Development and Manipulation of the Piglet Intestinal Microbiota

Tanya Louise Nowland

BSc. Honours (The University of Adelaide)

A thesis by publication submitted to The University of Adelaide in fulfilment of the requirements for the degree of Doctor of Philosophy (PhD)

THE UNIVERSITY of ADELAIDE

School of Animal and Veterinary Sciences

Roseworthy Campus

The University of Adelaide

March 2021

Table of Contents

List of tables

List of figures

Chapter 2: Faecal microbiota transplantation: is it the future for pig production?

Chapter 3: Characterisation of early microbial colonisers within the spiral colon of pre- and post-natal piglets

Chapter 5: Maternal supplementation with phytogenic additives influenced the faecal microbiota and reproductive potential in sows

Chapter 6: Faecal microbiota analysis of piglets during lactation

Chapter 7: A single faecal microbiota transplantation altered the microbiota of weaned pigs

Chapter 8: Discussion

List of publications included in this thesis

Chapter 1

Nowland TL, Plush KJ, Barton M and Kirkwood RN 2019. Development and function of the intestinal microbiome and potential implications for pig production. Animals 9, 76. Doi: 10.3390/ani9030076.

Chapter 2

Nowland TL and Kirkwood RN 2020. Faecal microbiota transplantation: is it the future for pig production? Microbiology Australia 41, 91-94. Doi: 10.1071/MA20023.

Chapter 3

Nowland TL, Kirkwood RN, Torok VA, Plush KJ and Barton MD 2021. Characterisation of early microbial colonisers within the spiral colon of pre- and post-natal piglets. Life 11, 312. Doi: 10.3390/life11040312.

Chapter 4

Nowland TL, Kirkwood RN, Plush KJ, Barton MD and Torok VA 2021. Exposure to maternal faeces in lactation influences piglet enteric microbiota, growth and survival pre-weaning. Journal of Animal Science. Doi: 10.1093/jas/skab170.

Chapter 6

Nowland TL, Torok VA, Low WY, Barton MD, Plush KJ and Kirkwood RN 2020. Faecal microbiota analysis of piglets during lactation. Animals 10, 762. Doi: 0.3390/ani10050762.

Chapter 7

Nowland TL, Torok VA, Low WY, Plush KJ, Barton MD and Kirkwood RN 2020. A single faecal microbiota transplantation altered the microbiota of weaned pigs. Life 10, 203. Doi: 10.3390/life10090203.

Awards and publications written during candidature

Nowland TL, van Wettere WHEJ and Plush KJ 2019. Allowing sows to farrow unconfined has positive implications for sow and piglet welfare. Applied Animal Behaviour Science 221, 1-9. Doi: 10.1016/j.applanim.2019.104872.

Nowland TL, Kind K, Hebart ML and van Wettere WHEJ 2019. Caffeine supplementation at birth, but not 8 to 12 h post-birth, increased 24 h pre-weaning mortality in piglets. Animal 14, 1529-1535. Doi: 10.1017/S175173111900301X.

Plush KJ, McKenny LA, **Nowland TL** and van Wettere WHEJ 2021. The effect of hessian and straw as nesting materials on sow behaviour and piglet survival and growth to weaning. Animal 15, 1-8. Doi: 10.1016/j.animal.2021.100273.

2018 – **Science and Innovation Award for Young People in Agriculture, Fisheries and Forestry:** Recognises innovative scientific projects that will contribute to the ongoing success and sustainability of Australia's agriculture industries.

2019 – **BIOMIN Young B.R.A.I.N. Award:** Supports young and highly motivated scientists.

Conference abstracts written during candidature

Postgraduate Conference 2018

Nowland TL, Low WY, Torok VA, Barton MD, Plush KJ and Kirkwood RN 2018. The effect of antibiotics on gut microbiota during lactation in pigs.

- Presented outcomes as a poster

Australasian Pig Science Association 2019

Nowland TL, Low WY, Torok VA, Barton MD, Plush KJ and Kirkwood RN 2019. Faecal microbiota transplantation during lactation had no effect on piglets to weaning. Advances in Animal Biosciences 10, s28.

- Presented outcomes as an oral presentation

McKenny LA, Plush KJ, **Nowland TL** and van Wettere WHEJ 2019. Hessian stimulates nest building behaviour in sows and the provision of straw improves traits important for piglet survival. Advances in Animal Biosciences 10, s94.

- Presented outcomes as a poster

McKenny LA, Plush KJ, **Nowland TL** and van Wettere WHEJ 2019. The provision of straw within a farrowing crate improves piglet survival. Advances in Animal Biosciences 10, s95.

- Presented outcomes as a poster

Terry R, **Nowland TL** and Plush KJ 2019. Use of synthetic olfactory agonist in the farrowing house. Advances in Animal Biosciences 10, s97.

- Presented outcomes as a poster

Asia Nutrition Forum 2019

Nowland TL, Torok VA, Barton MD, Plush KJ and Kirkwood RN 2019. Piglet management and the development of the intestinal microbiota.

- Invited speaker

Abstract

Piglet pre-weaning mortality is a major industry issue and one area that has not been explored is the role of the gastrointestinal tract (GIT) microbiota. The first microbial colonisers are the greatest determinants of health. However, little is known about the initial colonisers and the degree to which external factors influence GIT microbiota development within the piglet. Research reported in this thesis outlines GIT microbiota development and evaluates different methods for influencing the GIT microbiota and its effect on piglet growth and survival. Chapter 1.2 and 2 provide literature reviews in pigs, outlining the gap in knowledge surrounding the pre-weaning period and the use of faecal microbiota transplantation (FMT). Chapter 3 identified the presence of bacteria within the spiral colon of stillborn piglets demonstrating colonisation prior to birth and outlined the first colonisers in piglets that had or had not sucked. This study provides evidence surrounding the importance of the immediate post-natal environment. Chapter 4 focussed on the impact of pen environment and sow parity on microbial colonisation. Sow parity was assessed due to the differences between multiparous and primiparous sow progeny. This experiment found that the removal of faeces from the pen for the first 10 days of life increased piglet growth and survival to weaning, while the addition of multiparous sow faeces to a primiparous sow pen had no effect on the piglets. Chapter 5 assessed the effect of feeding a phytogenic additive (PA) to sows and found that the PA was successful in altering the microbiota of sows and this change influenced the piglets reared and persisted to at least two weeks post weaning. Sows fed PAs throughout gestation gave birth to more piglet's; however, this did not translate to more piglets born alive. Additionally, these sows tended to have a reduced wean to service interval. Currently no therapeutic alternatives to antibiotics exist for piglets, therefore, the evaluation of an alternative was assessed in Chapters 6 and 7. FMT has demonstrated success in the treatment of *Clostridium difficile* infections in humans, however there is limited research assessing its use in pigs in an industry appropriate manner. Chapter 6 was a proof-of-concept study that evaluated the use of a single FMT in 7-day-old antibiotic treated piglets. FMT at a young age was ineffective, however, requirements for optimisation in young pigs were gained and the key bacterial communities associated with age throughout lactation were identified. Chapter 7 evaluated the use of FMT post weaning and is the first study to demonstrate that a single FMT dose can elicit a change within

piglets. Collectively, the findings of this thesis present a comprehensive evaluation of microbial colonisation within the piglet prior to weaning, methods for altering piglet microbial colonisation via the sow and environment, and information surrounding the potential application of FMT on farm. This research has enabled the identification of possible industry applicable practices and interventions to enable optimal microbial colonisation within sows and piglets for the improvement of pig health and productivity.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

I acknowledge that copyright of published works contained within this thesis resides with the copyright holder(s) of those works. I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library Search and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

I acknowledge the support I have received for my research through the provision of an Australian Government Research Training Program Scholarship.

Signed: _

Tanya Nowland

Date: 31/03/2021

Acknowledgments

My deepest gratitude goes to my primary supervisor Associate Professor Roy Kirkwood, for his guidance and encouragement throughout my candidature. You have greatly contributed to my development as a researcher and your constructive criticism and energizing conversations will always be appreciated.

I would like to thank all of my co-supervisor's Dr Kate Plush, Dr Valeria Torok and Professor Mary Barton, for all of their help and encouragement throughout my candidature. Dr Kate Plush, thank you for being available whenever I needed and for always taking the time out of your busy schedule to meet up, I cherish our friendship. Dr Valeria Torok for providing me with an excellent education in microbiota analyses and interpretation. I will always be grateful of your support and positivity during my steep learning curve. Professor Mary Barton for your words and guidance during my candidature and Dr Wai Yee Low for answering my many questions and guiding me through the difficulties of code and bioinformatics.

This thesis could not have been completed without the support and contribution from Australian Pork Limited, BIOMIN and the University of Adelaide. Thank you to Australian Pork Limited for funding, as well as the networking opportunities provided throughout my candidature. Thank you, Darryl Meaney and Neil Gannon, from BIOMIN, you not only funded a project but went over and above in guiding and supporting me throughout my studies. Darryl Meaney, a special thank you for always being generous with your time and advice. Thank you to Sophia Ward and Bryony Tucker for taking time out of your own studies to assist me with my trial work. Thank you to the whole team at SARDI and the Roseworthy Piggery for your continued support throughout the many trials, particularly, Jessica Zemitis, Serena Barnes, Lisa McKenny, Simon Go and Carina Go.

I would also like to acknowledge the encouraging and constructive feedback obtained from editors and anonymous reviewers of the journal articles produced during this study; your inputs have greatly improved the quality of my work.

Finally, a special thank you to my friends and family, especially, Mum, Dad, Jason, Sherronna, Matt, Derek, Imogen, Kaylee, Pip, Ben, Nick, Tom and Aaron for their continued support, advice and love. Thank you to my sister Samantha Nowland for our daily chats and for always making sure I was on track; you are an incredible role model and sister. A huge thank you to my husband, Justin Ayris, for your continual encouragement and support throughout my entire studies, I would not have been able to do it without you.

My sincere gratitude to you all,

Tanya

Chapter 1.1: Introduction

General background

A microbiome is defined as all microorganisms, including their genomes and extra-chromosomal elements, present in and on the host (Dominguez-Bello *et al.*, 2019). These microorganisms include bacteria, archaea, fungi and viruses (Grice and Segre, 2011, Abeles and Pride, 2014, Hallen-Adams and Suhr, 2017). The initial microbiome is the result of an interaction of multiple factors including host genetics, the mode of delivery of the offspring (vaginal vs caesarean), feeding type (maternal vs formula), birth and living environment, maternal weight gain, stress and pre- and peri-natal antibiotic use (Arrieta *et al.,* 2015, Murphy *et al.*, 2015, Gomez-Gallego *et al.*, 2016). Microbiomes colonise all body surfaces and the communities they form are involved in host metabolism and gastrointestinal tract (GIT), immune and neural systems development (Arrieta *et al.*, 2014, Gensollen *et al.*, 2016). It is widely recognized that the microbiome is involved in a myriad of functions involving the production of antimicrobial compounds, nutrient metabolism, degradation of xenobiotics including hormones, as well as the competitive exclusion of potential pathogens (Bauer *et al.*, 2006, Rea MC, 2010, Schachtschneider *et al.*, 2013, Le Doare *et al.*, 2018). Research surrounding the GIT microbiota specifically has received a large amount of attention in recent years, with studies in humans demonstrating links with GIT microbiota dysbiosis and irritable bowel disease, necrotizing enterocolitis, eczema, obesity, autoimmunity, asthma and even autism (Kuitunen *et al.*, 2009, Cahenzli *et al.*, 2013, Wang *et al.*, 2016, Tang *et al.*, 2017, Carlson *et al.*, 2018). While studies in production animals are less common, research within the pork industry has expanded recently with studies demonstrating that the GIT microbiota is associated with the immune system (Bauer *et al.*, 2006), behaviour (Rabhi *et al.*, 2020), growth and feed efficiency (Mach *et al.*, 2015, Gaukroger *et al.*, 2020a).

Piglet pre-weaning mortality is a major industry issue with approximately 15% of all piglets born alive dying prior to weaning annually (Mota-Rojas *et al.*, 2012, Daigle, 2018, Nuntapaitoon *et al.*, 2018). As a result of this, much research investigating ways of reducing this mortality has been conducted, however, the issue persists. Additionally, as the industry continues to push towards greater sow prolificacy, piglet survival becomes an even larger issue, and so novel research investigating ways for improving piglet survival is warranted. One area that has not been extensively explored is the role the GIT microbiota has

in piglet health and survival. To date, research into the pig GIT microbiota is limited, with the majority of research assessing the microbiota of piglet's post weaning. Although this research has been beneficial, previous studies have demonstrated the importance of optimal colonisation during early life as disruptions during this time can have negative health consequences (Bauer *et al.*, 2006, Patil *et al.*, 2020). Although interest in this area has increased in recent times, research outlining the basics surrounding GIT microbiota colonisation and development, and potential management strategies for ensuring optimal colonisation in piglets for the improvement of health and survival is limited.

This thesis tested the unifying hypothesis that microbiota acquisition and development within the piglet occurs in late gestation and during the immediate post-natal period, allowing for the piglet's GIT microbiota to be influenced via the addition of a treatment either through the environment or the sow. This was explored through the investigation of microbial colonisation of piglets at different life stages, through the use of differing environments, sow dietary changes and through direct manipulation of the piglets GIT microbiota. Further information surrounding what was known in regard to microbial colonisation and development within the piglet at the beginning of my candidature is represented in a literature review in Chapter 1.2.

Thesis format

Chapter 1 covers the basic background and a general introduction into the topic of development and manipulation of the piglet intestinal microbiota and outlines the thesis format and overall aims of the thesis. It also includes a published literature review entitled "Development and function of the intestinal microbiome and potential implications for pig production" which discusses the development of the microbiota within piglets and also identifies key gaps within the literature that are then investigated within the experimental chapters.

Chapter 2 entitled "Faecal microbiota transplantation: is it the future for pig production?" is a second, shorter published review outlining the literature specifically surrounding the use of faecal microbiota

transplantation (FMT) as a tool for altering the gastrointestinal microbiota in pigs. This chapter identifies key gaps within the literature and areas to explore for later chapters within the thesis, Chapters 6 and 7.

Chapter 3 entitled “Characterisation of early microbial colonisers within the spiral colon of pre- and post-natal piglets” is the beginning of the experimental chapters and sets the scene for understanding how and when initial colonisation in the piglet occurs and what the first microbial colonisers are. The findings of this study demonstrated that microbial colonisation of the piglet likely occurs immediately prior to parturition and that the immediate post-natal environment largely influences the microorganisms colonising, while colostrum consumption further contributes to microbial community enrichment. The findings of Chapter 3 pose the question; how can the environment be altered in order to generate optimal microbiota development in the piglet during lactation? Therefore, given the natural differences already observed and well documented between primiparous and multiparous sows, Chapter 4 went on to investigate the effect of altering the pen environment by moving faeces from a multiparous sow to a primiparous sow’s pen.

Chapter 4 entitled “Exposure to maternal faeces in lactation influences piglet enteric microbiota, growth and survival pre-weaning” determined that the inclusion of multiparous sow faeces into the pen of a primiparous sow had no effect on the piglets. It was the removal of faeces from the pen that positively influenced piglet faecal microbiota characteristics, growth and survival at weaning. The findings of Chapters 3 and 4 demonstrate that the sow’s GIT microbiota seeds the piglet’s GIT microbiota during gestation and during lactation via maternal faeces. Therefore, the ability to manipulate the piglet’s microbiota through a dietary intervention given to the sow was tested.

This led to the development of Chapter 5 entitled “Maternal supplementation with phytogenic feed additives influenced the intestinal microbiota and reproductive potential in sows” which demonstrated that phytogenic feed additives have the potential to increase the number of piglets born per sow. Furthermore, sow gestation supplementation altered the microbiota of the sow and this change influenced their piglets.

This study also demonstrated that the change in piglet GIT microbiota generated by the sow persists beyond weaning, that is, even if the stimulus from the sow was no longer present. After identifying the possibility of changing the piglet GIT microbiota via dietary interventions through the sow and the environment as reported in previous chapters, we investigated the use of FMT as a means to alter the piglet's microbiota directly.

Chapter 6 entitled "Faecal microbiota analysis of piglets during lactation" determined that the faecal microbiota of piglets developed and stabilised with age and that the implementation of FMT at a young age was ineffective. This may have been because of the rapidly changing microbiota of young animals and therefore, a need for a greater number of doses for these piglets. Although no treatment effect was observed in this study, valuable information regarding microbial membership in the pre-weaning period was gained. As a result, Chapter 7 assessed the use of FMT in piglets post weaning.

Chapter 7 entitled "A single faecal microbiota transplantation altered the microbiota of weaned pigs" determined that a single FMT dose post weaning was effective in altering the microbiota of weaned pigs. This is the first study to demonstrate that a single FMT may be effective if given at the appropriate time. Chapter 7 concluded the experimental chapters.

Chapter 8 is the general discussion, integrating the five experimental chapters and exploring the outcomes and implications of the research.

This thesis is presented in a thesis-by-publication format. Each research chapter represents a distinct study that has either been published or is undergoing peer review. Manuscripts are formatted according to the journal requirements. Consequently, reference format and English spelling differ throughout this thesis. Additionally, each chapter that is either published or submitted for publication contains its own reference list and the collated reference list at the end of the thesis contains all references cited in the introduction and general discussion. The status of each chapter at the time of thesis submission is

indicated in the 'statement of authorship' form present at the beginning of each chapter. On this basis, there is some repetition of content between chapters.

Project aims

The overall hypothesis for this thesis was that enteric microbiota acquisition in piglets is initiated in late gestation and evolves rapidly during the immediate post-natal period from seeding by the sow. This, then, would allow for the piglet's GIT microbiota to be influenced via the addition of a treatment either through the environment or the sow. This information will assist in generating a collection of easy to implement industry applicable practices that enable the optimal development of the GIT microbiota. With the ultimate goal of improving piglet health and survival.

The individual experiments within this thesis aimed to:

1. Determine gastrointestinal tract colonisation before and immediately after birth in piglets that were stillborn or had or had not sucked.
2. Characterise the microbiota of primiparous and multiparous sows and determine whether contact with faeces from a multiparous sow can improve growth and survival of piglets born and reared on primiparous sows and if so, whether these differences are associated with the microbiota.
3. Determine whether the provision of a gestation and/or lactation diet containing phytogenic feed additives would alter the GIT microbiota of sows, and thus that of their piglets and so improve performance.
4. Provide a proof of concept for the application of FMT to control pre- and post weaning enteric disease in pigs.
5. Determine whether a dysbiosis caused by weaning could be corrected via FMT from healthy piglets from a previous weaning.

Chapter 1.2: Review of the literature:

Development and function of the intestinal microbiome and potential implications for pig production

Statement of Authorship

Title of Paper	Development and function of the intestinal microbiome and potential implications for pig production
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Nowland, T. L., K. J. Plush, M. Barton, and R. N. Kirkwood. 2019. Development and function of the intestinal microbiome and potential implications for pig production. *Animals* 9(3):76. doi: 10.3390/ani9030076

Principal Author

Name of Principal Author (Candidate)	Tanya Nowland
Contribution to the Paper	Investigation of the literature, conception of the theme, collation and interpretation of relevant information, writing and drafting of the paper.
Overall percentage (%)	80%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 16/02/2021

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Roy Kirkwood
Contribution to the Paper	Conception of the theme, evaluated and edited the manuscript.
Signature	Date 25/2/21

Name of Co-Author	Kate Plush
Contribution to the Paper	Evaluated and edited the manuscript.
Signature	Date 25/2/21

Name of Co-Author	Mary Barton			
Contribution to the Paper	Evaluated and edited the manuscript.			
Signature			Date	25/2/21

Please cut and paste additional co-author panels here as required.

animals

MDPI

Review

Development and Function of the Intestinal Microbiome and Potential Implications for Pig Production

Tanya L. Nowland [1,*], Kate J. Plush [2], Mary Barton [3] and Roy N. Kirkwood [1]

[1] School of Animal and Veterinary Sciences, The University of Adelaide, Roseworthy SA 5400, Australia; roy.kirkwood@adelaide.edu.au
[2] SunPork Solutions, Shea-oak Log SA 5371, Australia; kate.plush@sunporkfarms.com.au
[3] School of Pharmacy and Medical Sciences, University of South Australia, Adelaide SA 5000, Australia; mary.barton@unisa.edu.au
* Correspondence: tanya.nowland@adelaide.edu.au

Received: 10 January 2019; Accepted: 20 February 2019; Published: 28 February 2019

Simple Summary: Piglet preweaning mortality is a major economic loss and welfare concern for the global pork industry, with the industry average sitting at approximately 15%. As such, novel methods for reducing this mortality are needed. Since research into the intestinal microbiota has provided advances in human health, in particular the impact of early life factors, it was the logical next step to synthesise the existing literature to determine the potential relevance to the pig industry. It is evident from the literature that this area of research provides promising results. However, a large gap within the literature currently exists within the lactation period in pigs. Since optimal development within early life is proving to be critical for human infants, it is crucial that further research is invested into understanding the impact of early life events on a piglet's microbiome. It is hoped that this review will enable access to critical information for those interested in the microbiome and its potential for improving herd health on the farm.

Abstract: The intestinal microbiota has received a lot of attention in recent times due to its essential role in the immune system development and function. Recent work in humans has demonstrated that the first year of life is the most critical time period for microbiome development with perturbations during this time being proven to have long term health consequences. In this review, we describe the literature surrounding early life events in humans and mice that contribute to intestinal microbiota development and function, and compare this to piglets predominantly during their lactation period, which focuses on the impact lactation management practices may have on the intestinal microbiota. Although extensive research has been conducted in this area in humans and mice, little research exists in pigs during perceivably the most critical time period of development, which is the lactation period. The research reviewed outlines the importance of appropriate intestinal microbiota development. However, further research is needed in order to understand the full extent routine farm practices have on a piglet's intestinal microbiota.

Keywords: intestinal microbiota; neonatal environment; management; piglet; performance

1. Introduction

At parturition, the neonate is exposed to a range of microorganisms and this initial exposure forms the basis of the microbiome. A microbiome is a community of microorganisms that together have a mutualistic relationship with the host [1]. There are multiple niches on the host that have their own characteristic microbiome, e.g., the skin, mouth, urogenital tract, and all levels of the gastrointestinal

tract (GIT) [2–4], with each characteristic microbiome maintaining a diverse and relatively stable population of bacteria, archaea, fungi, and viruses [2–4]. The types of microorganisms that make up the gastrointestinal microbiota play an integral role in host metabolism, the development of a healthy GIT, and immune and neural system development [5,6]. As such, the health of offspring largely depends on the microorganisms that the body is exposed to throughout life. Studies in humans have demonstrated that the first year of life is the most critical time period for microbial acquisition and development, with disruptions to the microbiome during this time causing long-term consequences [7]. Some of the consequences observed in humans are gastrointestinal and systemic disorders including inflammatory bowel disease, necrotizing enterocolitis, eczema, obesity, autoimmunity, asthma, and autism [8–10]. These findings serve to illustrate the power of an appropriate intestinal microbiota and have initiated the idea that fostering a healthy microbiome through optimal initial colonisation may improve health and limit disease.

Finding novel methods for improving health on the farm is of particular interest to the pork industry since pre-weaning mortality is a major issue with the industry average being around 15% [11–13]. To date, a large quantity of research exists surrounding the development of methods for reducing these mortalities. However, it has proven to be a complex issue with little progress [11,13,14]. Research investigating the impact of routine farm practices on piglet microbiome is limited, with the majority of research in this area focusing on the microbiome in pigs as a model for humans and in piglets post weaning [15–17]. Given our understanding of the importance of early microbial colonisation and GIT health in humans, it is reasonable to suggest that fostering a healthy microbiome may contribute to piglet viability and survival. This review summarises studies examining initial microbial acquisition and development, the impact of different factors on the microbiome, and the impact of the microbiome on health and methods of microbiome manipulation.

2. Acquisition of the Microbiome

2.1. Pre-Partum Microbial Acquisition

The development of the microbiome has long been thought to originate at birth when the fetus transits from a supposedly sterile environment within the amniotic sac through the birth canal, into a microbially dense environment. However, recent studies in mice and humans question these claims and have demonstrated the presence of bacteria within the amniotic fluid of pregnant mice and the meconium of infants, which suggests some colonisation in utero [18,19]. Whether in utero colonisation occurs in food species such as pigs remains unknown. However, the differences in placentations (haemochorial vs. epitheliochorial) would likely impact the ability for microbial transfer. Additional microbial colonisation occurs during the parturition process when microorganisms colonise the mucus membranes and skin epithelia. Factors such as transit time and mode of delivery have an influence on the colonising microbial populations [20]. Neonates born vaginally are colonised by microorganisms that are similar to their mother's vaginal microbiota, while those delivered by caesarean section (C-section) are colonised by bacterial communities similar to the mother's skin microbiota [21–23]. The impact of the delivery method on the infant has been extensively studied over recent years with accumulating evidence suggesting that C-sections that delivered human infants have reduced microbial richness and diversity compared to those delivered vaginally [23,24]. It is this reduced diversity that is suggested to be the main cause of the increased incidence of allergic disease often seen in human infants delivered by C-section [24,25]. While delivering pigs via C-section is not a likely option within the pork industry generally, this research provides evidence that the microorganisms harbored at birth have long-term implications for health. This is of particular interest since C-sections are often used when establishing new farms, especially at the nucleus level where high health is the required outcome. The implications of an 'abnormal' microbiome may be detrimental to this requirement.

2.2. Post-Partum Microbial Acquisition

The post-partum GIT microbiota has three essential roles, which include protective, metabolic, and trophic roles [26]. First, the microorganisms act as a barrier against pathogenic organisms by competitive exclusion. Then, they aid in digestion and metabolism of colostrum and milk, they break down toxins and drugs, synthesise vitamins, and absorb ions. Lastly, they support the growth and differentiation of the epithelial cells lining the intestinal lumen and support homeostasis of the immune system [26]. Human post-natal factors such as feeding type (breast vs. formula), maternal weight gain, stress and prenatal and perinatal antibiotic use all influence the populations of bacteria colonising, which alters the way the microbiota performs these functions [7,27,28]. Although the microbiome is influenced via a variety of external factors in pigs, two predominant immediate postnatal factors that determine initial postnatal microbial colonisation are likely colostrum, milk quality, and the neonatal environment.

2.2.1. Colostrum

The importance of colostrum and milk for human and animal health has been extensively studied with a number of reviews available [9,27–30]. Maternal milk provides energy, nutrients, and bioactive compounds such as immunoglobulins, cytokines, chemokines, growth factors, hormones, and antibodies that directly influence development [27,29]. It also contains other compounds such as peptides, lactoferrin and other whey proteins, oligosaccharides, and a large number of bacteria [27]. Maternal milk is an important postnatal element for establishing an appropriate intestinal microbiota [31]. Studies in humans have demonstrated that being breast-fed is associated with a lower incidence of diabetes, obesity, celiac disease, multiple sclerosis, and asthma [29,32]. These associations are primarily driven by the protective effects of milk against early infections, its anti-inflammatory properties, antigen specific tolerance induction, and regulation of the infant's microbiome [29]. While clearly important in humans, the significance of the enteromammary axis in food animals is likely limited by the relatively short lacations, but likely becomes more significant with longer lactations. In pigs, longer lactations and higher weaning weights have been associated with improved health outcomes and fewer days to market. A role for the enteromammary axis in these benefits from longer lactations cannot be discounted.

The microbiome of the breastfed infant is very different from that of formula fed infants [21,33]. There is also a demonstrated specificity between the microbiome and suckling with a study in rodents demonstrating that milk cells contain a number of bacterial DNA signatures found in maternal peripheral blood mononuclear cells during pregnancy and lactation, which suggests bacterial translocation [34]. Other studies in humans and mice have suggested that this is a result of dendritic cells sampling the luminal microbiome and translocating it into the milk [29,34,35]. Although currently speculative, it seems reasonable to suggest a similar differential effect of maternal milk and formula would also occur in pigs and should be considered when providing supplemental nutrition to compromising pigs, such as those with low birth weights.

Compared to humans, pigs are born with relatively low body energy stores and are immunologically naive due to the epitheliochorial nature of the porcine placenta [36]. This means that, for the piglet, the consumption of colostrum immediately after parturition is essential to survival [37]. Colostrum not only provides a supply of warmth, energy, and immunity, it also enables the establishment of commensal microbes. The ability to acquire colostrum is largely dependent on piglet weight at birth [38] and, as the industry pushes towards improved sow prolificacy and a greater number of newborn piglets per litter, the proportion of low birth weight piglets is increased [38]. Morissette et al. [31] suggested colostrum and milk intakes (as measured by weight gain) within the first two weeks of life influenced the development of the microbiota. High weight gain piglets have higher levels of *Bacteroidetes*, *Bacteroides*, and *Ruminoccocaceae* and lower proportions of *Actinobacillus porcinus* and *Lactobacillus amylovorus* compared with low weight gain piglets. These data suggest that the quantity of milk ingested within the first two weeks of life has the potential to not only impact

weight gain but also influence long-term animal health and performance via the microbial populations colonising. Low birth weight piglets do not reach the udder as fast and have reduced competitiveness for teats [12,38], and a potential lack of maturity of the GIT may also impact the outcomes observed. It is evident that further research is required in order to establish the etiologic influence colostrum acquisition has on the development of the microbiome in piglets. Although an interaction exists between milk consumption and the microbiome, the impact of the quantity and quality of the milk obtained and its effects on the microbiome is yet to be investigated in the piglet.

2.2.2. Environment

Both pathogenic and non-pathogenic bacteria are ubiquitous in the environment. It is the combination of the environment, diet, and genetics that determine which microbes colonise the epithelial surfaces of the body [7,27,30]. In humans, it is relatively difficult to completely eliminate the confounding factors of differences in diet, genetics, gestation, and the delivery method from the impact of the environment alone. As such, animal studies have been conducted in order to fill these gaps.

In mice, it has been demonstrated that immunological development is largely dependent on the initial GIT microbial colonisation, which is determined by the environment. Cahenzli et al. [8] demonstrated that mice that were germ-free at birth and that were maintained in a germ-free environment had an increased antigen-induced oral anaphylaxis incidence, which demonstrates the importance of an appropriate intestinal microbial stimulus for immune system development. In pigs, the influence of low hygiene (farm housed, sow-fed) or high hygiene (isolator housed, milk formula-fed) environments influenced piglet immunological development. Piglets reared on the sow have a more diverse intestinal microbiota than the siblings reared in isolators [39]. It is impossible to determine the direct effect nutrition has on this. However, the latter study further corroborates the findings from previous studies, which indicate that the microorganisms that colonise the GIT influence immune development and subsequent health.

When considering the development of the microbiome in pigs, an understanding around the piglet postnatal environment is essential. Since pigs are produced within an intensive production system where they are housed in pens in contact with the mother's feces, skin, and mucosal surfaces until weaning, it is likely that the microbiome of a newborn piglet is largely dependent on the sow. When considering the opportunity for microbial manipulation through early life exposure, this may provide an effective arena, with studies suggesting that the pre-weaning period is critical for appropriate colonisation and immune system development [8,21,30]. Further investigations of the lactation period should prove fruitful. The development and variation of the microbiome in pigs is starting to gain understanding since a number of studies are investigating this [40,41]. However, relatively little is known about what impact general farm practices, including sow nutrition and parity, farrowing crate cleanliness, sow skin and udder cleanliness, piglet fostering, iron and penicillin injections at 24 h old, and age of weaning are having on the microbiome and individual piglet performance.

3. Impact of Different Factors on the Microbiome

The initial colonising bacteria largely drive microbiome establishment and development. However, the microbiome is a dynamic system that is continuously changing and is influenced by a variety of factors. Some of these factors include antibiotic use, stress, diet, age, and the rearing environment [7,27,28]. Previous studies in humans have suggested that the most important period for microbial establishment is the first 1 to 3 years of life since it is during this time that the microbiome is more dynamic and susceptible to change [42,43]. Disruption or dysbiosis during this period result in disease [8,44,45]. Thereafter, the microbiome changes toward a more adult-like state where it becomes more stable and resistant to change [42,43]. Accumulating evidence suggests that the shift in the microbial state may be attributed to the transition from a primarily liquid milk diet to one that relies on solid food [9,43]. From these data values, it can be assumed that, in the case of the pig, the most critical

time for microbial establishment would be prior to weaning while they still maintain a predominantly milk-based diet. As such, the practices undertaken during lactation should be critically reviewed in order to establish the potential impact they are having on the microbiome with the ultimate goal promoting the establishment of a healthy microbiome.

3.1. Antibiotics

Antibiotic use during the pre-natal and post-natal period has been demonstrated to negatively impact GIT microbial diversity and increase the number of resistant bacteria [46–48]. Antibiotics are commonly used for the control of pathogenic bacteria. However, they are non-specific and have the potential to perturb beneficial commensal bacteria in the GIT and elsewhere [49,50]. In humans, when administered during the early postnatal period and while the initial microbial establishment is occurring, these disruptions can lead to overgrowth of pathogenic bacteria and to long-term health problems such as asthma, necrotising enterocolitis, and late-onset sepsis [5,49,51].

In recent times, the importance of populating a healthy microbiome has become increasingly evident. As a result, a multitude of reviews surrounding the use of antibiotics on the microbiome have been conducted [26,46,49,50]. What can be taken collectively from these reviews is that microbial disruption during the perinatal period has detrimental effects on microbial establishment and metabolism, which often leads to long-term health problems. When investigating the effect of antibiotics in the pig specifically, a similar effect on microbial diversity and quantity is observed. Gao et al. [15] investigated the time-course effect of antibiotics on microbial composition and metabolism in pigs fed a standard diet with or without antibiotics. Their findings support the human literature where antibiotic administration leads to changes in microbial GIT communities and metabolism. These differences are noticeable as soon as two days in the ilium and seven days in the faeces. Similarly, studies conducted by Looft et al. [52,53] demonstrated that in-feed antibiotics for piglets caused divergence in microbiome membership and reduced microbial population quantity and diversity. These studies also demonstrated that *Escherichia coli* populations in the ileum increased with antibiotic exposure. Antimicrobial resistance genes to antibiotics that were not administered were identified. Furthermore, a study conducted by Kim et al. [53] demonstrated similar changes in microbiota populations in pigs when administered antibiotics were in the feed. It is common practice globally for piglets to receive antibiotic treatment at 24 h of age but little research has investigated its consequences with regards to the microbiome.

3.2. Stress

There is a growing body of evidence linking the GIT microbiota to the central nervous system function [26,54,55]. O'Mahony et al. [56] observed that rat pups exposed to maternal separation stress for three hours daily from two to 12 days of age and then exposed to a novel stressor had an increased number of faecal boli, increased plasma corticosterone levels, an increased visceral sensation, and an altered faecal microbiota when compared to undisturbed rats. Additionally, Bailey et al. [57] found that mice exposed to a social disruption stressor had large shifts in the microbiota community structure, which decreased the relative abundance of bacteria genus *Bacteriodes* while increasing the relative abundance of *Clostridium*. Additionally, the stressor also increased circulating levels of IL-6 and MCP-1, which correlates with changes in three bacteria genera *Coprococcus*, *Pseudobutyrivibrio*, and *Dorea*. Additional studies have also demonstrated that chronic stress affects the abundance and diversity of the GIT microbiota, which have long-term effects on the immune system [26,49,54].

Within the pork industry, weaning would be the most commonly studied event that causes stress in pigs. Weaning is a multifactorial stressor, including environmental, social, nutritional, and psychological disruptions [58]. The stress associated with weaning is a welfare concern and it causes a reduction in growth during the first days following weaning, which results in economic loss with increased days to market [58,59]. This reduced weight gain post weaning, commonly referred to as the 'post weaning growth check,' is thought to be a result of the reduced intestinal integrity caused

by stress, which causes leaky gut and diarrhoea as well as an increased susceptibility to colonisation with pathogenic bacteria such as *E. coli* [58–60]. Experiments in animals suggest that increased cortisol levels that accompany stress are the main driver for this, which increases gut permeability and bacterial lipopolysaccharide leakage across the intestinal wall [61,62]. The social stress from weaning is exacerbated by litters being mixed.

While stress affects the microbiota, evidence suggests that there is a bi-directional communication between the gut and the brain, which means that the intestinal microbiome can influence the animal's susceptibility to stress or anxiety [54,55,63]. When comparing germ-free (GF) mice with specific-pathogen free (SPF) mice, Sudo et al. [64] demonstrated that GF mice had elevated plasma adrenocorticotropic hormone and corticosterone levels in response to a restraint stress. GF mice also exhibited reduced brain-derived neurotrophic factor expression levels in the cerebral cortex and hippocampus. Furthermore, when the GF mice were reconstituted with *Bifidobacterium infantis*, the exaggerated HPA stress response observed could be reversed. Other such cases of this 'reversal' in behavior through microbial supplementation have been demonstrated. Messaoudi et al. [65] found that anxiety-like behavior was reduced in rats and physiological distress (depression and anger-hostility) was reduced in humans when given a combination of *Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175. The ability to alter an animal's response to stress by introducing different GIT bacteria is new and, although the results from these trials are promising, more research in this area is needed. Influencing the intestinal microbial composition in order to improve productivity and health are the ultimate objectives for the future and an improved ability to cope with stress would be beneficial to productivity.

Few studies to date have demonstrated the impact of prenatal stress on microbial establishment. Zijlmans et al. [66] demonstrated a link between maternal stress and microbiota colonization. Mothers who were identified as having high cumulative stress (high reported stress and high cortisol concentrations) during pregnancy had a significantly higher relative abundance of proteobacterial groups that are known to contain pathogens (i.e., *Escherichia*, *Serratia*, and *Enterobacter*), while beneficial bacteria such as *Lactobacillus*, *Lactococcus*, *Aerococcus*, and *Bifidobacteria* were reduced. Additionally, Gur et al. [67] not only demonstrated that prenatal stress resulted in different placental microbes in mice offspring, but also that prenatal stress led to long-term differences in behaviour and cognition with increased anxiety like behaviour in female mice and decreased social interaction in male mice. Within the pork industry, more research has been conducted into gestational group housing and farrowing accommodation in order to identify optimal housing for reduced sow stress and improved piglet welfare and survival. However, to our knowledge, no research to date has investigated the effect of housing during both the gestational and the pre-farrow period on the sow or piglet's microbiome and subsequent health. These studies provide precedent for further investigation into this area, especially in the case of intensive production systems with the increased risk for high stress.

3.3. Age and Diet

Diet represents one of the major factors contributing to intestinal microbial colonisation [68]. This is evident from research showing marked differences in the GIT microbiota community structure in pigs after only two weeks of feeding different experimental diets [69]. Similarly, a gradual taxonomic and functional rearrangement of the bacterial community in feces after feeding four different diets varying in protein source, calcium, and phosphorus concentration has been recorded [70], which indicates the importance of diet on microbial population modulation.

The largest and most dynamic change in microbiome transition, however, is during the weaning period. As such, studies have investigated the influence of weaning from an exclusive milk diet to a solid food diet on the microbiome [28,71]. In pigs, multiple authors have demonstrated that the microbiota of suckling piglets predominantly contained *Bacteroides*, *Oscillibacter*, *Escherichia/Shigella*, *Lactobacillus*, and unclassified *Ruminococcaceae* genera [40,72]. However, after weaning, the bacterial diversity increased linearly to be predominantly *Acetivibrio*, *Dialister*, *Oribacterium*, *Succinivibrio*,

and *Prevotella* genera. In contrast, others have observed a reduction in GIT microbial diversity until 11 days post weaning. Microbial diversity followed the trends observed by the previously mentioned studies [16]. These data provide an explanation for the reduction in weight gain and presence of diarrhea often observed in piglets post weaning. The differences observed between studies may be attributed to the fact that weaning ages and sample time points varied between studies. Hu et al. [16] conducted the only study to collect samples within eight days of weaning. This might indicate that the previous studies may have experienced the same drop in diversity, but they had no means for observing it since they had not investigated the microbiota at an early enough time point. Studies like those of Hu et al. [16] provide insights into possible methods that could be implemented to improve microbial diversity around weaning to enable increased stress tolerance for piglets.

Studies investigating the role diet has on modulating microbial populations and health provide promise for possible investigations in pigs. For example, an increase in fiber in the diet changed the GIT microbiota and increased protection against dysbiosis in mice, which prevented the development of hypertension and heart failure in hypertensive mice [73]. Studies in pigs are beginning to follow this trend and investigate the effect diet has on the microbiota and health. Heo et al. [74] fed piglets different protein levels post weaning and challenged them with an enterotoxigenic strain of *Escherichia coli*. They determined that those animals that were fed a reduced protein diet, had a reduced incidence of post weaning diarrhea in the face of an *E. coli* challenge. Unlike the previous authors, Qiu et al. [17] examined the microbiome and found that 65-day-old gilts fed diets with reduced crude protein levels had a shift in microbial composition in the ileum, which lead to enhanced microbial fermentation and short chain fatty acid production. Overall, these data support the suggestion that intestinal microbial colonisation is significantly influenced by diet, with age having an influence on its progression.

4. Impact of the Microbiome on Health

The intestinal microbiota has been demonstrated to be involved in the regulation and maintenance of overall health. Its initial colonisation mediates immune system development and long-term colonisation determines health and survival [30]. It has influences on susceptibility to enteric, autoimmune, cardiovascular, and atopic diseases [44,50,75]. It is also involved in cognitive development and can influence subsequent cognitive disorders [9]. There is a continuous interplay between the microbiota and health as well as the impact of pathogens and diet on the microbiota. It is an interconnected, multifactorial relationship, and understanding it is crucial for optimising current practices to enhance health.

Immune System

The intestinal microbiota has a demonstrated involvement in a myriad of functions. The interaction between the epithelial cells lining the intestine and the microbiota are essential for immune system development, maturation, regulation, and the maintenance of homeostasis [61,75,76]. Particularly, the haematopoietic and non-haematopoietic cells of the innate immune system have a unique positioning that allows them to have the ability to sense the microorganisms and their metabolic products for generation of a physiological response by the host [76]. The diversity, type, and quantity of microorganisms colonising impacts the way in which the microbiota regulates intestinal mucosal barriers, controls nutrient uptake and metabolism, assists with immune system development, and controls competitive exclusion of pathogenic microorganisms [6,30,50]. Not only do the microorganisms within the intestinal tract influence the innate immune system, they also communicate with and influence the adaptive immune system [76]. Research suggests that those animals that undergo disruptions in the microbiota or have a reduced intestinal microbial diversity are at an increased risk of cardiovascular disease, inflammatory bowel disease, necrotizing enterocolitis, eczema, obesity, malnutrition, autoimmunity, asthma, and autism [8,9,25]. Studies in germ-free animals have shown that the absence of an intestinal microbiota results in defects in lymphoid tissue development within the spleen, thymus, and lymph nodes and a reduction in lamina propria

CD4+ cells, IgA-producing cells, and hypoplastic Peyer's patches [8,20,77]. These studies have also demonstrated ileal and jejunal Peyer's patches in pigs to be shorter at 39 and 59 days of age, predominantly T cells rather than B cells at six weeks old, and have a similar cell yield at 45 days old as a five day old normal piglet [8,20,77].

The presence of microorganisms within the GIT are clearly essential and the types of microorganisms colonising also directly influences the immune system. A good example of this is seen in the acetogenic bacteria *B. longum* subsp. *Longum* and *B. longum* subsp. *Infanis*. These species produce the short chain fatty acid, acetate, which directly influences immune system regulation by inducing regulatory T cells [78,79]. Similarities can be seen for other bacteria producing the short chain fatty acids propionate and butyrate since they have functions that inhibit the growth of pathogens. For example, acetate when administered alone inhibits the growth of *Pseudomonas aeruginosa* [80], while acetate in combination with propionate and butyrate inhibit the growth of pathogenic *E. coli* O157 [81], *Proteus mirabilis*, *Klebsiella pneumonia*, and *P. aeruginosa* [80]. This is just one example of how the microbial populations have a direct influence on immune regulation.

In pigs, the development of the mucosal immune system occurs over a period of weeks and, from research done in germ-free pigs, it is evident that its development is largely dependent on microbial exposure [77]. This initial microbial exposure is primarily occurring at birth via urogenital and environmental exposure and at ingestion of colostrum and milk throughout lactation. Additionally, recent studies indicate that the microbiota within sow milk is dynamic and changing throughout lactation to support the piglets appropriate microbiome development, especially between the colostrum and milk phase of lactation [82]. The pig goes through a number of stressful events throughout its life and, therefore, a strong immune system is crucial for optimal growth and survival. It is understood that the stress associated with weaning, mixing of litters, and abrupt diet changes result in significant microbial shifts and dysbiosis, which reduces intestinal integrity and often leads to disease [61,83]. Dou et al. [84] demonstrated that those piglets that developed diarrhea post-weaning had a different microbiota than those who did not, with this difference being detectable as early as seven days of age (four weeks prior to diarrhea). At seven days of age, the non-diarrheic pigs displayed a higher abundance of *Prevotellaceae*, *Lachnospiraceae*, *Ruminocacaceae*, and *Lactobacillaceae* compared to diarrheic pigs. These data suggest that ensuring optimal microbial establishment in early life is essential for preventing disease during stressful periods in later life. This study did not investigate the effects of the sow or litter on this outcome. In order to understand the direct mechanisms for the differences in microbiota observed, it would be beneficial to establish if this microbial difference and susceptibility was attributed to the sow's microbiota or the housing environment during the lactation period. Hasan et al. [85] focused on both the sow and the piglet and demonstrated that, by influencing the sow diet through yeast derivatives (YD) (brewer's yeast hydrolysate) during pregnancy and lactation, the sow colostrum contained more fat and piglet performance was improved. Although sow fecal bacterial diversity was not different, those sows fed YD had higher levels of beneficial and fermentative bacteria and reduced numbers of opportunistic pathogenic bacteria. Furthermore, piglets from YD sows demonstrated a similar trend with increased numbers of beneficial bacteria and reduced opportunistic pathogens present in feces at one week of age. This study provides evidence that the sow's microbiota can be manipulated in order to positively influence their offspring.

