and P = .109, respectively. Jaccard distances were significantly different for the overall comparison of trials A, B, and C, P = .008, but not for pairwise comparisons A–B, P = .07, A–C, P = .09, or B–C, P = .09 (Fig. 1). There were no significant differences in homogeneity of variance between trials for all three β-diversity metrics when analyzed by the Levene test, P > .05 in each case.

Colonic microbiota composition

Sequences were clustered into 4520 OTUs and classified into 18 phyla, 35 classes, 54 orders, 108 families, and 214 genera. Of these, 4132 OTUs occurred in colonic samples from all trials at the genus level. The remaining OTUs were exclusive to colonic samples from pigs in trials A (112), B (104), and C (64; Fig. 2). Relative abundances of bacterial taxa at the phylum and genus level for colonic samples from the three separate trials are shown in Fig. 3. The predominant phyla were *Firmicutes* (55.68%), *Bacteroidetes* (33.68%), *Proteobacteria* (1.64%), and *Spirochaetes* (1.37%). Unclassified bacteria accounted for 6.22% of the total sequences. There were no significant differences in phyla abundance between trials when analyzed by Kruskal–Wallis rank sum tests, P > .05. At the genus level, the most abundant taxa identified were *Porphyromonadaceae* unclassified (15.81%), *Ruminococcaceae* unclassified, (12.78%), *Prevotella* (7.26%), *Clostridiales* unclassified (6.99%), *Lactobacillus* (6.58%), *Phascolarctobacterium* (6.52%), and *Firmicutes* unclassified (5.69%). The top 30 OTUs accounted for 95.69% of total relative abundance, in contrast to the remaining 4490 OTUs, which accounted for the remaining 4.31% total relative abundance indicating the nonparametric and skewed distribution of OTUs identified.

OTUs contributing to variation in the Bray–Curtis dissimilar- ity indices were identified by analysis of similarity percentages (SIMPER). For trial comparison A–B, 49 OTUs contributed up to 70.08% of the variation, for comparison A–C, 46 OTUs contributed up to 70.27% of the variation, and for comparison B–C, 47 OTUs contributed up to 70.10% of the variation. Overall, 65 different OTUs representing 26 genera contributed up to 70% variation across all three trials (Figure S2, Supporting Information). In order of rank, the top 10 OTUs contributing the most variance between trials A, B, and C were the *Porphyromonadaceae* unclassified (12.27%–15.91%), *Prevotella* (0.84%–9.18%), *Ruminococcaceae* unclassified (4.15%–9.16%), *Lactobacillus* (4.71%–8.38%), *Phascolarctobacterium* (3.15%–4.68%), *Clostridiales* unclassified (2.65%–5.16%), *Oscillibacter* (0.75%–2.37%), *Bacteroidetes* unclassified (1.12%–2.29%), *Firmicutes* unclassified (2.15%–5.16%), and *Faecalibacterium* (0.00%–2.11%). In addition, relative abundance of OTUs grouped into genera positively correlated with variance for each trial comparison

Table 1. Pig weights, ADG, and α-diversity of colonic samples.

[1]Trial	[2]Weight at day 5 (kg)	[2]Weight at day 12 (kg)	[2]Weight at day 19 (kg)	[2]Weight at day 22 (kg)	[2]ADG (d5–d22) kg/day	[2]Inverse Simpson Diversity	[2]Chao Richness
A	2.48 (0.27)	4.58 (0.42)	6.97 (0.60)	7.75 (0.43)	0.31 (0.02)	14.29 (2.20)	1241.08 (171.68)
B	2.34 (0.52)	4.38 (0.87)	6.40 (1.36)	7.38 (1.63)	0.30 (0.07)	17.97 (12.00)	1794.48 (250.51)
C	2.45 (0.46)	4.43 (0.50)	6.57 (0.86)	7.25 (0.75)	0.28 (0.04)	20.20 (7.94)	1679.88 (395.45)

[1]Trial A conducted January–February, B April–May, and C October–November 2010.
[2]Values are means (SD). Means are not significantly different between trials (ANOVA, $P = .92$, $P = .92$, $P = .78$, $P = .84$, $P = .79$, $P = .70$, and $P = .10$, respectively). Mean weight significantly increased with time (ANOVA, D5–D12 $P = .002$, D12–19 $P < .001$, and D19–D22 $P < .001$).

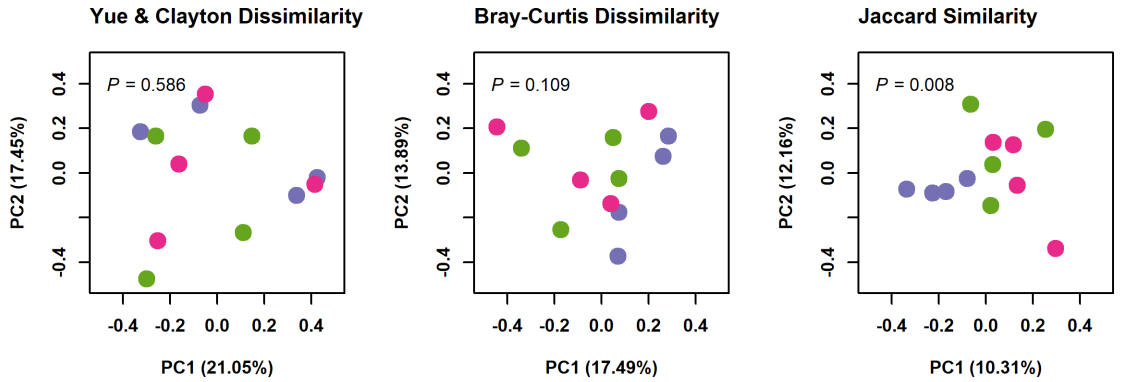

Figure 1. β-diversity of colonic samples from 22-day-old suckling pigs in three separate trials conducted at different times of year. Purple circles Trial A, pink Trial B, and green Trial C.

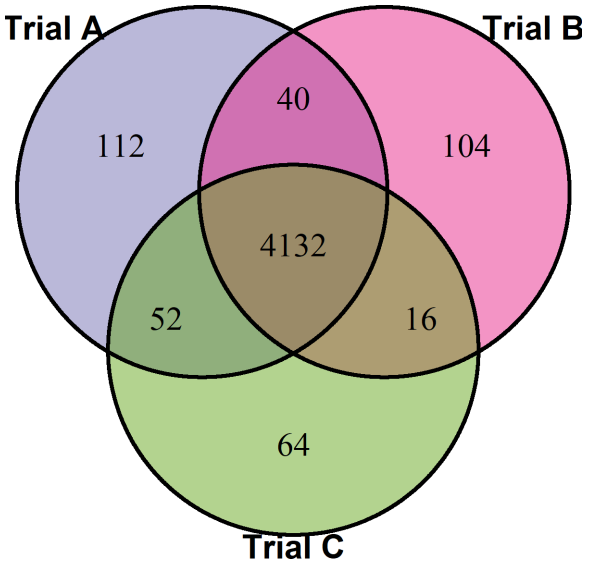

Figure 2. Venn diagram depicting unique and shared OTUs at the genus level in colonic samples from pigs in three separate trials, A, B, and C.

A–B, A–C, and B–C, $P < .001$ in each case. That is, the greater the relative abundance of named genera, the greater the variance lent to Bray–Curtis diversity measures (Figure S3, Supporting Information). Unclassified bacteria accounted for 2.57%–5.27% of the variation between trials. A total of 20 OTUs were identified by SIMPER as contributing variation to each trial comparison A–B, A–C, and B–C. However, their abundance across all three trials, A, B, and C was not significantly different following Kruskal–Wallis rank sum tests, $P > .05$ in each case. LEfSe did not identify differentially abundant OTUs occurring at ≥ 1% between trials with the exception of a greater abundance of one unclassified OTU at the genus level from trial A, $P = .01$.

Performance and diversity

Suckling pig weight at day 22 and Inverse Simpson Diversity correlated, where $r = 0.62$ (Pearson Correlation Coefficient), $R^2 = 0.38$ and $P = .032$ (linear modelling). Similarly, ADG and Inverse Simpson Diversity correlated, where $r = 0.59$, $R^2 = 0.35$, and $P = .042$ (Fig. 2). However, there was no correlation between weight at day 22 and Chao Richness where $r = 0.16$, $R^2 = 0.03$, and $P = .62$ or correlation between ADG and Chao Richness where $r = 0.10$, $R^2 = 0.01$, and $P = .75$ (Fig. 4).

Performance and abundance

Suckling pig weights at day 22 and ADG were correlated with abundance of phyla and OTUs at the genus level using Kendall rank correlations and assessed by subsequent linear modelling. The abundance of phyla did not correlate with weight or ADG where $P > .05$ in each case. However, the abundance of four OTUs at the genus level showed positive correlations with weight: *Terrisporobacter,* (Kendal Tau $\tau = 0.67$, $R^2 = 0.40$, and $P = .046$), *Ruminococcaceae* unclassified, ($\tau = 0.44$, $R^2 = 0.34$, and $P = .046$), *Intestinimonas,* ($\tau = 0.44$, $R^2 = 0.54$, and $P = .02$), and *Dorea*, ($\tau = 0.41$, $R^2 = 0.58$, and $P = .017$). A total of two OTUs at the genus level showed positive correlations with ADG: *Intestinimonas,* ($\tau = 0.27$, $R^2 = 0.53$, and $P = .024$) and *Dorea*, ($\tau = 0.36$, $R^2 = 0.51$, and $P = .024$; Fig. 5).

Discussion

The primary objective of this study was to compare variation in the microbiota of suckling pigs from three separate trials conducted at different times of year and to determine if common

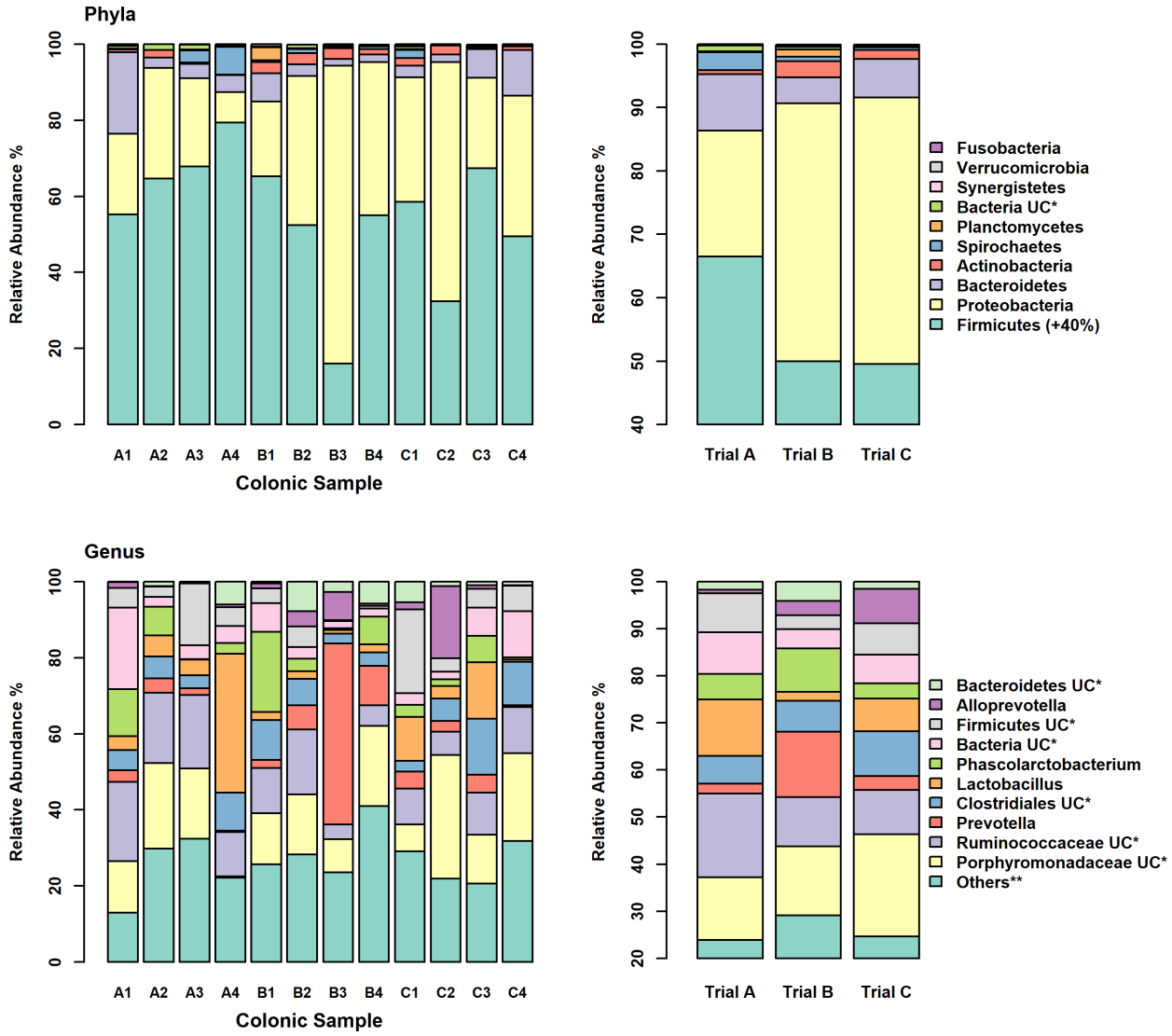

Figure 3. Relative abundance of bacterial taxa annotated to OTUs at the phyla and genus level as identified from colonic samples of 22-day-old suckling pigs in three separate trials conducted at different times of year. *UC = unclassified at the phylum or genus level. **Others = remaining 4490 OTUs comprising 4.31% of the total relative abundance.

colonic community indicators could be identified. Previous studies have analyzed pooled data from separate trials to establish community trends (Thompson *et al*. 2008; Kim *et al*. 2011; Pajarillo *et al*. 2015; Chen *et al*. 2017). This study has verified this approach with suckling pigs and demonstrated that whilst intertrial variation including significant differences in Jaccard Similarity exist, data may still be analyzed and compared to establish community relationships with attendant gains in statistical power. Whilst it is generally accepted that pig microbiota from different groups converge to a similar state over time (Kim *et al*. 2011; Bian *et al*. 2016) the variation needs to be more closely examined. In this study, each colonic sample was taken from suckling pigs in separate pens born from different sows. Cross-fostering for welfare issues may have introduced microbiota from other pens within the first 24 hours, but was limited to only one pen in trials A and B and two pens in Trial C. After this time, there was no physical contact between pigs in different pens. That is, neither sow nor piglet could influence one another across samples except for the direct effect of the nursing sow on the suckling piglet. This was probably the most influential factor for the development of neonatal bacteria during suckling (Bian *et al*. 2016). Other factors include the immediate early-life environment and the genetic background of the animals (Mulder *et al*. 2009; Merrifield *et al*. 2016), which were replicated, as far as possible, in these trials through use of the same facility, breed of sows, and batch of semen for artificial insemination. Long-term cohousing increases the similarity of pig faecal microbiota (Pajarillo *et al*. 2015), and there is a significant correlation between the intestinal microbiota of cohoused pigs particularly at 3–4 weeks postpartum (Thompson *et al*. 2008). These effects have been reduced in this study since samples relate to independently housed suckling pigs (n = 12 litters where "n" is the pen) rather than cohoused animals who might influence each other's microbiota as in other studies (Chen *et al*. 2017).

Results show that suckling pig weights at days 5–22 and ADG were normally distributed and not significantly different between trials indicating that animals could be compared. Furthermore, Inverse Simpson diversity and Chao Richness were normally distributed with no significant differences between trials (Table 1). In a meta-analysis of 91 pig colonic samples mean (SD), Inverse Simpson Diversity was reported to be 33.2 (22.6; Holman *et al*.

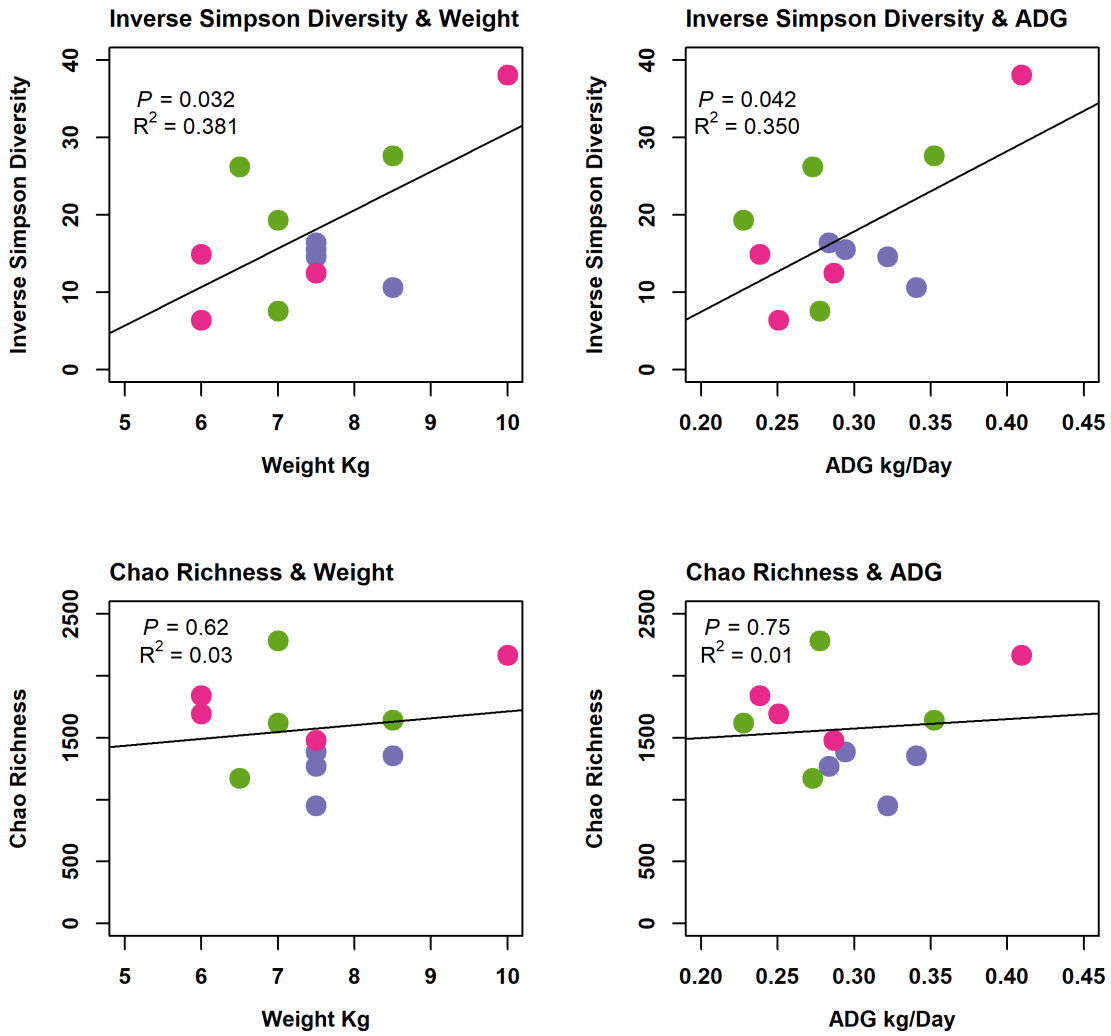

Figure 4. Correlations between performance and α-diversity of colonic samples from 22-day-old suckling pigs in three separate trials A, B, and C conducted at different times of year. Purple circles Trial A, pink Trial B, and green Trial C.

2017). However, these samples included older pigs, where the abundance and α-diversity of gut microbiota are known to significantly increase with age (Kim *et al*. 2011; Niu *et al*. 2015; Chen *et al*. 2017). Chao Richness results of other comparable studies with 21-day-old pigs are more variable, presumably due to differences in environmental conditions and/or breeds (Thompson *et al*. 2008; Bian *et al*. 2016). At suckling, Chao Richness was determined as 290.0 (Vo *et al*. 2017) and 1240.3 (Holman and Chénier 2014) for faecal samples and 1757 for colonic samples (Hoeflinger *et al*. 2015), the latter two in agreement with the present findings (Table 1). However, α-diversity metrics are highly dependent on the region sequenced, sequencing technology, depth, quality control postsequencing, and the reference database used. Thus, comparisons between studies may be confounded and difficult to compare.

β-diversity was modelled as Yue and Clayton Dissimilarity (θ_{YC}; Yue and Clayton 2005), Bray–Curtis Dissimilarity (Bray and Curtis 1957), and Jaccard Similarity (Jaccard 1901), with the model of best fit being θ_{YC} which explained 39% of the variance between trials in two dimensions. AMOVA of the θ_{YC} and Bray–Curtis metrics indicated no significant differences between trials, both metrics taking account of presence and abundance of OTUs. In contrast, there were significant differences between trials when using Jaccard Similarity as one of three metrics for analysis by AMOVA where $P = .008$ for overall comparisons. This metric compares samples based on the presence or absence of species and has revealed differences in colonic microbial community structure mainly between Trial A and Trials B and C. That is, Trial A was less similar to trials B and C, which had a greater similarity to each other (Fig. 1) in terms of species richness. This may be due to the greater abundance of *Proteobacteria* in colonic samples from trials B and C in contrast with Trial A as seen in Fig. 3. Nevertheless, community membership of the faecal microbiota, as measured by Jaccard Similarity and community structure as measured by θ_{YC}, significantly differ with pig age, underlying the importance of repeating studies with pigs of the same age if trials are to be compared (Slifierz *et al*. 2015). Likewise, interindividual Bray–Curtis distances between different pigs increased significantly during the suckling period and reduced postweaning, with no significant differences noted between two replicated trials (Chen *et al*. 2017).

In this study, a total of 4132 OTUs (91.4%) occurred in pigs in all trials A, B, and C, with *Firmicutes* and *Bacteroidetes* the dominant phyla, a result in keeping with previous studies of similarly aged suckling pigs for colonic (Jacobi *et al*. 2016; Zhang *et al*. 2016; Leblois *et al*. 2017) and faecal samples alike (Kim and Isaacson 2015; Chen *et al*. 2017; Vo *et al*. 2017). The fourth most abundant phylum were the *Proteobacteria* (1.64%), which are known to in-

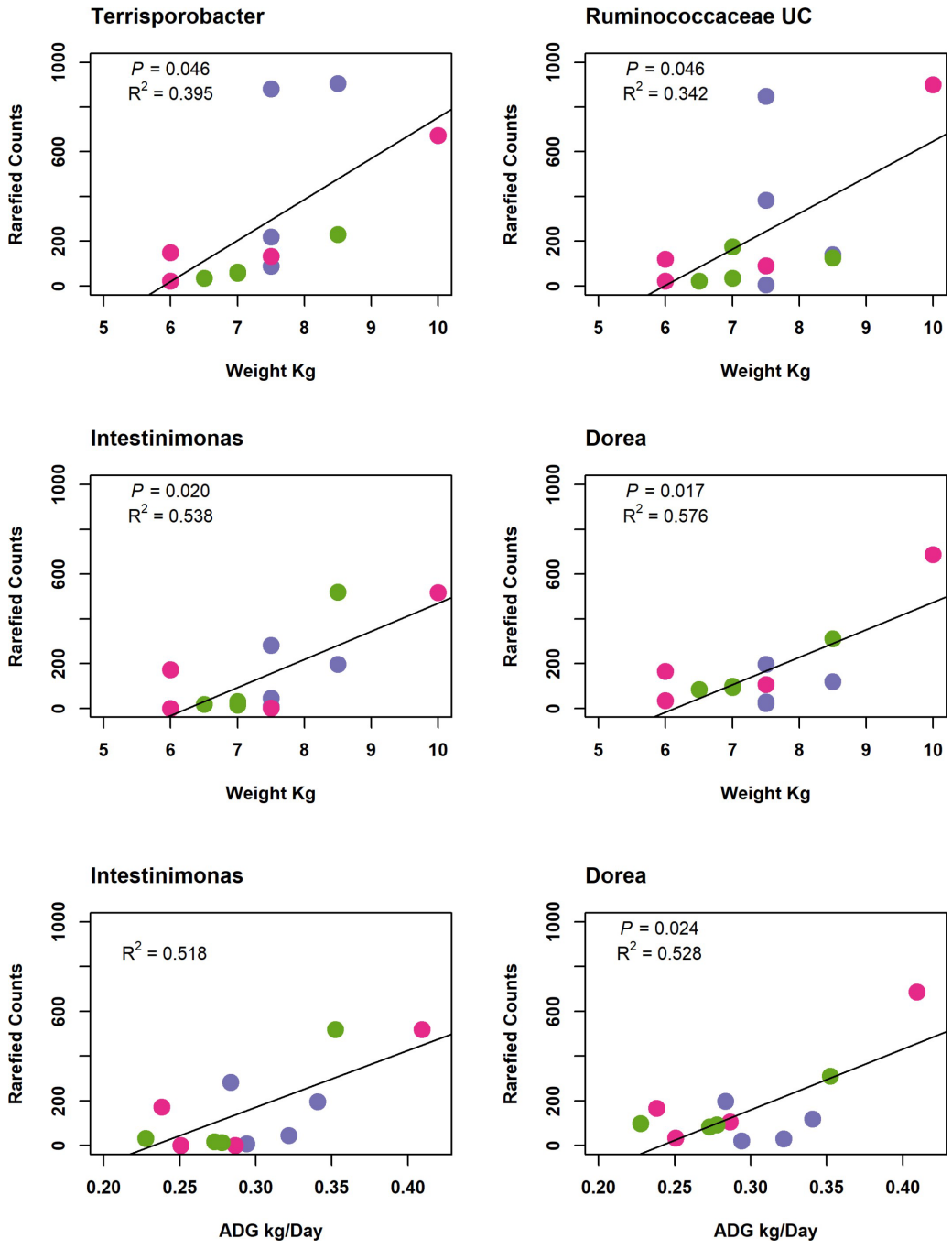

Figure 5. Correlations between performance and abundance of OTUs at the genus level of colonic samples from 22-day-old suckling pigs in three separate trials conducted at different times of year. Purple circles Trial A, pink Trial B, and green Trial C.

clude a wide variety of opportunistic, potentially pathogenic organisms such as *Campylobacter*, *Escherichia*, *Salmonella*, and *Helicobacter* (Madigan 2018). Although these OTUs occurred at very low relative abundances and may be commensal, their presence highlights the potential for the development of gut dysbiosis and the importance of a diverse microbiota at this stage of life.

Overall, there were no significant differences in the abundance of phyla between separate trials, further indicating the similarity of microbiota between trials. At the genus level, the most abundant taxa annotated to OTUs were the *Porphyromonadaceae* unclassified, *Ruminococcaceae* unclassified, *Prevotella*, *Clostridiales* unclassified, *Lactobacillus*, *Phascolarctobacterium*, and *Firmicutes* unclassified, which have been identified as predominant taxa in colonic (Hoeflinger *et al*. 2015; Zhang *et al*. 2016; Leblois *et al*. 2017) and faecal samples (Jacobi *et al*. 2016; Vo *et al*. 2017; Gaukroger *et al*. 2020) from preweaning pigs of a similar age to this study. Notably, these taxa were responsible for contributing the most variation between trials as analyzed by SIMPER (Figures S2 and S3, Supporting Information), but there were no significant differences in the abundance of the OTUs identified as contributing variation to each trial comparison. Neither did LEfSe identify differentially abundant OTUs occurring at ≥ 1% in each trial, except for a greater abundance of one unclassified OTU at the genus level in trial A, $P = .01$.

The secondary objective of this study was to determine if there were any associations between performance, microbial diversity, and abundance using pooled data from each trial. High diversity of GIT microbiota is considered beneficial for pig health (Gresse *et al*. 2017) and α-diversity expressed as Inverse Simpson Diversity correlated with weight at day 22 and ADG ($P = .032$ and $P = .042$, respectively). There were no correlations between performance and Chao Richness. This is probably explained by the difference in the two metrics used. Chao Richness estimates the total number of species, whereas Inverse Simpson Diversity estimates both richness and abundance of species (Morris *et al*. 2014). Thus, richness and abundance are possibly both factors relating to performance of suckling pigs of 22-days of age in this study. In contrast, α-diversity expressed as richness, evenness, and Shannon index were found not to be significantly different in colonic samples taken from low and high weight gain suckling piglets (Morissette *et al*. 2018). However, Shannon index was strongly correlated with back fat thickness and ADG in 15-week-old pigs (Lu *et al*. 2018), whereas abundance-based coverage and Chao richness estimators were significantly higher for weaned heavy pigs (~19 kg) compared with light pigs (~10 kg), but not Shannon and Simpson diversity indices (Han *et al*. 2017). These variations probably reflect the different ages of animals, environments, breeds, and evolution of the microbiota over time (Thompson *et al*. 2008; Kim *et al*. 2011; Bian *et al*. 2016).

This study identified four taxa from colonic samples, corresponding to OTUs at the genus level, which positively correlated with pig weight at day 22 and ADG. These were the *Ruminococcaceae* unclassified, *Terrisporobacter, Dorea*, and *Intestinimonas*, all of the order *Clostridiales* (Fig. 5). *Ruminococcaceae* are found in abundance in faecal samples from suckling pigs (Dou *et al*. 2017; Vo et al 2017) with their relative abundance positively linked to milk fat content of the nursing sow (Bian *et al*. 2016), better growth rates (Mach *et al*. 2015), and higher weight gain during the lactation period (Morissette *et al*. 2018). In adult pigs, OTUs annotated to *Ruminococcaceae* showed positive associations with fatness traits (He *et al*. 2016). *Ruminococcaceae* produce butyrate (Onrust *et al*. 2015), which is trophic to the colonic epithelium (Scheppach *et al*. 2001), their abundance in suckling pigs providing higher energy harvesting, prevention against pathogen infection and higher weight gain (Dou *et al*. 2017). *Terrisporobacter* is found in the ileum of adult pigs (Quan *et al*. 2018) and is a member of the family *Peptostreptococcaceae*, which are abundant in the GIT of suckling and weaning pigs (Li *et al*. 2017). These bacteria produce SCFAs from protein (Zhou *et al*. 2016) and their abundance in the GIT has been positively correlated with adult pig weight gain (Kim et al. 2016). *Dorea* belongs to the Lachnospiraceae family and ferments dietary carbohydrates to SCFAs (Vacca et al. 2020). *Intestinimonas* is a recently described bacterial genus with representative strains present in the GIT of humans and animals that produces SCFAs (Bui *et al*. 2016). Given that sows colostrum contains approximately 16% protein during the first 12 hours after parturition and milk 6%–7% 36 hours thereafter (Krogh *et al*. 2015), *Intestinimonas* and other protein fermenting, SCFA producers may have a nutritional role in the early, preweaning, suckling pig GIT that affects weight gain and development of the microbiota in later life and, in this respect, further research is required in the suckling pig. Notwithstanding the findings of this study, there are limitations. A larger sample size would have been preferable, but as discussed, this may be hard to achieve with large animals. Furthermore, only control samples and for one time-point have been analyzed. Future work may seek to address this by examining samples from test animals, e.g. those fed prebiotics and sampling postweaning to verify if common indicators can be identified across repeated trials in these conditions.

Conclusions

Reproducible small-scale suckling pig trials can be conducted in controlled environmental conditions, at different times of year without major differences in diversity, colonic microbiota composition, or OTU variation, except for a significant difference in Jaccard Similarity indicating species difference between trials. Regardless of intertrial variation, common colonic community indicators can be identified across repeated trials where pooling data supports the identification of performance related colonic microbiota. Correlations between α-diversity and performance show the abundance of common OTUs across trials are factors in the development of the suckling pig microbiota and weight gain. Correlation of the abundance of OTUs that relate to bacteria capable of protein digestion and SCFA production with performance, suggests a nutritional role for these community microbiota members in suckling pigs, which merits further investigation.

Authors' contributions

M.L.B. and K.H.M. conducted the animal research trials. A.L. performed the bioinformatics, analyzed the microbiota data, and wrote the manuscript. M.L.B., K.H.M., and I.F.C. designed the experiment and reviewed the data. All authors read and approved the final manuscript.

Supplementary data

Supplementary data is available at *FEMSEC* online.

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References

Anderson MJ. A new method for non-parametric multivariate analysis of variance. *Austral Ecol* 2001;**26**:32–46.

Bauer E, Williams BA, Smidt H *et al.* Influence of the gastrointestinal microbiota on development of the immune system in young animals. *Curr Issues Intest Microbiol* 2006;**7**:35–51. https://europepmc.org/article/med/16875418 (1 June 2021, date last accessed).

Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Stat Soc Series B Methodol* 1995;**57**:289–300. https://www.jstor.org/stable/2346101 (1 June 2021, date last accessed).

Bian G, Ma S, Zhu Z *et al.* Age, introduction of solid feed and weaning are more important determinants of gut bacterial succession in

piglets than breed and nursing mother as revealed by a reciprocal cross-fostering model. *Environ Microbiol* 2016;**18**:1566–77.

Bray JR, Curtis JT. An ordination of the upland forest communities of southern Wisconsin. *Ecol Monogr* 1957;**27**:326–49.

Brestoff JR, Artis D. Commensal bacteria at the interface of host metabolism and the immune system. *Nat Immunol* 2013;**14**: 676–84.

Bui TP, Shetty SA, Lagkouvardos I *et al.* Comparative genomics and physiology of the butyrate-producing bacterium *Intestinimonas butyriciproducens*. *Environ Microbiol Rep* 2016;**8**:1024–37.

Caporaso JG, Lauber CL, Walters WA *et al.* Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proc Natl Acad Sci USA* 2011;**108**:4516–22.

Chao A. Nonparametric estimation of the number of classes in a population. *Scand J Stat* 1984;**11**:265–270. https://www.jstor.org/stable/4615964 (1 June 2021, date last accessed).

Chen L, Xu Y, Chen X *et al.* The maturing development of gut microbiota in commercial piglets during the weaning transition. *Front Microbiol* 2017;**8**:1688.

Clarke KR. Non-parametric multivariate analyses of changes in community structure. *Austral Ecol* 1993;**18**:117–43.

Cole JR, Wang Q, Fish JA *et al.* Ribosomal database project: data and tools for high throughput rRNA analysis. *Nucleic Acids Res* 2014;**42**:D633–42.

Collins CL, Pluske JR, Morrison RS *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. *Anim Nutr* 2017;**3**:372–9.

Dill-McFarland KA, Breaker JD, Suen G. Microbial succession in the gastrointestinal tract of dairy cows from 2 weeks to first lactation. *Sci Rep* 2017;**7**:40864.

Ding X, Lan W, Liu G *et al.* Exploring possible associations of the intestine bacterial microbiome with the pre-weaned weight gaining performance of piglets in intensive pig production. *Sci Rep* 2019;**9**:15534.

Dou S, Gadonna-Widehem P, Rome V *et al.* Characterisation of early-life fecal microbiota in susceptible and healthy pigs to post-weaning diarrhoea. *PLoS ONE* 2017;**12**:e0169851.

Excoffier L, Smouse PE, Quattro JM. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics* 1992;**131**:479–91.

Frese SA, Parker K, Calvert CC *et al.* Diet shapes the gut microbiome of pigs during nursing and weaning. *Microbiome* 2015;**3**:28.

Frommlet F, Heinze G. Experimental replications in animal trials. *Lab Anim* 2021;**55**:65–75.

Gaukroger CH, Stewart CJ, Edwards SA *et al.* Changes in faecal microbiota profiles associated with performance and birthweight of piglets. *Front Microbiol* 2020;**11**:917.

Gresse R, Chaucheyras-Durand F, Fleury MA *et al.* Gut microbiota dysbiosis in postweaning piglets: understanding the keys to health. *Trends Microbiol* 2017;**25**:851–73.

Han GG, Lee JY, Jin GD *et al.* Evaluating the association between body weight and the intestinal microbiota of weaned piglets via 16S rRNA sequencing. *Appl Microbiol Biotechnol* 2017;**101**:5903–11.

He M, Fang S, Huang X *et al.* Evaluating the contribution of gut microbiota to the variation of porcine fatness with the cecum and fecal samples. *Front Microbiol* 2016;**7**:2108.

Hoeflinger JL, Kashtanov DO, Cox SB *et al.* Characterization of the intestinal lactobacilli community following galactooligosaccharides and polydextrose supplementation in the neonatal piglet. *PLoS ONE* 2015;**10**:e0135494.

Holman DB, Brunelle BW, Trachsel J *et al.* Meta-analysis to define a core microbiota in the swine gut. *mSystems* 2017;**3**:pii: e00004–17. https://doi.org/10.1128/mSystems.00004-17.

Holman DB, Chenier MR. Temporal changes and the effect of subtherapeutic concentrations of antibiotics in the gut microbiota of swine. *FEMS Microbiol Ecol* 2014;**90**:599–608.

Inoue R, Tsukahara T, Nakanishi N *et al.* Development of the intestinal microbiota in the piglet. *J Gen Appl Microbiol*. 2015;**51**: 257–65.

Jaccard P. Etude comparative de la distribution florale dans une portion des Alpes et du Jura. *Bulletin de la Société Vaudoise des Sciences Naturelles* 1901;**37**:547–79.

Jacobi SK, Yatsunenko T, Li D *et al.* Dietary isomers of sialyllactose increase ganglioside sialic acid concentrations in the corpus callosum and cerebellum and modulate the colonic microbiota of formula-fed piglets. *J Nutr* 2016;**146**:200–8.

Katouli M, Lund A, Wallgren P *et al.* Metabolic fingerprinting and fermentative capacity of the intestinal flora of pigs during pre- and post-weaning periods. *J Appl Microbiol* 1997;**83**:147–54.

Kim HB, Borewicz K, White BA *et al.* Longitudinal investigation of the age-related bacterial diversity in the feces of commercial pigs. *Vet Microbiol* 2011;**153**:124–33.

Kim HB, Isaacson RE. The pig gut microbial diversity: understanding the pig gut microbial ecology through the next generation high throughput sequencing. *Vet Microbiol* 2015;**177**:242–51.

Kim J, Guevarra RB, Nguyen SG *et al.* Effects of the antibiotics growth promoter tylosin on swine gut microbiota. *J Microbiol Biotechnol* 2016;**26**:876–82.

Konstantinov SR, Awati AA, Williams BA *et al.* Post-natal development of the porcine microbiota composition and activities. *Environ Microbiol* 2006;**8**:1191–9.

Kozich JJ, Westcott SL, Baxter NT *et al.* Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. *Appl Environ Microbiol* 2013;**79**:5112–20.

Krogh U, Bruun TS, Amdi C *et al.* Colostrum production in sows fed different sources of fiber and fat during late gestation. *Can J Anim Sci* 2015;**95**:211–23.

Kruskal WH, Wallis WA. Use of ranks in one-criterion variance analysis. *J Am Stat Assoc* 1952;**47**:583–621.

Leblois J, Massart S, Li B *et al.* Modulation of piglets' microbiota: differential effects by a high wheat bran maternal diet during gestation and lactation. *Sci Rep* 2017;**7**:7426.

Levene H. Robust tests for equality of variances. In: Olkin I (ed.), *Contributions to Probability and Statistics; Essays in Honour of Harold Hotelling*. Palo Alto: Stanford University Press, 1960, 278–292.

Li K, Xiao Y, Chen J *et al.* Microbial composition in different gut locations of weaning piglets receiving antibiotics. *Asian Aust J Anim Sci* 2017;**30**:78–84.

Lu D, Tiezzi F, Schillebeeckx C *et al.* Host contributes to longitudinal diversity of fecal microbiota in swine selected for lean growth. *Microbiome* 2018;**6**:4.

Mach N, Berri M, Estelle J *et al.* Early-life establishment of the swine gut microbiome and impact on host phenotypes. *Environ Microbiol Rep* 2015;**7**:554–69.

Madigan M, Martinko J. *Brock Biology of Microorganisms*. New York: Pearson, 2018.

Magurran AE. *Measuring Biological Diversity*. Oxford: Blackwell Science, 2004.

Merrifield CA, Lewis MC, Berger B *et al.* Neonatal environment exerts a sustained influence on the development of the intestinal microbiota and metabolic phenotype. *ISME J* 2016;**10**:145–57.

Morissette B, Talbot G, Beaulieu C *et al.* Growth performance of piglets during the first two weeks of lactation affects the development of the intestinal microbiota. *J Anim Physiol Anim Nutr* 2018;**102**: 525–32.

Morris EK, Caruso T, Buscot F *et al.* Choosing and using diversity indices: insights for ecological applications from the German Biodiversity Exploratories. *Ecol Evol* 2014;**4**:3514–24.

Mulder IE, Schmidt B, Stokes CR *et al.* Environmentally-acquired bacteria influence microbial diversity and natural innate immune responses at gut surfaces. *BMC Biol* 2009;**7**:79.

Niu Q, Li P, Hao S *et al.* Dynamic distribution of the gut microbiota and the relationship with apparent crude fiber digestibility and growth stages in pigs. *Sci Rep* 2015;**5**:9938.

Oksanen J, Guillaume B F, Friendly M *et al.* vegan: community ecology package. R package version 2. 4–4. 2017. https://CRAN.R-project.org/package=vegan (20 May 2021, date last accessed).

Onrust L, Ducatelle R, Van Driessche K *et al.* Steering endogenous butyrate production in the intestinal tract of broilers as a tool to improve gut health. *Front Vet Sci* 2015;**2**:75.

Pajarillo EA, Chae JP, Kim HB *et al.* Barcoded pyrosequencing-based metagenomic analysis of the faecal microbiome of three purebred pig lines after cohabitation. *Appl Microbiol Biotechnol* 2015;**99**:5647–56.

Petri D, Hill JE, Van Kessel AG. Microbial succession in the gastrointestinal tract (GIT) of the preweaned pig. *Livest Sci* 2010;**133**: 107–9.

Pluske JR, Turpin DL, Kim JC. Gastrointestinal tract (gut) health in the young pig. *Anim Nutr* 2018;**4**:187–96.

Pruesse E, Quast C, Knittel K *et al.* SILVA: a comprehensive on- line resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Res* 2007;**35**: 7188–96.

Quan J, Cai G, Ye J *et al.* A global comparison of the microbiome compositions of three gut locations in commercial pigs with extreme feed conversion ratios. *Sci Rep* 2018;**8**:4536.

R Core Team. R: a language and environment for statistical computing. Vienna: R Foundation for Statistical Computing. 2021. https://www.R-project.org/ (21 January 2022, date last accessed).

Salmon H, Berri M, Gerdts V *et al.* Humoral and cellular factors of maternal immunity in swine. *Dev Comp Immunol* 2009;**33**: 384–93.

Scheppach W, Luehrs H, Menzel T. Beneficial health effects of low-digestible carbohydrate consumption. *Br J Nutr* 2001;**85**: S23–30.

Schloss PD, Westcott SL, Ryabin T *et al.* Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl Environ Microbiol* 2009;**75**:7537–41.

Segata N, Izard J, Waldron L *et al.* Metagenomic biomarker discovery and explanation. *Genome Biol* 2011;**12**:R60.

Shapiro SS, Wilk MB. An analysis of variance test for normality (complete samples). *Biometrika* 1965;**52**:591–611.

Slifierz MJ, Friendship RM, Weese JS. Longitudinal study of the early-life fecal and nasal microbiotas of the domestic pig. *BMC Microbiol* 2015;**15**:184.

Thompson CL, Wang B, Holmes AJ. The immediate environment during postnatal development has long-term impact on gut community structure in pigs. *ISME J* 2008;**2**:739–48.

Vacca M, Celano G, Calabrese FM *et al.* The controversial role of human gut Lachnospiraceae. *Microorganisms* 2020;**8**:573.

Vo N, Tsai TC, Maxwell C *et al.* Early exposure to agricultural soil accelerates the maturation of the early-life pig gut microbiota. *Anaerobe* 2017;**45**:31–9.

Wang Q, Garrity GM, Tiedje JM *et al.* Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol* 2007;**73**:5261–7.

Westcott SL, Schloss PD. OptiClust, an improved method for assigning amplicon-based sequence data to operational taxonomic units. *mSphere* 2017;**2**:e00073–17.

Yue JC, Clayton MK. A similarity measure based on species proportions. *Commun Stat Theory Methods* 2005;**34**:2123–31.

Zhang L, Mu C, He X *et al.* Effects of dietary fibre source on microbiota composition in the large intestine of suckling piglets. *FEMS Microbiol Lett* 2016;**363**:fnw138.

Zhou L, Fang L, Sun Y *et al.* Effects of the dietary protein level on the microbial composition and metabolomic profile in the hindgut of the pig. *Anaerobe* 2016;**38**:61–9.

4 Chapter 4. Effects of prebiotic galacto-oligosaccharides in poorly performing pre-weaning piglets

The objectives of Chapter 4 were to investigate the effects of CMR supplemented with GOS on the microbiome, gut architecture and immunomodulatory goblet cell expression of poorly performing piglets that could benefit from milk replacement feeding when separated from sows and fit siblings in environmentally controlled pens. The most abundant taxa identified at genus level were *Lactobacillus*, *Streptococcus*, *Prevotella*, *Lactococcus* and *Leuconostoc*. This is one of the first studies to demonstrate the effects of prebiotic GOS in CMR when fed to poorly performing piglets. CMR plus GOS significantly improved gut architectural features and villus crypt ratio throughout the gastrointestinal tract, increased the number of goblet cells and differential abundance of beneficial probiotic bacteria, particularly *Lactobacillus* and *Bifidobacterium*. The paper was peer reviewed and accepted for publication by "Animals", an official journal of the publisher MDPI (Multidisciplinary Digital Publishing Institute) and is accessible at: https://www.mdpi.com/2076-2615/13/2/230 in portable document format.