5. How Can We Manipulate the Microbiome to Improve Health?

5.1. Prebiotics and Probiotics

Prebiotics and probiotics are two commonly used dietary additives in both human and animal nutrition. They have been extensively studied in recent years due to their perceived health benefits. A prebiotic is a substance that is not hydrolysed or absorbed in the first part of the digestive system and reaches the colon to selectively stimulate the proliferation of resident beneficial bacterial strains [86]. Probiotics are defined as cultures of potentially beneficial bacteria of healthy gut microflora that are

administered to colonise the large intestine and modify the composition of the microbiota [86,87]. To date, several reviews have investigated the effect of using both prebiotics and probiotics in treating human and animal disease [49,50,77].

Post-weaning diarrhea has been of particular concern to the pork industry for a long time. As such, many reviews of the literature surrounding the use of prebiotics and probiotics as a method of reducing post-weaning diarrhea have been conducted [1,58,68,83,88]. To date, the focus has been primarily around the use of prebiotics and probiotics for reducing post-weaning diarrhea, with little research surrounding their use during lactation. Hayakawa et al. [89] demonstrated that the administration of a probiotic containing *Bacillus mesentericus*, *Clostridum butyricum*, and *Enterococcus faecalis* (0.2% (*w*/*w*)) three weeks prior to farrowing and throughout lactation improved litter weight and sow return to oestrus (17% and 24% improvement, respectively). In addition to this, sow feed intake during late lactation, post weaning diarrhea incidence, and piglet growth performance were all improved. Additionally, another study found that inclusion of a prebiotic YD in a gestation and lactation diet resulted in shifts in the fecal microbiota so that the abundance of beneficial bacteria was supported and pathogenic bacteria reduced [85]. These fecal microbial differences were also associated with improved sow milk yield and piglet weight gain. To our knowledge, this is the only study that has assessed sow fecal microbial change as a result of a feed additive during lactation and its subsequent effect on offspring. From this, it is evident that prebiotics and probiotics have the potential to improve pig health. However, there is an obvious gap in the literature surrounding the use of these during the lactation period in pigs.

5.2. Fecal Microbiome Transplantation

Fecal microbiome transplantation (FMT) is the transplantation of a fecal suspension from a healthy individual into the gastrointestinal tract of another individual to cure a specific disease [90]. There has been recent interest in re-establishing a "good" microbiome via competitive exclusion using FMT. One example is the case of *Clostridium difficile* infections in humans. Overgrowths of *C. difficile* have achieved epidemic proportions associated primarily, but not exclusively, with hospitalization and specific antimicrobial treatments [91]. Treatment of human *C. difficile* with antibiotics often fails as antibiotics kill vegetative bacteria but not spores. With cessation of antibiotic treatment, spores germinate and recurrent *C. difficile* disease develops. To counter this, the ability to re-establish a "good" microbiome to competitively exclude *C. difficile* using FMT has been achieved. This procedure requires that feces from a healthy donor be inoculated into the patient either orally or via an enema [92]. The use of oral FMT for treating food poisoning or severe diarrhea was first described by Ge Hong in 4th century China [90]. In recent studies, the use of FMT for treating enteric diseases induced lasting changes in the patient's microbiome, with a >90% success rate observable within days and was without adverse side effects [90]. Brandt and Aroniadis [90] also described beneficial effects of FMT in non-enteric diseases such as Parkinson's disease, insulin resistance, multiple sclerosis, and childhood regressive autism.

Since antibiotic use is a growing global concern, the development of non-antibiotic techniques to treat animal disease needs to be explored. The potential of application of FMT to control preweaning and postweaning enteric diseases in pigs is intriguing. Studies by Hu et al. [93] and Xiao et al. [94] show promising results for the use of FMT within pigs. The administration of 1.5 mL of FMT daily to pigs from one to 11 days of age increased the average daily gain, reduced the incidence of diarrhea, and improved the intestinal barrier and immune system function [93]. Furthermore, positive outcomes were evident when performing FMT, with FMT from a colitis-resistant breed to an at-risk breed, which results in the improved resistance being transferred [94]. However, contrary to the results of Hu et al. [93] and Xiao et al. [94], others have demonstrated a negative effect in piglets receiving FMT four times throughout lactation directly or piglets reared on sows receiving FMT. The pigs are lighter at 70 and 155 days and have poorer absorptive capacity and intestinal health, as demonstrated through intestinal morphology and duodenal gene expression [95]. Although the results from these studies

are somewhat conflicting, they provide promise for the use of FMT in pigs. It is evident that the FMT donor chosen would also have an impact on the results obtained. Therefore, it may be that the negative results observed in the study by McCormack et al. [95] were a result of the donor animals chosen. To our knowledge, these are the only studies that have investigated the use of FMT in pigs and, from this, it is evident that this area of research provides promise.

6. Conclusions

It is evident that the intestinal microbiome plays an integral role in modulating health and disease. Although a large body of evidence has identified the ways in which the microbiome influences health, there is still much to learn about how we can utilise this knowledge for preventing and treating disease in humans and animals. The findings from this review demonstrate the lack of information covering the lactation period for pigs. With the critical time period for microbiome development likely occurring prior to weaning, it is crucial that further research is invested into understanding the impact routine farm practices are having on a piglet's microbiome. It is hoped that this review will enable access to critical information for those interested in the microbiome and enable its potential for improving herd health on the farm.

Author Contributions: All authors contributed to conceptualisation and preparing the manuscript.

Funding: This research received no external funding.

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

References

1. Young, V.B. The role of the microbiome in human health and disease: An introduction for clinicians. *Br. Med. J.* **2017**, *356*, j831. [CrossRef] [PubMed]
2. Abeles, S.R.; Pride, D.T. Molecular bases and role of viruses in the human microbiome. *J. Mol. Biol.* **2014**, *426*, 3892–3906. [CrossRef] [PubMed]
3. Grice, E.A.; Segre, J.A. The skin microbiome. *Nat. Rev. Microbiol.* **2011**, *9*, 244–253. [CrossRef] [PubMed]
4. Hallen-Adams, H.E.; Suhr, M.J. Fungi in the healthy human gastrointestinal tract. *Virulence* **2017**, *8*, 352–358. [CrossRef]
5. Arrieta, M.C.; Stiemsma, L.T.; Amenyogbe, N.; Brown, E.M.; Finlay, B. The intestinal microbiome in early life: Health and disease. *Front. Immunol.* **2014**, *5*, 427. [CrossRef] [PubMed]
6. Gensollen, T.; Iyer, S.S.; Kasper, D.L.; Blumberg, R.S. How colonization by microbiota in early life shapes the immune system. *Science* **2016**, *352*, 539–544. [CrossRef] [PubMed]
7. Murphy, K.; O'Shea, C.A.; Ryan, C.A.; Dempsey, E.M.; O'Toole, P.W.; Stanton, C.; Ross, R.P. The gut microbiota composition in dichorionic triplet sets suggests a role for host genetic factors. *PLoS ONE* **2015**, *10*, e0122561. [CrossRef] [PubMed]
8. Cahenzli, J.; Köller, Y.; Wyss, M.; Geuking, M.B.; McCoy, K.D. Intestinal microbial diversity during early-life colonization shapes long-term ige levels. *Cell Host Microbe* **2013**, *14*, 559–570. [CrossRef] [PubMed]
9. Carlson, A.L.; Xia, K.; Azcarate-Peril, M.A.; Goldman, B.D.; Ahn, M.; Styner, M.A.; Thompson, A.L.; Geng, X.J.; Gilmore, J.H.; Knickmeyer, R.C. Infant gut microbiome associated with cognitive development. *Biol. Psychiatry* **2018**, *83*, 148–159. [CrossRef] [PubMed]
10. Wang, M.; Monaco, M.H.; Donovan, S.M. Impact of early gut microbiota on immune and metabolic development and function. *Semin. Fetal. Neonat. Med.* **2016**, *21*, 380–387. [CrossRef] [PubMed]
11. Daigle, C. Parallels between postpartum disorders in humans and preweaning piglet mortality in sows. *Animals* **2018**, *8*, 22. [CrossRef] [PubMed]
12. Mota-Rojas, D.; Martinez-Burnes, J.; Villanueva-Garcia, D.; Roldan-Santiago, P.; Trujillo-Ortega, M.E.; Orozco-Gregorio, H.; Bonilla-Jaime, H.; Lopez-Mayagoitia, A. Animal welfare in the newborn piglet: A review. *Vet. Med.* **2012**, *57*, 338–349. [CrossRef]
13. 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] [PubMed]

14. Kirkden, R.D.; Broom, D.M.; Andersen, I.L. Invited review: Piglet mortality: Management solutions. *J. Anim. Sci.* **2013**, *91*, 3361–3389. [CrossRef] [PubMed]
15. Gao, K.; Pi, Y.; Peng, Y.; Mu, C.L.; Zhu, W.Y. Time-course responses of ileal and fecal microbiota and metabolite profiles to antibiotics in cannulated pigs. *Appl. Microbiol. Biotechnol.* **2018**, *102*, 2289–2299. [CrossRef] [PubMed]
16. Hu, J.; Nie, Y.F.; Chen, J.W.; Zhang, Y.; Wang, Z.C.; Fan, Q.W.; Yan, X.H. Gradual changes of gut microbiota in weaned miniature piglets. *Front. Microbiol.* **2016**, *7*, 1727. [CrossRef] [PubMed]
17. Qiu, K.; Zhang, X.; Jiao, N.; Xu, D.D.; Huang, C.Y.; Wang, Y.B.; Yin, J.D. Dietary protein level affects nutrient digestibility and ileal microbiota structure in growing pigs. *Anim. Sci. J.* **2018**, *89*, 537–546. [CrossRef] [PubMed]
18. Ardissone, A.N.; de la Cruz, D.M.; Davis-Richardson, A.G.; Rechcigl, K.T.; Li, N.; Drew, J.C.; Murgas-Torrazza, R.; Sharma, R.; Hudak, M.L.; Triplett, E.W.; et al. Meconium microbiome analysis identifies bacteria correlated with premature birth. *PLoS ONE* **2014**, *9*, e90784. [CrossRef] [PubMed]
19. Jimenez, E.; Marin, M.L.; Martin, R.; Odriozola, J.M.; Olivares, M.; Xaus, J.; Fernandez, L.; Rodriguez, J.M. Is meconium from healthy newborns actually sterile? *Res. Microbiol.* **2008**, *159*, 187–193. [CrossRef] [PubMed]
20. Macpherson, A.J.; Harris, N.L. Interactions between commensal intestinal bacteria and the immune system. *Nat. Rev. Immunol.* **2004**, *4*, 478–485. [CrossRef] [PubMed]
21. Backhed, F.; Roswall, J.; Peng, Y.Q.; Feng, Q.; Jia, H.J.; Kovatcheva-Datchary, P.; Li, Y.; Xia, Y.; Xie, H.L.; Zhong, H.Z.; et al. Dynamics and stabilization of the human gut microbiome during the first year of life. *Cell Host Microbe* **2015**, *17*, 690–703. [CrossRef] [PubMed]
22. Dominguez-Bello, M.G.; Costello, E.K.; Contreras, M.; Magris, M.; Hidalgo, G.; Fierer, N.; Knight, R. Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 11971–11975. [CrossRef] [PubMed]
23. Groer, M.W.; Luciano, A.A.; Dishaw, L.J.; Ashmeade, T.L.; Miller, E.; Gilbert, J.A. Development of the preterm infant gut microbiome: A research priority. *Microbiome* **2014**, *2*, 38. [CrossRef] [PubMed]
24. Bager, P.; Wohlfahrt, J.; Westergaard, T. Caesarean delivery and risk of atopy and allergic disesase: Meta-analyses. *Clin. Exp. Allergy* **2008**, *38*, 634–642. [CrossRef] [PubMed]
25. Kuitunen, M.; Kukkonen, K.; Juntunen-Backman, K.; Korpela, R.; Poussa, T.; Tuure, T.; Haahtela, T.; Savilahti, E. Probiotics prevent ige-associated allergy until age 5 years in cesarean-delivered children but not in the total cohort. *J. Allergy Clin. Immunol.* **2009**, *123*, 335–341. [CrossRef] [PubMed]
26. Yang, I.; Corwin, E.J.; Brennan, P.A.; Jordan, S.; Murphy, J.R.; Dunlop, A. The infant microbiome implications for infant health and neurocognitive development. *Nurs. Res.* **2016**, *65*, 76–88. [CrossRef] [PubMed]
27. Gomez-Gallego, C.; Garcia-Mantrana, I.; Salminen, S.; Collado, M.C. The human milk microbiome and factors influencing its composition and activity. *Semin. Fetal. Neonat. Med.* **2016**, *21*, 400–405. [CrossRef] [PubMed]
28. Laursen, M.F.; Bahl, M.I.; Michaelsen, K.F.; Licht, T.R. First foods and gut microbes. *Front. Microbiol.* **2017**, *8*, 356. [CrossRef]
29. Borba, V.V.; Sharif, K.; Shoenfeld, Y. Breastfeeding and autoimmunity: Programing health from the beginning. *Am. J. Reprod. Immunol.* **2018**, *79*, e12778. [CrossRef] [PubMed]
30. Le Doare, K.; Holder, B.; Bassett, A.; Pannaraj, P.S. Mother's milk: A purposeful contribution to the development of the infant microbiota and immunity. *Front. Immunol.* **2018**, *9*, 361. [CrossRef] [PubMed]
31. Morissette, B.; Talbot, G.; Beaulieu, C.; Lessard, M. 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*, 525–532. [CrossRef] [PubMed]
32. Iozzo, P.; Sanguinetti, E. Early dietary patterns and microbiota development: Still a way to go from descriptive interactions to health-relevant solutions. *Front. Nutr.* **2018**, *5*, 5. [CrossRef] [PubMed]
33. Martin, R.; Langa, S.; Reviriego, C.; Jimenez, E.; Marin, M.L.; Xaus, J.; Fernandez, L.; Rodriguez, J.M. Human milk is a source of lactic acid bacteria for the infant gut. *J. Pediatr.* **2003**, *143*, 754–758. [CrossRef] [PubMed]
34. Perez, P.F.; Dore, J.; Leclerc, M.; Levenez, F.; Benyacoub, J.; Serrant, P.; Segura-Roggero, I.; Schiffrin, E.J.; Donnet-Hughes, A. Bacterial imprinting of the neonatal immune system: Lessons from maternal cells? *Pediatrics* **2007**, *119*, E724–E732. [CrossRef] [PubMed]

35. Rescigno, M.; Urbano, M.; Valzasina, B.; Francolini, M.; Rotta, G.; Bonasio, R.; Granucci, F.; Kraehenbuhl, J.P.; Ricciardi-Castagnoli, P. Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. *Nat. Immunol.* **2001**, *2*, 361–367. [CrossRef] [PubMed]
36. Rootwelt, V.; Reksen, O.; Farstad, W.; Framstad, T. Associations between intrapartum death and piglet, placental, and umbilical characteristics. *J. Anim. Sci.* **2015**, *90*, 4289–4296. [CrossRef] [PubMed]
37. Carney-Hinkle, E.E.; Tran, H.; Bundy, J.W.; Moreno, R.; Miller, P.S.; Burkey, T.E. Effect of dam parity on litter performance, transfer of passive immunity, and progeny microbial ecology. *J. Anim. Sci.* **2013**, *91*, 2885–2893. [CrossRef] [PubMed]
38. Milligan, B.N.; Fraser, D.; Kramer, D.L. Within-litter birth weight variation in the domestic pig and its relation to pre-weaning survival, weight gain, and variation in weaning weights. *Livest. Prod. Sci.* **2002**, *76*, 181–191. [CrossRef]
39. Inman, C.F.; Haverson, K.; Konstantinov, S.R.; Jones, P.H.; Harris, C.; Smidt, H.; Miller, B.; Bailey, M.; Stokes, C. Rearing environment affects development of the immune system in neonates. *Clin. Exp. Immunol.* **2010**, *160*, 431–439. [CrossRef] [PubMed]
40. Chen, L.; Xu, Y.; Chen, X.; Fang, C.; Zhao, L.; Chen, F. The maturing development of gut microbiota in commercial piglets during the weaning transition. *Front. Microbiol.* **2017**, *8*, 1688. [CrossRef]
41. Kim, H.B.; Borewicz, K.; White, B.A.; Singer, R.S.; Sreevatsan, S.; Tu, Z.J.; Isaacson, R.E. Longitudinal investigation of the age-related bacterial diversity in the feces of commercial pigs. *Vet. Microbiol.* **2011**, *153*, 124–133. [CrossRef]
42. Koenig, J.E.; Spor, A.; Scalfone, N.; Fricker, A.D.; Stombaugh, J.; Knight, R.; Angenent, L.T.; Ley, R.E. Succession of microbial consortia in the developing infant gut microbiome. *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 4578–4585. [CrossRef] [PubMed]
43. Palmer, C.; Bik, E.M.; DiGiulio, D.B.; Relman, D.A.; Brown, P.O. Development of the human infant intestinal microbiota. *PLoS. Biol.* **2007**, *5*, 1556–1573. [CrossRef] [PubMed]
44. Arrieta, M.-C.; Stiemsma, L.T.; Dimitriu, P.A.; Thorson, L.; Russell, S.; Yurist-Doutsch, S.; Kuzeljevic, B.; Gold, M.J.; Britton, H.M.; Lefebvre, D.L. Early infancy microbial and metabolic alterations affect risk of childhood asthma. *Sci. Transl. Med.* **2015**, *7*, 307ra152. [CrossRef]
45. Mulligan, C.M.; Friedman, J.E. Maternal modifiers of the infant gut microbiota: Metabolic consequences. *J. Endocrinol.* **2017**, *235*, R1–R12. [CrossRef] [PubMed]
46. Fjalstad, J.W.; Esaiassen, E.; Juvet, L.K.; van den Anker, J.N.; Klingenberg, C. Antibiotic therapy in neonates and impact on gut microbiota and antibiotic resistance development: A systematic review. *J. Antimicrob. Chemother.* **2018**, *73*, 569–580. [CrossRef] [PubMed]
47. Willing, B.P.; Gill, N.; Finlay, B.B. The role of the immune system in regulating the microbiota. *Gut Microbes* **2010**, *1*, 213–223. [CrossRef] [PubMed]
48. Wlodarska, M.; Willing, B.; Keeney, K.M.; Menendez, A.; Bergstrom, K.S.; Gill, N.; Russell, S.L.; Vallance, B.A.; Finlay, B.B. Antibiotic treatment alters the colonic mucus layer and predisposes the host to exacerbated citrobacter rodentium-induced colitis. *Infect. Immun.* **2011**, *79*, 1536–1545. [CrossRef] [PubMed]
49. Rodriguez, J.; Jordan, S.; Mutic, A.; Thul, T. The neonatal microbiome implications for neonatal intensive care unit nurses. *Am. J. Matern. Child. Nurs.* **2017**, *42*, 332–337. [CrossRef] [PubMed]
50. Tang, W.H.W.; Kitai, T.; Hazen, S.L. Gut microbiota in cardiovascular health and disease. *Circ. Res.* **2017**, *120*, 1183–1196. [CrossRef] [PubMed]
51. Vangay, P.; Ward, T.; Gerber, J.S.; Knights, D. Antibiotics, pediatric dysbiosis, and disease. *Cell Host Microbe* **2015**, *17*, 553–564. [CrossRef] [PubMed]
52. Looft, T.; Johnson, T.A.; Allen, H.K.; Bayles, D.O.; Alt, D.P.; Stedtfeld, R.D.; Sul, W.J.; Stedtfeld, T.M.; Chai, B.L.; Cole, J.R.; et al. In-feed antibiotic effects on the swine intestinal microbiome. *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 1691–1696. [CrossRef] [PubMed]
53. Kim, H.B.; Borewicz, K.; White, B.A.; Singer, R.S.; Sreevatsan, S.; Tu, Z.J.; Isaacson, R.E. Microbial shifts in the swine distal gut in response to the treatment with antimicrobial growth promoter, tylosin. *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 15485–15490. [CrossRef] [PubMed]
54. Cong, X.M.; Henderson, W.A.; Graf, J.; McGrath, J.M. Early life experience and gut microbiome the brain-gut-microbiota signaling system. *Adv. Neonatal Care* **2015**, *15*, 314–323. [CrossRef] [PubMed]
55. Cryan, J.F.; Dinan, T.G. Mind-altering microorganisms: The impact of the gut microbiota on brain and behaviour. *Nat. Rev. Neurosci.* **2012**, *13*, 701–712. [CrossRef] [PubMed]

56. O'Mahony, S.M.; Marchesi, J.R.; Scully, P.; Codling, C.; Ceolho, A.M.; Quigley, E.M.M.; Cryan, J.F.; Dinan, T.G. Early life stress alters behavior, immunity, and microbiota in rats: Implications for irritable bowel syndrome and psychiatric illnesses. *Biol. Psychiatry* **2009**, *65*, 263–267. [CrossRef] [PubMed]
57. Bailey, M.T.; Dowd, S.E.; Galley, J.D.; Hufnagle, A.R.; Allen, R.G.; Lyte, M. Exposure to a social stressor alters the structure of the intestinal microbiota: Implications for stressor-induced immunomodulation. *Brain Behav. Immun.* **2011**, *25*, 397–407. [CrossRef] [PubMed]
58. Heo, J.M.; Opapeju, F.O.; Pluske, J.R.; Kim, J.C.; Hampson, D.J.; Nyachoti, C.M. Gastrointestinal health and function in weaned pigs: A review of feeding strategies to control post-weaning diarrhoea without using in-feed antimicrobial compounds. *J. Anim. Physiol. Anim. Nutr.* **2013**, *97*, 207–237. [CrossRef] [PubMed]
59. Nadeau, E.; Fairbrother, J.M.; Zentek, J.; Belanger, L.; Tremblay, D.; Tremblay, C.L.; Rohe, I.; Vahjen, W.; Brunelle, M.; Hellmann, K.; et al. Efficacy of a single oral dose of a live bivalent e. Coli vaccine against post-weaning diarrhea due to f4 and f18-positive enterotoxigenic e. Coli. *Vet. J.* **2017**, *226*, 32–39. [CrossRef] [PubMed]
60. Capozzalo, M.M.; Kim, J.C.; Htoo, J.K.; de Lange, C.F.M.; Mullan, B.P.; Hansen, C.F.; Resink, J.W.; Pluske, J.R. Pigs experimentally infected with an enterotoxigenic strain of escherichia coli have improved feed efficiency and indicators of inflammation with dietary supplementation of tryptophan and methionine in the immediate post-weaning period. *Anim. Prod. Sci.* **2017**, *57*, 935–947. [CrossRef]
61. Chase, C.C.L. Enteric immunity happy gut, healthy animal. *Vet. Clin. N. Am. Food Anim. Pract.* **2018**, *34*, 1–18. [CrossRef] [PubMed]
62. Santos, J.; Yang, P.C.; Soderholm, J.D.; Benjamin, M.; Perdue, M.H. Role of mast cells in chronic stress induced colonic epithelial barrier dysfunction in the rat. *Gut* **2001**, *48*, 630–636. [CrossRef] [PubMed]
63. Bravo, J.A.; Forsythe, P.; Chew, M.V.; Escaravage, E.; Savignac, H.M.; Dinan, T.G.; Bienenstock, J.; Cryan, J.F. Ingestion of lactobacillus strain regulates emotional behavior and central gaba receptor expression in a mouse via the vagus nerve. *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 16050–16055. [CrossRef] [PubMed]
64. Sudo, N.; Chida, Y.; Aiba, Y.; Sonoda, J.; Oyama, N.; Yu, X.N.; Kubo, C.; Koga, Y. Postnatal microbial colonization programs the hypothalamic–pituitary–adrenal system for stress response in mice. *J. Physiol.* **2004**, *558*, 263–275. [CrossRef] [PubMed]
65. Messaoudi, M.; Lalonde, R.; Violle, N.; Javelot, H.; Desor, D.; Nejdi, A.; Bisson, J.F.; Rougeot, C.; Pichelin, M.; Cazaubiel, M.; et al. Assessment of psychotropic-like properties of a probiotic formulation (lactobacillus helveticus r0052 and bifidobacterium longum r0175) in rats and human subjects. *Br. J. Nutr.* **2011**, *105*, 755–764. [CrossRef] [PubMed]
66. Zijlmans, M.A.C.; Korpela, K.; Riksen-Walraven, J.M.; de Vos, W.M.; de Weerth, C. Maternal prenatal stress is associated with the infant intestinal microbiota. *Psychoneuroendocrinology* **2015**, *53*, 233–245. [CrossRef] [PubMed]
67. Gur, T.; Rajasekera, T.; Vadodkar, A.; Allen, J.; Bailey, M. Prenatal stress leads to sex-specific changes in behavior, inflammation, and serotonergic dysfunction: Relevance to psychiatric disorders. *Biol. Psychiatry* **2018**, *83*, S85. [CrossRef]
68. Wang, C.; Shi, C.; Zhang, Y.; Song, D.; Lu, Z.; Wang, Y. Microbiota in fermented feed and swine gut. *Appl. Microbiol. Biotechnol.* **2018**, *102*, 2941–2948. [CrossRef] [PubMed]
69. Leser, T.D.; Lindecrona, R.H.; Jensen, T.K.; Jensen, B.B.; Moller, K. Changes in bacterial community structure in the colon of pigs fed different experimental diets and after infection with brachyspira hyodysenteriae. *Appl. Environ. Microbiol.* **2000**, *66*, 3290–3296. [CrossRef] [PubMed]
70. Tilocca, B.; Burbach, K.; Heyer, C.M.E.; Hoelzle, L.E.; Mosenthin, R.; Stefanski, V.; Camarinha-Silva, A.; Seifert, J. Dietary changes in nutritional studies shape the structural and functional composition of the pigs' fecal microbiome-from days to weeks. *Microbiome* **2017**, *5*, 144. [CrossRef] [PubMed]
71. Heinritz, S.N.; Mosenthin, R.; Weiss, E. Use of pigs as a potential model for research into dietary modulation of the human gut microbiota. *Nutr. Res. Rev.* **2013**, *26*, 191–209. [CrossRef] [PubMed]
72. Mach, N.; Berri, M.; Estelle, J.; Levenez, F.; Lemonnier, G.; Denis, C.; Leplat, J.J.; Chevaleyre, C.; Billon, Y.; Dore, J.; et al. Early-life establishment of the swine gut microbiome and impact on host phenotypes. *Environ. Microbiol. Rep.* **2015**, *7*, 554–569. [CrossRef] [PubMed]

73. Marques, F.Z.; Nelson, E.; Chu, P.Y.; Horlock, D.; Fiedler, A.; Ziemann, M.; Tan, J.K.; Kuruppu, S.; Rajapakse, N.W.; El-Osta, A.; et al. High-fiber diet and acetate supplementation change the gut microbiota and prevent the development of hypertension and heart failure in hypertensive mice. *Circulation* **2017**, *135*, 964–977. [CrossRef] [PubMed]
74. Heo, J.M.; Kim, J.C.; Hansen, C.F.; Mullan, B.P.; Hampson, D.J.; Pluske, J.R. Feeding a diet with decreased protein content reduces indices of protein fermentation and the incidence of postweaning diarrhea in weaned pigs challenged with an enterotoxigenic strain of escherichia coli. *J. Anim. Sci.* **2009**, *87*, 2833–2843. [CrossRef] [PubMed]
75. Yoo, B.B.; Mazmanian, S.K. The enteric network: Interactions between the immune and nervous systems of the gut. *Immunity* **2017**, *46*, 910–926. [CrossRef] [PubMed]
76. Thaiss, C.A.; Zmora, N.; Levy, M.; Elinav, E. The microbiome and innate immunity. *Nature* **2016**, *535*, 65–74. [CrossRef] [PubMed]
77. Bauer, E.; Williams, B.A.; Smidt, H.; Verstengen, M.W.; Mosenthin, R. Influence of the gastrointestinal microbiota on development of the immune system in young animals. *Curr. Issues Intest. Microbiol.* **2006**, *7*, 35–52. [PubMed]
78. Ishiguro, K.; Ando, T.; Maeda, O.; Watanabe, O.; Goto, H. Cutting edge: Tubulin alpha functions as an adaptor in nfat-importin beta interaction. *J. Immunol.* **2011**, *186*, 2710–2713. [CrossRef] [PubMed]
79. Smith, P.M.; Howitt, M.R.; Panikov, N.; Michaud, M.; Gallini, G.A.; Bohlooly, M.; Glickman, J.N.; Garrett, W.S. The microbial metabolites, short-chain fatty acids, regulate colonic treg cell homeostasis. *Science* **2013**, *341*, 569–573. [CrossRef] [PubMed]
80. Levison, M.E. Effect of colon flora and short-chain fatty-acids on growth in-vitro of pseudomonas-aeruginosa and enterobacteriaceae. *Infect. Immun.* **1973**, *8*, 30–35. [PubMed]
81. Shin, R.; Suzuki, M.; Morishita, Y. Influence of intestinal anaerobes and organic acids on the growth of enterohaemorrhagic escherichia coli o157: H7. *J. Med. Microbiol.* **2002**, *51*, 201–206. [CrossRef] [PubMed]
82. Chen, W.; Mi, J.; Lv, N.; Gao, J.; Cheng, J.; Wu, R.; Ma, J.; Lan, T.; Liao, X. Lactation stage-dependency of the sow milk microbiota. *Front Microbiol* **2018**, *9*, 945. [CrossRef] [PubMed]
83. Lalles, J.P.; Bosi, P.; Smidt, H.; Stokes, C.R. Nutritional management of gut health in pigs around weaning. *Proc. Nutr. Soc.* **2007**, *66*, 260–268. [CrossRef] [PubMed]
84. Dou, S.; Gadonna-Widehem, P.; Rome, V.; Hamoudi, D.; Rhazi, L.; Lakhal, L.; Larcher, T.; Bahi-Jaber, N.; Pinon-Quintana, A.; Guyonvarch, A.; et al. Characterisation of early-life fecal microbiota in susceptible and healthy pigs to post-weaning diarrhoea. *PLoS ONE* **2017**, *12*, e0169851. [CrossRef] [PubMed]
85. Hasan, S.; Junnikkala, S.; Peltoniemi, O.; Paulin, L.; Lyyski, A.; Vuorenmaa, J.; Oliviero, C. Dietary supplementation with yeast hydrolysate in pregnancy influences colostrum yield and gut microbiota of sows and piglets after birth. *PLoS ONE* **2018**, *13*, e0197586. [CrossRef] [PubMed]
86. Coppa, G.V.; Bruni, S.; Morelli, L.; Soldi, S.; Gabrielli, O. The first prebiotics in humans—Human milk oligosaccharides. *J. Clin. Gastroenterol.* **2004**, *38*, S80–S83. [CrossRef] [PubMed]
87. Kalliomaki, M.; Salminen, S.; Arvilommi, H.; Kero, P.; Koskinen, P.; Isolauri, E. Probiotics in primary prevention of atopic disease: A randomised placebo-controlled trial. *Lancet* **2001**, *357*, 1076–1079. [CrossRef]
88. Roselli, M.; Pieper, R.; Rogel-Gaillard, C.; de Vries, H.; Bailey, M.; Smidt, H.; Lauridsen, C. Immunomodulating effects of probiotics for microbiota modulation, gut health and disease resistance in pigs. *Anim. Feed Sci. Technol.* **2017**, *233*, 104–119. [CrossRef]
89. Hayakawa, T.; Masuda, T.; Kurosawa, D.; Tsukahara, T. Dietary administration of probiotics to sows and/or their neonates improves the reproductive performance, incidence of post-weaning diarrhea and histopathological parameters in the intestine of weaned piglets. *Anim. Sci. J.* **2016**, *87*, 1501–1510. [CrossRef] [PubMed]
90. Brandt, L.J.; Aroniadis, O.C. An overview of fecal microbiota transplantation: Techniques, indications, and outcomes. *Gastrointest. Endosc.* **2013**, *78*, 240–249. [CrossRef] [PubMed]
91. Bakken, J.S.; Borody, T.; Brandt, L.J.; Brill, J.V.; Demarco, D.C.; Franzos, M.A.; Kelly, C.; Khoruts, A.; Louie, T.; Martinelli, L.P.; et al. Treating clostridium difficile infection with fecal microbiota transplantation. *Clin. Gastroenterol. Hepatol.* **2011**, *9*, 1044–1049. [CrossRef] [PubMed]
92. Borody, T.J.; Khoruts, A. Fecal microbiota transplantation and emerging applications. *Nat. Rev. Gastroenterol. Hepatol.* **2012**, *9*, 88–96. [CrossRef] [PubMed]

93. Hu, L.S.; Geng, S.J.; Li, Y.; Cheng, S.S.; Fu, X.F.; Yue, X.J.; Han, X.Y. Exogenous fecal microbiota transplantation from local adult pigs to crossbred newborn piglets. *Front. Microbiol.* **2018**, *8*, 2663. [CrossRef] [PubMed]
94. Xiao, Y.; Yan, H.L.; Diao, H.; Yu, B.; He, J.; Yu, J.; Zheng, P.; Mao, X.B.; Luo, Y.H.; Chen, D.W. Early gut microbiota intervention suppresses dss-induced inflammatory responses by deactivating tlr/nlr signalling in pigs. *Sci. Rep.* **2017**, *7*, 3224. [CrossRef] [PubMed]
95. McCormack, U.M.; Curiao, T.; Wilkinson, T.; Metzler-Zebeli, B.U.; Reyer, H.; Ryan, T.; Calderon-Diaz, J.A.; Crispie, F.; Cotter, P.D.; Creevey, C.J.; et al. Fecal microbiota transplantation in gestating sows and neonatal offspring alters lifetime intestinal microbiota and growth in offspring. *mSystems* **2018**, *3*, e00134-17. [CrossRef] [PubMed]

Chapter 2:

Faecal microbiota transplantation: is it the future for pig production?

Statement of Authorship

Title of Paper	Faecal microbiota transplantation: is it the future for pig production?
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Nowland, T. L., and R. N. Kirkwood. 2020. Faecal microbiota transplantation: is it the future for pig production? *Microbiology Australia* 41(2):91-94. doi: 10.1071/MA20023

Principal Author

Name of Principal Author (Candidate)	Tanya Nowland
Contribution to the Paper	Investigation of the literature, collation and interpretation of relevant information, writing and drafting of the paper.
Overall percentage (%)	90%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 16/02/2021

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Roy Kirkwood
Contribution to the Paper	Evaluated and edited the manuscript.
Signature	Date 25/2/21

Please cut and paste additional co-author panels here as required.

Faecal microbiota transplantation: is it the future for pig production?

Tanya L Nowland

School of Animal and Veterinary Sciences, The University of Adelaide Roseworthy, SA 5400, Australia
Tel.: +61 8 8313 7664
Email: tanya.nowland@adelaide.edu.au

Roy N Kirkwood

School of Animal and Veterinary Sciences, The University of Adelaide Roseworthy, SA 5400, Australia

Abstract. Piglet mortality is a major issue for the pork industry globally and until recently, the main method for improving growth performance and reducing disease in commercial practice is centred on anti-microbial use. Antibiotic resistance is a global concern and, as such, animal production industries are seeking alternatives to antibiotics. Different approaches under investigation include but are not limited to management of the intestinal microbial environment. The gastrointestinal microbiota is involved in a myriad of processes that impact host health and well-being. Recently, interest in maintaining a healthy microbiome in order to improve herd health is increasing. In this article, we focus on faecal microbiota transplantation as a method for manipulating and improving the gastrointestinal microbiota in pigs in order to improve health and performance.

Currently, 11–15% of all piglets born alive die prior to weaning within the pork industry globally[1–3]. This represents a major welfare concern and economic loss to industry. To date, much research has gone into reducing this loss but with varied success. The current management methods for reducing piglet mortality caused by sickness, such as diarrhoea, and improving growth performance in weaned pigs, is the administration of antibiotics, with their use often being both therapeutic and prophylactic. Organisations such as the World Health Organization, the US Centres for Disease Control and Prevention, and the European Centre for Disease Prevention and Control have identified antibiotic resistance as a global concern, as what were once common treatable infections are now becoming life threatening[4]. As such, alternatives to antibiotics need to be explored.

The intestinal tract houses a community of microorganisms that has a mutualistic relationship with the host, known as the enteric microbiome[5]. These microorganisms include bacteria, fungi, archaea, protozoa and viruses[6–8]. The enteric microbiome is involved in a myriad of processes, some of which include immune system maintenance and development, intestinal barrier function, nutrient metabolism and competitive exclusion of pathogens[8–10]. While antibiotics are effective in pathogen removal, they also impact the commensal microbiome[11]. If a healthy microbiome is maintained, the need for therapeutic interventions such as antibiotic administration will be reduced as the animal will be better equipped to cope with external stressors. This is where the interest surrounding methods for influencing the microbiome, through management such as pre- and pro-biotics and faecal microbiota transfers, has expanded.

In particular, one such method that has demonstrated efficacy in treating *Clostridium difficile* infections in humans is faecal microbiota transplantation (FMT). FMT involves the transfer of faeces from a healthy donor into the gastrointestinal tract of a recipient. This can be done either orally (Figure 1) or rectally via an enema[12]. The objective being that the beneficial bacteria within the healthy donors' faeces will competitively exclude the pathogenic bacteria within the unhealthy or sick recipient, therefore altering the microbiota and in the case of *C. difficile* infections, treating the disease[12] (Figure 2). This method can also be used for altering the microbiota of the recipient to resemble that of the donor for the

Figure 1. Oral administration of faecal microbiome transplantation via a gastric tube to a 20-day-old piglet.

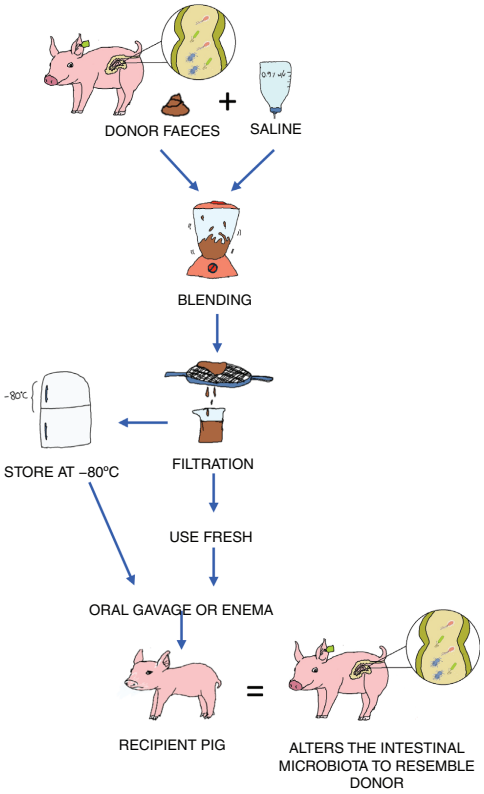


Figure 2. Schematic of faecal microbiota transplantation (FMT) in pigs.

objective of creating a phenotypic change[13]. FMT was first described by Ge Hong in 4th century China for the treatment of food poisoning and severe diarrhoea[12]. Today, FMT is commonly known for its efficacy for the treatment of *C. difficile* infections in humans. FMT has demonstrated a success rate of >90% in patients with reoccurring *C. difficile* where antibiotic use has been unsuccessful due to the formation of spores[14]. The use of FMT in other areas of human health and disease prevention are becoming increasingly popular; however, its efficacy in treating other diseases in humans to date is not as high. Although this is the case, investigation into its use within production animals such as pigs is increasing.

Recent studies investigating its use in pig production have shown promising but inconsistent results. Several research groups have demonstrated that the administration of multiple oral FMT to piglets from birth can increase average daily gain, reduce the incidence of diarrhoea and improve intestinal barrier and immune system function[15–18]. However, in contrast to this, others demonstrated a negative effect on intestinal integrity and growth when piglets received FMT directly or were reared on sows receiving FMT[19,20]. When examining the human literature, where additional phenotypic traits were transferred with FMT that mimicked the donor, it is evident that the donor used can significantly impact the results observed[21]. As such, particular care needs to be taken when selecting the appropriate donor as the risk of transferring undesirable traits is high. Further, Niederwerder *et al.*[22] found that FMT was an effective preventative effect against porcine circovirus associated disease in pigs co-infected with porcine circovirus type-2 and porcine reproductive and respiratory syndrome virus. The pigs that received one dose of FMT daily for seven days following weaning from healthy donor sows had a significant reduction in morbidity and mortality and increased antibody levels.

Studies where FMT in pigs was employed as a research model for humans have also found promising results that not only provide evidence for its effects on enteric microbiota modulation but also host metabolism. Wan *et al.*[23] demonstrated that oral FMT from 1 to 6 days of age reduced fatty acid oxidative catabolism and amino acid biosynthesis of piglets. Additionally, Brunse *et al.*[24] observed that rectal FMT from 10-day-old donor pigs to caesarean-derived preterm piglets changed their colonic carbohydrate metabolism from lactate to propionate production, increasing colonic pH. Rectal FMT also preserved goblet cell mucin stores and reduced the incidence of necrotizing enterocolitis. When comparing routes for FMT, it has been noted that when combining oral and rectal transplantation, piglet mortality increased. Conversely, those that

received only rectal administration did not suffer the same problems[24]. Further supporting the findings of the previous studies, Geng *et al.*[25] demonstrated that FMT reduced susceptibility to epithelial injury and modulated tryptophan metabolism in a piglet inflammatory bowel disease model. When taken collectively, it is evident that FMT in pigs not only alters microbial membership but also has effects on host metabolism, intestinal barrier function and the immune system.

Although FMT is a promising prospect it is not commercially applicable in its current form, with most studies administering multiple doses for 1–2 weeks in order to demonstrate an effect and fasting or stomach acid reduction protocols in place to improve post-gastric bacterial survival. Recently, our research group identified that the administration of a single FMT dose at weaning resulted in durable changes to 35 days of age (14 days post FMT) (TL Nowland *et al*., unpubl. data). To our knowledge, this is the first study to demonstrate changes to the microbiome of piglets after a single dose of FMT. However, whether this is possible in a younger pig and whether it lasts long term is yet to be determined. Additionally, some scepticism surrounds the use of FMT commercially due to the biosecurity risk that it entails as rigorous testing is needed in order to prevent the transfer of diseases[13]. If FMT is being considered in pigs for the treatment of a disease, then it is likely that the recipients are sick and probably relatively immunocompromised. Thus, the risk from possible transfer of pathogens will be increased. However, a possible refinement to FMT to minimise this risk is suggested by the work of Hu *et al.*[18]. These authors used a native Chinese pig breed with increased resistance to stress-induced diarrhoea to determine the identity of specific bacteria involved in this resistance. Such a targeted approach to disease control would have a major advantage over the 'shot gun' approach of conventional FMT. It is evident that research surrounding the use of FMT within pig production is still in its infancy. Although, an increasing number of studies are investigating the use of FMT as a tool for increasing growth, feed efficiency and treating enteric diseases in pigs, there is still a long way to go before it will be applicable to industry.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

The authors acknowledge the contributions to the project by Professor Mary Barton and Sophia Ward and the University of Adelaide and Australian Pork Limited for their support of this research.

References

1. Daigle, C. (2018) Parallels between postpartum disorders in humans and pre-weaning piglet mortality in sows. *Animals (Basel)* **8**, 22. doi:10.3390/ani8020022
2. Mota-Rojas, D. *et al*. (2012) Animal welfare in the newborn piglet: a review. *Vet. Med.* **57**, 338–349. doi:10.17221/6262-VETMED
3. Nuntapaitoon, M. *et al*. (2018) Newborn traits associated with pre-weaning growth and survival in piglets. *Asian-Australas. J. Anim. Sci.* **31**, 237–244. doi:10.5713/ajas.16.0962
4. Roca, I. *et al*. (2015) The global threat of antimicrobial resistance: science for intervention. *New Microbes New Infect.* **6**, 22–29. doi:10.1016/j.nmni.2015.02.007
5. Young, V.B. (2017) The role of the microbiome in human health and disease: an introduction for clinicians. *BMJ* **356**, j831. doi:10.1136/bmj.j831
6. Abeles, S.R. and Pride, D.T. (2014) Molecular bases and role of viruses in the human microbiome. *J. Mol. Biol.* **426**, 3892–3906. doi:10.1016/j.jmb.2014.07.002
7. Hallen-Adams, H.E. and Suhr, M.J. (2017) Fungi in the healthy human gastrointestinal tract. *Virulence* **8**, 352–358. doi:10.1080/21505594.2016.1247140
8. Cahenzli, J. *et al*. (2013) Intestinal microbial diversity during early-life colonization shapes long-term IgE levels. *Cell Host Microbe* **14**, 559–570. doi:10.1016/j.chom.2013.10.004
9. Gensollen, T. *et al*. (2016) How colonization by microbiota in early life shapes the immune system. *Science* **352**, 539–544. doi:10.1126/science.aad9378
10. Wang, M. *et al*. (eds) (2016) Impact of early gut microbiota on immune and metabolic development and function. Seminars in Fetal and Neonatal Medicine.
11. Looft, T. *et al*. (2014) Bacteria, phages and pigs: the effects of in-feed antibiotics on the microbiome at different gut locations. *ISME J.* **8**, 1566–1576. doi:10.1038/ismej.2014.12
12. Brandt, L.J. and Aroniadis, O.C. (2013) An overview of fecal microbiota transplantation: techniques, indications, and outcomes. *Gastrointest. Endosc.* **78**, 240–249. doi:10.1016/j.gie.2013.03.1329
13. Canibe, N. *et al*. (2019) Potential relevance of pig gut content transplantation for production and research. *J. Anim. Sci. Biotechnol.* **10**, 55. doi:10.1186/s40104-019-0363-4
14. Bakken, J.S. *et al*. (2011) Treating clostridium difficile infection with fecal microbiota transplantation. *Clin. Gastroenterol. Hepatol.* **9**, 1044–1049. doi:10.1016/j.cgh.2011.08.014
15. Cheng, C.S. *et al*. (2019) Early intervention with faecal microbiota transplantation: an effective means to improve growth performance and the intestinal development of suckling piglets. *Animal* **13**, 533–541. doi:10.1017/S1751731118001611
16. Hu, L. *et al*. (2018) Exogenous fecal microbiota transplantation from local adult pigs to crossbred newborn piglets. *Front. Microbiol.* **8**, 2663. doi:10.3389/fmicb.2017.02663
17. Xiao, Y. *et al*. (2017) Early gut microbiota intervention suppresses DSS-induced inflammatory responses by deactivating TLR/NLR signalling in pigs. *Sci. Rep.* **7**, 3224. doi:10.1038/s41598-017-03161-6
18. Hu, J. *et al*. (2018) A microbiota-derived bacteriocin targets the host to confer diarrhea resistance in early-weaned piglets. *Cell Host Microbe* **24**, 817–832.e8. doi:10.1016/j.chom.2018.11.006
19. McCormack, U.M. *et al*. (2018) Fecal microbiota transplantation in gestating sows and neonatal offspring alters lifetime intestinal microbiota and growth in offspring. *mSystems* **3**, e00134-17. doi:10.1128/mSystems.00134-17
20. McCormack, U.M. *et al*. (2019) Improvement of feed efficiency in pigs through microbial modulation via fecal microbiota transplantation in sows and dietary supplementation of inulin in offspring. *Appl. Environ. Microbiol.* **85**, e01255-19. doi:10.1128/AEM.01255-19
21. Maruvada, P. *et al*. (2017) The human microbiome and obesity: moving beyond associations. *Cell Host Microbe* **22**, 589–599. doi:10.1016/j.chom.2017.10.005
22. Niederwerder, M.C. *et al*. (2018) Fecal microbiota transplantation is associated with reduced morbidity and mortality in porcine circovirus associated disease. *Front. Microbiol.* **9**, 1631. doi:10.3389/fmicb.2018.01631
23. Wan, J.J. *et al*. (2019) Effects of early intervention with maternal fecal bacteria and antibiotics on liver metabolome and transcription in neonatal pigs. *Front. Physiol.* **10**, 171. doi:10.3389/fphys.2019.00171

24. Brunse, A. *et al*. (2019) Effect of fecal microbiota transplantation route of administration on gut colonization and host response in preterm pigs. *ISME J.* **13**, 720–733. doi:10.1038/s41396-018-0301-z

25. Geng, S. *et al*. (2018) Faecal microbiota transplantation reduces susceptibility to epithelial injury and modulates tryptophan metabolism of the microbial community in a piglet model. *J. Crohns Colitis* **12**, 1359–1374. doi:10.1093/ecco-jcc/jjy103

Biographies

Tanya Nowland is a PhD student at the University of Adelaide, Australia. Her research focuses on how the intestinal microbiome impacts piglet health and survival. Specifically, focusing on the development of non-antimicrobial industry applicable practices to help support healthy microbiome development and improve preweaning performance.

Roy Kirkwood is an Associate Professor in swine production medicine whose research areas include sow reproductive management and impacts on piglet health and survival. In particular, the involvement of microbiome structure on production outcomes is a current focus area.

Chapter 3:

Characterisation of early microbial colonisers within the spiral colon of pre- and post-natal piglets

Statement of Authorship

Title of Paper	Characterisation of early microbial colonisers within the spiral colon of pre- and post-natal piglets
Publication Status	☒ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Nowland TL, Kirkwood RN, Torok VA, Plush KJ and Barton MD 2021. Characterisation of early microbial colonisers within the spiral colon of pre- and post-natal piglets. Life 11, 312. Doi: 10.3390/life11040312.

Principal Author

Name of Principal Author (Candidate)	Tanya Nowland
Contribution to the Paper	Assisted with the experimental design, managed and carried out the experimental trial, analysed statistics, drafted and edited the manuscript.
Overall percentage (%)	85%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 16/02/2021

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Mary Barton
Contribution to the Paper	Assisted with the experimental design, data interpretation and editing manuscript.
Signature	Date 25/2/21

Name of Co-Author	Valeria Torok
Contribution to the Paper	Assisted with the experimental design, statistical analysis, data interpretation and editing manuscript.
Signature	Date 25/2/2021

Name of Co-Author	Roy Kirkwood		
Contribution to the Paper	Assisted with the experimental design, data collection and interpretation and editing manuscript.		
Signature		Date	25/2/21

Name of Co-Author	Kate Plush		
Contribution to the Paper	Assisted with the experimental design, evaluated and edited the manuscript.		
Signature		Date	25/2/21

Please cut and paste additional co-author panels here as required.

life

MDPI

Article

Characterisation of Early Microbial Colonisers within the Spiral Colon of Pre- and Post-Natal Piglets

Tanya L. Nowland [1,*], Roy N. Kirkwood [1], Valeria A. Torok [1,2], Kate J. Plush [3] and Mary D. Barton [4]

[1] School of Animal and Veterinary Sciences, The University of Adelaide, Roseworthy, SA 5371, Australia; roy.kirkwood@adelaide.eu.au (R.N.K.); valeria.torok@sa.gov.au (V.A.T.)
[2] South Australian Research and Development Institute, Food Sciences, Urrbrae, SA 5064, Australia
[3] SunPork Group, Murarrie, QLD 4172, Australia; kate.plush@sunporkfarms.com.au
[4] School of Pharmacy and Medical Sciences, University of South Australia, Adelaide, SA 5000, Australia; mary.barton@unisa.edu.au
* Correspondence: tanya.nowland@adelaide.edu.au; Tel.: +61-08-8313-7786

Abstract: Initial enteric microbial colonisation influences animal health and disease, hence an understanding of the first microbial colonisers within the piglet is important. The spiral colon of piglets that were stillborn (n = 20), born-alive (n = 10), and born alive and had sucked (n = 9) were collected from 28 sows to investigate whether initial microbial colonisation occurs pre- or post-partum and how it develops during the first 24 h post-partum. To examine this, DNA was extracted and 16S rRNA amplicon analysis was performed to allow analysis of microbial communities. The results indicate that microbial colonisation of the spiral colon had occurred in stillborn pigs, suggesting microbial exposure prior to birth. Alpha diversity metrics indicated that the number of taxa and community richness were higher in piglets that sucked ($p < 0.001$) and community evenness was lower in stillborns in comparison to born-alive ($p < 0.001$) but was not affected by colostrum consumption ($p < 0.001$). Additionally, when compared with stillborn piglets, the bacteria colonising the spiral colon during the first 24 h post-partum included the potentially pathogenic bacteria *Escherichia coli*, *Clostridium perfringens* and *Clostridium celatum*, and potentially beneficial bacteria *Lactobacillus reutueri* and *Faecalibacterium prausnitzii*. The relative presence of Archaea was high in stillborn piglets but decreased with post-natal environmental exposure. It is evident that stillborn piglets have bacteria present within their spiral colon, however further studies are needed in order to determine the time at which colonisation is initiated and the mechanisms determining how colonisation occurs. Additionally, as expected, the immediate post-natal environment largely influences the microorganisms colonising, while colostrum consumption further contributes to the microbial community enrichment.

Keywords: microbiota; stillborn piglets; bacterial abundance; archaea

Citation: Nowland, T.L.; Kirkwood, R.N.; Torok, V.A.; Plush, K.J.; Barton, M.D. Characterisation of Early Microbial Colonisers within the Spiral Colon of Pre- and Post-Natal Piglets. *Life* **2021**, *11*, 312. https://doi.org/10.3390/life11040312

Academic Editor: Einar Ringø

Received: 12 February 2021
Accepted: 31 March 2021
Published: 2 April 2021

1. Background

The first colonisers within the gastrointestinal tract (GIT) play a determinant role in the health of the host [1,2], therefore it is important to understand when colonisation occurs, and which are the main bacteria involved in early life colonisation. Advances in sequencing technologies have allowed for new observations in understanding the timing of initial microbial colonisation, as neonates were previously thought to be sterile until birth. Research in humans [3], mice [4] and rhesus macaques [5] indicate possible colonisation by bacteria *in utero*, with studies demonstrating the presence of bacteria in the amniotic fluid, placenta and meconium of healthy neonates. Even with these findings it is still a topic of debate with some studies refuting the likelihood of in utero colonisation [6,7]. Additionally, the fact that it is not clear how the microbes colonise these surfaces furthers this notion. Some studies suggest that the maternal oral or intestinal microbes may be selectively transported to the fetal-placental interface as has been shown previously for the transport of bacteria to mammary tissue in humans [3,8,9]. Additionally, studies in

humans have shown that fetuses ingest large amounts of amniotic fluid in late gestation which may aid GIT colonisation [10,11]. Few studies have investigated the bacteria present in the GIT within the first days of life in piglets [12,13]. Early microbial colonisation via the vagina, nipple surface and milk have been documented extensively in humans [14,15] with some work reported in pigs [16]. To our knowledge, no studies have documented microbial colonisation of piglets at any timepoint throughout gestation or in piglets that have not sucked. It is important to identify the first microbes colonising the GIT as it will allow for the planning of nutritional interventions in sows or newborn piglets to increase their survivability, feed efficiency and growth. Evidence suggests that pre and probiotics fed to the sow are effective in altering the microbiota of piglets during lactation [17,18] and so, therefore, the potential to utilise these interventions during gestation to foster the development of an advantageous intestinal microbiota in piglets is of interest. Therefore, microbial colonisation just prior to birth and during the immediate post-natal period was studied using the spiral colon of stillborn piglets and piglets prior to sucking. This study aimed to determine GIT colonisation before and immediately after birth in piglets that had or had not sucked. It was hypothesised that (1) passive transfer of microbes just prior to birth would occur and, therefore, microbes would be present in the spiral colon of stillborn piglets and (2) the composition, abundance and diversity of communities colonising the spiral colon would increase with birth, environmental exposure and the consumption of colostrum.

2. Materials and Methods

2.1. Animals and Experimental Procedures

All procedures were conducted at the University of Adelaide Roseworthy piggery, South Australia, with the approval of the University of Adelaide's Animal Ethics Committee (AEC number: S-2018-092). A total of 39 Large White x Landrace piglets born to 28 sows (parities 3.84 $\pm$ 0.34) were employed in this study over a series of two batches. All sows were group housed throughout gestation and did not receive antibiotics. They received 2.5 kg/d of commercial gestation diet (12.85 MJ DE/kg) throughout gestation. Upon entry into farrowing accommodation sows received a commercial lactation diet (14 MJ DE/kg) at 2.5 kg/d until farrowing, thereafter the feeding level was gradually increased until it reached 7 to 8 kg by day 7 of lactation. All sows had *ad libitum* access to water. Farrowing accommodation consisted of farrowing crates (1.7 m $\times$ 2.4 m) located in rooms that were climate controlled and had fully slatted plastic flooring. Sows were moved into farrowing accommodation five days prior to their expected due date. Sows farrowed naturally and were monitored during staffed hours from 8 a.m. to 3 p.m. On average, the sows gave birth to a total of 14.1 $\pm$ 0.6 piglets per litter, with an average gestation length of 115.2 $\pm$ 0.3 days. When sows were separated into Born-Alive, Sucked and Stillborn groups, no treatment differences existed for total born or gestation length. Of the 39 piglets employed, they consisted of 20 stage II stillborn piglets (stillborn), as defined previously [19], (9 female, 11 male), 10 euthanised or recently crushed 0–1 day old born-alive piglets that had not sucked (born-alive; 5 female, 5 male) and 9 euthanised or recently crushed 0–1 day old live-born but non-viable piglets that had sucked (sucked; 4 female, 5 male). Piglets were classified as stillborn if they had intact cartilaginous tips on their hooves and they had not taken a breath, as indicated by a lung float test. Piglets deemed as non-viable by the farm staff due to piglet size, splay legs or viability were euthanised by blunt force trauma to the skull with immediate exsanguination. The presence or absence of milk in the stomach was used as an indication of whether liveborn piglets had sucked or not. The average weights for piglets in the stillborn, born-alive and sucked groups were 1.03 $\pm$ 0.06 kg, 0.75 $\pm$ 0.04 kg and 1.14 $\pm$ 0.12 kg, respectively. Piglets were placed on ice immediately and transported to the laboratory for dissection within one hour of parturition or post-natal death. Piglets were weighed and cleaned with 70% alcohol and a midline incision was made from the sternum to the pubis. The spiral colon was lifted with sterile forceps and an incision was made at either end to remove it. The spiral colon was used as it was an

easy to identify area within the piglet that was in the lower region of the GIT. Therefore, if ingestion of fluid during parturition occurred, it would not have affected the results. Once dissected, the spiral colon segment was placed into a sterile tube and stored at −80 °C until DNA extraction. Utensils were changed between each incision in order to reduce the likelihood of contamination.

2.2. Extraction of DNA and 16S rRNA Amplicon Sequencing

Total nucleic acid was extracted and purified from freeze dried piglet spiral colon samples by a modification of a South Australian Research and Development Institute proprietary method [20–22]. Approximately 0.9 gm of freeze-dried spiral colon was added to 10 mL of extraction buffer (1.3 M guanidine thiocyanate, 1.5 M $NaCl_2$, 30 mM Tris-HCl, 65 mM phosphate buffer, 3.4% (w/v) sarkosyl and 1.7% (w/v) polyvinylpolypyrrolidone) and incubated for 1 h at 70 °C prior to proceeding with the proprietary extraction method.

PCR amplification and sequencing of the V3-V4 region of the 16S rRNA gene was done by the Australian Genome Research Facility (AGRF) Melbourne node. The V3-V4 region was PCR amplified over 29 cycles using forward primer 341-F (CCTAYGGGRBGCASCAG) and reverse primer 806-R (GGACTACNNGGGTATCTAAT). Amplicon sequencing was done on the illumina MiSeq platform (San Diego, CA, USA) with 2 × 300 bp paired-end chemistry. Both positive and negative controls were used on every plate processed by AGRF. The positive control used was ZymoBIOMICS Microbial Community DNA Standard II (Log Distribution). The obtained reads are available under the accession number PRJNA677620 of the Sequence Read Archive of the National Centre for Biotechnology Information. For bioinformatic analysis of raw sequence data performed by AGRF, the paired-end reads were assembled by aligning the forward and reverse reads using PEAR v0.9.5 [23]. Primers were identified and trimmed. All trimmed sequences were processed using Quantitative Insights into Microbial Ecology (QIIME 1.8.4) [24] and USEARCH v8.0.1623 [25,26] software and the UPARSE pipeline [27]. Using USEARCH tools, sequences were quality filtered, and full-length duplicate sequences were removed and sorted by abundance. Singletons or unique reads in the dataset were discarded. Additionally, chimeric sequences were clustered and removed using "rdp_gold" database as the reference. To obtain the number of reads in each operational taxonomic unit (OTU), reads were mapped back to OTUs with a minimum identity of 97%. Using QIIME, taxonomy was assigned with Greengenes database (version 13.8, August 2013) [28]. All sequences corresponding to mitochondria and chloroplasts were removed.

2.3. Statistical Analysis

The alpha diversity metrics, Shannon diversity (H') index, Pielou's evenness (J') and number of taxa (S), were calculated using DIVERSE (PRIMER6 PRIMER-E Ltd., Ivybridge, UK). Normality was tested within RStudio software (Version 1.1.456, Boston, MA, USA) using the Shapiro–Wilk test. Those alpha diversity metrics that were normally distributed were analysed using an analysis of variance (ANOVA) and those not normally distributed were analysed using the Kruskal–Wallis test, with corrections for multiple tests using false discovery rate (FDR) and a P-value threshold of 0.05. The fixed effects included in the model were group (stillborn, born-alive and sucked) and gender. The gender and the gender x group interaction were not significant ($p > 0.05$) so were removed from the final model.

Multivariate statistical techniques (PRIMER6, PRIMER-E Ltd., Ivybridge, UK) were used to analyse the spiral colon 16S rRNA bacterial taxonomic data. For phyla, family and genus levels species accumulation plots were generated on standardised by total and fourth root transformed data. Plots were generated on permuted (max 999) data. Indices investigated were: S, Chao, Jacknife, Bootstrap, Michaelis Menton and Ugland-Gray-Elligsen. Similarities among colonic bacterial communities of piglets from the 16S rRNA data metrics were analysed using Bray–Curtis measures of similarity [29] following standardisation by total and fourth-root transformation. One-way analysis of similarity (ANOSIM) [30] on

the Bray–Curtis similarity data was used to test if there were significant differences among colonic bacterial communities for piglets that were stillborn, born-alive or had sucked. If the global R statistic was significant ($p \leq 0.05$), then the significance of pairwise R statistics were investigated further. The R statistic value describes the extent of similarity among or between groups, with values close to unity (1) indicating that groups are entirely separate and a zero-value indicating that there is no difference among or between groups. To determine which individual bacterial taxa contributed most to the overall dissimilarity between significant pair-wise comparisons, similarity percentages (SIMPER) [30] analyses were done on the Bray–Curtis dissimilarity data. The percentage contributions (%) of significant taxa (average dissimilarity/standard deviation > 1) to the average dissimilarities were calculated. Non-metric multidimensional scaling (nMDS) [31,32] on Bray–Curtis similarity data was done to graphically illustrate relationships among the groups.

3. Results

Across all 39 samples, the total number of sequenced reads were 8,772,894, of which 4,648,730 reads were retained after quality control with an average of 119,198 sequenced reads per spiral colon sample. The number of reads clustered into OTUs were 4281. Species accumulation curves and richness indices of the bacterial communities in the spiral colon of piglets in the stillborn, born-alive and born-alive and sucked groups were performed. The calculated species accumulation indices reached an asymptote after ~9 samples, indicating that this number of samples allowed for the detection of most bacterial genera present and that the number of replicates per treatment were sufficient for statistical analysis (Supplementary Figure S1).

Alpha diversity metrics showed that the number of taxa and community richness were higher in piglets that sucked when compared with stillborn and born-alive animals ($p < 0.001$; Figure 1A,C). The community evenness was lower in stillborns in comparison to born-alive ($p < 0.001$; Figure 1B), but not effected by colostrum consumption (Pielou's evenness, $p < 0.001$; Figure 1B). Colonic bacterial genera significantly differed among piglets (ANOSIM, Global R = 0.552, $p = 0.001$), with significant pairwise difference between stillborn and those that had sucked (R = 0.804, $p = 0.001$), and stillborn and born-alive piglets (R = 0.441, $p = 0.001$). Piglets that were born-alive and either had or had not sucked did not differ (R = 0.103, $p = 0.112$). Non-metric multidimensional scaling (nMDS), on the Bray–Curtis similarity taxonomic data, generated an ordination where the closer samples are together in space, the more similar their GIT microbial communities are. This shows that the population composition of bacteria in the born-alive and sucked piglets differed from that of stillborn piglets. Furthermore, the born-alive and sucked piglets showed a highly diverse community composition, with each of these groups showing a distinct division; with some piglets grouping closer to the stillborn piglets and others not (Figure 2). At genus level, stillborn, born-alive and sucked piglets showed GIT microbial community similarities of 66%, 42% and 41%, respectively.

As shown in Figure 3, a total of 11 phyla were identified from all samples. The relative abundance of Proteobacteria remained stable as external exposure increased. Proportionally, Unclassified Archaea and Unclassified Bacteria decreased with external exposure. The abundance of Firmicutes gradually increased with external exposure while Actinobacteria, which were present in very small amounts in stillborn animals (0.85%), increased after the ingestion of milk, going from 0.97% in piglets that were born-alive to 7% in those that had sucked (Figure 3). At phylum level, the average dissimilarity between stillborn piglets and those that were born-alive and those that had sucked were 25% and 31%, respectively. The main phyla driving the differences between stillborn piglets and born-alive piglets were Firmicutes, Bacteroidetes, Unclassified Archaea, Crenarchaeota and Euryarchaeota, while those phyla driving the differences between stillborn piglets and those that had sucked were Unclassified Bacteria, Firmicutes, Actinobacteria, Unclassified Archaea, Crenarchaeota, Proteobacteria and Euryarchaeota.

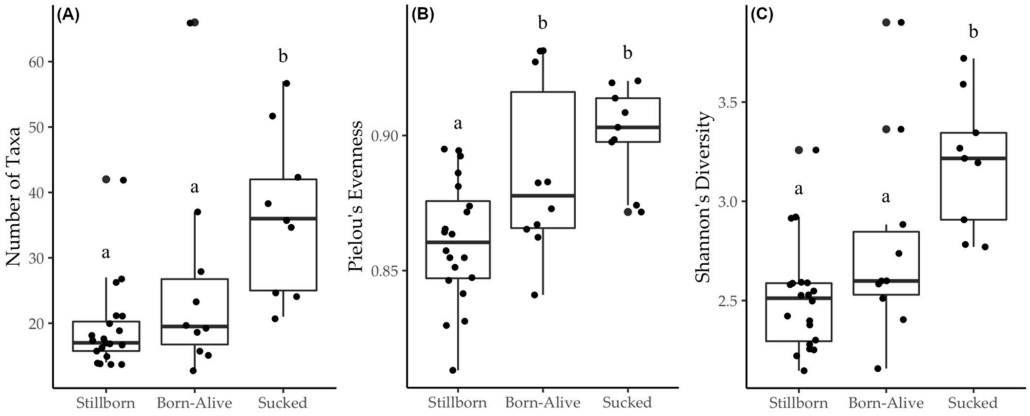


Figure 1. Boxplots demonstrating the change at genus level in (**A**) the number of taxa, (**B**) Pielou's evenness, and (**C**) Shannon's diversity for piglets that were stillborn, born-alive or had sucked. When subscripts differ, they denote a significant difference between treatments ($p < 0.001$).

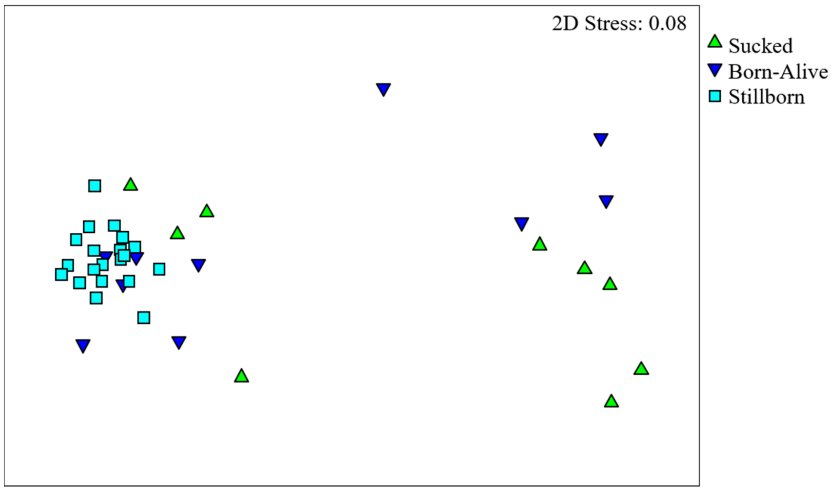


Figure 2. nMDS ordination showing the relationship of colonic bacterial genera from piglets that were stillborn (square), born-alive (inverted triangle) or had sucked (triangle), calculated using Bray–Curtis distances. Points on the ordination represent individual piglet samples which are positioned based on their similarity to all other samples in a two-dimensional space. The closer the samples are together in the ordination space, the more similar are their GIT microbial communities based on taxa composition and abundance.

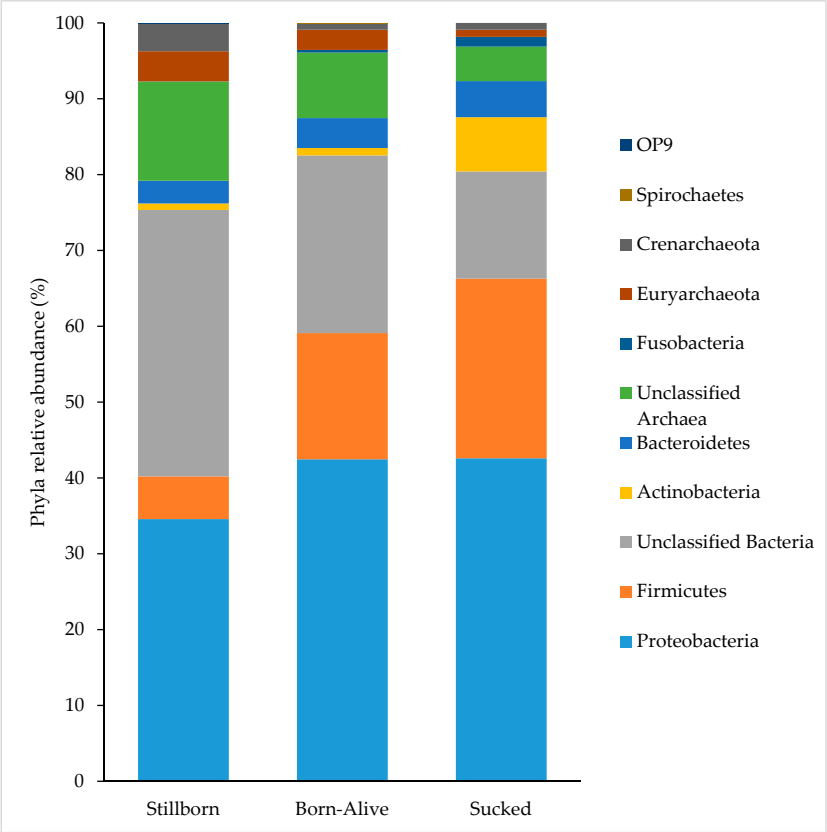


Figure 3. Abundance of microbial phyla present in the spiral colon of piglets that were stillborn, born-alive or those that had sucked. The bacterial phyla within the legend are arranged in the same order as they appear on the bar chart.

The dominant families identified in the spiral colon of piglets that were stillborn, born-alive or had sucked are shown in Figure 4. As post-natal exposure increased, the proportion of Unclassified Bacteria decreased from 27% to 21% in piglets that were born-alive and 10% in those that had sucked. Additionally, Unclassified Proteobacteria, Unclassified Alphaproteobacteria and Unclassified Archaea decreased with environmental exposure, while *Enterobacteriaceae* and *Clostridiaceae* increased with environmental exposure (Figure 4). At the family level, the average dissimilarity between piglets that were stillborn and those that were born-alive was 48%. Of the taxa that could be identified to family level, *Pseudomonadaceae* and *Bacteroidaceae* were significantly more abundant in those that were stillborn, while *Enterobacteriaceae*, *Clostridiaceae*, *Ruminococcaceae* and *Lachnospiraceae* were significantly more abundant in animals that were born-alive. When comparing stillborn piglets to animals that had sucked, the average dissimilarity at the family level was 60%. Of the taxa that could be identified to the family level, *Pseudomonadaceae* and *Ruminococcaceae* were more abundant in animals that were stillborn while *Enterobacteriaceae*, *Clostridiaceae*, *Pasteurellaceae*, *Streptococcaceae*, *Moraxellaceae*, *Lachnospiraceae*, *Lactobacillaceae*, *Micrococcaceae* and *Peptostreptococcaceae* were more abundant in animals that had sucked.

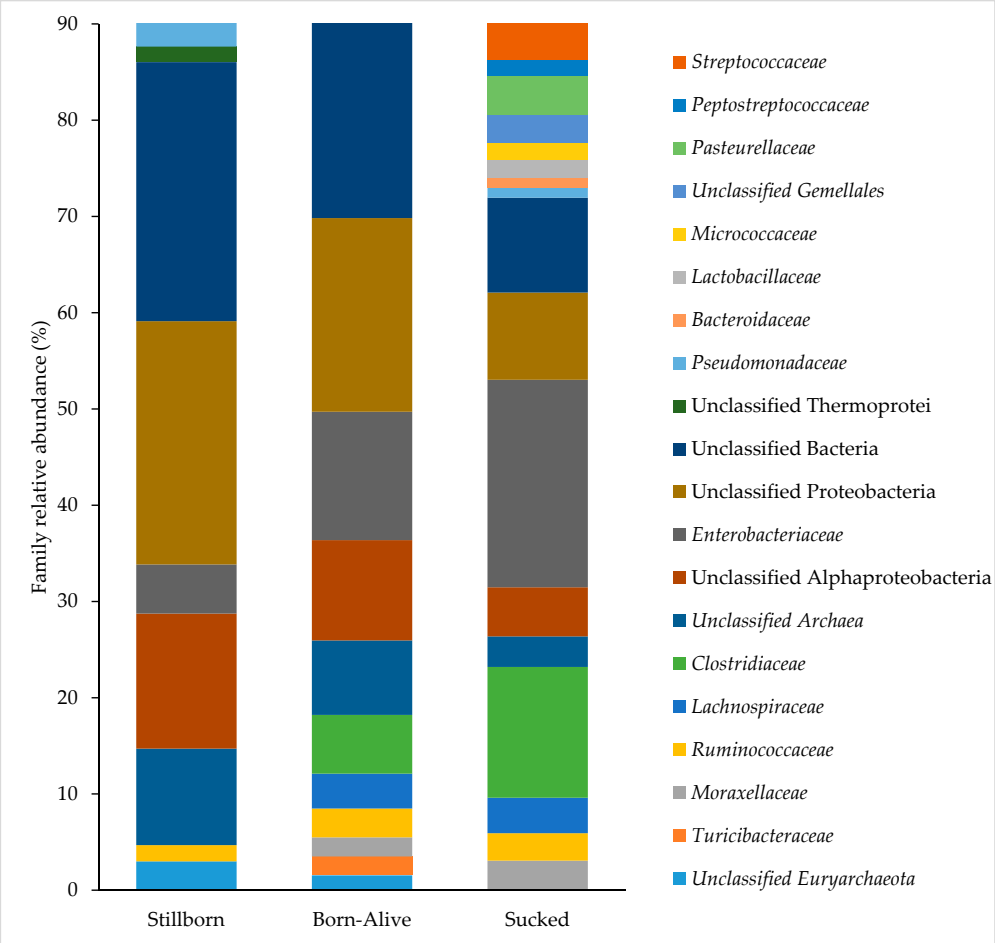


Figure 4. Abundance of the top 90% of microbial families present in the spiral colon of piglets that were stillborn, born-alive or those that have sucked. The bacterial families within the legend are arranged in the same order as they appear on the bar chart.

At genus level, the average dissimilarity in the spiral colon microbiota between piglets that were stillborn and those that were born-alive was 53%. Of the taxa that could be classified to the genus level and were significantly contributing to the dissimilarity, *Escherichia* and *Clostridium* were more abundant in animals that were born-alive, while *Pseudomonas*, *Bacteroides* and *Faecalibacterium* were more abundant in animals that were stillborn (Table 1). When assessing the spiral colon microbiota between piglets that were stillborn and those that had sucked, the average dissimilarity was 65%. Of the taxa that could be classified to the genus level and were significantly contributing to the dissimilarity, *Escherichia*, *Clostridium*, *Actinobacillus*, *SMB53*, *Streptococcus*, *Lactobacillus*, *Faecalibacterium* and *Roseburia* were more abundant in animals that had sucked and *Pseudomonas* were more abundant in those animals that were stillborn (Table 2). Of the taxa which could be classified to species level, *Escherichia coli*, *Clostridium perfringens* and *Prevotella copri* were

more abundant in animals that were born-alive then those that were stillborn. Additionally, *E. coli*, *Clostridium celatum*, *C. perfringens*, *Lactobacillus reutueri*, *Streptococcus luteciae* and *Faecalibacterium prausnitzii* were more abundant in animals that had sucked when compared with stillborn piglets, while *Bacteroides uniformis* was more abundant in stillborns than animals that had sucked.

Table 1. Taxa contributing significantly (average dissimilarity/standard deviation > 1) to the dissimilarity between born-alive and stillborn piglets as determined by SIMPER analysis at the genus level.

	Born-Alive	**Stillborn**	
Genus	**Average Abundance**	**Average Abundance**	**%**
Unclassified Archaea	0.67	1.03	2.15
Unclassified Thermoprotei	0.18	0.30	1.85
Unclassified Crenarchaeota	0.07	0.19	1.20
Unclassified Euryarchaeota	0.19	0.35	1.11
Clostridium	0.98	0.09	5.71
Faecalibacterium	0.10	0.11	0.79
Unclassified Alphaproteobacteria	1.00	1.44	2.91
Unclassified Rickettsiales	0.14	0.21	1.06
Escherichia	1.59	0.67	7.11
Pseudomonas	0.19	0.53	2.96
Bacteroides	0.14	0.15	0.82

Overall average dissimilarity between treatments was 53%. Only taxa that were significantly different between treatments are shown in this table. % represents the percentage contribution for these bacteria.

Table 2. Taxa contributing significantly (average dissimilarity/standard deviation > 1) to the dissimilarity between piglets that had sucked and those that were stillborn as determined by SIMPER analysis at the genus level.

	Sucked	**Stillborn**	
Genus	**Average Abundance**	**Average Abundance**	**%**
Unclassified Archaea	0.54	1.03	2.17
Unclassified Crenarchaeota	0.13	0.19	0.76
Unclassified Thermoprotei	0.10	0.30	1.16
Unclassified Euryarchaeota	0.16	0.35	0.92
Unclassified Bacteria	1.47	2.66	5.11
Unclassified Gemellales	0.45	0.03	1.85
Lactobacillus	0.39	0.04	1.53
Streptococcus	0.60	0.10	2.45
Unclassified *Clostridiaceae*	0.42	0.00	1.73
Clostridium	1.25	0.09	5.07
SMB53	0.74	0.04	3.19
Unclassified *Peptostreptococcaceae*	0.31	0.00	1.29
Unclassified *Lachnospiraceae*	0.16	0.02	0.69
Roseburia	0.11	0.06	0.45
Faecalibacterium	0.16	0.11	0.58
Unclassified Proteobacteria	1.39	2.50	4.78
Unclassified Alphaproteobacteria	0.80	1.44	2.89
Unclassified Rickettsiales	0.07	0.21	0.82
Escherichia	2.28	0.67	7.20
Actinobacillus	0.83	0.00	3.37
Pseudomonas	0.30	0.53	2.01
Unclassified *S24-7*	0.13	0.02	0.55

Overall average dissimilarity between treatments is 65%. Only taxa that were significantly different between treatments are shown in this table. % represents the percentage contribution for these bacteria.

4. Discussion

It is known that the initial microorganisms colonising the GIT impacts animal health and survival, and as a result, understanding the timing of initial colonisation is crucial [1]. Additionally, as the regulation of intestinal immunity relies largely on the GIT microbiota attained by neonates in early life, piglet survival depends on 'optimal' microbial colonisation occurring [33]. Until recently, neonates were presumed sterile until parturition, but, studies in humans [3], mice [4] and rhesus macaques [5] have identified microorganisms within the GIT prior to parturition. To our knowledge, no other studies have investigated the intestinal microbiota of piglets prior to colostrum ingestion. The present study identified bacteria in the spiral colon of stillborn piglets, therefore the hypothesis that passive transfer of microbes would occur in the developing fetus, at least immediately prior to birth, is supported. Further, the data largely supports the second hypothesis that the composition, abundance and diversity of microbes colonising the spiral colon would increase with birth, environmental exposure and colostrum consumption.

Alpha and beta diversity metrics indicated that rapid and diverse microbial colonisation of the GIT occurred within a few hours of birth. This observation of rapid and diverse post-natal colonisation was expected and has been documented previously [13,34]. These latter authors observed the formation of dominant populations of bacteria within the first few days of life with a gradual increase in minor populations, thus increasing diversity as time progressed. In the present study, however, temperature and oxygenation of digestive tissues may have also impacted the differences observed in microbial populations in piglets that were born-alive when compared to those that were stillborn.

When comparing sample diversity, the between-sample variation was relatively low for stillborn animals, even with the majority of piglets born to different sows, while those that were born alive, regardless of whether they had sucked, showed very large between-sample variation. Additionally, a distinct split in microbial communities were observed for born-alive animals regardless of whether they had sucked, with one cluster within each group being similar to stillborn animals. The separation in microbial communities observed is likely caused by the sampling time for piglets. As some sows farrowed overnight, animals may have differed in age by as much as 12 h, therefore, it is likely that the digesta did not have enough time to reach the spiral colon prior to sampling in some animals. Additionally, there may be differences in GIT transit time between animals that had sucked and those that had not, or in the case of piglets within the sucked group, it is likely that some animals may have sucked earlier than others. Overall, this indicates that a rapid change in spiral colon microbiota occurs within the first day of life and possibly identifies a crucial time for manipulation of the microbiota through the addition of environmental substrates or diet in order to ensure it establishes in a positive state.

Interestingly, although Pielou's evenness and taxonomic differences existed between stillborn animals and those that were born alive, regardless of whether they had sucked, stillborn and born-alive animals did not differ in Shannon's diversity and the number of taxa. This suggests that colostrum had a major contribution to community richness but it was somewhat unexpected that animals that were born-alive did not differ in diversity and number of taxa from stillborn animals. These animals would have not only received potential exposure prior to and during parturition but would have also gained external exposure from the environment they were born into. It is likely that the amount of environmental exposure may have been insufficient to result in a significant difference in taxa number and diversity or this similarity may be a result of stillborn piglets having received some degree of microbial exposure from the vaginal fluids during parturition. However, exposure to the environment was sufficient to result in a significant change in the dominant bacteria colonising the GIT.

Stillborn piglets were used as an indicator of microbial inoculation immediately prior to birth for this experiment. It is possible that the place and time of piglet death within the reproductive tract could have influenced the colonisers observed. Studies in pigs demonstrate that the risk of stillbirth is increased by a number of factors including large

litter sizes, prolonged parturition, placental detachment and umbilical cord occlusions, ruptures and breaks [19]. The majority of stillbirths are likely to occur in or above the cervix, while the vagina is the predominant location for inoculation of neonates [14]. Late-term fetuses ingest large amounts of amniotic fluid in utero and it is the ingestion of this fluid that is presumed to facilitate the colonisation of the GIT prior to birth [10]. It is possible that piglets may have ingested fluid within the cervix or vagina prior to death so, to attain the most accurate representation of microbial colonisation of the GIT in late gestation, the spiral colon of the piglet was sampled as it was assumed unlikely that any microbes ingested during parturition would not have arrived in the spiral colon within such a short time. When comparing the spiral colon microbiota of stillborn animals to the vaginal microbiota of sows from other studies it is evident that they share some dominant phyla (Firmicutes and Proteobacteria). Other phyla that are observed in dominant populations within the sow vagina are only present in very low amounts (Actinobacteria and Bacteriodetes) within stillborn piglets or are not present at all (Tenericutes) [35]. Additionally, as the variability in microbial communities between stillborn animals was low regardless of the fact that sampling time in relation to piglet death likely differed due to this death occurring during parturition. This low variability between animals suggests that the samples likely represents the environment immediately prior to birth rather than any microbes that may have been ingested during parturition. Alternately, due to the pressure exerted during parturition, the possibility of microorganisms being forced through the anus into the colon cannot be ignored. Further studies should investigate piglets prior to the onset of parturition to evaluate this. Nevertheless, if colonisation does occur during late gestation this raises the question, is there a way of influencing the microbiota of a piglet prior to parturition through the sow? It is well established within the literature that the initial microbes colonising the GIT are important for long-term health, therefore, the obvious next step would be to investigate piglets prior to parturition and whether the microbiota of a piglet can be altered in utero through sow nutritional management.

Research investigating sow microbiota identified specific bacteria present within their GIT that influence oxidative stress status and, therefore, potential stillbirth rate [36], but the taxa observed to cause this effect were not detected in the present study. Stillborn animals had consistently higher *Pseudomonadaceae*, however, information on the role it has within the gastrointestinal microbiota is not fully understood and its documentation within the GIT of pigs is limited [37]. Additionally, the present study only investigated stage II stillbirth, which are those that die during parturition, not stage I, which die prior to the onset of parturition. In the present study, there was no evidence to suggest that these bacteria are passed onto the piglet to cause stage II stillbirth, but rather it is possible that these bacteria influence internal mechanisms within the sow to cause stillbirth [36]. Alternately, the dissimilar microbial community may be a consequence of intrauterine death, however it is hoped that the sampling technique used in the present study did eliminate this possibility.

Similar to previous research, the present study demonstrated that the piglet is colonised by *Clostridiaceae* and *Enterobacteriaceae* species during the first day of postnatal life, with these being the predominant families in piglets that were born-alive and had sucked [34,38,39]. As these families were only present in small amounts within stillborn animals and increased substantially with environmental exposure and then sucking, it indicates that these taxa are most likely present within the environment and sow colostrum. This is further supported by the identification of these bacteria in piglets as prominent taxa throughout lactation [13,40]. The microbiota of sow colostrum was not evaluated, so any contribution it made remains speculative in the current study. Interestingly, high levels of archaea were observed within stillborn piglets, but these proportionally decreased as external exposure increased. Little research exists documenting the presence of archaea in piglets. Similar to the present study, Mao et al. [41] documented the presence of Euryarchaeota, which includes methanogenic archaea, and Su et al. [42] documented the presence of methanogenic archaea in the piglet from 1 day of age and saw a decrease in diversity of archaea as piglets aged. Crenarchaeota

were detected in the faeces of humans, however, its mechanism of action was not completely understood [43]. While these studies identified the presence of archaea in piglets, details regarding the specific abundance and richness of archaea observed in stillborn piglets cannot be addressed by the present study as archaea-specific primers were not employed.

When examining the taxa that could be identified to the species level, it was evident that a variety of potentially pathogenic bacteria colonise very early in life and although they may not be harmful initially, if they become present in high amounts they can become pathogenic. For example, *E. coli*, *C. perfringens* and *C. celatum* were present within those animals that were born alive regardless of whether they had sucked or not, suggesting that they were likely colonised from the environment and highlighting the importance of the environmental microbiota that the piglet is born into. Similarly, a study by Chen et al. [16], identified that the microbiota from the floor, sow milk and sow nipple surface were the earliest colonisers of the piglet faecal microbiota during early lactation. These results support the importance of farrowing crate cleanliness but also highlight the possible impact of sow faeces on the initial bacteria colonising the GIT. Indeed, piglets are born onto the region of the crate where the sow urinates and defecates, however, whether this is positive or negative is yet to be elucidated as studies within our research group and others are conflicting [44]. Previous studies have also documented the high prevalence of potentially pathogenic bacteria in early life, particularly in regard to *C. perfringens* in pigs [39,45]. These latter studies also documented that as time progressed, potentially beneficial bacteria outnumbered those that were potentially pathogenic. One explanation for this is that maternal immunoglobulin A (IgA) inhibits colonisation of harmful pathogens and by day seven post-partum, IgA is the major immunoglobulin isotype identified in breast milk [46]. Additionally, the main bacteria identified in the milk of sows were lactic acid bacteria such as *Lactobacilli* and *Bifidobacterium* [45]. Therefore, it is likely that as milk consumption increases, the bacteria present within the milk dominate the bacterial shift in GIT microbiota observed as piglets age. Although previous studies demonstrated this milk microbiota shift [47], research in humans and animals suggests that it is still important for positive microbial colonisation to occur at birth in order to support long-term health and productivity [1]. Although this cannot be confirmed within the present study, further investigations into environmental bacterial exposure and the effect it has on piglet microbiota and how it impacts survival and productivity are warranted.

5. Conclusions

To our knowledge, this is the first study to characterise the microbiota of piglets that are stillborn or born-alive prior to sucking, facilitating the identification of bacteria that colonise the spiral colon immediately prior to birth and the initial colonisers following parturition. The results suggest that the colonisation of the GIT of a piglet occurs immediately prior to birth and that following parturition, rapid and diverse colonisation of the GIT occurs, with this colonisation being driven by the environment and the consumption of colostrum. Further investigation into the role the vaginal and environmental microbiota have on these initial colonisers is needed in order to understand the origin of the potentially pathogenic bacteria observed in the current study. Indeed, a potential limitation of this study is that piglets were not sampled pre-partum. This could be addressed in the future by sampling in late gestation. Investigation of the amniotic and placental microbiota of pre-term piglets would help to understand the accuracy surrounding the stillborn samples attained in the present study and to determine the origin of the bacteria and archaea colonising. This will aid in determining the potential to influence the gastrointestinal microbiota of the developing fetus.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/life11040312/s1, Supplementary Figure S1: Species accumulation curves and richness indices of the bacterial communities in the spiral colon of piglets in the stillborn, born-alive and sucked groups.