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Galacto-Oligosaccharides Increase the Abundance of Beneficial Probiotic Bacteria and Improve Gut Architecture and Goblet Cell Expression in Poorly Performing Piglets, but Not Performance

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Simple Summary: New-born piglets often fail to thrive during suckling without any obvious signs of clinical disease, which causes significant economic loss and suffering. This is, in part, caused by enhanced production values, with too many piglets produced for the sow to provide adequate nutrition to its offspring. These piglets may not be receiving enough milk from the sow and may be removed to controlled environment pens and fed a complete commercial milk replacer to provide adequate nutrition and enhanced care. However, milk replacers do not traditionally contain the milk sugars found in sow milk that stimulate the development of a healthy immune system and gut microbiota. In this study, the effects of supplementing milk replacer with simple milk sugars on gut health, the microbiome and immune-protective goblet cells were investigated. Commercial milk replacer supplemented with milk sugars significantly increased the abundance of beneficial gut bacteria, improved gut health and the numbers of protective immune goblet cells. Results indicate that milk sugars given in milk replacer may be a useful addition in the husbandry of non-thriving, poorly performing piglets when moved to environmentally controlled pens away from sows and fit siblings by modulating their microbiome and gut health performance.

Abstract: Poorly performing piglets receiving commercial milk replacers do not benefit from the naturally occurring probiotic galacto-oligosaccharides otherwise found in sow milk. Study objectives were to investigate the effects of complete milk replacer supplemented with galacto-oligosaccharides on the microbiome, gut architecture and immunomodulatory goblet cell expression of poorly performing piglets that could benefit from milk replacement feeding when separated from sows and housed with fit siblings in environmentally controlled pens. The study is novel in that it is one of the first to investigate the effects of supplementing complete milk replacer with galacto-oligosaccharides in poorly performing piglets. Gastrointestinal tract samples were collected from piglets, and the microbiome composition was assessed by 16s ribosomal ribonucleic acid gene sequencing. Gut architectural features, villus/crypt ratio and enumeration of goblet cells in tissues were assessed by histopathological techniques. The most abundant taxa identified at the genus level were *Lactobacillus*, *Streptococcus*, *Prevotella*, *Lactococcus* and *Leuconostoc*. Milk replacer plus galacto-oligosaccharides significantly improved gut architectural features and villus/crypt ratio throughout the gastrointestinal tract, increased the number of goblet cells and revealed a differential abundance of beneficial probiotic bacteria, particularly *Lactobacillus* and *Bifidobacterium*. In these respects, galacto-oligosaccharide-supplemented milk replacer may be a useful addition to animal husbandry in poorly performing, non-thriving animals when moved to environmentally controlled pens away from sows and fit siblings, thereby modulating the microbiome and gastrointestinal tract performance.

Keywords: pigs; microbiota; suckling; galacto-oligosaccharides; probiotics; histology; goblet cells; milk replacer; lactic acid bacteria

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1. Introduction

The establishment and maintenance of beneficial gastrointestinal tract (GIT) microbiota during suckling is essential for the future performance, growth, health and welfare of animals [1]. Moreover, the GIT microbiota contributes to the developmental and metabolic needs of animals through short-chain fatty acid (SCFA) production, vitamin synthesis, complex carbohydrate digestion and immune system regulation [2,3]. However, poor pre-weaning performance and failure to thrive without obvious signs of clinical disease during lactation are of concern in piglet production, with estimated mortality rates of at least 12.6% representing significant economic loss [4], and this is notwithstanding gut dysbiosis due to known bacterial pathogens and/or scour from rotavirus [5]. Neonatal piglet viability has decreased in relation to selection for greater numbers of piglets born per sow. The focus on larger litter sizes has increased the number of piglets with decreased viability, lighter birth weights and a reduced ability to thrive in early life [6]. It is also recognised that weaning weight has a significant and profound effect on lifetime growth and performance, with lower birth-weight piglets achieving sub-optimal performance [7].

Microbiome manipulation through the addition of pre- and/or probiotic feeds, without pharmaceutical zinc oxide, particularly in healthy post-weaning pigs, is established, with an emphasis on beneficial lactic acid fermenting taxa, notably *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Bifidobacterium* and *Streptococcus* [8]. However, these approaches require further investigation in pre-weaning piglets with below-expected performance and economic return. Commercially available prebiotic galacto-oligosaccharides (GOS) comprising two to eight polymerised galactose units with terminal glucose moieties are functionally similar to those of mammalian milk and modulate gut architecture and intestinal microbiota in healthy pigs [9]. GOS is a major component of mammalian milk [10,11], which stimulates the development of the microbiota in neonates whilst conferring a variety of health benefits, including innate and adaptive immune development [12,13]. Naturally occurring milk GOS are typically composed of three to ten monosaccharide units, including galactose (Gal), glucose (Glc) and N-acetyl-glucosamine (GlcNAc), with some fucose and sialic acids. The core moiety present at the reducing end of milk oligosaccharides is either lactose (Gal(β1–4)Glc) or N-acetyl-lactosamine (Gal(β1–4)GlcNAc). Most animal milk oligosaccharides are sialylated (sialic acid at glycoprotein terminal ends), containing N-acetylneuraminic acid (Neu5Ac) and/or N-glycolylneuraminic acid (Neu5Gc) [14,15].

Porcine milk oligosaccharides (PMOs) contain the highest percentage of neutral oligosaccharides (20 %) in comparison with other domestic farm animals, the most abundant variety of mono and di-sialylated large oligosaccharides, and are most similar to the composition of human milk oligosaccharides [11]. However, PMOs decrease in abundance by approximately 43% during the first week of lactation, with the relative concentration of acidic PMOs decreasing and neutral PMOs increasing [16], suggesting a change in functionality during lactation and possibly the need for GOS early rather than later in life.

In pigs, GOS is fermented by GIT bacteria, increasing beneficial probiotic populations and SCFA concentrations [9,17,18]. Studies have also shown that GOS inhibits pathogen adhesion and colonisation and reduces the expression of pro-inflammatory cytokines [9,18,19]. A healthy, well-differentiated intestinal mucosa has long, regular villi and high villus-to-crypt ratios [20], with the villus epithelium lining consisting mainly of absorptive enterocytes and specialised secretory goblet cells (GCs) [21]. GCs throughout the entirety of the GIT secrete mucins, forming a protective mucus layer against enteric pathogens. That is, they are critical to barrier function, maintenance of intestinal homeostasis, integrity of the GIT epithelium and physical lubrication of luminal contents [21,22]. GCs also form GC-associated antigen passages (GAPs) that deliver soluble luminal antigens to lamina propria dendritic cells [23]. GC intrinsic sensing of the GIT microbiota is now consid- ered to play a critical role in regulating the exposure of the immune system to microbial challenges [24,25].

However, the modulatory effects of probiotics and prebiotics on GCs in suckling pigs remain largely uninvestigated. It is suggested that *Bacillus* probiotics can modulate and

enhance GC function in *E. coli*-challenged pigs [26]. In vitro, GOS is considered to enhance mucosal barrier function through direct modulation of GC function and upregulation of GC secretory product genes [27,28]. In vivo, daily GOS dosing in piglets improves barrier function and relieves colonic inflammation via modulation of the mucosal microbiota [29]. Nevertheless, more in vivo studies are required to investigate the effects of GOS on the piglet microbiome, gut architecture and GC expression throughout the GIT, particularly in poorly performing pigs.

For animals receiving sub-optimal nutrition from the sow, it is industry practice to remove them from farrowing pens and feed with a commercial milk replacer in controlled pen environments with no access to the sow. In contrast to natural sow colostrum and milk, commercial milk replacers have not traditionally contained GOS, although studies have demonstrated that formulas supplemented with GOS are safe and well tolerated in neonatal piglets [30]. Therefore, the objectives of this study were to investigate the effects of GOS on the microbiome, gut architecture and GC expression in poorly performing (non-thriving) piglets with below average birthweight who may benefit from milk replacement feeding alone or supplemented with GOS in four separate and repeated trials.

2. Materials and Methods

2.1. Ethics Approval Statement

All methods were carried out in accordance with the relevant guidelines and regulations of the Harper Adams University Research Ethics Committee and approved by them. The study is reported in accordance with ARRIVE guidelines. All animals were sacrificed using a schedule 1 method, the UK Animals (Scientific Procedures) Act 1986.

2.2. Animals and Trial Design

For all trials, piglets were derived from five to seven Landrace x Large white sows (JSR 9T; JSR Genetics, Driffield, UK) of similar parity (1–4), which were artificially inseminated with JSR 900 sire line semen (JSR Genetics, Driffield, UK). On day 100 (±3 d) of gestation, sows were moved to individual 1.81 × 2.61 m farrowing pens with a 2.2 × 0.63 m farrowing crate. Sows were housed in a single facility in separate pens. Trial 1 was conducted during March 2018, Trial 2 August 2018, Trial 3 October 2018 and Trial 4 March 2019 under identical housing and environmental conditions. Farrowing pens contained a 1.2 × 0.47 m piglet box heated with an industry standard heat lamp. Sows received a wheat-based lactation diet (BOCM Pauls Ltd., Wherstead, UK) containing 20.1% protein, 5.5 % oil, 3.5% crude fibre, 5.3% ash, 1.15% lysine, 3.1% methionine, 7.0% calcium, 1.6% phosphorous and 1.9% sodium plus water ad libitum. For prevention of iron deficiency, new-born piglets received a 1 mL intramuscular iron injection of 200 mg/mL (Ferroferon, Iron4u, Holte, Denmark) 24 h after birth. Sows were vaccinated with Porcilis® Porcoli Diluvac Forte suspension for injection (Intervet International BV; Vm:EU/2/96/001/003-008) 3-weeks prior to farrowing for the passive immunisation of piglets by active immunisation of sows/gilts to reduce mortality and the clinical signs of neonatal enterotoxicosis. No vaccinations were given to the experimental animals directly. Piglets were ear-tagged on day 1 for identification. Poorly performing piglets potentially receiving sub-optimal nutrition from the sow were selected within the first seven days of life by visual assessment and the appearance of "non-thriving" by qualified animal technicians. This was based upon poor weight gain, a high degree of contamination with faecal material, the presence of watery faeces and overall health. Animals displayed no clinical symptoms of underlying disease, for example, scour or lameness, but were considered to benefit from a complete milk replacement feeding program. Piglets were group housed in 2.3 × 0.89 m slatted plastic isolation pens heated by industry standard lamps, with water ad libitum through a nipple drinker and twice daily feeding to appetite with either complete porcine milk replacer (CMR) (Faramate, Volac International Ltd., Royston, UK) alone (Table S1) or supplemented with 1% (*w*/*w*) DP2 + GOS (Nutrabiotic®, Saputo Dairy UK, Weybridge, UK) with no access to a sow. Diets were designed to meet or exceed the nutrient requirements recommended

for piglets. An acidifier (benzoic acid) was not included as a preservative in CMR due to possible interferences with the microbiome. Metal chain toys with plastic balls were provided in orphan pens as environmental enrichment. Piglets did not receive any creep feed supplementation, growth promoter or any other prophylactic antibiotic treatment during the studies. The temperature was kept within the range of 18–20 °C for sows and 23–24 °C for piglets, with light periods from 8:00 am to 16:30 pm. Piglets were weighed within 24 h of birth, on the day of recruitment to the trial (within 3–7 days of life) and then at weekly intervals terminating at the time of euthanasia when they were not considered suitable for economic production (week 4 of life). Each pen had a dedicated weight bucket to avoid microbiome contamination. Pens were deep cleaned by spraying with TopFoam, pressure washing and disinfection with MegaDes Novo (both from MS Schippers, Hapert, Netherlands) and left to dry. Pens were not used for any other experiments in between trials. Milk input and output, on a pen basis, were recorded daily and daily feed intake (DFI) was calculated.

2.3. Sample Collection and DNA Extraction

For trials 1 to 4, samples of digesta were aseptically collected post-mortem from piglets at anatomical sites throughout the GIT. Duodenal, jejunal, ileal, colonic, caecal and rectal lumen samples were held on dry ice prior to transfer to the laboratory and storage at −80 °C until bacterial DNA isolation. Bacterial DNA was isolated from 200 mg of luminal contents for each sample using the MP Biomedicals Fast DNA Kit for Feces (MP Biomedicals, Solon, OH, USA) according to the manufacturer's instructions.

2.4. PCR Amplification of 16S rRNA Gene Sequences

Using the isolated DNA as a template, the V4 region of the bacterial 16S rRNA genes was PCR amplified using primers 515f (5^t GTGCCAGCMGCCGCGGTAA 3^t) and 806r (5^t GGACTACHVGGGTWTCTAAT 3^t) [31]. Amplicons were sequenced on the Illumina MiSeq platform (Illumina, Inc., San Diego, CA, USA) using 2 × 250 bp cycles according to the MiSeq Wet Lab SOP [32] separately for trials 1 to 4. Sequence data were deposited in the NCBI database within the Bioproject PRJNA866473, with SRA records available at: (https://www.ncbi.nlm.nih.gov/sra/PRJNA866473, accessed online 5 August 2022).

2.5. Microbiota Diversity Analysis

For each trial, the 16S rRNA gene sequence analysis was performed using mothur v.1.46.1, using default settings [33]. Analysis was performed according to the MiSeq SOP (https://www.mothur.org/wiki/MiSeq_SOP, accessed online 9 February 2022) [32]. The 16S rRNA gene sequences were aligned against a reference alignment based on the SILVA rRNA database [34] for use in mothur (release 132), available at: (https://www.mothur.org/wiki/Silva_reference files, accessed online 9 February 2022). The similarity cutoff for OTUs was 0.03. The consensus taxonomy of the OTUs was generated using the "classify.otu" command in mothur with reference data from the Ribosomal Database Project (version 14) [35,36] adapted for use in mothur available at: (https://www.mothur.org/wiki/RDP_reference_files, accessed online 9 February 2022). The relative abundance of OTUs annotated to taxa at the phylum and genus level were analysed from mothur output files using bespoke code written in R v4.1.1 using R Studio (2021.09.0) [37] and deposited at: (https://github.com/AdamLeeNottinghamUniversity/Piglets, accessed online 12 September 2022)

2.6. Histology

For all trials, immediately after excision, jejunal, ileal, colonic and caecal tissue samples from each piglet were fixed in 10% neutral buffered formalin in 40 mL prefilled specimen jars (Leica Microsystems UK, Ltd., Milton Keynes, UK). These were dehydrated through a series of alcohol solutions, cleared in xylene and embedded in paraffin wax. Sections 3 to 5 µm thick were prepared and stained with either haematoxylin and eosin (HE) to

elucidate villus crypt architecture or periodic acid-Schiff (PAS) staining to enumerate mucin-producing GCs (VPG Histology, Bristol, UK). After staining, all HE and PAS slides were scanned using the NanoZoomer digital pathology system (Hamamatsu, Welwyn Garden City, UK). For the jejunum and ileum, measurements of villus height, crypt depth and villus and crypt area were made using the NanoZoomer digital pathology image programme (Hamamatsu). Ten well-oriented villi and crypts per tissue section of each piglet GIT sample from each trial were scanned at 40× resolution. Villus height was measured from the tip of the villus to the crypt opening, with the associated crypt depth measured from the base of the crypt to the level of the crypt opening. The villus/crypt ratio (VCR) was calculated by dividing the villus height by the crypt depth. The GCs were enumerated from ten well-oriented villi and crypts of jejunal, ileal, colonic and caecal sections stained with PAS, and the area of each was measured with individual GCs counted and pinned on each slide. For all tissue samples, both HE and PAS, well oriented villi and crypts were chosen using a random number generator at: (https://www.random.org/, accessed online 7 October 2019) from 1 to 10.

2.7. Statistical Analyses

Good's coverage [38] and α-diversity expressed as Inverse Simpson diversity [39], and Chao richness [40] were calculated using the "summary.single" command in mothur [33]. Shapiro–Wilk tests [41] were used to determine normality for piglet weights at 24 h post-partum, day of trial 1, 7, 14 and 21, total weight gain, ADG and α-diversity metrics. Significant differences were tested using ANOVA with repeated measures for weight. Kruskal–Wallis tests were used to test for differences in α-diversity metrics. Estimates of β-diversity were calculated in mothur as Yue and Clayton dissimilarity (θ_{YC}) [42], Bray–Curtis dissimilarity [43], and Jaccard similarity [44]. Analysis of molecular variance executed in mothur (AMOVA) was used to test for differences in β-diversity between samples [45,46]. Linear discriminant analysis effect size (LEfSe) was used to examine differential OTU abundances in mothur [47]. Where appropriate, multiple comparisons (ANOVA and AMOVA) were adjusted for false discovery rates using the Benjamini and Hochberg procedure [48]. All post-mothur statistical analyses were performed in R Studio (2021.09.0) unless otherwise stated [37]. For histological sections, ileal and villus height, crypt depth, VCR and number of GCs per mm^2 tissues were analysed by Wilcoxon rank sum exact tests.

3. Results

3.1. Production Criteria: Weight, ADG and DFI

This study was performed in a commercial facility with pigs destined to go through the full production process. There were a limited number of farrowing pens (28) and only two isolation pens for poorly performing piglets. The number of poorly performing pre-weaning piglets differed between trials and could not be predicted in advance. Differences in "n" across trials one to four arose from those piglets that were visually assessed as poorly performing and subsequently randomly allocated to either receive milk replacer without GOS or milk replacer plus GOS in milk-replacer pens. Four non-GOS-fed piglets and four GOS-fed piglets were included in trial 1. Five non-GOS-fed piglets and four GOS-fed piglets were included in trial 2. Four non-GOS-fed piglets and four GOS-fed piglets were included in trial 3. Five non-GOS-fed piglets and five GOS-fed piglets were included in trial 4. In total, eighteen "poorly performing" non-GOS-fed piglets were included in analyses as opposed to seventeen "poorly performing" GOS-fed piglets across trials 1 to 4. A further eight pigs were physically removed during the study period (and excluded from analysis) after meeting the set humane endpoint threshold or requiring antibiotic treatment. Piglet weights at 24 h after birth; trial days 1, 7, 14 and 21; total weight gain and ADG were normally distributed according to Shapiro–Wilk tests ($p > 0.05$ in each case) for non-GOS and GOS-fed piglets alike in all four trials. These metrics were not significantly different across trials one to four or between non-GOS and GOS-fed piglets ($p > 0.05$ in each

case, ANOVA, Table 1). Weight significantly increased with time for all trials and treatment groups (ANOVA, Day 1 to 21, $p < 0.05$ in each case). Piglet weights at 24 h *post-partum* significantly correlated with final weight at day 21 with no significant differences between treatment groups (linear modelling, $R^2 = 0.5$, $p < 0.001$ for non-GOS piglets, $R^2 = 0.48$, $p < 0.001$ for GOS-fed piglets, Figure S1). DFI was not significantly different between trials one to four for non-GOS-fed piglets and GOS-fed piglets alike (ANOVA, $p = 0.709$ and $p = 0.343$, respectively). The mean DFI (SD) for non-GOS-fed piglets increased from 0.311 (0.135) kg/piglet on day 1 of trials to 1.17 (0.476) kg/piglet on day 21 of trials. For GOS-fed piglets, the mean DFI (SD) at day one of trials was 0.493 (0.444), increasing to 1.547 (0.447) kg/piglet on day 21 of trials (Figure S2). Mean DFI and feed conversion ratio (FCR) across all four trials were not significantly different between treatments (*t*-test, $p = 0.802$ and $p = 0.783$, respectively).

Table 1. Pig weights at 24 h *post-partum*, days 1 to 21 of trials, total weight gain, ADG and FCR.

	Trial 1		**Trial 2**		**Trial 3**		**Trial 4**	
Weight kg	**Non-GOS**	**GOS**	**Non-GOS**	**GOS**	**Non-GOS**	**GOS**	**Non-GOS**	**GOS**
24 h	1.34 (0.19)	1.30 (0.20)	1.09 (0.37)	1.11 (0.28)	1.16 (0.25)	1.21 (0.24)	0.97 (0.15)	0.90 (0.19)
Day 1	1.48 (0.23)	1.43 (0.27)	1.15 (0.37)	1.22 (0.28)	1.41 (0.45)	1.36 (0.38)	1.12 (0.18)	1.16 (0.11)
Day 7	2.17 (0.43)	2.32 (0.26)	2.17 (0.72)	2.17 (0.52)	2.31 (0.66)	2.10 (0.48)	1.77 (0.37)	1.92 (0.53)
Day 14	2.93 (0.45)	3.23 (0.24)	3.44 (1.33)	3.26 (0.94)	3.25 (0.52)	2.74 (0.54)	2.35 (0.76)	3.13 (0.81)
Day 21	4.41 (0.85)	4.53 (0.20)	4.30 (1.73)	3.91 (1.44)	4.58 (0.44)	3.71 (0.75)	3.11 (0.99)	3.93 (0.92)
Total gain	2.93 (0.81)	3.10 (0.28)	3.15 (1.49)	2.69 (1.18)	3.17 (0.43)	2.35 (0.50)	1.99 (0.95)	2.77 (0.85)
ADG kg/d	0.15 (0.04)	0.15 (0.01)	0.14 0.07)	0.12 (0.05)	0.16 (0.02)	0.12 (0.02)	0.10 (0.04)	0.14 (0.04)
DFI kg	0.84 (0.40)	1.03 (0.44)	1.04 (0.38)	0.98 (0.30)	1.23 (0.41)	1.00 (0.25)	0.83 (0.17)	0.91 (0.29)
FCR	5.60	6.87	7.43	8.17	7.69	8.33	8.30	6.50

Values are means (SD). The means are not significantly different across trials or between non-GOS and GOS-fed piglets (ANOVA, $p > 0.05$, in each case). The mean weight significantly increased with time for all trials and treatment groups (ANOVA, Day 1 to 21, $p < 0.01$ in each case).

3.2. GIT Microbiota Diversity

A total of 2,380,409 high-quality V4 16s rRNA sequence reads were obtained from 210 piglet GIT samples, with a Good's coverage of 97.1 to 99.8% (minimum to maximum across all samples and four separate trials). The number of high-quality sequences obtained from each trial 1 to 4 and each section of the GIT, from the duodenum to the rectum, is shown in Table S2. Inverse Simpson diversity and Chao richness were normally distributed for the majority of samples except for rectal samples from GOS-fed pigs in trial 1 ($p = 0.042$ and $p = 0.006$, respectively, Shapiro–Wilk tests); inverse Simpson diversity for ileal samples in non-GOS-fed piglets in trial 2 ($p = 0.01$); Chao richness for ileal samples from non-GOS- fed piglets in trial 3 ($p = 0.04$); inverse Simpson diversity for ileal samples from GOS-fed piglets in trial 4 ($p = 0.01$) and Chao richness for jejunal samples from GOS-fed piglets in trial 4 ($p = 0.05$). Inverse Simpson diversity and Chao richness (Table 2) were significantly different between trials one to four in some but not all comparisons (Kruskal–Wallis tests). Chao richness for duodenal samples from non-GOS piglets was significantly different across trials one to four ($p = 0.015$) as was the inverse.

Table 2. Alpha diversity. Mean (SD).

	Trial 1		Trial 2		Trial 3		Trial 4	
	Non-GOS [3,4]	**GOS [3,4]**	**Non-GOS**	**GOS [3]**	**Non-GOS [3,4]**	**GOS [3,4]**	**Non-GOS [3,4]**	**GOS [3]**
				Inverse Simpson Diversity				
Duodenum [1,2]	5.11 (2.31)	2.93 (1.27)	21.16 (16.21)	6.52 (0.50)	6.28 (1.36)	5.13 (1.79)	7.47 (2.28)	14.08 (5.83)
Jejunum	8.12 (2.85)	6.36 (4.50)	17.39 (10.20)	7.60 (2.13)	5.76 (1.13)	4.99 (1.45)	8.49 (2.73)	9.61 (0.80)
Ileum	15.42 (10.12)	6.17 (3.18)	12.04 (9.18)	14.51 (10.88)	5.04 (2.28)	8.48 (3.67)	10.52 (3.53)	15.22 (7.29)
Caecum [2]	17.56 (6.04)	20.63 (4.65)	29.31 (15.82)	22.64 (4.81)	18.96 (4.24)	17.22 (3.55)	21.49 (13.62)	20.44 (3.31)
Colon [2]	18.89 (4.41)	12.65 (2.81)	38.27 (12.78)	27.41 (8.09)	21.56 (2.70)	14.03 (8.11)	21.36 (10.81)	25.32 (3.38)
Rectum	21.00 (9.77)	22.08 (12.31)	26.14 (7.96)	25.14 (5.94)	23.11 (5.76)	17.86 (3.53)	23.91 (11.07)	26.05 (1.46)
				Chao Richness				
Duodenum [1]	113.37 (43.90)	95.53 (11.59)	301.92 (99.28)	213.78 (43.28)	192.39 (61.81)	161.19 (64.40)	132.45 (25.48)	178.14 (59.61)
Jejunum [1]	87.97 (10.65)	111.59 (34.05)	278.74 (76.24)	169.04 (38.75)	122.98 (37.74)	122.26 (63.35)	110.23 (8.81)	123.39 (36.81)
Ileum	207.53 (96.48)	104.44 (24.26)	256.66 (79.15)	248.67 (183.61)	116.42 (25.74)	146.11 (87.06)	184.46 (82.81)	177.95 (45.65)
Caecum	237.49 (70.67)	213.63 (32.34)	257.02 (64.16)	226.92 (62.83)	279.81 (52.19)	237.44 (23.60)	260.98 (66.12)	220.23 (55.32)
Colon	197.52 (49.21)	179.42 (43.35)	310.12 (49.66)	259.70 (60.27)	295.06 (49.14)	258.41 (20.57)	315.40 (95.66)	243.22 (47.65)
Rectum [2]	264.23 (132.91)	261.21 (29.00)	324.84 (30.92)	286.94 (76.27)	307.68 (71.12)	314.12 (46.32)	243.34 (63.14)	156.87 (34.07)

[1] Significant differences between trials 1 to 4 for non-GOS piglets ($p < 0.05$ in each case, Kruskal–Wallis tests). [2] Significant differences between trials 1 to 4 for GOS piglets ($p < 0.05$ in each case, Kruskal–Wallis tests). [3] Significant differences across GIT for inverse Simpson diversity ($p < 0.05$ in each case, Kruskal–Wallis tests). [4] Significant differences across GIT for Chao richness ($p < 0.05$ in each case, Kruskal–Wallis tests).

Simpson diversity for GOS-fed piglets ($p = 0.03$); for jejunal samples from non-GOS piglets, Chao richness ($p = 0.008$); for colonic samples from GOS-fed piglets, inverse Simpson diversity ($p = 0.02$); for rectal samples, Chao richness for GOS-fed piglets ($p = 0.03$). Alpha diversity significantly increased from duodenal to rectal samples throughout the GIT in trials 1 to 4 ($p < 0.05$ in each case, Kruskal–Wallis tests) and in non-GOS piglets and GOS-fed piglets. There were exceptions for the trial 2 inverse Simpson diversity and Chao richness tests in non-GOS-fed pigs and Chao richness in GOS-fed piglets and the trial 4 Chao richness test for GOS-fed piglets. There were no significant differences in alpha diversity between non-GOS-fed piglets and GOS-fed piglets in trials 1 to 4. Table 3 shows the results of AMOVA used to test for significant differences in calculated β-diversity measures between trials one to four for non-GOS and GOS-fed piglets. All trials showed at least one significant difference in AMOVA for one or more β-diversity measures between non-GOS and GOS-fed piglets ($p < 0.05$ in each case). Complementary PCA plots for β-diversity are shown in Figure S3A–D.

Table 3. β-diversity showing significant differences in AMOVA between non-GOS and GOS-fed piglets in four separate trials.

Trial	Duodenum	Jejunum	Ileum	Caecum	Colon	Rectum
			Yue & Clayton Dissimilarity (θ_{YC})			
1	0.246	0.293	0.373	0.517	0.173	0.031
2	0.012	0.015	0.284	0.011	0.047	0.313
3	0.486	0.513	0.085	0.494	0.392	0.034
4	0.062	0.209	0.185	0.005	0.05	0.121
			Bray-Curtis Dissimilarity			
1	0.186	0.217	0.173	0.427	0.254	0.062
2	0.01	0.012	0.155	0.006	0.087	0.197
3	0.456	0.327	0.069	0.131	0.241	0.034
4	0.001	0.252	0.257	0.004	0.01	0.061
			Jaccard Similarity			
1	0.062	0.660	0.09	0.106	0.106	0.241
2	0.031	0.025	0.126	0.016	0.140	0.006
3	0.456	0.017	0.145	0.034	0.034	0.034
4	0.018	0.006	0.173	0.005	0.005	0.012

3.3. GIT Microbiota Composition

The number of unique, high-quality sequences clustered into OTUs obtained from each trial and each section of the GIT, from the duodenum to the rectum, is shown in Table S3 without discriminating between non-GOS-fed and GOS-fed piglets. Analyses were based on all sequences so that OTU numbers were consistent across all trials. In total, 3274 unique OTUs were identified from 210 piglet GIT samples across trials 1 to 4 and all GIT sections. 1279 OTUs were identified in trial 1, 1726 in trial 2, 1273 in trial 3 and 1216 in trial 4. 1062 OTUs were identified from the duodenum, 1000 from the jejunum, 1182 from the ileum, 1339 from the caecum, 1474 from the colon and 1503 from the rectum across trials 1 to 4. Figure 1 shows the number of OTUs shared by each trial and for each section of the piglet GIT. Only 176 OTUs were shared between trials 1 to 4 in the duodenum (16.6%), 118 in the jejunum (11.8%), 155 in the ileum (13.1%), 245 in the caecum (18.3%), 272 in the colon (18.5%) and 296 in the rectum (19.7%). However, these OTUs accounted for at least 97.9% of the total relative abundance of bacteria taxa at the genus level in each case. When analysed, on a trial-by-trial basis, predominant phyla in trial 1 across all samples were *Firmicutes* (77.08%), *Bacteroidetes* (16.07%), *Proteobacteria* (3.34%) and *Actinobacteria* (3.03%). Unclassified bacteria accounted for 0.25% of sequences. In trial 2, predominant phyla were *Firmicutes* (69.98%), *Proteobacteria* (13.59%), *Bacteroidetes* (9.69%), *Actinobacteria* (3.97%) and *Fusobacteria* (1.22%). Unclassified bacteria accounted for 0.73% of sequences. In trial 3, the predominant phyla were *Firmicutes* (77.15%), *Bacteroidetes* (14.67%), *Actinobacteria* (4.077%) and *Proteobacteria* (3.32%). Unclassified bacteria accounted for 0.44% of sequences. In trial 4, the predominant phyla were *Firmicutes* (78.70%), *Proteobacteria* (10.00%), *Bac- teroidetes* (5.98%), *Actinobacteria* (2.69%) and *Deferribacteres* (1.34%). Unclassified bacteria accounted for 0.44% of sequences. At the genus level for trial 1, the most abundant taxa identified were *Lactobacillus* (22.94%), *Streptococcus* (19.85%), *Prevotella* (8.83%), *Leuconostoc* (7.72%), *Megasphaera* (3.11%), *Veillonella* (2.74%), *Bacteroides* (2.53%**),** *Phascolarctobacterium* (2.51%), *Alloprevotella* (1.83%) and *Ruminococcaceae* unclassified (1.53%). For trial 2, the most abundant taxa identified at the genus level were *Veillonella* (14.84%), *Lactobacillus* (11.68%), *Pasteurellaceae* unclassified (11.32%), *Streptococcus* (9.27%), *Leuconostoc* (6.50%), *Lactococcus* (3.20%), *Bacteroides* (2.98%), *Prevotella* (2.53%), *Phascolarctobacterium* (2.37%) and *Megasphaera* (2.31%). For trial 3, the most abundant taxa identified at the genus level were *Lactobacillus* (27.25%), *Streptococcus* (13.55%), *Prevotella* (5.91%), *Phascolarctobacterium* (4.59%), *Blautia* (3.48%), *Veillonella* (3.38%), *Subdoligranulum* (3.25%), *Alloprevotella* (3.03%), *Ruminococcaceae* unclassified (2.95%) and *Collinsella* (2.67%). For trial 4, the most abundant taxa identified at the genus level were *Lactobacillus* (25.50%), *Streptococcus* (8.57%), *Veillonella* (7.70%), *Leuconostoc* (7.39%), *Lactococcus* (6.84%), *Enterobacteriaceae* unclassified (6.52%), *Megasphaera* (3.57%), *Blautia* (3.24%), *Prevotella* (2.43%) and *Phascolarctobacterium* (1.97%). Relative abundances of the top ten bacterial taxa at the phylum and genus levels for GIT samples from trials 1 to 4 for non-GOS and GOS-fed piglets are shown in Figures 2 and 3, respectively. Significant differences in the differential abundance of OTUs ascribed to bacterial taxa at the genus level were calculated using LEfSe for each trial. Table 4 shows a summary of results for LEfSe for those OTUs ascribed to lactic acid fermenting bacteria throughout the GIT for non-GOS and GOS-fed piglets, trials 1 to 4, these being *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Bifidobacterium* and *Streptococcus* and the relative abundance of each OTU in parentheses, with a cut-off of 0.1% relative abundance. In total, across all GIT sections and trials 1 to 4, twenty-five linear discriminant features for all lactic acid fermenting bacteria occurred with non-GOS-fed piglets as opposed to forty-seven for GOS-fed piglets. In total, thirteen linear discriminant features for *Lactobacillus* occurred in non-GOS-fed piglets as opposed to twenty-eight for GOS-fed piglets. For *Leuconostoc*, this was two in non-GOS-fed piglets and three in GOS-fed piglets. For *Lactococcus*, this was one in non-GOS-fed piglets and two in GOS-fed piglets. For *Streptococcus*, this was eight in non-GOS-fed piglets and six in GOS-fed piglets. Only one linear discriminant feature ascribed to *Bifidobacterium* occurred in non-GOS-fed piglets, in contrast to eight in GOS-fed piglets. There was variation between trials. In trial 1, nine linear discriminant features ascribed to lactic acid fermenting

bacteria were identified for non-GOS-fed piglets compared with eight for GOS-fed piglets. In trial 2, this was four for non-GOS-fed piglets and eighteen for GOS-fed piglets. In trial 3, three for non-GOS piglets and three for GOS-fed piglets. In trial 4, nine for non-GOS-fed piglets and eighteen for GOS-fed piglets. However, these results need to be interpreted in consideration of the relative abundance of each OTU. A full description of all differentially abundant OTUs and linear discriminant effect size (LEfSe) ascribed to all bacterial taxa at the genus level for all GIT sections and each trial one to four are shown in Figure S4A–D, with a cut-off of either the top ten taxa or relative abundance by 0.1%, whichever occurred first, and showing unique OTUs for each trial.

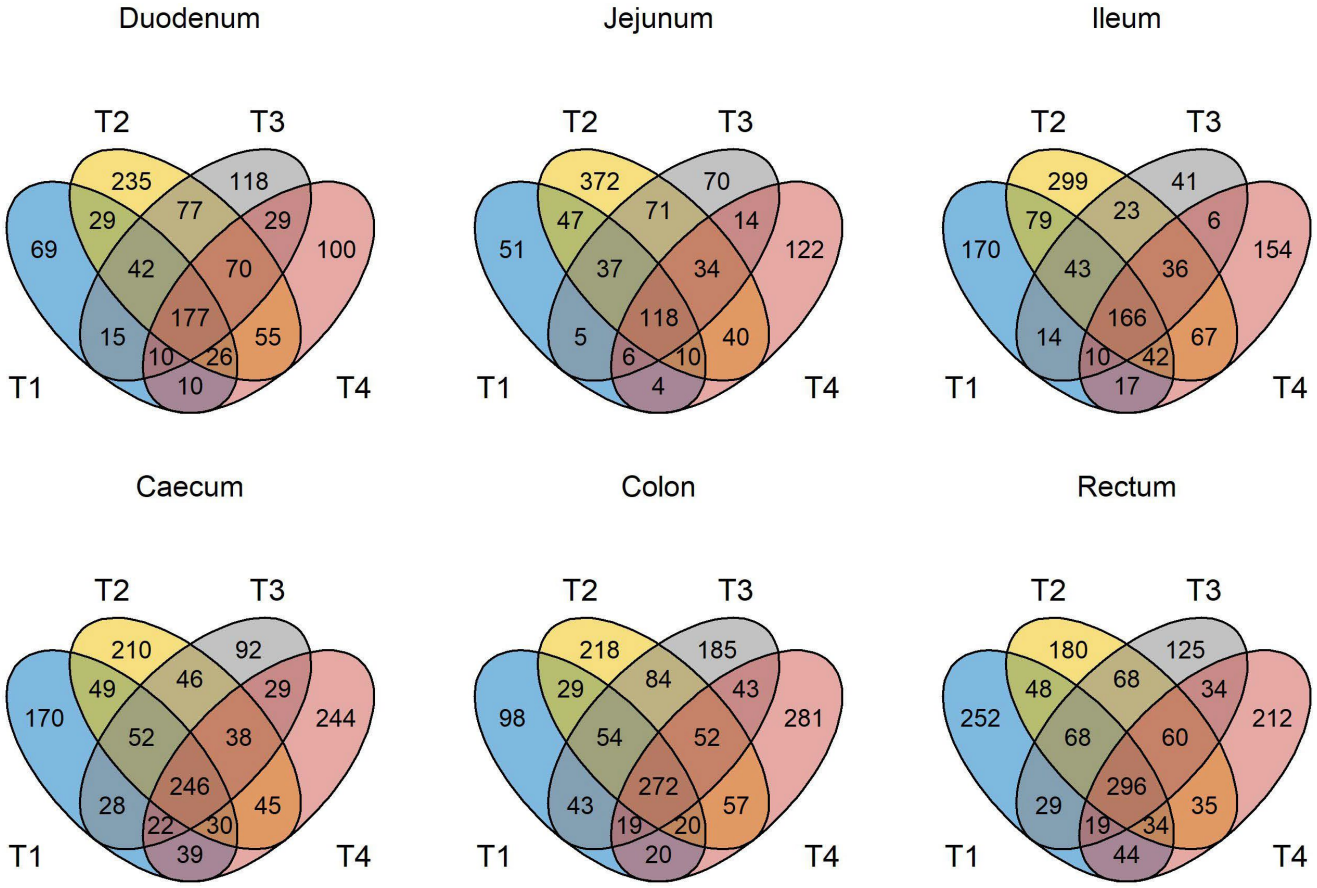

Figure 1. Venn diagram depicting unique and shared OTUs at the genus level in GIT samples from pigs in trials 1 to 4. T1 = trial 1; T2 = trial 2; T3 = trial 3; T4 = trial 4.

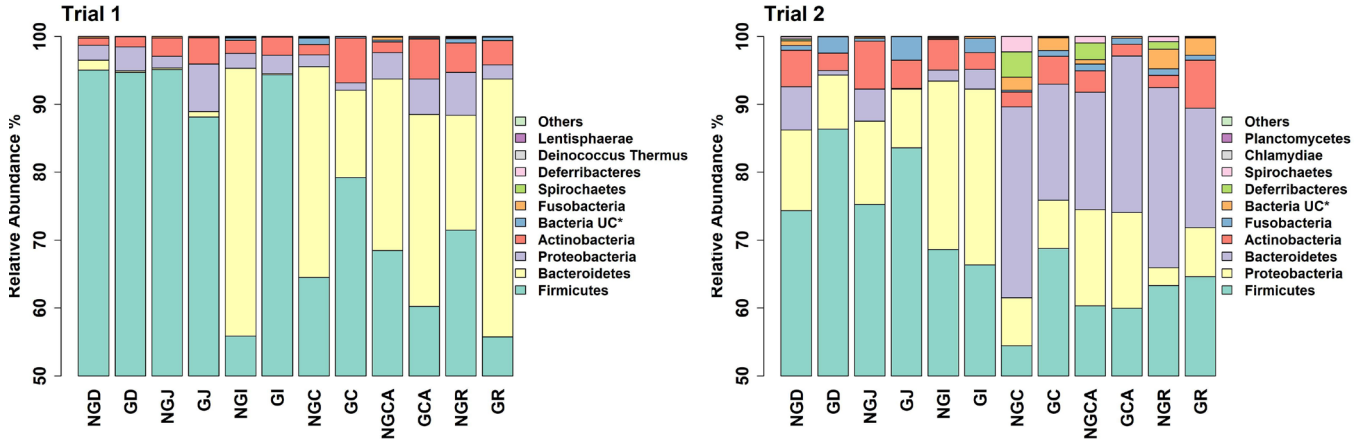

Figure 2. *Cont.*

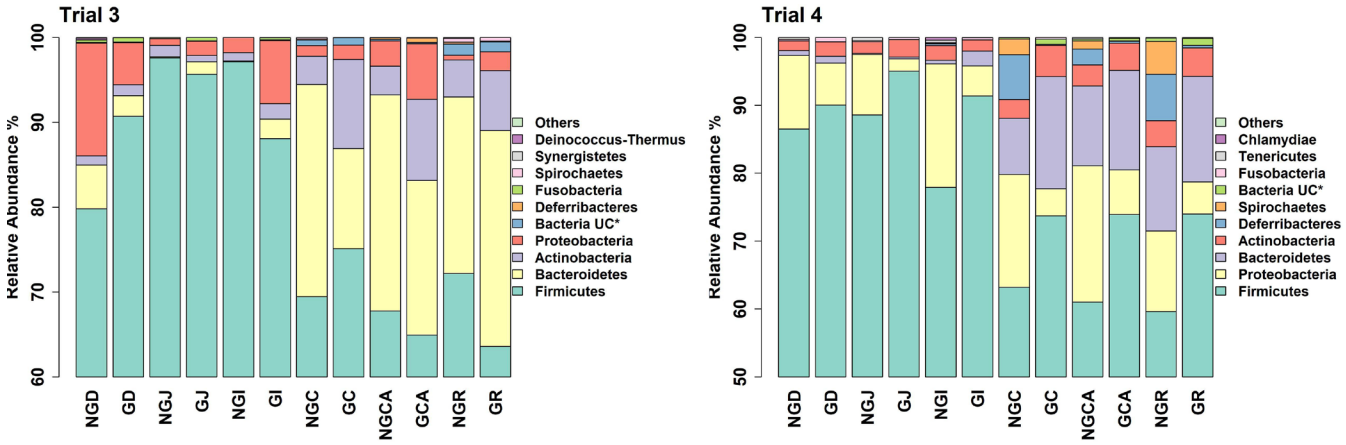

Figure 2. Relative abundance of bacterial taxa annotated to OTUs at the phylum level as iden- tified from GIT samples of piglets fed milk replacer alone or milk replacer with GOS in trials 1 to 4. * UC = unclassified at the phylum level. NGD = Non-GOS Duodenum; GD = GOS Duo- denum; NGJ = Non-GOS Jejunum; GJ = GOS Jejunum; NGI = Non-GOS Ileum; GI = GOS Ileum; NGC = Non-GOS Colon; GC = GOS Colon; NGCA = Non-GOS Caecum; GCA = GOS Caecum; NGR = Non-GOS Rectum; GR = GOS Rectum.

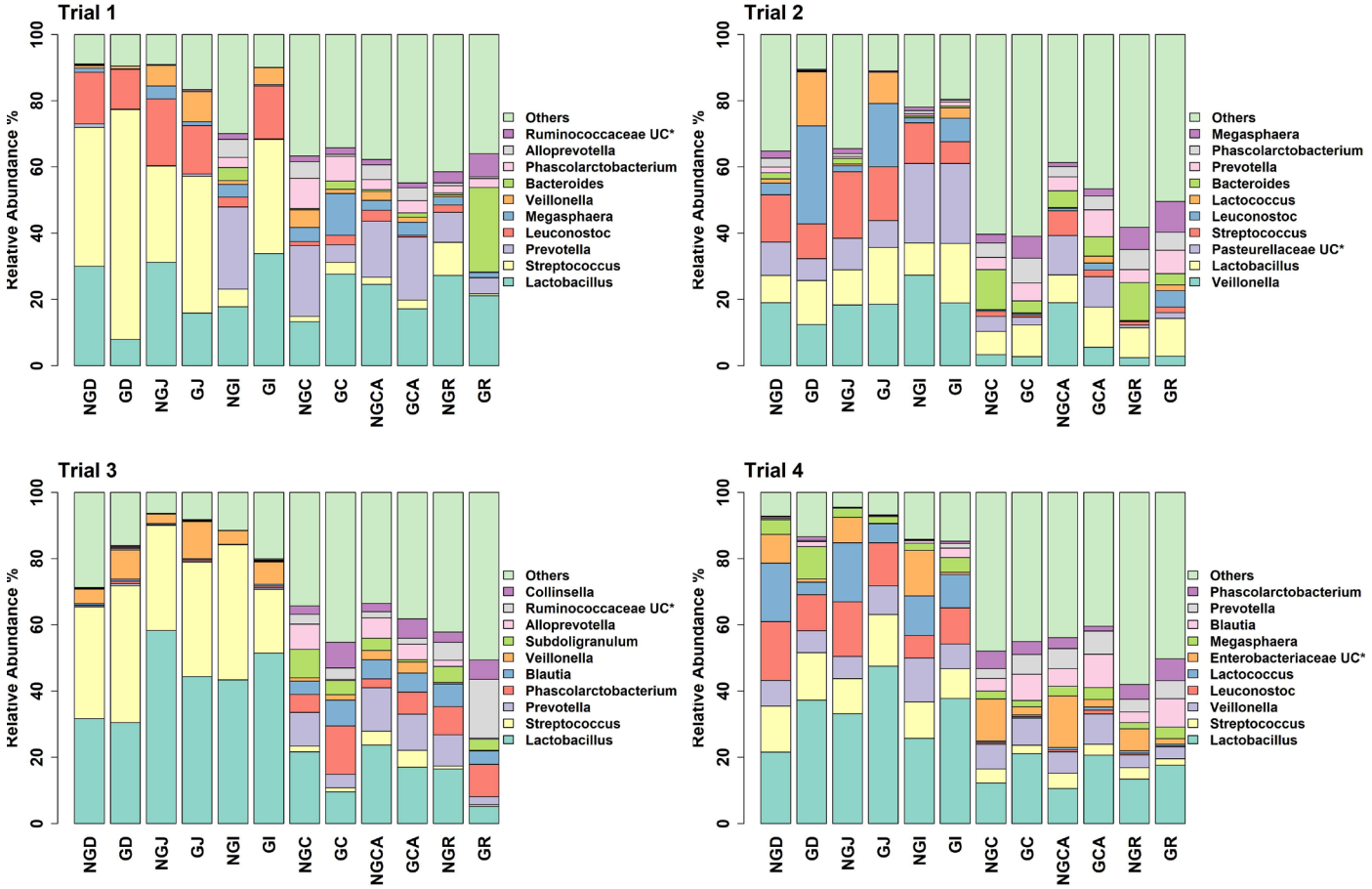

Figure 3. Relative abundance of bacterial taxa annotated to OTUs at the genus level as identi- fied from GIT samples of piglets fed milk replacer alone or milk replacer with GOS in trials 1 to 4. * UC = unclassified at the genus level. NGD = Non-GOS Duodenum; GD = GOS Duode- num; NGJ = Non-GOS Jejunum; GJ = GOS Jejunum; NGI = Non-GOS Ileum; GI = GOS Ileum; NGC = Non-GOS Colon; GC = GOS Colon; NGCA = Non-GOS Caecum; GCA = GOS Caecum; NGR = Non-GOS Rectum; GR = GOS Rectum.