Author Contributions: Conceptualization, T.L.N., R.N.K., K.J.P. and M.D.B.; Data curation, T.L.N.; Formal analysis, T.L.N. and V.A.T.; Methodology, T.L.N., R.N.K. and M.D.B.; Project administration, T.L.N.; Supervision, R.N.K., V.A.T., K.J.P. and M.D.B.; Writing—original draft, T.L.N.; Writing—review and editing, T.L.N., R.N.K., V.A.T., K.J.P. and M.D.B. All authors have read and agreed to the published version of the manuscript.

Funding: This work received no specific grant from any funding agency in the public, commercial or not-for-profit sectors, however T.L. Nowland was supported by Australian Pork Limited and the University of Adelaide.

Institutional Review Board Statement: This study was conducted according to to the guidelines of the University of Adelaide Roseworthy piggery, South Australia and approved by the University of Adelaide's Animal Ethics Committee (AEC number: S-2018-092) in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (NHMRC, 2013).

Informed Consent Statement: Not applicable.

Data Availability Statement: All data generated or analysed during this study are included in this published article or can be found in the Sequence Read Archives of the NCBI database under accession number PRJNA677620.

Acknowledgments: The authors wish to thank Sophia Ward and Jessica Zemitis for their expert technical assistance.

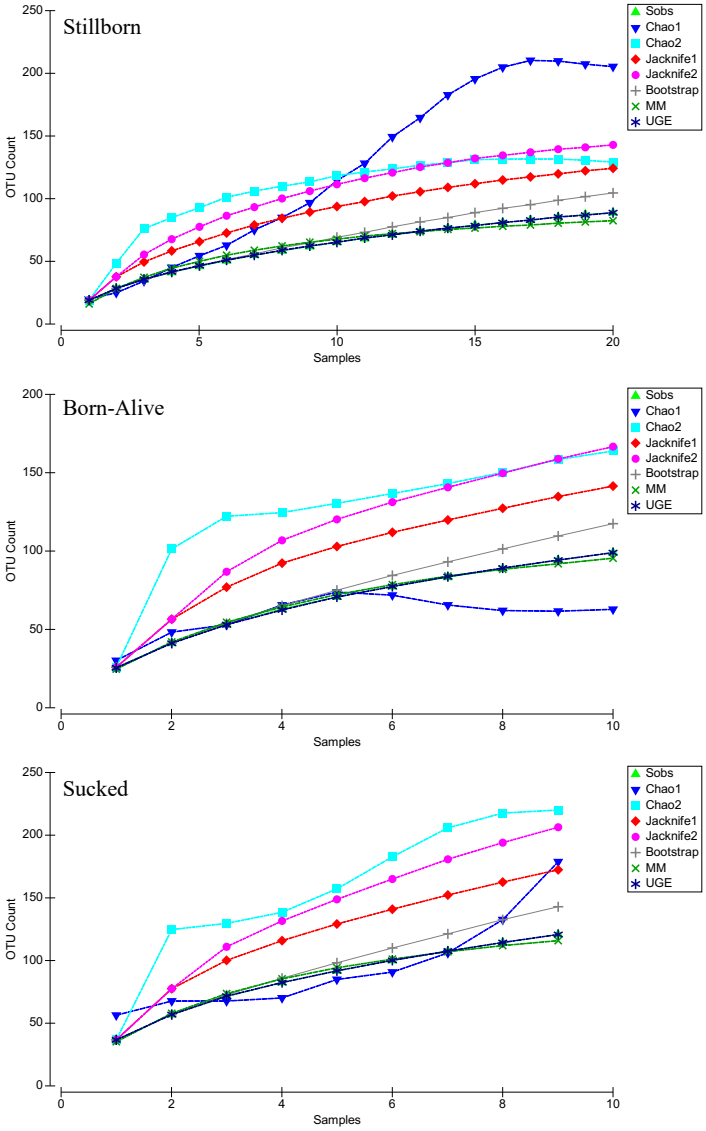
Conflicts of Interest: The authors declare no conflict of interest.

References

1. Nowland, T.L.; Plush, K.J.; Barton, M.; Kirkwood, R.N. Development and function of the intestinal microbiome and potential implications for pig production. *Animals* **2019**, *9*, 76. [CrossRef] [PubMed]
2. Rautava, S.; Luoto, R.; Salminen, S.; Isolauri, E. Microbial contact during pregnancy, intestinal colonization and human disease. *Nat. Rev. Gastroenterol. Hepatol.* **2012**, *9*, 565–576. [CrossRef] [PubMed]
3. Collado, M.C.; Rautava, S.; Aakko, J.; Isolauri, E.; Salminen, S. Human gut colonisation may be initiated in utero by distinct microbial communities in the placenta and amniotic fluid. *Sci. Rep.* **2016**, *6*, 23129. [CrossRef]
4. Younge, N.; McCann, J.R.; Ballard, J.; Plunkett, C.; Akhtar, S.; Araujo-Perez, F.; Murtha, A.; Brandon, D.; Seed, P.C. Fetal exposure to the maternal microbiota in humans and mice. *JCI Insight* **2019**, *4*, 1–14. [CrossRef] [PubMed]
5. Chu, D.; Prince, A.; Ma, J.; Baquero, K.; Blundell, P.; Takahashi, D.; Dean, T.; Grove, K.; Aagaard, K. 114: Evidence of fetal microbiota and its maternal origins in a non-human primate model. *Am. J. Obstet. Gynecol.* **2017**, *216*, S80. [CrossRef]
6. Perez-Munoz, M.E.; Arrieta, M.C.; Ramer-Tait, A.E.; Walter, J. A critical assessment of the "sterile womb" and "in utero colonization" hypotheses: Implications for research on the pioneer infant microbiome. *Microbiome* **2017**, *5*, 48. [CrossRef] [PubMed]
7. De Goffau, M.C.; Lager, S.; Sovio, U.; Gaccioli, F.; Cook, E.; Peacock, S.J.; Parkhill, J.; Charnock-Jones, D.S.; Smith, G.C.S. Human placenta has no microbiome but can contain potential pathogens. *Nature* **2019**, *572*, 329–334. [CrossRef]
8. Perez, P.F.; Dore, J.; Leclerc, M.; Levenez, F.; Benyacoub, J.; Serrant, P.; Segura-Roggero, I.; Schiffrin, E.J.; Donnet-Hughes, A. Bacterial imprinting of the neonatal immune system: Lessons from maternal cells? *Pediatrics* **2007**, *119*, E724–E732. [CrossRef]
9. Gomez-Arango, L.F.; Barrett, H.L.; McIntyre, H.D.; Callaway, L.K.; Morrison, M.; Nitert, M.D. Contributions of the maternal oral and gut microbiome to placental microbial colonization in overweight and obese pregnant women. *Sci. Rep.* **2017**, *7*, 2860. [CrossRef]
10. Ardissone, A.N.; de la Cruz, D.M.; Davis-Richardson, A.G.; Rechcigl, K.T.; Li, N.; Drew, J.C.; Murgas-Torrazza, R.; Sharma, R.; Hudak, M.L.; Triplett, E.W.; et al. Meconium microbiome analysis identifies bacteria correlated with premature birth. *PLoS ONE* **2014**, *9*, e90784. [CrossRef]
11. Jimenez, E.; Marin, M.L.; Martin, R.; Odriozola, J.M.; Olivares, M.; Xaus, J.; Fernandez, L.; Rodriguez, J.M. Is meconium from healthy newborns actually sterile? *Res. Microbiol.* **2008**, *159*, 187–193. [CrossRef]
12. Inman, C.F.; Haverson, K.; Konstantinov, S.R.; Jones, P.H.; Harris, C.; Smidt, H.; Miller, B.; Bailey, M.; Stokes, C. Rearing environment affects development of the immune system in neonates. *Clin. Exp. Immunol.* **2010**, *160*, 431–439. [CrossRef]
13. Frese, S.A.; Parker, K.; Calvert, C.C.; Mills, D.A. Diet shapes the gut microbiome of pigs during nursing and weaning. *Microbiome* **2015**, *3*, 28. [CrossRef]

14. Dominguez-Bello, M.G.; Costello, E.K.; Contreras, M.; Magris, M.; Hidalgo, G.; Fierer, N.; Knight, R. Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 11971–11975. [CrossRef]
15. Martín, V.; Maldonado-Barragán, A.; Moles, L.; Rodriguez-Baños, M.; Campo, R.D.; Fernández, L.; Rodríguez, J.M.; Jiménez, E. Sharing of bacterial strains between breast milk and infant feces. *J. Hum. Lact.* **2012**, *28*, 36–44. [CrossRef] [PubMed]
16. Chen, X.; Xu, J.; Ren, E.; Su, Y.; Zhu, W. Co-occurrence of early gut colonization in neonatal piglets with microbiota in the maternal and surrounding delivery environments. *Anaerobe* **2018**, *49*, 30–40. [CrossRef]
17. Baker, A.A.; Davis, E.; Spencer, J.D.; Moser, R.; Rehberger, T. The effect of a bacillus-based direct-fed microbial supplemented to sows on the gastrointestinal microbiota of their neonatal piglets. *J. Anim. Sci.* **2013**, *91*, 3390–3399. [CrossRef] [PubMed]
18. Jiang, L.; Feng, C.; Tao, S.; Li, N.; Zuo, B.; Han, D.; Wang, J. Maternal imprinting of the neonatal microbiota colonization in intrauterine growth restricted piglets: A review. *J. Anim. Sci. Biotechnol.* **2019**, *10*, 88. [CrossRef]
19. Langendijk, P.; Plush, K. Parturition and its relationship with stillbirths and asphyxiated piglets. *Animals* **2019**, *9*, 885. [CrossRef] [PubMed]
20. Stirling, G.R.; Griffin, D.; Ophel-Keller, K.; McKay, A.; Hartley, A.; Curran, J.; Stirling, A.M.; Monsour, C.; Winch, J.; Hardie, B. Combining an initial risk assessment process with DNA assays to improve prediction of soilborne diseases caused by root-knot nematode (*meloidogyne* spp.) and fusarium *oxysporum* f. Sp. Lycopersici in the queensland tomato industry. *Australas. Plant Path.* **2004**, *33*, 285–293. [CrossRef]
21. Torok, V.A.; Ophel-Keller, K.; Loo, M.; Hughes, R.J. Application of methods for identifying broiler chicken gut bacterial species linked with increased energy metabolism. *Appl. Environ. Microbiol.* **2008**, *74*, 783–791. [CrossRef]
22. Torok, V.A.; Percy, N.J.; Moate, P.J.; Ophel-Keller, K. Influence of dietary docosahexaenoic acid supplementation on the overall rumen microbiota of dairy cows and linkages with production parameters. *Can. J. Microbiol.* **2014**, *60*, 267–275. [CrossRef] [PubMed]
23. Zhang, J.; Kobert, K.; Flouri, T.; Stamatakis, A. Pear: A fast and accurate illumina paired-end read merger. *Bioinformatics* **2014**, *30*, 614–620. [CrossRef] [PubMed]
24. Caporaso, J.G.; Kucztnski, J.; Stombaugh, J.; Bittinger, K.; Bushman, F.D.; Costello, E.K.; Fierer, N.; Peña, A.G.; Goodrich, J.K.; Gordon, J.I.; et al. Qiime allows analysis of high-throughput community sequencing data. *Nat. Methods* **2010**, *7*, 335–336. [CrossRef] [PubMed]
25. Edgar, R.C. Search and clustering orders of magnitude faster than blast. *Bioinformatics* **2010**, *26*, 2460–2461. [CrossRef]
26. Edgar, R.C.; Haas, B.J.; Clemente, J.C.; Quince, C.; Knight, R. Uchime improves sensitivity and speed of chimera detection. *Bioinformatics* **2011**, *27*, 2194–2200. [CrossRef]
27. Edgar, R.C. Uparse: Highly accurate otu sequences from microbial amplicon reads. *Nat. Methods* **2013**, *10*, 996–998. [CrossRef]
28. DeSantis, T.Z.; Hugenholtz, P.; Larsen, N.; Rojas, M.; Brodie, E.L.; Keller, K.; Huber, T.; Dalevi, D.; Hu, P.; Andersen, G.L. Greengenes, a chimera-checked 16s rrna gene database and workbench compatible with arb. *Appl. Environ. Microbiol.* **2006**, *72*, 5069–5072. [CrossRef] [PubMed]
29. Bray, J.R.; Curtis, J.T. An ordination of the upland forest communities of southern wisconsin. *Ecol. Monogr.* **1957**, *27*, 325–349. [CrossRef]
30. Clarke, K.R. Non-parametric multivariate analyses of changes in community structure. *J. Ecol.* **1993**, *18*, 117–143. [CrossRef]
31. Kruskal, J.B. Multidimensional scaling by optimizing a goodness of fit to a nonmetric hypothesis. *Psychometrics* **1964**, *29*, 1–28. [CrossRef]
32. Shepard, R.N. The analysis of proximities: Multidimensional scaling with an unknown distance function. I. *Psychometrika* **1962**, *27*, 125–140. [CrossRef]
33. Patil, Y.; Gooneratne, R.; Ju, X.-H. Interactions between host and gut microbiota in domestic pigs: A review. *Gut Microbes* **2020**, *11*, 310–334. [CrossRef]
34. Petri, D.; Hill, J.E.; Van Kessel, A.G. Microbial succession in the gastrointestinal tract (git) of the preweaned pig. *Livest. Sci.* **2010**, *133*, 107–109. [CrossRef]
35. Sanglard, L.P.; Schmitz-Esser, S.; Gray, K.A.; Linhares, D.C.L.; Yeoman, C.J.; Dekkers, J.C.M.; Niederwerder, M.C.; Serão, N.V.L. Investigating the relationship between vaginal microbiota and host genetics and their impact on immune response and farrowing traits in commercial gilts. *J. Anim. Breed. Genet.* **2020**, *137*, 84–102. [CrossRef]
36. Wang, H.; Hu, C.; Cheng, C.; Cui, J.; Ji, Y.; Hao, X.; Li, Q.; Ren, W.; Deng, B.; Yin, Y.; et al. Unraveling the association of fecal microbiota and oxidative stress with stillbirth rate of sows. *Theriogenology* **2019**, *136*, 131–137. [CrossRef] [PubMed]
37. Spring, S.; Premathilake, H.; Bradway, C.; Shili, C.; DeSilva, U.; Carter, S.; Pezeshki, A. Effect of very low-protein diets supplemented with branched-chain amino acids on energy balance, plasma metabolomics and fecal microbiome of pigs. *Sci. Rep.* **2020**, *10*, 15859. [CrossRef] [PubMed]
38. Konstantinov, S.R.; Awati, A.A.; Williams, B.A.; Miller, B.G.; Jones, P.; Stokes, C.R.; Akkermans, A.D.; Smidt, H.; de Vos, W.M. Post-natal development of the porcine microbiota composition and activities. *Environ. Microbiol.* **2006**, *8*, 1191–1199. [CrossRef] [PubMed]
39. Inoue, R.; Tsukahara, T.; Nakanishi, N.; Ushida, K. Development of the intestinal microbiota in the piglet. *J. Gen. Appl. Microbiol.* **2005**, *51*, 257–265. [CrossRef] [PubMed]

40. 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*, 1–10. [CrossRef]
41. Mao, S.Y.; Yang, C.F.; Zhu, W.Y. Phylogenetic analysis of methanogens in the pig feces. *Curr. Microbiol.* **2011**, *62*, 1386–1389. [CrossRef]
42. Su, Y.; Bian, G.; Zhu, Z.; Smidt, H.; Zhu, W. Early methanogenic colonisation in the faeces of meishan and yorkshire piglets as determined by pyrosequencing analysis. *Archaea* **2014**, *2014*, 547908. [CrossRef] [PubMed]
43. Rieu-Lesme, F.; Delbes, C.; Sollelis, L. Recovery of partial 16s rdna sequences suggests the presence of crenarchaeota in the human digestive ecosystem. *Curr. Microbiol.* **2005**, *51*, 317–321. [CrossRef]
44. Aviles-Rosa, E.O.; Rakhshandeh, A.; McGlone, J.J. Preliminary study: Depriving piglets of maternal feces for the first seven days post-partum changes piglet physiology and performance before and after weaning. *Animals* **2019**, *9*, 268. [CrossRef] [PubMed]
45. Knecht, D.; Cholewińska, P.; Jankowska-Ma, K.A.; Czyź, K. Development of swine's digestive tract microbiota and its relation to production indices—a review. *Animals* **2020**, *10*, 527. [CrossRef] [PubMed]
46. Butler, J.E.; Brown, W.R. The immunoglobulins and immunoglobulin genes of swine. *Vet. Immunol. Immunopathol.* **1994**, *43*, 5–12. [CrossRef]
47. Chen, W.; Mi, J.; Lv, N.; Gao, J.; Cheng, J.; Wu, R.; Ma, J.; Lan, T.; Liao, X. Lactation stage-dependency of the sow milk microbiota. *Front. Microbiol.* **2018**, *9*, 945. [CrossRef] [PubMed]



Supplementary Figure 1. Species accumulation curves and richness indices of the bacterial communities in the spiral colon of piglets in the stillborn, born-alive and sucked groups. Green triangle represent the observed accumulated species richness (Sobs). Blue upside-down triangle and blue square represent the abundance-based estimator of species richness (Chao). Red diamond and pink circle represent the absence or presence-based estimator of species richness (Jacknife). Grey plus sign represents an estimator of true richness (Bootstrap). Green "X" sign representing Michaelis-Menten (MM), a parametric approach to richness and the blue star represents true regional richness given by the Ugland-Gray-Elligsen (UGE) index.

Chapter 4:

Exposure to maternal faeces in lactation influences piglet enteric microbiota, growth and survival pre-weaning

Statement of Authorship

Title of Paper	Exposure to Maternal Faeces in Lactation Influences Piglet Enteric Microbiota, Growth and Survival pre-weaning
Publication Status	☐ Published ☒ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Nowland TL, Kirkwood RN, Plush KJ, Barton MD and Torok VA 2021. Exposure to maternal faeces in lactation influences piglet enteric microbiota, growth and survival pre-weaning. Journal of Animal Science. Doi: 10.1093/jas/skab170.e

Principal Author

Name of Principal Author (Candidate)	Tanya Nowland
Contribution to the Paper	Assisted with the experimental design, managed and carried out the experimental trial, analysed statistics, drafted and edited the manuscript.
Overall percentage (%)	85%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 16/2/2021

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Roy Kirkwood
Contribution to the Paper	Assisted with the experimental design, data collection and interpretation and editing manuscript.
Signature	Date 25/2/21

Name of Co-Author	Kate Plush
Contribution to the Paper	Assisted with the experimental design, evaluated and edited the manuscript.
Signature	Date 25/2/21

Name of Co-Author	Mary Barton		
Contribution to the Paper	Assisted with the experimental design, data interpretation and editing manuscript.		
Signature		Date	25/2/21

Name of Co-Author	Valeria Torok		
Contribution to the Paper	Assisted with the experimental design, statistical analysis, data interpretation and editing manuscript.		
Signature		Date	25/2/21

Please cut and paste additional co-author panels here as required.

Exposure to maternal faeces in lactation influences piglet enteric microbiota, growth and survival pre-weaning

Tanya L. Nowland[*,#], Roy N. Kirkwood[*], Kate J. Plush[‡], Mary D. Barton[§] and Valeria A. Torok[*,†]

[*]School of Animal and Veterinary Sciences, The University of Adelaide, Roseworthy, SA 5371, Australia: tanya.nowland@adelaide.edu.au and roy.kirkwood@adelaide.edu.au

[‡]SunPork Group, Murarrie, QLD 4172, Australia: kate.plush@sunporkfarms.com.au

[§]School of Pharmacy and Medical Sciences, University of South Australia, Adelaide SA 5000, Australia: mary.barton@unisa.edu.au

[†]South Australian Research and Development Institute, Food Sciences, SA 5064, Australia: valeria.torok@sa.gov.au

[#]Correspondence: tanya.nowland@adelaide.edu.au; 08 8313 7786

Abstract

It is known that gilt progeny performance is reduced compared to sow progeny. Previous research suggests that the presence of maternal faeces in early life improves the health and survival of offspring. Therefore, we aimed to determine whether contact with faeces from multiparous (MP) sows would improve the growth and survival of piglets born and reared on primiparous (P1) sows and if so, whether these differences are associated with the gut microbiota. Four treatments were applied for 10 days: Donor (n = 29) piglets had limited access to maternal faeces as, each morning, sow faeces were removed and placed in the crate of a P1 sow (P1-FT; n = 30 piglets); and P1-Con (n = 29) and MP-Con (n = 33) piglets had access to their own mothers' faeces. All piglets were weighed on days 1, 3, 10 and 18. Faecal samples were collected from a subset of sows (n = 10/treatment) 3 days post-farrow and from 2 female piglets/litter on day 10 and 18 (n = 20/treatment) and subject to 16S rRNA amplicon analysis. Escherichia, Clostridium, Campylobacter and Treponema were more abundant in MP sows, while P1 sows had a higher abundance of Lactobacillus and Prevotella. At 10 days, P1 progeny faecal microbiota differed, and growth and survival were reduced when compared to MP progeny. No treatment effect was observed for P1-FT piglets ($P > 0.05$). Donor piglets had a different faecal microbiota and improved weight and survival then all other treatments ($P < 0.05$). Overall, the removal of sow faeces from the farrowing crate improved piglet microbiota development, growth and survival.

Keywords: Health, Microbiota, Parity, Pig, Postpartum, Progeny

List of abbreviations

rRNA: Ribosomal ribonucleic acid

DNA: Deoxyribonucleic acid

OTU: Operational taxonomic unit

nMDS: Non-metric multidimensional scaling

SIMPER: Similarity percentages

ANOSIM: Analysis of similarity

ANOVA: analysis of variance

AGRF: Australian Genome Research Facility

SARDI: South Australian Research and Development Institute

MJ: Megajoules

DE: Digestible energy

1. Introduction

The progeny of primiparous (P1) sows are born lighter and remain lighter throughout each phase of production and have a higher rate of mortality than multiparous (MP) sow progeny (Craig et al., 2017). Therefore, new methods for improving gilt progeny performance are needed. Recent research with humans and animals indicates that the gastrointestinal microbiota has a major role in health and survival (Nowland et al., 2019). To our knowledge, only one study investigating the differences in microbiota between P1 and MP sows has been published and it demonstrated a significant difference in faecal microbiota between P1 and MP sows within a UK herd (Gaukroger et al., 2020a). A preliminary study conducted by Aviles-Rosa et al. (2019) demonstrated that feed intake, growth and white blood cell count were affected by whether the piglets had access to maternal faeces during their first 7 days of life, or not, with those being exposed to maternal faeces exhibiting improvements. However, since no investigation into the specifics surrounding the gastrointestinal microbiota were conducted, the cause of these differences remains to be determined.

Evidence suggests that piglets develop their gastrointestinal microbiota from contact with the sow and their environment and as piglets are housed exclusively with their sow in individual pens throughout lactation, it is likely that the sow drives this development. Therefore, the current study aimed to determine whether contact with faeces from an older parity sow can improve growth and survival of piglets born and reared on primiparous sows and if so, whether these differences are associated with the gut microbiota. We hypothesised that (1) MP sows would have a faecal microbiota that is more diverse then P1 sows and (2) piglets born and reared on a P1 sow but exposed to faeces from a MP sow would have an enteric microbiota similar to progeny of the older sows and would demonstrate improved growth and survival.

2. Materials and methods

All procedures were conducted at the University of Adelaide Roseworthy piggery, South Australia, with the approval of the University of Adelaide's Animal Ethics Committee (AEC number: S-2019-053).

2.1. Animals and experimental procedures

A total of 121 Large White x Landrace primiparous (P1) and multiparous (MP) sows (parities 2 – 4; 2.95 ± 0.09) were employed in a series of 4 batches from September 2019 to January 2020. All sows were group housed and received 2.5 kg of commercial gestation diet daily (12.9 MJ DE/kg) throughout gestation. Sows were moved into a farrowing shed approximately 5 days before their expected farrow date and were housed in individual commercial farrowing crates (1.7 m x 2.4 m). The farrowing shed consisted of climate controlled and fully slatted plastic floored rooms. Upon entry into farrowing accommodation, sows received a commercial lactation diet (14.0 MJ DE/kg) at 2.5 kg/d until farrowing, thereafter the feeding level was gradually increased until it reached 7-8 kg by day 7 of lactation. All sows had ad libitum access to water. Two days before their due date, sows were induced to farrow by vulva injection of 100 µg cloprostenol at 8 am and again at 2 pm. Farrowing was monitored during staffed hours from 8 am to 3 pm daily. Sows were allocated to one of four treatments on farrowing house entry:

- P1 control: maternal faeces moved to each side at the rear of the pen to allow piglets easier access to faeces (n = 29; P1-Con).
- P1 faecal transfer: maternal faeces removed from the pen twice daily and a pooled faecal mixture from MP donor sows placed on each side at the rear of the pen to allow piglets easier access to faeces (n = 30; P1-FT).
- MP control (parity 3 ± 0.7): maternal faeces moved to each side at the rear of the pen to allow piglets easier access to faeces (n = 33; MP-Con).
- MP donor (parities 3 ± 0.7): Sow faeces collected from the crate after feeding at 7 am and 3 pm daily for placement in P1-FT pens. Therefore, these litters had reduced access to maternal faeces (n = 29; Donor).

Treatments were imposed from farrowing for ten days. The objective of the study was to assess the potential benefits to P1 progeny from exposure to MP sow faeces due to the previously documented superior performance of MP sow progeny, and as such no treatment where MP sows received P1 sow faeces were applied. Cross fostering was permitted within treatment at 24 h according to teat capacity (average litter size = 10.6 ± 1.2). All piglets within the litter were tagged with an individual identification number and weighed on days 1, 3, 10 and 18. Faecal samples were collected from a subset of sows 3 days post farrow (n = 10/treatment) and from 2 female focal piglets from each litter at 10 and 18 days of age (n = 20 piglets/treatment/timepoint). Sow faecal samples were collected by rectal stimulation with a gloved hand and direct collection into a sterile sample container. Piglet faecal samples were collected by isolating piglets in a sterile pen until defecation, whereby the faeces were collected either directly from the rectum or off of the floor of the sterile container immediately after defecation. Once collected, faecal samples were placed on ice immediately, transported to a laboratory within 4 h, and stored at -80^{o}C until required for microbial analysis. Sows and litters had no contact with antibiotics during lactation and the prior gestation. All piglet deaths were recorded. If a live-born piglet death occurred within the first 24 h of life it was classified as pre-foster mortality and any that occurred after 24 h and prior to weaning were classified as post-foster mortality. While total pre-weaning mortality was the sum of both pre- and post-foster deaths. Weaning occurred when piglets reached 18 days old.

2.2. Donor sample preparation and administration

All sows were fed at 7 am and 2 pm daily to encourage defecation. Upon standing, all faeces present in the Donor sows pen and any fresh faecal material was collected at 8 am and 3 pm daily (n = 7-8 sows per batch). Once collected, the faeces from all donor sows were immediately mixed in a bucket and evenly distributed to each P1-FT treated pen where it was placed at the rear right and left corners of the pen to allow piglets to access easily. Approximately 2-4 kg of faeces was administered per day. The quantity of faeces administered to each pen differed daily as it depended on the amount of excreta present at the time of collection.

2.3. DNA Extraction and 16S rRNA amplicon analysis

Total nucleic acid was extracted from freeze dried piglet faecal samples by a modification of a South Australian Research and Development Institute (SARDI, Adelaide, Australia) proprietary method. Approximately 2 gm of freeze-dried faecal sample was added to 20 mL extraction buffer (1.3 M guanidine thiocyanate, 1.5 M $NaCl_2$, 30 mM Tris-HCl, 65 mM phosphate buffer, 3.4% (w/v) sarkosyl and 1.7% (w/v) polyvinylpolypyrrolidone) and incubated for 1 h at 70^{o}C prior to proceeding with the proprietary extraction method (Haling et al., 2011).

PCR amplification and sequencing of the V3-V4 region of the 16S rRNA gene was done by the Australian Genome Research Facility (AGRF). The V3-V4 region was PCR amplified over 29 cycles using forward primer 341-F (CCTAYGGGRBGCASCAG) and reverse primer 806-R (GGACTACNNGGGTATCTAAT). Amplicon sequencing was done on the illumina MiSeq platform (San Diego, CA, USA) with two by 300 bp paired-end chemistry. Both positive and negative controls were used on every plate processed by AGRF. The positive control used was ZymoBIOMICS Microbial Community DNA Standard II (Log Distribution). The obtained reads are available under the accession number PRJNA682009 of the Sequence Read Archive of the National Centre for Biotechnology Information. Bioinformatic analysis of raw sequence data was done by the AGRF as follows. The paired-end sequences were assembled by aligning the forward and reverse reads using PEAR (Zhang et al., 2014) (version 0.9.5) and the primers were identified and trimmed. All trimmed sequences were processed using Quantitative Insights into Microbial Ecology (QIIME 1.8) (Caporaso et al., 2010) USEARCH (version 8.0.1623) (Edgar, 2010; Edgar et al., 2011) and UPARSE software (Edgar, 2013). Sequences were quality filtered, full length duplicate sequences were removed and sorted by abundance. Singletons or unique reads in the dataset were discarded. Additionally, chimeric sequences were clustered and removed using “rdp_gold” database as the reference. Sequences were grouped into operational taxonomic units (OTUs) based on 97% sequence similarity. Using QIIME, taxonomy was assigned using the Greengenes database (Version 13.8, Aug 2013) (DeSantis et al., 2006). All sequences corresponding to chloroplasts were removed.

2.4. Statistical analysis

All production data were tested for the normality of residuals and outliers before analysis. All weight data were analysed using RStudio software (Version 1.1.456, Boston, MA, USA). The "lmer" package was used to perform a general linear model to assess the effect of treatment on piglet weight. The fixed effects included in the model were treatment, sex, litter size weaned, age and treatment*age, with piglet ID specified as the random effect. However, sex and litter size weaned were not found to be significant so were removed from the final model. Data were expressed as estimated marginal means ± standard error of the mean (SEM), and a P-value threshold of 0.05. In SPSS v26 (IBM, USA) a generalized linear mixed model was fit to total pre-weaning mortality using a Poisson regression with treatment as a fixed effect and block as the random term.

The alpha diversity metrics, Shannon diversity (H') index, Pielou's evenness (J') and number of taxa (S), were calculated using DIVERSE (PRIMER6 PRIMER-E Ltd., Ivybridge, UK). Normality was tested within RStudio software (Version 1.1.456, Boston, MA, USA) using the Shapiro-Wilk test. Those alpha diversity metrics that were found to be normally distributed were analysed using an analysis of variance (ANOVA) and those not normally distributed were analysed using the Kruskal-Wallis test, with corrections for multiple tests using false discovery rate and a P-value threshold of 0.05.

Multivariate statistical techniques (PRIMER6, PRIMER-E Ltd., Ivybridge, UK) were used to analyse the faecal 16S rRNA bacterial taxonomic data. Similarities among faecal bacterial communities of sows and piglets from the 16S rRNA data metrics were analysed using Bray–Curtis measures of similarity (Bray and Curtis, 1957), following standardisation by sample total and fourth-root transformation. One-way analysis of similarity (ANOSIM) (Clarke, 1993) on the Bray-Curtis similarity data was used to test if there were significant treatment and sow parity differences among faecal bacterial communities. If the global R statistic was significant ($P \leq 0.05$), then the significance of pairwise R statistics were investigated further. The R statistic value describes the extent of similarity among or between groups, with values close to unity (1) indicating that groups are entirely separate and a zero-value indicating that there is no difference

among or between groups. In order to determine which individual bacterial taxa contributed most to the overall dissimilarity between statistically different groups, similarity percentages (SIMPER) (Clarke, 1993) analyses were done and the overall average dissimilarity between sow or piglet faecal bacterial communities were calculated. The percentage contributions of significant taxa (average dissimilarity/standard deviation > 1) to the top 60% of the average dissimilarities were calculated. Non-metric multidimensional scaling (nMDS) (Shepard, 1962; Kruskal, 1964) on Bray-Curtis similarity data was done to graphically illustrate relationships with parity.

3. Results

3.1. Performance

Significant treatment related differences in piglet weight at 1, 3, 10 and 18 days of age were observed ($P < 0.001$; Fig. 1). Consistently, Donor and MP-Con piglets were heavier than P1-Con and P1-FT piglets (Fig. 1). Differences were small at day 1 but became larger with increasing age and by day 18 Donor piglets were heavier than piglets from all other treatments (Fig. 1). There was a treatment effect on piglet pre-weaning mortality ($P = 0.008$). Piglets in the Donor treatment had a lower total pre-weaning mortality (0.89 ± 0.25 pigs per litter), than animals in the P1-Con (1.67 ± 0.30), P1-FT (1.82 ± 0.30) and MP-Con (1.41 ± 0.27) treatments.

3.2. The effect of parity on the sow's faecal microbiota

Across all 40 sow faecal samples, the total number of 16S rRNA sequenced reads were 2,458,821 with 1,869,533 reads retained after quality control, and an average of 46,738 16S rRNA sequenced reads per sow. Reads were clustered into 2,369 OTUs and assigned taxonomic classification.

For alpha diversity metrics, Shannon's diversity and the number of taxa, no significant differences were observed between parities ($P = 0.641$ and $P = 0.896$, respectively), while Pielou's evenness tended to be higher for P1 sows compared to MP sows ($P = 0.056$). Faecal bacterial genera differed between P1 and MP sows (ANOSIM, Global $R = 0.124$, $P = 0.004$) and is graphically demonstrated in Fig. 2. At the genus

level, the average dissimilarity between the faecal microbiota of P1 and MP sows was 23%. Of the taxa that could be classified to the genus level and were contributing significantly to the average dissimilarity between parity, those in the top 60% are displayed in Fig. 3.

3.3. Treatment related effects on the piglet's faecal microbiota

Across all 160 piglet faecal samples, the total number of 16S rRNA sequenced reads were 12,677,307 with 9,508,933 reads retained after quality control, and an average of 59,430 16S rRNA sequenced reads per piglet faecal sample. Reads clustered into 2,305 OTUs and assigned taxonomic classification.

3.3.1. Day 10

No genus level significant differences were observed between treatments for Shannon's diversity, Pielou's evenness and the number of taxa ($P = 0.210$, $P = 0.419$ and $P = 0.539$, respectively). However, for beta diversity metrics, piglet faecal bacterial genera differed significantly with treatment (ANOSIM, Global $R = 0.112$, $P = 0.010$), with all pairwise comparisons being significantly different ($P < 0.010$), with the exception of P1-Con versus P1-FT ($R = 0.007$, $P = 0.329$). The average abundance of phyla present within these piglet treatments are shown in Fig. 4. The top 6 phyla in all treatments were Bacteroidetes, Firmicutes, Proteobacteria, Fusobacteria, Actinobacteria and Unclassified Bacteria, accounting for over 80% of the microbial community population (Fig. 4). All treatments except P1-Con and P1-FT differed in the abundance of Bacteroidetes, Proteobacteria, Fusobacteria, Actinobacteria, Unclassified Bacteria, Spirochaetes, Synergistetes, Lentisphaerae, Euryarchaeota and Tenericutes. In addition to these differences, Donor piglets were significantly higher in Firmicutes then all other treatment groups and higher in TM7 when compared with P1-Con piglets, while Unclassified Archaea were higher in piglets reared on P1 sows (Fig. 4).

The top 60% of genera driving the differences observed between Donor vs MP-Con piglets as determined by SIMPER analysis are listed in Table 1. The mean relative abundance of multiple genera differed between 10-day old, P1-Con and Donor piglets and had an average dissimilarity of 35%. Of those that

were in the top 60% and could be classified to the genus level, Bacteroides, Prevotella, Butyricimonas, Lactobacillus, Odoribacter, Blautia, Clostridium, Peptostreptococcus, Actinomyces, Sutterella, Phascolarctobacterium, Dorea and Flexispira were more abundant in Donor piglets, while Escherichia, Turicibacter, Roseburia, Sphaerochaeta, Synergistes, Parabacteroides, Campylobacter, Enterococcus, Eubacterium, Actinobacillus, Bulleidia, Ruminococcus and Butyricicoccus were more abundant in P1-Con piglets. Furthermore, when assessing the difference between P1-Con and MP-Con piglets, the average dissimilarity of bacteria was 37% and the differences observed were similar to those differences observed between P1-Con and Donor piglets with exception to, Fusobacterium, Oscillospira, Turicibacter, Roseburia, Clostridium, Campylobacter, Dialister and Butyricicoccus being more abundant in MP-Con animals and Blautia, Collinsella and Dorea being more abundant in P1-Con treated animals.

<Insert Table 1 here>

There was an average dissimilarity of 33% between the faecal microbiota of 10-day old piglets in the Donor and P1-FT treatments at genus level. When assessing the top 60% of genera, Fusobacterium, Oscillospira, Lactobacillus, Odoribacter, Butyricimonas, Actinomyces, Sutterella, Ruminococcus, Parabacteroides, Blautia, Eubacterium, Dorea and Anaerotruncus, were more abundant in Donor piglets, while Bacteroides, Prevotella, Escherichia, Campylobacter, Roseburia, Sphaerochaeta, Peptostreptococcus, Flexispira, Actinobacillus, Turicibacter, Paludibacter, Faecalibacterium, Synergistes, Bulleidia and Mogibacterium were more abundant in P1-FT piglets. The differences observed were similar to those observed between P1-FT and MP-Con piglets, with exception to Bacteroides, Actinobacillus, Collinsella, Dialister and Streptococcus being more abundant in MP-Con animals and Oscillospira, RFN20 and Blautia being more abundant in P1-FT animals. The average dissimilarity between the faecal microbiota of piglets at 10 days of age for MP-Con and P1-FT was 36%.

3.3.2. Day 18

At day 18 alpha diversity metric, Shannon's diversity differed with MP-Con piglets having a higher diversity than P1-FT piglets (P = 0.024; Fig. 5), while all other comparisons were not significantly different. Bacterial community evenness and the number of taxa also differed, with MP-Con piglets having a lower evenness but a higher number of taxa than all other treatments (P < 0.001; Fig. 5). Treatment associated differences for beta diversity metrics were observed in 18-day old piglet faecal bacterial genera (ANOSIM, Global R=0.041, P=0.016). The significant pairwise differences observed were between the Donor versus P1-FT (R=0.082, P=0.015) and MP-Con versus P1-FT (R=0.112, P=0.005). The top 60% of genera contributing significantly to the difference between 18-day old piglets within the P1-FT and Donor treatment groups are shown in Table 2.

<Insert Table 2 here>

Differences also existed between piglets in the P1-FT and MP-Con treatment groups, the top 60% of the differences in genera are shown in Table 3.

<Insert Table 3 here>

4. Discussion

As the lactation environment involves the housing of piglets in a pen exclusively with one sow, the sow's faeces will influence the developing intestinal microbiota within her piglets via coprophagy, which has been documented as a natural phenomenon in pigs (Aviles-Rosa et al., 2019). Additionally, sow parity differences have been noted for their piglet's nasal mucosal bacterial colonisation (Brean et al., 2016), so an expectation of sow parity differences on piglet's enteric bacterial colonisation is reasonable. In our study, MP and P1 sows had significantly different faecal microbiota 3 days post-partum, with the differences observed presented in the faeces of their piglets at day ten of lactation. These data are similar to the findings of Gaukroger et al. (2020a) who demonstrated differences between MP and P1 sow faecal

microbiota both prior to and post farrowing. Additionally, similar to previous literature, the present study observed significantly lower growth and survival throughout lactation in P1 progeny when compared with MP sow progeny (Carney-Hinkle et al., 2013; Craig et al., 2017). The production improvements in MP sow progeny in the present study are interesting as MP sows had a higher abundance of potentially pathogenic bacteria such as Escherichia, Clostridium, Campylobacter and Treponema compared to P1 sows, while P1 sows had a higher abundance of beneficial bacteria, Lactobacillus and Prevotella, with these same differences observed in the progeny of MP and P1-Con animals at ten days of age. It is likely that passive immunity transferred from sows to their piglets could compensate for these differences. In addition, previous work has shown that gilt progeny have a number of anatomical differences indicative of delayed development that persist to weaning when compared to sow progeny (Craig et al., 2019), and in some cases MP sows have increased IgG and IgA concentrations in serum and milk/colostrum (Carney-Hinkle et al., 2013). Therefore, given the fact that we were able to demonstrate differences in microbiota, it may be more complex than originally thought and it is likely that it is a combination of these differences that collectively contribute to the parity differences observed in piglet performance.

The higher abundance of potentially pathogenic bacteria observed within MP sow faeces and piglets within the MP-Con treatment may also provide some insight as to why the piglets in the Donor treatment performed better than all other treatment groups. Their improved growth performance is possibly due to them having limited exposure to the potentially pathogenic bacteria within the sow's faeces for the first ten days of life, arguably during the time of the highest risk of disease for the piglet (Lay et al., 2015). The reduction in pre-weaning mortality in these pigs further supports this suggestion. Our findings contrast those of Aviles-Rosa et al. (2019), who documented poorer performance for pigs deprived of maternal faeces. Although Aviles-Rosa et al. (2019) recorded weight throughout lactation, no treatment effects were seen in weight until 56 days post-weaning, while we only measured growth to 18 days. In contrast, studies comparing flooring type observed similar findings to the present study and demonstrated the positive effects of crate cleanliness on production outcomes (Mabry et al., 1982; Rantzer and Svendsen, 2001).

That the addition of MP faeces to the pen of P1 piglets provided no evident advantage or disadvantage to the piglets is intriguing. This implies that either sow faeces do not impact piglet performance or that, in our study, the piglets had inadequate contact with minimal coprophagy. It is also possible that the quantity of faeces added to the pen was not sufficient, especially early in lactation when sows did not defecate often so the amount of fresh faeces to deliver was sometimes limited. To ensure that faeces were present within the pen at birth, the faecal transfers started prior to the onset of parturition. It is possible that the freshness of the faeces at the time of birth could have influenced this. Additionally, it is possible that the amount of time the piglets spent interacting with the faeces could have had an influence and since piglets can differentiate their sow's faeces (Horrell and Hodgson, 1992), donor sow faeces may not have been as attractive as their own mothers would have been.

In the present study, MP and P1 progeny maintained production differences throughout lactation, and previous studies demonstrate that these deficits remain beyond weaning (Craig et al., 2017). The parity specific differences observed in the piglet faecal microbiota at day ten were not as evident by day 18 as control animals did not differ (MP-Con and P1-Con). Previous studies by our research group and others have documented this age-related change in faecal microbiota of piglets during lactation (Gaukroger et al., 2020b; Nowland et al., 2020a; Nowland et al., 2020b). However, to our knowledge, no analysis of faecal microbiota between piglets reared on different parity sows have been documented. Diet and environment shape the developing intestinal microbiota of the neonate (Nowland et al., 2019). Therefore, it is likely that the sow's microbiota has a greater influence on development of the piglet's microbiota early in lactation but, as the piglets age and are exposed to more environmental stimuli (handling by stock people, eating the sows feed, etc.), the impact of the sow diminishes.

5. Conclusion

To our knowledge, this is the first study to characterise and compare the faecal microbiota of different parity sows with their piglets, and to document how the addition of MP sow faeces to the pen of P1 sows

influences piglet development. The identification of parity specific microbial differences throughout lactation may allow for the development of easy to implement on-farm approaches to improve gut health and performance of the sow during lactation and in turn influence piglet's growth and survival. The present results suggest that MP and P1 sows do have a significantly different faecal microbiota that influences the piglet faecal microbiota until at least ten days of age. As other studies have also demonstrated, the growth and survival of P1 sow progeny was significantly reduced pre-weaning when compared to MP sow progeny, however it is uncertain as to whether differences in microbiota cause these production differences. It is evident that the inclusion of MP faeces to the pen of a P1 sow provided no benefit or hinderance to the piglets reared in that environment. However, the removal of faeces from the pen for the first ten days significantly improved piglet weight and survival to weaning. Further investigation into the possibility of altering the sow's faecal microbiota through dietary manipulation to positively influence the piglet's microbiota and growth are needed.

Conflict of interest statement

The authors declare that they have no conflicts of interests.

Funding

This work received no specific grant from any funding agency in the public, commercial or not-for-profit sectors, however T. Nowland was supported by Australian Pork Limited and The University of Adelaide.

Acknowledgments

The authors wish to thank Sophia Ward, Serena Barnes and Jessica Zemitis for their expert technical assistance.

Literature Cited

Aviles-Rosa, E. O., A. Rakhshandeh, and J. J. McGlone. 2019. Preliminary study: Depriving piglets of maternal feces for the first seven days post-partum changes piglet physiology and performance before and after weaning. Animals 9(5):1-12. doi: 10.3390/ani9050268

Bray, J. R., and J. T. Curtis. 1957. An ordination of the upland forest communities of southern Wisconsin. Ecol Monogr 27(4):325-349. doi: 10.2307/1942268

Brean, M., S. Abraham, M. Hebart, and R. N. Kirkwood. 2016. Influence of parity of birth and suckled sows on piglet nasal mucosal colonization with Haemophilus parasuis. Can Vet J 57:1281-1283.