Table 4. Significant differences in differential abundance of lactic acid fermenting bacteria throughout the GIT for non-GOS and GOS-fed piglets, trials 1 to 4.

	Trial 1		**Trial 2**		**Trial 3**		**Trial 4**	
	Non-GOS	**GOS**	**Non-GOS**	**GOS**	**Non-GOS**	**GOS**	**Non-GOS**	**GOS**
Duodenum	Otu008 LB (2.71) * Otu015 LB (0.88) * Otu017 LB (0.70) * Otu024 LB (0.30) *	Otu005 SC (8.4) *	Otu024 LB (1.1) *	Otu002 LN (29.5) * Otu004 LC (16.3) * Otu017 LB (2.6) ** Otu038 BB (0.8) ** Otu074 LB (0.22) **	NS	Otu074 SC (0.11) *	Otu002 LC (15.9) * Otu003 SC (10.6) **	Otu018 LB (1.9) * Otu025 LB (1.1) ** Otu067 LB (0.11) *
	Otu003 LB (14.6)*			Otu003 LN (18.2) *			Otu024 BB (0.56) **	Otu032 SC (0.36) *
Jejunum	Otu005 LB (8.1) * Otu017 LB (1.4) *	Otu031 SC (0.26) *	NS	Otu004 LC (9.4) * Otu023 BB (1.5) ** Otu024 LB (1.6) **	Otu017 LB (0.9) *	NS	Otu025 LB (0.48) * Otu028 LB (0.39) * Otu042 SC (0.21) **	Otu035 LB (0.36) ** Otu036 LB (0.36) ** Otu054 LB (0.12) **
Ileum	NS	Otu011 SC (3.3) * Otu091 SC (0.13) *	Otu060 SC (0.15) **	Otu007 LB (5.1) ** Otu008 LB (4.9) ** Otu016 LB (2.3) * Otu030 BB (0.92) * Otu054 LB 0.30) **	Otu038 SC (0.17) *	Otu027 BB (0.3) *	Otu018 SC (2.5) * Otu045 SC (0.33) ** Otu062 LB (0.18) *	Otu005 LB (10.0) * Otu068 LB (0.20) **
Caecum	Otu016 LN (3.3) *	Otu020 BB (2.6) *	Otu058 SC (0.62) ** Otu135 SC (0.10) *	Otu006 LB (4.39) ** Otu134 BB (0.12) *	Otu117 LB (0.13) *	Otu031 LN (1.2) **	NS	Otu002 LB (9.7) * Otu003 LB (7.2) ** Otu008 LB (4.1) * Otu019 LB (2.25) * Otu020 LB (2.48) * Otu059 LB (0.48) * Otu076 LB (0.36) **
Colon	NS	Otu009 BB (4.1) ** Otu033 LB (1.1) *	NS	Otu003 LB (4.7) * Otu038 BB (0.54) *	NS	NS	NS	Otu003 LB (6.2) ** Otu020 LB (2.1) *
Rectum	Otu021 LN (1.7) *	Otu022 LB (1.6) *	NS	NS	NS	NS	NS	NS

LB = *Lactobacillus*; LC = *Lactococcus*; LN = *Leuconostoc*; BB = *Bifidobacterium*; SC = *Streptococcus*. * $p < 0.05$, ** $p < 0.01$, NS = no significant difference in differential abundance between non-GOS and GOS-fed piglets (LEfSe). Figures in brackets are the relative abundance of named Otu for each GIT section and trial.

3.4. Histology and Gut Architecture

Measurement of gut architecture parameters was determined using HE-stained slides and the enumeration of GCs was performed using PAS-stained slides, examples of which are shown in Figure 4. Upper intestinal villus height, crypt depth VCR and the number of GCs per mm^2 GIT tissues were not normally distributed in most cases in trials 1 to 4 according to Shapiro–Wilk tests ($p < 0.05$) and therefore analysed non-parametrically on a trial-by-trial basis. Results for gut architecture are shown in Figure 5. The jejunal villus height was significantly greater for GOS-fed piglets in trials 1, 3 and 4 ($p = 0.005$, emphp = 6.4×10^{-16}, $p = 5.3 \times 10^{-8}$, Wilcoxon rank sum exact tests). Jejunal villus height was significantly greater for non-GOS-fed piglets in trial 2 ($p = 0.005$). Ileal villus height was significantly greater for GOS-fed piglets in trials 3 and 4 ($p = 61.6 \times 10^{-6}$ and $p = 1.1 \times 10^{-10}$, Wilcoxon rank sum exact tests). There were no significant differences in jejunal or ileal crypt depth between treatments. Jejunal VCR was significantly greater for GOS-fed piglets in trials 1, 3 and 4 ($p = 0.034$, $p = 5.1 \times 10^{-15}$, $p = 1.1 \times 10^{-8}$, Wilcoxon rank sum exact tests). Jejunal VCR was significantly greater for non-GOS-fed piglets in trial 2 ($p = 0.004$). Ileal VCR was significantly greater for GOS-fed piglets in trials 3 and 4 ($p = 0.0007$ and $p = 1.2 \times 10^{-9}$, Wilcoxon rank sum exact tests). The number of GCs per mm^2 GIT tissue per trial was determined from PAS-stained slides as shown in Figure 6. The number of GCs per mm^2 in the jejunal villus was significantly greater in GOS-fed piglets in trials 1, 3 and 4 ($p = 0.004$, $p = 1.8 \times 10^{-12}$ and $p = 1.1 \times 10^{-9}$, respectively; in the jejunal crypt of GOS-fed piglets in trials 1 and 3 ($p = 3.3 \times 10^{-9}$ and $p = 3.2 \times 10^{-12}$, respectively); in the ileal villus in trials 2, 3 and 4 ($p = 0.035$, $p = 1.8 \times 10^{-12}$ and $p = 1.2 \times 10^{-9}$ respectively); in the ileal crypt in trials 3 and 4 ($p = 0.0008$ and $p = 1.9 \times 10^{-5}$, respectively); in the colonic crypt in trial 2 ($p = 7.5 \times 10^{-6}$); and in the caecal crypt in trial 3 ($p = 0.0008$, Wilcoxon rank sum exact tests). Regardless of inter-trial variation, it was possible to pool data and plot the area of the jejunal and ileal villi versus height and the area of the jejunal, ileal, colonic and caecal crypts versus depth (Figure S5). There was a significant correlation between the jejunal and ileal villus area and height and jejunal, ileal, colonic and caecal crypt area and depth ($p < 0.001$ in each case using linear modelling). For pooled data, jejunal villus height was significantly greater for GOS-fed piglets as opposed to non-GOS-fed piglets ($p = 1.7 \times 10^{-11}$) as was ileal villus height ($p = 2.1 \times 10^{-8}$), caecal crypt depth ($p = 1.8 \times 10^{-4}$) and colonic crypt depth ($p = 0.008$, Wilcoxon rank sum exact tests). There were no significant differences in jejunal and ileal crypt depth between non-GOS-fed and GOS-fed piglets. Similarly, there was a significant correlation between the jejunal and ileal villus areas and the jejunal, ileal, caecal and colonic crypt areas with GC density expressed as number of GCs per mm^2 sectioned GIT tissues ($p < 0.001$ in each case, linear modelling, (Figure S6). Moreover, the number of GCs per mm^2 GIT tissue was significantly higher in GOS-fed piglets than non-GOS-fed piglets for the jejunal villus ($p = 7.4 \times 10^{-6}$), the jejunal crypt ($p = 1.4 \times 10^{-4}$), the ileal villus ($p = 2.8 \times 10^{-5}$), the ileal crypt ($p = 3.8 \times 10^{-7}$), the colonic crypt ($p = 5.3 \times 10^{-3}$) and the caecal crypt ($p = 0.003$, Wilcoxon rank sum exact tests).

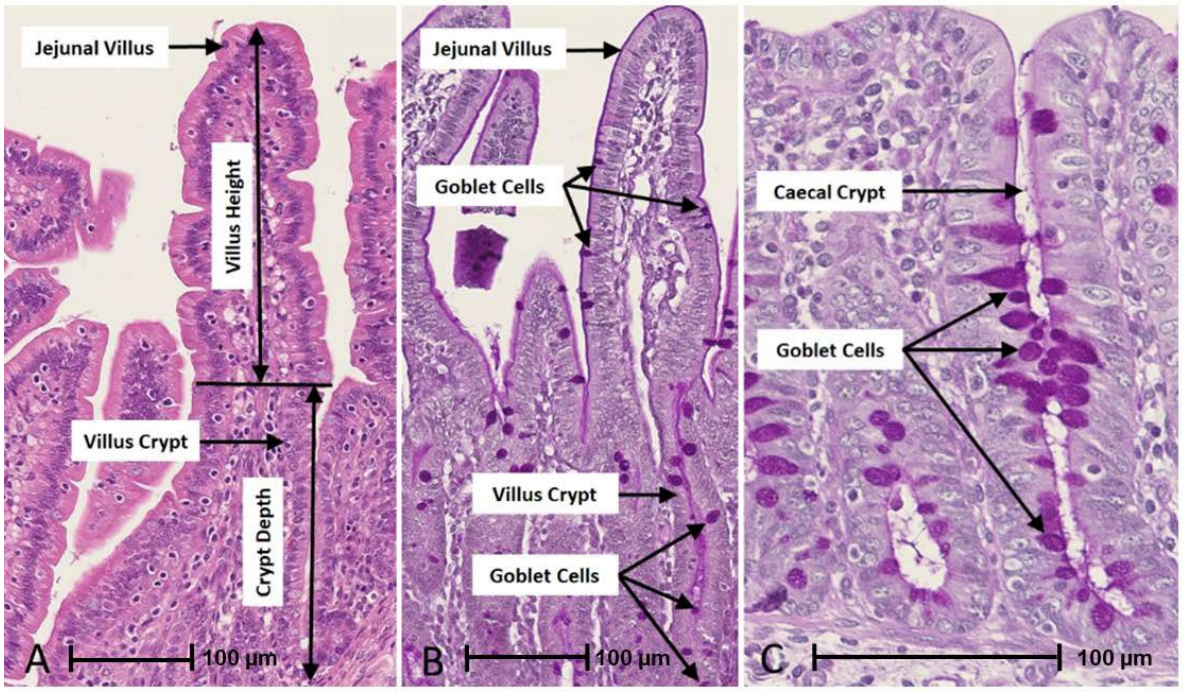

Figure 4. Example of gut architecture and GC enumeration in GIT sections taken with the NanoZoomer digital pathology system. (**A**) = HE-stained jejunal villus and crypt used for determination of villus height, crypt depth and VCR. (**B**) = PAS-stained jejunal villus and crypt used for GC enumeration. (**C**) = PAS-stained caecal crypt used for GC enumeration.

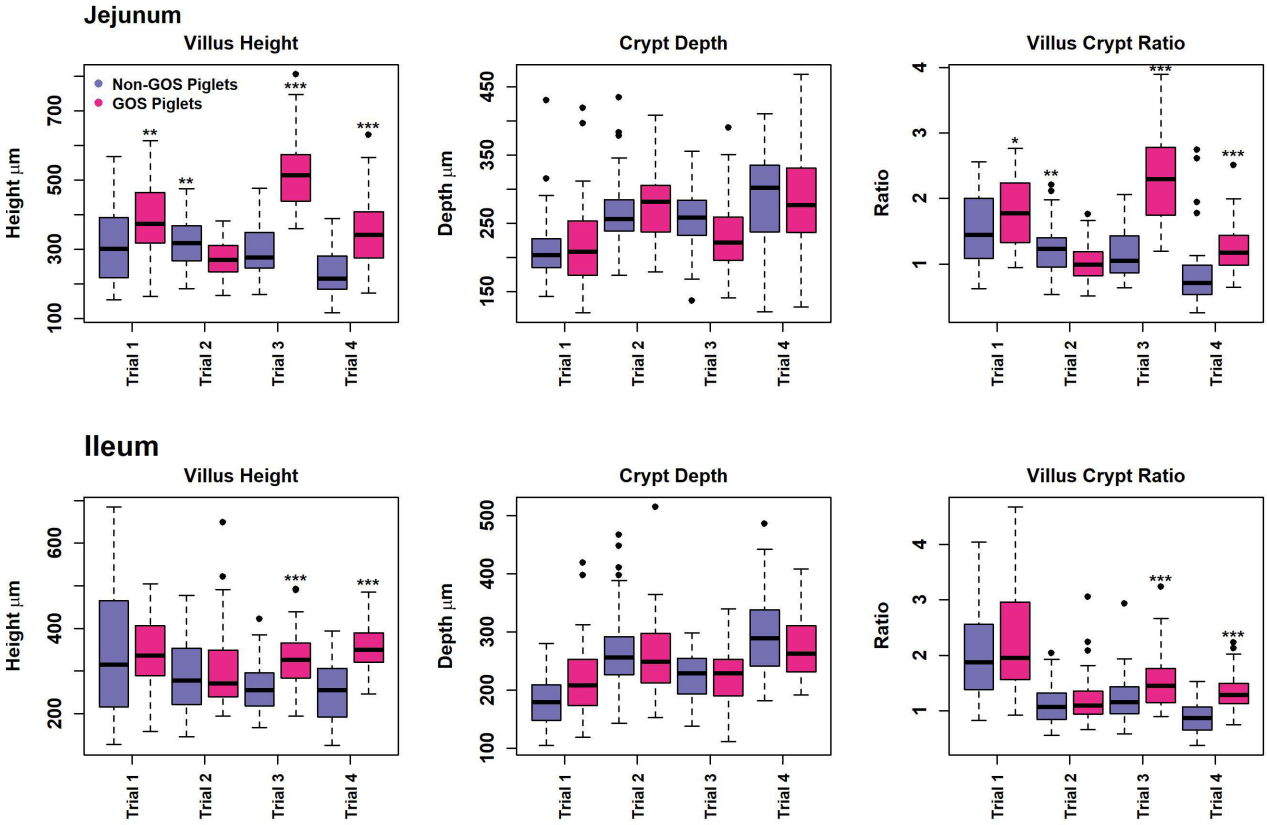

Figure 5. Upper intestinal gut architecture of non-GOS and GOS-fed piglets for trials 1 to 4 showing

villus height, crypt depth and villus crypt ratio. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Wilcoxon rank sum exact tests). Dots show outliers above maximum interquartile range.

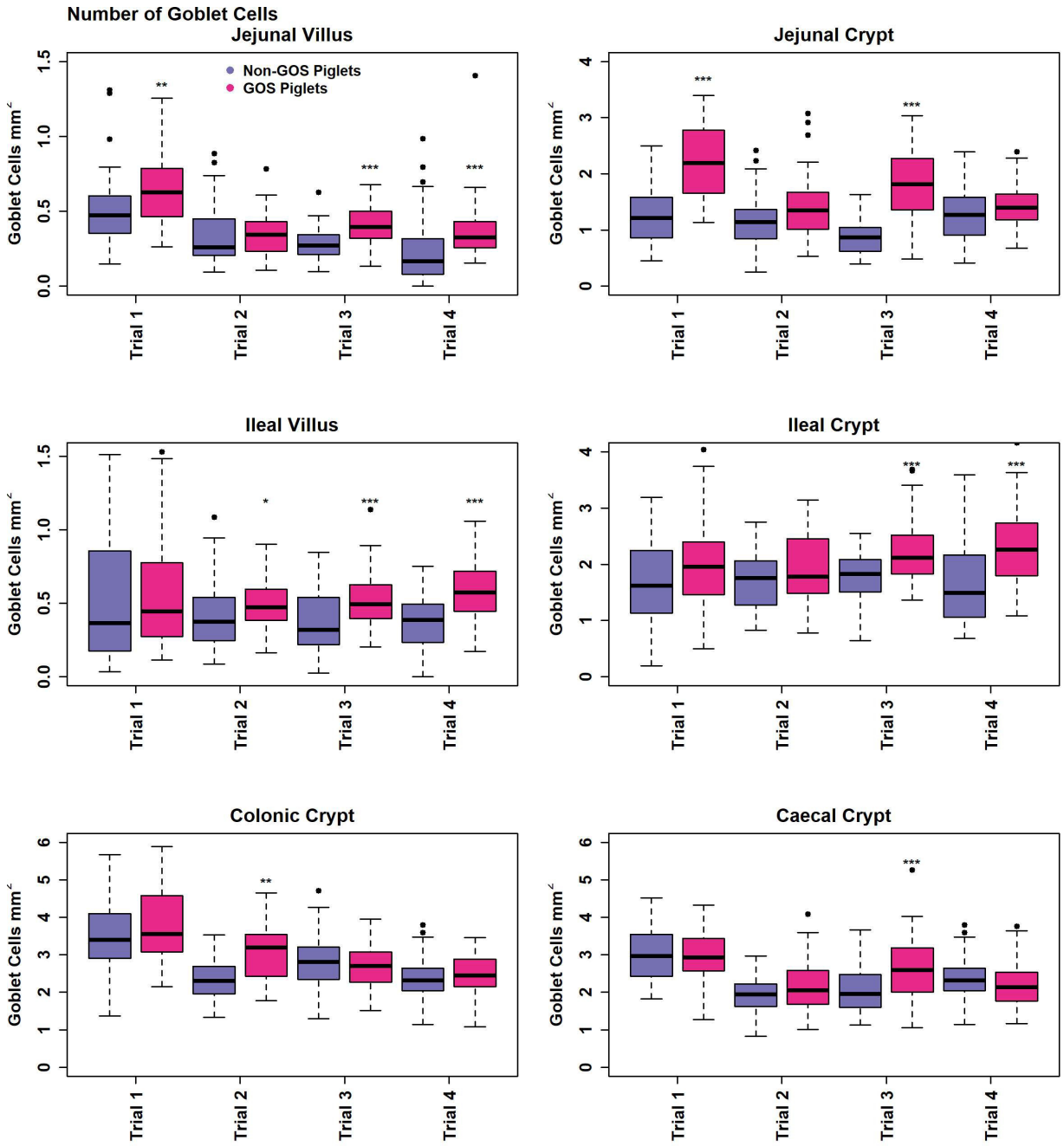

Figure 6. Number of goblet cells per mm^2 GIT tissue of non-GOS and GOS-fed piglets for trials 1 to 4. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Wilcoxon rank sum exact tests). Dots show outliers above maximum interquartile range.

4. Discussion

This study is novel in that it is one of the first to investigate the effects of supplementing complete milk replacer with galacto-oligosaccharides in poorly performing piglets. The objectives were to investigate if industry standard milk replacer supplemented with GOS as opposed to milk replacer alone affected the microbiome, gut architecture, GC expression and performance of poorly performing, non-thriving piglets who were unlikely to receive adequate nutrition from the sow. Studies were conducted in a commercial facility with animals destined for the food chain. However, there were only a limited number of

farrowing pens and a limited number of experimental isolation pens for poorly performing piglets. Therefore, trials had to be replicated. There were no significant differences in piglet weight at 24 h *post-partum*, trial days 1 to 21, total weight gain and ADG between the four repeated trials (Table 1). Neither was mean DFI (Figure S2) significantly different between trials and/or treatments, indicating that performance, food intake and animal husbandry were consistent between trials. However, supplementation of milk replacer with GOS had no significant effect on performance, with the main predictor of end-of-study weight (d21) being weight at 24 h *post-partum* (Figure S1). By way of comparison, it is reported that healthy GOS-fed piglets of the same age as this study showed no significant difference in body weight compared with the control groups but a significant increase in ADG [49]. However, weight at day 1 was, on average, 1.55 kg and at day 21~6 kg, with ADG at over 0.2 kg per day, values were much higher than in this study and for all trials and treatments (Table 1). Similarly, healthy GOS-fed piglets challenged with lipopolysaccharide endotoxin showed no significant difference in body weight compared with control groups but a significant increase in ADG [50]. Weight at day 1 was over 1.5 kg and at day 14 was over 4 kg, with an ADG of over 0.2 kg per day, with values again being higher than in this study. However, these studies administered a known dose of GOS at 1 g per kg body weight by manual oral infusion with continued access to the sow, as opposed to this study where animals were provided with milk replacer supplemented with GOS ad libitum with no access to the sow. In this respect, the addition of maternal GOS may have had a significant effect in contrast to the animals in this study that were removed from sows. Moreover, the animals in this study were initially underweight and poorly performing, which may explain why GOS had no effect on performance in terms of final body weight and ADG. Indeed, higher energy diet intake in the grower phase does not improve the performance of low-birth-weight pigs, which are less efficient than their heavier counterparts and are unable to show compensatory gains [51]. Studies have indicated that dietary supplementation with GOS post-weaning increases performance, and in this respect, it cannot be ruled out that GOS could positively affect performance in studies of longer duration than 21 days for poorly performing piglets [52].

There were no significant differences in α-diversity between non-GOS and GOS-fed piglets in trials 1 to 4 (Table 2). Previous studies have shown that intervention with GOS pre-weaning does not affect α-diversity, but post-weaning significantly increases the ACE and Chao1 indices of colonic mucosal communities in pigs [53]. Similarly, there were no significant differences in the Shannon, Simpson, Ace or Chao1 indices reported for colonic mucosal communities in lipopolysaccharide challenged piglets fed GOS compared with controls [29]. In this study, there were significant differences between trials in terms of α-diversity ($p < 0.05$ in each case), indicating that data could not be pooled but rather analysed on a trial-by-trial basis. The variation in data does not allow data to be pooled, in contrast to the study by Lee et al. [54], where trials were rigorously repeated in highly controlled conditions with a view to pooling data for the sake of performance-related measures. However, α-diversity expressed as inverse Simpson diversity or Chao richness significantly increased from the duodenum to the rectum in trials and for non-GOS and GOS-fed piglets alike, indicating the establishment of more diverse communities throughout the lower and upper GIT, consistent with previous work [55,56]. In Lee et al. (2022) [54], reported colonic inverse Simpson diversity ranged from (mean ± SD) 14.29 ± 2.20 to 20.20 ± 7.94, values that are broadly comparable with this study's range of 12.65 ± 2.81 to 27.41 ± 8.09. For colonic Chao richness, this was 1241.08 ± 171.68 to 1794.48 ± 250.51 compared with 179.42 ± 43.35 to 315.40 ± 95.66 for the present study. Similarly, at suckling, Chao richness was determined as 1240.3 for faecal samples [57], and 1757 for colonic samples [58]. This large difference in species richness may be explained by the "poorly performing", non-thriving nature of the piglets, since it is recognised that lower-weight, intrauterine growth-restricted piglets have lower microbial diversity [59]. Lower GIT diversity may be a result of poor performance or possibly a function of removing piglets from the sow to a controlled environment where they are not subjected to the microbiome of maternal sows and fit siblings.

In contrast to α-diversity, there were significant differences in β-diversity as measured by three metrics: Yue and Clayton dissimilarity (θ_{YC}) [42], Bray–Curtis dissimilarity [43] and Jaccard similarity [44] between non-GOS and GOS-fed pigs in trials 1 to 4 (Table 3 and Figure S3A–D). Differences in β-diversity were most prevalent for trials 2 and 4, and for Jaccard similarity, indicating that GOS possibly had more of an effect on microbial community membership, rather than community structure. Nevertheless, results demonstrate that early-life GOS intervention modulated GIT microbial composition, as in other studies [49,53].

The number of OTUs shared by all four trials (duodenum 177, jejunum 118, ileum 166, caecum 246, colon 272 and rectum 296) accounted for 97.9 to 99.76 % of the total relative abundance of all taxa at the genus level (Figure 1). Whilst the number of unique and/or partially shared OTUs may be much larger than those common between all four trials, they only account for 0.24 to 2.1 % of the total relative abundance of taxa across GIT samples. It is suggested there is a core microbiota in the GIT of healthy pigs, which can be a potential target for nutritional regulation and benefit the growth and GIT health of the animal [60,61]. If the definition of a core GIT microbiota is accepted as those being present in 90 % of samples [60], then the number of OTUs shared between trials 1 to 4 may be considered core to the suckling piglet microbiota. However, the core microbiota in pigs may be defined as those that are resident in the GIT throughout the lifetime of the animal as opposed to those that are "stage associated" and only occur at certain growth stages such as suckling [62]. At the phylum level, *Firmicutes, Bacteroidetes*, *Proteobacteria* and *Actinobacteria* were considered core to the lifetime pig GIT microbiome [62], in keeping with trials 1 to 4 of this study, where these phyla occurred in all samples. In nursing pigs, the three most abundant core genera were *Prevotella*, *Lactobacillus* and *Oscillispira*, with *Blautia* identified as a nursery stage-associated genus [61]. In this study, *Prevotella*, *Lactobacillus* and *Streptococcus* occurred in all samples, confirming them as core microbiota but *not Oscillispira*. Perhaps it is useful to consider not only the "core" and "stage" microbiota but also the "peripheral" OTUs, which occur in low abundances but nevertheless contribute to the diversity of the whole microbiome.

Analysis of the taxa annotated to OTUs at the phylum level in the intestinal contents of piglets fed milk replacer alone or milk replacer with GOS in trials 1 to 4 enabled comparison of the relative abundance of taxa present at anatomical sites throughout the length of the GIT (Figure 2). *Bacteroidetes* were more prevalent in lower rather than upper GIT samples as opposed to *Firmicutes,* which were more prevalent in upper GIT samples, as reported by Crespo-Piazuelo et al., 2018 [55]. Predominant phyla in all trials were *Firmicutes*, *Bacteroidetes*, *Proteobacteria* and *Actinobacteria* in keeping with previous observations [54,62,63]. *Proteobacteria* are found in abundance during suckling and decline post-weaning [64]. They usually include commensal but opportunistic and potentially pathogenic organisms from the genera *Campylobacter*, *Escherichia*, *Salmonella* and *Helicobacter* [65]. Although occurring at very low relative abundances, their presence highlights the potential for the development of gut dysbiosis considering that low diversity bacterial ecosystems have reduced colonisation resistance to pathogens [66]. The relative abundance of bacterial taxa annotated to OTUs at the genus level show *Lactobacillus, Streptococcus*, *Prevotella* and *Leuconostoc* were highly prevalent throughout all the trials (Figure 3). Although occurring throughout the GIT, *Streptococcus* was more prevalent in the upper GIT samples. LEfSe gives a clearer distinction between non-GOS and GOS-fed piglets (Table 4) and highlights those lactic acid fermenting and beneficial OTUs occurring between treatments and across the GIT for trials 1 to 4. Dietary supplementation with GOS increases *Lactobacillus* and *Bifidobacterium* populations in pigs, as confirmed in this study [9,52]. In trial 1, GOS significantly increased *Streptococcus* in the upper GIT as opposed *to Lactobacillus*, suggesting competition between these organisms. Nevertheless, GOS significantly increased *Bifidobacterium and Lactobacillus* in the lower GIT. Trials 2 and 4 identified a number of linear discriminant features attributable to all five lactic acid bacteria, confirming the lactogenic and bifidogenic effects of GOS throughout the GIT of suckling pigs.

Despite a large degree of variation between trials, this study has demonstrated that GOS significantly affects gut architecture and VCR in poorly performing piglets (Figure 5 and Figure S5). Not only does GOS protect against mucosal GIT damage in lipopolysaccharide-challenged pigs [29], but it also promotes higher villus height and VCR in *E. coli*-challenged pigs [67], suggesting physiological and nutritional health benefits for non-healthy animals and those with compromised upper GIT mucosal surfaces. However, the effects of GOS on production remain to be seen, presumably due to the non-thriving nature of animals in this study, the study length of 21 days and the inability of low-birth-weight animals to make compensatory weight gains [51]. Nevertheless, GC density per mm^2 tissue significantly increased throughout the GIT in GOS-fed piglets compared with non-GOS-fed piglets (Figure 6 and Figure S6). This is an important finding since GCs are known to be essential to barrier function and immune regulation in animals [21,22,25]. The physiological effect of GOS on GIT architecture and GC expression is not limited to pigs, with significant increases in villus height, caecal crypt depth and increased GIT GC numbers found in GOS-fed chickens [68,69]. GOS directly modulates the expression of GC secretory products that contribute to the production of barrier-enhancing mucins via cell surface receptors [27]. However, this may be indirectly modulated by the microbiota, particularly the lactic acid-producing bacteria *Bifidobacterium* and *Lactobacillus* spp., as significantly increased in this study by GOS, which support intestinal cell regeneration and the proliferation of intestinal stem, Paneth and GCs through the action of lactic acid [70].

5. Conclusions

GOS, as a supplement to milk replacer formula for piglets separated from sows, is palatable and well tolerated, with significant increases in weight during trials, but no significant performance difference between treatments. Administration of GOS had no significant effect on α-diversity, for which Chao richness appeared to be low, but may be a function of inherent poor performance and/or removing piglets from sows and healthy siblings to controlled environments, thus possibly being a product of methodology. Nevertheless, GOS significantly modulated GIT microbial communities as demonstrated by β-diversity measures, with key effects on microbial community membership rather than structure, demonstrating that GOS is effective in promoting more diverse, beneficial communities. GOS significantly increased linear discriminant features attributable to lactic acid-producing bacteria, notably *Lactobacillus* and *Bifidobacterium* throughout the GIT, demonstrating benefits as a supplement to milk replacer in poorly performing piglets. GOS significantly improved GIT architectural features and VCR throughout the upper GIT as well as increasing the number of barrier-enhancing and immunomodulatory GCs, possibly through direct modulation by GOS or indirectly by the lactogenic effect of lactic acid-producing bacteria. The significance of these indicators lies in the improvement of GIT health in poorly performing animals, giving them a better chance of survival in controlled environments. In conclusion, GOS significantly increases the differential abundance of beneficial probiotic bacteria, particularly *Lactobacillus* and *Bifidobacterium*, and improves gut architecture and goblet cell expression in poorly performing piglets. In these respects, a GOS-supplemented milk replacer may be a useful addition to animal husbandry for poorly performing, non-thriving animals when moved to environmentally controlled pens away from sows and their thriving siblings, thereby modulating the microbiome and GIT performance. Future applications may include the addition of GOS in milk replacers for healthy piglets requiring additional nutrition from the sow, but this would require further research.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ani13020230/s1, Table S1: Composition of Faramate milk replacer feed for piglets (10 kg); Table S2: Number of high-quality sequences obtained from each trial and section of the piglet GIT; Table S3: Number of high-quality sequences clustered into OTUs obtained from each trial and section of the piglet GIT; Figure S1: Piglet weight at 24 h correlates with the final end-of-study weight; Figure S2: Daily feed intake; Figure S3A–S3D: PCA plots for β-diversity; Figure S4A–S4D: Linear discriminant effect size; Figure S5: Area of the jejunal and ileal villi versus height and the area of the jejunal, ileal, colonic and caecal crypts versus depth; Figure S6: GC density expressed as the number of GCs per mm^2 sectioned GIT tissues.

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Institutional Review Board Statement: All methods were carried out in accordance with the relevant guidelines and regulations of the Harper Adams University Research Ethics Committee and approved by them. Approved on 'Thu 26/04/2018 09:42' with project identification code '0221-201803-STAFF'.

Informed Consent Statement: Informed consent was obtained from Harper Adams University, Shropshire, TF10 8NB UK under the project title: The effect of the addition of Nutrabiotic® to milk replacer on the health and growth of poor performing piglets from one week of age (Unique Trial Code: HAUDC004/2018/Pig).

Data Availability Statement: Sequence data were deposited in the NCBI database within the Bioproject PRJNA866473 with SRA records available at: (https://www.ncbi.nlm.nih.gov/sra/PRJNA866473, accessed online 5 August 2022). R-code for analyses are deposited at: (https://github.com/AdamLeeNottinghamUniversity/Piglets, accessed online 12 September 2022).

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Conflicts of Interest: Stephen Mansbridge was a paid consultant to Saputo Dairy UK (formerly Dairy Crest) at the time of this work.

References

1. Guevarra, R.B.; Lee, J.H.; Lee, S.H.; Seok, M.J.; Kim, D.W.; Kang, B.N.; Johnson, T.J.; Isaacson, R.E.; Kim, H.B. Piglet gut microbial shifts early in life: Causes and effects. *J. Anim. Sci. Biotechnol.* **2019**, *10*, 1. [CrossRef]
2. Brestoff, J.R.; Artis, D. Commensal bacteria at the interface of host metabolism and the immune system. *Nat. Immunol.* **2013**, *14*, 676–684. [CrossRef]
3. Kim, H.B.; Isaacson, R.E. The pig gut microbial diversity: Understanding the pig gut microbial ecology through the next generation high throughput sequencing. *Vet. Microbiol.* **2015**, *177*, 242–251. [CrossRef]
4. Nuntapaitoon, M.; Muns, R.; Tummaruk, P. Newborn traits associated with pre-weaning growth and survival in piglets. *Asian-Australas. J. Anim. Sci.* **2018**, *31*, 237–244. [CrossRef]
5. Monteagudoo, L.; Benito, A.A.; Lázaro-Gaspar, S.; Arnal, J.L.; Martin-Jurado, D.; Menjon, R.; Quílez, J. Occurrence of rotavirus A genotypes and other enteric pathogens in diarrheic suckling piglets from Spanish swine farms. *Animals* **2022**, *12*, 251. [CrossRef]
6. Tucker, B.S.; Craig, J.R.; Morrison, R.S.; Smits, R.J.; Kirkwood, R.N. Piglet viability: A review of identification and pre-weaning management strategies. *Animals* **2021**, *11*, 2902. [CrossRef]

7. Collins, C.L.; Pluske, J.R.; Morrison, R.S.; McDonald, T.N.; Smits, R.J.; Henman, D.J.; Stensland, I.; Dunshea, F.R. 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. *Anim. Nutr.* **2017**, *3*, 372–379. [CrossRef]
8. Liao, S.F.; Nyachoti, M. Using probiotics to improve swine gut health and nutrient utilization. *Anim. Nutr.* **2017**, *3*, 331–343. [CrossRef]
9. Alizadeh, A.; Akbari, P.; Difilippo, E.; Schols, H.A.; Ulfman, L.H.; Schoterman, M.H.; Garssen, J.; Fink-Gremmels, J.; Braber, S. The piglet as a model for studying dietary components in infant diets: Effects of galacto-oligosaccharides on intestinal functions. *Br. J. Nutr.* **2016**, *115*, 605–618. [CrossRef]
10. Ninonuevo, M.R.; Park, Y.; Yin, H.; Zhang, J.; Ward, R.E.; Clowers, B.H.; German, J.B.; Freeman, S.L.; Killeen, K.; Grimm, R.; et al. A strategy for annotating the human milk glycome. *J. Agric. Food Chem.* **2006**, *54*, 7471–7480. [CrossRef]
11. Albrecht, S.; Lane, J.A.; Mariño, K.; Al Busadah, K.A.; Carrington, S.D.; Hickey, R.M.; Rudd, P.M. A comparative study of free oligosaccharides in the milk of domestic animals. *Br. J. Nutr.* **2014**, *111*, 1313–1328. [CrossRef]
12. Bode, L. Human milk oligosaccharides: Every baby needs a sugar mama. *Glycobiology* **2012**, *22*, 1147–1162. [CrossRef]
13. Donovan, S.M.; Comstock, S.S. Human milk oligosaccharides influence neonatal mucosal and systemic immunity. *Ann. Nutr. Metab.* **2016**, *69* (Suppl 2), 42–51. [CrossRef] [PubMed]
14. Urashima, T.; Saito, T.; Nakamura, T.; Messer, M. Oligosaccharides of milk and colostrum in non-human mammals. *Glycoconj. J.* **2001**, *18*, 357–371. [CrossRef] [PubMed]
15. Urashima, T.; Taufik, E.; Fukuda, K.; Asakuma, S. Recent advances in studies on milk oligosaccharides of cows and other domestic farm animals. *Biosci. Biotechnol. Biochem.* **2013**, *77*, 455–466. [CrossRef] [PubMed]
16. Difilippo, E.; Bettonvil, M.; Willems, R.H.; Braber, S.; Fink-Gremmels, J.; Jeurink, P.V.; Schoterman, M.H.C.; Gruppen, H.; Schols, H.A. Oligosaccharides in urine, blood, and feces of piglets fed milk replacer containing galacto-oligosaccharides. *J. Agric. Food Chem.* **2015**, *64*, 2127–2133. [CrossRef]
17. Difilippo, E.; Pan, F.; Logtenberg, M.; Willems, R.; Braber, S.; Fink-Gremmels, J.; Schols, H.A.; Gruppen, H. In vitro fermentation of porcine milk oligosaccharides and galactooligosaccharides Using Piglet Fecal Inoculum. *J. Agric. Food. Chem.* **2016**, *63*, 10862–10872. [CrossRef]
18. Yan, Y.L.; Hu, Y.; Simpson, D.J.; Gänzle, M.G. Enzymatic synthesis and purification of galactosylated chitosan oligosaccharides reducing adhesion of enterotoxigenic Escherichia coli K88. *J. Agric. Food Chem.* **2017**, *65*, 5142–5150. [CrossRef]
19. Bouwhuis, M.A.; McDonnell, M.J.; Sweeney, T.; Mukhopadhya, A.; O'Shea, C.J.; O'Doherty, J.V. Seaweed extracts and galacto-oligosaccharides improve intestinal health in pigs following Salmonella Typhimurium challenge. *Animal* **2017**, *11*, 1488–1496. [CrossRef]
20. Epple, H.J.; Kreusel, K.M.; Hanski, C.; Schulzke, J.D.; Riecken, E.O.; Fromm, M. Differential stimulation of intestinal mucin secretion by cholera toxin and carbachol. *Pflugers Arch.* **1997**, *433*, 638–647. [CrossRef]
21. McCauley, H.A.; Guasch, G. Three cheers for the goblet cell: Maintaining homeostasis in mucosal epithelia. *Trends Mol. Med.* **2015**, *21*, 492–503. [CrossRef]
22. Forder, R.E.; Howarth, G.S.; Tivey, D.R.; Hughes, R.J. Bacterial Modulation of small intestinal goblet cells and mucin composition during early post hatch development of poultry. *Poult. Sci.* **2007**, *86*, 2396–2403. [CrossRef]
23. McDole, J.R.; Wheeler, L.W.; McDonald, K.G.; Wang, B.; Konjufca, V.; Knoop, K.A. Goblet cells deliver luminal antigen to CD103+ dendritic cells in the small intestine. *Nature* **2012**, *483*, 345–349. [CrossRef]
24. Knoop, K.A.; McDonald, K.G.; McCrate, S.; McDole, J.R.; Newberry, R.D. Microbial sensing by goblet cells controls immune surveillance of luminal antigens in the colon. *Mucosal Immunol.* **2015**, *8*, 198–210. [CrossRef]
25. Knoop, K.A.; Newberry, R.D. Goblet cells: Multifaceted players in immunity at mucosal surfaces. *Mucosal Immunol.* **2018**, *11*, 1551–1557. [CrossRef]
26. Zhang, W.; Zhu, Y.H.; Zhou, D.; Wu, Q.; Song, D.; Dicksved, J.; Wang, J.F. Oral administration of a select mixture of Bacillus probiotics affects the gut microbiota and goblet cell function following *Escherichia coli* challenge in newly weaned pigs of genotype MUC4 that are supposed to be enterotoxigenic *E. coli* F4ab/ac receptor negative. *Appl. Environ. Microbiol.* **2017**, *83*, e02747-16. [CrossRef] [PubMed]
27. Bhatia, S.; Nagendra Prabhu, P.; Benefiel, A.C.; Miller, M.J.; Chow, J.; Davis, S.R.; Gaskins, H.R. Galacto-oligosaccharides may directly enhance intestinal barrier function through the modulation of goblet cells. *Mol. Nutr. Food. Res.* **2015**, *59*, 566–573. [CrossRef] [PubMed]
28. Cheng, L.; Kong, C.; Walvoort, M.T.C.; Faas, M.M.; de Vos, P. Human milk oligosaccharides differently modulate goblet cells under homeostatic, proinflammatory conditions and ER Stress. *Mol. Nutr. Food Res.* **2020**, *64*, e1900976. [CrossRef]
29. Gao, R.; Tian, S.; Wang, J.; Zhu, W. Galacto-oligosaccharides improve barrier function and relieve colonic inflammation via modulating mucosa-associated microbiota composition in lipopolysaccharides-challenged piglets. *J. Anim. Sci. Biotechnol.* **2021**, *12*, 92. [CrossRef]
30. Kruger, C.; Zhou, Y.; Thorsrud, B.A.; Morel-Despeisse, F.; Chappuis, E. Safety evaluation of α-galacto-oligosaccharides for use in infant formulas investigated in neonatal piglets. *Toxicol. Res. Appl.* **2017**, *1*, 1–10. [CrossRef]
31. Caporaso, J.G.; Lauber, C.L.; Walters, W.A.; Berg-Lyons, D.; Lozupone, C.A.; Turnbaugh, P.J.; Fierer, N.; Knight, R. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proc. Natl. Acad. Sci. USA.* **2011**, *108* (Suppl. 1), 4516–4522. [CrossRef] [PubMed]

32. Kozich, J.J.; Westcott, S.L.; Baxter, N.T.; Highlander, S.K.; Schloss, P.D. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. *Appl. Environ. Microbiol.* **2013**, *79*, 5112–5120. [CrossRef] [PubMed]
33. Schloss, P.D.; Westcott, S.L.; Ryabin, T.; Hall, J.R.; Hartmann, M.; Hollister, E.B.; Lesniewski, R.A.; Oakley, B.B.; Parks, D.H.; Robinson, C.J. Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl. Environ. Microbiol.* **2009**, *75*, 7537–7541. [CrossRef]
34. Pruesse, E.; Quast, C.; Knittel, K.; Fuchs, B.M.; Ludwig, W.; Peplies, J.; Glöckner, F.O. SILVA: A comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Res.* **2007**, *35*, 7188–7196. [CrossRef] [PubMed]
35. 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. *Appl. Environ. Microbiol.* **2007**, *73*, 5261–5267. [CrossRef]
36. Cole, J.R.; Wang, Q.; Fish, J.A.; Chai, B.; McGarrell, D.M.; Sun, Y.; Brown, C.T.; Porras-Alfaro, A.; Kuske, C.R.; Tiedje, J.M. Ribosomal Database Project: Data and tools for high throughput rRNA analysis. *Nucleic Acids Res.* **2014**, *42*, D633–D642. [CrossRef]
37. R Core Team. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. 2021. Available online: https://www.R-project.org/ (accessed on 14 July 2022).
38. Good, I.J.; Toulmin, G.H. The number of new species, and the increase in population coverage, when a sample is increased. *Biometrika* **1956**, *43*, 45–63. [CrossRef]
39. Magurran, A.E. *Measuring Biological Diversity*; Blackwell Science: Oxford, UK, 2004.
40. Chao, A. Nonparametric estimation of the number of classes in a population. *Scand. J. Stat.* **1984**, *11*, 265–270. Available online: https://www.jstor.org/stable/4615964 (accessed on 14 July 2022).
41. Shapiro, S.S.; Wilk, M.B. An analysis of variance test for normality (complete samples). *Biometrika* **1965**, *52*, 591–611. [CrossRef]
42. Yue, J.C.; Clayton, M.K. A similarity measure based on species proportions. *Commun. Stat. – Theory Methods* **2005**, *34*, 2123–2131. [CrossRef]
43. Bray, J.R.; Curtis, J.T. An ordination of the upland forest communities of southern Wisconsin. *Ecol. Monogr.* **1957**, *27*, 326–349. [CrossRef]
44. Jaccard, P. Etude comparative de la distribution florale dans une portion des Alpe's et du Jura. *Bull. Soci'et'e Vaud. Sci. Nat.* **1901**, *37*, 547–579. [CrossRef]
45. Excoffier, L.; Smouse, P.E.; Quattro, J.M. Analysis of molecular variance inferred from metric distances among DNA haplotypes: Application to human mitochondrial DNA restriction data. *Genetics* **1992**, *131*, 479–491. [CrossRef] [PubMed]
46. Anderson, M.J. A new method for non-parametric multivariate analysis of variance. *Austral. Ecology.* **2001**, *26*, 32–46. [CrossRef]
47. Segata, N.; Izard, J.; Waldron, L.; Gevers, D.; Miropolsky, L.; Garrett, W.S.; Huttenhower, C. Metagenomic biomarker discovery and explanation. *Genome Biol.* **2011**, *12*, R60. [CrossRef] [PubMed]
48. Benjamini, Y.; Hochberg, Y. Controlling the false discovery rate: A practical and powerful approach to multiple testing. *J. R. Stat. Soc. Series B Methodol.* **1995**, *57*, 289–300. Available online: https://www.jstor.org/stable/2346101 (accessed on 11 July 2022). [CrossRef]
49. Tian, S.; Wang, J.; Yu, H.; Wang, J.; Zhu, W. Effects of galacto-oligosaccharides on growth and gut function of newborn suckling piglets. *J. Anim. Sci. Biotechnol.* **2018**, *9*, 75. [CrossRef] [PubMed]
50. Tian, S.; Wang, J.; Gao, R.; Wang, J.; Zhu, W. Early-life galacto-oligosaccharides supplementation alleviates the small intestinal oxidative stress and dysfunction of lipopolysaccharide-challenged suckling piglets. *J. Anim. Sci. Biotechnol.* **2022**, *13*, 70. [CrossRef] [PubMed]
51. Douglas, S.L.; Edwards, S.A.; Kyriazakis, I. Too late to catch up: A high nutrient specification diet in the grower phase does not improve the performance of low birth weight pigs. *J. Anim. Sci.* **2014**, *92*, 4577–4584. [CrossRef] [PubMed]
52. Xing, Y.; Li, K.; Xu, Y.; Wu, Y.; Shi, L.; Guo, S.; Yan, S.-M.; Jin, X.; Shi, B.-L. Effects of galacto-oligosaccharide on growth performance, feacal microbiota, immune response and antioxidant capability in weaned piglets. *J. Appl. Anim. Res.* **2020**, *48*, 63–69. [CrossRef]
53. Tian, S.; Wanj, J.; Wang, J.; Zhu, W. Differential effects of early-life and postweaning galacto-oligosaccharide intervention on colonic bacterial composition and function in weaning piglets. *Appl. Environ. Microbiol.* **2022**, *88*, e01318-21. [CrossRef] [PubMed]
54. Lee, A.; Le Bon, M.; Connerton, I.F.; Mellits, K.H. Common colonic community indicators of the suckling pig microbiota where diversity and abundance correlate with performance. *FEMS Microbiol. Ecol.* **2022**, *98*, fiac048. [CrossRef] [PubMed]
55. Crespo-Piazuelo, D.; Estellé, J.; Revilla, M.; Criado-Mesas, L.; Ramayo-Caldas, Y.; Óvilo, C.; Fernández, A.I.; Ballester, M.; Folch, J.M. Characterization of bacterial microbiota compositions along the intestinal tract in pigs and their interactions and functions. *Sci. Rep.* **2018**, *8*, 12727. [CrossRef] [PubMed]
56. Qi, R.; Qiu, X.; Du, L.; Wang, J.; Wang, Q.; Huang, J.; Liu, Z. Changes of gut microbiota and its correlation with short chain fatty acids and bioamine in piglets at the early growth stage. *Front. Vet. Sci.* **2021**, *7*, 617259. [CrossRef]
57. Holman, D.B.; Chénier, M.R. Temporal changes and the effect of subtherapeutic concentrations of antibiotics in the gut microbiota of swine. *FEMS Microbiol. Ecol.* **2014**, *3*, 599–608. [CrossRef]

58. Hoeflinger, J.L.; Kashtanov, D.O.; Cox, S.B.; Dowd, S.E.; Jouni, Z.E.; Donovan, S.M.; Miller, M.J. Characterization of the intestinal lactobacilli community following galactooligosaccharides and polydextrose supplementation in the neonatal piglet. *PLoS One.* **2015**, *10*, e0135494. [CrossRef]
59. Zhang, W.; Ma, C.; Xie, P.; Zhu, Q.; Wang, X.; Yin, Y.; Kong, X. Gut microbiota of newborn piglets with intrauterine growth restriction have lower diversity and different taxonomic abundances. *J. Appl. Microbiol.* **2019**, *127*, 354–369. [CrossRef]
60. Holman, D.B.; Brunelle, B.W.; Trachsel, J.; Allen, H.K. Meta-analysis to define a core microbiota in the swine gut. *mSystems* **2017**, *3*, e00004-17. [CrossRef]
61. Luo, Y.; Ren, W.; Smidt, H.; Wright, A.G.; Yu, B.; Schynns, G.; McCormack, U.M.; Cowieson, A.J.; Yu, J.; He, J. Dynamic distribution of gut microbiota in pigs at different growth stages: Composition and contribution. *Microbiol. Spectr.* **2022**, *10*, e0068821. [CrossRef]
62. Wang, X.; Tsai, T.; Deng, F.; Wei, X.; Chai, J.; Knapp, J. Longitudinal investigation of the swine gut microbiome from birth to market reveals stage and growth performance associated bacteria. *Microbiome* **2019**, *7*, 109. [CrossRef]
63. Zhang, L.; Mu, C.; He, X.; Su, Y.; Mao, S.; Zhang, J.; Smidt, H.; Zhu, W. Effects of dietary fibre source on microbiota composition in the large intestine of suckling piglets. *FEMS Microbiol. Lett.* **2016**, *363*, fnw138. [CrossRef] [PubMed]
64. Vo, N.; Tsai, T.C.; Maxwell, C.; Carbonero, F. Early exposure to agricultural soil accelerates the maturation of the early-life pig gut microbiota. *Anaerobe* **2017**, *45*, 31–39. [CrossRef]
65. Madigan, M.; Martinko, J. *Brock Biology of Microorganisms*, 15th ed.; Pearson: New York, NY, USA, 2018.
66. Stecher, B.; Chaffron, S.; Käppeli, R.; Hapfelmeier, S.; Freedrich, S.; Weber, T.C.; Kirundi, J.; Suar, M.; McCoy, K.D.; von Mering, C.; et al. Like will to like: Abundances of closely related species can predict susceptibility to intestinal colonization by pathogenic and commensal bacteria. *PLoS Pathog.* **2010**, *6*, e1000711. [CrossRef]
67. Zou, Y.; Wang, J.; Wang, Y.; Peng, B.; Liu, J.; Zhang, B. Protection of galacto-oligosaccharide against *E. coli* O157 colonization through enhancing gut barrier function and modulating gut microbiota. *Foods* **2020**, *9*, 1710. [CrossRef]
68. Lafontaine, G.M.F.; Richards, P.J.; Connerton, P.L.; O'Kane, P.M.; Ghaffar, N.M.; Cummings, N.J.; Fish, N.M.; Connerton, I. Prebiotic driven increases in IL-17A do not prevent *campylobacter jejuni* colonization of chickens. *Front. Microbiol.* **2020**, *10*, 3030. [CrossRef] [PubMed]
69. Richards, P.J.; Lafontaine, G.M.F.; Connerton, P.L.; Liang, L.; Asiani, K.; Fish, N.M.; Connerton, I.F. Galacto-oligosaccharides modulate the juvenile gut microbiome and innate immunity to improve broiler chicken performance. *mSytems.* **2020**, *5*, e00827-19. [CrossRef]
70. Lee, Y.; Kim, T.; Kim, Y.; Lee, S.; Kim, S.; Kang, S.; Yang, J.-Y.; Baek, I.-J.; Sung, Y.H.; Park, Y.-Y.; et al. Microbiota-derived lactate accelerates intestinal stem-cell-mediated epithelial development. *Cell Host Microbe.* **2018**, *24*, 833–846.e6. [CrossRef]

5 Chapter 5. Effects of prebiotic galacto-oligosaccharides on rotavirus antibodies in sow colostrum, reduced infectivity and microbiome modulation in neonatal piglets.