Caporaso, J. G., J. Kucztnski, J. Stombaugh, K. Bittinger, F. D. Bushman, E. K. Costello, N. Fierer, A. G. Peña, J. K. Goodrich, J. I. Gordon, G. A. Huttley, S. T. Kelley, D. Knights, J. E. Koenig, R. E. Ley, C. A. Lozupone, D. McDonald, B. D. Muegge, M. Pirrung, J. Reeder, J. R. Sevinsky, P. J. Turnbaugh, W. A. Walters, J. Widmann, T. Yatsunenko, J. Zaneveld, and R. Knight. 2010. QIIME allows analysis of high-throughput community sequencing data. Nat Methods 7(5):335-336. doi: 10.1038/nmeth0510-335

Carney-Hinkle, E. E., H. Tran, J. W. Bundy, R. Moreno, P. S. Miller, and T. E. Burkey. 2013. Effect of dam parity on litter performance, transfer of passive immunity, and progeny microbial ecology. J Anim Sci 91(6):2885-2893. doi: 10.2527/jas.2011-4874

Clarke, K. R. 1993. Non-parametric multivariate analyses of changes in community structure. J Ecol 18(1):117-143.

Craig, J. R., C. L. Collins, K. L. Bunter, J. J. Cottrell, F. R. Dunshea, and J. R. Pluske. 2017. Poorer lifetime growth performance of gilt progeny compared with sow progeny is largely due to weight differences at birth and reduced growth in the preweaning period, and is not improved by progeny segregation after weaning. J Anim Sci 95(11):4904-4916. doi: 10.2527/jas2017.1868

Craig, J. R., F. R. Dunshea, J. J. Cottrell, J. B. Furness, U. A. Wijesiriwardana, and J. R. Pluske. 2019. A comparison of the anatomical and gastrointestinal functional development between gilt and sow progeny around birth and weaning1. J Anim Sci 97(9):3809-3822. doi: 10.1093/jas/skz217

DeSantis, T. Z., P. Hugenholtz, N. Larsen, M. Rojas, E. L. Brodie, K. Keller, T. Huber, D. Dalevi, P. Hu, and G. L. Andersen. 2006. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. App Environ Microbiol 72(7):5069-5072. doi: 10.1128/AEM.03006-05

Edgar, R. C. 2010. Search and clustering orders of magnitude faster than BLAST. Bioinform 26(19):2460-2461. doi: 10.1093/bioinformatics/btq461

Edgar, R. C. 2013. UPARSE: highly accurate OTU sequences from microbial amplicon reads. Nat Methods 10(10):996-998. doi: 10.1038/nmeth.2604

Edgar, R. C., B. J. Haas, J. C. Clemente, C. Quince, and R. Knight. 2011. UCHIME improves sensitivity and speed of chimera detection. Bioinform 27(16):2194-2200. doi: 10.1093/bioinformatics/btr381

Gaukroger, C. H., S. A. Edwards, J. Walshaw, A. Nelson, I. P. Adams, C. J. Stewart, and I. Kyriazakis. 2020a. Shifting sows: longitudinal changes in the periparturient faecal microbiota of primiparous and multiparous sows. Animal 15(3):1-8. doi: 10.1016/j.animal.2020.100135

Gaukroger, C. H., C. J. Stewart, S. A. Edwards, J. Walshaw, I. P. Adams, and I. Kyriazakis. 2020b. Changes in faecal microbiota profiles associated with performance and birthweight of piglets. Frontiers in Microbiology 11:917. doi: 10.3389/fmicb.2020.00917

Haling, R. E., R. J. Simpson, A. C. McKay, D. Hartley, H. Lambers, K. Ophel-Keller, S. Wiebkin, Herdina, I. T. Riley, and A. E. Richardson. 2011. Direct measurement of roots in soil for single and mixed species using a quantitative DNA-based method. Plant and Soil 348(1-2):123-137. doi: 10.1007/s11104-011-0846-3

Horrell, I., and J. Hodgson. 1992. The bases of sow-piglet identification. 2. Cues used by piglets to identify their dam and home pen. App Anim Behav Sci 33(4):329-343. doi: 10.1016/s0168-1591(05)80070-x

Kruskal, J. B. 1964. Multidimensional scaling by optimizing a goodness of fit to a nonmetric hypothesis. Psychometrics 29:1-28.

Lay, D. C., Jr., R. L. Matteri, J. A. Carroll, T. J. Fangman, and T. J. Safranski. 2015. Preweaning survival in swine. J Anim Sci 80:74-86. doi: 10.2527/animalsci2002.0021881200800ES10011x

Mabry, J. W., R. D. Jones, and R. W. Seerley. 1982. Effects of adaptation of a solid-floor farrowing facility utilizing elevated farrowing crates. J Anim Sci 55(3):484-488.

Nowland, T. L., K. J. Plush, M. Barton, and R. N. Kirkwood. 2019. Development and function of the intestinal microbiome and potential implications for pig production. Animals 9(3):76. doi: 10.3390/ani9030076

Nowland, T. L., V. A. Torok, W. Y. Low, M. D. Barton, K. J. Plush, and R. N. Kirkwood. 2020a. Faecal microbiota analysis of piglets during lactation. Animals 10(5):762. doi: 10.3390/ani10050762

Nowland, T. L., V. A. Torok, W. Y. Low, K. J. Plush, M. D. Barton, and R. N. Kirkwood. 2020b. A single faecal microbiota transplantation altered the microbiota of weaned pigs. Life 10(9):203. doi: 10.3390/life10090203

Rantzer, D., and J. Svendsen. 2001. Slatted versus solid floors in the dung area of farrowing pens: effects on hygiene and pig performance, birth to weaning. Acta Agri Scand, Sect. A, Animal Sci 51(3):167-174. doi: 10.1080/09064700117298

Shepard, R. N. 1962. The analysis of proximities: multidimensional scaling with an unknown distance function. I. Psychometrika 27(2):125-140. doi: 10.1007/BF02289630

Zhang, J., K. Kobert, T. Flouri, and A. Stamatakis. 2014. PEAR: a fast and accurate Illumina Paired-End reAd mergeR. Bioinform 30(5):614-620. doi: 10.1093/bioinformatics/btt593

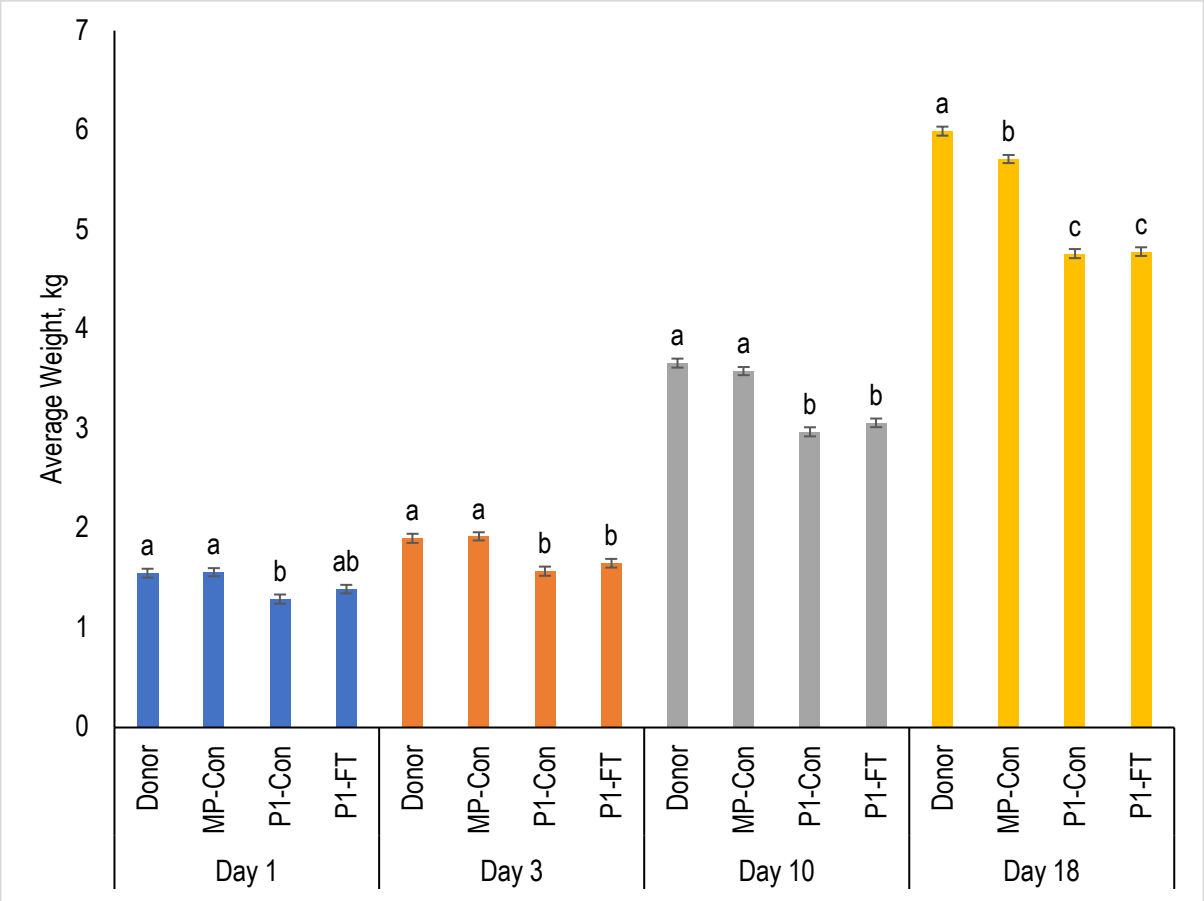


Figure 1. The effect of treatment (Donor, MP-Con, P1-Con and P1-FT) on average piglet weight (kg ± SEM) at 1, 3, 10 and 18 days of age. Within age, means with differing letters are significantly different ($P < 0.05$).

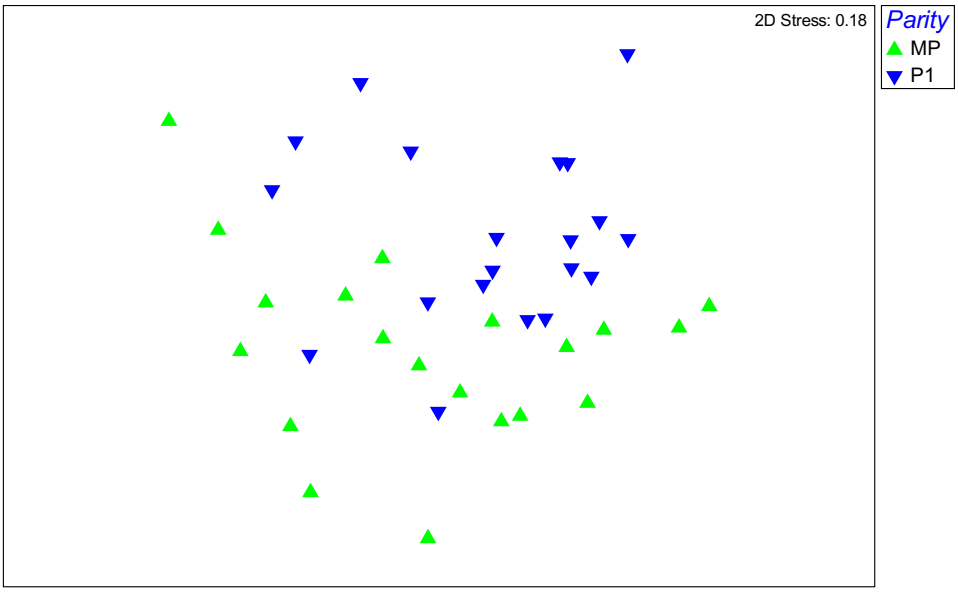


Figure 2. nMDS ordination showing the differences in relatedness of faecal bacterial genera from multiparous (MP) (triangle) or primiparous (P1) (inverted triangle) sows, calculated using Bray-Curtis distances. Points on the ordination represent individual sow faecal samples which are positioned based on their similarity to other communities in a two-dimensional space. Points more closely clustered represent microbial communities more closely related to one another based on taxa composition and abundance.

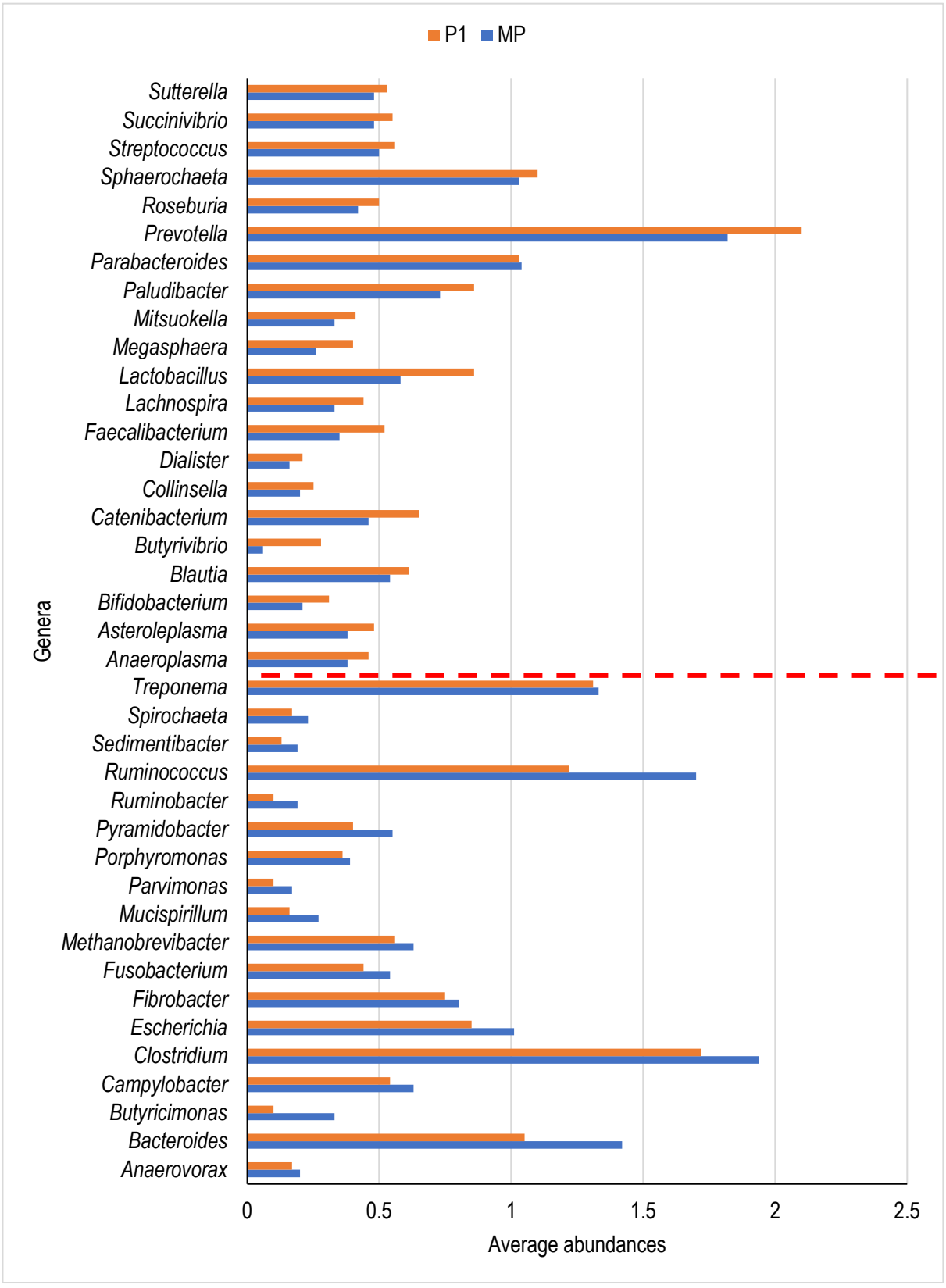


Figure 3. Average abundance of bacterial genera contributing significantly (average dissimilarity/standard deviation > 1) to the top 60% of dissimilarity between MP and P1 sows 3 days post-partum. Genera above the broken line were more abundant in P1 sows and all genera below the broken line were more abundant in MP sows.

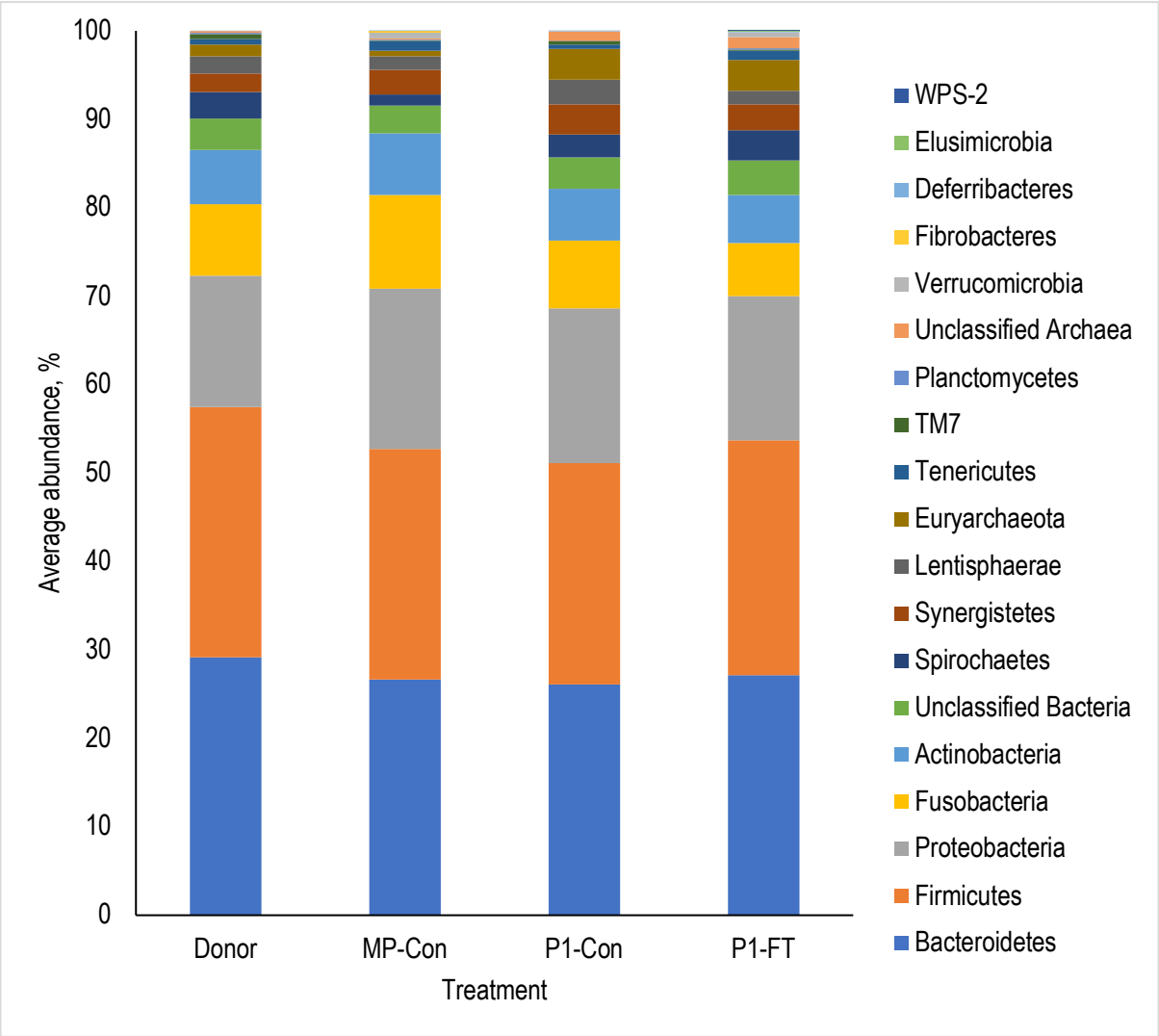


Figure 4. Average abundance of the phyla present within the faeces of 10 day old piglets in the Donor, MP-Con, P1-Con and P1-FT treatments.

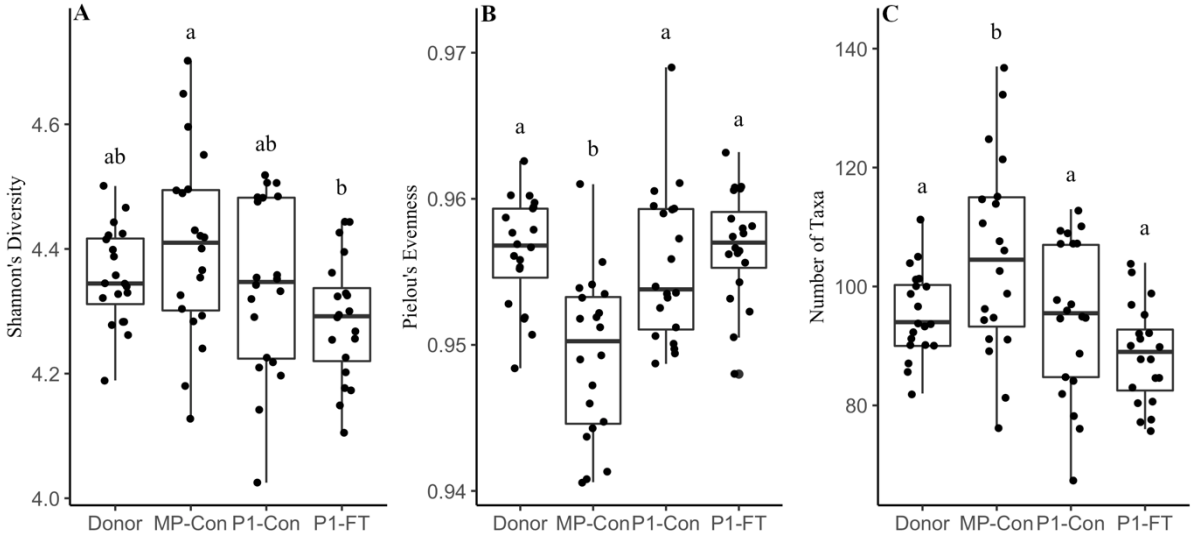


Figure 5. Boxplots demonstrating the differences between bacterial genera for piglets in treatments Donor, MP-Con, P1-Con and P1-FT for (A) Shannon's Diversity, (B) Pielou's Evenness and (C) number of taxa. Subscripts without a common letter denote a significant difference between treatments ($P < 0.05$).

Table 1. Genera contributing to the top 60% of dissimilarity of bacteria between Donor and MP-Con treated 10-day old piglets as determined by SIMPER. Overall, average dissimilarity between treatments was 34%.

Genera	Donor Average abundance	MP-Con Average abundance	Contribution %
Bacteroides	**1.64**	**2.05**	**2.01**
Escherichia	**1.17**	**1.47**	**1.77**
Unclassified _Rikenellaceae_	**0.61**	**0.77**	**1.76**
Clostridium	**1.81**	**1.85**	**1.59**
Lactobacillus	**1.18**	**1.24**	**1.47**
Campylobacter	**0.44**	**0.61**	**1.15**
Roseburia	**0.28**	**0.36**	**1.11**
Butyricimonas	**1.04**	**1.13**	**1.05**
Actinobacillus	**0.50**	**0.60**	**0.98**
Sutterella	**0.65**	**0.74**	**0.94**
Turicibacter	**0.28**	**0.38**	**0.94**
Eubacterium	**0.50**	**0.51**	**0.90**
Collinsella	**0.22**	**0.24**	**0.87**
Unclassified _Comamonadaceae_	**0.26**	**0.28**	**0.86**
Actinomyces	**0.39**	**0.41**	**0.83**
SMB53	**0.41**	**0.57**	**0.82**
Synergistes	**0.29**	**0.39**	**0.81**
Unclassified _Clostridiaceae_	**0.35**	**0.43**	**0.80**
Butyricicoccus	**0.44**	**0.48**	**0.70**
Streptococcus	**0.53**	**0.58**	**0.70**
Dialister	**0.10**	**0.26**	**0.71**
Prevotella	1.93	1.53	2.90
Unclassified _S24-7_	1.75	1.25	2.47
Unclassified _Bacteroidales_	1.42	1.20	1.61
Oscillospira	1.98	1.70	1.46
_CF231_	0.49	0.29	1.45
Fusobacterium	0.89	1.15	1.42
Unclassified Clostridiales	0.89	0.72	1.40
RFN20	0.72	0.62	1.40
_p-75-a5_	0.73	0.62	1.21
Sphaerochaeta	0.38	0.26	1.13
Odoribacter	0.38	0.31	0.97
Unclassified _Ruminococcaceae_	1.44	1.42	0.94
Unclassified _Christensenellaceae_	0.61	0.55	0.94
Parabacteroides	1.13	1.09	0.93
Unclassified Bacteria	0.46	0.45	0.90
Unclassified GMD14H09	0.38	0.29	0.89
Unclassified _Lachnospiraceae_	0.97	0.8	0.85

Ruminococcus	1.19	1.08	0.85
Flexispira	0.25	0.16	0.78
Bulleidia	0.26	0.16	0.76
vadinCA11	0.20	0.19	0.73
Dorea	0.75	0.62	0.72
Unclassified *Paraprevotellaceae*	0.22	0.17	0.72
Unclassified *Mogibacteriaceae*	0.68	0.52	0.72
Unclassified Firmicutes	0.24	0.16	0.70
Blautia	0.54	0.44	0.70

Bold depicts those genera that have a higher abundance in MP-Con treated piglets.

Table 2. Genera contributing to the top 60% of dissimilarity between Donor and P1-FT treated 18-day old piglets as determined by SIMPER. Overall, average dissimilarity between treatments was 31%.

Genera	Donor Average abundance	P1-FT Average abundance	Contribution %
Escherichia	**1.29**	**1.4**	**1.95**
Prevotella	**1.19**	**1.47**	**1.85**
Bacteroides	**1.26**	**1.42**	**1.56**
Unclassified *Christensenellaceae*	**1.17**	**1.22**	**1.56**
p-75-a5	**1.05**	**1.15**	**1.53**
Unclassified *S24-7*	**1.66**	**1.67**	**1.34**
Prevotella	**0.61**	**0.72**	**1.13**
Campylobacter	**0.48**	**0.65**	**1.08**
Ruminococcus	**1.09**	**0.97**	**1.05**
CF231	**0.62**	**0.65**	**1.00**
Roseburia	**0.48**	**0.42**	**0.99**
Clostridium	**0.91**	**0.92**	**0.98**
Unclassified *Lachnospiraceae*	**0.98**	**1.09**	**0.96**
Anaerovibrio	**0.18**	**0.32**	**0.90**
Synergistes	**0.56**	**0.58**	**0.85**
Streptococcus	**0.50**	**0.56**	**0.79**
Faecalibacterium	**0.38**	**0.42**	**0.77**
Unclassified *Erysipelotrichaceae*	**0.24**	**0.36**	**0.75**
Turicibacter	**0.27**	**0.29**	**0.74**
Unclassified *Peptostreptococcaceae*	**0.21**	**0.31**	**0.73**
Butyricimonas	**0.80**	**0.87**	**0.71**
Dialister	**0.14**	**0.21**	**0.67**
Unclassified Clostridiales	1.33	1.18	1.63
Unclassified Bacteroidales	1.51	1.25	1.29
Unclassified *Rikenellaceae*	0.44	0.41	1.23
Unclassified p-2534-18B5	0.52	0.39	1.19
Paludibacter	0.41	0.19	1.17
Lactobacillus	1.11	0.98	1.07
Dorea	0.62	0.51	1.06
Unclassified *Ruminococcaceae*	1.71	1.53	1.02
Clostridium	0.85	0.61	0.98
Ruminococcus	0.90	0.87	0.98
Sphaerochaeta	0.62	0.54	0.94
Oscillospira	1.99	1.85	0.92
Blautia	0.73	0.70	0.90
Collinsella	0.42	0.22	0.88
Unclassified RF39	0.55	0.46	0.88
Megasphaera	0.35	0.31	0.86
Treponema	0.51	0.33	0.86

L7A_E11	0.34	0.23	0.83
Catenibacterium	0.33	0.23	0.83
Odoribacter	0.35	0.19	0.82
Flexispira	0.47	0.46	0.82
Unclassified Clostridiales	1.24	1.12	0.82
Sutterella	0.59	0.47	0.82
Parabacteroides	1.15	1.10	0.80
RFN20	0.69	0.62	0.79
Acidaminococcus	0.24	0.21	0.78
Unclassified GMD14H09	0.34	0.32	0.78
Pyramidobacter	0.29	0.22	0.75
Peptococcus	0.34	0.18	0.69
Unclassified Firmicutes	0.36	0.32	0.69
Eubacterium	0.68	0.58	0.68
Unclassified *Victivallaceae*	0.34	0.24	0.68
Unclassified *Lachnospiraceae*	0.52	0.49	0.67
Actinobacillus	0.47	0.54	0.66

Bold depicts those species that have a higher abundance in P1-FT treated piglets.

Table 3. Genera contributing to the top 60% of dissimilarity between MP-Con and P1-FT treated 18-day old piglets as determined by SIMPER. Overall, average dissimilarity between treatments was 32%.

Genera	MP-Con Average abundance	P1-FT Average abundance	Contribution %
Prevotella	**1.51**	**2.19**	**3.30**
Escherichia	**1.32**	**1.40**	**1.76**
Unclassified *S24-7*	**1.36**	**1.67**	**1.67**
p-75-a5	**1.13**	**1.15**	**1.62**
CF231	**0.56**	**0.65**	**1.09**
Roseburia	**0.37**	**0.42**	**1.00**
Campylobacter	**0.57**	**0.65**	**0.93**
Flexispira	**0.34**	**0.46**	**0.92**
Megasphaera	**0.21**	**0.31**	**0.89**
Anaerovibrio	**0.13**	**0.32**	**0.84**
Unclassified RF39	**0.46**	**0.46**	**0.78**
Unclassified GMD14H09	**0.29**	**0.32**	**0.74**
Streptococcus	**0.50**	**0.56**	**0.73**
RFN20	**0.53**	**0.62**	**0.73**
Unclassified *Paraprevotellaceae*	**0.34**	**0.47**	**0.72**
Treponema	**0.27**	**0.33**	**0.72**
Unclassified *Peptostreptococcaceae*	**0.25**	**0.31**	**0.71**
Peptostreptococcus	**0.25**	**0.25**	**0.68**
Unclassified *Erysipelotrichaceae*	**0.30**	**0.36**	**0.68**
Dialister	**0.15**	**0.21**	**0.63**
Unclassified *Christensenellaceae*	1.26	1.22	1.68
Bacteroides	1.62	1.42	1.57
Unclassified Clostridiales	1.25	1.18	1.50
Unclassified *Rikenellaceae*	0.51	0.41	1.20
Enterococcus	0.34	0.27	1.14
Unclassified p-2534-18B5	0.47	0.39	1.13
Unclassified Bacteroidales	1.37	1.25	1.10
Synergistes	0.71	0.58	1.09
Dorea	0.71	0.51	1.05
Ruminococcus	1.99	1.84	2.03
Clostridium	0.90	0.61	1.00
Oscillospira	1.94	1.85	0.99
Blautia	0.72	0.70	0.91
Unclassified *Coriobacteriaceae*	0.36	0.30	0.91
Unclassified *Ruminococcaceae*	1.60	1.53	0.90
Unclassified *Lachnospiraceae*	1.10	1.09	0.89
Lactobacillus	1.03	0.98	0.89
Clostridium	0.99	0.92	0.86
Sphaerochaeta	0.55	0.54	0.85

Parabacteroides	1.20	1.10	0.84
Faecalibacterium	0.44	0.42	0.83
Unclassified Clostridiales	1.27	1.12	0.80
Catenibacterium	0.29	0.23	0.80
Unclassified *Clostridiaceae*	0.25	0.12	0.80
Turicibacter	0.43	0.29	0.78
Paludibacter	0.22	0.19	0.75
Sutterella	0.56	0.47	0.75
Unclassified *Comamonadaceae*	0.64	0.58	0.73
Butyricimonas	0.92	0.87	0.73
Collinsella	0.32	0.22	0.72
Odoribacter	0.29	0.19	0.72
L7A_E11	0.27	0.23	0.71
Actinomyces	0.34	0.19	0.70
Moryella	0.31	0.16	0.70
Eubacterium	0.63	0.58	0.66
Methanobrevibacter	0.35	0.21	0.64
Pyramidobacter	0.24	0.22	0.63

Bold depicts those species that have a higher abundance in P1-FT treated piglets.

Chapter 5:

Maternal supplementation with phytogenic additives influenced the faecal microbiota and reproductive potential in sows

Statement of Authorship

Title of Paper	Maternal supplementation with phytogenic additives influenced the faecal microbiota and reproductive potential in sows
Publication Status	☐ Published ☐ Accepted for Publication ☒ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Submitted for publication in the journal: Applied Microbiology and Biotechnology.

Principal Author

Name of Principal Author (Candidate)	Tanya Nowland		
Contribution to the Paper	Assisted with the experimental design, managed and analysed statistics, drafted and edited the manuscript.		
Overall percentage (%)	80%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	23/03/2021

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Dana Stanley		
Contribution to the Paper	Assisted with statistical analysis, data interpretation and editing manuscript.		
Signature		Date	24/03/21

Name of Co-Author	Roy Kirkwood
Contribution to the Paper	Assisted with the experimental design and interpretation and editing manuscript.

Signature		Date	23/03/2021

Name of Co-Author	Kate Plush		
Contribution to the Paper	Assisted with the experimental design, conducted the experiment, evaluated and edited the manuscript.		
Signature		Date	23/03/2021

Name of Co-Author	Valeria Torok		
Contribution to the Paper	Assisted with the experimental design, evaluated and edited the manuscript.		
Signature		Date	25/03/2021

Name of Co-Author	Yadav Bajagai		
Contribution to the Paper	Assisted with statistical analysis, data interpretation, evaluated and edited the manuscript.		
Signature		Date	23/03/2021

Please cut and paste additional co-author panels here as required.

Name of Co-Author	Neil Gannon		
Contribution to the Paper	Assisted with experimental design, funding provision, evaluated and edited the manuscript.		
Signature		Date	25/03/2021

Maternal supplementation with phytogenic additives influenced the faecal microbiota and reproductive potential in sows

Tanya L. Nowland[a,*], Dragana Stanley[b], Roy N. Kirkwood[a], Valeria A. Torok[a,c], Yadav S. Bajagai[b], Neil J. Gannon[d] and Kate J. Plush[e]

[a] School of Animal and Veterinary Sciences, The University of Adelaide, Roseworthy, SA 5371, Australia

[b] Institute for Future Farming Systems, Central Queensland University, Rockhampton, QLD 4701, Australia

[c] South Australian Research and Development Institute, Food Sciences, SA 5064, Australia

[d] BIOMIN Pte. Ltd., Singapore, 159741, Singapore

[e]SunPork Group, Eagle Farm, QLD 4009, Australia

*Corresponding author:

Tanya Nowland

ORCHID ID: 0000-0001-8812-5948

School of Animal and Veterinary Sciences

The University of Adelaide

Roseworthy SA 5371, Australia

Telephone: 08 8313 7786

E-mail: tanya.nowland@adelaide.edu.au

Abstract

Sows undergo physiological stress during gestation and lactation, potentially leading to enteric dysbiosis and reduced reproductive potential. Phytogenic additives (PAs) may improve performance via their antioxidant, anti-inflammatory and antimicrobial properties. This study determined whether the provision of a gestation/lactation diet containing PAs would alter the gastrointestinal microbiota of sows and their piglets, and improve performance. Sows received a commercial diet throughout gestation and lactation (CTR; n = 64), a commercial diet throughout gestation and a diet containing PAs in lactation (CTR-PA; n = 63) or a commercial diet containing PAs in gestation and lactation (PA; n = 90). Sows were weighed and P2 backfat recorded after mating and at entry and exit from the farrowing house and piglets were weighed on days 1 and 21 of life. Faecal samples collected from sows at farrowing house entry and piglets at 21 and 35 d were subjected to 16S rRNA gene amplicon analysis. The addition of PAs to sow diets resulted in more piglets born (P = 0.03), however, it did not improve the number of liveborn piglets (P = 0.14). There were no differences in sow weight, P2 backfat depth or lactation feed intake observed. PAs had no effect on piglet weight or survival to weaning but did alter the faecal microbiota of sows, and this change was observed in piglets at 21 and 35 d. PA supplementation to sows has the potential to increase litter size, while also potentially influencing gastrointestinal tract health of the sow and piglets reared.

Keywords: bacteria, gut health, production, pig

Key points

- Number of piglets born was increased through phytogenic supplementation to sows.
- *Oscillospira, Roseburia* and *Ruminococcus* were increased in sows fed phytogenics.
- Sow phytogenic supplementation increased *Faecalibacterium* in piglets post weaning.

1. Introduction

Gestation and lactation are both times of high physiological stress for sows. Gestation involves the partitioning of nutrients for the development of multiple foetuses, while lactation has great demands on sows as they produce enough milk to feed their litter. Often due to this highly taxing process, sows lose from 5 to 20% of their body weight (Thaker & Bilkei, 2005). Stress can also decrease food intake and induce enteric dysbiosis in pigs, which can cause suboptimal digestion and poor nutrient utilisation and negatively affect intestinal health (Gresse *et al.*, 2017). Impaired nutrient intake and utilisation increases weight loss and can have a negative effect on their ability to rear their litter and to return to oestrus after their litter is weaned (Thaker & Bilkei, 2005). Additionally, sows undergo large shifts in the gastrointestinal tract (GIT) microbiota throughout this time (Gaukroger *et al.*, 2020) and exhibit metabolic syndrome in late gestation and early lactation (Cheng *et al.*, 2018). Nutritional interventions may improve sow rearing ability and reduce negative effects on their health.

Phytogenics are a group of natural flavour and sensory compounds derived from plants and include herbs, spices and essential oils (Windisch *et al.*, 2008). When added to feed, they improve animal performance via three main mechanisms; flavour properties which enhance feed intake, biological activity that aids digestion, and improving GIT health via modulation of the GIT microbiota (Windisch *et al.*, 2008, Murugesan *et al.*, 2015). The proposed drivers for these influences on performance are the antioxidant, anti-inflammatory and antimicrobial properties they exhibit (Windisch *et al.*, 2008).

Recent work in pigs demonstrated that a phytogenic additive (PA) which includes a combination of essential oils, maintained finisher performance when protein and energy specifications in the diet were reduced, and improved performance when dietary specifications were maintained (Walker *et al.*, 2019). However, there is little published data on the effect of phytogenics on sow reproduction or the GIT microbiota. Additionally, given that piglets are raised within a farrowing crate in direct contact with their sow, it is likely that the establishment of the piglet GIT microbiota is dependent on contact with their mother. We aimed to determine whether the provision of gestation and/or lactation diets containing PAs

would alter the GIT microbiota of sows, and thus that of their piglets, and so improve performance. It was hypothesised that (1) the provision of a diet containing PAs during gestation would increase litter birth weight, and when fed during lactation would increase sow feed intake and lactation performance; (2) the provision of a gestation/lactation diet containing PAs would alter the GIT microbiota of the sow, with this change transmitted to their piglets causing a shift in piglet GIT microbiota and improvements in their growth and survival.

2. Materials and methods

2.1. Sow housing and feeding management

After mating, 351 sows (parity 2 to 4) were allocated to one of six identical, naturally ventilated gestation pens (1.8m^2 per sow) based on mating date and parity. The pens had partially slatted concrete flooring with eight drinkers per pen. Sows were housed in groups of ~60 and fed via electronic sow feeders (ESF; MPS Agri Ltd, Suffolk, UK). The electronic sow feeders enabled the feeding of two separate diets to pigs within the same pen. Sows were allowed 2.2 kg/day of a commercial gestation diet formulated to provide 13.0 MJ DE/kg, 13.1% total protein and 0.55% standardised ileal digestible (SID) lysine unless their P2 backfat depth (P2; 65 mm off the midline at the last rib curve) at breeding was < 14 mm, when the allowance was increased to 2.8 kg/day for the first 30 days and then subsequently reduced to 2.2 kg/day until moved to farrowing accommodation. Pregnancy confirmation was performed by B-mode ultrasonography at 35 d and 70 d post-breeding and any non-pregnant sows removed from the pen.

At 5.7 ± 0.4 d prior to their calculated farrowing date, sows were moved into naturally ventilated farrowing accommodations and housed in individual farrowing crates (1.8 x 2.4m). Each farrowing crate contained its own lamp heated creep area for the piglets and two water nipples for the sow and one for the piglets. Prior to farrowing, sows were fed 2.4 kg/d of a commercial lactation diet formulated to provide 14 MJ DE/kg, 17.3% total protein and 0.84% SID lysine. After farrowing, sows were fed the lactation diet to-appetite up to 16 kg/d delivered in two meals until weaning at 22.4 ± 0.1 d.

At the time of breeding, sows were assigned to one of three dietary treatments to have equal parity distributions, previous litter size and wean-to-serve intervals. Treatments were:

1. CTR: fed a commercial diet in gestation and lactation (n = 64)
2. PA: fed a commercial diet containing a phytogenic additive (700 g/t) in gestation and lactation (n = 90)
3. CTR-PA: fed a commercial diet in gestation and a diet containing a PA (700 g/t) in lactation (n = 63)

Base diet specifications used are outlined in supplementary table 1. The PA used throughout the study was Digestarom® DC Xcel 1000 provided by BIOMIN (BIOMIN Animal Nutrition GmbH, Getzersdorf, Austria) and contained a proprietary mix of essential oil extracts and herbs with menthol, carvacrol, carvone as major bioactive compounds. 700 g of the proprietary mix was added to each tonne of base diet via micro dispenser. The proprietary mix was microencapsulated to ensure heat stability during pelleting.

2.2. Data recorded

All sows were weighed and their P2 backfat depths recorded at entry into the gestation housing and on entry and exit from the farrowing house. Sow feed intakes in the farrowing house were measured by weighing all leftover feed and all new feed into the feeder when sows were fed twice daily. On the day of farrowing, the total born and live-born litter sizes and individual birth weights were recorded. At farrowing, two live female focal piglets per litter were tagged to allow individual identification. At 13 h and within 24 h of farrowing, fostering occurred within treatment based on the sows rearing capacity (functional teat number) and all piglet movement was noted. Litter weight was recorded on day 1 and 21 of lactation. Individual piglet weights on day 1 were used to determine the total litter weight, minimum and maximum piglet weight and the percentage of piglets within the litter weighing less than 1.1 kg. All mortalities and removals for ill thrift were recorded, as were the number of pigs weaned per sow and the time from weaning to mating. Faecal samples were collected from sows at weighing prior to farrowing house entry and from tagged focal piglets at 21 (prior to weaning) and at 35 days of age (~2 weeks postweaning). The

focal piglets were individually weighed at 21 and at 35 days of age. Faeces were placed on ice immediately and stored at -80^{o}C within four hours of collection.

2.3. DNA extraction and 16S rRNA Amplicon Analysis

Approximately 0.2 g from each sample was used for the DNA extraction using the modified repeated bead beating plus column method (Yu & Morrison, 2004) and the quantity of DNA was estimated using a NanoDrop spectrophotometer (ThermoFisher Scientific, Massachusetts, USA).

The forward and reverse primers used for amplification of the V3-V4 region of the 16S rRNA gene were: ACTCCTACGGGAGGCAGCAG and GGACTACHVGGGTWTCTAAT, respectively. The 16S rRNA gene amplicon sequencing library was prepared by amplifying the V3-V4 region of the gene with the primers containing linker sequences, index sequences and heterogenicity spacers (Fadrosh *et al.*, 2014). The amplified amplicon library was cleaned up using AMPure XP clean up kit (Beckman Coulter, Lane Cost West, NSW, Australia). Sequencing was conducted on the Illumina MiSeq platform using 2x300 bp paired-end sequencing at the Genewiz sequencing facility (GENEWIZ Suzhou, China).

The microbial communities were analysed using QIIME 2 v2020.6 (Bolyen *et al*., 2019). The dereplicating of sequences and OTU (operational taxonomic unit) clustering at 97% identity was done using the VSEARCH plugin (Rognes *et al.*, 2016). Representative sequences for each OTU were assigned taxonomy using q2-feature-classifier (Bokulich *et al*., 2018) with the classifier pre-trained on GreenGenes v13.8 with 99% OTUs. GreenGenes taxonomy was used provisionally (DeSantis *et al*., 2006, Balvociute & Huson, 2017) up to the genus level; species level was not inferred from 16S rRNA data. After quality filtering, 16S rRNA gene amplicon data for 322 samples were included in the analysis with an average of 9560 reads per sample and a minimum of 1036 reads per sample. The sequence data is publicly available at the MG-RAST database under library accession number mgl837686 (https://www.mg-rast.org/).

2.4. Statistical Methods

All production data were analysed in SPSS v25 (IBM, Armonk, NY, USA) and significance was established at $P < 0.05$. Normally distributed data were analysed using a general linear mixed model. Generalised linear mixed models were applied to binary data (pregnancy and farrowing rate) using binary logistic regression and to count data (all piglet mortalities) using Poisson regression. Gestation and lactation periods were analysed as separate datasets. The model applied to the gestation data included gestation pen as a random term and treatment (CTR and PA) as a fixed effect. The model applied to lactation data included farrowing shed as a random term, and treatment (CTR, CTR-PA and PA) as a fixed effect. All data is expressed as mean ± standard error of the mean (SEM) unless it was binary data, whereby the confidence intervals are presented.