The objectives of this study were to determine if GOS supplementation of late gestational sows (7 days before parturition) on a commercial farm with natural endemic rotavirus challenge could improve neonatal immunity, reduce rotavirus infection and modulate the microbiota. This is one of the first papers to demonstrate that GOS fed to sows during late gestation significantly increased RVA specific IgG and IgA in sow colostrum. Another major and novel finding was that 65% of non-GOS piglet faecal samples tested positive for RVA as opposed to 45% for GOS-fed piglet faecal samples representing a significant reduction in infectivity of RVA in the maternally GOS fed group. Predominant phyla in sows and piglets irrespective of GOS supplementation to sows were *Firmicutes*, *Bacteroidetes, Proteobacteria* and *Actinobacteria.* Irrespective of GOS supplementation, *Clostridium sensu stricto*, *Acinetobacter*, *Enterobacteriaceae* unclassified, *Terrisporobacter* and *Lactobacillus* dominated taxa at genus level in sow faecal samples as did *Bacteroides*, *Clostridium senso stricto*, *Enterobacteriaceae* unclassified, *Lactobacillus* and *Streptococcus* in piglet faecal samples. Differential abundance of potentially pathogenic organisms was lower in GOS fed sows demonstrating modulation of the maternal microbiome. A higher differential abundance of virally suppressant *Collinsella* in faeces of piglets born to GOS fed sows suggests modulation of the piglet microbiome through late gestational feeding with GOS. The paper was peer reviewed and accepted for publication by Frontiers in Veterinary Science, an official journal of the publisher Frontiers Media S.A. and is accessible at:

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Galacto-oligosaccharides fed during gestation increase Rotavirus A specific antibodies in sow colostrum, modulate the microbiome, and reduce infectivity in neonatal piglets in a commercial farm setting

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Introduction: Rotavirus A is a major cause of acute dehydrating diarrhea in neonatal pigs resulting in significant mortality, morbidity, reduced performance and economic loss. Commercially available prebiotic galacto-oligosaccharides are similar to those of mammalian milk and stimulate the development of the microbiota and immune system in neonates. Little is known about the effects of supplementing sows' diets with galacto-oligosaccharides during gestation. This study aimed to determine if dietary galacto-oligosaccharide supplementation during gestation could improve immunity, reduce rotavirus infection and modulate the microbiota in sows and neonates in a commercial farm setting with confirmed natural endemic rotavirus challenge.

Methods: In a randomized controlled trial, control sows received lactation diet with no galacto-oligosaccharide supplementation and test sows received lactation diet with 30 g/day galacto-oligosaccharide top-dressed into feed daily, seven days before farrowing. Colostrum was collected from sows 24 hours *post-partum* and tested for rotavirus specific antibodies. Fecal samples were collected from sows and piglets three days *post-partum*, tested for rotavirus A by qPCR and the microbiome composition assessed by 16s rRNA gene sequencing.

Results: Supplementation with galacto-oligosaccharides during gestation significantly increased rotavirus-specific IgG and IgA in sow colostrum and reduced the number of rotavirus positive piglet fecal samples. Abundance of potential pathogens *Treponema* and *Clostridiales* were higher in fecal samples from non-galacto-oligosaccharide fed sows, their piglets and rotavirus positive samples.

Discussion: This study demonstrates that galacto-oligosaccharide supplementation during gestation significantly increases rotavirus specific IgG and IgA in sow colostrum thereby reducing neonatal rotavirus infection and suppresses potential pathogenic bacteria in nursing sows and neonatal piglets.

KEYWORDS

rotavirus, microbiota, pigs, galacto-oligosaccharides, antibodies, colostrum

Introduction

Rotaviruses are classified into at least ten serogroups (1, 2) with A, B, and C affecting humans (3), whilst groups A to H have been found in pigs (2). The most common groups are A, B and C, with Rotavirus A (RVA) representing the most prevalent group causing acute dehydrating diarrhea in public and veterinary health settings (2). RVA fecal-oral infection results in destruction of small intestinal enterocytes, the development of malabsorptive diarrhea (4) and promotes gut dysbiosis through alteration of the microbiota (5). The effects on pigs are significant mortality and morbidity in neonates, reduced performance in surviving growers and significant economic loss (1, 2, 6). RV is endemic in UK pig farms. A range of RVA genotypes has been identified in UK pigs: six G types (VP7); G2, G3, G4, G5, G9, and G11 and six P types (VP4); P6, P7, P8, P13, P23, and P32 (7). Furthermore, the common human genotype P8 can infect pigs highlighting the need for surveillance of porcine rotavirus genotypes to safeguard human and porcine health (7).

Previous livestock vaccination strategies have focussed on the induction of active (immune cell mediated) and passive (antibody mediated) immunity by oral administration of attenuated RV vaccines (8). However, these have lacked efficacy, in contrast to engineered virus-like particles (VLP) designed as vaccines to boost antibodies in bovine and porcine mammary secretions which have shown promise when administered with attenuated vaccines (9). The wide variety of RV genotypes in pigs complicates effective vaccine production. This is further complicated by attenuated replicating porcine RVA vaccines which may contribute to the diversity of porcine RVs, through re-assortment of vaccine strains with wild type strains and the emergence of novel genetic variants that can evade herd immunity (2, 7). Whilst vaccination remains popular in the farming community, a more pragmatic view may be to focus on cleaning and disinfection with efficacious detergents that not only limit the spread and infectivity of RV but also other microbial pathogens (10, 11). Nevertheless, endemic porcine RV infection still needs alternative strategies to boost lactogenic immunity in sows, thus providing RV antibodies to the neonate with colostrum and milk (2).

Galacto-oligosaccharides (GOS) are a major constituent of mammalian milk (12, 13) primarily stimulating the development of the microbiota in neonates and conferring a variety of health benefits including innate and adaptive immune development (14, 15). Milk oligosaccharides are typically composed of three to ten monosaccharide units, including glucose (Glc), galactose (Gal) and N-acetyl-glucosamine (GlcNAc) as well as fucose and sialic acids. The core moiety present at the reducing end of milk oligosaccharides is either lactose (Gal(β1–4)Glc) or N-acetyl-lactosamine (Gal(β1–4)GlcNAc) (16). Most animal milk oligosaccharides are sialylated, containing N-acetylneuraminic acid (Neu5Ac) and/or N-glycolylneuraminic acid (Neu5Gc) (17). Compared with other domestic animals, porcine milk contains the highest percentage of neutral oligosaccharides (20%), the most abundant variety of mono-sialylated and di-sialylated large oligosaccharides and are the closest to human milk oligosaccharide composition (13). In addition, porcine milk oligosaccharides (PMOs) decrease in abundance by ∼43% during the first week of lactation with the relative concentration of acidic PMOs decreasing and neutral PMOs increasing (18), indicating a change in functionality during lactation.

In pigs there is evidence that GOS is readily fermented in the gastrointestinal tract (GIT) increasing short-chain fatty acid (SCFA) concentrations and increasing beneficial probiotic bacteria numbers (19, 20). Furthermore, GOS may reduce adhesion of pathogens to cells, (21) inhibit pathogen colonization (21), improve gut architecture (20) and reduce expression of pro- inflammatory cytokines (22). Specific effects of GOS on RVs have been demonstrated. For example, GOS/fructo-oligosaccharide mixtures reduce RV induced diarrhea and modulate dysbiosis in suckling rats (5, 23). Human milk oligosaccharides (HMOs) inhibit RV infectivity *in vitro* (24, 25), in acutely infected piglets (24) and reduce the duration of RV-induced diarrhea in piglets whilst modulating the colonic microbiota *in vivo*. (26). Also, RV specific antibodies from Human breast milk neutralize RV infectivity *in vitro* (27). However, most studies have focussed on feeding neonatal to pre-weaning piglets GOS, whilst few have considered supplementing the diets of gestational sows to determine effects on the neonate. It has been reported that the combination of GOS and casein glycomacropeptides (CGMP) fed to gestational sows modulated the neonatal microbiota colonization, promoted gut development and growth performance of piglets, thus demonstrating that manipulation of the maternal gestational immune/microbiome axis has positive effects on offspring, but without RVA challenge (28). The aims of this study were to determine if GOS supplementation in gestational sows conferred immunity, reduced infectivity and modulated the microbiome in neonatal piglets in a commercial pig farm where RV challenge is naturally endemic and as confirmed by previous veterinary reports.

Materials and methods

Experimental design

Animals

A randomized controlled trial was performed on a commercial farrow-to-finish pig farm in Yorkshire UK, between October and December 2018. The trial was approved by the farm veterinary consultant and by the University of Nottingham ethics committee on 12-9-18, approval reference number 190. Landrace x Large white sows crossed with a Piétran boar were paired with respect to parity. Gestating sows of similar weight were moved to 3.0 × 1.8 m farrowing pens with a 0.8 × 2.2 m farrowing crate, seven days before farrow. Pens had a slatted floor and were heated with industry standard heat lamps. Temperature was kept at range 18–20°C for sows and 23–24°C for piglets with light periods from 8:00 am to 17:00 pm. Relative humidity was 50 to 70% for farrowing units and 24 to 30% for weaning units. Metal chain toys with plastic balls were provided as environmental enrichment. Sows received a wheat-based lactation diet (Gold Lactator, Noble Foods, Stokesley, UK) containing 18.4% protein, 5.6% ash, 4.6% oil, 4.1% fiber, 1.13% lysine, 0.9% calcium, 0.34% methionine and 0.49% phosphorous. New-born pigs received a 1 ml intramuscular iron injection (Gleptosil, Alstoe Ltd, York, UK) 24 h after birth. Sows and gilts were vaccinated with a combined Rotavirus OSU 6 strain and *E. coli* strains 0101:K99 vaccine two weeks prior to farrowing as per manufacturer's instructions and as according to standard farm practice (Rokovac Neo, Bioveta, Czech Republic). Piglets and sows did not receive any creep feed supplementation or prophylactic

antibiotic treatment during the trials. Sows were individually housed and randomized in a homogenous pattern to either basal control diet or supplementation with GOS powder (DP2+ GOS, Nutrabiotic, Saputo Dairy UK, Weybridge UK). Sows received the lactation diet with no GOS supplementation (non-GOS sows) or received the lactation diet with 30 g/day GOS top-dressed into feed daily, seven days before farrowing (GOS sows). Piglets born to non-GOS sows were referred to as non-GOS piglets and those born to GOS sows were referred to as GOS piglets. Trial size was determined using a power calculation accessed at: https://clincalc.com/stats/samplesize.aspx on 02-08-18, where $\alpha = 0.05$, $\beta = 0.2$, and power = 0.8, giving thirty-six replicates per control and treatment groups with a total of seventy-two pens, with one sow per pen. Trials were repeated six times, from week one to week six, in order to obtain the desired number of replicates. Models were fixed effect, whereby sows from the production cohort were randomly allocated to farrowing pens pre-assigned for non-GOS or GOS feed (independent variables). All animals were kept in identical environmental conditions, housed in identical pens and in the same building. Pens were cleaned and disinfected prior to trial replicates from the end of week one to week six onwards throughout the entire standard farm production methods. Once born, neonatal piglets were cross fostered within treatment groups, as per commercial farm standard practice, to equilibrate litter size and for welfare reasons. All trial personnel, including investigators were blinded to treatment allocation. All animals were monitored daily by trained farm personnel for any signs of scour, disease, lameness and/or distress. No animals were euthanized, or invasive samples taken during studies.

Sample collection

Trained farm personnel collected samples for biosecurity reasons. Colostrum from sows was collected within 24 hours post parturition by massaging the two teats closest to the head of sows and immediately frozen at −20°C, in a freezer, in 30 ml sterile plastic universal tubes (Thermo Scientific, Loughborough, UK). Approximately 2–3 g of freshly voided fecal samples were collected from sows and piglets per pen, in sterile nuclease free 2 ml micro tubes (Sarstedt, Leicester, UK) three days post partition and immediately frozen at−20°C. Fecal samples from piglets were pooled from each pen, whilst those of sows were kept separately. Frozen samples were delivered by refrigerated courier service to the University of Nottingham for storage at −80°C and further laboratory analyses.

ELISA for RVA IgG and IgA in colostrum

Samples were defrosted and 1 ml aliquots centrifuged at 13,000 g for 15 min to separate the fat from the colostrum. Aqueous phase colostrum was pipetted from underneath the fat layer and into sterile 2 ml micro tubes for subsequent analysis. The Ingezim rotavirus porcine ELISA kit (Immunologia Y Genetica Aplicada S.A. Madrid, Spain) was used to determine specific anti-RVA IgG and anti-RVA IgA activity in the colostrum samples according to manufacturer's instructions. For the detection of anti-RVA IgA antibodies, ELISA was performed as with IgG, but the secondary antibody was substituted with peroxidise-labeled goat anti-porcine IgA (Thermo Fisher Scientific, Bonn, Germany) at a dilution of 1/10,000 as according to Kreuzer et al. (29). The positive control serum supplied with the kit, was assayed on each occasion and the mean value from these measurements used to obtain a normalized absorbance ratio to reduce assay-to-assay variation (30). Total non-specific IgG and IgA in colostrum were assayed using IgG and IgA Pig ELISA Kits obtained from (Abcam plc, Cambridge, UK).

DNA and RNA extraction

Bacterial DNA was extracted from 200 mg sow and piglet fecal samples using the QIAamp PowerFecal QIAcube HT Kit and QIAcube HT robot according to manufacturer's instructions (Qiagen, Hilden, Germany). Viral RNA was extracted from sow and piglet feces by mixing 100 mg with 900 µl isotonic 0.9% NaCl (Merck, Gillingham, UK), prepared in diethyl pyrocarbonate (DEPC) treated nuclease free water (Fisher Scientific UK Ltd, Loughborough UK), vortexed and centrifuged at 16,000 g for 5 min. All glassware was treated with 0.1% v/v DEPC (Merck, Gillingham, UK), to remove RNase enzymes and autoclaved at 121°C at 15 psi to eliminate residual DEPC. 200 µl of the clear supernatant was used for viral nucleic acid extraction using the QIAamp 96 Virus QIAcube HT Kit and QIAcube HT robot according to manufacturer's instructions (Qiagen, Hilden, Germany). DNA was digested in samples by including an optional DNase digestion step in the QIAcube HT protocol using the Qiagen RNase Free DNase Set (Qiagen, Hilden, Germany) to prevent the possibility of interference with RNA assays in downstream applications. Bespoke software for loading onto the QIAcube HT robot was provided by Qiagen for this step. During viral RNA extraction 4 µl per sample of a Techne qPCR Rotavirus A kit internal extraction control RNA was spiked into the lysis buffer as a positive control for the extraction process (Cole-Parmer, Stone, Staffordshire UK).

Detection of RVA RNA in RNA samples

The Techne qPCR Rotavirus A kit was used to detect the presence of RVA in samples with an amplification protocol using OneStep 2x Reverse Transcription-qPCR MasterMix according to manufacturer's instructions (Cole-Parmer, Stone, Staffordshire UK). RVA specific primer probe mix was used to detect the presence of RVA non-structural protein 5 (NSP5) genomes. Standard curves were prepared with RVA positive control template with copy numbers from 2×10^5 per µl to 2 per ul. Real-time quantitative PCR data were collected using the Roche LightCycler 480 (Hoffman La Roche, Basel, Switzerland). The amplification protocol was reverse transcription for 10 min at 42°C, enzyme activation for 2 min at 95°C, then 50 cycles of denaturation for 10 s at 95°C and fluorogenic data collection for 60 s at 60°C followed by one cycle of cooling. The detection format was dual color hydrolysis/Universal Probe Library (UPL), with dynamic integration time mode and a filter combination of duplexing TaqMan probes, FAM and VIC. Amplification curves were initially analyzed using the LightCycler 480 Software release 1.5.0.39. as obtained from https://pim-eservices.roche.com/eLD/web/gb/en/products/3.8.1.4.4.8 accessed 20-02-20.

PCR amplification of 16S rRNA gene sequences

Using the extracted DNA as a template, the V4 region of the bacterial 16S rRNA genes were PCR amplified using primers 515f (5' GTGCCAGCMGCCGCGGTAA 3') and 806r (5' GGACTACHVGGGTWTCTAAT 3') (31). The full preparation and sequencing of 16S rRNA gene sequencing libraries were conducted according to the MiSeq Wet Lab SOP accessed at https://github.com/SchlossLab/MiSeq_WetLab_SOP/blob/master/MiSeq_WetLab_SOP on the 19-02-20. Amplicons were sequenced on the Illumina MiSeq platform (Illumina, San Diego, CA, USA) using 2 × 250 bp cycles (32). Sequence data were deposited in the NCBI database within Bioproject PRJNA884280.

Microbiota diversity analysis

The 16S rRNA sequence analyses were performed using Mothur v. 1.43, (33) open source software and accessed at: (https://github.com/mothur/mothur/releases accessed 12-03-20). Analysis was performed according to the MiSeq SOP accessed at: (https://mothur.org/wiki/miseq_sop/ accessed 12-03-20). The 16S rRNA gene sequences were aligned against a reference alignment based on the SILVA rRNA database for use in Mothur available at: (https://mothur.org/wiki/silva_reference_files accessed 12-03-20) (34) and clustered into OTUs using the "opticlust" clustering algorithm (35). The consensus taxonomy of the OTUs was generated using the "classify.otu" command in Mothur with reference data from the Ribosomal Database Project (version 14) (36, 37) adapted for use in Mothur available at: (https://mothur.org/wiki/rdp_reference_files accessed 12-03-20).

Statistical analyses

Analyses were performed in R version 4.1.1 using R Studio (2021.09.0) (38) unless otherwise stated. Shapiro Wilk tests (39) were used to determine normality for the results of ELISA, $\log_{10}$ copy numbers for RVA positive fecal samples and microbiota α-diversity metrics. For ELISA and $\log_{10}$ copy numbers of RVA positive samples, significant differences between groups were tested using Mann–Whitney *U*-tests. Significant differences in the number of RVA infected piglet fecal samples were tested using the Binomial test. Coverage and α-diversity expressed as Inverse Simpson diversity (40), Chao (41) Richness, Shannon (42) Index, and ACE Estimator (43), were calculated using the "summary.single" command in Mothur (33). Significant differences were tested for using Kruskal–Wallis rank sum tests. Estimates of β-diversity were calculated in Mothur as Yue and Clayton (44) Dissimilarity (θ_{YC}), Bray and Curtis (45) Dissimilarity and Jaccard (46) Similarity. Analysis of molecular variance executed in Mothur (AMOVA) was used to test for differences in β-diversity between samples (47, 48). Linear discriminant analysis effect size (LEfSe) was used to examine differential OTU abundances at genus level in Mothur (49). Where appropriate, multiple comparisons (AMOVA, Kruskal–Wallis rank sum tests) were adjusted for false discovery rates (FDR) by the Benjamini and Hochberg procedure (50) ($P = 0.05$, FDR = 25%).

Results

RVA specific and total antibody titres in sow colostrum

RVA specific and total antibody levels in sow colostrum are shown in Figure 1. Median RVA specific antibody levels in sow colostrum were, IgG non-GOS sows 0.179, IgG GOS sows 0.285, IgA non-GOS sows 1.771, IgA GOS sows 2.182 (normalized absorbance ratios). Median total antibody levels in sow colostrum were, IgG non-GOS sows 38.06, IgG GOS sows 33.25, IgA non-GOS sows 12.83, IgA GOS sows 11.55 (mg/ml colostrum). Shapiro-Wilk normality tests indicated colostrum concentrations of RVA specific and non-specific antibodies were not normally distributed ($P < 0.05$ in each case). Colostrum RVA specific IgG and IgA concentrations expressed as ELISA normalized absorbance ratio were significantly higher in GOS fed sows compared with non-GOS sows ($P = 0.03$ and $P = 0.049$ respectively, Mann–Whitney *U*-tests). However, total IgG and IgA colostrum contents were not significantly different between GOS fed sows compared with non-GOS sows ($P = 0.587$ and $P = 0.886$ respectively, Mann–Whitney *U*-tests) (Figure 1).

qPCR identification of RVA infected fecal samples

Internal extraction control RNA spiked into lysis buffer during viral RNA extraction was positive for all samples indicating successful RNA extraction and qPCR amplification using the LightCycler 480 VIC channel. Log_{10} copy numbers per g of fecal material for RVA positive samples from non-GOS piglets and GOS piglets were non-normally distributed ($P = 5.7 \times 10^{-4}$ and $P = 0.024$, respectively using Shapiro–Wilk tests). Median $\log_{10}$ copy numbers per g of fecal material were 16.25 for non-GOS piglets and 17.12 for GOS piglets. There was no significant difference in the RVA $\log_{10}$ copy number between non-GOS piglets or GOS piglets ($P = 0.7007$, Mann–Whitney *U*-tests). Out of thirty-four non-GOS piglet fecal samples, twelve (35%) tested negative and twenty-two (65%) positive for RVA. Out of thirty-six GOS piglet fecal samples, twenty (55%) tested negative and sixteen (45%) positive for RVA. There was a significant difference in the number of piglet fecal samples testing RVA positive between groups, $P = 0.0085$, Binomial test. Out of seventy-one sow fecal samples analyzed seven proved RVA positive, four non-GOS sow fecal samples (8.15–14.23 $\log_{10}$ copy number per g) and three GOS sow fecal samples (7.72–11.75 $\log_{10}$ copy number per g).

Fecal microbiota diversity and composition

In total 3,333,385 high quality 16S rRNA, V4 sequences were obtained from 141 sow and piglet fecal samples. Of these, 2,189,090 were recovered from seventy-one sow fecal samples and 1,144,295 from seventy piglet fecal samples. By treatment groups, 1,021,516 sequences were recovered from thirty-five non-GOS fed sows,

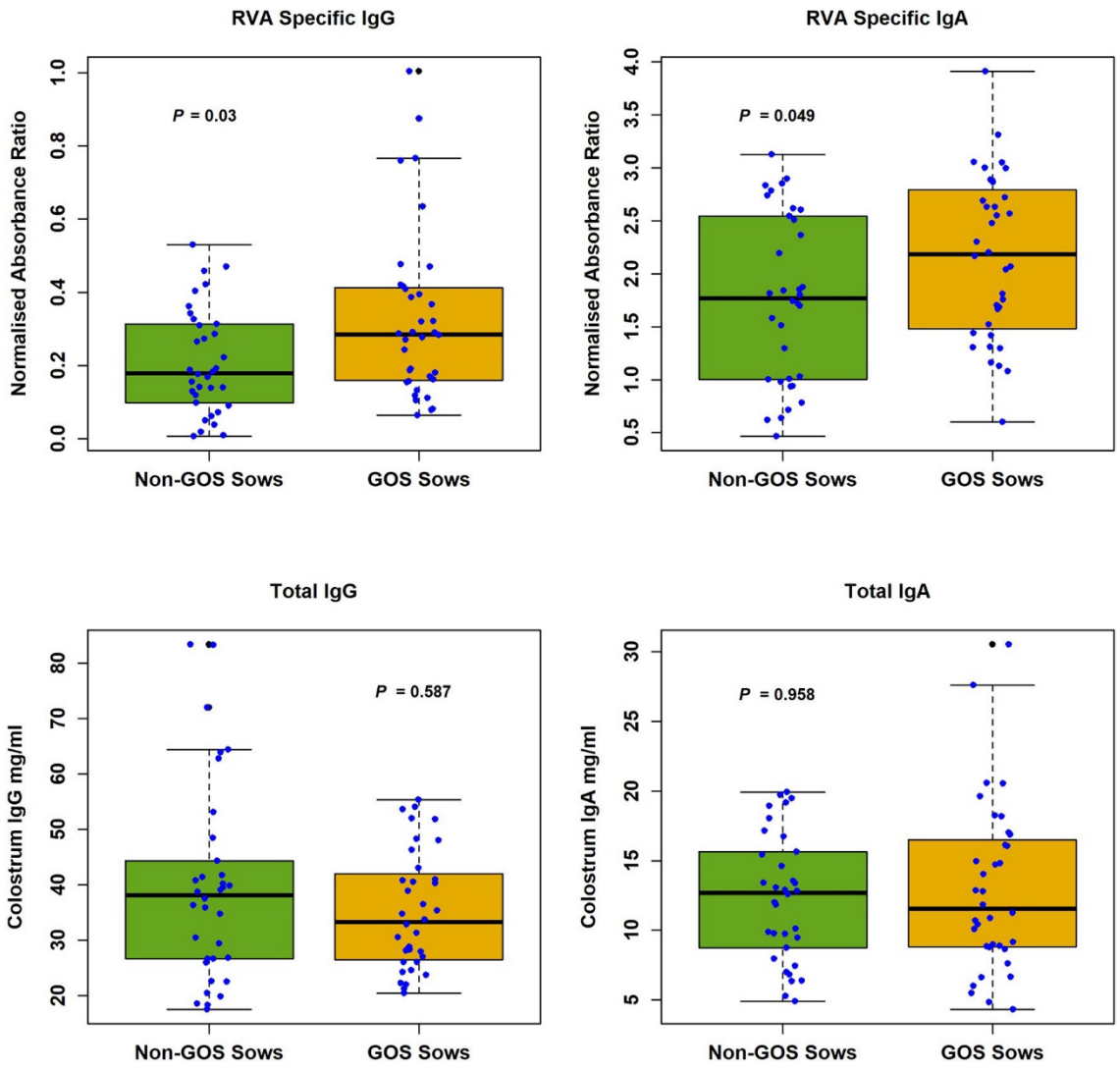

FIGURE 1
RVA specific IgG and IgA and total IgG and IgA in colostrum from non-GOS and GOS fed sows.

1,167,574 from 36 GOS fed sows, 449,463 from thirty-four piglets born to non-GOS fed sows and 694,832 from thirty-six piglets born to GOS fed sows. Sequences were subsampled to 11,210 per sample with a Good's coverage (51) of 97.8 to 99.9%. Metrics for α-diversity were not normally distributed (Shapiro–Wilk tests). There were no significant differences in α-diversity metrics between non-GOS fed sows and GOS fed sows, or piglets born to non-GOS fed sows and piglets born to GOS fed sows, $P > 0.05$ in each case (Kruskal–Wallis rank sum tests). α-diversity for all four metrics were significantly higher in non-GOS sows as opposed to non-GOS piglets and GOS sows as opposed to GOS piglets $P < 0.005$ in each case (Figure 2). Calculated β-diversity θ_{YC}, Bray and Curtis (45) and Jaccard (46) distances between non-GOS fed sows and GOS fed sows were not significantly different, as determined by AMOVA (48), $P = 0.707$, $P = 0.581$, and $P = 0.285$, respectively. θ_{YC}, Bray-Curtis and Jaccard distances were not significantly different between non-GOS piglets and GOS piglets, $P = 0.11$, $P = 0.102$, and $P = 0.075$. There was a highly significant difference between sows and piglets for all three β-diversity metrics, $P < 0.001$ in each case (Figure 3).

Figure 4 shows relative abundance of bacterial taxa at phylum and genus level for fecal samples from non-GOS sows, GOS sows, non-GOS piglets and GOS piglets. For sow fecal samples, sequences were clustered into 5629 OTUs and classified into 19 unique phyla, 43 classes, 80 orders, 171 families and 397 genera. In total, the top ten taxa allocated to OTUs at phylum level were *Firmicutes* (60.09%), *Proteobacteria* (17.23%), *Bacteroidetes* (9.10%), *Actinobacteria* (5.71%), *Spirochaetes* (4.98%), *Planctomycetes* (1.37%), Bacteria unclassified (1.18%), *Synergistetes* (0.11%), *Verrucomicrobia* (0.06%) and *Fusobacteria* (0.03%). The top ten taxa allocated to OTUs at genus level were, *Clostridium sensu stricto* (18.63%), *Acinetobacter* (6.89%), *Enterobacteriaceae* unclassified (6.43%), *Terrisporobacter* (5.19%), *Lactobacillus* (4.85%), *Romboutsia* (3.07%), *Planococcaceae* unclassified (3.02%), *Turicibacter* (2.02%), *Streptococcus* (1.99%) and *Bacteroides* (0.95%). For piglet fecal samples, sequences were clustered into 2273 OTUs and classified into 19 unique phyla, 40 classes, 73 orders, 154 families and 349 genera. The top ten taxa allocated to OTUs at phylum level were *Firmicutes* (46.66%), *Bacteroidetes* (25.03%), *Proteobacteria* (15.21%), *Fusobacteria*

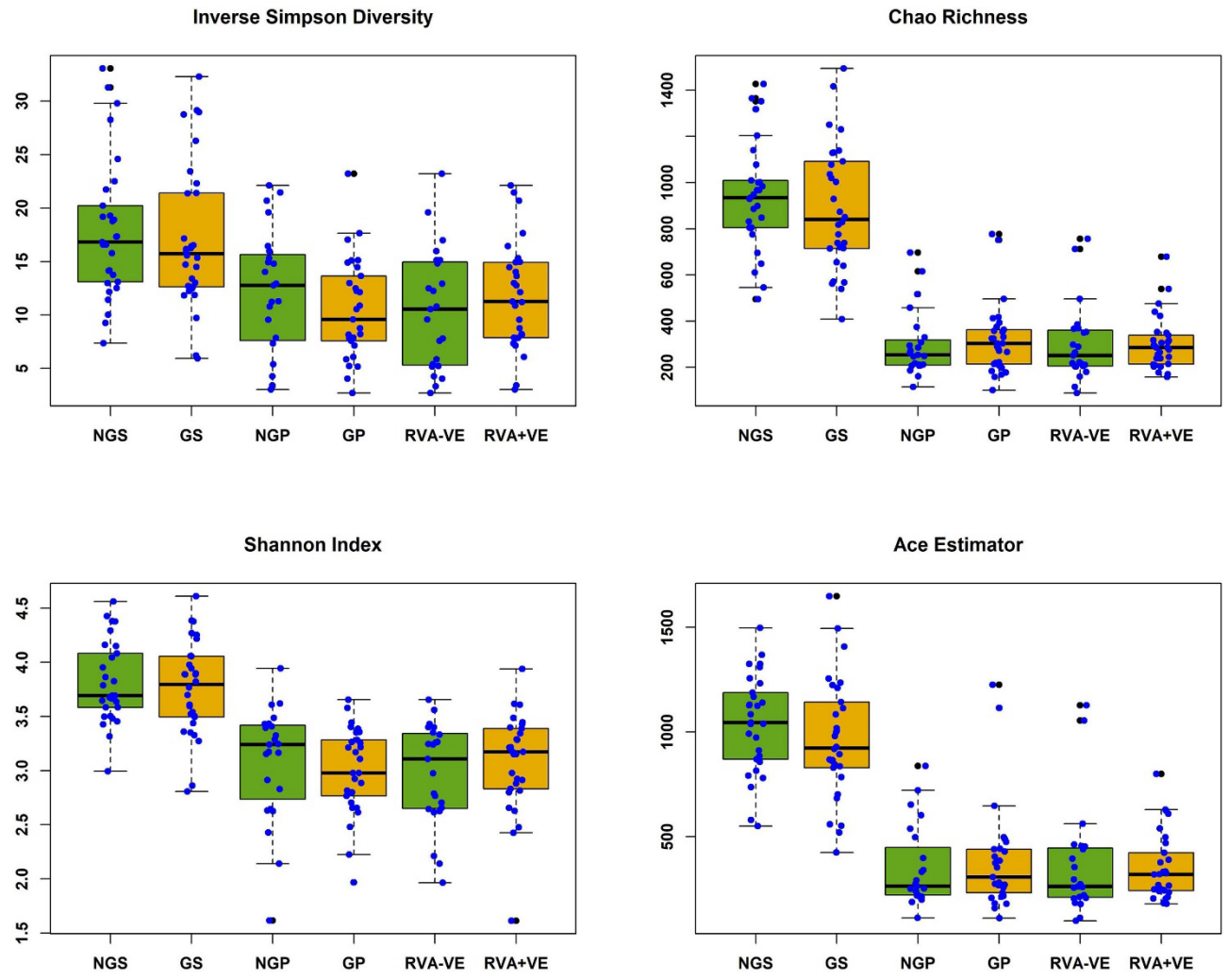

FIGURE 2
Alpha diversity of fecal samples collected from sows and piglets during suckling. NGS, non-GOS fed sow; GS, GOS fed sow; NGP, non-GOS piglet; GP, GOS piglet; RVA-VE, RVA negative piglet; RVA+VE, RVA positive piglet. Significant difference between NGS and NGP, GS, and GP in all cases $P < 0.005$.

(10.01%), *Actinobacteria* (2.76%), *Verrucomicrobia* (0.14%), Bacteria unclassified (0.09%), *Synergistetes* (0.04%), *Spirochaetes* (0.02%) and *Planctomycetes* (0.002%). The top ten taxa allocated to OTUs at genus level were, *Bacteroides* (20.47%), *Clostridium Senso Stricto* (13.17%), *Enterobacteriaceae* unclassified (12.37%), *Lactobacillus* (8.85%), *Streptococcus* (2.59%), *Terrisporobacter* (0.29%), *Romboutsia* (0.21%), *Planococcaceae* unclassified (12%), *Acinetobacter* (0.11%) and *Turicibacter* (0.05%). LEfSe identified significant differences in the abundance of differential OTUs annotated to taxa at genus level between treatment groups (Figure 5). In total non-GOS sows had eight OTUs occurring at significantly higher relative abundance compared with GOS sows, five of these being *Treponema* and one each to *Phascolarctobacterium*, *Megasphaera,* and *Clostridiales* unclassified. Non-GOS piglets had seven OTUs occurring at significantly higher relative abundance compared with GOS piglets, two of these being *Ruminococcaceae* unclassified and one each to *Lactobacillus*, *Phascolarctobacterium*, *Aerococcus*, *Actinobacillus,* and *Clostridiales* unclassified. GOS piglets had three OTUs occurring at a differentially higher abundance than non-GOS piglets, these being *Peptoniphilus, Lachnospiriaceae* unclassified, and *Collinsella.*

Fecal microbiota diversity and composition in non-infected and RVA infected piglets

In separate analyses by Mothur, 1,144,334 high quality 16S rRNA, V4 sequences were obtained from seventy piglet fecal samples. Of these, 531,797 were recovered from thirty-two RVA negative samples and 612,537 from thirty-eight RVA positive samples. Sequences were subsampled to 8078 per sample with a Good's coverage of 97.8 to 99.9%. Metrics for α-diversity were not normally distributed (Shapiro–Wilk tests). There were no significant differences in α diversity (Kruskal–Wallis Rank sum tests) or β-diversity (AMOVA) (48). Sequences were clustered into 2188 OTUs and classified into 19 unique phyla, 40 classes, 74 orders, 157 families and 348 genera. Figure 4 shows relative abundance of bacterial taxa at phylum and genus level for RVA negative and RVA positive fecal samples. In total, relative abundance of the top ten OTUs annotated to taxa at phylum level were *Firmicutes* (46.68%), *Bacteroidetes* (25.03%), *Proteobacteria* (15.21%), *Fusobacteria* (10.01%), *Actinobacteria* (2.76%), *Verrucomicrobia* (0.14%), Bacteria unclassified (0.07%), *Chloroflexi* (0.04%), *Synergistetes* (0.03%), and *Spirochaetes* (0.02%).

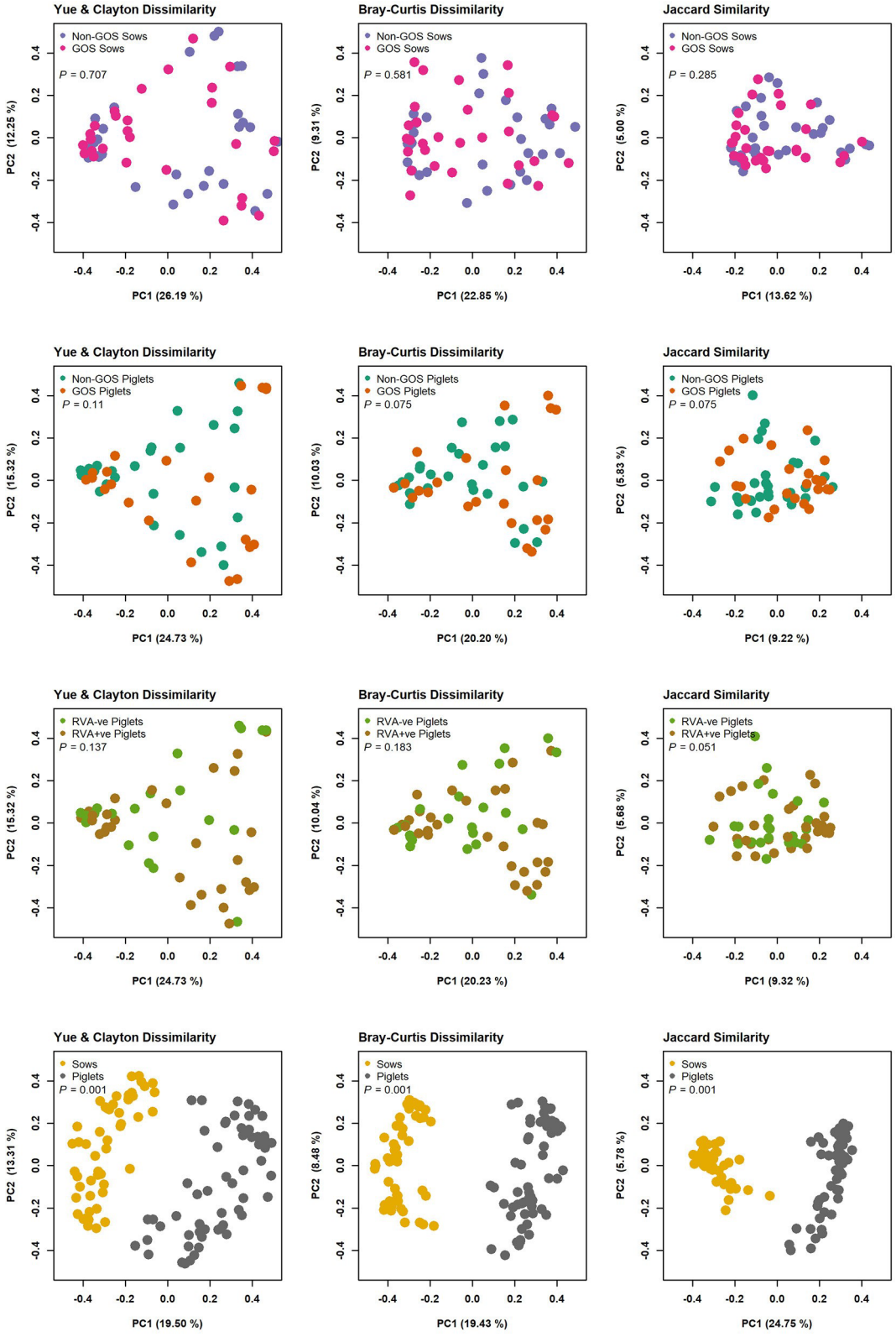

FIGURE 3
β-diversity measures for fecal samples from non-GOS sows vs. GOS fed sows, non-GOS piglets vs. GOS piglets, RVA negative vs. RVA positive piglets and sows vs. piglets.

The top ten OTUs annotated to taxa at genus level were Bacteroides (20.47%), *Clostridium sensu stricto* (13.17%), *Enterobacteriacea* unclassified (12.37%), *Fusobacterium* (9.42%), *Lactobacillus* (8.85%), *Prevotella* (3.14%), *Streptococcus* (2.59%), *Peptostreptococcus* (2.43%),

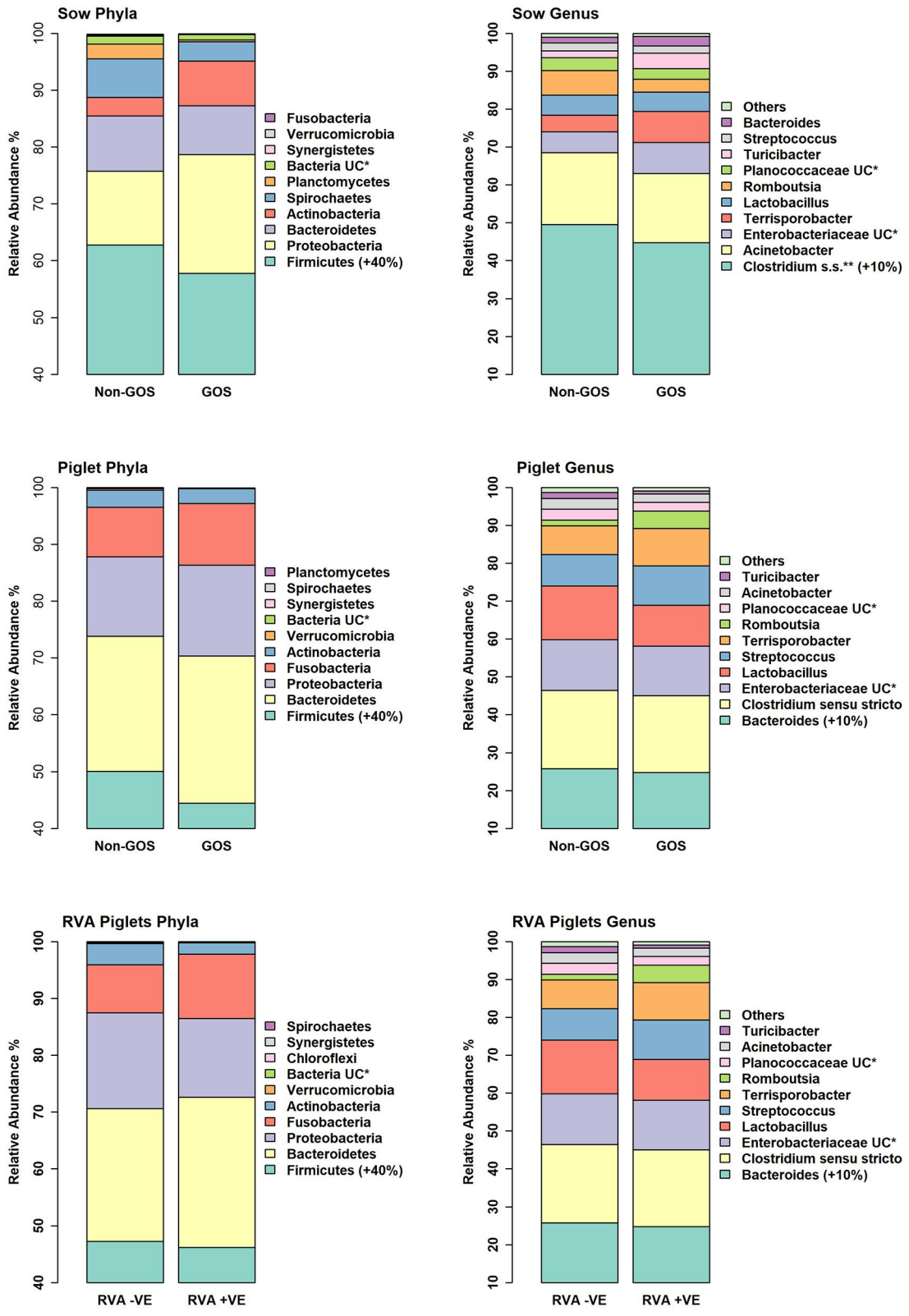

FIGURE 4
Relative abundance of bacterial taxa at phylum and genus level for fecal samples from non-GOS sows, GOS sows, non-GOS piglets, GOS piglets, RVA negative piglets, and RVA positive piglets.

Enterococcus (1.20%), and *Phascolarctobacterium* (1.11%). LEfSe identified significant differences in the abundance of differential OTUs annotated to taxa at genus level between RVA negative piglets and RVA positive piglets (Figure 5). RVA negative piglets

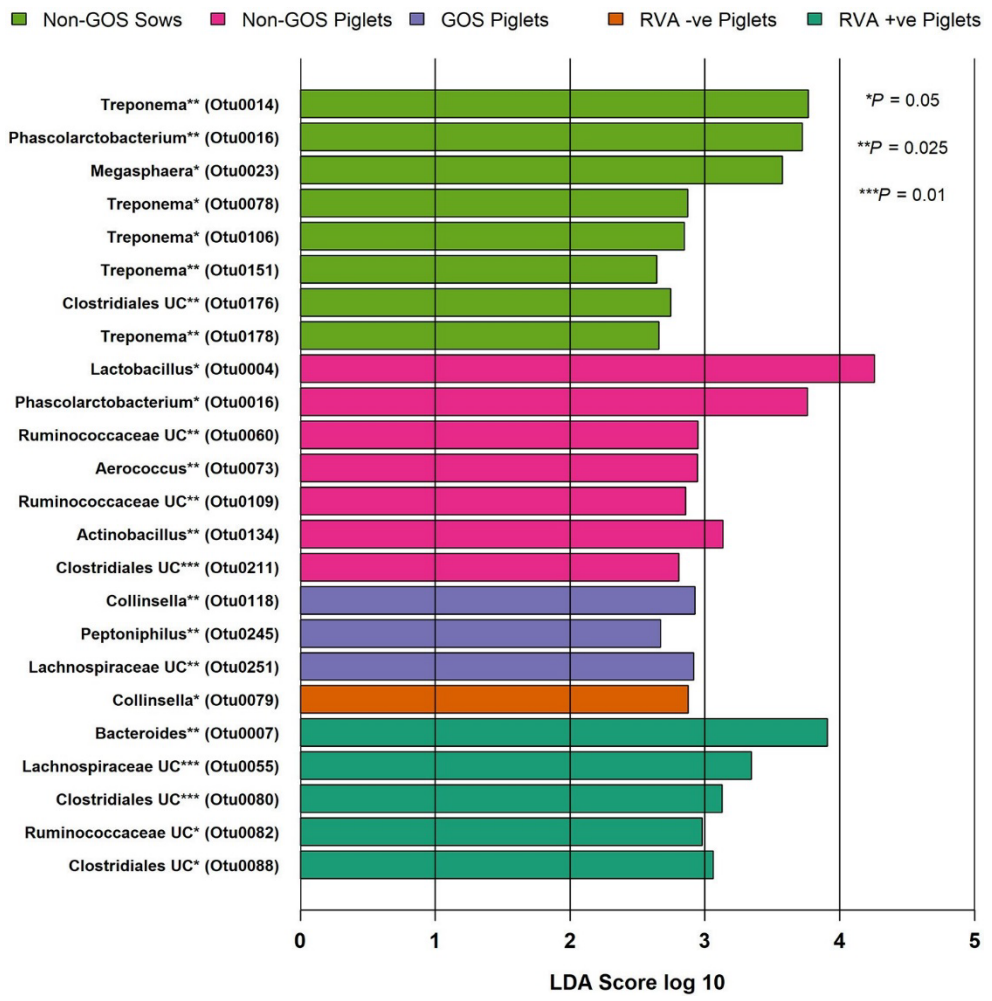

FIGURE 5
Significant differences in differential abundance of taxa at genus level for fecal samples from non-GOS sows, non-GOS piglets, GOS piglets, RVA negative piglets, and RVA positive piglets (LEfSe). No attributable differential abundance features for GOS sows, therefore not shown. UC = unclassified.

expressed an increased differential abundance of *Collinsella* in contrast with RVA positive piglets. RVA positive piglets expressed a significant differential abundance in five OTUs, two being ascribed to *Clostridiales* unclassified and three others being *Bacteroides*, *Lachnospiraceae* unclassified, and *Ruminococcacae* unclassified.

Discussion

The objectives of this study were to determine if GOS supplementation in gestational sows conferred immunity, reduced infectivity and modulated the microbiome in neonatal piglets in a commercial pig farm where RVA challenge was endemic. Whilst PMOs are expressed naturally in sow colostrum (13), supplementation with GOS top-fed at 30 g per day was associated with significantly increased RVA specific IgG and IgA in sow colostrum ($P = 0.03$ and $P = 0.049$ respectively), but not the expression of total IgG and IgA (Figure 1). The maternal gut microbiome breast axis and the importance of entero-mammary pathways in programming the mammary gland to face the nutritional, microbiological, immunological, and neuroendocrine requirements of the growing infant have been well described in humans (52). However, humans possess a hemochorial placenta whereas pigs have an epitheliochorial placenta (53), one which, in contrast, is a relatively impenetrable barrier to maternal immunoglobulins during gestation, particularly IgG. Thus, piglets are born "agammaglobulinemic" and survival depends on early acquisition of maternal immunity through colostrum (54) before gut closure within 24 to 48 h *post-partum* and reduced intestinal enterocyte ability to sequester immunoglobulins from protein rich colostrum (55). Moreover, colostrum intake is the main determinant of piglet survival through energy provision and immune protection with long-term effects on growth and immunity (56). Few animal studies have investigated how pre- and/or probiotics fed to epitheliochorial pregnant mammals interact with the immune composition of mammary secretions. In dogs, pregnant bitches fed a mixture of fructo-oligosaccharides, mannan-oligosaccharides, *E. faecium* and *L. acidophilus* expressed significantly more IgG, IgM and IgA in colostrum (57). Possible mechanisms are the modulation of immunoglobulin secretion by the maternal microbiome. In murine models, gut microbiome induced maternal IgG is transferred to the neonatal intestine through milk *via* neonatal Fc receptors and directly

inhibits pathogen colonization (58). For IgA, the gut microbiota induces Peyer's-patch dependent secretion of maternal IgA into milk. Antigen sampling by M cells in Peyer's-patches are the major source of migratory IgA plasma cells in mammary glands that produce maternal IgA found in milk (59). Similar mechanisms are found in sows with IgA secreted by mammary gland recruited plasma cells exhibiting specificity for antigens in the maternal digestive tract. This "entero-mammary" link is due to the migration of lymphocytes originating in gut associated lymphoid tissue via the bloodstream to the mammary gland (54). Other mechanisms may include viral triggering of goblet cell associated pathways, which present antigens to the immune system and serve as mechanisms of tolerance or translocation outside the gut (60).

In this study 65% of non-GOS piglet fecal samples tested positive for RVA as opposed to 45% for GOS-fed piglet fecal samples representing a significant reduction in infectivity of RVA in the maternally GOS fed group ($P = 0.008$). This reduction in infectivity can be explained by the significantly higher levels of RVA specific IgG and IgA expressed in the GOS fed sows colostrum as possibly modulated by entero-mammary pathways. Nevertheless, there may be other factors affected and/or modulated by GOS feeding such as the many unique proteins, cytokines, exosomes and leucocytes found in sow colostrum (61), which may require further investigation. Previous work has shown that human milk oligosaccharide supplementation can protect pigs against RV infection, as evidenced by shorter diarrhea duration, inhibiting RV binding and/or replication, enhancing mucosal T helper cell and T helper cell 2 cytokine responses and modulating microbiota composition (24). However, this is with direct feeding of GOS to piglets in contrast to the present study where colostrum and then milk were the only source of nutrition for piglets during the study period. In this respect, this study may be one of the first to demonstrate a significant increase in RVA colostrum viral specific immunoglobulins expressed following prebiotic gestational feeding with GOS to sows and concomitant reduction in infectivity in neonates in a commercial farm setting. Out of seventy-one sow fecal samples only seven (9.9%) were RVA positive with no significant difference between non-GOS and GOS fed sows. RVA prevalence rates in pigs varies from 3.3 to 67.3% (2) and prevalence in this study may have been low. Sows are usually immune to RVA, but the virus has been detected in the feces of sows as early as 5 days before farrowing and up to 2 weeks thereafter. Moreover, sows immune to RVA can shed the virus as a result of transient re-infection, or as asymptomatic carriers and at a time when piglets are susceptible to infection (62). Nevertheless, piglets may acquire RVA from their immediate environment given the prevalence of the virus and its stability in feces over time and at ambient temperatures (63). This demonstrates the circulation of RVA from adult sows to piglets and to the environment with resultant re-infection from environmental sources contaminated with RVA positive fecal matter. Animal and environmental RVA reservoirs indicate the need for efficacious detergents that limit the spread and infectivity of RVA and other microbial pathogens as previously described (10, 11) and in this respect GOS supplementation of gestational sows as an adjunct to these practices to reduce the RVA burden in neonates may be useful.

There were no significant differences in α or β-diversity metrics between non-GOS fed sows and GOS fed sows, or piglets born to non-GOS fed sows and piglets born to GOS fed sows. However, highly significant differences in α and β-diversity were seen between non-GOS fed sows and their piglets and GOS fed sows and their piglets (Figures 2, 3) demonstrating major differences in richness, evenness, community membership and structure. Notably, 2.5 times the number of OTUs were recovered from sow fecal samples as opposed to piglet fecal samples. The suckling pig microbiota is particularly different from that of sows and shows a lower bacterial diversity (64). This is not unexpected since piglets have high a high protein and PMO diet compared with the fiber rich diet of sows that support different microbial communities. Moreover, microbial gut diversity increases with age and with longitudinal changes in structure at different growth stages (65). However, it should be considered that both the environment and the sow influence the development of the piglet microbiome. In early lactation, the piglets' GIT microbiota composition is similar to the bacteria found on pen floors, in sow's milk and the nipple surface with the fecal microbiota of piglets becoming more similar to the sow as lactation progresses (66).

Predominant phyla in sows and piglets irrespective of GOS supplementation to sows were *Firmicutes*, *Bacteroidetes*, *Proteobacteria,* and *Actinobacteria* in keeping with other studies (65, 67, 68) (Figure 4). However, piglets had a higher relative abundance of taxa at phylum level belonging to *Fusobacteria* (10%) compared with sows (0.3%), which are associated with diarrhea and may be indicative of infection with enteric viruses such as porcine epidemic diarrhea virus which is known to affect the balance of beneficial gut bacteria as opposed to potential bacterial pathogens (69). Irrespective of GOS supplementation, *Clostridium sensu stricto*, *Acinetobacter*, *Enterobacteriaceae* unclassified, *Terrisporobacter,* and *Lactobacillus* dominated taxa at genus level in sow fecal samples as did *Bacteroides*, *Clostridium Senso Stricto*, *Enterobacteriaceae* unclassified*, Lactobacillus,* and *Streptococcus* in piglet fecal samples (Figure 4). These results were consistent with those from sow and piglet fecal microbiota taken from commercial pig farms (64) and as analyzed by similar methods. However, analyses of differential abundance of taxa at genus level by LEfSe revealed a significant increase in five OTUs belonging to the genus *Treponema* in non-GOS fed sows, but not GOS fed sows (Figure 5). *Treponema* spp are a cause of ear necrosis and shoulder ulcers in pigs leading to animal welfare problems and economic losses for producers (70). LEfSe also indicated a significant and increased differential abundance of *Clostridiales* in both non-GOS sows and non-GOS piglets (Figure 5). Whilst the majority of these organisms are commensal, some have potential to cause severe and sometimes lethal enteric infections in pigs (71). These results may indicate a direct effect of GOS in the sow GIT, thus indicating the capacity for GOS to inhibit pathogen colonization (20, 21). Reduction of *Clostridial* spp in GOS piglets may be explained by piglets inheriting fewer organisms from GOS fed sows with low abundance. Alternatively, sampling and translocation of maternal gut bacteria into colostrum and presentation of antigens to T helper cells by migratory dendritic cells may explain the reduction in *Clostridiales* in piglets (72). In non-GOS fed sows the occurrence of OTUs attributed to *Treponema* and *Clostridia* may indicate that sows harbor potentially pathogenic organisms that may cause pathologies in down-stream production and therefore, GOS supplementation to sows may suppress potential bacterial pathogens in the GIT microbiome, that otherwise may be transmitted allochthonously.

There were no significant differences in microbiota diversity and composition of RVA negative and RVA positive fecal samples taken from piglets when analyzed separately from sow fecal samples (Figure 3). Predominant phyla were *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Fusobacteria,* and *Actinobacteria* in keeping with other studies (65, 67, 68) (Figure 4). Abundance of *Fusobacterium* at genus level was higher than that of *Lactobacillus,* which is indicative of viral enteric infection (69). The only OTU occurring at significantly differential levels in RVA negative fecal samples from piglets was *Collinsella* (Figure 5). This bacterium is a member of the *Coriobacteriaceae* and has been strongly and positively correlated with intestinal and circulating rotavirus specific IFN-γ producing CD8+ T helper cell responses, which are known to correlate with protection against rotavirus diarrhea (73). Moreover, *Collinsella* produces ursodeoxycholate which reportedly inhibits binding of SARS-CoV-2 to angiotensin-converting enzyme, suppresses pro-inflammatory cytokines such as TNF-α, IL-1β, IL-2, IL-4, IL-6, and is protective against COVID-19 infection reducing mortality rates (74, 75). *Collinsella* also occurred at significantly differential levels in GOS piglets as opposed to non-GOS piglets although any true link between GOS feeding to gestational sows and occurrence of *Collinsella* in piglets requires further research. In RVA positive piglets two OTUs attributed to *Clostridiales* occurred at significantly differential levels possibly indicating how enteric viruses can favor potential pathogens as opposed to beneficial community members (69, 76) (Figure 5). Indeed, RVA infection favors shifts in ileal microbiome structure with a significant increase in mucin digesting *Bacteroides* as verified by this study in RVA positive piglets (76).

Conclusions

This study is one of the first to demonstrate that GOS supplementation to sows during gestation significantly increases RVA specific IgG and IgA in colostrum, which confers immunity to neonates and reduces infectivity presumably through the effect of GOS on entero-mammary pathways. The implications for commercial pig farming are that gestational fed GOS could be used as a useful adjunct to other anti-virals and/or cleaning with efficacious detergents that can reduce infectivity in neonates by 20%, which would represent a significant economic gain for commercial herds. Whilst there was no demonstrable effect on microbial diversity of GOS in sows and their offspring, it should be considered that only fecal samples were collected in this study and may not be a true proxy of intestinal contents, which may be different in community membership and structure. In this respect, more research is required. However, non-GOS sows compared with GOS fed sows had a significant and increased differential abundance of potentially pathogenic organisms *Treponema* and *Clostridiales* suggesting GOS modulates the maternal microbiome by suppressing these organisms. The occurrence of *Collinsella* at significantly differential levels in GOS and RVA negative piglet fecal samples as opposed to the occurrence of *Clostridiales* and *Bacteroides* in non-GOS and RVA positive samples suggests modulation of the piglet microbiome through gestational feeding with GOS. Nevertheless, any true link between gestational GOS feeding to sows and occurrence of viral suppressing *Collinsella* in piglets, or indeed any other member of the microbiota requires further research.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih. gov/, PRJNA884280.

Ethics statement

The animal study was reviewed and approved by the Farm Veterinary Consultant and by the University of Nottingham Ethics Committee.

Author contributions

AL, IC, and KM: conceptualization. AL, LL, and PC: methodology. AL: bioinformatics, formal analyses, and writing–original draft. AL, LL, PC, IC, and KM: validation. AL and KM: investigation. IC and KM: writing–review and editing. IC: funding acquisition. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

1. Estes M, Kapikian A. "Rotaviruses". In: Knipe DM, Howley PM, Griffin DE, Lamb RA, Martin MA, Roizman B, editors. *Fields Virology*. Philadelphia, PA: Kluwer Health/Lippincott, Williams and Wilkins (2007). p. 1917–74.

2. Vlasova AN, Amimo JO, Saif LJ. Porcine rotaviruses: Epidemiology, immune responses and control strategies. *Viruses*. (2017) 9:E48. doi: 10.3390/v9030048

3. Matthijnssens J, Ciarlet M, McDonald SM, Attoui H, Bányai K, Brister JR. et al. Uniformity of rotavirus strain nomenclature proposed by the Rotavirus classification working group (RCWG). *Arch Virol*. (2011) 156:1397–413. doi: 10.1007/s00705-011-1006-z

4. Estes MK, Kang G, Zeng CQ, Crawford SE, Ciarlet M. Pathogenesis of rotavirus gastroenteritis. *Novartis Found Symp.* (2001) 238:82–96. doi: 10.1002/0470846534.ch6

5. Azagra-Boronat I, Massot-Cladera M, Knipping K. van't Land B, Stahl Bernd, Garssen J, et al. Supplementation with 2'-FL and sc GOS/IcFOS ameliorates Rotavirus-induced diarrhea in suckling rats. *Front Cell Infect Microbiol.* (2018) 8:372. doi: 10.3389/fcimb.2018.00372

6. Svensmark B, Jorsal SE, Nielsen K, Willeberg P. Epidemiological studies of piglet diarrhoea in intensively managed Danish sow herds. *I Pre-weaning diarrhoea Acta Vet Scand.* (1989) 30:43–53. doi: 10.1186/BF03548067

7. Chandler-Bostock R, Hancox LR, Nawaz S, Watts O, Iturriza-Gomara M, Mellits KH. Genetic diversity of porcine group A rotavirus strains in the UK. *Vet Microbiol*. (2014) 173:27–37. doi: 10.1016/j.vetmic.2014.06.030

8. Saif LJ, Fernandez FM. Group A rotavirus veterinary vaccines. *J Infect Dis*. (1996) 174:S98–S106. doi: 10.1093/infdis/174.Supplement_1.S98

9. Azevedo M. Vlasova, AN, Saif LJ. Human rotavirus virus-like particle vaccines evaluated in a neonatal gnotobiotic pig model of human rotavirus disease. *Expert Rev Vaccines*. (2013) 12:169–81. doi: 10.1586/erv.13.3

10. Chandler-Bostock R, Mellits KH. Efficacy of disinfectants against porcine rotavirus in the presence and absence of organic matter. *Lett Appl Microbiol.* (2015) 61:538–43. doi: 10.1111/lam.12502

11. Hancox LR, Le Bon M, Dodd CE, Mellits KH. Inclusion of detergent in a cleaning regime and effect on microbial load in livestock housing. *Vet Rec.* (2013) 173:167. doi: 10.1136/vr.101392

12. Ninonuevo MR, Park Y, Yin H, Zhang J, Ward RE, Clowers BH, et al. A strategy for annotating the human milk glycome. *J Agric Food Chem.* (2006) 54:7471–80. doi: 10.1021/jf0615810

13. Albrecht S, Lane JA, Mariño K, Al Busadah KA, Carrington SD, Hickey RM, et al. A comparative study of free oligosaccharides in the milk of domestic animals. *Br J Nutr.* (2014) 111:1313–28. doi: 10.1017/S0007114513003772

14. Bode L. Human milk oligosaccharides: every baby needs a sugar mama. *Glycobiology.* (2012) 22:1147–62. doi: 10.1093/glycob/cws074

15. Donovan SM1, Comstock SS. Human milk oligosaccharides influence neonatal mucosal and systemic immunity. *Ann Nutr Metab*. (2016) 69:42–51. doi: 10.1159/000452818

16. Urashima T, Saito T, Nakamura T, Messer M. Oligosaccharides of milk and colostrum in non-human mammals. *Glycoconj J.* (2001) 18:357–71. doi: 10.1023/A:1014881913541

17. Urashima T, Taufik E, Fukuda K, Asakuma S. Recent advances in studies on milk oligosaccharides of cows and other domestic farm animals. *Biosci Biotechnol Biochem.* (2013) 77:455–66. doi: 10.1271/bbb.120810

18. Difilippo E, Bettonvil M, Willems RH, Braber S, Fink-Gremmels J, Jeurink PV, et al. Oligosaccharides in urine, blood, and feces of piglets fed milk replacer containing galacto-oligosaccharides. *J Agric Food Chem.* (2015) 63:10862–72. doi: 10.1021/acs.jafc.5b04449

19. Difilippo E, Pan F, Logtenberg M, Willems RH, Braber S, Fink-Gremmels J, et al. (2016). *In vitro* fermentation of porcine milk oligosaccharides and galacto-oligosaccharides using piglet fecal inoculum. *J Agric Food Chem*. (2016) 64:2127–33. doi: 10.1021/acs.jafc.5b05384

20. Lee A, Mansbridge SC, Liang L, Connerton IF, Mellits KH. Galacto-oligosaccharides increase the abundance of beneficial probiotic bacteria and improve gut architecture and goblet cell expression in poorly performing piglets, but not performance. *Animals.* (2023) 13:230. doi: 10.3390/ani13020230

21. Yan YL, Hu Y, Simpson DJ, Gänzle MG. Enzymatic synthesis and purification of galactosylated chitosan oligosaccharides reducing adhesion of enterotoxigenic *Escherichia coli* K88. *J Agric Food Chem.* (2017) 65:5142–50. doi: 10.1021/acs.jafc.7b01741

22. Bouwhuis MA, McDonnell MJ, Sweeney T, Mukhopadhya A, O'Shea CJ, O'Doherty JV. Seaweed extracts and galacto-oligosaccharides improve intestinal health in pigs following Salmonella Typhimurium challenge. *Animal.* (2017) 11:1488–96. doi: 10.1017/S1751731117000118

23. Azagra-Boronat I, Massot-Cladera M, Knipping K, Van Land B, Tims S, Stahl B, et al. Oligosaccharides modulate Rotavirus-associated dysbiosis and TLR gene expression in neonatal rats. *Cells.* (2019) 8:876. doi: 10.3390/cells8080876

24. Hester SN, Chen X, Li M, Monaco MH, Comstock SS, Kuhlenschmidt TB, et al. Human milk oligosaccharides inhibit rotavirus infectivity *in vitro* and in acutely infected piglets. *Br J Nutr.* (2013) 110:1233–42. doi: 10.1017/S0007114513000391

25. Laucirica DR, Triantis V, Schoemaker R, Estes MK, Ramani S. Milk oligosaccharides inhibit human rotavirus infectivity in MA104 cells. *J Nutr.* (2017) 147:1709–14. doi: 10.3945/jn.116.246090

26. Li M, Monaco MH, Wang M, Comstock SS, Kuhlenschmmidt TB, Fahey GC, et al. Human milk oligosaccharides shorten rotavirus-induced diarrhea and modulate piglet mucosal immunity and colonic bacteria. *ISME J.* (2014) 8:1609–20. doi: 10.1038/ismej.2014.10

27. Asensi MT, Martínez-Costa C, Buesa J. Anti-rotavirus antibodies in Human milk: Quantification and neutralizing activity. *J Pediatr Gastroenterol Nutr.* (2006) 42:560–7. doi: 10.1097/01.mpg.0000221892.59371.b3

28. Wu Y, Zhang X, Tao S, Pi Y, Han D, Ye H. Maternal supplementation with combined galactooligosaccharides and casein glycomacropeptides modulated microbial colonization and intestinal development of neonatal piglets. *J Funct Foods.* (2020) 74:104170. doi: 10.1016/j.jff.2020.104170

29. Kreuzer S, Machnowska P, Aßmus, Sieber M, Pieper R, Schmidt MFG, et al. Feeding of the probiotic bacterium *Enterococcus faecium* NCIMB 10415 differentially affects shedding of enteric viruses in pigs. *Vet Res*. (2012) 43:58. doi: 10.1186/1297-9716-43-58

30. Ramanakumar AV, Thomann P, Candeias JM, Ferreira S, Villa LL, Franco EL. Use of the normalized absorbance ratio as an internal standardization approach to minimize measurement error in enzyme-linked immunosorbent assays for diagnosis of human papillomavirus infection. *J Clin Microbiol.* (2010) 48:791–6. doi: 10.1128/JCM.00844-09

31. Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Lozupone CA, Turnbaugh PJ, et al. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proc Natl Acad Sci USA.* (2011) 108:4516–22. doi: 10.1073/pnas.1000080107

32. Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. *Appl Environ Microbiol.* (2013) 79:5112–20. doi: 10.1128/AEM.01043-13

33. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, et al. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl Environ Microbiol.* (2009) 75:7537–41. doi: 10.1128/AEM.01541-09

34. Pruesse E, Quast C, Knittel K, Fuchs BM, Ludwig W, Peplies J, et al. SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Res.* (2007) 35:7188–96. doi: 10.1093/nar/gkm864

35. Westcott SL, Schloss PD. OptiClust, an improved method for assigning amplicon-based sequence data to operational taxonomic units. *mSphere.* (2017) 2:17. doi: 10.1128/mSphereDirect.00073-17

36. Cole JR, Wang Q, Fish JA, Chai B, McGarrell DM, Sun Y, et al. Ribosomal Database Project: data and tools for high throughput rRNA analysis. *Nucleic Acids Res.* (2014) 42:D633–42. doi: 10.1093/nar/gkt1244

37. Wang Q, Garrity GM, Tiedje JM, Cole J. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol.* (2007) 73:5261–7. doi: 10.1128/AEM.00062-07

38. Development Core Team R. *R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria*. (2013). Available online at: http://www.R-project.org (accessed December 15, 2017).

39. Shapiro SS, Wilk MB. An analysis of variance test for normality (complete samples). *Biometrika.* (1965) 52:591–611. doi: 10.1093/biomet/52.3-4.591

40. Magurran AE. *Measuring Biological Diversity*. Oxford: Blackwell Science. (2004).

41. Chao A. Nonparametric estimation of the number of classes in a population. *Scand J Stat*. (1984) 11:265–70.

42. Shannon CE. A mathematical theory of communication. *Bell System Tech J*. (1948) 27:379–423. doi: 10.1002/j.1538-7305.1948.tb00917.x

43. Chao A, Lee SM. Estimating the number of classes via sample coverage. *J Am Stat Assoc.* (1992) 87:210–17. doi: 10.1080/01621459.1992.10475194

44. Yue JC, Clayton MK, A. similarity measure based on species proportions. *Commun Stat Theor Methods.* (2005) 34:2123–31. doi: 10.1080/STA-200066418

45. Bray JR, Curtis JT. An ordination of the upland forest communities of southern Wisconsin. *Ecol Monogr.* (1957) 27:326–49. doi: 10.2307/1942268

46. Jaccard P. Etude comparative de la distribution florale dans une portion des Alpe's et du Jura. *Bullet Soci Vaudoise Sci Nat*. (1901) 37:547–79. doi: 10.5169/seals-266450

47. Excoffier L, Smouse PE, Quattro JM. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics.* (1992) 131:479–91. doi: 10.1093/genetics/131.2.479

48. Anderson MJ, A. new method for non-parametric multivariate analysis of variance. *Austral Ecol.* (2001) 26:32–46. doi: 10.1046/j.1442-9993.2001.01070.x

49. Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, et al. Metagenomic biomarker discovery and explanation. *Genome Biol.* (2011) 12:R60. doi: 10.1186/gb-2011-12-6-r60

50. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Stat Soc Series B Methodol*. (1995) 57:289–300. doi: 10.1111/j.2517-6161.1995.tb02031.x

51. Good IJ, Toulmin GH. The number of new species, and the increase in population coverage, when a sample is increased. *Biometrika.* (1956) 43:45–63. doi: 10.1093/biomet/43.1-2.45

52. Rodríguez JM, Fernández L, Verhasselt V. The gut-breast axis: Programming health for life. *Nutrients.* (2021) 13:606. doi: 10.3390/nu13020606

53. Furukawa S, Kuroda Y, Sugiyama A. A comparison of the histological structure of the placenta in experimental animals. *J Toxicol Paothol*. (2014) 27:11–18. doi: 10.1293/tox.2013-0060

54. Salmon, H. Mammary gland immunology and neonate protection in pigs. In: Clegg RA, editor. *Advances in Experimental Medicine and Biology. Biology of the Mammary Gland: Homing of lymphocytes into the MG*. Boston, MA: Springer (2002). p. 279–86. doi: 10.1007/0-306-46832-8_32

55. Weström BR, Ohlsson BG, Svendsen J, Tagesson C, Karlsson BW. Intestinal transmission of macromolecules (BSA and FITC-Dextran) in the neonatal pig: enhancing effect of colostrum, proteins and proteinase inhibitors. *Biol Neonate.* (1985) 47:359–66. doi: 10.1159/000242140

56. Devillers N, Le Dividich J, Prunier A. Influence of colostrum intake on piglet survival and immunity. *Animal.* (2011) 5:1605–12. doi: 10.1017/S175173111100067X

57. Alonge S, Aiudi GG, Lacalandra GM, Leoci MM. Pre-and Probiotics to increase the immune power of colostrum in dogs. *Front Vet Sci.* (2020) 7:570414. doi: 10.3389/fvets.2020.570414

58. Sanidad KZ, Amir M, Ananthanarayanan, Singaraju A, Shiland NB, Hong HS, et al. Maternal gut microbiome–induced IgG regulates neonatal gut microbiome and immunity. *Sci Immunol*. (2022) 7:eabh3816 doi: 10.1126/sciimmunol.abh3816

59. Usami K, Niimi K, Matsuo A, Suyama Y, Sakai Y, Sato S, et al. The gut microbiota induces Peyer's-patch-dependent secretion of maternal IgA into milk. *Cell Rep.* (2021) 36:109655. doi: 10.1016/j.celrep.2021.109655

60. Cortez V, Schultz-Cherry S. The role of goblet cells in viral pathogenesis. *FEBS J.* (2021) 288:7060–72. doi: 10.1111/febs.15731

61. Inoue R, Tsukahara T. Composition and physiological functions of the porcine colostrum. *Anim Sci J*. (2021) 92:e13618 doi: 10.1111/asj.13618

62. Benfield DA, Stotz I, Moore R, McAdaragh JP. Shedding of rotavirus in feces of sows before and after farrowing. *J Clin Microbiol.* (1982) 16:186–90. doi: 10.1128/jcm.16.1.186-190.1982

63. Woode GN, Bohl EH. Rotavirus. In: Leman AD, Glock RD, Mengeling WL, Penny RHE, Scholl E, and Straw B, editors. *Diseases of Swine*. Ames, IA: Iowa State University Press (1981). p. 310–22.

64. Lührmann A, Ovadenko K, Hellmich J, Sudendey C, Belik V, Zentek J. et al. Characterization of the fecal microbiota of sows and their offspring from German commercial pig farms. *PLoS ONE.* (2021) 16:e0256112. doi: 10.1371/journal.pone.0256112

65. Wang X, Tsai T, Deng F, Wei X, Chai J, Knapp J. Longitudinal investigation of the swine gut microbiome from birth to market reveals stage and growth performance associated bacteria. *Microbiome.* (2019) 7:109. doi: 10.1186/s40168-019-0721-7

66. Cheng C, Wei H, Yu H, Xu C, Jiang S, Peng J. Co-occurrence of early gut colonisation in neonatal piglets with microbiota in the maternal and surrounding delivery environments. *Anaerobe.* (2018) 49:30–40. doi: 10.1016/j.anaerobe.2017.12.002

67. Zhang L, Mu C, He X, Su Y, Mao S, Zhang J, et al. Effects of dietary fibre source on microbiota composition in the large intestine of suckling piglets. *FEMS Microbiol Lett*. (2016) 363:fnw138. doi: 10.1093/femsle/fnw138

68. Lee A, Le Bon M, Connerton IF, Mellits KH. Common colonic community indicators of the suckling pig microbiota where diversity and abundance correlate with performance. *FEMS Microbiol Ecol*. (2022) 98:48. doi: 10.1093/femsec/fiac048

69. Tan Z, Dong W, Ding Y, Ding X, Zhang Q, Jiang L. Porcine epidemic diarrhea altered colonic microbiota communities in suckling piglets. *Genes.* (2020) 11:44. doi: 10.3390/genes11010044

70. Svartström O, Karlsson F, Fellstr?m C, Pringle M. Characterization of Treponema spp. isolates from pigs with ear necrosis and shoulder ulcers. *Vet Microbiol.* (2013) 166:617–23. doi: 10.1016/j.vetmic.2013.07.005

71. Songer JG, Uzal FA. Clostridial enteric infections in pigs. *J Vet Diagn Invest*. (2005) 17:528–36. doi: 10.1177/104063870501700602

72. Aliberti J. Immunity and tolerance induced by intestinal mucosal dendritic cells. *Mediat Inflamm.* (2016) 2016:3104727. doi: 10.1155/2016/3104727

73. Twitchell EL, Tin C, Wen K, Zhang H, Becker-Dreps S, Azcarate-Peril MA. Modeling human enteric dysbiosis and rotavirus immunity in gnotobiotic pigs. *Gut Pathog.* (2016) 8:51. doi: 10.1186/s13099-016-0136-y

74. Ko WK, Lee SH, Kim SJ, Jo MJ, Kumar H, Han IB, et al. Anti-inflammatory effects of ursodeoxycholic acid by lipopolysaccharide-stimulated inflammatory responses in RAW 264.7 macrophages. *PLoS ONE.* (2017) 12:e0180673. doi: 10.1371/journal.pone.0180673

75. Hirayama M, Nishiwaki H, Hamaguchi T, Ito M, Ueyama J, Maeda T, et al. Intestinal Collinsella may mitigate infection and exacerbation of COVID-19 by producing ursodeoxycholate. *PloS ONE.* (2021) 16:e0260451. doi: 10.1371/journal.pone.0260451

76. Engevik MA, Banks LD, Engevik KA, Chang-Graham AL, Perry JL, Hutchinson DS, et al. Rotavirus infection induces glycan availability to promote ileum-specific changes in the microbiome aiding rotavirus virulence. *Gut Microbes*. (2020) 11:1324–47 doi: 10.1080/19490976.2020.1754714

6 Chapter 6. Summary and conclusion

Suckling is a unique period in porcine development where the early life environment significantly affects the development and composition of the adult microbiota. The GIT microbiota contribute to the metabolic and developmental needs of animals through SCFA production, vitamin synthesis, complex carbohydrate digestion and immune regulation thought the life-time of the animal (Brestoff & Artis 2013; Kim & Isaacson 2015). Development of the GIT microbiota is a gradual and sequential process (Inoue, *et al*. 2015), where the pre-weaning suckling period offers an opportunity to study the microbiome in healthy control animals (Chapter 3), but also poorly performing animals (Chapter 4) and those with enteric infections (Chapter 5). There is a "continuum" between basic study design (Chapter 3), the inclusion of test products GOS in poorly performing animals (Chapter 4) and the investigation of late gestational feeding of GOS to sows leading to reduction in RVA infection in neonates (Chapter 5). In this work, the methods in Mothur (Schloss *et al*., 2009) were used throughout, with updates implemented to improve bioinformatics analyses of the microbiome and the SILVA taxonomy database updated allowing greater granularity in the description of bacterial taxa using OTUs (Pruesse *et al*., 2007). In this respect, the field of 16S rRNA community analyses and bioinformatics are constantly evolving. Techniques can be applied to any bacterial organism. Moreover, constant submission of meta-data for 16S rRNA gene sequencing of GIT bacterial communities to the National Center for Biotechnology Information NCBI sequence read archive database (https://www.ncbi.nlm.nih.gov/sra) as applied to these studies, allows inclusion of data for meta-analyses such as that exemplified by Holman *et al* 2017, whereby the community structure and bacterial ecology of the pig GIT may be interrogated in future.

Pigs are large animals and controlled rearing environments for research purposes may be limited due to the number of pens available and cost. Thus, trials may have to be repeated and the data pooled as in the studies of Thompson, Wang and Holmes 2008; Pajarillo *et al*. 2015; Chen *et al*. 2017. There has been little discussion of this approach, if trials can be truly compared and the data pooled for further analyses. Thus, in Chapter 3, three-suckling pig trials were reproduced where influences on microbial diversity and composition were controlled for as far as possible. Not all variables could be controlled, but examining animals of the same age, similar genetic traits, reproducing environmental and housing conditions, diets and reducing physical contact between animals has allowed the trials to be compared. The primary objective of this study was to compare variation in the microbiota of suckling pigs from three separate trials conducted at different times of year and to determine if common colonic community members of the bacterial microbiome could be identified. In this respect, reproducible small-scale suckling pig trials can be conducted in highly controlled environmental conditions, at different times of year without major differences in diversity, colonic microbiota composition or OTU variation, except for a significant difference in Jaccard Similarity indicating some microbial species differences between trials. Moreover, regardless of inter-trial variation, common colonic community indicators could be identified across repeated trials, the most abundant taxa identified at genus level being *Porphyromonadaceae* unclassified (15.81%), *Ruminococcaceae* unclassified, (12.78%), *Prevotella* (7.26%), *Clostridiales* unclassified (6.99%), *Lactobacillus* (6.58%), *Phascolarctobacterium* (6.52%) and *Firmicutes* unclassified (5.69%). Having established a high degree of similarity between studies, the secondary objective of this study was to determine if there were any associations between animal performance, microbial diversity and

abundance using pooled data from each trial. Using pooled data, this study is one of the first to show a significant correlation between animal performance in terms of suckling pig weight and ADG with bacterial community diversity as in Inverse Simpson Diversity (Magurran, 2004). This is an important finding, since it suggests that microbial community richness and evenness in suckling pigs is associated with development and performance in the first 22 days after birth. Taxa from colonic samples, corresponding to OTUs at genus level, were identified, whose relative abundance (rarefied counts) positively correlated with animal performance measures. These were the *Ruminococcaceae* unclassified, *Terrisporobacter, Dorea* and *Intestinimonas*, all of the order *Clostridiales,* which produce SCFA from carbohydrate and/or protein. These organisms may confer beneficial effects and contribute a nutritional role in the development of suckling pigs, possibilities that merit further investigation. However, it should be noted that this study was only performed with healthy piglets in the absence of any challenge that might be present on farm. The microbiome was compared with suckling pigs receiving colostrum, then milk from sows in each trial. The trial conditions and data analyses were exacting, to demonstrate the data from three separate trials could be truly pooled. These are documented in the extensive materials and methods section plus statistical analyses, particularly the SIMPER analysis and the homogeneity of variance tests (Clarke, 1993*;* Levene, 1960) to thoroughly examine variance between trials. Although pooling data to achieve statistical numbers for analyses, the "n" is still small compared to the number of pigs in a commercial setting. Moreover, it would be unlikely for any commercial pig farm to consider and/or implement the trial conditions as set out in Chapter 3 for economic reasons. Nevertheless, the findings remain relevant for research practice to allow the pooling of data to make significant inferences between repeated trials, which remains an important issue for trial design

and reporting of highly controlled and refined porcine studies. Given the exacting standards of the paper, it may be considered that the methodology could be adopted for future research studies.

However, not all pre-weaning piglets are healthy. The very nature of intensive pig production produces animals requiring extra husbandry and nurturing to survive and thrive. Not all results can be reproduced in different settings with different pigs and/or pooled to achieve statistical significance. Poor pre-weaning performance and failure to thrive without signs of clinical disease during suckling is of concern in piglet production, with estimated losses of 12% (Nuntapaitoon *et al*., 2018). Failure to thrive is not an economic problem, but rather a physiological problem due to current intensive production techniques (Panzardi *et al*., 2013). In this respect, the objectives of Chapter 4 were to investigate the effects of CMR supplemented with GOS on the microbiome, gut architecture and immunomodulatory goblet cell expression of poorly performing piglets that could benefit from milk replacement feeding when separated from sows and fit siblings in environmentally controlled pens. CMR supplemented with GOS was palatable and well tolerated by piglets. This is important since animals will not thrive on feedstuffs they do not like. There were no significant differences in piglet weight 24 hours *post-partum*, trial days1 to 21, total weight gain and ADG between the four repeated trials. Neither was mean DFI significantly different between trials and/or treatments indicating that performance, food intake and animal husbandry were consistent between trials. However, supplementation of CMR with GOS had no significant effect on performance, which was probably due to the short length of the trials and the poorly performing nature of the piglets. It would have been highly unusual for GOS fed poorly performing piglets to make compensatory weight gains

(Douglas *et al*., 2014) and show better performance than controls in only twenty one days. Indeed, the main predictor of end of study weight at twenty one days was weight at 24 h *post-partum*.

There were no significant differences in α-diversity between non-GOS and GOS fed piglets in trials although Chao Richness (Chao, 1984) was somewhat lower compared healthy piglets in other studies (Hoeflinger *et al*., 2015). Nevertheless, there were significant differences in β-diversity between non-GOS and GOS fed pigs in trials with differences in β-diversity most prevalent for trials 2 and 4 and for Jaccard Similarity (Jaccard, 1901). This indicated that GOS possibly had more of an effect on microbial community membership, rather than community structure and demonstrated that early life GOS intervention modulated GIT microbial composition and increased diversity. The number of OTUs shared by all four trials and across GIT sample sites accounted for 97.9 to 99.76 % of the total relative abundance of all taxa at genus level indicating a core microbiota. Whilst the total number of unique and/or partially shared OTUs may be much larger than those common between all four trials, they only accounted for 0.24 to 2.1 % of the total relative abundance of taxa across GIT samples at day twenty-one of life. Nevertheless, these "peripheral" microbiota at suckling may be important at later age in the life of the pig through post-weaning and grower finishing. They should not be neglected in their capacity to form important members of the GIT microbial community in more mature animals being "stage related" organisms that are suited to more complex carbohydrate metabolism and digestion in contrast to the LABs found during suckling (Wang *et al*., 2019). It is possible that these "peripheral" microbiota are but being nurtured during suckling and may become dominant and beneficial members of the pig GIT community when diet changes from milk based to creep feed and a much higher fibre and carbohydrate diet found in mature pigs. For Chapter 4 (paper 2) predominant

phyla in trials were *Firmicutes*, *Bacteroidetes*, *Proteobacteria* and *Actinobacteria*. At genus level for these trials, the most abundant taxa identified were *Lactobacillus*, *Streptococcus*, *Prevotella*, *Leuconostoc*, *Veillonella* and *Phascolarctobacterium*.