All of the downstream statistical microbial data analysis and visualisation were done using Calypso Version 8.84 (Zakrzewski *et al.*, 2016) on a Hellinger transformed abundance table (Legendre & Gallagher, 2001). Statistical analysis on alpha diversity metrics of Shannon's index, Richness and Chao1 were performed. Multivariate data visualisations and multivariate statistical testing among treatment groups were performed using redundancy analysis (RDA), discriminatant anlaysis of principal components (DAPC) and Adonis analysis based on Bray-Curtis distance matrices. Univariate non-parametric Wilcoxon-rank tests were also applied to the data to identify the differences between specific taxa for each treatment. Core microbiota Venn diagram was also generated and plotted in Calypso Version 8.84 (Zakrzewski *et al*., 2016).

3. Results

3.1. Sow and litter performance

There was no effect of treatment on gestation weight gain (CTR: 59.3 ± 3.6, PA: 59.4 ± 3.6, $P = 0.967$) or P2 backfat gain (mm; CTR: 1.5 ± 0.6, PA: 1.2 ± 0.6, $P = 0.411$) during gestation. Pregnancy and farrowing rates were unaffected by gestation treatment ($P > 0.05$; Table 1). Litter size was increased by 0.8 pigs per litter in PA sows compared with CTR ($P < 0.05$; Table 1) however, this did not translate to a

higher number of piglets born alive (P = 0.141) as stillbirths were higher in PA sows (P = 0.03). Additionally, the number of piglets born at less than 1.1kg was significantly higher for PA sows (P = 0.015; Table 1).

Piglets were fostered to achieve the same litter size (11.7 ± 0.1 piglets per sow), but PA litters tended to exhibit a lower litter weight than CTR and CTR-PA post-foster (CTR: 16.8 ± 0.6, CTR-PA: 16.5 ± 0.6, PA: 15.7 ± 0.6, P = 0.080). There was no treatment effect on average daily gain (CTR: 0.215 ± 0.01, CTR-PA: 0.210 ± 0.02, PA: 0.214 ± 0.02, P = 0.797) and litter size (CTR: 10.1 ± 0.5, CTR-PA: 10.2 ± 0.5, PA: 10.0 ± 0.5, P = 0.713) or weight of piglets at weaning (day 21; CTR: 59.3 ± 5.1, CTR-PA: 60.3 ± 5.1, PA: 57.5 ± 5.1, P = 0.345).

There was no difference between treatments for pre-foster (CTR: 0.9 ± 0.2, CTR-PA: 0.7 ± 0.2, PA: 0.8 ± 0.2, P = 0.288), post-foster (CTR: 1.1 ± 0.1, CTR-PA: 1.0 ± 0.1, PA: 0.9 ± 0.1, P = 0.709) or total liveborn piglet mortality (CTR: 1.7 ± 0.4, CTR-PA: 1.4 ± 0.3, PA: 1.5 ± 0.4, P = 0.313). There were no treatment effects on sow feed intake, body weight or P2 backfat in lactation (P > 0.05; Table 2). There was a tendency for sows from the PA treatment to display the shortest rebreeding interval (P < 0.1).

3.2. Impact of gestation diet on sow faecal microbiota

The administration of PAs to the gestation diet did not affect major alpha diversity metrics; Shannon's index (P = 0.51), Chao1 (P = 0.46) and Richness (P = 0.59). Redundancy analysis (RDA) indicated a significant difference between the faecal microbiota of sows fed the CTR and PA diets in gestation (P = 0.001). Likewise, when assessing the microbiota structure differences using Adonis permutational multivariate analysis of variance based on the Bray-Curtis distance matrix, significant differences between the CTR and PA treatments existed (R^2 = 0.02, P = 0.0003).

Differences in community structure were evident at the genus level, with 18 genera significantly affected by diet (Wilcoxon rank test; P < 0.05). Specifically, Unclassified p253418B5, Unclassified Bacteria,

Enterococcus, Sporobacter, Succinispira and the archaea *Methanobrevibacter* were more abundant in control sows (CTR), while *Roseburia, Subdoligranulum, Lactonifactor, Oscillospira, Coprococcus, Pediococcus, p75a5, CF231, Prevotella, Ruminococcus*, Unclassified *S247* and *Butyrivibrio* were more abundant in the faeces of PA sows. Those bacteria that contributed to $P < 0.01$ are presented in Fig. 1.

3.3. Maternal influence on the core piglet microbiota at different ages

The influence of sow microbiota on the development and maturation of piglet intestinal microbial communities is presented in the Venn diagram depicting the core microbiota (Fig. 2). Of 77 total core genera, 36 (46%) were core genera shared among sows, piglets at day 21, and piglets at day 35. Sows and piglets (including both day 21 and day 35) shared 62% (48) of bacterial core genera, indicating the influence of maternal microbiota on piglets. A genus was considered a member of the group's core microbiota if it was present in more than 40% of the samples of that group.

3.4. Impact of sow diet on piglet faecal microbiota

A significant shift in the microbial community occurred between day 21 and day 35 in piglets, moving their microbiota structure further away from the maternal influence; thus, we will present these separately.

3.4.1. In 21-day-old piglets

In 21 d old piglets, there was no effect of sow diet on faecal alpha diversity measures (Shannon's index, $P = 0.48$; Chao1, $P = 0.38$; and Richness, $P = 0.88$). A range of multivariate analyses and corresponding visualisation indicated some degree of overlapping occurred between treatments (CTR-PA, PA and CTR). Discriminant analysis of principal components (DAPC) showed that each treatment segregated from one another (Fig. 3). Additionally, Adonis permutational multivariate analysis of variance based on Bray-Curtis distance demonstrated a significant difference among the treatments ($R^2 = 0.02$, $P = 0.05$).

Of the differences in community structure observed in the faeces of 21-day old piglets reared on sows fed differing diets, 8 genera differed significantly (Wilcoxon rank test; $P < 0.05$; Fig. 4). *Succinivibrio, Shuttleworthia* and *Marvinbryantia* were most abundant in CTR-PA piglets, while *Treponema* were most abundant in CTR-PA and PA piglets, *Lactobacillus, Chlamydia* and *Pediococcus* were most abundant in PA piglets and *Odoribacter* were most abundant in CTR piglets.

3.4.2. In 35-day-old piglets

Alpha diversity analysis showed that Shannon's index ($P = 0.02$) and Richness ($P = 0.001$) were higher for those piglets reared on sows being fed PA, regardless of how long the sows received PAs for (PA and CTR-PA), while Chao1 tended to be higher for piglets reared on control sows (CTR; $P = 0.07$; Fig. 5). DAPC showed that piglets in the CTR-PA and PA treatment were more similar and clustered away from CTR piglets at 35-day of age (Fig. 6). Additionally, Adonis analysis based on Bray-Curtis distance matrices observed significant differences among treatments ($R^2 = 0.03$, $P = 0.002$).

There were 11 genera within the faeces of 35-day old piglets significantly affected by treatment, ten of which are presented in Fig. 7. Bacterial genera *Prevotella, Succinispira* and *Faecalibacterium* were most abundant in piglets reared on sows fed PAs regardless of the intervention length (CTR-PA and PA). *Lactobacillus* and *Bifidobacterium* were most abundant in piglets reared on sows fed a control diet throughout gestation (CTR-PA and CTR). *Proteocatella* and *Collinsella* were more abundant in CTR and PA piglets, while Unclassified *Lachnospiraceae* were more abundant in CTR-PA piglets. PA piglets had a higher abundance of *Macellibacteroides* and CTR piglets were more abundant in *Cloacibacillus* and archaea *Methanobrevibacter*.

4. Discussion

4.1. Sow gestation performance

Pregnancy is a time of high metabolic demand as fertilisation, implantation and embryo development occur. As a result, oxidative stress is a common by-product of these processes (Wang *et al.*, 2018). Free-

radical production is associated with many reproductive disorders (Berchieri-Ronchi *et al.*, 2011) and therefore, a reduction in free-radicals would have positive implications for the sow. A key finding in the current study was an increase in litter size observed for those sows that were fed PAs in gestation. This increase in litter size has been documented previously in studies investigating the use of a different combination of PAs supplied during gestation (Reyes-Camacho *et al.*, 2020). One possible explanation for how these additives influence litter size is their anti-inflamatory and antioxidative capacity. Supporting this notion, Reyes-Camacho *et al.* (2020) observed improvements in litter size and increased antioxidant enzyme activity as well as nitrous oxide levels during early gestation (d 35) when sows were fed PAs.

The PAs used may have caused an increase in litter size via two mechanisms. Although the essential oil components of the PA used are different from the study above, they may have effected litter size via their anti-inflammatory and antioxidant ability in the same way discussed above (Windisch *et al.*, 2008, Karásková *et al*., 2016) or they may have influenced litter size via modulation of the GIT microbiota. Previous studies have identified specific bacteria associated with oxidative stress in sows (Wang *et al*., 2018, Wang *et al*., 2019). Wang *et al.* (2018) identified antioxidant capacity was positively correlated with *Bacteroidaceae* but negatively with *Phascolarctobacterium* and *Streptococcus*, while Wang *et al.* (2019) reported correlations between *Ruminococcaceae* and *Coprococcus* with sows who gave birth to a higher number of stillborn piglets. In the present study, *Coprococcus* was increased in PA sows when compared to controls, and these animals had a significant increase in stillbirth rate, however, no other bacteria identified previously as being correlated with oxidative stress were observed. Additionally, sows that received PAs during gestation had a higher abundance of the potentially beneficial bacteria, *Oscillospira*, which is strongly correlated with the formation of secondary bile acids (Cheng *et al*., 2018) and *Roseburia* and *Ruminococcus*, known as butyrate-producing bacteria (Wang *et al*., 2018). Butyrate exerts a variety of functions that aid in maintaining GIT barrier function, it is an important energy source for colonocytes/epithelial cells, protects against inflammation and decreases oxidative stress, which can all lead to an improvement in feed efficiency (Hamer *et al*., 2007). Interestingly, CTR sow faeces were more abundant in genera *Enterococcus*, which has been associated with necrotising enterocolitis (Wang *et al.*,

2016) and several *Enterococcus* species are associated with pathogenicity causing urinary tract infections, endocarditis and bacteremia (Singh *et al*., 2017). Additionally, PA sows had a higher abundance of potentially beneficial bacterial genera *Prevotella*, which has a unique ability to degrade mucin glycoproteins and increase weight and survival in pigs (McCormack *et al.*, 2017, Wang *et al.*, 2017). However, Wang *et al.* (2018) has demonstrated that it is correlated with 8-hydroxy-deoxyguanosine which is a marker for oxidative damage in sows. Together, the results suggest that PA may be beneficial by reducing potentially pathogenic *Enterococcus* and enhancing butyrate-producing bacteria and hence improve intestinal barrier function, decreasing oxidative stress. However, further research is needed to assess these effects directly.

4.2. Sow lactation performance

Whilst the total number of piglets born increased in the PA treated sows, this failed to translate to an increase in the number of piglets born alive. This contrasts with other published studies with PAs that utilised a similar experimental design (Reyes-Camacho *et al.*, 2020). An increase in the number of piglets born dead in the PA treated group was observed, which likely explains why no improvement in born alive was observed. However, no autopsy was completed on dead piglets, and rather piglets were classified as dead at birth by the presence of caps on feet (i.e., they had not walked). The sows farrowed in naturally ventilated rooms throughout the trial and the average minimum temperature was 6°C (maximum 16°C), and except for creep heat lamps, no additional heat was provided in the farrowing shed. The 60 g reduction in average birth weight in piglets from PA sows, likely due to the increased litter size in this group, also increases the probability that these piglets died from exposure, as low-birth-weight piglets are naturally at a higher risk of mortality (Baxter *et al.*, 2008). Taken collectively, the reduced birthweight in PA piglets and the low ambient temperature during the experimental period may have increased the risk of deaths from exposure which were incorrectly categorised as stillbirths. Thus, these piglets might have survived if the farrowing room environment was optimised.

There was no impact of the PA on lactation sow feed intake, litter weight or sow body condition at weaning. Surprisingly, there was a tendency for a 2 to 3-day reduction in the interval from weaning to breeding. It is unknown why this improvement in reproductive performance was observed in the absence of significant changes in total feed intake and body condition. Presumably, it involves a positive effect on ovarian follicular growth. Others have postulated that the anti-inflammatory and antioxidant properties of the phytogenics containing oregano fed around the time of farrowing improve uterine involution, and this is what leads to the reduction in wean to service interval (Kis & Bilkei, 2003). Regardless, this finding has implications for non-productive days in sow herds and potentially for subsequent fertility.

4.3. Piglet performance

It is well established that the development of the GIT microbiota is important for health and survival in all species. The farrowing house provides the first place to influence the development of the microbiota of the piglet as the piglet is housed exclusively with their sow. It is well understood in commercial operations that the sow's microbiota can have positive and negative impacts on the piglet if not well managed. Finding that PA fed sows, regardless of whether it was fed in gestation and/or lactation or just lactation, altered the microbiota of piglets at 21 days of age was somewhat expected but has not been widely demonstrated. Previous research suggests that the GIT microbiota develops rapidly during early lactation and is influenced by a combination of factors, including the sow's urogenital microbiota, colostrum and milk consumption, the pen microbiota, and interaction with the sow's faeces (Nowland *et al.*, 2019). Therefore, it is likely that the piglet's microbiota was modulated via one or more of these processes. This is further substantiated by the finding that sows shared 62% of core genera with their piglets in the present study. Additionally, previous research investigating the use of PAs in sow diets throughout gestation or gestation and lactation demonstrated that phytogenic volatile compounds were present in the placental fluid of those animals fed the additive throughout gestation and were present in the milk of those fed the additive throughout lactation (Reyes-Camacho *et al.*, 2020). Hence, it is possible that GIT modulation was initiated before parturition in the PA piglets and persisted throughout lactation from its presence in the milk in the

PA and CTR-PA sows. Additionally, piglets have been known to exhibit coprophagy and hence it would be expected that this would have contributed to the change observed (Aviles-Rosa *et al.*, 2019).

Although the faecal microbiota of piglets was altered by the inclusion of PAs in sow diets, no improvements in production parameters such as piglet weight and survival were observed. This contrasts with a previous study where PA fed grower-finisher pigs demonstrated improvements in growth (Walker *et al.*, 2019). However, dosage may have affected this outcome as the PA concentration in milk is likely lower than what they would have received in the feed. Additionally, a milk fed animal is very different from one on solid feed and hence this may have also had an impact. When investigating the faecal microbiota at 21 days of age, a combination of potentially beneficial and potentially pathogenic bacteria were present in piglets reared on PA and CTR-PA sows. 21-day-old PA piglets were more abundant in *Lactobacillus*, which is known for its probiotic attributes, being associated with improved GIT health, feed efficiency and growth in pigs (Shu *et al.*, 2001). While *Chlamydia*, a potentially pathogenic bacteria, was also more abundant in PA piglets at 21 days of age. Additionally, *Treponema*, a potentially pathogenic bacterial genus, previously associated with swine dysentery (Rees *et al.*, 1989) was more abundant in piglets reared on CTR-PA and PA sows. These results suggest that although the faecal microbiota of 21-day old piglets was influenced by PAs, no apparent advantage or disadvantage for piglet growth performance was evident.

Interestingly, differences in faecal microbiota between piglets reared on PA and CTR-PA sows when compared with CTR animals existed two weeks post weaning (d35) even when the influence of the sow was removed. Additionally, the faecal microbiota of piglets from sows fed PAs also tended to cluster closer together and become more similar postweaning. Weaning is a time of high stress and can cause postweaning diarrhoea and often results in a postweaning growth check (Pluske *et al.*, 2018). Therefore, the presence of an optimal microbiota during this time may be beneficial. Unfortunately, no post weaning pig weights could be collected on these pigs, so no assessment of piglet productivity occurred. Regardless, piglets reared on sows fed PAs were colonised by multiple potentially beneficial bacteria

postweaning. At 35 d, PA and CTR-PA piglets had an increased abundance of *Faecalibacterium*, which is a butyrate-producing bacteria with anti-inflammatory effects (Singh *et al.*, 2017), and a short chain fatty acid producing bacteria, *Succinispira* (Janssen & O'Farrell, 1999). While potentially beneficial bacteria, *Prevotella* and *Bifidobacterium*, which are positively correlated with body weight (Shu *et al.*, 2001, McCormack *et al.*, 2017) and likely butyrate-producing bacteria, Unclassified *Lachnospiraceae* (Cheng *et al.*, 2018), were assessed as explaining some of the microbial differences between piglets and were most abundant in CTR-PA 35-day old piglets. This indicates potentially improved intestinal health and an associated growth in these animals. Additionally, CTR-PA and CTR piglets shared a higher abundance of potentially beneficial bacteria, *Bifidobacterium* and *Lactobacillus*, at 35-days of age. The 35-day old CTR piglets had a higher abundance of the potentially pathogenic bacteria, *Cloacibacillus*, which is a potential human pathogen associated with bacteremia (Domingo *et al.*, 2015). Overall, without the added production characteristics it is difficult to distinguish whether the PA provided any benefit to the piglets. This study provides evidence that microbiota manipulation of the sow influences the piglet microbiota and that this influence persists for at least two weeks beyond weaning.

5. Conclusions

Our findings demonstrate that PAs altered the microbiota of sows and that this change was transferred to their piglets and was maintained for up to 14 days post weaning. Additionally, the inclusion of PAs to a gestation diet increased the number of piglets born, presumably via its antioxidant effects, however, this was not evident as liveborn piglets. While no further improvements in weight or survival parameters were observed in the sows and piglets during lactation, the wean to oestrus interval tended to be reduced in sows fed the PA throughout gestation and lactation. Therefore, the inclusion of PAs in a sow diet throughout gestation and lactation has the potential to increase the number of piglets born per sow and reduce the number of non-productive days. Further research investigating how PAs influence litter size and what effect it is having on the GIT microbiota of piglets reared post weaning in relation to performance parameters is warranted.

Acknowledgments

The authors wish to acknowledge the expert technical assistance of Jessica Zemitis and Dannielle Glencorse for assistance in conducting the experiment.

Authors' Contributions

Conceptualisation, R.N.K., K.J.P and N.J. G; methodology, R.N.K., K.J.P., N.J.G. and T.L.N.; software, T.N.K and D.S.; validation, R.N.K., K.J.P., T.L.N. and D.S.; formal analysis, T.L.N., Y.S.B. and D.S.; investigation, R.N.K., K.J.P. and T.L.N.; resources, R.N.K., K.J.P., V.A.T and T.L.N; data curation, T.L.N. and D.S.; writing—original draft preparation, T.L.N.; writing—review and editing, R.N.K., K.J.P., V.A.T., Y.S.B., N.J.G. and D.S.; visualisation, T.L.N. and D.S.; supervision, R.N.K., K.J.P., V.A.T. and D.S.; project administration, K.J.P and R.N.K.; funding acquisition, K.J.P and N.J.G.. All authors have read and agreed to the published version of the manuscript.

Funding

This project was partly funded by BIOMIN (www.biomin.net).

Data availability statement

Sequencing data is publicly available on MG-RAST metagenomic data server database (https://www.mg-rast.org/) under library accession number MGL mgl837686.

Code availability

Not applicable.

Declarations

Ethics approval

Animal ethics approvals were obtained from the University of Adelaide Animal Ethics committee, approval number S-2019-044.

Conflicts of interest/Competing interests

This project was partly funded by BIOMIN (www.biomin.net). The authors declare no other conflicts of interest.

Consent to participate

Not applicable.

Consent for publication

Not applicable.

References

Aviles-Rosa EO, Rakhshandeh A and McGlone JJ 2019. Preliminary study: Depriving piglets of maternal feces for the first seven days post-partum changes piglet physiology and performance before and after weaning. Animals 9, 1-12.

Balvociute M and Huson DH 2017. SILVA, RDP, Greengenes, NCBI and OTT - how do these taxonomies compare? BMC Genomics 18, 114.

Baxter EM, Jarvis S, D'Eath RB, Ross DW, Robson SK, Farish M, Nevison IM, Lawrence AB and Edwards SA 2008. Investigating the behavioural and physiological indicators of neonatal survival in pigs. Theriogenology 69, 773-783.

Berchieri-Ronchi CB, Kim SW, Zhao Y, Correa CR, Yeum KJ and Ferreira ALA 2011. Oxidative stress status of highly prolific sows during gestation and lactation. Animal 5, 1774-1779.

Bokulich NA, Kaehler BD, Rideout JR, Dillon M, Bolyen E, Knight R, Huttley GA and Caporaso JG 2018. Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's q2-feature-classifier plugin. Microbiome 6, 1-17.

Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, Alexander H, Alm EJ, Arumugam M, Asnicar F, Bai Y, Bisanz JE, Bittinger K, Brejnrod A, Brislawn CJ, Brown CT, Callahan BJ, Caraballo-Rodriguez AM, Chase J, Cope EK, Da Silva R, Diener C, Dorrestein PC, Douglas GM, Durall DM, Duvallet C, Edwardson CF, Ernst M, Estaki M, Fouquier J, Gauglitz JM, Gibbons SM, Gibson DL, Gonzalez A, Gorlick K, Guo J, Hillmann B, Holmes S, Holste H, Huttenhower C, Huttley GA, Janssen S, Jarmusch AK, Jiang L, Kaehler BD, Kang KB, Keefe CR, Keim P, Kelley ST, Knights D, Koester I, Kosciolek T, Kreps J, Langille MGI, Lee J, Ley R, Liu YX, Loftfield E, Lozupone C, Maher M, Marotz C, Martin BD, McDonald D, McIver LJ, Melnik AV, Metcalf JL, Morgan SC, Morton JT, Naimey AT, Navas-Molina JA, Nothias LF, Orchanian SB, Pearson T, Peoples SL, Petras D, Preuss ML, Pruesse E, Rasmussen LB, Rivers A, Robeson MS, 2nd, Rosenthal P, Segata N, Shaffer M, Shiffer A, Sinha R, Song SJ, Spear JR, Swafford AD, Thompson LR, Torres PJ, Trinh P, Tripathi A, Turnbaugh PJ, Ul-Hasan S, van der Hooft JJJ, Vargas F, Vazquez-Baeza Y, Vogtmann E, von Hippel M, Walters W, Wan Y, Wang M, Warren J, Weber KC, Williamson CHD, Willis AD, Xu ZZ, Zaneveld JR, Zhang Y, Zhu Q, Knight R and Caporaso JG 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat Biotechnol 37, 852-857.

Cheng C, Wei H, Yu H, Xu C, Jiang S and Peng J 2018. Metabolic syndrome during perinatal period in sows and the link with gut microbiota and metabolites. Front Microbiol 9, 1-13.

DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, Huber T, Dalevi D, Hu P and Andersen GL 2006. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Appl Environ Microbiol 72, 5069-5072.

Domingo MC, Yansouni C, Gaudreau C, Lamothe F, Lévesque S, Tremblay C and Garceau R 2015. Cloacibacillus sp., a potential human pathogen associated with bacteremia in quebec and new brunswick. J Clinic Microbiol 53, 3380-3383.

Fadrosh DW, Ma B, Gajer P, Sengamalay N, Ott S, Brotman RM and Ravel J 2014. An improved dual-indexing approach for multiplexed 16S rRNA gene sequencing on the Illumina MiSeq platform. Microbiome 2, 6.

Gaukroger CH, Edwards SA, Walshaw J, Nelson A, Adams IP, Stewart CJ and Kyriazakis I 2020. Shifting sows: longitudinal changes in the periparturient faecal microbiota of primiparous and multiparous sows. Animal, 1-8.

Gresse R, Chaucheyras-Durand F, Fleury MA, Van de Wiele T, Forano E and Blanquet-Diot S 2017. Gut microbiota dysbiosis in postweaning piglets: Understanding the keys to health. Trends Microbiol 25, 851-873.

Hamer HM, Jonkers D, Venema K, Vanhoutvin S, Troost FJ and Brummer RJ 2007. Review article: the role of butyrate on colonic function. Aliment Pharmacol. Ther 27, 104-119.

Janssen PH and O'Farrell KA 1999. Succinispira mobilis gen. nov., sp. nov., a succinate-decarboxylating anaerobic bacterium. Int J Syst Evol Microbiol 49, 1009-1013.

Karásková K, Suchý P and Straková E 2016. Current use of phytogenic feed additives in animal nutrition: a review. Czech J Anim Sci 60, 521-530.

Kis RK and Bilkei G 2003. Effect of a phytogenic feed additive on weaning-to-estrus interval and farrowing rate in sows. J Swine Health Prod 11, 296-299.

Legendre P and Gallagher ED 2001. Ecologically meaningful transformations for ordination of species data. Oecologia 129, 271-280.

McCormack UM, Curiao T, Buzoianu SG, Prieto ML, Ryan T, Varley P, Crispie F, Magowan E, Metzler-Zebeli BU, Berry D, O'Sullivan O, Cotter PD, Gardiner GE and Lawlor PG 2017. Exploring a possible link between the intestinal microbiota and feed efficiency in pigs. App Environ Microbiol 83, 1-16.

Murugesan GR, Syed B, Haldar S and Pender C 2015. Phytogenic feed additives as an alternative to antibiotic growth promoters in broiler chickens. Front Vet Sci 2, 1-6.

Nowland TL, Plush KJ, Barton M and Kirkwood RN 2019. Development and function of the intestinal microbiome and potential implications for pig production. Animals 9, 76.

Pluske JR, Turpin DL and Kim J-C 2018. Gastrointestinal tract (gut) health in the young pig. Anim Nutr 4, 187-196.

Rees AS, Lysons RJ, Stokes CR and Bourne FJ 1989. Antibody production by the pig colon during infection with treponema hyodyseneriae. Res Vet Sci 47, 263-269.

Reyes-Camacho D, Vinyeta E, Pérez JF, Aumiller T, Criado L, Palade LM, Taranu I, Folch JM, Calvo MA, Van Der Klis JD and Solà-Oriol D 2020. Phytogenic actives supplemented in hyperprolific sows: effects on maternal transfer of phytogenic compounds, colostrum and milk features, performance and antioxidant status of sows and their offspring, and piglet intestinal gene expression. J Anim Sci 98, 1-13.

Rognes T, Flouri T, Nichols B, Quince C and Mahé F 2016. VSEARCH: a versatile open source tool for metagenomics. PeerJ 4, e2584.

Shu Q, Qu F and Gill HS 2001. Probiotic treatment using Bifidobacterium lactis HN019 reduces weanling diarrhea associated with rotavirus and *Escherichia coli* infection in a piglet model. J Pediatr Gastroenterol Nutr 33, 171-177.

Singh RK, Chang HW, Yan D, Lee KM, Ucmak D, Wong K, Abrouk M, Farahnik B, Nakamura M, Zhu TH, Bhutani T and Liao W 2017. Influence of diet on the gut microbiome and implications for human health. J Transl Med 15, 17.

Thaker MYC and Bilkei G 2005. Lactation weight loss influences subsequent reproductive performance of sows. Anim Repro Sci 88, 309-318.

Walker J, Henman DJ, Beer SL, Meaney D and Gannon N 2019. Inclusion of a phytogenic feed additive maintains growth performance in grower-finisher pigs when diets are down-specified in energy and protein. Adv Anim Biosci 10, s56.

Wang H, Ji Y, Yin C, Deng M, Tang T, Deng B, Ren W, Deng J, Yin Y and Tan C 2018. Differential analysis of gut microbiota correlated with oxidative stress in sows with high or low litter performance during lactation. Front Microbiol 9, 1-11.

Wang H, Hu C, Cheng C, Cui J, Ji Y, Hao X, Li Q, Ren W, Deng B, Yin Y, Deng J and Tan C 2019. Unraveling the association of fecal microbiota and oxidative stress with stillbirth rate of sows. Theriogenology 136, 131-137.

Wang J, Han Y, Zhao JZ, Zhou ZJ and Fan H 2017. Consuming fermented distillers' dried grains with solubles (DDGS) feed reveals a shift in the faecal microbiota of growing and fattening pigs using 454 pyrosequencing. J Integr Agric 16, 900-910.

Wang M, Monaco MH and Donovan SM 2016. Impact of early gut microbiota on immune and metabolic development and function. Semin Fetal Neonat Med 21, 380-387.

Windisch W, Schedle K, Plitzner C and Kroismayr A 2008. Use of phytogenic products as feed additives for swine and poultry. J Anim Sci 86, E140-E148.

Yu Z and Morrison M 2004. Improved extraction of PCR-quality community DNA from digesta and fecal samples. Biotechniques 36, 808-812.

Figure Captions

Fig. 1: Classified genera significantly altered (P < 0.01) in the faeces of sows fed two different diets: a control diet (CTR), and a control diet supplemented with a PA during gestation.

Fig. 2: Venn diagram of core microbiota at the genus level between sows and piglets at 21 and 35 days of age.

Fig. 3: Discriminant analysis of principal components (DAPC) showing the relationship among 21-day old piglets reared on sows fed different dietary treatments (CTR, CTR-PA and PA). Each dot represents the microbiota profile from one piglet, while each ellipse represents the groups. Discriminant analysis (DA) eigenvalues of the analysis are displayed inset.

Fig. 4: Genera significantly altered (P < 0.05) in the faeces of 21-day old piglets reared on sows fed different dietary treatments (CTR, CTR-PA and PA). Subscripts that differ denote a significant difference.

Fig. 5: Boxplots demonstrating the change at genus level in (A) Chao1, (B) Richness and (C) Shannon's diversity for 35-day old piglets that were reared on sows fed different dietary treatments (CTR, CTR-PA and PA). Subscripts that differ denote a significant difference.

Fig. 6: Discriminant analysis of principal components (DAPC) showing the relationships among 35-day old piglets reared on sows fed different dietary treatments (CTR, CTR-PA and PA). Each dot represents the microbiota profile from one piglet, while each ellipse represents the groups. Discriminant analysis (DA) eigenvalues of the analysis are displayed inset.

Fig. 7: Genera significantly altered ($P < 0.05$) in the faeces of 35-day old piglets reared on sows fed different dietary treatments (CTR, CTR-PA and PA). Subscripts that differ denote a significant difference.

Table 1. Reproductive performance of sows fed a control diet (CTR) or the control diet supplemented with a PA during the gestation period.

	CTR	PA	P-value
Pregnancy rate (%)*	85.9 (79.9 - 90.4)	86.8 (80.5 - 91.2)	0.831
Farrowing rate (%)*	83 (76.4 - 88.1)	79.3 (72.0 - 85.0)	0.380
Total pigs born#	**12.7 ± 0.3**	**13.5 ± 0.3**	**0.034**
Total pigs born alive#	11.8 ± 0.2	12.3 ± 0.3	0.141
Total pigs born dead#	**0.90 ± 0.1**	**1.2 ± 0.1**	**0.030**
Day 1 average piglet weight (kg) #	**1.42 ± 0.04**	**1.34 ± 0.04**	**0.016**
Number of piglets less than 1.1kg#	**3.6 ± 0.4**	**4.2 ± 0.5**	**0.015**

#Data are expressed as mean ± SEM

*Confidence intervals rather than SEM presented for binary data

Table 2. Weight, P2 backfat change in lactation, and wean to service interval of sows fed different dietary treatments (CTR, CTR-PA and PA).

	CTR	CTR-PA	PA	P-value
Average daily feed intake	7.1 ± 0.3	7.0 ± 0.3	7.2 ± 0.3	0.200
Weight (kg)				
Entry	280.1 ± 3.7	274.1 ± 3.7	277.9 ± 2.9	0.504
Exit	243.3 ± 9.4	237.6 ± 9.4	238.5 ± 9.2	0.443
Lactation change	-36.6 ± 8.5	-36.7 ± 8.5	-38.6 ± 8.4	0.778
Backfat thickness (mm)				
Entry	19.1 ± 1.4	18.2 ± 1.4	18.2 ± 1.4	0.259
Exit	18.8 ± 0.1	18.8 ± 0.1	18.5 ± 0.1	0.221
Lactation change	-0.7 ± 0.7	-0.6 ± 0.7	-0.4 ± 0.7	0.750
Wean to service interval (days)	9.0 ± 0.9	8.3 ± 0.9	6.4 ± 0.8	0.061

All data are expressed as mean ± SEM

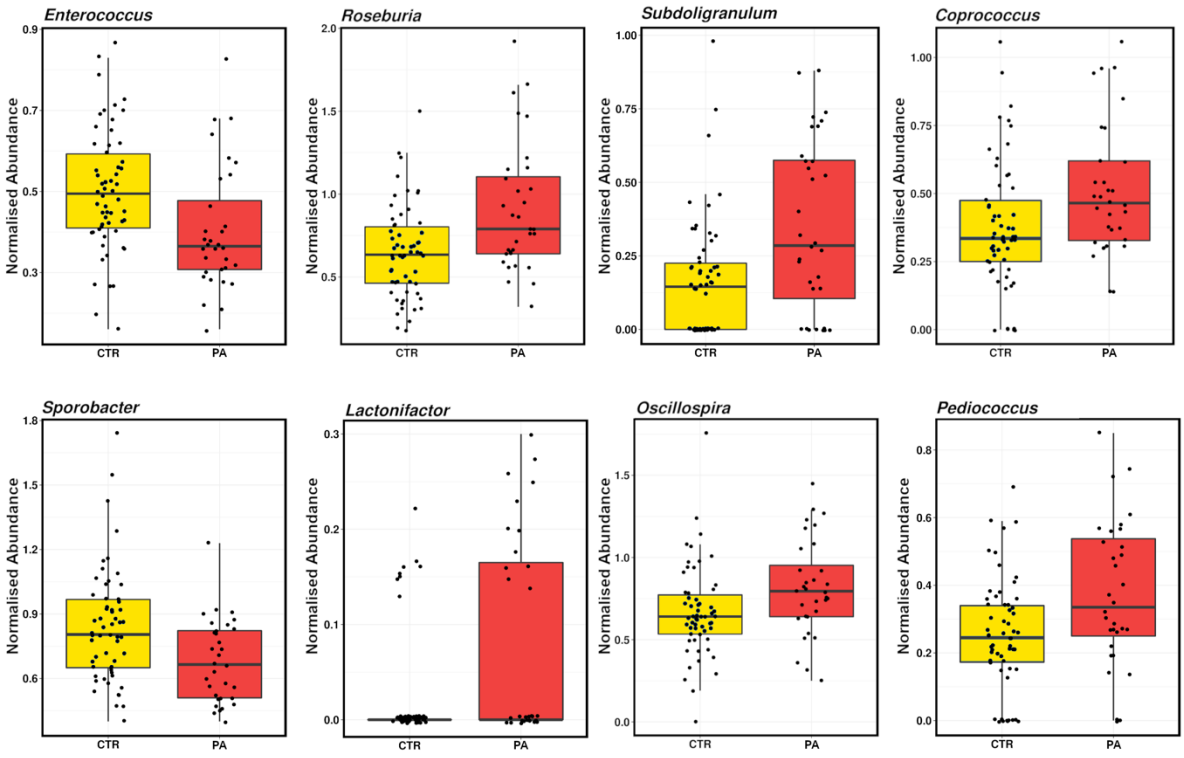

Enterococcus
Roseburia
Subdoligranulum
Coprococcus
Sporobacter
Lactonifactor
Oscillospira
Pediococcus
Normalised Abundance
CTR
PA

Fig. 1

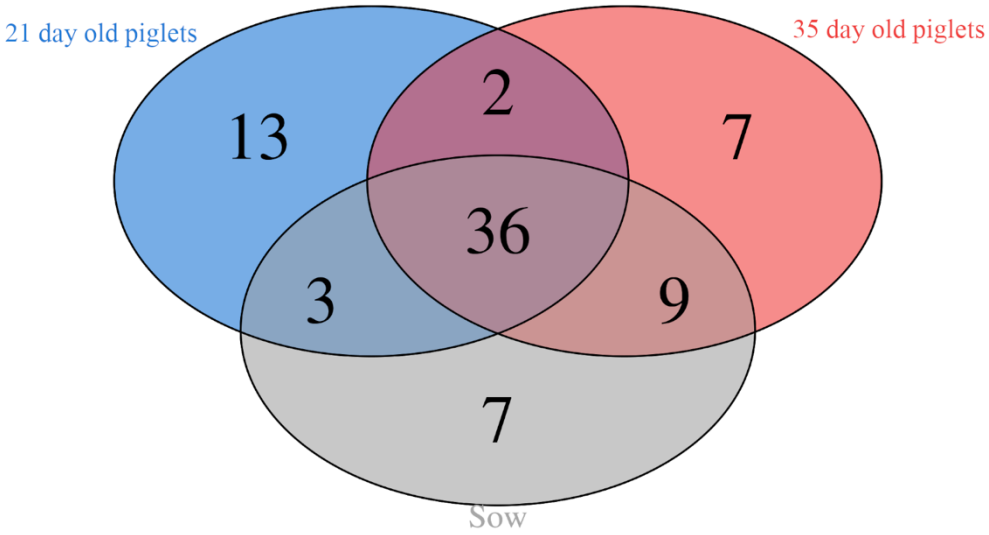


Fig. 2

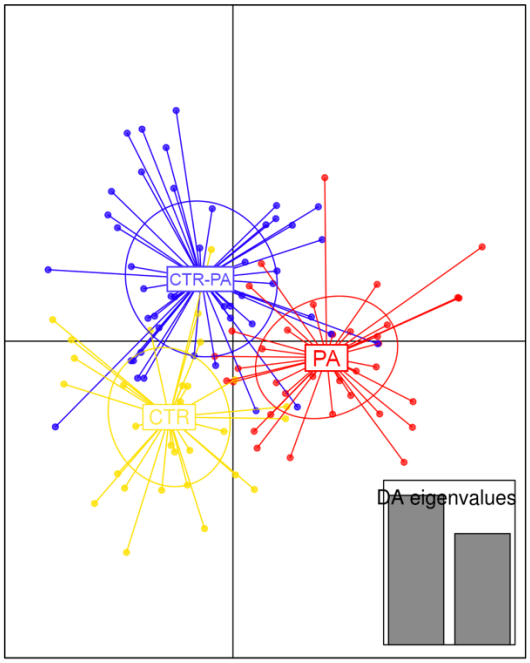


Fig. 3

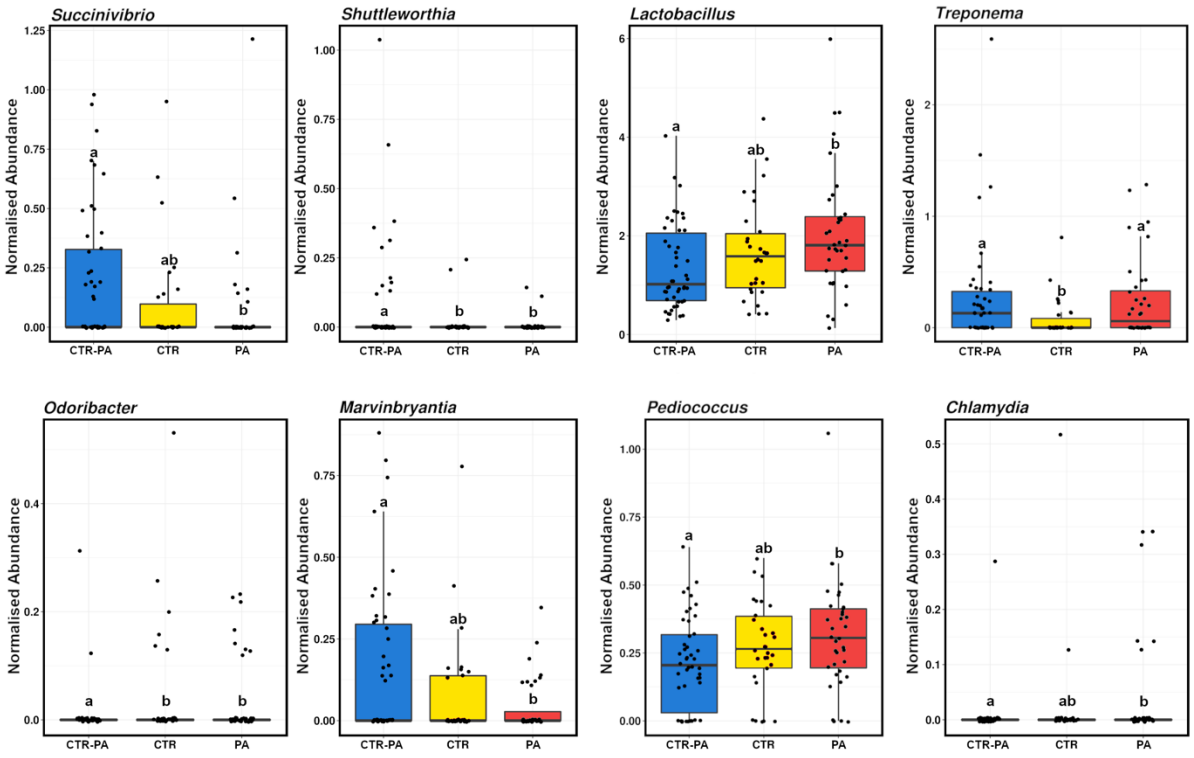

Succinivibrio
Shuttleworthia
Lactobacillus
Treponema
Odoribacter
Marvinbryantia
Pediococcus
Chlamydia
Normalised Abundance
CTR-PA
CTR
PA

Fig. 4

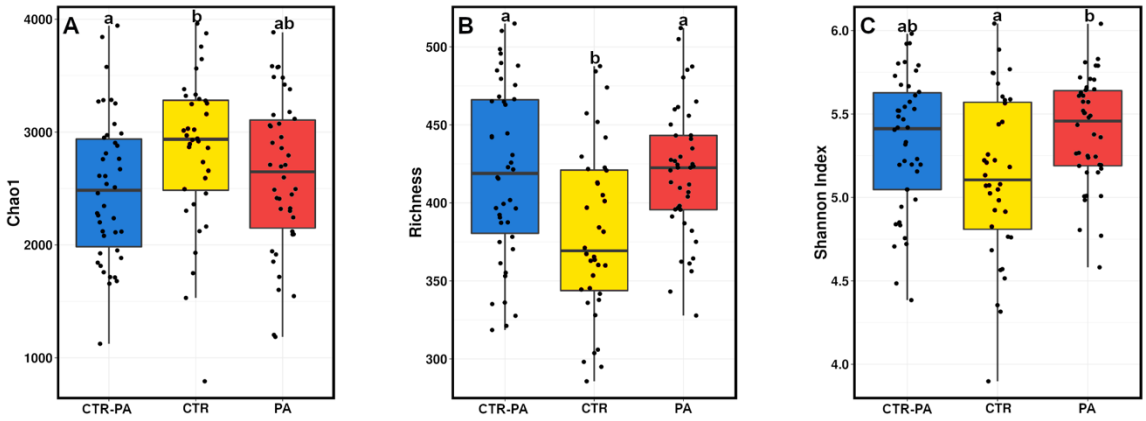

A
B
C
Chao1
Richness
Shannon Index
a
b
ab
ab
a
b
a
ab
a
b
CTR-PA
CTR
PA
4000
3000
2000
1000
500
450
400
350
300
6.0
5.5
5.0
4.5
4.0

Fig. 5

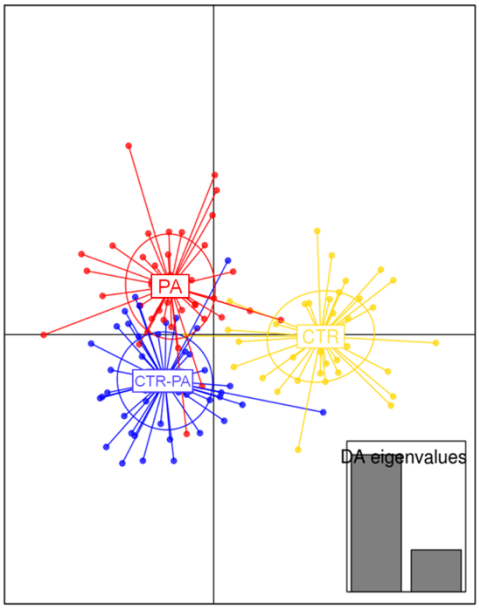


Fig. 6

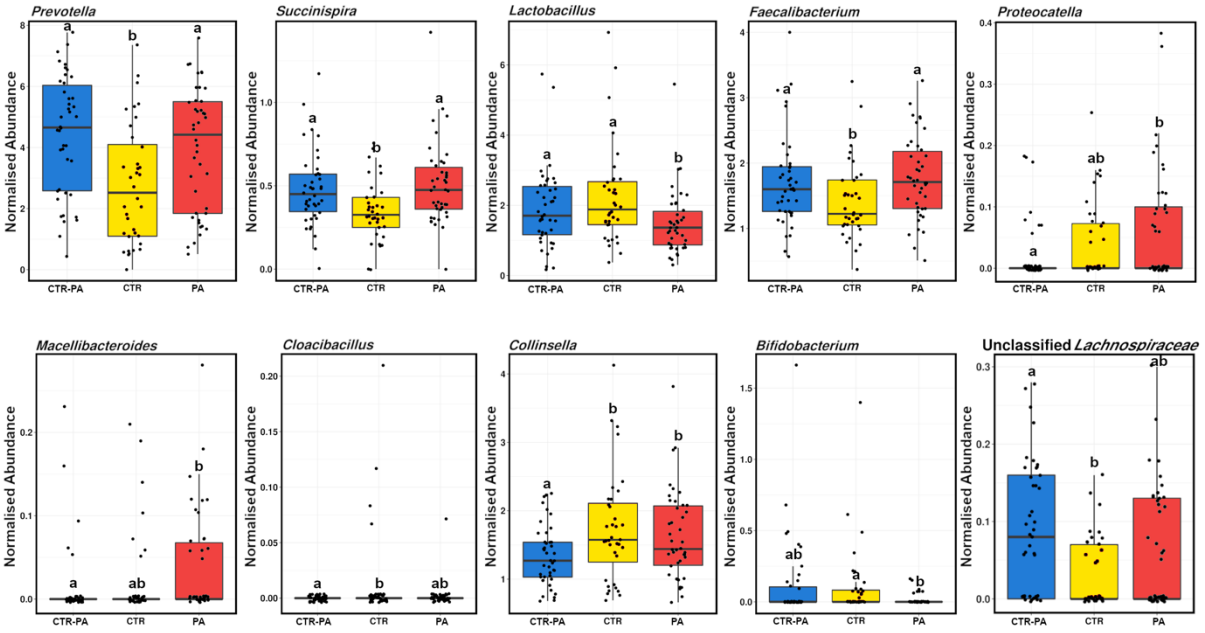

Prevotella
Succinispira
Lactobacillus
Faecalibacterium
Proteocatella
Macellibacteroides
Cloacibacillus
Collinsella
Bifidobacterium
Unclassified Lachnospiraceae
Normalised Abundance
CTR-PA
CTR
PA

Fig. 7

Supplementary table 1. Gestation and lactation base diet specifications.