These results show a high relative abundance of beneficial LABs throughout piglet GIT sites. LEfSe gave a clearer distinction between non-GOS and GOS fed piglets. Dietary supplementation with GOS significantly increased beneficial *Lactobacillus* and *Bifidobacterium* populations in pigs. In total, across all GIT sections and trials 1 to 4, twenty-five linear discriminant features for all lactic acid fermenting bacteria occurred with non-GOS piglets as opposed to forty-seven for GOS fed piglets. Although SCFA concentrations in GIT luminal contents of piglets were not determined in this work, GOS is fermented to SCFA in the GIT of pigs (Alizadeh *et al*., 2016). However, different taxa at genus level produce different SCFA profiles with some producing intermediary lactate and succinate (Markowiak-Kopeć & Śliżewska, 2020). Considering the significant increase in LABs in GOS fed animals, it is not unlikely that this was associated with increased SCFA production, although this would have to be confirmed in further research. Of these LABs, *Biofidobacterium* spp., produce acetic and lactic acid, *Streptococcus* spp., acetic acid and *Lactobacillus* spp., acetic, propionic, butyric and lactic acids (Markowiak-Kopeć & Śliżewska, 2020). In addition, *Leconostoc* spp., are known to produce formic, propionic and butyric acids (Silva *et al*., 2017), whereas *Lactococcus* spp., are known to produce acetic, propionic and butyric acids (Fang *et al*., 2022). This should not give the impression that only the LABs produce SCFA. Other highly abundant taxa at genus level identified in this study, notably *Prevotella*,, *Veillonella* and *Phascolarctobacterium* are all known SCFA producers (Markowiak-Kopeć & Śliżewska, 2020).

GOS also affected gut histology and architecture. Ileal and jejunal villus heights

and VCR were significantly higher in GOS fed piglets as were the number of GCs per mm^2 GIT tissues indicating a physiological and beneficial effect of GOS on GIT architecture, although trials were too short for these effects to translate into positive production values. In conclusion, GOS significantly increased differential abundance of beneficial probiotic bacteria, particularly *Lactobacillus* and *Bifidobacterium*, improved gut architecture and goblet cell expression in poorly performing piglets, this being a pronounced physiological effect. Thus, GOS supplemented milk replacer may be a useful addition to animal husbandry in poorly performing, non-thriving piglets when moved to environmentally controlled pens away from sows and thriving siblings, thereby modulating the microbiome and GIT performance. Nevertheless, findings are in contrast to those in Chapter 3. Replicates of four studies were significantly different in terms of α-diversity, β-diversity and differential abundance. Therefore, replicates could not be pooled due to variance between trials and thus, had to be considered separately. This can be explained by the commercial nature of the trials, the inclusion of only "poorly performing piglets" (as opposed to healthy piglets) and the introduction of a "test product", notably GOS. Chapter 3 only considered control animals who were not receiving any test feed and were still suckling with sows, which was acknowledged in Chapter 3, with the need for further research with regards to the inclusion of test products, rather than controls alone.

Notwithstanding poor physiological performance, piglets are challenged by a variety of infectious diseases one of the most endemic being RVA (Vlasova, Amimo & Saif, 2017). Whilst GOS inhibit rotaviruses directly (Hester *et al*., 2013), little is known about the effects of supplementing sows' diets with GOS during late gestation and if there are effects on neonates. Recent studies have shown that direct GOS supplementation is able to reduce the incidence and severity of RV-associated diarrhea and influence the immune response against RV infections in suckling rats

(Massot-Cladera *et al*, 2022). GOS was fed to non-RV challenged and RV challenged neonates and it was concluded that RV infection could be ameliorated by nutritional intervention with bioactive compounds, such as prebiotics. The objectives of Chapter 5 were to determine if GOS supplementation of late gestational sows on a commercial farm with natural endemic rotavirus challenge could improve maternal and neonatal immunity, reduce rotavirus infection, modulate the microbiota through entero-mammary pathways and confer immunity to neonates. A major finding was that GOS supplementation of late gestational sows significantly increased RVA specific IgG and IgA in colostrum. This is probably due to the existence of entero-mammary pathways that program the mammary gland to serve the nutritional, microbiological and immunological requirements of the neonate (Rodríguez, Fernández & Verhasselt, 2021). This finding possibly explains the second major finding that 65% of non-GOS piglet faecal samples tested positive for RVA as opposed to 45% for GOS-fed piglet faecal samples representing a significant reduction in infectivity of RVA in the maternally GOS fed group. This is possibly the first time that the effects of late gestational GOS feeding in sows has been shown to reduce RVA in neonates.

Whilst studies of GOS administered to neonatal mammals directly reduces RV infection (Azagra-Boronat *et al*., 2018; Azagra-Boronat *et al*., 2019), this study has shown that late gestational feeding of sows with GOS significantly affects pathogen (RVA) infection and modulates the microbiome of both nursing sows and neonatal piglets. This suggests that prebiotic intervention during late gestation can significantly affect not just late gestational animals, but also their neonates through entero-programming immune mammary pathways as described by Rodríguez, Fernández & Verhasselt, 2021, whereby colostrum and milk provide infants with gut microbes, immune cells and stem cells from the mother. Recent studies strongly

suggest the existence of an endogenous entero-mammary pathway for some bacteria, including *Lactobacillus*, during lactation in the sow (Greiner *et al*., 2022). Given the prebiotic effect of GOS as established in Chapter 4, it is not unreasonable to assume that late gestational feeding of GOS to sows would increase the abundance of probiotic bacteria such as *Lactobacillus* and *Bifidobacterium* in the sow GIT and that these could be translocated from the GIT to the mammary gland and then to suckling neonates via colostrum. This could improve intestinal epithelial and mucus barrier development, antimicrobial peptide expression and innate immune cell expression in the neonate (Macpherson *et al*., 2017) and plausibly reduce RVA infection. However, the mechanism by which GOS increases IgG and IgA in sow colostrum remains to be elucidated. It could be speculated that GOS acting as soluble cell surface decoys (Newburg *et al*., 2005; Bode, 2012; Li *et al*., 2014) reduce RVA burden in the sow allowing RVA antigen sequestration by the immune system. Possible mechanisms are the modulation of immunoglobulin secretion by the maternal microbiome. In murine models, gut microbiome induced maternal IgG is transferred to the neonatal intestine through milk via neonatal Fc receptors directly inhibiting pathogen colonisation (Sanidad *et al*., 2022). For IgA, gut microbiota induce Peyer's-patch dependent secretion of maternal IgA into milk. Antigen sampling by M cells in Peyer's-patches are the major source of migratory IgA plasma cells in mammary glands that produce maternal IgA found in milk (Usami *et al*., 2021). Similar mechanisms are found in sows with IgA secreted by mammary gland recruited plasma cells exhibiting specificity for antigens in the maternal GIT, e.g. RVA. This entero-mammary link is due to the migration of lymphocytes originating in gut associated lymphoid tissue via the bloodstream to the mammary gland (Salmon, 2002). Other mechanisms may include viral triggering of GC associated pathways, which present antigens to the immune system and serve as

mechanisms of tolerance or translocation outside the GIT (Cortez & Schultz-Cherry, 2021). GOS is known to upregulate secretory goblet cell lineage gene transcription factor 1, ATOH1, (Zhang *et al*., 2017) and Chapter 4 (Paper 2) demonstrated that GOS significantly increases GC expression in the GIT of suckling pigs. Therefore, it is not unreasonable to speculate that GOS fed to sows also increases GC expression in the maternal GIT. Given the endemic nature of RVA in pigs, the presence of RVA in sows and or RVA antigens, albeit the majority remain immune and asymptomatic, it may be plausible that increased GC expression in sows following GOS feeding increases RVA antigen sequestration and presentation to the immune system thereby increasing RVA specific IgG and IgA in colostrum. Thus it may be speculated that there are at least two mechanisms whereby GOS fed to late gestational sows significantly reduces RVA infection in neonates, these being translocation of GOS induced probiotic bacteria to the neonate via entero-mammary pathways and induction of colostral RVA specific IgG and IgA via increased immunomodulatory GC expression due to GOS.

The predominant phyla in sows and piglets irrespective of GOS supplementation to sows were *Firmicutes*, *Bacteroidetes, Proteobacteria* and *Actinobacteria.* Irrespective of GOS supplementation, *Clostridium sensu stricto*, *Acinetobacter*, *Enterobacteriaceae* unclassified, *Terrisporobacter* and *Lactobacillus* dominated taxa at genus level in sow faecal samples as did *Bacteroides*, *Clostridium senso stricto*, *Enterobacteriaceae* unclassified, *Lactobacillus* and *Streptococcus* in piglet faecal samples. However, LEfSe indicated an increased differential abundance of potentially pathogenic organisms *Treponema* and *Clostridiales* in non-GOS versus GOS-fed sows, suggesting that GOS modulates the maternal microbiome by suppressing these organisms. LEfSe also indicated a higher differential abundance of virally suppressant *Collinsella* (Hirayama, *et al*., 2021) in faeces of piglets born to

GOS fed sows suggesting modulation of the piglet microbiome through late gestational feeding with GOS. In conclusion, this study has demonstrated that GOS supplementation during late gestation significantly increases RVA specific antibodies in colostrum which is associated with protection of neonates neonates and a reduction in infection. This is accompanied by changes in the microbial communities from GOS-fed sows and their piglets with the potential to suppress pathogenic organisms.

Whilst it is acknowledged that the leading cause of gastroenteritis amongst young children, worldwide are the group RVA rotaviruses, producing a range of symptoms from limited debilitating diarrhea to severe dehydration and death and that these infections could possibly be reduced by prebiotic intervention (Massot-Cladera *et al*, 2022), the reviewers of Chapter 5 (paper 3) chose not to include any implications for the potential effects of GOS in late gestational human mothers, but rather focus on animal production alone. Whilst this is accepted, RVA has been a major cause of morbidity and mortality in children under five years of age with an estimated 440,000 deaths annually pre-vaccine introduction in 2006 (Parashar, *et al*., 2003) and is still a major causative agent of lethal gastroenteritis in young children globally (Tate *et al*., 2012; Tate *et al*., 2016; Burnett *et al*., 2017) Where RVA is endemic, other opportunistic, enteric pathogens of livestock origin are prevalent, indicating the need for integrated strategies to combat RVA in susceptible communities (Msolo *et al*., 2020), and in this respect there is the possibility that GOS as a safe (GRAS) compound may offer a prophylactic intervention not only in animal production but as an intervention in human pregnant mothers susceptible to RVA infection in communities with compromised sanitation, nutrition and healthcare and at risk of RVA infection.

There are several areas of future work that may need to be addressed. In

Chapter 3 (Paper 1), only control samples and at one time-point were taken. No test product was administered. The question regarding combining small-scale experimental trials is relevant to the field (as identified by reviewers for Chapter 3, Paper 1), but how far can this approach be extended? Future work may seek to address this by examining samples from test as well as control animals. For example, test animals could be fed a prebiotic and sampling undertaken at least at two time-points, one during suckling (pre-weaning) and one post-weaning to verify if common community indicators can be identified across repeated trials in these conditions for both control and test animals. It would also be useful to sample more than one site in the pig GIT, such as the bacterial rich caecum to observe if common community indicators could be identified there. Perhaps there may be common indicators of the upper GIT (duodenum, jejunum, ileum) that could be also be studied, but their relative abundance may be low, lending weight to the argument for pooling data across studies to achieve statistical significance. If common community indicators could be identified, then pooled data could be used to identify any taxa correlating with animal performance in control and test animals, pre- and post-weaning.

For Chapter 4 (Paper 2), further studies of a longer duration may be required to fully investigate the effect of GOS on performance in poorly performing pigs. Studies accommodating GOS feeding to suckling pre-weaning piglets, continuing through weaning and into the post-weaning grower stage would be welcomed, mainly through age development, to elucidate effects of GOS on animal performance over time. GOS could be administered into solid food post-weaning. Future applications may include the addition of GOS in milk replacers for healthy piglets requiring some additional nutrition from the sow. This may allow some comparison of the healthy suckling piglet microbiome with the poorly-performing piglet microbiome. Preferably “n” needs to be increased, where “n” is the pen for any

future studies. Studies could include SCFA analyses of pig GIT samples to gain a better understanding of the effects of GOS supplemented CMR given to piglets.

In Chapter 5 (Paper 3), there was no demonstrable effect on microbial diversity of GOS in sows and their offspring. However, it should be considered that only faecal samples were collected in this study and may not be a true proxy of intestinal contents, which may be different in community membership and structure. It may be of interest to study the piglet microbiota throughout the GIT (as in Chapter 4) and the log copy numbers of RVA to give a better understanding of the relationship between the piglet microbiota, RVA and non-GOS or GOS maternal feeding. However, no commercial operation would wish to sacrifice piglets, so the study would have to be repeated in a suitable research setting, one with natural RVA challenge or one where animals could be challenged with RVA. It should be considered that the sheer expense of such a study may outweigh its benefits.

Having demonstrated that GOS increases RVA specific antibodies in colostrum, after late gestational feeding in pigs and reduces infectivity in neonates it may be hypothesised that similar effects could be seen in late gestational human mothers. Further research is required, but there may be the possibility that GOS fed to human pregnant mothers in high RVA risk communities may be a useful, affordable and effective simple adjunct to current vaccination and public health strategies to combat RVA in newborn children. One possible research path is that high RVA risk areas/populations would need to be identified and pregnant mothers recruited to a double-blind randomised, controlled trial based in a maternity unit, or at least a medical setting with basic facilities as afforded by health services in developing countries. Extensive study design and ethical approval would be required, but otherwise no invasive samples would be taken. As in the pig study, mothers could be safely administered GOS pre-birth and colostrum and faecal samples collected post

partum for immunoglobulin, microbiome and RVA analyses. Or quite simply, all pregnant mothers could be administered GOS during late pregnancy and the prevalence of neonates with RVA in high risk areas compared with that pre-GOS intervention. This would be more ethical. GOS is a safe food additive. It is not a novel medicinal product requiring licencing. Whilst not a controlled study, it would be ethically and practically easier than clinical trials but perhaps could be confirmed by more epidemiological studies which are established in RVA prevalent regions. In some respects, simple GOS intervention is possibly similar to oral rehydration solution (ORS) intervention in under five children, in developing countries, that can benefit from this simple therapy to prevent severe diarrhoea, dehydration and death (Wiens *et al*., 2020). Perhaps addition of GOS to ORS could be a simple and safe adjunct to rehydration therapy, thereby replenishing and or fortifying the neonatal to infant microbiome following gut dysbiosis in challenged situations. This may be pure conjecture, but as in the foreword to this work, the overriding desire is that this work translates into further studies that not only improve animal production welfare, but may help to reduce the burden of preventable viral and enteric diseases in children.

In final conclusion, this work has demonstrated that small scale porcine research trials can be adequately reproduced to pool data allowing significant inferences on bacterial colonic community membership, structure and animal performance to be made. This is important since it defines and refines future work with large animals and informs future studies in terms of study design and analyses, but also identifies taxa at genus level, which are correlated with animal performance. The research (Chapters 4 and 5, papers 2 and 3) are appropriate since they are based on the domestic pig (*sus scrofa domesticus*) rather than murine models as required by EU regulations for investigation of feed additives in animals (EC, 2008). Further to studies of bacterial ecology in suckling pigs, with no test-product intervention (but

with the benefit of methodology), GOS significantly increases beneficial probiotic members of the GIT bacterial community, improves gut architecture in poorly performing pigs and that late gestational feeding of GOS to sows significantly reduces RVA infection in neonates in a commercial setting where RVA is endemic. Given that the definition of a prebiotic is "a substrate that is selectively utilized by host microorganisms conferring a health benefit." (Gibson *et al*., 2017), Chapters 4 and 5 (papers 2 and 3) have clearly demonstrated the prebiotic effects of GOS and in particular against endemic rotavirus infection in commercial pig herds. Moreover, it has been demonstrated that late gestational feeding of prebiotic GOS modulates the microbiome of sows and confers benefits to neonates, in terms of viral and bacterial pathogen suppression. In this respect, the summary of the main physiological and patho-physiological targets for prebiotic effects (Roberfroid *et al*., 2010) which include "reduction of risk of intestinal infections" have been proven to be true and may in future, perhaps, include suppression of enteric viral diseases such as rotavirus and that late gestational effects of prebiotics may confer benefits to neonates.

7 References

1. Abou-Nader, J.A., Sauer, M. A., Steele, A. D., Tate, J. E., Atherly, D., Parashar, U. D., Santosham, M., & Nelson, E. A. S. (2018). Global rotavirus vaccine introductions and coverage: 2006 - 2016. *Human Vaccines & Immunotherapeutics*, 14(9):2281-296. https://doi.org/10.1080/21645515.2018.1470725
2. Akbari, P., Braber, S., Alizadeh, A., Verheijden, K. A. T., Schoterman, M. H. C., Kraneveld, A. D., Garssen, J., & Fink-Gremmels, J. (2015). Galacto-oligosaccharides protect the intestinal barrier by maintaining the tight junction network and modulating the inflammatory responses after a challenge with the mycotoxin deoxynivalenol in human caco-2 cell monolayers and B6C3F1 mice. *The Journal of Nutrition*, 145(7):1604-1613. https://doi.org/10.3945/jn.114.209486
3. Albrecht, S., Lane, J. A., Mariño, K., Al Busadah, K. A., Carrington, S. D., Hickey, R. M., & Rudd, P. M. (2014). A comparative study of free oligosaccharides in the milk of domestic animals. *British Journal of Nutrition*, 111(7):1313-1328. https://doi.org/10.1017/s0007114513003772
4. Alizadeh, A., Akbari, P., Difilippo, E., Schols, H. A., Ulfman, L. H., Schoterman, M. H. C., Garssen, J., Fink-Gremmels, J., & Braber, S. (2016). The piglet as a model for studying dietary components in infant diets: effects of galacto-oligosaccharides on intestinal functions. *British Journal of Nutrition,* 115(4):605-618. https://doi.org/10.1017/S0007114515004997

5. Anee, I. J., Alam, S., Begum, R. A., Shahjahan, R. M., & Khandaker, A. M. (2021). The role of probiotics on animal health and nutrition. *The Journal of Basic and Applied Zoology*, 82(52). https://doi.org/10.1186/s41936-021-00250-x
6. Angelakis, E. (2017). Weight gain by gut microbiota manipulation in productive animals. *Microbial Pathogenesis*, 106:162-170. https://doi.org/10.1016/j.micpath.2016.11.002
7. APHA. (2022). Livestock demographic data group: Pig population report. Livestock population density maps for GB, using 2018/19 data. http://apha.defra.gov.uk/documents/surveillance/diseases/lddg-pig-pop-report-2021.pdf
8. Apte, A., Roy, S., Bavdekar, A., Juvekar, S., & Hirve, S. (2018). Facilitators and barriers for use of rotavirus vaccine amongst various stakeholders and its implications for Indian context - A systematic review. *Human Vaccines and Immunotherapeutics*, 14(11):2760-2767. https://doi.org/10.1080/21645515.2018.1489190
9. Arslanoglu, S., Moro G. E., & Boehm G. (2007). Early supplementation of prebiotic oligosaccharides protects formula-fed infants against infections during the first 6 months of life. *The Journal of Nutrition*, 137:2420–2424. https://doi.org/10.1093/jn/137.11.2420
10. Asadpoor, M., Varasteh, S., Pieters, R. J., Folkerts, G., & Braber, S. (2021). Differential effects of oligosaccharides on the effectiveness of ampicillin against *Escherichia coli* in vitro. *PharmaNutrition*, 16:100264. https://doi.org/10.1016/j.phanu.2021.100264

11. Azagra-Boronat, I., Massot-Cladera, M., Knipping, K., Van't Land, B., Stahl, B., Garssen, J., Rodríguez-Lagunas, M. R., Franch, A., Castell, M., & Pérez-Cano, F. J. (2018). Supplementation with 2'-FL and sc GOS/IcFOS ameliorates Rotavirus-induced diarrhea in suckling rats. *Frontiers in Cellular and Infection Microbiology*, 8(372). https://doi.org/10.3389/fcimb.2018.00372

12. Azagra-Boronat, I., Massot-Cladera, M., Knipping, K., Van't Land, B., Tims, S., Stahl, B., Knol, J., Garssen, J., Franch, À, Castell, M., Pérez-Cano, F. J., & Rodríguez-Lagunas, M. J. (2019). Oligosaccharides modulate rotavirus-associated dysbiosis and TLR gene expression in neonatal rats. *Cells*, 8(8): 876. https://doi.org/10.3390/cells8080876

13. Azevedo, M. P., Vlasova, A. N., & Saif, L. J. (2013). Human rotavirus virus-like particle vaccines evaluated in a neonatal gnotobiotic pig model of human rotavirus disease. *Expert Review of Vaccines*, 12(2):169181. https://doi.org/10.1586/erv.13.3

14. Bajagai, Y. S., Klieve, A. V., Dart, P. J., & Bryden, W. L. (2016). Probiotics in animal nutrition: production, impact and regulation. In: Makkar HPS, editor. FAO animal production and health paper No. 179. Rome, Italy: Food and Agriculture Organization of the United Nations. https://www.fao.org/3/i5933e/i5933e.pdf

15. Bauer, E., Williams, B. A., Smidt, H., Verstegen, M. W. A., & Mosenthin, R. (2006). Influence of the gastrointestinal microbiota on development of the immune system in young animals. *Current Issues in Intestinal Microbiology*, 7(2):35-51. https://europepmc.org/article/med/16875418

16. Bava, L., Zucali, M., Sandrucci, A., & Tamburini, A. (2017). Environmental impact of the typical heavy pig production in Italy. *Journal of Cleaner Production*, 140(2):685-691. https://doi.org/10.1016/j.jclepro.2015.11.029

17. Bedu-Ferrari, C., Biscarrat, P., Langella, P., & Cherbuy, C. (2022). Prebiotics and the human gut microbiota: From breakdown mechanisms to the impact on metabolic health. *Nutrients*, 14(10):2096. https://doi.org/10.3390/nu14102096

18. Bhatia, S., Prabhu P. N., Benefiel, A. C., Miller, M. J., Chow, J., Davis, S. R., & Gaskins, H. R. (2015). Galacto-oligosaccharides may directly enhance intestinal barrier function through the modulation of goblet cells. *Molecular Nutrition and Food Research*, 59(3):566–573. https://doi.org/10.1002/mnfr.201400639

19. Bindels, L. B., Delzenne, N. M., Cani, P. D., & Walter, J. (2015). Towards a more comprehensive concept for prebiotics. *Nature Reviews. Gastroenterology & Hepatology*, 12(5):303-310. https://doi.org/10.1038/nrgastro.2015.47

20. Bode L. (2012). Human milk oligosaccharides: every baby needs a sugar mama. *Glycobiology*, 22(9):1147-1162. https://doi.org/10.1093/glycob/cws074

21. Bonetti, A., Tugnoli, B., Piva, A., & Grilli, E. (2021). Towards zero zinc oxide: Feeding strategies to manage post-weaning diarrhoea in piglets. *Animals*, 11(3):642. https://doi.org/10.3390/ani11030642

22. Bouwhuis, M. A., McDonnell, M. J., Sweeney, T., Mukhopadhya, A., O`Shea, C. J., & O`Doherty, J. V. (2017). Seaweed extracts and galacto-oligosaccharides improve intestinal health in pigs following *Salmonella Typhimurium* challenge. *Animal*, 11(9):1488-1496. https://doi.org/10.1017/s1751731117000118

23. Brestoff, J. R. & Artis, D. (2013). Commensal bacteria at the interface of host metabolism and the immune system. *Nature Immunology*, 14(7):676-684. https://doi.org/10.1016/j.vetmic.2015.03.014

24. Bryda, E. C. (2013). The mighty mouse: The impact of rodents on advances in biomedical research. *Missouri Medicine*, 110(3):207-211. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3987984/

25. Buddington, R. K. & Sangild, P. T. (2011). Companion animals symposium: development of the mammalian gastrointestinal tract, the resident microbiota, and the role of diet in early life. *Journal of Animal Science*, 89(5):1506-1519. https://doi.org/10.2527/jas.2010-3705

26. Burnett, E., Jonesteller, C. L., Tate, J. E., Yen, C., & Parashar, U. D. (2017). Global impact of rotavirus vaccination on childhood hospitalizations and mortality from diarrhea. *The Journal of Infectious Diseases*, 215(11):1666-1672. https://doi.org/10.1093/infdis/jix186

27. Burnett, E., Parashar, U. D., & Tate, J. E. (2020). Global impact of rotavirus vaccination on diarrhea hospitalizations and deaths among children <5 years old: 2006 -2019. *The Journal of Infectious Diseases*, 222(10):1731-1739. https://doi.org/10.1093/infdis/jiaa081

28. Butaye, P., Devriese, L. A., & Haesebrouck, F. (2003). Antimicrobial growth promoters used in animal feed: Effects of less well known antibiotics on Gram-positive bacteria. *Clinical Microbiology Reveiws*, 16(2):175-188. https://doi.org/10.1128/cmr.16.2.175-188.2003

29. Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Lozupone, C. A., Turnbaugh, P. J., Fierer, N., & Knight, R. (2011). Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proceedings of the National Academy of Sciences of the United States of America*, 108(Suppl 1):4516-22. https://doi.org/10.1073/pnas.1000080107

30. Casewell, M., Friis, C., Marco, E., McMullin, P., & Phillips, I. (2003). The European ban on growth-promoting antibiotics and emerging consequences for human and animal health. *Journal of Antimicrobial Chemotherapy*, 52(2): 159-161. https://doi.org/10.1093/jac/dkg313

31. Cavaco, L. M., Hasman, H., Stegger, M., Andersen, P. S., Skov, R., Fluit, A. C., Ito, T., & Aarestrup, F. M. (2010). Cloning and occurrence of czrC, a gene conferring cadmium and zinc resistance in methicillin-resistant *Staphylococcus aureus* CC398 isolates. *Antimicrobial. Agents Chemotherapy*, 54(9):3605–3608. https://doi.org/10.1128/aac.00058-10

32. Cavalcante, T., Medeiros, M. M., Mule, S. N., Palmisano, G. & Stolf, B. S. (2021). The role of sialic acids in the establishment of infections by pathogens, with special focus on Leishmania. *Frontiers in Cellular and Infection Microbiology*, 11:671913. https://doi.org/10.3389/fcimb.2021.671913

33. Chandler-Bostock, R., Hancox, L. R., Nawaz, S., Watts, O., Iturriza-Gomara, M., & Mellits, K. H. (2014). Genetic diversity of porcine group A rotavirus strains in the UK. *Veterinary Microbiology*, 173(1-2):27-37. https://doi.org/10.1016/j.vetmic.2014.06.030

34. Chao, A. (1984). Nonparametric estimation of the number of classes in a population. *Scandinavian Journal of Statistics*, 11(4):265-270. http://dx.doi.org/10.2307/4615964

35. Chen, L., Xu, Y., Chen, X., Fang, C., Zhao, L., & Chen, F. (2017). The maturing development of gut microbiota in commercial piglets during the weaning transition. *Frontiers in Microbiology*, 8:1688. https://doi.org/10.3389/fmicb.2017.01688

36. Cheng, L., Xu, Q., Yang, K., He, J., Chen, D., Du, Y., & Yin, H. (2016). Annotation of porcine milk oligosaccharides throughout lactation by hydrophilic interaction chromatography coupled with quadruple time of flight tandem mass spectrometry. *Electrophoresis*, 37(11):1525-1531. https://doi.org/10.1002/elps.201500471

37. Cheng, L., Chunli, K., Walvoort, M. T. C., Faas, M. M., & de Vos, P. (2020). Human milk oligosaccharides differently modulate goblet cells under homeostatic, proinflammatory conditions and ER Stress. *Molecular Nutrition and Food Research*, 64(5):e1900976. https://doi.org/10.1002/mnfr.201900976

38. Ciarlet, M., & Estes, M. K. (2003). Rotaviruses: basic biology, epidemiology and methodologies. In: Bitton G, editor. Encyclopedia of Environmental Microbiology. New York: John Wiley and Sons. pp. 2573-2773. https://doi.org/10.1002/0471263397.env089

39. Clarke, K. R. (1993). Non-parametric multivariate analyses of changes in community structure. *Austral Ecology*, 18(1):117-143. http://dx.doi.org/10.1111/j.1442-9993.1993.tb00438.x

40. Clarke, E., & Desselberger, U. (2014). Correlates of protection against human rotavirus disease and the factors influencing protection in low-income settings. *Mucosal Immunology*, 8(1):1-17. https://doi.org/10.1038/mi.2014.114

41. Cortez, V., & Schultz-Cherry, S. (2021) The role of goblet cells in viral pathogenesis. *The FEBS Journal*, 288(24):7060-7072. https://doi.org/ 10.1111/febs.15731.

42. Dai, Z., Feng, S., Liu, A., Wang, H., Zeng, X., & Yang, C. S. (2018). Anti-inflammatory effects of newly synthesized α-galacto-oligosaccharides on dextran sulfate sodium-induced colitis in C57BL/6J mice. *Food Research International*, 109:350-357. https://doi.org/10.1016/j.foodres.2018.04.054

43. Damtie, D., Melku, M., Tessema, B., & Vlasova, A. N. (2020). Prevalence and genetic diversity of rotaviruses among under-five children in Ethiopia: A systematic review and meta-analysis. *Viruses*, 12(1):62. https://doi.org/10.3390/v12010062

44. Decaluwé, R., Maes, D., Wuyts, B. Cools, A., Piepers, S., & G. P. J., Janssens. (2014). Piglets' colostrum intake associates with daily weight gain and survival until weaning. *Livestock Science*, 162:185-192. https://doi.org/10.1016/j.livsci.2014.01.024

45. Devillers, N., Le Dividich, J., & Prunier, A. (2011). Influence of colostrum intake on piglet survival and immunity. *Animal*, 5(10):1605-1612 https://doi.org/10.1017/s175173111100067x

46. Dibner, J.J., & Richards, J. D. (2005). Antibiotic growth promoters in agriculture: History and mode of action. *Poultry Science*, 84(4):634-643. https://doi.org/10.1093/ps/84.4.634

47. Difilippo, E., Bettonvil, M., Willems, H. A. M., Braber, S., Fink-Gremmels, J., Jeurink, P. V., Schoterman, M. H. C., Gruppen, H & Schols, H. A. (2015). Oligosaccharides in urine, blood, and feces of piglets fed milk replacer containing galacto-oligosaccharides. *Journal of Agricultural and Food Chemistry*, 63(50):10862-10872. https://doi.org/10.1021/acs.jafc.5b04449

48. Difilippo, E., Pan, F., Logtenberg, M., Willems, R. H. A. M., Braber, S., Fink-Gremmels, J., Schols, H. A., & Gruppen, H. (2016a). Milk oligosaccharide variation in sow milk and milk oligosaccharide fermentation in piglet intestine. *Journal of Agricultural and Food Chemistry*, 64(10):2087-2093. https://doi.org/10.1021/acs.jafc.6b00497

49. Difilippo, E., Pan, F., Logtenberg, M., Willems, R. H. A. M., Braber, S., Fink-Gremmels, J., Schols, H. A., & Gruppen, H. (2016b). In vitro fermentation of porcine milk oligosaccharides and galacto-oligosaccharides using piglet fecal inoculum. *Journal of Agricultural and Food Chemistry*, 64(10):2127-2133. https://doi.org/10.1021/acs.jafc.5b05384

50. Do, T. N., Cu, P. H., Nguyen, H. X., Au, T. X., Vu, Q. N., Driesen, S. J., Townsend, K. M., Chin, J. J. C., & Trott, D. J. (2006). Pathotypes and serogroups of enterotoxigenic *Escherichia coli* isolated from pre-weaning pigs in North Vietnam. *Journal of Medical Microbiology*, 55(1):93-99. https://doi.org/10.1099/jmm.0.46247-0

51. Donovan, S. M., & Comstock, S. S. (2016). Human milk oligosaccharides influence neonatal mucosal and systemic immunity. *Annals of Nutrition & Metabolism*, 69(Suppl 2):42-51. https://doi.org/10.1159/000452818

52. Dou, S., Gadonna-Widehem, P., Rome, V., Hamoudi, D., Rhazi, L., Lakhal, L., Larcher, T., Bahi-Jaber, N., Pinon-Quintana, A., Guyonvarch, A., Huërou-Luron, I. L. E., & Abdennebi-Najar, L. (2017). Characterisation of early-life fecal microbiota in susceptible and healthy pigs to post-weaning diarrhoea. *PLoS One*, 12(1):e0169851. https://doi.org/10.1371/journal.pone.0169851

53. Douglas, S.L., Edwards, S.A., & Kyriazakis, I. (2014). Too late to catch up: A high nutrient specification diet in the grower phase does not improve the performance of low birth weight pigs. *Journal of Animal Science*, 92(10):4577-4584. https://doi.org/10.2527/jas.2014-7793

54. Dourmad, J. Y., Ryschawy, J., Trousson, T., Bonneau, M., Gonzàlez, J., Houwers, H. W. J., Hviid, M., Zimmer, C., Nguyen, T. L. T., & Morgensen, L. (2014). Evaluating environmental impacts of contrasting pig farming systems with life cycle assessment. *Animal*, 8(12):2027-2037. https://doi.org/10.1017/S1751731114002134

55. EC. (2003). Council Regulation (EC) 1831/2003 of 2003 on additives for use in animal nutrition. https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32003R1831&rid=10

56. EC. (2008). Commission Regulation (EC) No 429/2008 of 25 April 2008 on detailed rules for the implementation of Regulation (EC) No 1831/2003 of the European Parliament and of the Council as regards the preparation and the presentation of applications and the assessment and the authorisation of feed additives. OJ L 133 22.05.2008, p. 1, ELI: http://data.europa.eu/eli/reg/2008/429/oj

57. EC. (2021). EU agricultural outlook 2021-31: Consumer behaviour to influence meat and dairy markets. https://ec.europa.eu/info/news/eu-agriculturaloutlook-2021-31-consumer-behaviour-influence-meat-and-dairy-markets-2021-dec-09_en.pdf

58. EFSA. (2021). European Food Safety Authority. Administrative guidance for the preparation of applications on additives for use in animal nutrition. *EFSA Journal*, 18(3):EN-6508. https://doi.org/10.2903/sp.efsa.2021.EN-6508

59. EMA. (2000). The European Agency for the Evaluation of Medicinal Products. Veterinary Medicines and Information Technology Unit. VICH Topic GL9 Guidelines on Good Clinical Practices. CVMP/VICH/595/98-FINAL London, 4 July 2000. https://www.ema.europa.eu/en/documents/scientific-guideline/vich-gl9-good-clinical-practices-step-7_en.pdf

60. EMA. (2017). Questions and answers on veterinary medicinal products containing zinc oxide to be administered orally to food-producing species Outcome of a referral procedure under Article 35 of Directive 2001/82/EC (EMEA/V/A/118) 26 June 2017. https://www.ema.europa.eu/en/documents/referral/zinc-oxide-article-35-referral-questions-answers-veterinary-medicinal-products-containing-zinc-oxide_en.pdf

61. Epple, H. J., Kreusel, K, M., Hanski, C., Schulzke, J. D., Riecken, E. O., & Fromm, M. (1997). Differential stimulation of intestinal mucin secretion by cholera toxin and carbachol. *Pflugers Archiv*, 433(5):638-647. https://doi.org/10.1007/s004240050325

62. Estes, M. K., & Kapikian, A. Z. (2007). Rotaviruses. In: Knipe, D. M., Howley, M., Griffin, D.E., Lamb, R. A., Martin, M. A., Roizman, B., Straus, S. E. editors. Fields Virology 5th edition. Kluwer Health/Lippincott, Williams and Wilkins; Philadelphia; pp. 1917-1974.

63. Estes, M. K., Kang, G., Zeng, C. Q., Crawford, S. E., & Ciarlet, M. (2001). Pathogenesis of rotavirus gastroenteritis. *Novartis Foundation Symposium*, 238:82 - 96. https://doi.org/10.1002/0470846534.ch6

64. Estorninos, E., Lawenko, R. B., Palestroque, E., Sprenger, N., Benyacoub, J., Kortman, G. A. M., Boekhorst, J., Bettler, J., Cercamondi, C. I., & Berger, B. (2022). Term infant formula supplemented with milk-derived oligosaccharides shifts the gut microbiota closer to that of human milk-fed infants and improves intestinal immune defense: a randomized controlled trial. *American Journal of Clinical Nutrition*, 115(1):142-153. https://doi.org/10.1093/ajcn/nqab336

65. EU. (2015). Regulation (EU) 2015/2283 of the European Parliament and of the Council of 25 November 2015 on novel foods, amending Regulation (EU) No 1169/2011 of the European Parliament and of the Council and repealing Regulation (EC) No 258/97 of the European Parliament and of the Council and Commission Regulation (EC) No 1852/2001. https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32015R2283

66. Fairbrother, J. M., Nadeau, E., & Gyles, C. L. (2005). *Escherichia coli* in postweaning diarrhea in pigs: an update on bacterial types, pathogenesis, and prevention strategies. *Animal Health Research Reviews*, 6(1):17-39. https://doi.org/10.1079/ahr2005105

67. Fairbrother J. M., & Gyles, C. L. (2012). Colibacillosis. In: Zimmerman J. J., Karriker, L. A., Ramirez, A., Schwartz, K. J., & Stevenson, G. W., editors. Disease of Swine. 10th ed. UK: Wiley-Blackwell. p. 723–47. https://doi.org/10.1002/9781119350927.ch52

68. Fang, S., Qin, T., Yu, T., & Zhang, G. (2022). Improvement of the gut microbiota in vivo by a short-chain fatty acids-producing strain *Lactococcus garvieae* CF11. *Processes*, 10:604. https://doi.org/10.3390/pr10030604

69. Ferrari, C. V., Sbardella, P. E., Bernardi, M. L., Coutinho, M. L., Vaz Jr, I. S., Wentz, I., & Bortolozzo, F. P. (2014). Effect of birth weight and colostrum intake on mortality and performance of piglets after cross-fostering in sows of different parities. *Preventive Veterinary Medicine*, 114(3-4):259-266. https://doi.org/10.1016/j.prevetmed.2014.02.013

70. Ferrari, E., Salogni, C., Martella, V., Alborali, G. L., Scaburri, A., & Boniotti, M. B. (2022). Assessing the epidemiology of rotavirus A, B, C and H in diarrheic pigs of different ages in northern Italy. *Pathogens*, 11(4):467. https://doi.org/10.3390%2Fpathogens11040467

71. Fitzgerald, G. R., Garland, P. G., & Fahy, V. A. (1988). Diarrhea in young pigs: comparing the incidence of the five most common infectious agents. *Veterinary Medicine Food and Animal Practice*, 1:80–86.

72. Forder, R. E., Howarth, G. S., Tivey, D. R., & Hughes, R. J. (2007). Bacterial modulation of small intestinal goblet cells and mucin composition during early posthatch development of poultry. *Poultry Science*, 86(11):2396–2403. https://doi.org/10.3382/ps.2007-00222

73. Frommlet, F., & Heinze, G. (2021). Experimental replications in animal trials. *Laboratory Animals*, 55(1):65-75. https://doi.org/10.1177/0023677220907617

74. Fuller, R. (1989). Probiotics in man and animals. *Journal of Applied Bacteriology*, 66(5):365-378. https://sfamjournals.onlinelibrary.wiley.com/doi/pdf/10.1111/j.1365-2672.1989.tb05105.x

75. Gao, R., Tian, S., Wang, J., & Zhu, W. (2021). Galacto-oligosaccharides improve barrier function and relieve colonic inflammation via modulating mucosa-associated microbiota composition in lipopolysaccharides-challenged piglets. *Journal of Animal Science and Biotechnology*, 12(1):92. https://doi.org/10.1186/s40104-021-00612-z

76. Gibson, G. R., & Roberfroid, M. B. (1995). Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. *The Journal of Nutrition*, 125(6):1401-1412. https://doi.org/10.1093/jn/125.6.1401

77. Gibson, G. R. (1999). Dietary modulation of the human gut microflora using the prebiotics oligofructose and inulin. *The Journal of Nutrition*, 129(7 Suppl):1438S – 1441S. https://doi.org/10.1093/jn/129.7.1438s

78. Gibson, G. R, Probert, H. M., Van Loo, J., Rastall, R. A., & Roberfroid, M. B. (2004). Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. *Nutrition Research Reviews*, 17(2):259-275. https://doi.org/10.1079/nrr200479

79. Gibson, G.R., Scott, K. P., Rastall, R. A., Tuohy, K. M., Hotchkiss, A., Dubert-Ferrandon, A., Gareau, M., Murphy, E. F., Saulnier, D., Loh, G., Macfarlane, S., Delzenne, N., Ringel, Y., Kozianowski, G., Dickmann, R., Lenoir-Wijnkoop, I., Walker, C., & Buddington, R. (2010). Dietary prebiotics: current status and new definition. *Food Science and Technology Bulletin: Functional Foods*, 7(1):1-19 https://doi.org/10.1616/476-2137.15880

80. Gibson, G. R., Hutkins, R., Sanders, M. E., Prescott, S. L., Reimer, R, A., Salminen, S. J., Scott, K., Stanton, C., Swanson, K. S., Cani, P. D., Verbeke, K., & Reid G. (2017). Expert consensus document: The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. *Nature Reviews. Gastroenterology & Hepatology*, 14(8):491-502. https://doi.org/10.1038/nrgastro.2017.75

81. Giraldi-Díaz, M. R., Castillo-González, E., De Medina-Salas, L., Velásquez-De la Cruz, R., & Huerta-Silva, H. D. (2021). Environmental impacts associated with intensive production in pig farms in Mexico through life cycle assessment. *Sustainability*, 13(20):11248. https://doi.org/10.3390/su132011248

82. Greiner, L. L., Humphrey, D. C., Holland, S. N., Anderson, C. J., & Schmitz-Esser, S. (2022) The validation of the existence of the entero-mammary pathway and the assessment of the differences of the pathway between first and third parity sows. *Translational Animal Science*, 6:1-7. https://doi.org/10.1093/tas/txac407

83. Gresse, R., Chaucheyras-Durand, F., Fleury, M. A., Van de Wiele, T., Forano, E., & Blanquet-Diot, S. (2017). Gut microbiota dysbiosis in postweaning piglets: Understanding the keys to health. *Trends in Microbiology*, 25(10): 851-873. https://doi.org/10.1016/j.tim.2017.05.004

84. Gustafson, R. H., & Bowen, R. E. (1997). Antibiotic use in animal agriculture. *Journal of Applied Microbiology*, 83:531-541. https://doi.org/10.1046/j.1365-2672.1997.00280.x

85. Hancox, L. R., Le Bon, M., Dodd, C. E., & Mellits, K. H. (2013). Inclusion of detergent in a cleaning regime and effect on microbial load in livestock housing. *The Veterinary Record*. 173(7):167. https://doi.org/10.1136/vr.101392

86. Harastani, H. H., Reslan, L., Sabra, A., Ali, Z., Hammadi, M., Ghanem, S., Hajar, F., Matar, G. M., Dbaibo, G. S., & Zaraket, H. (2020). Genetic diversity of human rotavirus A among hospitalized children under-5 years in Lebanon. *Frontiers in Immunology*, 11:317. https://doi.org/10.3389/fimmu.2020.00317

87. Hester, S. N., Chen, X., Li, M., Monaco, M. H., Comstock, S. S., Kuuhlenschmidt, T. B., Kuhlenschmidt, M. S., & Donovan, S. M. (2013). Human milk oligosaccharides inhibit rotavirus infectivity *in vitro* and in acutely infected piglets. *British Journal of Nutrition*, 110(7):1233-42. https://doi.org/10.1017/s0007114513000391

88. Hiergeist, A., Rueischl, U., Priority Program 1656 Intestinal Microbiota Consortium/quality assessment participants, Gessner, A. (2016). Multicenter quality assessment of 16S ribosomal DNA-sequencing for microbiome analyses reveals high inter-center variability. *International Journal of Medical Microbiology*, 306(5):334-342. https://doi.org/10.1016/j.ijmm.2016.03.005

89. Hill, G. M., & Shannon, M. C. (2019). Copper and zinc nutritional issues for agricultural animal production. *Biological Trace Element Research*, 188(1):148-159. https://doi.org/10.1007/s12011-018-1578-5

90. Hirayama, M., Nishiwaki, H., Hamaguchi, T., Ito, M., Ueyama, J., Maeda, T., Kashihara, K., Tsuboi, Y., & Ohno, K. (2021). Intestinal Collinsella may mitigate infection and exacerbation of COVID-19 by producing ursodeoxycholate. *PLoS One*, 23;16(11):e0260451. https://doi.org/10.1371/journal.pone.0260451

91. Hoeflinger, J. L., Kashtanov, D. O., Cox, S. B., Dowd, S. E., Jouni, Z. E., Donovan, S. M., & Miller, M. J. (2015). Characterization of the intestinal lactobacilli community following galactooligosaccharides and polydextrose supplementation in the neonatal piglet. *PLoS One*. 10(8):e0135494. https://doi.org/10.1371/journal.pone.0135494

92. Holman, D. B., Brunelle, B. W., Trachsel, J., & Allen, H. K. (2017). Meta-analysis to define a core microbiota in the swine gut. *mSystems*, 2(3):e00004-17. https://doi.org/10.1128/mSystems.00004-17

93. Holscher, H. D., Davis, R. S., & Tappenden, K. A. (2014). Human milk oligosaccharides influence maturation of human intestinal Caco-2Bbe and HT-29 cell lines. *The Journal of Nutrition*, 144(5):586-591. https://doi.org/10.3945/jn.113.189704

94. Hong, T. T. T., Linh, N, Q., Ogle, B., & Lindberg, J. E. (2006). Survey on the prevalence of diarrhoea in pre-weaning piglets and on feeding systems as contributing risk factors in smallholdings in Central Vietnam. *Tropical Animal Health and Production*, 38(5):397-405. https://doi.org/10.1007/s11250-006-4399-z

95. Inoue, R., Tsukahara, T., Nakanishi, N., & Ushida, K. (2015). Development of the intestinal microbiota in the piglet. *The Journal of General and Applied Microbiology*, 51(4):257-265. https//doi.org/10.2323/jgam.51.257

96. Intanon, M., Arreola, S. L., Pham, N. H., Kneifel, W., Haltrich, D., & Nguyen, T. (2014). Nature and biosynthesis of galacto-oligosaccharides related to oligosaccharides in human breast milk. *FEMS Microbiology Letters*, 353(2):89-97. https://doi.org/10.1111%2F1574-6968.12407

97. Jaccard, P. (1901). Etude comparative de la distribution florale dans une portion des Alpe´s et du Jura. *Bulletin de la Soci´et´e Vaudoise des Sciences Naturelles*, 37:547–79. https://dx.doi.org/10.5169/seals-266450

98. Jensen, J., Kyvsgaard, N. C., Battisti, A., & Baptiste K. E. (2018). Environmental and public health related risk of veterinary zinc in pig production – Using Denmark as an example. *Environment International*, 114:181-190. https://doi.org/10.1016/j.envint.2018.02.007

99. Jensen-Waern, M., Melin, L., Lindberg, R., Johannisson, A., Petersson, L., & Wallgren, P. (1998). Dietary zinc oxide in weaned pigs - effects on performance, tissue concentrations, morphology, neutrophil functions and faecal microflora. *Research in Veterinary Science*, 64(3):225-31. https://doi.org/10.1016/s0034-5288(98)90130-8

100. Jeurissen, S. H. M., Lewis, F., Dirk van der Klis, J., Mroz, Z., Rebel, J. M. J., & ter Huurne, A. A. H. M. (2002). Parameters and techniques to determine intestinal health of poultry as constituted by immunity, integrity, and functionality. *Current Issues in Intestinal Microbiology*, 3(1):1-14. https://pubmed.ncbi.nlm.nih.gov/12022808/

101. Kapikian, A. Z., Hoshino, Y, Chanock, R. M. Rotaviruses. (2001). In: Knipe, D.M., Howley, M., Griffin, D. E., Lamb, R. A., Martin, M. A., Roizman, B., Straus, S. E., eds. Fields Virology. 4th ed. Philadelphia: Lippincott Williams, Wilkins:1787–1833.