	Gestation	Lactation
Barley %	59.6	24
Wheat %	19.2	40.8
Millrun %	8.2	5
Peas %	-	10.3
Canola meal %	3	-
Soybean meal %	-	3.4
Meat meal %	2.5	5.4
Blood meal %	-	0.5
Vegetable oil blend %	1.4	3
Salmon oil %	-	0.4
Limestone %	1.2	0.8
DE MJ/kg	13	14
Protein %	13.1	17.3
Calcium %	0.9	0.97
Phosphorus %	0.6	0.64
SID Lysine %	0.55	0.84
Methionine %	0.3	0.34
Threonine %	0.55	0.67
Tryptophan %	0.15	0.2
Isoleucine %	0.47	0.62
Valine %	0.63	0.82
Leucine %	-	1.18

Chapter 6:

Faecal microbiota analysis of piglets during lactation

Statement of Authorship

Title of Paper	Faecal microbiota analysis of piglets during lactation
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Nowland, T. L., V. A. Torok, W. Y. Low, M. D. Barton, K. J. Plush, and R. N. Kirkwood. 2020. Faecal microbiota analysis of piglets during lactation. *Animals* 10(5):762. doi: 10.3390/ani10050762

Principal Author

Name of Principal Author (Candidate)	Tanya Nowland
Contribution to the Paper	Assisted with the experimental design, managed and carried out the experimental trial, analysed statistics, drafted and edited the manuscript.
Overall percentage (%)	85%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 16/02/2021

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Roy Kirkwood
Contribution to the Paper	Assisted with the experimental design, data collection and interpretation and editing manuscript.
Signature	Date 25/2/21

Name of Co-Author	Kate Plush
Contribution to the Paper	Assisted with the experimental design, evaluated and edited the manuscript.
Signature	Date 25/2/21

Name of Co-Author	Mary Barton		
Contribution to the Paper	Assisted with the experimental design, data interpretation and editing manuscript.		
Signature		Date	25/2/2021

Name of Co-Author	Valeria Torok		
Contribution to the Paper	Assisted with the experimental design, statistical analysis, data interpretation and editing manuscript.		
Signature		Date	25/2/21

Name of Co-Author	Wai Yee low		
Contribution to the Paper	Assisted with the experimental design, statistical analysis, data interpretation and editing manuscript.		
Signature		Date	25/2/21

Please cut and paste additional co-author panels here as required.

animals

MDPI

Article

Faecal Microbiota Analysis of Piglets During Lactation

Tanya L. Nowland [1,*], Valeria A. Torok [2], Wai Y. Low [3], Mary D. Barton [4], Kate J. Plush [5] and Roy N. Kirkwood [1]

1 School of Animal and Veterinary Sciences, The University of Adelaide, Roseworthy, SA 5400, Australia; roy.kirkwood@adelaide.edu.au
2 South Australian Research and Development Institute, Food Sciences, SA 5064, Australia; valeria.torok@sa.gov.au
3 The Davies Research Centre, School of Animal and Veterinary Sciences, University of Adelaide, Roseworthy, SA 5371, Australia; wai.low@adelaide.edu.au
4 School of Pharmacy and Medical Sciences, University of South Australia, Adelaide, SA 5000, Australia; mary.barton@unisa.com.au
5 SunPork Group, Murarrie, QLD 4172, Australia; kate.plush@sunporkfarms.com.au
* Correspondence: tanya.nowland@adelaide.edu.au; Tel.: +8-8313-7664

Received: 11 March 2020; Accepted: 24 April 2020; Published: 27 April 2020

Abstract: Antimicrobial use in animals and the potential development of antimicrobial resistance is a global concern. So, non-antimicrobial techniques for animal disease control are needed. This study aimed to determine whether neonatal ceftiofur (CF) treatment affects piglet faecal microbiomes and whether faecal microbiome transplantation (FMT) can correct it. Two focal piglets per sow were assigned to treatments as follows: cffresh (n = 6) received CF (3 mg/kg intramuscular) at 7 d and fresh FMT at 13 d; cffrozen (n = 7) received CF at 7 d and frozen FMT at 13 d; CF (n = 8) received CF at 7 d and no FMT; and no CF (n = 5) received no CF or FMT. DNA was extracted from faecal samples collected on days 7, 13, and 18 for 16S rRNA amplicon analysis. All faecal blends used for the FMT consisted of pooled donor pig faeces at 1:2 ratio with saline, delivered orally at 3 mL/kg. Alpha and beta diversity metrics increased with age ($p < 0.05$). However, no effect of antibiotic or FMT treatment was evident in 13 and 18 d old piglets ($p > 0.05$). Although no effect of treatment was observed, information regarding microbial membership during lactation was gained.

Keywords: faecal microbiome transplantation; ceftiofur; antibiotic; bacteria; diversity

1. Introduction

The intestinal tract houses a large, diverse, and relatively stable population of bacteria, archaea, fungi, and viruses, together called the enteric microbiome [1]. Different components of the gut microbiome are involved in numerous functions including the production of antimicrobial compounds [2], nutrient metabolism, degradation of xenobiotics including hormones and development of the immune system [3,4], as well as the established property of competitive exclusion of pathogens [5]. The potential benefits of a normal gut microbiome in animal production have only recently been explored, with the focus historically being on pathogens and their control and particularly the use of antibiotics. While antibiotics are efficacious in pathogen removal, they also impact the normal commensal microbiome. Microbes are needed for maintenance of innate mucosal defenses [6] and antibiotics have been shown to reduce host expression of antimicrobial peptides [7]. Further, microbiome perturbations were shown to occur in pigs after a single amoxicillin injection which were still evident after 5 weeks [8].

An example of a successful alternative to antibiotics in treating human disease is faecal microbiome transplantation (FMT) for the treatment of *Clostridium difficile* infections. The use of antibiotics to treat *C. difficile* infections often results in failure as the antibiotics kill the vegetative bacteria but not their spores [9]. At cessation of antibiotic treatment, spores germinate and recurrent *C. difficile* disease develops. To counter this, FMT has been successfully used to re-establish a "good" gut microbiome to competitively exclude *C. difficile*. This procedure requires that faeces from a healthy donor be inoculated into the patient either orally or via an enema. The use of oral FMT for the treatment of food poisoning or severe diarrhoea was first described by Ge Hong in 4th century China [10]. In recent studies, the use of FMT for the treatment of enteric diseases induced durable changes in the patient's enteric microbiome, with a more than 90% success rate observable within days and was without adverse side effects [10]. Interestingly, Brandt and Aroniadis [10] also described beneficial effects of FMT on non-enteric diseases such as Parkinson's disease, insulin resistance, multiple sclerosis, and childhood regressive autism.

The present study aimed to provide a proof of concept for the application of FMT to control pre- and post-weaning enteric disease in pigs. Our intent was not to treat piglet diarrhoea but to confirm an ability to (re)-establish an appropriate enteric microbiome, potentially informing the ability to apply later as a technique to treat animals at times of greatest risk of enteric disease, particularly in early lactation and post-weaning phases. We hypothesised that piglets treated with the antibiotic ceftiofur, a critically important antibiotic, would undergo a reduction in the diversity and quantity of beneficial bacteria and that the treatment of these animals with fresh or previously frozen faeces would result in a re-established microbiome that resembles the microbial composition of the faeces transplanted.

2. Materials and Methods

This experiment was conducted at the University of Adelaide Roseworthy piggery with the approval of the University of Adelaide's Animal Ethics Committee (AEC number: S-2017-063).

2.1. Experimental Design and Sample Collection

A total of 15 Large White x Landrace sows (parities 1–2: 1.5 ± 0.5) and their litters were included in the experiment. All sows were group housed and had not received any antibiotics during gestation. Sows were moved into the farrowing shed at day 110 of gestation where they received a commercial lactation diet (14.2 MJ DE/kg) twice daily and had *ad libitum* access to water. Prior to farrowing, sows were fed 2.5 kg/d, which was gradually increased to 7–8 kg/d by day 7 after farrowing. Sows were induced to farrow using cloprostenol two days before their estimated due date. Sows farrowed over two days and piglets cross-fostered as necessary to teat capacity approximately 24 h post-partum. Thereafter, two piglets per sow were randomly selected where possible to be focal pigs and were assigned to one of four treatments:

- Injection of ceftiofur (3 mg/kg intramuscular injection (IM)) at 7 d and fresh FMT at 13 d (n = 4 litters, n = 6 piglets; cffresh)
- Ceftiofur (3 mg/kg IM) at 7 d and frozen FMT at 13 d (n = 4 litters, n = 7 piglets; cffrozen)
- Ceftiofur (3 mg/kg IM) at 7 d and no FMT (n = 4 litters, n = 8 piglets; CF)
- No ceftiofur and no FMT (n = 3 litters, n = 5 piglets; no CF).

When ceftiofur was administered, all piglets in the litter were treated. FMT was administered to randomly selected piglets within the designated treatment groups. Weaning occurred on day 20 or 21 of age. Faecal samples were collected from each focal piglet at 7 d (prior to ceftiofur administration), 13 d (prior to FMT administration), and at 18 d (prior to weaning). Faeces were collected by separating the focal piglet into a clean crate, stimulating the rectum with a sterile swab and collecting the faeces directly into a sterile container in order to limit contamination. Faeces were placed on ice immediately, transported to the laboratory within 4 h, and stored at −80 °C until required for microbial analyses. For frozen FMT, faeces were collected from 8 clinically healthy 13 d-old donor piglets. After collection, faeces were blended at 1-part faeces, 2-parts saline, with glycerol added to 10%, and stored at −80 °C

until required for use the following day. Faecal samples for fresh FMT were collected from the same donor pigs and blended 1:2 in saline without glycerol, however, to ensure the same volume was administered, an additional 10% saline was added. Donor piglets had no previous contact with antibiotics or antibiotic-treated animals. All faecal blends were brought to room temperature before being delivered by oral gavage at 3 mL/kg (Brandt and Aroniadis, 2013). Focal piglets were fasted for 3 h before FMT to minimise gastric acidity. A sample was collected from both the fresh and frozen pooled donor faeces for microbial analysis.

2.2. DNA Extraction and 16S rRNA Amplicon Sequencing

DNA was extracted and purified using a MagMAX^{TM} DNA Multi-Sample Ultra Kit Protocol for Faecal Samples (ThermoFisher Scientific, Australia) following the manufacturer's instructions. 16S rRNA metagenomic sequencing and library preparations were performed at the AMRID Laboratory at Murdoch University on the Illumia MiSeq platform following the "16S Metagenomic Sequencing Library Preparation" guide [11]. The forward primer (5' TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG) and reverse primer (5' GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C) were used to amplify the V3 through V4 hypervariable regions of the 16S rRNA gene. The obtained reads are available under the accession number PRJNA622643 of the Sequence Read Archive (SRA) of the NCBI. Bioinformatic analysis of raw sequence data was done by the Australian Genome Research Facility as follows. The paired-end sequences were merged by aligning the forward and reverse reads using PEAR [12] (version 0.9.5) and the primers were identified and trimmed. All trimmed sequences were processed using Quantitative Insights into Microbial Ecology (QIIME 1.8) [13] USEARCH (version 8.0.1623) [14,15] and UPARSE software [16]. Sequences were quality filtered, full length duplicate sequences were removed and sorted by abundance. Singletons or unique reads in the dataset were discarded using USEARCH tools. Additionally, chimeric sequences were clustered and removed using "rdp_gold" database as the reference. Sequences were grouped into operational taxonomic units (OTUs) based on 97% sequence similarity. Using QIIME, taxonomy was assigned using the Silva database (Version 132) [17].

2.3. Statistical Analysis

All weight data were analysed using SPSS, version 26 (IBM, Armonk, NY, USA). All data were tested for normality of residuals and outliers before analysis. A linear mixed model was used to assess the effect of treatment on piglet weight and average daily gain. The fixed effects included in the model were sex, age (7, 13 or 18 days), litter size and treatment (CF, no CF, cffresh, cffrozen). Age was fitted as a repeated measure and sow was included as a random effect. Data are expressed as estimated marginal means ± SEM.

The faecal 16S rRNA bacterial taxonomic data were analysed using multivariate statistical techniques (PRIMER6, PRIMER-E Ltd., Ivybridge, UK). Bray–Curtis measures of similarity [18] were calculated to examine similarities between faecal bacterial communities of piglets from the 16S rRNA data matrices, following standardisation and fourth-root transformation. Analysis of similarity (ANOSIM) [19] was used to test if faecal bacterial communities were significantly different between treatment and age. Similarity percentages (SIMPER) [19] analyses were done to determine which individual bacterial taxa contributed most to the overall dissimilarity among age groups. The overall average dissimilarity between faecal bacterial communities of piglets on the treatments were calculated. The percent contributions of significant OTUs (average dissimilarity/standard deviation > 1) to the top 70% of the average dissimilarities were calculated. Unconstrained ordinations were done to graphically illustrate relationships between treatments using non-metric multidimensional scaling (nMDS) [20–22] and principal coordinate analysis (PCO) [23]. Subsets of OTUs found to best represent results from ordinations on the full set of OTU data were also determined by using the BVSTEP procedure [24] on a random selection of starting variables. Matches of ordination produced from the subset of OTUs to

the full set of OTUs were determined by Spearman rank correlation (Rho) of elements from the two underlying Bray-Curtis similarity matrices.

Alpha diversity metrics were calculated using the Shannon diversity (H') index, Pielou's Evenness (J') and Number of taxa (S) using DIVERSE (PRIMER6 PRIMER-E Ltd., Ivybridge, UK). Using the Shapiro-Wilk test implemented within the RStudio software (Version 1.1.456, Boston, MA, USA), those alpha diversity metrics that were found to be not normally distributed were analysed using a non-parametric analysis, the Kruskal-Wallis test, with corrections for multiple tests using false discovery rate (FDR) with p-value threshold of 0.05. Alpha diversity metrics were found to be normally distributed and were analysed using analysis of variance (ANOVA).

3. Results

3.1. Body Weight

No significant difference between weights were visible at 13 days of age for animals treated with CF (5.42 ± 0.18 kg) vs those not treated with CF (5.04 ± 0.37 kg; $p > 0.05$). Additionally, no treatment differences existed between treatments at 18 days of age (no CF: 6.82 ± 0.45 kg, CF: 7.22 ± 0.34 kg, cffresh: 7.09 ± 0.39 kg, cffrozen: 6.49 ± 0.36 kg; $p > 0.05$) or for average daily gain to 13 or 18 days of age ($p > 0.05$).

3.2. Treatment Effects on Diversity Metrics

No significant differences were observed between genera for ceftiofur (CF) and non-ceftiofur (no CF)-treated animals for beta diversity metrics at 13 days of age (Global R = 0.181, $p = 0.144$). Additionally, no treatment differences existed at 18 days of age for beta diversity metrics (Global R = 0.033, $p = 0.255$). When assessing bacterial genera richness as measured by Shannon's diversity index, Pielou's Evenness and the number of genera, no treatment differences were observed between CF and no CF-treated animals at 13 days and no treatment differences were observed at 18 days of age ($p > 0.05$).

3.3. Age Effects on Diversity Metrics

Faecal bacterial genera significantly differed with age (Global R = 0.411, $p = 0.001$) with all pairwise comparison being significantly different (day 7 versus day 18 R = 0.635, $p = 0.001$; day 7 versus day 13 R = 0.494, $p = 0.001$; and day 13 versus day 18 R = 0.121 $p = 0.001$). These age-related differences are graphically presented for the bacterial taxa at the genus level in Figure 1, which also shows the donor faeces in relation to all piglets in the study and proximity to 13-day old piglet faecal microbiota. Bacterial genera richness, as measured by Shannon's diversity index and the number of genera, significantly increased with age ($p < 0.001$; Figure 2). Bacterial community evenness also increased with age with significant differences observed between day 7 and day 13 or day 18 ($p = 0.002$; Figure 2).

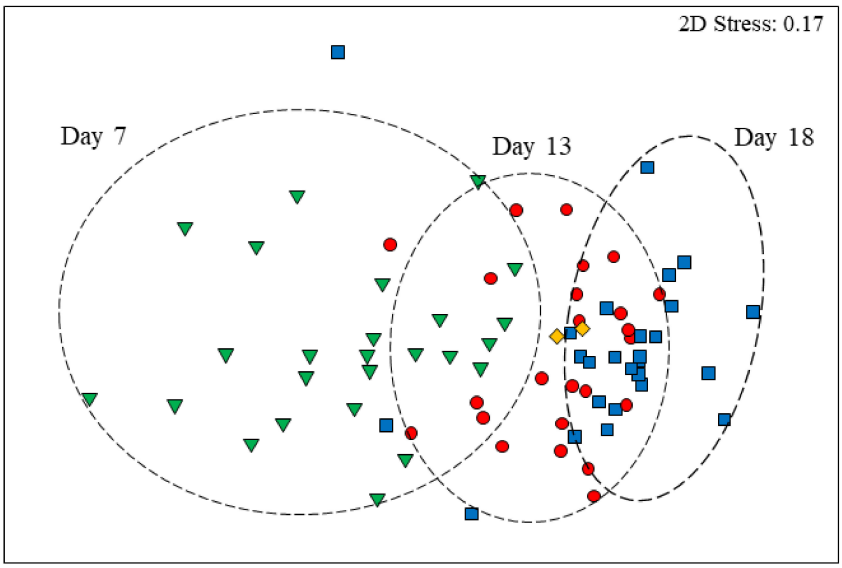


Figure 1. Non-metric multidimensional scaling (nMDS) ordination of faecal bacterial genera from piglets at 7 (inverted triangle), 13 (circle), and 18 (square) days of age along with donor piglets (diamond). All nMDS ordinations attempt to place all samples in an arbitrary two-dimensional space such that their relative distances apart match the corresponding pairwise similarities. Hence, the closer the two samples are in the ordination, the more similar their overall bacterial communities. "Stress" values (Kruskal's formula 1) reflect the difficulty involved in compressing the sample relationship into the two-dimensional ordination.

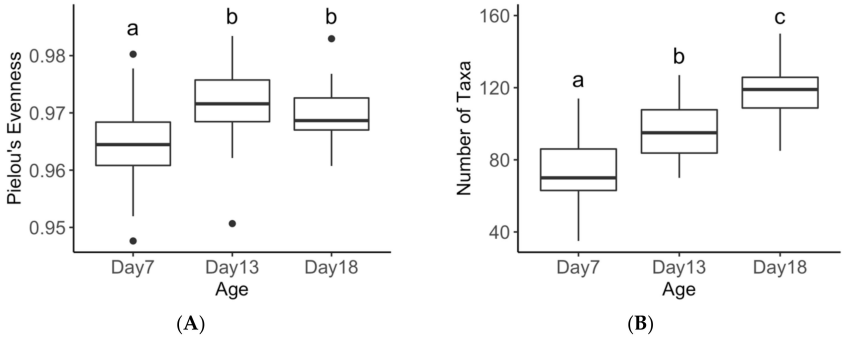
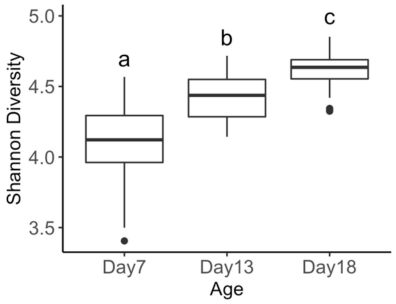


Figure 2. *Cont.*



(C)

Figure 2. Comparison of bacterial Pielou's Evenness (**A**), Number of taxa (**B**) and Shannon Diversity (**C**) between piglets at age 7, 13, and 18 days at genus level. Subscripts of differing letters are significantly different from one another ($P < 0.05$).

3.4. Age-Related Taxonomic Composition of Bacterial Communities

The dominant phyla in piglet faecal microbiota were Bacteroidetes, Firmicutes, Fusobacteria, Proteobacteria, Actinobacteria and Epsilonbacteraeota. However, the proportion of these phyla decreased with age, collectively representing 98%, 91%, and 80% of the microbiota at day 7, 13, and 18, respectively. As piglets aged, both the number and overall proportion of less dominant phyla increased (Figure 3). The average dissimilarity in bacterial phyla between age groups ranged from 23 to 28%. The main phyla driving significant change in faecal microbiota between 7 and 18 days of age were increases in Synergistetes, Epsilonbacteraeota, Lentisphaerae, Spirochaetes, Tenericutes, Firmicutes, and Planctomycetes and decreases in *Fusobacteria* and *Proteobacteria* at 18 days of age.

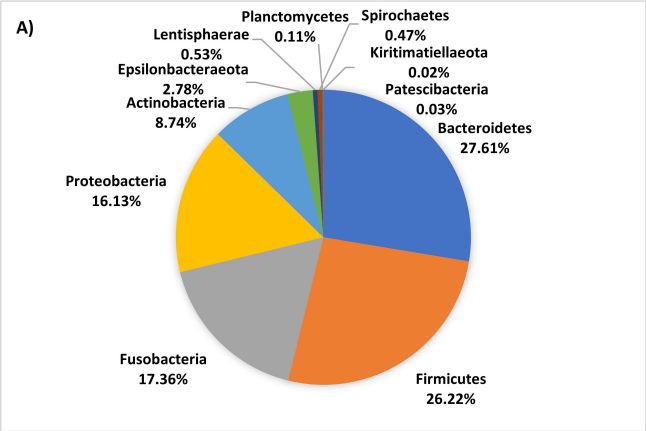


Figure 3. *Cont.*

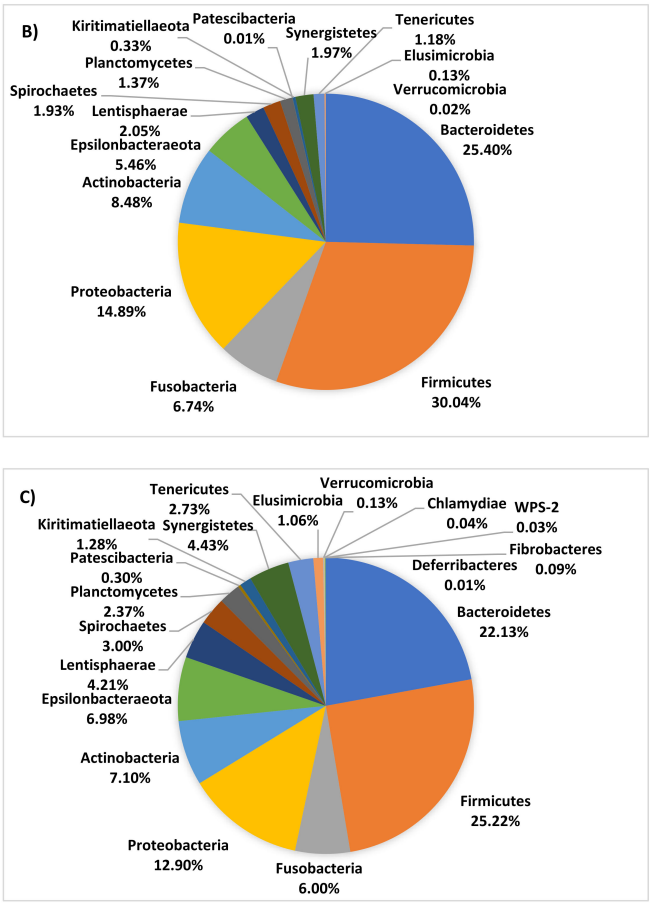


Figure 3. Pie charts of faecal bacteria phyla present at 7 (**A**), 13 (**B**), and 18 (**C**) days of age.

The average dissimilarity in bacterial families between piglets aged 7 and 18 days was 36%. Nine age-associated families were confirmed by both the SIMPER (Table 1) and BVSTEP (Figure 4) analyses, with four (*Fusobacteriaceae*, *Clostridiaceae*, *Bacteroidaceae*, and *Enterobacteriaceae*) of these having a strong association with the faecal microbiota of 7 day old piglets and five (*Christensenellaceae*, *Muribaculaceae*, *Rikenellaceae*, *Synergistaceae* and *Spirochaetaceae*) having a strong association with the faecal microbiota of day 18 piglets. At the genus level, the average dissimilarity in faecal microbiota between piglets aged 7 and 18 days was 51% (Table S1). Of the genera significantly contributing to the top 70% of dissimilarity *Fusobacterium*, *Bacteriodes*, *Lactobacillus*, *Escherichia-Shigella*, *Butyricimonas*, *Peptostreptococcus*, *Lachnoclostridium*, *Actinomyces*, *Tyzzerella*, *Veillonella*, *Ruminococcus*, *Eisenbergiella*, *Enterococcus*, *Streptococcus*, *Butyricicoccus*, *Allisonella*, *Actinobacillus*, and *Hungatella* were more abundant in day 7 piglets and *Prevotella*, *Campylobacter*, *Pyramidobacter*, *Alloprevotella*, *Oscillospira*, *Roseburia*, *Alistipes*, *Dorea*, *Oscillibacter*, *Intestinimonas*, *Treponema*, *Helicobacter*, *Sanguibacteroides*, *Synergistes*, *Bilophila*, *Collinsella*, *Hydrogenoanaerobacterium*, *Phascolarctobacterium*, *Vitivallis*, *Sphaerochaeta*, *Blautia*, *Faecalibacterium*, *Mailhella*, *Sutterella*, *Holdemanella*, *Catenibacterium*, *Romboutsia*, and *Clostridiodes* were more abundant in the day 18 piglets (Table S1. Of those taxa which could be classified to the

species level *Bacteroides fragilis*, *Bacteroides uniformis*, *Clostridium perfringens*, *Bacteroides vulgarus*, *Escherichia coli*, *Fusobacterium gastrosuis*, *Bacteroides plebeius*, *Actinomyces hyovaginalis*, *Clostridum baratii*, *Lactobacillus johnsonii* and *Lactobacillus mucosae* were more abundant in the 7 day old piglets and *Campylobacter jejuni*, *Megasphaera elsdenii*, *Lactobacillus reuteri*, *Lactobacillus salivarius*, *Lactobacillus coleohominis*, and *Sanguibacteroides justesenii* were more abundant in 18 day old piglets, contributing significantly to the top 50% of dissimilarity between these age groups.

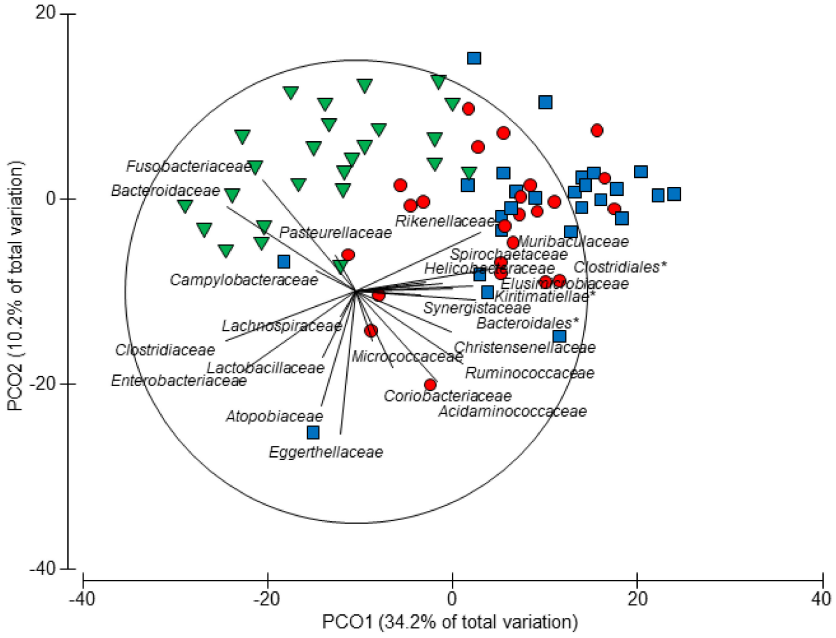


Figure 4. Principal coordinate analysis (PCO) ordination of faecal bacterial families from piglets at 7 (inverted triangle), 13 (circle), and 18 (square) days of age. Overlaid onto the PCO are vectors of the subset of 24 taxa identified by the BVSTEP procedure (Rho = 0.951, P = 0.001) to best represent the overall community pattern from the full set of 81 identified families. Vectors indicate the association of the families with a particular diet. * Uncharacterised family.

Table 1. Family contributing to the top 70% of significant dissimilarity of bacteria between 7 and 18 day old piglets as determined by SIMPER. Overall, average dissimilarity between ages is 36%.

	Day 7	Day 18	
Family	**Average Abundance**	**Average Abundance**	**%**
Uncharacterised Clostridiales	0.22	1.21	4.27
Christensenellaceae	0.38	1.37	8.47
Fusobacteriaceae	1.71	0.88	12.59
Muribaculaceae	0.86	1.52	16.25
Clostridiaceae	1.35	0.61	19.56
Prevotellaceae	1.32	1.45	22.5
Uncharacterised Bacteroidales	0.06	0.7	25.32
Synergistaceae	0.02	0.69	28.11
Bacteroidaceae	2.1	1.63	30.86
Lactobacillaceae	1.4	1.24	33.52

Table 1. *Cont.*

Family	Day 7	Day 18	%
	Average Abundance	Average Abundance	
Campylobacteraceae	0.58	0.83	36.13
Enterobacteriaceae	1.37	1.09	38.62
Rikenellaceae	0.88	1.24	41.03
Marinifilaceae	0.83	0.9	43.36
Ruminococcaceae	1.61	1.94	45.44
Actinomycetaceae	0.58	0.28	47.37
Spirochaetaceae	0.17	0.53	49.29
Victivallaceae	0.16	0.5	51
Enterococcaceae	0.36	0.31	52.7
Helicobacteraceae	0.12	0.41	54.31
Veillonellaceae	0.88	0.68	55.87
Lachnospiraceae	1.7	1.75	58.98
Oligosphaeraceae	0.04	0.38	60.52
Coriobacteriaceae	0.51	0.61	62.05
Acidaminococcaceae	0.86	1.13	63.55
Uncharacterised Mollicutes	0	0.36	65.05
Pirellulaceae	0.09	0.38	66.52
Streptococcaceae	0.82	0.58	67.94
Uncharacterised Bradymonadales	0.12	0.33	69.34
Pasteurellaceae	0.63	0.53	70.73

4. Discussion

FMT is an effective tool for the treatment of enteric clostridial disease in humans [1,9]. Consequently, this study aimed to provide a proof of concept for the use of FMT in pigs. An antibiotic was administered to piglets with the aim of disrupting their gastrointestinal microbiota and either fresh or frozen FMT was applied to re-establish a normal microbiota. The results showed that faecal diversity increased with age. However, the antibiotic administration had no impact on the faecal microbiota, and in contrast to our hypothesis, FMT had no effect on altering the faecal microbiota of piglets at weaning.

In the present study, the faecal microbiota of piglets increased in diversity and richness as age increased, irrespective of treatment. This finding is supported by the literature [25,26]. It is well established that the period preceding birth is the point whereby human neonates develop their gastrointestinal microbiome [27,28]. As such, an increase in microbial species number and diversity during the weeks following birth is expected. This early period is considered the most critical time for human gastrointestinal microbiome development, with disruptions to the microbiome during this time having consequences for long-term health [29]. Interestingly, we are aware of no research to date that has identified a critical time period for gastrointestinal microbiome development for the pig. However, it is well established that the main factors influencing the development of the microbiota of piglets as they age are the environment and diet to which they are exposed [30,31]. It is evident from the present study that large changes in microbial composition and diversity occur within the first two weeks following birth and then undergoes little change during the last week prior to weaning. It is interesting that such a large shift occurs between 7 and 13 days in the present study as no change in environment occurred during this time and the piglets had no access to creep feed throughout lactation. It is possible that the piglets may have been able to access the sow's feed, however, previous research investigating creep feed usage suggests that feed consumption is low and variable with it increasing linearly from 2 weeks of age [32]. Although the present study did not go beyond weaning, in other studies investigating the microbiota of piglets post-weaning, the microbiota continues to undergo changes beyond 18 days of age [26,33]. This is to be expected as the diet changes significantly post-weaning. Alternately, piglets are known to exhibit coprophagy [34], which in turn

would aid in the development of the microbiota and suggests that this change may be a function of natural gut maturation as piglets age.

One of the main differences observed with age at the phyla level were increased Synergistetes and decreased Fusobacteria. Interestingly, unlike in the present study, Fusobacteria and Synergistetes were not observed in the faeces of 21 day old nursed piglets in a study conducted by Guevarra, et al. [26]. Furthermore, Spirochaetes which were classified as one of the main phyla in the study conducted by Guevarra, et al. [26], were present only in low amounts in the present study. Additionally, McCormack, et al. [35] found similar findings to Guevarra, et al. [26], whereby Fusobacteria were only found in small amounts and Synergistetes were not documented in piglets prior to weaning. Although no production differences can be observed between studies, this provides further evidence to demonstrate that the microbiota of individuals not only differs with age but also differs between farms and locations, suggesting environmental and possibly genetic components. Similar to other studies, Bacteriodetes and Firmicutes were the two most abundant phyla present at all age stages prior to weaning. Although the ratio of Bacteriodetes to Firmicutes was similar in the present study, they were present in comparatively much lower amounts.

Of the potentially pathogenic bacteria detected in the faeces of piglets, it is evident that younger aged piglets had significantly more *Escherichia-Shigella* than those at 18 days of age. Other potentially pathogenic bacteria detected were *Streptococcaceae*, *Fusobacterium*, and *Bacteroides* however these are also common gut commensals so their presence would be expected, and it is not until an imbalance of bacteria occurs that they may exhibit a more pathogenic nature. However, *Clostridia* was also detected which has the potential to be an opportunistic pathogen. It is known that an increase in bacterial diversity is generally associated with a reduction in diarrhea in pigs [36] and improved gastrointestinal health in humans [30]. It is important to note that the *E. coli-Shigella* detected may be commensal strains and not necessarily pathogenic, and the fact that these bacteria decrease as pigs age may be a byproduct of the natural increase in microbial diversity as pigs age and their gastrointestinal tract matures.

It is well established that the administration of antibiotics to animals alters their gastrointestinal microbiota, with marked reductions in population diversity being detected in the faeces from 7 days post-treatment [37]. However, this response did not occur within the present study. Those animals that received antibiotics via an intramuscular injection at 7 days of age showed no differences in Shannon diversity, Pielou's evenness or number of taxa 6 or 11 days after antibiotic administration. This indicates that those animals that were treated with antibiotics had a similar community structure to one another. However, regardless of antibiotic exposure they were both equally as diverse, even in distribution, and had a similar number of taxa composing them. These findings contrast with Gao, et al. [37], who observed a significant reduction in Shannon diversity and evenness within the faecal microbiota of pigs provided with in-feed antibiotics 7 days after the beginning of the treatment. Although that study differed from the present study as to the antibiotic type, route, and duration of administration, a change to diversity and evenness of the faecal microbiota would still have been expected. Janczyk, et al. [8], noted differences in the faecal microbiota 5 weeks after administering one dose of intramuscular amoxicillin to a 1-day old piglet. However, the latter study did not investigate how quickly the differences became established. More recently, Ruczizka, et al. [38] observed differences in the microbiota of piglets given a single intramuscular injection of ceftiofur 12 h post-partum, with differences evident at 12, 28 and 97 days of age. This approach was conducted in a more similar manner to the one implemented in the current study, therefore, it is likely that our inability to detect an effect of antibiotic may be due to a need for a longer period of time after antibiotic administration in order to see a change in the faeces. Additionally, Ruczizka, et al. [38], also observed sex-specific differences and had 16 piglets of each sex per treatment. So, the lower number of replicates in the present study may have impacted the results.

FMT has been demonstrated to be an effective tool in the treatment of *Clostridium difficile* infections in humans [9], with studies showing that FMT changes the microbiota of the sick recipient to become more similar to that of the faeces they were treated with [39]. Even more recently within the pig

industry, investigation into the use of FMT as a tool for improving feed conversion efficiency has been conducted. However, contradictory results have been observed, with some showing positive and other negative impacts on feed conversion efficiency of the treated pigs. It has been suggested that the donor faeces used impacted the outcomes observed [35,40]. In contrast to previous studies, no differences in faecal microbiota were observed between treatments in the current study. This may be due to not enough time being allowed after FMT for a greater change to be observed, and suggests that the administration of a single FMT to 13-day old piglets does not produce changes in the faecal microbiota to weaning at 18 days of age, or our replicates were insufficient to assess this. Studies conducted in humans investigating FMT as a treatment for *Clostridium difficile* infections found that one to two FMT doses was sufficient [1,9]. However, unlike the studies in humans where the patient's microbiome would have been unstable due to the excessive antibiotic use beforehand, the microbiota of our recipient piglets would have been relatively stable, and the antibiotic had no impact on the piglet's faecal microbiota. Therefore, it is likely that the ability for the donor faeces to competitively exclude the bacteria already present within the GIT would have been limited. Furthermore, the donor animals used for the preparation of the FMT were a similar age to those animals that were receiving the FMT. Therefore, because the antibiotics had no effect on the microbiota as expected, the FMT given may not have been different enough from the piglets receiving it to see an effect. Overall, further research is likely necessary in order to understand the best timing for dosing with FMT and the likely number of FMT doses required in order to create a long-lasting change within the microbiota of piglets.

5. Conclusions

Significant differences in faecal bacterial communities associated with age were observed. Specifically, a reduction in the dominant phyla, particularly Fusobacteria and Proteobacteria, and an increase in less dominant phyla by 18 days of age. However, the finding that animals treated with the antibiotic ceftiofur had no reduction in alpha diversity metrics was unexpected, as other studies have shown that antibiotics reduced both alpha and beta diversity metrics. Additionally, FMT had no influence on piglets to weaning, which may be attributed to the fact that antibiotic administration did not disrupt the microbiota as initially intended and the donor faeces used may not have been different enough to elicit an effect that was detectable. Although no treatment effect was observed, information regarding microbial membership in the pre-weaning period for piglets was gained.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-2615/10/5/762/s1, Table S1: Genus contributing to the top 70% of significant dissimilarity of bacteria between day 7 and 18 age groups as determined by SIMPER. Overall average dissimilarity between ages is 51%.

Author Contributions: Conceptualization, M.D.B. and R.N.K.; Data curation, T.L.N.; Formal analysis, T.L.N., V.A.T. and W.Y.L.; Funding acquisition, T.L.N. and R.N.K.; Investigation, R.N.K.; Methodology, T.L.N., M.D.B., K.J.P. and R.N.K.; Project administration, T.L.N. and R.N.K.; Supervision, V.A.T., W.Y.L., M.D.B., K.J.P. and R.N.K.; Writing—original draft, T.L.N.; Writing—review & editing, T.L.N., V.A.T., W.Y.L., M.D.B., K.J.P. and R.N.K. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Department of Agriculture and Water resources ABARES and Australian Pork Limited under the Science and Innovation Awards for Young People in Agriculture, Forestry and Fisheries 2018 (Grant number: 2018094).

Acknowledgments: The authors wish to thank Australian Pork Limited and the Science and Innovation Awards for Young People in Agriculture, Fisheries and Forestry for funding this project. Additionally, we thank Sophia Ward and Bryony Tucker for their technical assistance and Mark O'Dea and Sam Abraham for the 16S rRNA amplicon analysis.

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

References

1. Borody, T.J.; Khoruts, A. Fecal microbiota transplantation and emerging applications. *Nat. Rev. Gastroenterol. Hepatol.* **2012**, *9*, 88–96.
2. Rea, M.C.; Sit, C.S.; Clayton, E.; O'Connor, P.M.; Whittal, R.M.; Zheng, J.; Vederas, J.; Ross, R.P.; Hill, C. Thuricin cd, a posttranslationally modified bacteriocin with a narrow spectrum of activity against clostridium difficile. *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 9352–9357. [PubMed]
3. Bauer, E.; Williams, B.A.; Smidt, H.; Verstengen, M.W.; Mosenthin, R. Influence of the gastrointestinal microbiota on development of the immune system in young animals. *Curr. Issues Intest. Microbiol.* **2006**, *7*, 35–52.
4. Schachtschneider, K.M.; Yeoman, C.J.; Isaacson, R.E.; White, B.A.; Schook, L.B.; Pieters, M. Modulation of systemic immune responses through commensal gastrointestinal microbiota. *PLoS ONE* **2013**, *8*, 16.
5. La Fata, G.; Weber, P.; Mohajeri, M.H. Probiotics and the gut immune system: Indirect regulation. *Probiotics Antimicrob. Prot.* **2018**, *10*, 11–21.
6. Willing, B.P.; Gill, N.; Finlay, B.B. The role of the immune system in regulating the microbiota. *Gut Microbes* **2010**, *1*, 213–223.
7. Wlodarska, M.; Willing, B.; Keeney, K.M.; Menendez, A.; Bergstrom, K.S.; Gill, N.; Russell, S.L.; Vallance, B.A.; Finlay, B.B. Antibiotic treatment alters the colonic mucus layer and predisposes the host to exacerbated citrobacter rodentium-induced colitis. *Infect. Immun.* **2011**, *79*, 1536–1545.
8. Janczyk, P.; Pieper, R.; Souffrant, W.B.; Bimczok, D.; Rothkotter, H.J.; Smidt, H. Parenteral long-acting amoxicillin reduces intestinal bacterial community diversity in piglets even 5 weeks after the administration. *ISME J.* **2007**, *1*, 180–183.
9. Bakken, J.S.; Borody, T.; Brandt, L.J.; Brill, J.V.; Demarco, D.C.; Franzos, M.A.; Kelly, C.; Khoruts, A.; Louie, T.; Martinelli, L.P.; et al. Treating clostridium difficile infection with fecal microbiota transplantation. *Clin. Gastroenterol. Hepatol.* **2011**, *9*, 1044–1049.
10. Brandt, L.J.; Aroniadis, O.C. An overview of fecal microbiota transplantation: Techniques, indications, and outcomes. *Gastrointest. Endosc.* **2013**, *78*, 240–249.
11. Illumina. 16s metagenomic sequencing library preparation (15044223 b). Available online: https://www.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf (accessed on 1 September 2019).
12. Zhang, J.; Kobert, K.; Flouri, T.; Stamatakis, A. Pear: A fast and accurate illumina paired-end read merger. *Bioinformatics* **2014**, *30*, 614–620. [PubMed]
13. Caporaso, J.G.; Kucztnski, J.; Stombaugh, J.; Bittinger, K.; Bushman, F.D.; Costello, E.K.; Fierer, N.; Peña, A.G.; Goodrich, J.K.; Gordon, J.I.; et al. Qiime allows analysis of high-throughput community sequencing data. *Nat. Methods* **2010**, *7*, 335–336. [PubMed]
14. Edgar, R.C. Search and clustering orders of magnitude faster than blast. *Bioinformatics* **2010**, *26*, 2460–2461. [PubMed]
15. Edgar, R.C.; Haas, B.J.; Clemente, J.C.; Quince, C.; Knight, R. Uchime improves sensitivity and speed of chimera detection. *Bioinformatics* **2011**, *27*, 2194–2200.
16. Edgar, R.C. Uparse: Highly accurate otu sequences from microbial amplicon reads. *Nat. Methods* **2013**, *10*, 996–998. [PubMed]
17. DeSantis, T.Z.; Hugenholtz, P.; Larsen, N.; Rojas, M.; Brodie, E.L.; Keller, K.; Huber, T.; Dalevi, D.; Hu, P.; Andersen, G.L. Greengenes, a chimera-checked 16s rrna gene database and workbench compatible with arb. *Appl. Environ. Microbiol.* **2006**, *72*, 5069–5072. [PubMed]
18. Bray, J.R.; Curtis, J.T. An ordination of the upland forest communities of southern wisconsin. *Ecol. Monogr.* **1957**, *27*, 325–349.
19. Clarke, K.R. Non-parametric multivariate analyses of changes in community structure. *Aust. J. Ecol.* **1993**, *18*, 117–143.
20. Kruskal, J.B. Multidimensional scaling by optimizing a goodness of fit to a nonmetric hypothesis. *Phsychometrics* **1964**, *29*, 1–28.
21. Shepard, R.N. The analysis of proximities: Multidimensional scaling with an unknown distance function. I. *Psychometrika* **1962**, *27*, 125–140.