102. Katouli, M., Melin, L., Jensen-Waern, M., Wallgren, P., & Möllby, R. (1999). The effect of zinc oxide supplementation on the stability of the intestinal flora with special reference to composition of coliforms in weaned pigs. *Journal of Applied Microbiology*, 87(4):564–573. https://doi.org/10.1046/j.1365-2672.1999.00853.x

103. Kim, H. B., & Isaacson, R. E. (2015). The pig gut microbial diversity: Understanding the pig gut microbial ecology through the next generation high throughput sequencing. *Veterinary Microbiology*, 177(3-4):242-251. https://doi.org/10.1016/j.vetmic.2015.03.014

104. Kim, K., Song, M., Liu, Y., & Ji, P. (2022). Enterotoxigenic *Escherichia coli* infection of weaned pigs: Intestinal challenges and nutritional intervention to enhance disease resistance. *Frontiers in Immunology*, 13:885253. https://doi.org/10.3389/fimmu.2022.885253

105. Knoop, K. A., McDonald, K. G., McCrate, S., McDole, J. R., & Newberry, R. D. (2015). Microbial sensing by goblet cells controls immune surveillance of luminal antigens in the colon. *Mucosal Immunology*, 8(1):198–210. https://doi.org/10.1038/mi.2014.58

106. Knoop, K. A., & Newberry, R. D. (2018). Goblet cells: multifaceted players in immunity at mucosal surfaces. *Mucosal Immunology*, 11(6):1551-1557. https://doi.org/10.1038/s41385-018-0039-y

107. Koketsu, Y., Takenobu, S., & Nakamura, R. (2006). Preweaning mortality risks and recorded causes of death associated with production factors in swine breeding herds in Japan. *The Journal of Veterinary Medical Science*, 68(8):821-826. https://doi.org/10.1292/jvms.68.821

108. Kopeloff, N., Blackman, N., & McGinn, B. (1932). The incidence of *Lactobacillus acidophilus* in adults. *The Journal of Infectious Diseases*, 50:426-429.

109. Kozich, J. J., Westcott, S. L., Baxter, N. T., Highlander, S. K., & Schloss, P. D. (2013). Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. *Applied and Environmental Microbiology*, 79(17):5112-20 https://doi.org/10.1128/AEM.01043-13

110. Kreuzer, S., Machnowska, P., Aßmus, J., Sieber, M., Pieper, R., Schmidt, M. F. G., Brockmann, G. A., Scharek-Tedin, L., & Reimar, J. (2012). Feeding of the probiotic bacterium *Enterococcus faecium* NCIMB 10415 differentially affects shedding of enteric viruses in pigs. *Veterinary Research*, 43(1):58. https://doi.org/10.1186/1297-9716-43-58

111. Kruger, C., Zhou, Y., Thorsrud, B. A., Morer-Despeisse, F., & Chappuis, E. (2017). Safety evaluation of α-galacto-oligosaccharides for use in infant formulas investigated in neonatal piglets. *Toxicology Research and Application,* 1:1-10 https://doi.org/10.1177/2397847317722828

112. Lafontaine, G. M. F., Richards, P. J., Connerton, P. L., O'Kane, P. M., Ghaffar, N. M., Cummings, N. J., Fish, N. M., & Connerton, I. F. (2020). Prebiotic driven increases in IL-17A do not prevent *Campylobacter jejuni* colonization of chickens. *Frontiers in Microbiology*, 10:3030. https://doi.org/10.3389/fmicb.2019.03030

113. Lai, J., Nguyen, C., Tabwaia, B., Nikuata, A, Baueri N., Timeon, E., Diaaldeen, M., Iuta, T., Ozturk, M. H., Moore, A., Hall, A., Nyambat, B., Davis, S., Rahman, A., Erasmus, W., Fox, K., & Russell, F. (2020). Temporal decline in diarrhea episodes and mortality in Kiribati children two years following rotavirus vaccine introduction, despite high malnutrition rates: a retrospective review. *BMC Infectious Diseases*, 20(1):207. https://doi.org/10.1186/s12879-020-4874-6

114. Lallès, J., Bosi, P., Smidt, H., & Stokes, C. R. (2007). Nutritional management of gut health in pigs around weaning. *Proceedings of the Nutrition Society*, 66(2):260-268. https://doi.org/10.1017/s0029665107005484

115. Laucirica, D. R., Triantis, V., Schoemaker, R., Estes, M. K., & Ramani, S. (2017). Milk oligosaccharides inhibit human rotavirus infectivity in MA104 cells. *The Journal of Nutrition*, 147(9):1709-1714. https://doi.org/10.3945%2Fjn.116.246090

116. Levene, H. (1960). Robust tests for equality of variances. In: Olkin I (ed.), Contributions to Probability and Statistics; Essays in Honour of Harold Hotelling. Palo Alto: Stanford University Press, 278–292.

117. Li, B. T., Van Kessel, A. G., Caine, W. R., Huang, S. X., & Kirkwood, R. N. (2001). Small intestinal morphology and bacterial populations in ileal digesta and feces of newly weaned pigs receiving a high dietary level of zinc oxide. *Canadian Journal of Animal Science*, 81(4):511-516. https://doi.org/10.4141/A01-043

118. Li, M., Monaco, M. H., Wang, M., Comstock, S. S., Kuhlenschmmidt, T. B., Fahey, G. C., Miller, M. J., Kuhlenschmidt, M. S., & Donovan, S. M. (2014). Human milk oligosaccharides shorten rotavirus-induced diarrhea and modulate piglet mucosal immunity and colonic bacteria. *The ISME Journal*, 8(8):1609-1620. https://doi.org/10.1038/ismej.2014.10

119. Liao, S. F., & Nyachoti, M. (2017) Using probiotics to improve swine gut health and nutrient utilization. *Animal Nutrition*, 3(4):331-343. https://doi.org/10.1016/j.aninu.2017.06.007

120. Lilly, D. M., & Stillwell, R. H. (1965). Probiotics: growth-promoting factors produced by microorganisms. *Science*, 147:747-748.

121. Lu, D., Tiezzi, F., Schillebeeckx, C., McNulty, N. P., Schwab, C., Shull, C., & Maltecca, C. (2018). Host contributes to longitudinal diversity of fecal microbiota in swine selected for lean growth. *Microbiome*, 6(1):4. https://doi.org/10.1186/s40168-017-0384-1

122. Luppi, A., Gibellini, M., Gin, T., Vangroenweghe. F., Vandenbroucke, V., Bauerfiend, R., Bonilauri P., Labarque, G., & Hidalgo, A. (2016). Prevalence of virulence factors in enterotoxigenic *Escherichia coli* isolated from pigs with post-weaning diarrhoea in Europe. *Porcine Health Management*, 2(20). https://doi.org/10.1186/s40813-016-0039-9

123. Lymbery, P. (2022). Sixty harvests left: How to reach a nature-friendly future. Bloomsbury, London. https://www.bloomsbury.com/uk/sixty-harvests-left-9781526654694/

124. Macfarlane, G. T., Steed, H., & Macfarlane, S. (2008). Bacterial metabolism and health-related effects of galacto-oligosaccharides and other prebiotics. *Journal of Applied Microbiology*, 104(2):305-44. https://doi.org/10.1111/j.1365-2672.2007.03520.x

125. Macpherson, A. J., de Agüero, M. G., & Ganal-Vonarburg, S. C. (2017) How nutrition and the maternal microbiota shape the neonatal immune system. *Nature Reviews Immunology*. 17(8):508-517. https://doi.org/ 10.1038/nri.2017.58.

126. Magurran, A. E. (2004). Measuring Biological Diversity. Oxford: Blackwell Science.

127. Markowiak-Kopeć, P., & Śliżewska, K. (2020). The effect of probiotics on the production of short-chain fatty acids by human intestinal microbiome. *Nutrients*, 12(4):1107. https://doi.org/10.3390/nu12041107

128. Martinez, R. C. R., Cardarelli, H. R., Borst, W., Albrecht, S., Schols, H., Gutiérrez, O. P., Maathuis, A. J. H., De Melo Franco, B. D. G., De Martinis, E. C. P., Zoetendal, E. G., Venema, K., Saad, S. M. I., & Smidt, H. (2013). Effect of galactooligosaccharides and *Bifidobacterium animalis* Bb-12 on growth of *Lactobacillus amylovorus* DSM 16698, microbial community structure, and metabolite production in an *in vitro* colonic model set up with human or pig microbiota. *FEMS Microbiology Ecology*, 84(1):110-123. https://doi.org/10.1111/1574-6941.12041

129. Massot-Cladera, M., Rigo-Adrover, M. M., Herrero, L., Franch, A., Castell, M., Vulevic, J., Pérez-Cano, F. J., & Rodríguez Lagunas, M. J. (2022). A galactooligosaccharide product decreases the rotavirus infection in suckling rats. *Cells*, 11(10):1669. https://doi.org/10.3390/cells11101669

130. Matthijnssens, J., Ciarlet, M., Rahman, M., Attoui, H., Bányai, K., Estes, M. K., Gentsch, J. R., Iturriza-Gómara, M., Kirkwood, C. D., Martella, V., Mertens, P. P., Nakagomi, O., Patton, J. T., Ruggeri, F. M., Saif, L. J., Santos, N., Steyer, A., Taniguchi, K., Desselberger, U., & Van Ranst, M. (2008). Recommendations for the classification of group A rotaviruses using all 11 genomic RNA segments. *Archives of Virology*, 153(8):1621-1629. https://doi.org/10.1007/s00705-008-0155-1

131. Matthijnssens, J., Ciarlet, M., McDonald, S. M., Attoui, H., Bányai, K., Brister, J. R., Buesa, H., Esona, M. D., Estes, M. K., Gentsch, J. R., Iturriza-Gómara, M., Johne, R., Kirkwood, C. D., Martella, V., Mertens, P. P. C., Nakagomi, O., Parreño, V., Rahman, M., Ruggeri, F. M., Saif, L. J., Santos, N., Steyer, A., Taniguchi, K., Patton, J. T., Desselberger, U., & Van Ranst, M. (2011). Uniformity of rotavirus strain nomenclature proposed by the Rotavirus Classification Working Group (RCWG). *Archives of Virology*, 156(8):1397-1413. https://doi.org/10.1007/s00705-011-1006-z

132. Matthijnssens, J., Otto, P. H., Ciarlet, M., Desselberger, U., Ranst, M. V., & Johne, R. (2012). VP6-sequence-based cutoff values as a criterion for rotavirus species demarcation. *Archives of Virology*, 157(6):1177-1182. https://doi.org/10.1007/s00705-012-1273-3

133. McCance, R. A., & Widdowson, E. M. (2015). McCance and Widdowson's the composition of foods. Royal Society of Chemistry, Cambridge, UK.

134. McCauley, H. A., & Guasch, G. (2015). Three cheers for the goblet cell: maintaining homeostasis in mucosal epithelia. *Trends in Molecular Medicine,* 21(8):492–503. https://doi.org/10.1016/j.molmed.2015.06.003

135. McDole, J. R., Wheeler, L. W., McDonald, K. G., Wang, B., Konjufca, V., & Knoop, K. A. (2012). Goblet cells deliver luminal antigen to CD103+ dendritic cells in the small intestine. *Nature*, 483(7389):345-349 https://doi.org/10.1038/nature10863

136. McDonnell, M. J., Bouwhuis, M. A., Sweeney, T., O`Shea, C. J., & O`Doherty, J. V. (2016). Effects of dietary supplementation of galactooligosaccharides and seaweed – derived polysaccharides on an experimental *Salmonella* Typhimurium challenge in pigs. *Journal of Animal Science*, 94(3):153-156. https://doi.org/10.2527/jas.2015-9779

137. Megahed, A., Zeineldin, M., Evans, K., Maradiaga, N., Blair, B., Aldridge, B. & Lowe, J. (2019). Impacts of environmental complexity on respiratory and gut microbiome community structure and diversity in growing pigs. *Scientific Reports*, 9(1):13773. https://doi.org/10.1038/s41598-019-50187-z

138. Merrifield, C. A., Lewis, M. C., Berger, B., Cloarec, O., Heinzmann, S. S., Charton, F., Krause, L., Levin, N. S., Duncker, S, Mercenier, A., Holmes, E., Bailey, M., & Nicholson, J. K. (2016). Neonatal environment exerts a sustained influence on the development of the intestinal microbiota and metabolic phenotype. *The ISME Journal*, 10(1):145-57. https://doi.org/10.1038/ismej.2015.90

139. Metchnikoff, E. (1908). Chapter V. Lactic acid as inhibiting intestinal putrefaction. In: The prolongation of life. Optimistic studies. New York, London: G.P. Putnam's Sons:161–83.

140. Milligan, B. N., Fraser, D., & Kramer, D. L. (2002). Within-litter birth weight variation in the domestic pig and it's relation to pre-weaning survival, weight gain, and variation in weaning weights. *Livestock Production Science*, 76(1-2):181-191. https://doi.org/10.1016/S0301-6226(02)00012-X

141. Monteagudoo, L. V., Benito, A. A., Lázaro-Gaspar, S., Arnal, J. L., Martin-Jurado, D., Menjon, R., & Quílez, J. (2022). Occurrence of rotavirus A genotypes and other enteric pathogens in diarrheic suckling piglets from Spanish swine farms. *Animals*, 12(3):251 https://doi.org/10.3390/ani12030251

142. Moxley, R.A., Duhamel, G.E. (1999). Comparative pathology of bacterial enteric diseases of swine. In: Paul, P.S., Francis, D.H. (eds) Mechanisms in the pathogenesis of enteric diseases 2. Advances in experimental medicine and biology, vol 473. Springer, Boston, MA. https://doi.org/10.1007/978-1-4615-4143-1_7

143. Msolo, L., Iweriebor, B. C., & Okoh, A. I. (2020). Rotavirus and Cryptosporidium pathogens as etiological proxies of gastroenteritis in some pastoral communities of the Amathole District Municipality, Eastern Cape, South Africa. *BMC Research Notes*, 13(1):187. https://doi.org/10.1186/s13104-020-05024-2

144. Mudd, A. T., Salcedo, J., Alexander, L. S., Johnsom, S. K., Getty, C. M., Chichlowski, M., Berg, B. M., Barile, D., & Dilger, R. N. (2016). Porcine milk oligosaccharides and sialic acid concentrations vary throughout lactation. *Frontiers in Nutrition*, 3(39). https://doi.org/10.3389/fnut.2016.00039

145. Mulder, I. E., Schmidt, B., Stokes, C. R., Lewis, M., Bailey, M., Aminov, R. I., Proseer, J. I., Gill, B. P., Pluske, J. R., Mayer, C., Musk, C. C., & Kelly, D. (2009). Environmentally-acquired bacteria influence microbial diversity and natural innate immune responses at gut surfaces. *BMC Biology*, 7:79. https://doi.org/10.1186/1741-7007-7-79

146. Muns, R., Nuntapaitoon, M., & Tummaruk, P. (2016). Non-infectious causes of pre-weaning mortality in piglets. *Livestock Science*, 184:46-57. https://doi.org/10.1016/j.livsci.2015.11.025

147. NRC. (2012). Nutrient Requirements of Swine. Washington, DC: The National Academies Press. https://img1.wsimg.com/blobby/go/cef62d35-7a84-4a76-a0b6-562062e3ac2e/downloads/NRC%20Cerdos%202012.pdf?ver=1614196783937

148. Newburg, D. S., Ruiz-Palacious, G. M., & Morrow, A. L. (2005). Human milk glycans protect infants against enteric pathogens. *Annual Review of Nutrition*, 25:37-58. https://doi.org/10.1146/annurev.nutr.25.050304.092553

149. Niñonuevo, M. R., Park, Y., Yin, H. F., Zhang, J., Ward, R. E., Clowers, B. H., German, J. B., Freeman S. L., Killeen, K., Grimm, R., & Lebrilla, C. B. (2006). A strategy for annotating the human milk glycome. *Journal of Agricultural and Food Chemistry*, 54(20):7471-7480. https://doi.org/10.1021/jf0615810

150. Nuntapaitoon, M., Muns, R., & Tummaruk, P. (2018). Newborn traits associated with pre-weaning growth and survival in piglets. *Asian-Australasian Journal of Animal Sciences*, 31(2):237-344. https://doi.org/10.5713%2Fajas.16.0962

151. OECD/FAO. (2022). Meat. In *OECD-FAO Agricultural Outlook 2022-2031*, OECD Publishing, Paris. https://doi.org/10.1787/ab129327-en

152. ONS. (2020). National and subnational mid-year population estimates for the UK and its constituent countries by administrative area, age and sex. https://www.ons.gov.uk/peoplepopulationandcommunity/populationandmigration/populationestimates/bulletins/annualmidyearpopulationestimates/mid2020#the-uk-population-at-mid-2020Population estimates.

153. Pajarillo, E. A, Chae, J. P., Kim, H. B., Kim, I. H., & Kang, D. (2015). Barcoded pyrosequencing-based metagenomic analysis of the faecal microbiome of three purebred pig lines after cohabitation. *Applied Microbiology and Biotechnology*, 99(13):5647-5656. https://doi.org/10.1007/s00253-015-6408-5

154. Panzardi, A., Bernardi, M. L., Mellagi, A. P., Bierhals, T., Bortolozzo, F. P., & Wentz, I. (2013). Newborn piglet traits associated with survival and growth performance until weaning. *Preventive Veterinary Medicine*, 110(2):206-213. https://doi.org/10.1016/j.prevetmed.2012.11.016

155. Parashar, U. D., Hummelman, E. G., Bresee, J. S., Miller, M. A., & Glass, R. I. (2003). Global illness and deaths caused by rotavirus disease in children. *Emerging Infectious Diseases*, 9(5):565-572. https://doi.org/10.3201/eid0905.020562

156. Percie du Sert, N., Hurst, V., Ahluwalia, A., Alam, S., Avey, M. T., Baker, M., Browne, W. J., Clark, A., Cuthill, I. C., Emerson, M., Garner, P., Holgate, S.T., Howelss, D. W., Karp, N. A., Lazic, S. E., Lidster, K., MacCallum, C. J., Macleod, M., Pearl, E. J., Peterson, O., Rawle, F., Peynolds, P., Rooney, K., Sena, E. S., Silberberg, S. D., Steckler, T., & Wurbel, H. (2020). The ARRIVE guidelines 2.0: updated guidelines for reporting animal research. *PLos Biology*, 18(7):e3000410. http://doi.org/10.1371/journal.pbio.3000410

157. Piekkola, S., Turner, P. C., Abdel-Hamid, M., Ezzat, S., El-Daly, M., El-Kafrawy, S., Savchenko, E., Poussa, T., Woo, J. C. S., Mykkänen, H., & El-Nezami, H. (2012). Characterisation of aflatoxin and deoxynivalenol exposure among pregnant Egyptian women. *Food Additives and Contaminants*, 29(6):962-971. https://doi.org/10.1080/19440049.2012.658442

158. Pineiro, M., Asp, N. G., Reid, G., Macfarlane, S., Morelli, L., Brunser, O., & Tuohy K. (2008). FAO Technical meeting on prebiotics. *Journal of Clinical Gastroenterology*, 42 (Suppl 3 Pt 2):S156-159. https://doi.org/10.1097/mcg.0b013e31817f184e

159. Pluske, J. R., Hampson, D. J., & Williams, I. H. (1997). Factors influencing the structure and function of the small intestine in the weaned pig: a review. *Livestock Production Science*, 51(1-3):215-236. https://doi.org/10.1016/S0301-6226(97)00057-2

160. Pluske, J. R. (2013). Feed- and feed additives-related aspects of gut health and development in weanling pigs. *Journal of Animal Science and Biotechnology*, 4(1). https://doi.org/10.1186/2049-1891-4-1

161. Pluske, J. R., Turpin, D. L., & Kim, J. C. (2018). Gastrointestinal tract (gut) health in the young pig. *Animal Nutrition*, 4(2):187-196. https://doi.org/10.1016/j.aninu.2017.12.004

162. Proorocu, M., Petrescu, D. C., Burny, P., & Petrescu-Mag, R. M. (2021). Pork meat consumption, from statistics to consumer behaviour: a review. *Porcine Research*, 11(1):2-8. http://www.porc.bioflux.com.ro/

163. Pruesse, E., Quast, C., Knittel, L., Fuchs, B. M., Ludwig, W., Peplies, J., Glöckner, F. O. (2007). SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Research*, 35(21):7188-96. https://doi.org/10.1093/nar/gkm864

164. Pujari, R., & Banerjee, G. (2021). Impact of prebiotics on immune response: from the bench to the clinic. *Immunology and Cell Biology*, 99:255-273. https://doi.org/10.1111/imcb.12409

165. Ramanakumar, A. V., Thomann, P., Candeias, J. M., Ferreira, S., Villa, L. L., Franco, E. L. (2010). Use of the normalized absorbance ratio as an internal standardization approach to minimize measurement error in enzyme-linked immunosorbent assays for diagnosis of human papillomavirus infection. *Journal of Clinical Microbiology*, 48(3):791-796. https://doi.org/10.1128/JCM.00844-09.

166. Red Tractor. (2021). Red Tractor Pigs Standards, Version 5: 1 November 2021. chrome-extension://efaidnbmnnnibpcajpcglclefindmkaj/https://redtractorassurance.org.uk/wp-content/uploads/2021/08/RTStandardsV5_PIGS_SINGLES.pdf

167. Rettger, L. F., & Cheplin, H. A. (1922). *Bacillus acidophilus* and its therapeutic application. *Archives of Internal Medicine*, 29:357-367.

168. Richards, P. J., Lafontaine, G. M. F., Connerton, P. L., Liang, L., Asiani, K., Fish, N. M., & Connerton, I. F. (2020). Galacto-oligosaccharides modulate the juvenile gut microbiome and innate immunity to improve broiler chicken performance. *mSytems*, 5(1):e00827-19. https://doi.org/10.1128/msystems.00827-19

169. Roberfroid, M., Gibson, G. R., Hoyles, L., McCartney, A. L., Rastall, R., Rowland, I., Wolvers, D., Watzl, B., Szajewska, H., Stahl, B., Guarner, F., Respondek, F., Whelan, K., Coxam V., Davicco, M., Léotoing, L., Wittrant, Y., Delzenne, N.M., Cani, P. D., Neyrinck, A. M., & Meheust, A. (2010). Prebiotic effects: metabolic and health benefits. *British Journal of Nutrition*, 104 Suppl 2:S1-63. https://doi.org/10.1017/s0007114510003363

170. Rodríguez, J. M., Fernández, L., & Verhasselt, V. (2021). The gut-breast axis: Programming health for life. *Nutrients*, 13(2):606. https://doi.org/10.3390%2Fnu13020606

171. Ruiz-Palacios, G . M., Pérez-Schael, I., Velázquez, F. R., Abate, H., Breuer, T., Clemens, S. C., Cheuvart, B., Espinoza, F., Gillard, P., Innis, B. L., Cervantes, Y., Linhares, A. C., López, P., Macías-Parra, M., Ortega-Barría, E., Richardson, V., Rivera-Medina, D. B., Rivera, L., Salinas, B., Pavía-Ruz, N., Salmerón, J., Rüttimann, R., Tinoco, J. C., Rubio, P., Nuñez, E., Guerrero, M. L., Yarzábal, J. P., Damaso, S., Tornieporth, N., Sáez-Llorens, X., Vergara, R. F., Vesikari, T., Bouckenooghe, A., Clemens, R., De Vos, B., O'Ryan, M., & Human Rotavirus Vaccine Study Group. (2006). Safety and efficacy of an attenuated vaccine against severe rotavirus gastroenteritis. *The New England Journal of Medicine*, 354(1):11-22. https://doi.org/10.1056/NEJMoa052434

172. Saif, L. J., & Fernandez, F. M. (1996). Group A rotavirus veterinary vaccines. *The Journal of infectious diseases*, 174(Suppl 1):S98-106. https://doi.org/10.1093/infdis/174.supplement_1.s98

173. Salcedo, J., Frese, S. A., Mills, D. A., & Barile, D. (2016). Characterization of porcine milk oligosaccharides during early lactation and their relation to the fecal microbiome. *Journal of Dairy Science*, 99(10):7733-7743. https://doi.org/10.3168/jds.2016-10966

174. Salminen, S., Bouley, C., Boutron-Ruault, M. C., Cummings, J. H., Franck, A., Gibson, G. R., Isolauri, E., Moreau, M. C., Roberfroid, M., & Rowland, I. R. (1998). Functional food science and gastrointestinal physiology and function. *British Journal of Nutrition*, 80(Suppl 1):S147-S171. https://doi.org/10.1079/bjn19980108

175. Salmon, H. (2002). Mammary gland immunology and neonate protection in pigs. In: Mol, J.A., Clegg, R.A. (eds) Biology of the Mammary Gland: Homing of lymphocytes into the MG. Advances in Experimental Medicine and Biology, vol 480. Springer, Boston, MA. https://doi.org/10.1007/0-306-46832-8_32

176. Sanidad, K. Z., Amir, M., Ananthanarayanan, A., Singaraju, A., Shiland, N. B., Hong, H. S., Kamada, N., Inohara, N., Núñez, G., & Zeng, M. Y. (2022). Maternal gut microbiome-induced IgG regulates neonatal gut microbiome and immunity. *Science Immunology*, 7(72):eabh3816. https://doi.org/10.1126/sciimmunol.abh3816

177. Scheppach, W., Luehrs, H., & Menzel, T. (2001). Beneficial health effects of low-digestible carbohydrate consumption. *British Journal of Nutrition*, 85(Suppl 1):S23-30. https://doi.org/10.1079/BJN2000259

178. Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., Lesniewski, R. A., Oakly, B. B., Parks, D. H., Robinson, C. J., Sahl, J. W., Stres, B., Thallinger, G. G., Van Horn, D. J., & Weber, C. F. (2009). Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Applied and Environmental Microbiology*, 75(23):7537-7541. https://doi.org/10.1128/AEM.01541-09

179. Shaw, D. P., Morehouse, L. G., & Solorzano, R. F. (1989) Experimental rotavirus infection in three-week old pigs. *American Journal of Veterinary Research*, 50(11):1961–1965.

180. Shoaf, K., Mulvey, G. L., Armstrong, G. D., & Hutkins, R. W. (2006). Prebiotic galactooligosaccharides reduce adherence of enteropathogenic *Escherichia coli* to tissue culture cells. *Infection and Immunity*, 74(12):6920-6928. https://doi.org/10.1128/iai.01030-06

181. Silva, M. S., Ramos, C. L., González-Avila, M., Gschaedler, A., Arrizon, J., Schwan, R. F., & Dias, D. R. (2017). Probiotic properties of *Weissella cibaria* and *Leuconostoc citreum* isolated from *tejuino* – A typical Mexican beverage. *LWT – Food Science and Technology*, 86:227-232. https://doi.org/10.1016/j.lwt.2017.08.009

182. Skansberg, A., Sauer, M., Tan, M., Santosham, M., & Jennings, M. C. (2021). Product review of the rotavirus vaccines ROTASIIL, ROTAVAC, and Rotavin-M1. *Human Vaccines and Immunotherapeutics*, 17(4):1223-1234. https://doi.org/10.1080/21645515.2020.1804245

183. Slade, R. D., Kyriazakis, I., Carroll, S. M., Reynolds F. H., Wellock, I. J., Broom, L. J, & Miller, H.M. (2011). Effect of rearing environment and dietary zinc oxide on the response of group-housed weaned pigs to enterotoxigenic *Escherichia coli* 0149 challenge. *Animal*, 5(8):1170-1178. https://doi.org/10.1017/s1751731111000188

184. Slifierz, M. J., Friendship, R., & Weese J. S. (2014). Zinc oxide therapy increases prevalence and persistence of methicillin-resistant *Staphylococcus* in pigs: a randomized controlled trial. *Zoonoses Public Health*, 62(4):301-308. https://doi.org/10.1111/zph.12150

185. Statista. (2021). Projected pig meat consumption worldwide from 2021 to 2030. https://www.statista.com/statistics/739879/pork-consumption-worldwide/

186. Stewart, C., Piernas, C., Cook, B., & Jebb S. A. (2021). Trends in UK meat consumption: analysis of data from years 1–11 (2008–09 to 2018–19) of the National Diet and Nutrition Survey rolling programme. *Lancet Planet Health*, 5:e699-708. https://doi.org/10.1016/S2542-5196(21)00228-X

187. Stokes, C.R. (2017). The development and role of microbial host interactions in gut mucosal immune development. *Journal of Animal Science and Biotechnology*, 8(12). https://doi.org/10.1186/s40104-016-0138-0

188. Šuligoj, T., Vigsnæs, L. K., Abbeele, P. V. D., Apostolou, A., Karalis, K., Savva, G. M., McConnell, B., & Juge, N. (2020). Effects of human milk oligosaccharides on the adult gut microbiota and barrier function. *Nutrients*, 12(9):2808. https://doi.org/10.3390/nu12092808.

189. Svensmark, B., Jorsal, S. E., Nielsen, K., & Willeberg, P. (1989a). Epidemiological studies of piglet diarrhoea in intensively managed Danish sow herds. 1. Pre-weaning diarrhoea. *Acta Veterinaria Scandinavica*, (30)1:43-53. https://doi.org/10.1186/bf03548067

190. Svensmark, B., Askaa, J., Wolstrup, C., & Nielsen, K. (1989b) Epidemiological studies of piglet diarrhea in intensively managed Danish sow herds. IV. Pathogenicity of porcine rotavirus. *Acta Veterinaria Scandinavica*, 30(1):71–76. https://doi.org/10.1186/bf03548070

191. Tallaksen, J., Johnston, L., Sharpe, K., Reese, M., & Buchanan, E. (2020). Reducing life cycle fossil energy and greenhouse gas emissions for Midwest swine production systems. *Journal of Cleaner Production*, 246(10):118998. https://doi.org/10.1016/j.jclepro.2019.118998

192. Tanner, S.A., Chassard, C., Berner, A. Z., & Lacroix C. (2014). Synergistic effects of *Bifidobacterium thermophilum* RBL67 and selected prebiotics on inhibition of *Salmonella* colonization in the swine proximal colon PolyFermS model. *Gut Pathogens*, 6(1):44. https://doi.org/10.1186/s13099-014-0044-y

193. Tao, N., Ochonicky, K. L., German, J. B., Donovan, S. M., & Lebrilla, C. B. (2010). Structural determination and daily variations of porcine milk oligosaccharides. *Journal of Agricultural and Food Chemistry*, 58(8):4653-4659. https://doi.org/10.1021/jf100398u

194. Tate, J. E., Burton, A. H., Boschi-Pinto, C., Steele, A. D., Duque, J., & Parashar, U. D. (2012). 2008 estimate of worldwide rotavirus-associated mortality in children younger than 5 years before the introduction of universal rotavirus vaccination programmes: a systematic review and meta-analysis. *The Lancet. Infectious Diseases*, 12(2):136-141. https://doi.org/10.1016/s1473-3099(11)70253-5

195. Tate, J. E., Burton, A. H., Boschi-Pinto, C., & Parashar, U. D. (2016). Global, regional, and national estimates of rotavirus mortality in children <5 years of age, 2000-2013. *Clinical Infectious Diseases*, 62(suppl 2):S96-S105. https://doi.org/10.1093/cid/civ1013

196. The Guardian (2022). UK has more than 1,000 livestock mega-farms, investigation reveals. https://www.theguardian.com/environment/2022/aug/18/uk-has-more-than-1000-livestock-mega-farms-investigation-reveals Accessed 18/08/2022.

197. Thompson, C. L., Wang, B., & Holmes, A. J. (2008). The immediate environment during postnatal development has long-term impact on gut community structure in pigs. *The ISME Journal*, 2(7):739-748. https://doi.org/10.1038/ismej.2008.29

198. Tian, S., Wang, J., Yu, H., Wang, J., & Zhu, W. (2018). Effects of galacto-oligosaccharides on growth and gut function of newborn suckling piglets. *Journal of Animal Science and Biotechnology*, 13:70. https://doi.org/10.1186%2Fs40104-022-00711-5

199. Tian, S., Wang, J., Yu, H., Wang, J., & Zhu, W. (2019). Changes in ileal microbial composition and microbial metabolism by an early-life galacto-oligosaccharides intervention in a neonatal porcine model. *Nutrients*, 11(8): 1753. https://doi.org/10.3390/nu11081753

200. Tian, S., Wang, J., Gao, R., Wang, J., & Zhu, W. (2021). Early-life galacto-oligosaccharides supplementation alleviates the small intestinal oxidative stress and dysfunction of lipopolysaccharide-challenged suckling piglets. *Journal of Animal Science and Biotechnology*, 13(1):70. https://doi.org/10.1186/s40104-022-00711-5

201. Tucker, B. S., Craig, J. R., Morrison, R. S., Smits, R. J., & Kirkwood, R. N. (2021). Piglet viability: A review of identification and pre-weaning management strategies. *Animals*, 11(10):2902. https://doi.org/10.3390%2Fani11102902

202. Tzortzis, G., Goulas, A. K., Gee, J. M., & Gibson, G. R. (2005). A novel galactooligosaccharide mixture increases the bifidobacterial population numbers in a continuous in vitro fermentation system and in the proximal colonic contents of pigs in vivo. *The Journal of Nutrition*, 135(7):1726-1731. https://doi.org/10.1093/jn/135.7.1726

203. Underwood, M. A., German, J. B., Lebrilla, C. B., & Mills, D. A. (2015). *Bifidobacterium longum* subspecies infantis: champion colonizer of the infant gut. *Pediatric Research*, 77(1-2):229-35. https://doi.org/10.1038/pr.2014.156

204. UNFAO. (2002). Guidelines for the evaluation of probiotics in food. https://4cau4jsaler1zglkq3wnmje1-wpengine.netdna-ssl.com/wp-content/uploads/2019/04/probiotic_guidelines.pdf

205. Urashima, T., Saito, T., Nakamura, T., & Messer, M. (2001). Oligosaccharides of milk and colostrum in non-human mammals. *Glycoconjugate Journal*, 18(5):357-371. https://doi.org/10.1023/a:1014881913541

206. Urashima, T., Asakima, S., & Messer, M. (2007). Milk oligosaccharides. In: Comprehensive Glycoscience. From Chemistry to System Biology, pp. 695-722. J. P. Kamerling, G. J. Boons, Y. C. Lee, Suzuki A, Taniguchi N, & A. G. J. Voragen, editors, Oxford, UK: Elsevier.

207. Urashima, T., Taufik, E., Fukuda, K., & Asakuma, S. (2013). Recent advances in studies on milk oligosaccharides of cows and other domestic farm animals. *Bioscience, Biotechnology and Biochemistry*, 77(3):455-466. https://doi.org/10.1271/bbb.120810

208. Usami, K., Niimi, K., Matsuo, A., Suyama, Y., Sakai, Y., Sato, S., Fujihashi, K., Kiyono, H., Uchino, S., Furukawa, M., Islam, J., Ito, K., Moriya, T., Kusumoto, Y., Tomura, M., Hovey, R. C., Sugawara, J., Yoneyama, H., Kitazawa, H., Watanabe, K., Aso, H., & Nochi, T. (2021). The gut microbiota induces Peyer's-patch-dependent secretion of maternal IgA into milk. *Cell Reports*, 36(10):109655. https://doi.org/10.1016/j.celrep.2021.109655

209. USDA. (2019). U. S. Department of Agriculture. Food Data Central. Pork, cured bacon, unprepared. https://fdc.nal.usda.gov/fdc-app.html#/food-details/168277/nutrients

210. USFDA. (2019). U. S. Food and Drug Administration. Generally Regarded as Safe (GRAS). https://www.fda.gov/food/food-ingredients-packaging/generally-recognized-safe-gras

211. Vaillancourt, J. P., Stein, T. E., Marsh, W. E., Leman, A. D., & Dial, G. D. (1990). Validation of producer-recorded causes of preweaning mortality in swine. *Preventive Veterinary Medicine*, 10:119-130. https://doi.org/10.1016/0167-5877(90)90057-O

212. Van Boeckel, T. P., Glennon, E. E., Chen, D., Gilbert, M., Robinson, T. P., Grenfell, B. T., Levin, S. A., Bonhoeffer, S., & Laxminarayan, R. (2017). Reducing antimicrobial use in food animals. *Science*, 357(6358):1350-1352. https://doi.org/10.1126/science.aao1495

213. Van Boeckel, T. P., Pires, J., Silvester, R., Zhao, C., Song, J., Criscuolo, N. G., Gilbert, M., Bonhoeffer, S., & Laxminarayan, R. (2019). Global trends in antimicrobial resistance in low- and middle- income countries. *Science*, 365(6459):eaaw1944. https://doi.org/10.1126/science.aaw1944

214. Verheijden, K. A. T., Akbari, P., Willemsen, L. E. M., Kraneveld, A. D., Folkerts, G., Garssen, J., Fink-Gremmels, J., & Braber, S. (2015). Inflammation-induced expression of the alarmin interleukin 33 can be suppressed by galacto-oligosaccharides. *International Archives of Allergy and Immunology*, 167(2):127-136. https://doi.org/10.1159/000437327

215. Viaene, J., & Verbeke, W. (1998). Economic feasibility of the use of feed additives. *Pig Progress*, 14(3):11-14. https://oa.mg/work/2620267596

216. Vesikari, T., Matson, D. O., Dennehy, P., Van Damme, P., Santosham, M., Rodriguez Z., Dallas, M. J., Heyse, J. F., Goveia, M. G., Black, S. B., Shinefield, H. R., Christie, C. D. C., Ylitalo, S., Itzler, R. F., Coia, M. L., Onorato, M. T., Adeyi, B. A., Marshall, G. S., Gothefors, L., Campens, D., Karvonen, A., Watt, J. P., O'Brien, K, L., DiNubile, M. J., Clark, H. F., Boslego, J. W., Offit, P. A., Heaton, P. M., & Rotavirus Efficacy and Safety Trial (REST) Study Team. (2006). Safety and efficacy of a pentavalent human-bovine (WC3) reassortant rotavirus vaccine. *The New England Journal of Medicine*, 354(1). 23-33. https://doi.org/10.1056/nejmoa052664

217. Vlasova, A. N., Amimo, J. O., & Saif, L. J. (2017). Porcine rotaviruses: Epidemiology, immune responses and control strategies. *Viruses*, 9(3):48. https://doi.org/10.3390/v9030048

218. Vondruskova, H., Slamova, R., Trckova, M., Zraly, Z., & Pavlik, I. (2010). Alternatives to antibiotic growth promoters in prevention of diarrhoea in weaned piglets: a review. *Veterinarni Medicina*, 55:199-224. https://doi.org/10.17221/2998-VETMED

219. Wang, J., Tian, S., Yu, H., Wang, J., & Zhu, W. (2019). Response of colonic mucosa-associated microbiota composition, mucosal immune homeostasis, and barrier function to early life galactooligosaccharides intervention in suckling piglets. Journal of Agricultural and Food Chemistry, 67:578-588. https://doi.org/10.1021/acs.jafc.8b05679

220. Wang, X., Tsai, T., Deng, F., Wei, X., Chai, J., Knapp, J., Apple, J., Maxwell, C. V., Ae Lee, J., Li, Y., & Zhao, J. (2019) Longitudinal investigation of the swine gut microbiome from birth to market reveals stage and growth performance associated bacteria. *Microbiome*, 7(1):109. https://doi.org/10.1186/s40168-019-0721-7

221. WATOK. (2015). The welfare of animals at the time of killing (England) regulations 2015. https://www.legislation.gov.uk/uksi/2015/1782/contents/made

222. Wiens, K. E., Schaeffer, L. E., Sow, S. O., Ndoye, B., Cain, C. J., Baumann, M. M., Johnson, K. B., Lindstedt, P. A., Blacker, B. F., Bhutta, Z. A., Cormier, N. M., Daoud, F., Earl, L., Farag, T., Khalil, I. A., Kinyoki, D. K., Larson, H. J., LeGrand, K. E., Cook, A. J., Malta, D. C., Månsson, J. C., Mayala, B. K., Mokdad, A. H., Ogbuanu, I. U., Sankoh, O., Sartorius, B., Topor-Madry, R., Troeger, C. E., Welgan, C. A., Werdecker, A., Hay, S. I., & Reiner, R. C. Jr. (2020). Oral rehydration therapies in Senegal, Mali, and Sierra Leone: a spatial analysis of changes over time and implications for policy. *BMC Medicine*, 18(1):405. https://doi.org/10.1186/s12916-020-01857-7

223. Weström, B. R., Ohlsson, B. G., Svendsen, J., Tagesson, C., & Karlsson, B. W. (1985). Intestinal transmission of macromolecules (BSA and FITC-Dextran) in the neonatal pig: Enhancing effect of colostrum, proteins and proteinase inhibitors. *Biology of the Neonate*, 47:359-366. https://doi.org/10.1159/000242140

224. Wei, J., Wang, Z. A., Wang, B., Jahan, M., Wang, Z., Wynn, P. C., & Du, Y. (2018). Characterization of porcine milk oligosaccharides over lactation between primiparous and multiparous female pigs. *Scientific Reports*, 8(1):4688. https://doi.org/10.1038/s41598-018-23025-x

225. Whittemore, C., & Green, D. (2001). Growth of the young weaned pig. In: M. Varley and J. Wiseman, eds. The weaner pig. Nutrition and management. Wallingford: CABI Publishing. pp. 1-16. https://www.cabi.org/ahpc/FullTextPDF/2001/20013155799.pdf

226. Wu, F., Liu, L. T., Jiang, B., Kuo, T., Wu, C., & Liao, M. (2022). Prevalence and diversity of rotavirus A in pigs: Evidence for a possible reservoir in human infection. *Infection, Genetics and Evolution: Journal of Molecular Epidemiology and Evolutionary Genetics in Infectious Diseases*, 98:105198. https://doi.org/10.1016/j.meegid.2021.105198

227. Xing, Y., Li, K., Xu, Y., Wu, Y., Shi, L., Guo, S., Yan, S., Jin, X., & Shi, B. (2020). Effects of galacto-oligosaccharide on growth performance, feacal microbiota, immune response and antioxidant capability in weaned piglets. *Journal of Applied Animal research*, 48(1):63-69. https://doi.org/10.1080/09712119.2020.1732394

228. Xu, S., Lane, J. A., Chen, J., Zheng, Y., Wang, H., Fu, X., Huang, Q., Dhital, S., Liu, F & Zhang, B. (2022). In vitro infant fecal fermentation characteristics of human milk oligosaccharides were controlled by initial microbiota composition more than chemical structure. *Molecular Nutrition and Food Research*, 66(19):e2200098. https://doi.org/10.1002/mnfr.202200098

229. Yan, Y. L., Hu, Y., Simpsom, D. J., & Gänzle, G. (2017). Enzymatic synthesis and purification of galactosylated chitosan oligosaccharides reducing adhesion of enterotoxigenic *Escherichia coli* K88. *Journal of Agricultural and Food Chemistry*, 65(25):5142-5150. https://doi.org/10.1021/acs.jafc.7b01741

230. Yang, Y., Hou, C., Zeng, X., & Qiao, S. (2015). The use of lactic acid bacteria as a probiotic in swine diets. *Pathogens*, 41(1):34-45. https://doi.org/10.3390%2Fpathogens4010034

231. Zira, S., Rydhmer, L., Ivarsson, E., Hoffmann, & R., Röös, E. (2021). A life cycle sustainability assessment of organic and conventional pork supply chains in Sweden. *Sustainable Production and Consumption*, 28:21–38. https://doi.org/10.1016/j.spc.2021.03.028

232. Zhang, W., Zhu, Y., Zhou, D., Wu, Q., Song, D., Dicksved, J., & Wang, J. (2017). Oral administration of a select mixture of Bacillus probiotics affects the gut microbiota and goblet cell function following *Escherichia coli* challenge in newly weaned pigs of genotype MUC4 that are supposed to be enterotoxigenic *E. coli* F4ab/ac receptor negative. *Applied and Environmental Microbiology*, 83(3):e02747-16. https://doi.org/10.1128/aem.02747-16

233. Zhang, Z., Yang, L., He, Y., Luo, X., Zhao, S., & Jia, X. (2021). Composition of fecal microbiota in grazing and feedlot angus beef cattle. *Animals*, 11(11):3167. https://doi.org/10.3390/ani11113167

234. Zhang, M., Li, Q., Wang, J., Sun, J., Xiang, Y., & Jin, X. (2022). Aflatoxin B1 disrupts the intestinal barrier integrity by reducing junction protein and promoting apoptosis in pigs and mice. *Ecotoxicology and Environmental Safety*, 1(247):114250. https://doi.org/10.1016/j.ecoenv.2022.114250

235. Zmora, N., Zilberman-Schapira, G., Suez, J., Mor, U., Dori-Bachash, M. D., Bashiardes, S., Kotler, E., Zur, M., Regev-Lehavi, D., Brik, R. B., Federici, S., Cohen, Y., Linevsky, R., Rothschild, D., Moor, A. E., Ben-Moshe, S., Harmelin, A., Itzkovitz, S., Maharshak, N., Shibolet, O., Shapiro, H., Pevsner-Fischer, M., Sharon, I., Halpern, Z., Segal, E., & Elinav, E. (2018). Personalized gut mucosal colonisation resistance to empiric probiotics is associated with unique host and microbiome features. *Cell*, 174(6):1388-1405. https://doi.org/10.1016/j.cell.2018.08.041

236. Zuurveld, M., van Witzenburg, N. P., Garssen, J., Folkerts, G., Stahl, B., Van't Land, B., & Willemsen, E. M. (2020). Immunomodulation by human milk oligosaccharides: The potential role in prevention of allergic diseases. *Frontiers in Immunology*, 11:801. https://doi.org/10.3389/fimmu.2020.00801

APENDICES

Appendix 1. Primer design

Generic PCR primer design:

AATGATACGGCGACCACCGAGATCTACAC <i5><pad><link><16Sf>

VX.N5??

CAAGCAGAAGACGGCATACGAGAT <i7><pad><link><16Sr> VX.N7??

Generic read 1 primer design

<pad><link><16Sf> VX.read1

Generic read 2 primer design

<pad><link><16Sr> VX.read2

Generic index read primer design

Reverse complement of (<pad><link><16Sr>) VX.p7_index

The listed sequences in the generic design above are the adapter sequences to allow annealing of the amplicons to the Illumina MiSeq flow cell. The i5 and i7 sequences are the 8-nt index sequences. The pad is a 10-nt sequence to boost the sequencing primer melting temperatures. The link is a 2-nt sequence that is anti-complementary to the known sequences.

16S forward read

V4: GTGCCAGCMGCCGCGGTAA

16S reverse read

V4: GGACTACHVGGGTWTCTAAT

Link:

V4 forward: GT

V4 reverse: CC

Pad:

Forward: TATGGTAATT

Reverse: AGTCAGTCAG

8-nt index sequences i5

IA501: ATCGTACG

IA502: ACTATCTG

IA503: TAGCGAGT

IA504: CTGCGTGT

IA505: TCATCGAG

IA506: CGTGAGTG

IA507: GGATATCT

IA508: GACACCGT

IB501: CTACTATA

IB502: CGTTACTA

IB503: AGAGTCAC

IB504: TACGAGAC

IB505: ACGTCTCG

IB506: TCGACGAG

IB507: GATCGTGT

IB508: GTCAGATA

8-nt index sequences i7

IA701: AACTCTCG

IA702: ACTATGTC

IA703: AGTAGCGT

IA704: CAGTGAGT

IA705: CGTACTCA

IA706: CTACGCAG

IA707: GGAGACTA

IA708: GTCGCTCG

IA709: GTCGTAGT

IA710: TAGCAGAC

IA711: TCATAGAC

IA712: TCGCTATA

IB701: AAGTCGAG

IB702: ATACTTCG

IB703: AGCTGCTA

IB704: CATAGAGA

IB705: CGTAGATC

IB706: CTCGTTAC

IB707: GCGCACGT

IB708: GGTACTAT

IB709: GTATACGC

IB710: TACGAGCA

IB711: TCAGCGTT

IB712: TCGCTACG

Primers used to amplify samples using the V4 region:

I5

v4.IA501:

AATGATACGGCGACCACCGAGATCTACACATCGTACGTATGGTAATTGTG

TGCCAGCMGCCGCGGTAA

v4.IA502:

AATGATACGGCGACCACCGAGATCTACACACTATCTGTATGGTAATTGTG

TGCCAGCMGCCGCGGTAA

v4.IA503:

AATGATACGGCGACCACCGAGATCTACACTAGCGAGTTATGGTAATTGTG

TGCCAGCMGCCGCGGTAA

v4.IA504:

AATGATACGGCGACCACCGAGATCTACACCTGCGTGTTATGGTAATTGTG

TGCCAGCMGCCGCGGTAA

v4.IA505:

AATGATACGGCGACCACCGAGATCTACACTCATCGAGTATGGTAATTGTG

TGCCAGCMGCCGCGGTAA

v4.IA506:

AATGATACGGCGACCACCGAGATCTACACCGTGAGTGTATGGTAATTGTG

TGCCAGCMGCCGCGGTAA

v4.IA507:

AATGATACGGCGACCACCGAGATCTACACGGATATCTTATGGTAATTGTG

TGCCAGCMGCCGCGGTAA

v4.IA508:

AATGATACGGCGACCACCGAGATCTACACGACACCGTTATGGTAATTGTG

TGCCAGCMGCCGCGGTAA

v4.IB501:

AATGATACGGCGACCACCGAGATCTACACCTACTATATATGGTAATTGTG

TGCCAGCMGCCGCGGTAA

v4.IB502:

AATGATACGGCGACCACCGAGATCTACACCGTTACTATATGGTAATTGTG

TGCCAGCMGCCGCGGTAA

v4.IB503:

AATGATACGGCGACCACCGAGATCTACACAGAGTCACTATGGTAATTGT

GTGCCAGCMGCCGCGGTAA

v4.IB504:

AATGATACGGCGACCACCGAGATCTACACTACGAGACTATGGTAATTGT

GTGCCAGCMGCCGCGGTAA

v4.IB505:

AATGATACGGCGACCACCGAGATCTACACACGTCTCGTATGGTAATTGTG

TGCCAGCMGCCGCGGTAA

v4.IB506:

AATGATACGGCGACCACCGAGATCTACACTCGACGAGTATGGTAATTGT

GTGCCAGCMGCCGCGGTAA

v4.IB507:

AATGATACGGCGACCACCGAGATCTACACGATCGTGTTATGGTAATTGTG

TGCCAGCMGCCGCGGTAA

v4.IB508:

AATGATACGGCGACCACCGAGATCTACACGTCAGATATATGGTAATTGTG

TGCCAGCMGCCGCGGTAA

i7

v4.IA701:

CAAGCAGAAGACGGCATACGAGATAACTCTCGAGTCAGTCAGCCGGACT

ACHVGGGTWTCTAAT

v4.IA702:

CAAGCAGAAGACGGCATACGAGATACTATGTCAGTCAGTCAGCCGGACT

ACHVGGGTWTCTAAT

v4.IA703:

CAAGCAGAAGACGGCATACGAGATAGTAGCGTAGTCAGTCAGCCGGACT

ACHVGGGTWTCTAAT

v4.IA704:

CAAGCAGAAGACGGCATACGAGATCAGTGAGTAGTCAGTCAGCCGGACT

ACHVGGGTWTCTAAT

v4.IA705:

CAAGCAGAAGACGGCATACGAGATCGTACTCAAGTCAGTCAGCCGGACT

ACHVGGGTWTCTAAT

v4.IA706:

CAAGCAGAAGACGGCATACGAGATCTACGCAGAGTCAGTCAGCCGGACT

ACHVGGGTWTCTAAT

v4.IA707:

CAAGCAGAAGACGGCATACGAGATGGAGACTAAGTCAGTCAGCCGGACT

ACHVGGGTWTCTAAT

v4.IA708:

CAAGCAGAAGACGGCATACGAGATGTCGCTCGAGTCAGTCAGCCGGACT

ACHVGGGTWTCTAAT

v4.IA709:

CAAGCAGAAGACGGCATACGAGATGTCGTAGTAGTCAGTCAGCCGGACT

ACHVGGGTWTCTAAT

v4.IA710:

CAAGCAGAAGACGGCATACGAGATTAGCAGACAGTCAGTCAGCCGGACT

ACHVGGGTWTCTAAT

v4.IA711:

CAAGCAGAAGACGGCATACGAGATTCATAGACAGTCAGTCAGCCGGACT

ACHVGGGTWTCTAAT

v4.IA712:

CAAGCAGAAGACGGCATACGAGATTCGCTATAAGTCAGTCAGCCGGACT

ACHVGGGTWTCTAAT

v4.IB701:

CAAGCAGAAGACGGCATACGAGATAAGTCGAGAGTCAGTCAGCCGGACT

ACHVGGGTWTCTAAT

v4.IB702:

CAAGCAGAAGACGGCATACGAGATATACTTCGAGTCAGTCAGCCGGACT

ACHVGGGTWTCTAAT

v4.IB703:

CAAGCAGAAGACGGCATACGAGATAGCTGCTAAGTCAGTCAGCCGGACT

ACHVGGGTWTCTAAT

v4.IB704:

CAAGCAGAAGACGGCATACGAGATCATAGAGAAGTCAGTCAGCCGGACT

ACHVGGGTWTCTAAT

v4.IB705:

CAAGCAGAAGACGGCATACGAGATCGTAGATCAGTCAGTCAGCCGGACT

ACHVGGGTWTCTAAT

v4.IB706:

CAAGCAGAAGACGGCATACGAGATCTCGTTACAGTCAGTCAGCCGGACT

ACHVGGGTWTCTAAT

v4.IB707:

CAAGCAGAAGACGGCATACGAGATGCGCACGTAGTCAGTCAGCCGGACT

ACHVGGGTWTCTAAT

v4.IB708:

CAAGCAGAAGACGGCATACGAGATGGTACTATAGTCAGTCAGCCGGACT

ACHVGGGTWTCTAAT

v4.IB709:

CAAGCAGAAGACGGCATACGAGATGTATACGCAGTCAGTCAGCCGGACT

ACHVGGGTWTCTAAT

v4.IB710:

CAAGCAGAAGACGGCATACGAGATTACGAGCAAGTCAGTCAGCCGGACT

ACHVGGGTWTCTAAT

v4.IB711:

CAAGCAGAAGACGGCATACGAGATTCAGCGTTAGTCAGTCAGCCGGACT

ACHVGGGTWTCTAAT

v4.IB712:

CAAGCAGAAGACGGCATACGAGATTCGCTACGAGTCAGTCAGCCGGACT

ACHVGGGTWTCTAAT

Read 1 primer for V4 region:

TATGGTAATTGTGTGCCAGCMGCCGCGGTAA

Read 2 primer for V4 region:

AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT

Index primer for V4 region:

ATTAGAWACCCBDGTAGTCCGGCTGACTGACT

Appendix 2. Code for analysis of 16S rRNA gene sequences in Mothur

For Chapters 3 and 5 the 16S rRNA gene sequence analysis was performed using Mothur v. 1.39 and for Chapter 4, v.1.46.1 (Schloss *et al*., 2009). Analysis was performed according to the MiSeq SOP https://www.mothur.org/wiki/MiSeq_SOP (Kozich *et al*., 2013). The 16S rRNA gene sequences were aligned against a reference alignment based on the SILVA rRNA database (Pruesse *et al*., 2007) for use in Mothur, available at: https://www.mothur.org/wiki/Silva_reference files. Only one example for analyses of jejunal sequences from paper 2, Chapter 4 is presented here since the code for all chapters would be too extensive to include and run into more pages than necessary.

#Mothur Code

#Create an index of fastq files

make.file(inputdir=F:\Jejunum, type=fastq, prefix=stability)

#Combine forward and reverse read fastq files

make.contigs(file=stability.files, processors=8)

#Interrogate contig assembly read

summary.seqs(fasta=stability.trim.contigs.fasta, count=stability.contigs.count_table)

#Remove duplicate sequences

unique.seqs(fasta=stability.trim.contigs.fasta, count=stability.contigs.count_table)

#Create SILVA alignment database

pcr.seqs(fasta=silva.bacteria.fasta, start=11894, end=25319, keepdots=F)

#Rename the output file

rename.file(input=silva.bacteria.pcr.fasta, new=silva.v4.fasta)

#Interrogate the SILVA output file

summary.seqs(fasta=silva.v4.fasta)

#Align sequences to SILVA alignment database

align.seqs(fasta=stability.trim.contigs.unique.fasta, reference=silva.v4.fasta)

#Interogate the output file

summary.seqs(fasta=stability.trim.contigs.unique.align,

count=stability.trim.contigs.count_table)

#Ensure sequences overlap the same region

screen.seqs(fasta=stability.trim.contigs.unique.align,

count=stability.trim.contigs.count_table, start=1969, end=11551)

#Interrogate the output file

summary.seqs(fasta=current, count=current)

#Filter sequences to remove overhangs at both ends

filter.seqs(fasta=stability.trim.contigs.unique.good.align, vertical=T, trump=.)

#Remove duplicate sequences

unique.seqs(fasta=stability.trim.contigs.unique.good.filter.fasta,

count=stability.trim.contigs.good.count_table)

#Pre-cluster the sequences

pre.cluster(fasta=stability.trim.contigs.unique.good.filter.unique.fasta,

count=stability.trim.contigs.unique.good.filter.count_table, diffs=2)

#Interrogate the output file

summary.seqs(fasta=current, count=current)

#Classify sequences

classify.seqs(fasta=stability.trim.contigs.unique.good.filter.unique.precluster.denovo.

vsearch.fasta,

#Count the number of sequences

count=stability.trim.contigs.unique.good.filter.unique.precluster.denovo.vsearch.count_table,reference=trainset16_022016.pds.fasta,taxonomy=trainset16_022016.pds.tax)

#remove non-bacterial lineages

(fasta=stability.trim.contigs.unique.good.filter.unique.precluster.denovo.vsearch.fasta,count=stability.trim.contigs.unique.good.filter.unique.precluster.denovo.vsearch.count_table,taxonomy=stability.trim.contigs.unique.good.filter.unique.precluster.denovo.vsearch.pds.wang.taxonomy, taxon=Chloroplast-Mitochondria-unknown-Archaea-Eukaryota)

#Create taxonomy file

summary.tax(taxonomy=current, count=current)

#Extract sequences from MOCK synthetic sample

get.groups(count=stability.trim.contigs.unique.good.filter.unique.precluster.denovo.vsearch.pick.count_table,
fasta=stability.trim.contigs.unique.good.filter.unique.precluster.denovo.vsearch.pick.fasta, groups=MOCK)

#Assess error rates

seq.error(fasta=stability.trim.contigs.unique.good.filter.unique.precluster.denovo.vsearch.pick.pick.fasta,
count=stability.trim.contigs.unique.good.filter.unique.precluster.denovo.vsearch.pick.pick.count_table, reference=HMP_MOCK.v35.fasta, aligned=F)

#Cluster sequences into OTUs

dist.seqs(fasta=stability.trim.contigs.unique.good.filter.unique.precluster.denovo.vsearch.pick.pick.fasta, cutoff=0.03)

cluster(column=stability.trim.contigs.unique.good.filter.unique.precluster.denovo.vsearch.pick.pick.dist,
count=stability.trim.contigs.unique.good.filter.unique.precluster.denovo.vsearch.pick.pick.count_table)

make.shared(list=stability.trim.contigs.unique.good.filter.unique.precluster.denovo.vsearch.pick.pick.opti_mcc.list,
count=stability.trim.contigs.unique.good.filter.unique.precluster.denovo.vsearch.pick.pick.count_table, label=0.03)

rarefaction.single(shared=stability.trim.contigs.unique.good.filter.unique.precluster.denovo.vsearch.pick.pick.opti_mcc.shared)

#Remove MOCK sequences

remove.groups(count=stability.trim.contigs.unique.good.filter.unique.precluster.denovo.vsearch.pick.count_table,
fasta=stability.trim.contigs.unique.good.filter.unique.precluster.denovo.vsearch.pick.fasta,
taxonomy=stability.trim.contigs.unique.good.filter.unique.precluster.denovo.vsearch.pds.wang.pick.taxonomy, groups=MOCK)

#Rename the output file

rename.file(fasta=current, count=current, taxonomy=current, prefix=final)

#Split sequences into taxonomic bins

cluster.split(fasta=final.fasta, count=final.count_table, taxonomy=final.taxonomy, taxlevel=4, cutoff=0.03)

 # Count sequences in each OTU group

make.shared(list=final.opti_mcc.list, count=final.count_table, label=0.03)

 # Generate consensus taxonomy for each OTU

classify.otu(list=final.opti_mcc.list, count=final.count_table,

taxonomy=final.taxonomy, label=0.03)

#Count number of sequences in each sample

count.groups(shared=final.opti_mcc.shared)

#Subsample number of sequences

sub.sample(shared=final.opti_mcc.shared, size=6068)

#Describe number of OTUs observed as a function of sampling effort

rarefaction.single(shared=final.opti_mcc.shared, calc=sobs, freq=100)

#Generate coverage & alpha diversity

summary.single(shared=final.opti_mcc.shared, calc=coverage-invsimpson-chao,

subsample=6068)

#Generate beta diversity

dist.shared(shared=final.opti_mcc.shared, calc=thetayc-braycurtis-jclass,

subsample=6068)

#Generate PCoA distances

pcoa(phylip=final.opti_mcc.thetayc.0.03.lt.ave.dist)

pcoa(phylip=final.opti_mcc.braycurtis.0.03.lt.ave.dist)

pcoa(phylip=final.opti_mcc.jclass.0.03.lt.ave.dist)

#Generate analysis of molecular variance results

amova(phylip=final.opti_mcc.thetayc.0.03.lt.ave.dist, design=Design.txt)

amova(phylip=final.opti_mcc.braycurtis.0.03.lt.ave.dist, design=Design.txt)

amova(phylip=final.opti_mcc.jclass.0.03.lt.ave.dist, design=Design.txt)

#Generate linear discriminant analysis effect size results

lefse(shared=final.opti_mcc.0.03.subsample.shared, design=Design.txt)

Appendix 3. Sample code for microbiome analyses in R

The full code for Chapter 4 can be found at https://github.com/AdamLeeNottinghamUniversity/Piglets. Sample code for the jejunal microbiome analyses in R v4.1.1 using R Studio 2021.09.0 is shown below. The sample code gives the basics of R coding for microbiome analyses and forms the basis of coding for all studies. Only one example for analyses of jejunal sequences from paper 2, Chapter 4 is presented here since the code for all chapters would be too extensive to include and run into more pages than necessary.

```
#Load packages
set.seed(3711)
library(dplyr)
library(tidyr)
library(stringr)
library(openxlsx)
library(RColorBrewer)
options(scipen=1000)

#Load OTU data
testbiome = read.table("F:/Jejunum/final.opti_mcc.shared", header=TRUE, sep="\t")
row.names(testbiome) = testbiome$Group
testbiome = testbiome[,-which(names(testbiome) %in% c("label", "numOtus",
"Group"))]
testbiome = testbiome[-c(5, 6),]
testbiome = t(testbiome)

#Load taxonomy data
testax = read.table("F:/Jejunum/final.opti_mcc.0.03.cons.taxonomy", header=TRUE,
sep="\t")
testax <- testax %>% mutate(Taxonomy = str_replace_all(Taxonomy,
"\\s*\\([^\\)]+\\)", ""))
testax = separate(testax, Taxonomy, into = c("Kingdom", "Phylum", "Class",
"Order", "Family", "Genus"), sep=";")

#Bind OTU & taxonomy data
testbiome <-cbind(testax, testbiome)

#Remove kit control data
testbiome$count <- rowSums(testbiome =="0")
testbiome = filter(testbiome, count < 8)
testbiome <- testbiome[ -c(17)]

#Sum relative abundance of OTUs and convert to % abundance
testbiome$CONTROL <- rowSums(testbiome[ , c(9,10,11,12)],na.rm=TRUE)
```

```
testbiome$TEST <- rowSums(testbiome[ , c(13,14,15,16)],na.rm=TRUE)
testbiome$TOTAL <- rowSums(testbiome[ ,
c(9,10,11,12,13,14,15,16)],na.rm=TRUE)
colPerc <- function(x){x / sum(x) * 100}
sapply(testbiome[, 9:19], colPerc)
testbiome[, 9:19] <- sapply(testbiome[, 9:19], colPerc)
```

#Create tables of relative abundance of OTUs

```
dir.create("F:/Jejunum/Results")
write.table(testbiome, file = "F:/Jejunum/Results/Relative_Abundance_Jejunum.txt",
sep = "\t", row.names = TRUE, col.names = TRUE)
write.xlsx(testbiome, file =
"F:/Jejunum/Results/Relative_Abundance_Jejunum.xlsx", sheetName = "RA
Jejunum", append = FALSE)
```

#Determine number of unique taxa at phylum level

```
testbiomePhylum <- testbiome %>%
    group_by(Phylum) %>%
    summarise_if(is.numeric, sum)
testbiomePhylum <- testbiomePhylum[ -c(2)]
testbiomePhylum <- testbiomePhylum %>%
    mutate(rank_order = min_rank(-TOTAL)) %>%
    arrange(rank_order)
write.table(testbiomePhylum, file = "F:/Jejunum/Results/Phylum_Jejunum.txt", sep =
"\t", row.names = TRUE, col.names = TRUE)
write.xlsx(testbiomePhylum, file = "F:/Jejunum/Results/Phylum_Jejunum.xlsx",
sheetName = "Phylum Jejunum", append = FALSE)
```

#Determine number of unique taxa at genus level

```
testbiomeGenus <- testbiome %>%
    group_by(Genus) %>%
    summarise_if(is.numeric, sum)
testbiomeGenus <- testbiomeGenus[ -c(2)]
testbiomeGenus <- testbiomeGenus %>%
    mutate(rank_order = min_rank(-TOTAL)) %>%
    arrange(rank_order)
write.table(testbiomeGenus, file = "F:/Jejunum/Results/Genus_Jejunum.txt", sep =
"\t", row.names = TRUE, col.names = TRUE)
write.xlsx(testbiomeGenus, file = "F:/Jejunum/Results/Genus_Jejunum.xlsx",
sheetName = "Genus Jejunum", append = FALSE)
```

#Determine total number of unique taxa at phylum level

```
TotalPhylum <- select(testbiomePhylum, Phylum, CONTROL, TEST, TOTAL)
write.table(TotalPhylum, file = "F:/Jejunum/Results/Total_Phylum_Jejunum.txt",
sep = "\t", row.names = TRUE, col.names = TRUE)
write.xlsx(TotalPhylum, file = "F:/Jejunum/Results/Total_Phylum_Jejunum.xlsx",
sheetName = "Total Phylum Jejunum", append = FALSE)
```

#Determine total number of unique taxa at genus level

```
TotalGenus <- select(testbiomeGenus, Genus, CONTROL, TEST, TOTAL)
```

```
write.table(TotalGenus, file = "F:/Jejunum/Results/Total_Genus_Jejunum.txt", sep =
"\t", row.names = TRUE, col.names = TRUE)
write.xlsx(TotalGenus, file = "F:/Jejunum/Results/Total_Genus_Jejunum.xlsx",
sheetName = "Total Genus Jejunum", append = FALSE)
```

#Select top ten taxa at phylum level

```
TotalPhylumGraph <- select(testbiomePhylum, Phylum, CONTROL, TEST)
OtherPhylum <- TotalPhylumGraph[-c(1:10), ]
OtherPhylum <- OtherPhylum %>%
  bind_rows(summarise(.,
                      across(where(is.numeric), sum),
                      across(where(is.character), ~"Others")))
OtherPhylum <- OtherPhylum %>% slice_tail(n = 1)
TotalPhylumGraph <- TotalPhylumGraph %>% slice(1:10)
TotalPhylumGraph <- rbind(TotalPhylumGraph,OtherPhylum[1,])
write.table(TotalPhylumGraph, file =
"F:/Jejunum/Results/Total_Phylum_Graph.txt", sep = "\t", row.names = TRUE,
col.names = TRUE)
write.xlsx(TotalPhylumGraph, file =
"F:/Jejunum/Results/Total_Phylum_Graph.xlsx", sheetName = "Total Phylum
Jejunum", append = FALSE)
```

#Select top ten taxa at genus level

```
TotalGenusGraph <- select(testbiomeGenus, Genus, CONTROL, TEST)
OtherGenus <- TotalGenusGraph[-c(1:10), ]
OtherGenus <- OtherGenus %>%
  bind_rows(summarise(.,
                      across(where(is.numeric), sum),
                      across(where(is.character), ~"Others")))
OtherGenus <- OtherGenus %>% slice_tail(n = 1)
TotalGenusGraph <- TotalGenusGraph %>% slice(1:10)
TotalGenusGraph <- rbind(TotalGenusGraph,OtherGenus[1,])
write.table(TotalGenusGraph, file = "F:/Jejunum/Results/Total_Genus_Graph.txt",
sep = "\t", row.names = TRUE, col.names = TRUE)
write.xlsx(TotalGenusGraph, file = "F:/Jejunum/Results/Total_Genus_Graph.xlsx",
sheetName = "Total Genus Jejunum", append = FALSE)
```

#Prepare phylum data for graphing

```
TotalPhylumGraph <- as.data.frame(TotalPhylumGraph)
row.names(TotalPhylumGraph) = TotalPhylumGraph$Phylum
TotalPhylumGraph$Phylum <- NULL
TotalPhylumGraph <- as.matrix(TotalPhylumGraph)
row.names(TotalPhylumGraph)[6] <-  "Bacteria UC*"
row.names(TotalPhylumGraph)[9] <-  "Candidatus Saccaribacteria"
TotalPhylumGraph[1, ] <- TotalPhylumGraph[1, ] - 70
```

#Prepare genus data for graphing

```
TotalGenusGraph <- as.data.frame(TotalGenusGraph)
row.names(TotalGenusGraph) = TotalGenusGraph$Genus
TotalGenusGraph$Genus <- NULL
```

```
TotalGenusGraph <- as.matrix(TotalGenusGraph)
row.names(TotalGenusGraph)[5] <-  "Pasteurellaceae UC*"
TotalGenusGraph[1, ] <- TotalGenusGraph[1, ] - 10
```

#Create bargraphs and export to Tiff File

```
tiff('F:/Jejunum/Results/Relative Abundance.tiff', pointsize=8, width=2100,
height=2000, res=300)
par(mfrow = c(1, 2))
par(mar = c(4,4,4,2) + 0.15)
barplot(TotalPhylumGraph,
        width = c(1.5,1.5),
        names.arg = c("CONTROL", "TEST"),
        ylab = "Relative Abundance %",
        ylim = c(0, 30),
           xlim = c(0, 6),
        font.lab = 2,
        font = 2,
        col = brewer.pal(n = 11, name = "Set3"),
        axes = F,
        cex.lab = 1,
        cex.axis = 1,
        cex.names = 1,
        legend.text = rownames(TotalPhylumGraph),
        args.legend=list(x = "topright", inset = c(-0.2, 0.3), cex=0.9, text.font = 2, bty =
"n"))
axis(2, at = seq(0, 30, by = 10), labels = seq(70, 100, by = 10), cex.axis = 1, font = 2)
mtext("Jejunum", side=3, adj=0, line=2, cex=1.1, font=2, font.lab = 2)
mtext("Phyla", side=3, adj=0, line=0.8, cex=1, font=2, font.lab = 2)
barplot(TotalGenusGraph,
        width = c(1.5,1.5),
        names.arg = c("CONTROL", "TEST"),
        ylab = "Relative Abundance %",
        ylim = c(0, 90),
           xlim = c(0, 6),
        font.lab = 2,
        font = 2,
        col = brewer.pal(n = 11, name = "Set3"),
        axes = F,
        cex.lab = 1,
        cex.axis = 1,
        cex.names = 1,
        legend.text = rownames(TotalGenusGraph),
        args.legend=list(x = "topright", inset = c(-0.08, 0.3), cex=0.9, text.font = 2, bty
= "n"))
axis(2, at = seq(0, 90, by = 10), labels = seq(10, 100, by = 10), cex.axis = 1, font = 2)
mtext("Genus", side=3, adj=0, line=0.8, cex=1, font=2, font.lab = 2)
dev.off()
```

#Analyse alpha diversity data

```
library(matrixStats)
AlphaDiversityJejunum = read.table("F:/Jejunum/final.opti_mcc.groups.ave-std.summary", header=TRUE, sep="\t")
AlphaDiversityJejunum = AlphaDiversityJejunum[,-which(names(AlphaDiversityJejunum) %in% c("label", "method", "invsimpson_lci", "invsimpson_hci", "chao_lci", "chao_hci"))]
AlphaDiversityJejunum = AlphaDiversityJejunum[-c(9:16),]
row.names(AlphaDiversityJejunum) = AlphaDiversityJejunum$group
AlphaDiversityJejunum$group<- NULL
AlphaDiversityJejunum = t(AlphaDiversityJejunum)
AlphaDiversityJejunum <- as.data.frame(AlphaDiversityJejunum)
AlphaDiversityJejunum$CONTROLMean <- rowMeans(AlphaDiversityJejunum[ , c(1,2,3,4)],na.rm=TRUE)
AlphaDiversityJejunum$CONTROLsd <- rowSds(as.matrix(AlphaDiversityJejunum[,c(1,2,3,4)]))
AlphaDiversityJejunum$TESTMean <- rowMeans(AlphaDiversityJejunum[ , c(5,6,7,8)],na.rm=TRUE)
AlphaDiversityJejunum$TESTsd <- rowSds(as.matrix(AlphaDiversityJejunum[,c(5,6,7,8)]))
AlphaDiversityJejunum = t(AlphaDiversityJejunum)
AlphaDiversityJejunum <- as.data.frame(AlphaDiversityJejunum)
write.table(AlphaDiversityJejunum, file = "F:/Jejunum/Results/Alpha_Diversity_Jejunum.txt", sep = "\t", row.names = TRUE, col.names = TRUE)
write.xlsx(AlphaDiversityJejunum, file = "F:/Jejunum/Results/Alpha_Diversity_Jejunum.xlsx", sheetName = "Alpha Diversity", row.names = TRUE)
```

#Format alpha diversity data for Shapiro-Wilk tests

```
SWAlphaDiversityJejunum = read.table("F:/Jejunum/final.opti_mcc.groups.ave-std.summary", header=TRUE, sep="\t")
SWAlphaDiversityJejunum = SWAlphaDiversityJejunum[,-which(names(SWAlphaDiversityJejunum) %in% c("label", "method", "invsimpson_lci", "invsimpson_hci", "chao_lci", "chao_hci"))]
SWAlphaDiversityJejunum = SWAlphaDiversityJejunum[-c(9:16),]
OtherSWAlphaDiversityJejunum <- SWAlphaDiversityJejunum[-c(1:4), ]
SWAlphaDiversityJejunum = SWAlphaDiversityJejunum[-c(5:8),]
SWAlphaDiversityJejunum <- cbind(SWAlphaDiversityJejunum, OtherSWAlphaDiversityJejunum)
colnames(SWAlphaDiversityJejunum) <- c("Control", "CovCon", "ISCon", "ChaoCon", "TEST", "CovTEST", "ISTEST", "ChaoTEST")
SWAlphaDiversityJejunum = SWAlphaDiversityJejunum[,-which(names(SWAlphaDiversityJejunum) %in% c("Control", "TEST"))]
apply(SWAlphaDiversityJejunum,2,shapiro.test)
```

#Analyse beta diversity data

#Theta YC Dissimilarity

```
TYCJejunum = read.table("F:/Jejunum/final.opti_mcc.thetayc.0.03.lt.ave.pcoa.axes", header=TRUE, sep="\t")
```

```
TYCJejunum = TYCJejunum[,-which(names(TYCJejunum) %in% c("axis3",
"axis4", "axis5", "axis6", "axis7", "axis8"))]
TYCJejunum$Group <-  c("CONTROL", "CONTROL", "CONTROL",
"CONTROL", "TEST", "TEST", "TEST", "TEST")
TYCJejunum <- TYCJejunum[ -c(1)]
my_palette3 <- c("#7570B3", "#E7298A")
```

#Create PCoA plots for Theta YC Dissimilarity

```
tiff('F:/Jejunum/Results/Beta Diversity.tiff', pointsize=10, width=2400, height=900,
res=300)
par(mfrow=c(1,3))
plot(TYCJejunum$axis1, TYCJejunum$axis2,
     col = my_palette3[as.factor(TYCJejunum$Group)],
     xlim = c(-0.6, 0.6),
     ylim = c(-0.6, 0.6),
     cex = 3,
     cex.lab = 1,
     cex.axis = 1,
     pch = 20,
     xlab = "PC1 (43.10 %)",
     ylab = "PC2  (35.80 %)",
     font.lab = 2,
     font = 2,
     text(x = 0.5, y = 0.5, label = substitute(paste(italic("P"), " = 0.053")), font = 2,
col = "black"))
legend("topleft", c("Control", "Test"), bty = "n", cex = 1, pch = 19, col =
my_palette3)
mtext("Yue & Clayton Dissimilarity", side=3, adj=0, line=0.6, cex=0.8, font=2)
```

#Create PCoA plots for Bray-Curtis Dissimilarity

```
BCJejunum =
read.table("F:/Jejunum/final.opti_mcc.braycurtis.0.03.lt.ave.pcoa.axes",
header=TRUE, sep="\t")
BCJejunum = BCJejunum[,-which(names(BCJejunum) %in% c("axis3", "axis4",
"axis5", "axis6", "axis7", "axis8"))]
BCJejunum$Group <-  c("CONTROL", "CONTROL", "CONTROL", "CONTROL",
"TEST", "TEST", "TEST", "TEST")
BCJejunum <- BCJejunum[ -c(1)]
my_palette3 <- c("#7570B3", "#E7298A")
plot(BCJejunum$axis1, BCJejunum$axis2,
     col = my_palette3[as.factor(BCJejunum$Group)],
     xlim = c(-0.6, 0.6),
     ylim = c(-0.6, 0.6),
     cex = 3,
     cex.lab = 1,
     cex.axis = 1,
     pch = 20,
     xlab = "PC1 (44.66 %)",
     ylab = "PC2  (27.72 %)",
     font.lab = 2,
```

```
    font = 2,
text(x = 0.5, y = 0.5, label = substitute(paste(italic("P"), " = 0.034")), font = 2, col =
"black"))
legend("topleft", c("Control", "Test"), bty = "n", cex = 1, pch = 19, col =
my_palette3)
mtext("Bray Curtis Dissimilarity", side=3, adj=0, line=0.6, cex=0.8, font=2)
```

#**Create PCoA plots for Jaccard Similarity**

```
JSJejunum = read.table("F:/Jejunum/final.opti_mcc.jclass.0.03.lt.ave.pcoa.axes",
header=TRUE, sep="\t")
JSJejunum = JSJejunum[,-which(names(JSJejunum) %in% c("axis3", "axis4",
"axis5", "axis6", "axis7", "axis8"))]
JSJejunum$Group <-  c("CONTROL", "CONTROL", "CONTROL", "CONTROL",
"TEST", "TEST", "TEST", "TEST")
JSJejunum <- JSJejunum[ -c(1)]
my_palette3 <- c("#7570B3", "#E7298A")
plot(JSJejunum$axis1, JSJejunum$axis2,
    col = my_palette3[as.factor(JSJejunum$Group)],
    xlim = c(-0.6, 0.6),
    ylim = c(-0.6, 0.6),
    cex = 3,
    cex.lab = 1,
    cex.axis = 1,
    pch = 20,
    xlab = "PC1 (32.66 %)",
    ylab = "PC2  (14.72 %)",
    font.lab = 2,
    font = 2,
    text(x = 0.5, y = 0.5, label = substitute(paste(italic("P"), " = 0.034")), font = 2,
col = "black"))
legend("topleft", c("Control", "Test"), bty = "n", cex = 1, pch = 19, col =
my_palette3)
mtext("Jaccard Similarity", side=3, adj=0, line=0.6, cex=0.8, font=2)
dev.off()
```

#Linear Discriminant Analysis Effect Size (LEfSe)

```
LEFSE =
read.table("F:/Jejunum/final.opti_mcc.0.03.subsample.0.03.lefse_summary",
header=TRUE, sep="\t")
LEFSE$logMaxMean <- NULL
RelativeAbundance =
read.table("F:/Jejunum/Results/Relative_Abundance_Jejunum.txt", header=TRUE,
sep="\t")
RelativeAbundance = RelativeAbundance <- select(RelativeAbundance, Genus,
CONTROL, TEST,TOTAL)
RelativeAbundance <- RelativeAbundance[RelativeAbundance$TOTAL > 0.1, ]
nrow <- nrow(RelativeAbundance)
LEFSE <- LEFSE %>% slice(1:nrow)
LEFSE <-cbind(LEFSE, RelativeAbundance)
LEFSE <- na.omit(LEFSE)
```

```
write.table(LEFSE, file = "F:/Jejunum/Results/LEFSE.txt", sep = "\t", row.names =
TRUE, col.names = TRUE)
write.xlsx(LEFSE, file = "F:/Jejunum/Results/LEFSE.xlsx", sheetName = "LEFSE",
append = FALSE)
LEFSE$pValue <- round(LEFSE$pValue, digits = 3)
LEFSE$significance <- ifelse(LEFSE$pValue < "0.001", "****",
                  ifelse(LEFSE$pValue < "0.01", "***",
                  ifelse(LEFSE$pValue < "0.025", "**",
                  ifelse(LEFSE$pValue <= "0.05", "*"))))
LEFSE$Names = paste(LEFSE$OTU, LEFSE$Genus, LEFSE$significance)
LEFSE$LDA = ifelse(LEFSE$Class == "CONTROL", LEFSE$LDA*-
1,LEFSE$LDA*1)
LEFSE <- arrange(LEFSE, desc(LDA))
```

#Create LEFSE graph

```
LEFSEGraph <- select(LEFSE, Names, LDA, Class)
my_palette3 <- c("#7570B3", "#E7298A")
LEFSEGraph$Names [9] <- "Otu057 Clostridiales UC*"
LEFSEGraph$Names [18] <- "Otu029 Enterobacteriaceae UC*"
tiff('F:/Jejunum/Results/LEFSE.tiff', pointsize=10, width=2400, height=2400,
res=300)
par(mar = c(4, 16, 4, 0.5))
barplot(LEFSEGraph$LDA,
        horiz = TRUE,
        col = my_palette3[as.factor(LEFSEGraph$Class)],
        xlim = c(-5, 5),
        xlab ="LDA Score log 10",
        font.lab = 2,
        font = 2,
        names.arg = LEFSEGraph$Names,
           las = 1,
        cex.names = 0.9,
        cex.lab = 1.0)
axis(1, at = seq(-5, 5, by = 1), labels = seq(-5, 5, by = 1), cex.axis = 1, font = 2)
legend("topright", c("Control", "Test"), bty = "n", cex = 1.2, pch = 19, col =
my_palette3)
mtext("Linear Discriminant Analysis Effect Size", side=3, adj=0, line=0.6, cex=1.2,
font=2)
text(3.9, 20, label = substitute(paste(italic("*P"), " = 0.05")), font = 2, col = "black")
text(3.9, 18.5, label = substitute(paste(italic("**P"), " = 0.025")), font = 2, col =
"black")
text(3.8, 17, label = substitute(paste(italic("***P"), " = 0.01")), font = 2, col =
"black")
text(3.8, 15.5, label = substitute(paste(italic("****P"), " = 0.001")), font = 2, col =
"black")
abline (v = 0)
dev.off()
```

#Export LEFSE data

```
LEFSEGraph$Class <- NULL
write.table(LEFSEGraph, file = "F:/Jejunum/Results/LEFSE_Graph.txt", sep = "\t",
row.names = TRUE, col.names = TRUE)
write.xlsx(LEFSEGraph, file = "F:/Jejunum/Results/LEFSE_Graph.xlsx",
sheetName = "LEFSE Graph", append = FALSE)
```

Appendix 4. Composition of buffers and solutions

Macherey-Nagel NucleoSpin Tissue Genomic DNA Purification Kit	
Wash buffer B5	80% ethanol, 20 mM NaCl, 2 mM Tris-HCl
Proteinase buffer	30 mM Tris-HCl
Elution buffer BE	5 mM Tris-HCL
Buffer TE	10 mM Tris-HCl, 1 mM EDTA
Lysis buffer T1	Composition not available
Buffer B3	50% v/v guanidine HCL, 50% v/v H_2O
Wash buffer BW	50% v/v guanidine HCL, 50% v/v isopropanol
Wash buffer B5	80% ethanol v/v, 20 mM NaCl, 2 mM Tris-HCl
Biomedicals FastDNA SPIN Kit for Feces	
Wash buffer 2	Composition not available
Pre-lysis solution	Composition not available
Sodium phosphate buffer	5% sodium phosphate w/v, 95% H_2O v/v
Buffer MT	1% sodium dodecyl sulphate v/v, 99% v/v H_2O
Protein precipitate solution PPS	5% acetic acid v/v, 95% v/v H_2O
Binding matrix solution	87.8% silicon dioxide v/v, 12.2% H_2O v/v
Wash buffer 1	Guanidine thiocyanate 30% v/v, 70% H_2O v/v
Tris EDTA solution TES	Composition not available
QIAmp 96 PowerFecal QIA cube HT Kit	
Buffer AW1	Guanidine HCL 50% v/v, 50% H_2O
Buffer AW2	70% ethanol v/v, 30% H_2O v/v
Buffer PW1	Guanidine thiocyanate 2.5% v/v, 97.5% H_2O
Buffer C3	Composition not available
Proteinase K	20 mg/mL in 30 mM Tris-HCL
Solution C4	50% v/v guanidine HCL, 50% v/v isopropanol
Top elute fluid	100% white mineral oil
Buffer ATE	10 mM Tris-HCl, 0.1 mM EDTA, 0.04% v/v sodium azide
QIAmp 96 Virus QIA cube HT Kit	
Buffer ACB	Guanidine thiocyanate 50% v/v, 50% H_2O v/v
Buffer AW1	Guanidine HCL 50% v/v, 50% H_2O v/v
Buffer AW2	70% ethanol v/v, 30% H_2O v/v
Buffer AVE	0.04% sodium azide v/v, 99.96% H_2O v/v
Buffer RDD	Composition not available
Buffer ACL	Guanidine thiocyanate 30% v/v, 70% H_2O v/v
Carrier RNA solution	1550 µL buffer AVE, 310 µg lyophilized carrier RNA
DNase I stock solution	RNase free H_2O 550 µL in 1 vial lyophilized RNA
Illumina MiSeq: Library generation, quality control and DNA sequencing	
Accuprime Pfx Supermix	22U/mL Thermococcus species KOD (Pyrococcus kodakaraensis) thermostable polymerase complexed with anti-KOD antibodies, 66mM Tris-SO_4, 30.8mM $(NH_4)2SO_4$, 11mM KCl, 1.1mM $MgSO_4$, 330µM dNTPs
Tris acetate solution TAE	Tris 11.31% v/v, EDTA disodium salt 0.87% v/v, acetic acid sodium salt 7.66% v/v, HCl 3.74% v/v
Ethidium bromide	10 mg/mL in H_2O
SequalPrep binding buffer	HCl 10% v/v, 90% H_2O v/v

SequalPrep wash buffer	Composition not available
SequalPrep elution buffer	10 mM Tris-HCl, pH 8.5
Agilent Tape Station sample buffer	20mM KCl, 60mM PO_4 buffer, 60mM guanidine-HCl, 240mM NaCl, 60mM NaOAc
Qubit HS reagent	Composition not available
Qubit HS buffer	Composition not available
Illumina HT1 buffer	10 mM Tris-HCl, 0.1% v/v Tween 20
0.2N NaOH	200 µL 1N NaOH to 800 µL PCR grade water
Techne qPCR kit for rotavirus A	
qPCR Master Mix	Composition not available
Template preparation buffer	Composition not available
ELISA for rotavirus IgG and IgA in sow colostrum	
All solutions	Composition not available
Miscellaneous solutions	
Virkon disinfectant Chapter 4	1% w/w solid Virkon, 99% H_2O v/v, 10 g in 1 L H_2O, dry product potassium peroxymonosulfate 21.45% w/w, sodium dodecylbenzenesulfonate w/w, sulfamic acid 77.05% w/w, sodium chloride 1.5% w/w
Neutral buffered formalin Chapter 4	100 mL Formaldehyde (37-40% stock solution), 900ml H_2O, 4g/L NaH_2PO_4 (monobasic), 6.5g/L Na_2HPO_4 (dibasic/anhydrous)
Faramate Milk Replacer Feed for Piglets (10 kg)	
Crude protein (whey)	22% w/w
Crude fibre	0% w/w
Crude oils & fats	14% w/w
Crude ash	7.5% w/w
Lysine	2% w/w
Calcium	0.9% w/w
Sodium	0.5% w/w
Phosphorous	0.7% w/w
Buylated hydroxytoluene (antioxidant)	150 mg
Citric acid (preservative)	1000 mg
Vitamin A (alphatocopherol acetate)	25,000 iu
Vitamin D3	10,000 iu
Vitamin E	500 iu
Vitamin K	3 mg
Vitamin B1	5 mg
Vitamin B2	3 mg
Vitamin B6	3 mg
Vitamin B12	60 µg
Vitamin C	100 mg
Nicotinic acid	20 mg
Biotin	50 µg

Pantothenic acid	10 mg
Copper (copper sulphate pentahydrate)	10 mg
Iodine (potassium iodide)	0.25 mg
Iron (ferrous sulphate monohydrate)	100 mg
Manganese (manganese sulphate monohydrate)	40 mg
Selenium (sodium selenite)	0.4 mg
Zinc (sulphate monohydrate)	50 mg