22. Shepard, R.N. The analysis of proximities: Multidimensional scaling with an unknown distance function. II. *Psychometrika* **1962**, *27*, 219–246.
23. Gower, J.C. Some distance properties of latent root and vector methods used in multivariate analysis. *Biometrics* **1966**, *53*, 325–338.
24. Clarke, K.R.; Warwick, R.M. Quantifying structural redundancy in ecological communities. *Oecology* **1998**, *113*, 278–289.
25. Hu, J.; Nie, Y.F.; Chen, J.W.; Zhang, Y.; Wang, Z.C.; Fan, Q.W.; Yan, X.H. Gradual changes of gut microbiota in weaned miniature piglets. *Front. Microbiol.* **2016**, *7*, 15.
26. Guevarra, R.B.; Hong, S.H.; Cho, J.H.; Kim, B.R.; Shin, J.; Lee, J.H.; Kang, B.N.; Kim, Y.H.; Wattanaphansak, S.; Isaacson, R.E.; et al. The dynamics of the piglet gut microbiome during the weaning transition in association with health and nutrition. *J. Anim. Sci. Biotechnol.* **2018**, *9*, 54. [PubMed]
27. Arrieta, M.C.; Stiemsma, L.T.; Amenyogbe, N.; Brown, E.M.; Finlay, B. The intestinal microbiome in early life: Health and disease. *Front. Immunol.* **2014**, *5*, 18.
28. Gensollen, T.; Iyer, S.S.; Kasper, D.L.; Blumberg, R.S. How colonization by microbiota in early life shapes the immune system. *Science* **2016**, *352*, 539–544.
29. Murphy, K.; O'Shea, C.A.; Ryan, C.A.; Dempsey, E.M.; O'Toole, P.W.; Stanton, C.; Ross, R.P. The gut microbiota composition in dichorionic triplet sets suggests a role for host genetic factors. *PLoS ONE* **2015**, *10*, e0122561.
30. Nowland, T.L.; Plush, K.J.; Barton, M.; Kirkwood, R.N. Development and function of the intestinal microbiome and potential implications for pig production. *Animals Basel* **2019**, *9*, 76.
31. Frese, S.A.; Parker, K.; Calvert, C.C.; Mills, D.A. Diet shapes the gut microbiome of pigs during nursing and weaning. *Microbiome* **2015**, *3*, 28.
32. Wattanakul, W.; Bulman, C.A.; Edge, H.L.; Edwards, S.A. The effect of creep feed presentation method on feeding behaviour, intake and performance of suckling piglets. *Appl. Anim. Behav. Sci.* **2005**, *92*, 27–36.
33. 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. [PubMed]
34. Aviles-Rosa, E.O.; Rakhshandeh, A.; McGlone, J.J. Preliminary study: Depriving piglets of maternal feces for the first seven days post-partum changes piglet physiology and performance before and after weaning. *Animals Basel* **2019**, *9*.
35. McCormack, U.M.; Curiao, T.; Wilkinson, T.; Metzler-Zebeli, B.U.; Reyer, H.; Ryan, T.; Calderon-Diaz, J.A.; Crispie, F.; Cotter, P.D.; Creevey, C.J.; et al. Fecal microbiota transplantation in gestating sows and neonatal offspring alters lifetime intestinal microbiota and growth in offspring. *Am. Soc. Microbiol.* **2018**, *3*, 268.
36. Dou, S.; Gadonna-Widehem, P.; Rome, V.; Hamoudi, D.; Rhazi, L.; Lakhal, L.; Larcher, T.; Bahi-Jaber, N.; Pinon-Quintana, A.; Guyonvarch, A.; et al. Characterisation of early-life fecal microbiota in susceptible and healthy pigs to post-weaning diarrhoea. *PLoS ONE* **2017**, *12*, e0169851.
37. Gao, K.; Pi, Y.; Peng, Y.; Mu, C.L.; Zhu, W.Y. Time-course responses of ileal and fecal microbiota and metabolite profiles to antibiotics in cannulated pigs. *Appl. Microbiol. Biotechnol.* **2018**, *102*, 2289–2299.
38. Ruczizka, U.; Metzler-Zebeli, B.; Unterweger, C.; Mann, E.; Schwarz, L.; Knecht, C.; Hennig-Pauka, I. Early parenteral administration of ceftiofur has gender-specific short- and long-term effects on the fecal microbiota and growth in pigs from the suckling to growing phase. *Animals Basel* **2019**, *10*, 17.
39. Khoruts, A.; Dicksved, J.; Jansson, J.K.; Sadowsky, M.J. Changes in the composition of the human fecal microbiome after bacteriotherapy for recurrent clostridium difficile-associated diarrhea. *J. Clin. Gastroenterol.* **2010**, *44*, 354–360. [PubMed]
40. Hu, L.S.; Geng, S.J.; Li, Y.; Cheng, S.S.; Fu, X.F.; Yue, X.J.; Han, X.Y. Exogenous fecal microbiota transplantation from local adult pigs to crossbred newborn piglets. *Front. Microbiol.* **2018**, *8*, 2663.

Table S1: Genus contributing to the top 70% of significant dissimilarity of bacteria between day 7 and 18 age groups as determined by SIMPER. Overall average dissimilarity between ages is 51%.

Phyla	Class	Order	Family	Genus	Day 7 Average Abundance	Day 18 Average Abundance	%
Actinobacteria	Actinobacteria	Actinomycetales	*Actinomycetaceae*	*Actinomyces*	0.57	0.24	0.74
	Coriobacteriia	Coriobacteriales	*Coriobacteriaceae*	*Collinsella*	0.51	0.61	0.57
Bacteroidetes	Bacteroidia	Bacteroidales	*Bacteroidaceae*	*Bacteroides*	2.1	1.63	1.03
			Marinifilaceae	*Butyricimonas*	0.82	0.81	0.9
				Sanguibacteroides	0.08	0.42	0.59
				CAG-873	0.01	1.1	1.68
			Marinifilaceae		0.38	0.78	0.91
			Marinifilaceae		0.78	1.04	0.97
			p-2534-18B5 gut group		0.06	0.7	1.04
			Prevotellaceae	*Alloprevotella*	0.46	0.83	0.89
				Prevotella 2	0.95	1.11	1.3
				Prevotella 7	0.22	0.42	0.56
				Prevotellaceae NK3B31 group	0.62	0.68	0.77
				Prevotellaceae UCG-003	0.26	0.64	0.89
				Prevotellaceae UCG-004	0.11	0.48	0.68
			Prevotellaceae		0.64	0.52	0.89
			Prevotellaceae		0.27	0.42	0.55
			Rikenellaceae	*Alistipes*	0.39	0.62	0.77
				dgA-11 gut group	0.04	0.38	0.56
				RC9 gut group	0.79	1.17	0.95
Epsilonbacteraeota	Campylobacteria	Campylobacterales	*Campylobacteraceae*	*Campylobacter*	0.58	0.83	0.97
			Helicobacteraceae	*Helicobacter*	0.12	0.41	0.6
Firmicutes	Bacilli	Lactobacillales	*Enterococcaceae*	*Enterococcus*	0.36	0.31	0.64
			Lactobacillaceae	*Lactobacillus*	1.4	1.24	1
			Streptococcaceae	*Streptococcus*	0.82	0.58	0.53
Firmicutes	Clostridia	Clostridiales	*Christensenellaceae*	*Christensenellaceae R-7 group*	0.38	1.36	1.56
			Clostridiaceae	*Clostridium sensu stricto 1*	1.16	0.58	1

	Clostridium sensu stricto 2	0.92	0.14	1.31
Clostridiales vadinBB60 group		0.05	0.67	0.97
Clostridiales vadinBB60 group		0.18	1.15	1.56
Family XIII	*[Eubacterium] nodatum group*	0.29	0.46	0.51
	Family XIII AD3011 group	0.15	0.59	0.72
Lachnospiraceae	*[Eubacterium] fissicatena group*	0.92	0.61	0.67
	[Ruminococcus] gauvreauii group	0.32	0.53	0.68
	Blautia	0.44	0.49	0.51
	Dorea	0.51	0.72	0.72
	Eisenbergiella	0.73	0.45	0.65
	Hungatella	0.56	0.43	0.48
	Lachnoclostridium	1.44	1.43	0.75
	Lachnospiraceae FCS020 group	0.15	0.39	0.53
	Lachnospiraceae UCG-002	0.07	0.31	0.47
	Lachnospiraceae UCG-010	0.11	0.4	0.56
Lachnospiraceae		0.2	0.74	0.98
	Roseburia	0.47	0.51	0.78
	Tyzzerella	0.59	0.31	0.72
Peptostreptococcaceae	*Clostridioides*	0.3	0.13	0.47
	Peptostreptococcus	0.66	0.21	0.81
	Romboutsia	0.48	0.55	0.47
Ruminococcaceae	*[Eubacterium] coprostanoligenes group*	1.14	1.14	0.62
	Butyricicoccus	0.59	0.48	0.51
	Faecalibacterium	0.08	0.33	0.5
	GCA-900066225	0.23	0.35	0.45
	Hydrogenoanaerobacterium	0.38	0.64	0.56
	Intestinimonas	0.62	0.97	0.66
	Oscillibacter	0.12	0.52	0.7
	Oscillospira	0.22	0.65	0.83
	Ruminiclostridium 9	0.57	0.83	0.71
	Ruminococcaceae NK4A214 group	0.51	1.01	0.9
	Ruminococcaceae UCG-002	0.75	1.24	1.04
	Ruminococcaceae UCG-003	0.26	0.54	0.72
	Ruminococcaceae UCG-005	0.22	0.83	0.98

				Ruminococcaceae UCG-010	0.46	0.83	0.74
				Ruminococcus 2	0.92	0.81	0.68
				Subdoligranulum	0.08	0.45	0.66
				UBA1819	0.6	0.48	0.45
			Ruminococcaceae		0.77	0.97	0.68
	Erysipelotrichia	Erysipelotrichales	*Erysipelotrichaceae*	*Catenibacterium*	0.17	0.33	0.48
				Erysipelotrichaceae UCG-004	0.19	0.47	0.65
				Holdemanella	0.52	0.56	0.49
			Erysipelotrichaceae		0.12	0.71	0.97
	Negativicutes	Selenomonadales	*Acidaminococcaceae*	*Phascolarctobacterium*	0.85	1.1	0.54
			Veillonellaceae	*Allisonella*	0.31	0.12	0.49
				Megasphaera	0.18	0.43	0.68
				Veillonella	0.77	0.42	0.69
Fusobacteria	Fusobacteriia	Fusobacteriales	*Fusobacteriaceae*	*Fusobacterium*	1.71	0.88	1.54
Lentisphaerae	Lentisphaeria	Victivallales	*Victivallaceae*	*Victivallis*	0.12	0.38	0.54
	Oligosphaeria	Oligosphaerales	*Oligosphaeraceae*	*Z20*	0.04	0.38	0.57
Planctomycetes	Planctomycetacia	Pirellulales	*Pirellulaceae*	*p-1088-a5 gut group*	0.09	0.38	0.55
Proteobacteria	Deltaproteobacteria	Bradymonadales			0.12	0.33	0.52
		Desulfovibrionales	*Desulfovibrionaceae*	*Bilophila*	0.32	0.59	0.57
				Mailhella	0.17	0.36	0.5
			Desulfovibrionaceae		0.06	0.31	0.47
	Gammaproteobacteria	Betaproteobacteriales	*Burkholderiaceae*	*Sutterella*	0.8	0.73	0.49
		Enterobacteriales	*Enterobacteriaceae*	*Escherichia-Shigella*	1.37	1.09	0.94
		Pasteurellales	*Pasteurellaceae*	*Actinobacillus*	0.58	0.5	0.48
Spirochaetes	Spirochaetia	Spirochaetales	*Spirochaetaceae*	*Sphaerochaeta*	0.13	0.36	0.53
				Treponema 2	0.08	0.45	0.64
Synergistetes	Synergistia	Synergistales	*Synergistaceae*	*Pyramidobacter*	0.02	0.6	0.91
				Synergistes	0	0.37	0.57
Tenericutes	Mollicutes	Mollicutes RF39			0	0.36	0.55

Chapter 7:

A single faecal microbiota transplantation altered the microbiota of weaned pigs

Statement of Authorship

Title of Paper	A Single Faecal microbiota Transplantation Altered the Microbiota of Weaned pigs
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Nowland, T. L., V. A. Torok, W. Y. Low, K. J. Plush, M. D. Barton, and R. N. Kirkwood. 2020. A Single Faecal Microbiota Transplantation Altered the Microbiota of Weaned Pigs. Life (Basel) 10(9) doi: 10.3390/life10090203

Principal Author

Name of Principal Author (Candidate)	Tanya Nowland
Contribution to the Paper	Assisted with the experimental design, managed and carried out the experimental trial, analysed statistics, drafted and edited the manuscript.
Overall percentage (%)	85%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 16/02/2021

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Roy Kirkwood
Contribution to the Paper	Assisted with the experimental design, data collection and interpretation and editing manuscript.
Signature	Date 25/2/21

Name of Co-Author	Kate Plush
Contribution to the Paper	Assisted with the experimental design, evaluated and edited the manuscript.
Signature	Date 25/2/21

Name of Co-Author	Mary Barton		
Contribution to the Paper	Assisted with the experimental design, data interpretation and editing manuscript.		
Signature		Date	25/2/2021

Name of Co-Author	Valeria Torok		
Contribution to the Paper	Assisted with the experimental design, statistical analysis, data interpretation and editing manuscript.		
Signature		Date	25/2/21

Name of Co-Author	Wai Yee low		
Contribution to the Paper	Assisted with the experimental design, statistical analysis, data interpretation and editing manuscript.		
Signature		Date	25/2/21

Please cut and paste additional co-author panels here as required.

life

MDPI

Article

A Single Faecal Microbiota Transplantation Altered the Microbiota of Weaned Pigs

Tanya L. Nowland [1,*], Valeria A. Torok [1,2], Wai Y. Low [3], Kate J. Plush [4], Mary D. Barton [5] and Roy N. Kirkwood [1]

1 School of Animal and Veterinary Sciences, The University of Adelaide, Roseworthy 5371, Australia; valeria.torok@sa.gov.au (V.A.T.); roy.kirkwood@adelaide.edu.au (R.N.K.)
2 Food Sciences, South Australian Research and Development Institute, Waite Campus, Urrbrae 5064, Australia
3 The Davies Research Centre, School of Animal and Veterinary Sciences, The University of Adelaide, Roseworthy 5371, Australia; wai.low@adelaide.edu.au
4 SunPork Group, Murarrie 4172, Australia; kate.plush@sunporkfarms.com.au
5 School of Pharmacy and Medical Sciences, University of South Australia, Adelaide 5000, Australia; mary.barton@unisa.edu.au
* Correspondence: tanya.nowland@adelaide.edu.au; Tel.: +61-08-83-13-76-17

Received: 21 August 2020; Accepted: 14 September 2020; Published: 15 September 2020

Abstract: Weaning is a stressful time for piglets, often leading to weight loss and is associated with increased morbidity and mortality. A leading cause for these post-weaning problems is enteric dysbiosis and methods to improve piglet health at this crucial developmental stage are needed. This study aimed to determine whether an enteric dysbiosis caused by weaning could be corrected via a faecal microbiota transplantation (FMT) from healthy piglets from a previous wean. Two or four focal piglets per litter were assigned to one of two treatments; FMT two days post weaning (n = 21; FMT) or a control which received saline two days post weaning (n = 21; CON). FMT consisted of homogenised donor faeces administered orally at 3 mL/kg. Weaning occurred at 18 days of age and weights and faecal samples were collected on days 18, 20, 24 and 35. 16S rRNA amplicon analysis was used to assess the faecal microbiota of piglets. FMT increased Shannon's diversity post weaning ($p < 0.001$) and reduced the scratch score observed at 24 days of age ($p < 0.001$). The bacterial populations significantly differed in composition at each taxonomic level. In FMT pigs, significant increases in potentially pathogenic *Escherichia coli* were observed. However, increases in beneficial bacteria *Lactobacillus mucosae* and genera *Fibrobacteres* and *Bacteroidetes* were also observed in FMT treated animals. To our knowledge, this is the first study to observe a significant effect on piglet faecal microbiota following a single FMT administered post weaning. Therefore, FMT post weaning can potentially alleviate enteric dysbiosis.

Keywords: pigs; weaning; enteric dysbiosis; microbiota transplantation; enteric microbiota

1. Introduction

Weaning is one of the most stressful times in a piglet's life. It involves separation from the sow, a change in diet from primarily milk to solid feed and the mixing of litters [1]. As a result of this stress, piglets have reduced daily feed intake following weaning which results in villus atrophy, increased intestinal permeability and may result in post-weaning diarrhoea (PWD) [2]. Much research has investigated ways for making the weaning transition easier for piglets, including provision of creep feed during lactation [3] and the inclusion of ZnO or in-feed antibiotics in post weaning diets as a preventative for PWD [4,5]. Research has demonstrated some benefit from creep feeding;

however, with weaning ages as young at 18 to 21 days, the impact of feeding creep is minimal [3]. While ZnO provides benefits to piglets by reducing PWD and increasing growth performance, it is an environmental pollutant and as such its use is restricted in some countries [6]. Both antibiotics and high levels of ZnO are associated with antimicrobial resistance [7] and this necessitates the development of alternatives.

Microbiota are communities of microorganisms colonising all body surfaces. These microorganisms include bacteria, fungi, archaea and viruses [8,9]. The microbiota within the gastrointestinal tract has been demonstrated to be involved in nutrient metabolism and immune system development and function, and disruptions of the microbiota cause long-term health problems [10]. Some of these problems include necrotising enterocolitis, a reduced immune response following infection and an associated higher susceptibility to disease, reduced growth and diarrhoea in pigs [11–14]. As such, the development or maintenance of a healthy gut microbiota is important for health and survival.

One method that has been successful for the treatment of enteric diseases such as *Clostridium difficile* infections in humans is faecal microbiota transplantation (FMT) [15]. FMT was first described by Ge Hong in fourth century China for treating food poisoning and severe diarrhoea [16]. FMT involves transferring faeces from a healthy donor to a sick recipient with the aim of re-establishing a healthy microbiota through the competitive exclusion of pathogenic bacteria. The aim of the present study was to determine whether a dysbiosis caused by weaning could be corrected via FMT from healthy piglets from a previous weaning. We hypothesised that the microbiota of the donor pigs would be in a state optimal for the post-weaning changes of diet and environment, therefore providing a benefit to the naïve, newly weaned piglet. As a result, the weaning stress associated with enteric dysbiosis would be corrected by administration of FMT post weaning.

2. Materials and Methods

All procedures were conducted at the University of Adelaide Roseworthy piggery with the approval of the University of Adelaide's Animal Ethics Committee (AEC number: S-2017-063).

2.1. Experimental Design and Sample Collection

A total of 19 Large White x Landrace sows (parities 2 to 4; mean ± standard deviation: 2.8 ± 0.8) were used in the study. All sows were group housed during gestation and received no antibiotics. Sows were moved into the farrowing house at day 110 of gestation where they received a commercial lactation diet (14.2 MJ DE/kg) twice daily and had free access to water. Prior to farrowing, sows were fed 2.5 kg/d, which was gradually increased to 7 to 8 kg/d by day 7 after farrowing. Two days before their due date, sows were induced to farrow using split-dose vulva injections of cloprostenol (125 µg at 7 a.m. and 2 p.m.). At weaning (18 days of age), 2 or 4 male piglets per sow were selected at random to be focal pigs and were assigned equally to one of two treatments:

- FMT: Faecal transplant administered two days post weaning (n = 21; FMT)
- Control: Saline administered two days post weaning (n = 21; CON)

To ensure that the effect of sow was not confounding, the focal piglets per litter were assigned equally to treatments. Sows farrowed over two days and piglets were cross fostered to achieve 10 or 11 piglets per litter 24 h post-partum. Weaning occurred on day 18. Focal piglets were weaned into two pens with slatted flooring according to their treatment. Both pens were in the same room and had a walkway separating them to prevent faecal transfer between pens. Heat lamps were set over one corner of the pens and feeders were placed in the other. Piglets had *ad libitum* access to antibiotic-free weaner feed (14.5 MJ DE/kg) and water throughout the experiment. All focal piglets were individually weighed at 3, 18, 20, 24 and 35 days of age. Faecal samples were collected from each focal piglet at weaning (18 days), prior to FMT (20 days of age) and 4 and 15 days post FMT (age 24 and 35 days). Faeces were placed on ice immediately, transported to the laboratory within 4 h and stored at −80 °C. For FMT, donor faeces were collected from eight clinically healthy male piglets

from a previous weaning (7 weeks old) and blended 1:2 with saline. Donor piglets were housed in a slatted floored pen with *ad libitum* access to antibiotic-free feed and had no previous contact with antibiotics or antibiotic-treated animals. All faecal blends were collected within an hour of FMT and were at room temperature before being delivered by oral gavage at 3 mL/kg [16]. Piglets were fasted for 3 h before FMT to minimise gastric acidity. All piglets were monitored for any adverse reactions, such as vomiting, but none were noted. Scratch scores as an indicator of fighting and the presence of diarrhoea were recorded each time the piglets were weighed post weaning. Diarrhoea was defined as the presence of liquid faeces as described previously [17] and scratch scores were recorded based on the system used by Seyfang et al. [18]. A schematic of the experimental timeline can be seen in Figure 1.

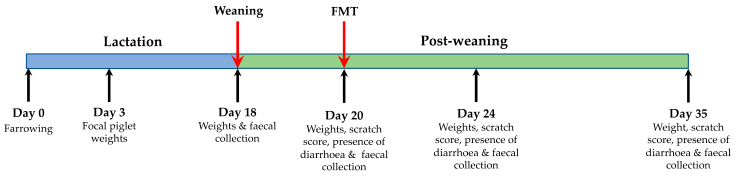


Figure 1. Schematic of the experimental timeline. Piglets were divided into two groups at weaning, control animals (CON; $n = 21$) or faecal microbiota transplantation (FMT; $n = 21$), the FMT procedure is described in the materials and methods section. On day 3, 18, 20, 24 and 35, all piglets were weighed. Faecal samples, scratch scores and diarrhoea incidence were collected from day 18 onwards as stated in the figure above.

2.2. *DNA Extraction and 16S rRNA Amplicon Sequencing*

Total nucleic acid was extracted from freeze dried piglet faecal samples using a proprietary method (South Australian Research and Development Institute, Adelaide, Australia) [19–21]. The V3–V4 region of the 16S rRNA gene was sequenced using the illumina MiSeq platform using 300 bp paired end reads (forward primer: CCTAYGGGRBGCASCAG and reverse primer: GGACTACNNGGGTATCTAAT), following a standard protocol by the Australian Genome Research Facility for next generation sequencing (Melbourne, Australia). The obtained reads are available under the accession number PRJNA634575 of the Sequence Read Archive of the NCBI. For bioinformatic analysis of raw sequence data performed by AGRF, the paired-end sequences were merged by aligning the forward and reverse reads using PEAR v0.9.5 [22] and the primers were identified and trimmed. All trimmed sequences were processed using Quantitative Insights into Microbial Ecology (QIIME 1.8) [23], USEARCH (version 8.0.1623) [24,25], and UPARSE software [26]. Sequences were quality filtered and full-length duplicate sequences were removed and sorted by abundance. Singletons or unique reads in the dataset were discarded. Additionally, chimeric sequences were clustered and removed using the "rdp_gold" database as the reference. Sequences were grouped into operational taxonomic units (OTUs) based on 97% sequence similarity. Using QIIME, taxonomy was assigned using the Silva database v132 [27].

2.3. *Statistical Methods*

All data were tested for the normality of residuals and outliers before analysis, no outliers were found or removed, and all weight and average daily gain data were normally distributed. Outliers were defined as data points greater than two standard deviations from the mean and were deemed as aberrant data. All weight, average daily gain, diarrhoea incidence and scratch score data were analysed using SPSS, v26 (IBM, Armonk, NY, USA). A linear mixed model with repeated measures design was used to assess the effect of treatment on piglet weight and average daily gain. The fixed effects included in the model were treatment, age and treatment*age, with age specified as the repeated measure. Scratch score data were analysed using a generalised linear model, repeated measures design with a Poisson distribution, and diarrhoea incidence data were analysed using a generalised linear

model, repeated measures design with a binary logistic regression using the same model. Data were expressed as estimated marginal means ± standard error of the mean, and a p-value threshold of 0.05.

Multivariate statistical techniques (PRIMER6, PRIMER-E Ltd., Ivybridge, UK) were used to analyse the faecal 16S rRNA bacterial taxonomic data. Similarities between faecal bacterial communities of piglets from the 16S rRNA data metrics were analysed using Bray–Curtis measures of similarity [28], following standardisation and fourth-root transformation. Analysis of similarity (ANOSIM) [29] was used to test if there were significant treatment and age differences between faecal bacterial communities. In order to determine which individual bacterial taxa contributed most to the overall dissimilarity among treatment and age groups, similarity percentages (SIMPER) [29] analyses were done and the overall average dissimilarity between piglet faecal bacterial communities were calculated. The contributions (%) of significant OTUs (average dissimilarity/standard deviation > 1) to the top 60% of the average dissimilarities were calculated. Non-metric multidimensional scaling (nMDS) [30,31] was done to graphically illustrate relationships between ages.

Alpha diversity metrics; Shannon diversity (H′) index, Pielou's evenness (J′) and number of taxa (S) were calculated using DIVERSE (PRIMER6 PRIMER-E Ltd., Ivybridge, UK). Normality was tested within RStudio software (Version 1.1.456, Boston, MA, USA) using the Shapiro–Wilk test. Those alpha diversity metrics that were found to be normally distributed were analysed using an analysis of variance (ANOVA) and those not normally distributed were analysed using the Kruskal–Wallis test, with corrections for multiple tests using false discovery rate (FDR) and a p-value threshold of 0.05.

3. Results

3.1. Production Characteristics

No significant differences in piglet weights were evident between treatments at 24 (CON: 6.8 ± 0.2 kg, FMT: 6.5 ± 0.2 kg; $p = 0.391$) and 35 (CON: 9.0 ± 0.3 kg, FMT 8.9 ± 0.3 kg; $p = 0.776$) days of age. Average daily gain was higher for control animals from 20 to 24 days of age (CON: 0.07 ± 0.01 kg, FMT: 0.02 ± 0.01 kg; $p = 0.003$), but no significant difference between treatments was observed for average daily gain between 24 and 35 days of age (CON: 0.20 ± 0.02 kg, FMT: 0.21 ± 0.02 kg; $p = 0.664$). A post-weaning growth check was observed for piglets regardless of treatment; average daily again from 3 days old to weaning (18 days) was 0.30 ± 0.01 kg, from weaning to 20 days was −0.12 ± 0.01 kg, 20 to 24 days old was 0.04 ± 0.01 kg and from 24 to 35 days of age was 0.21 ± 0.01 kg. No significant difference for the occurrence of diarrhoea was observed at 24 days or 35 days of age (25% CI (14, 40) and 0.9% CI (0.01, 88), respectively; $p = 0.385$). Those animals treated with a faecal transplant had significantly lower scratch scores at 24 days of age when compared to control animals (CON: 0.86 ± 0.19, FMT: 0.05 ± 0.04; $p < 0.001$).

3.2. Age Related Effects

On average, 50,578 16S rRNA sequenced reads were retained after quality control per faecal sample. Faecal bacterial genera significantly differed as piglets aged, with all age-wise comparisons being different (Global R = 0.538, $p < 0.001$; Figure 2). Alpha diversity metrics such as Shannon's diversity, Pielou's evenness and number of taxa, differed between ages ($p < 0.001$; Figure 3). Shannon's diversity and number of taxa increased from 18 to 20 days of age ($p < 0.001$), thereafter decreasing to levels similar to 18-day-old animals by 35 days of age. Pielou's evenness increased significantly as piglets aged ($p < 0.001$; Figure 3).

The dominant phyla in the faecal microbiota of piglets at 18 days of age were Bacteroidetes (26.69%), Firmicutes (29.72%), Proteobacteria (15.67%), Fusobacteria (6.30), Actinobacteria (6.22%) Synergistetes (3.78%), Tenericutes (3.34%), and Spirochaetes (2.61%). These made up 94.55% of all bacteria found in piglet faeces at 18 days of age, with 10 bacterial phyla comprising the remaining 5.45% (Figure 4). The proportions of these phyla changed with age, collectively representing 91.51%, 90.95%, 92.24% at 20, 24 and 35 days of age, respectively. The three most dominant phyla represented as piglets aged

(Bacteroidetes, Firmicutes and Proteobacteria) remained the same; however, Fusobacteria, which was the fourth most abundant phylum at 18 and 20 days of age, declined and was not represented at 35 days of age. The average dissimilarity in bacterial phyla between age groups ranged from 18 to 24%. The main phyla driving significant change between 18 and 35 days of age were increases in Spirochaetes, Tenericutes, TM7, Bacteroidetes, Deferribacteres and Fibrobacteres, and decreases in Fusobacteria, Proteobacteria, Synergistetes, Lentisphaerae and Firmicutes at 35 days of age.

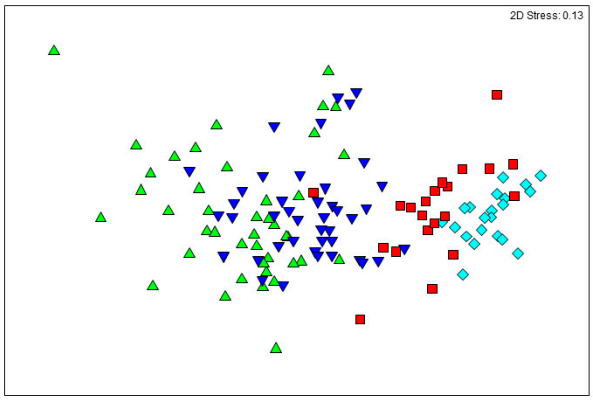
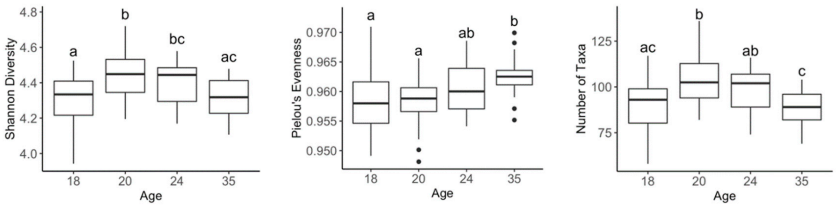


Figure 2. nMDS ordination of faecal bacterial genera from piglets at 18 (triangle), 20 (inverted triangle), 24 (square) and 35 (diamond) days of age. nMDS ordinations attempt to place all samples in an arbitrary two-dimensional space such that their relative distances apart match the corresponding pairwise similarities. Hence, the closer the two samples are in the ordination, the more similar their overall bacterial communities. "Stress" values (Kruskal's formula 1) reflect the difficulty involved in compressing the sample relationship into the two-dimensional ordination.



(**a**) (**b**) (**c**)

Figure 3. Comparison of Shannon diversity (**a**), Pielou's evenness (**b**), and number of taxa (**c**) between piglets at age 18, 20, 24 and 35 days at the genus level. Means with different superscripts (a, b, c) are significantly different ($p < 0.05$).

At the genus level, the average dissimilarity in faecal microbiota between 18- and 35-day-old piglets was 44.21%. Of the genera significantly contributing to the top 60% of dissimilarity, *Bacteroides*, *Butyricimonas*, *Escherichia*, *Parabacteroides*, *Fusobacterium*, *Lactobacillus*, *Bilophila*, *Oscillospira*, *Enterococcus*, *Clostridium*, *Ruminococcus*, *Veillonella*, *Streptococcus*, *Rothia* and *Collinsella* were more abundant at 18 days and *Roseburia*, *Prevotella*, *Lachnospira*, *Succinivibrio*, *Lachnobacterium*, *Treponema*, *Anaerovibrio*, *Sarcina*, *Bulleidia*, *Coprococcus*, *Butyrivibrio*, *Mitsuokella*, *Megasphaera*, *Faecalibacterium*, *Campylobacter*, *Acidaminococcus*, *Catenibacterium*, *Turicibacter* and *Dialister* were more abundant at 35 days. Of the taxa which could be classified to the species level, *Bacteroides fragilis*, *Escherichia coli*, *Parabacteroides distansonis*, *Lactobacillus delbrueckii*, *Clostridium perfringens*, *Streptococcus luteciae*, *Clostridium hathewayi*, and *Bacteroides uniformis* were more abundant in the 18 day old piglets and *Prevotella copri*, *Roseburia*

faecis, *Faecalibacterium prausnitzii*, *Prevotella stercorea*, *Ruminococcus bromii*, and *Ruminococcus flavefaciens* were more abundant in 35 day old piglets, contributing to the top 50% of dissimilarity between these groups.

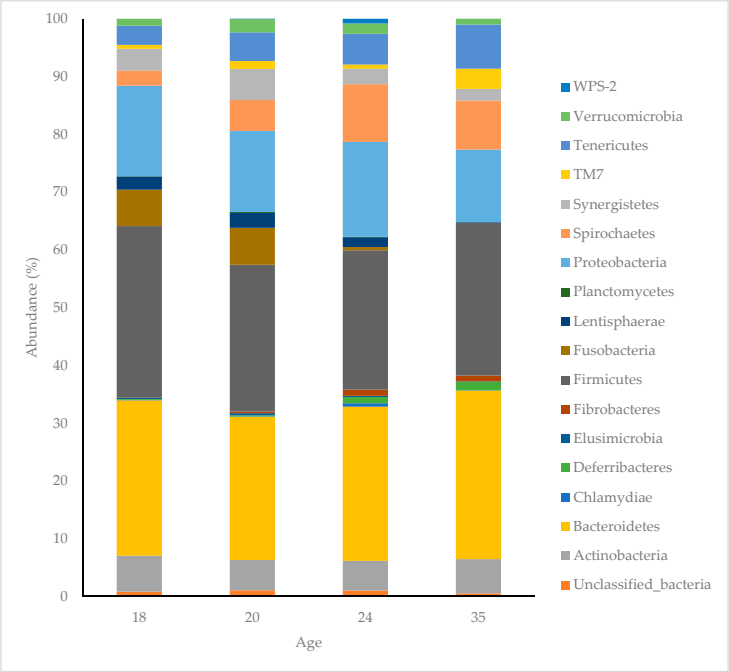


Figure 4. Abundance (%) of bacterial phyla present in the faeces of pigs at 18, 20, 24 and 35 days of age. The bacterial phlya within the legend are arranged in the same order as they appear in the bar chart.

3.3. Donor Sample Composition

The main phyla present within the pooled homogenate of eight donor pigs were Actinobacteria, Bacteroidetes, Fibrobacteres, Firmicutes, Proteobacteria, Spirochaetes, TM7 and Tenericutes. Of the taxa that could be identified to genus level, the top 6 bacteria present were *Prevotella* (60.16%), *Roseburia* (6.36%), *Oscillospira* (2.63%), *Faecalibacterium* (1.7%), *Lachnospira* (1.54%) and *Dialister* (1.06%), while 53 other genera comprised the remaining bacteria present and all individually represented less than 1% of the total bacteria present. Of the taxa that could be identified to species level, the donor sample homogenate contained *Prevotella ruminicola*, *Streptococcus luteciae*, *Defluviitalea saccharophila*, *Bacteroides plebeius*, *Helicobacter equorum*, *Clostridium hathewayi*, *Asteroleplasma anaerobium*, *Coprococcus eutactus*, *Ruminococcus callidus*, *Oxalobacter formigenes*, *Clostridium piliforme*, *Eubacterium biforme*, *Mitsuokella multacida*, *Lactobacillus reuteri*, *Butyricicoccus pullicaecorum*, *Ruminococcus gnavus*, *Faecalibacterium prausnitzii*, *Roseburia faecis*, *Collinsella aerofaciens*, *Escherichia coli*, *Eubacterium cylindroides*, *Lactobacillus mucosae*, *Ruminococcus flavefaciens*, *Ruminococcus bromii*, *Desulfovibrio D168*, *Prevotella stercorea* and *Prevotella copri*.

3.4. Treatment by Age Effects

Faecal bacterial genera differed between treatments at 24 (Global R = 0.168, $p = 0.002$; Figure 5) and 35 days of age (Global R = 0.110, $p = 0.001$; Figure 5). For alpha-diversity metrics, Shannon's diversity was also different between treatments in both age groups ($p < 0.001$; Figure 6), while Pielou's

evenness and number of taxa only differed with treatment in the 24-day-old piglets ($p = 0.040$ and $p < 0.001$, respectively; Figure 6).

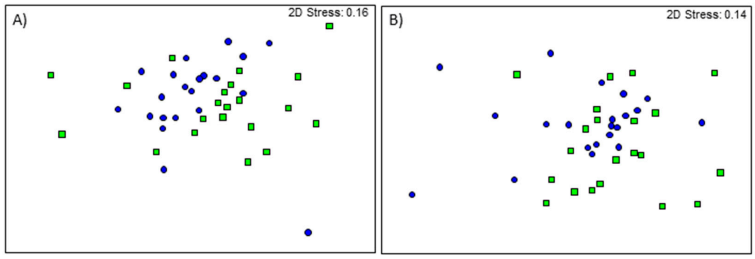
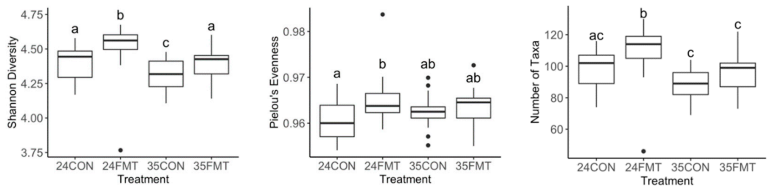


Figure 5. nMDS ordination of faecal bacterial genera from control (square) and FMT (circle) piglets at (**A**) 24 days of age and (**B**) 35 days of age.



(**a**) (**b**) (**c**)

Figure 6. Comparison of Shannon diversity (**a**), Pielou's evenness (**b**), and number of taxa (**c**) between piglets in treatments CON and FMT at 24 and 35 days of age at the genus level. Means with different superscripts (a, b, c) are significantly different ($p < 0.05$).

The main phyla driving the change between treatments at 24 days of age were increases in Spirochaetes, Deferrbacteres, Synergistetes, TM7, Elusimicrobia, WPS-2, Fibrobacteres, Tenericutes, Firmicutes, Verrucomicrobia and Lentisphaerae, and decreases in Proteobacteria, Bacteroidetes and Chlamydiae for those animals treated with FMT. The main phyla driving the difference between treatments at 35 days of age were an increase in Proteobacteria, Deferribacteres, Fibrobacteres, Elusimicrobia, Actinobacteria and Bacteroidetes and decreases in Spirochaetes, TM7, Synergistetes, Terenicutes, Verrucomicrobia and Firmicutes for those animals treated with FMT.

The average dissimilarity in bacterial genera between treatments in piglets at 24 days of age was 27% (Table 1). Of those taxa which could be classified to genus level and significantly contributed to the top 60% of dissimilarity, *Acidaminococcus*, *Dorea*, *YRC22*, *Butyrivibrio*, *Pyramidobacter*, *Mucispirillum*, *Streptococcus*, *Actinobacillus*, *Anaerovibrio*, *Butyricimonas*, *Oscillospira*, *Lachnobacterium*, *SMB53*, *Fibrobacter*, *Lachnospira* and *rc4-4* were more abundant in animals treated with the faecal transplant and *Succinivibrio*, *Prevotella*, *Mitsuokella*, *Lactobacillus*, *Faecalibacterium*, *Megasphaera*, *Catenibacterium*, *Collinsella* and *Roseburia* were more abundant in control animals of the same age. The average dissimilarity in bacterial genera between treatments in piglets aged 35 days was 22% (Table 2). Of those taxa which could be classified to genus level and significantly contributed to the top 60% of dissimilarity, *Dialister*, *Shuttleworthia*, *Acidaminococcus*, *Mitsuokella*, *Campylobacter*, *Megasphaera*, *Catenibacterium*, *Bulleidia*, *Streptococcus*, *Mucispirillum*, *Actinobacillus*, *Anaerostipes*, *Faecalibacterium*, *Escherichia*, *Ruminococcus*, *Fibrobacter*, *Collinsella*, *Oscillospira* and *L7A_E11* were more abundant in animals treated with the faecal transplant and *Succinivibrio*, *Treponema*, *Lachnobacterium*, *Sarcina*, *Prevotella*, *Roseburia*, *Phascolarctobacterium*, *Parabacteroides*, *YRC22*, *rc4-4*, *SMB53*, *Lachnospira*, *Dorea*, *p-75-a5*, *CF231*, *Oxalobacter* and *Coprococcus* were more abundant in control animals.

Table 1. Genus contributing to the top 60% of significant dissimilarity of bacteria between FMT and CON at 24 days of age as determined by similarity percentages (SIMPER). Overall average dissimilarity between treatments is 27%.

					Group FMT	**Group CON**	
Phyla	**Class**	**Order**	**Family**	**Genus**	**Average Abundance**	**Average Abundance**	**%**
Actinobacteria	Coriobacteriia	Coriobacteriales	*Coriobacteriaceae*	*Collinsella*	0.26	0.27	0.69
Bacteroidetes	Bacteroidia	Bacteroidales	*Bacteroidaceae*	*Bacteroides*	0.82	0.68	1.24
			Odoribacteraceae	*Butyricimonas*	0.27	0.25	0.75
			p-2534-18B5		0.46	0.29	1.09
			Paraprevotellaceae	*YRC22*	0.61	0.38	0.94
				CF231	0.9	0.9	0.81
			Porphyromonadaceae	*Parabacteroides*	1.02	0.79	1.25
				Paludibacter	0.37	0.24	1.18
			Prevotellaceae	*Prevotella*	2.12	2.31	1.13
				Prevotella	1.19	1.03	1.09
			Prevotellaceae		0.69	0.58	0.82
			RF16		0.47	0.36	0.81
			Rikenellaceae		0.25	0.2	0.73
			S24-7		1.77	1.64	0.99
Deferribacteres	Deferribacteres	Deferribacterales	*Deferribacteraceae*	*Mucispirillum*	0.4	0.21	0.8
Elusimicrobia	Elusimicrobia	Elusimicrobiales	*Elusimicrobiaceae*		0.26	0.12	0.77
Fibrobacteres	Fibrobacteria	Fibrobacterales	*Fibrobacteraceae*	*Fibrobacter*	0.36	0.22	0.7
Firmicutes	Bacilli	Lactobacillales	*Lactobacillaceae*	*Lactobacillus*	0.46	0.5	0.98
			Streptococcaceae	*Streptococcus*	0.41	0.32	0.78
	Clostridia	Clostridiales	*Christensenellaceae*		1.1	0.9	1.27
			Clostridiaceae	*Sarcina*	0.82	0.67	1.02
			Clostridiaceae		0.49	0.41	0.81
				SMB53	0.48	0.37	0.72
			Lachnospiraceae		1.43	1.2	1.16
				Shuttleworthia	0.37	0.13	1.02
				Dorea	0.75	0.63	0.96
				Butyrivibrio	0.54	0.4	0.94
				Lachnobacterium	0.38	0.25	0.73
				Roseburia	0.84	0.93	0.69
				Lachnospira	0.71	0.65	0.69
			Mogibacteriaceae		0.82	0.72	0.8
			Peptococcaceae	*rc4-4*	0.33	0.26	0.66
			Ruminococcaceae	*Faecalibacterium*	1.01	1.14	0.91
				Oscillospira	1.52	1.45	0.73
			Ruminococcaceae		1.69	1.56	0.66
			Veillonellaceae	*Mitsuokella*	0.43	0.53	1.07
				Dialister	0.54	0.2	1.06

Table 1. *Cont.*

					Group FMT	Group CON	
Phyla	**Class**	**Order**	**Family**	**Genus**	**Average Abundance**	**Average Abundance**	**%**
			Veillonellaceae		0.63	0.61	1
				Acidaminococcus	0.57	0.43	0.98
				Megasphaera	0.63	0.64	0.9
				Anaerovibrio	0.95	0.91	0.75
		Clostridiales			1.07	0.91	1.01
		Clostridiales			1.17	1.02	0.66
Firmicutes	Erysipelotrichi	Erysipelotrichales	*Erysipelotrichaceae*	*p-75-a5*	0.77	0.67	0.82
				Catenibacterium	0.65	0.71	0.8
Proteobacteria	Alphaproteobacteria	RF32			0.33	0.21	0.82
	Alphaproteobacteria				0.3	0.16	0.76
	Deltaproteobacteria	GMD14H09			0.72	0.68	1.1
	Epsilonproteobacteria	Campylobacterales	*Campylobacteraceae*	*Campylobacter*	0.91	0.75	1.08
	Gammaproteobacteria	Aeromonadales	*Succinivibrionaceae*	*Succinivibrio*	1.18	1.44	1.85
			Succinivibrionaceae	*Ruminobacter*	0.63	0.39	1.51
			Succinivibrionaceae		0.52	0.15	1.5
		Enterobacteriales	*Enterobacteriaceae*	*Escherichia*	0.76	0.54	1.33
		Pasteurellales	*Pasteurellaceae*	*Actinobacillus*	0.37	0.3	0.76
Spirochaetes	Spirochaetes	Spirochaetales	*Spirochaetaceae*	*Treponema*	1.31	1.16	1.14
Synergistetes	Synergistia	Synergistales	*Dethiosulfovibrionaceae*	*Pyramidobacter*	0.36	0.26	0.87
			Dethiosulfovibrionaceae		0.33	0.27	0.66
Tenericutes	RF3	ML615J-28			0.38	0.27	0.75
TM7	TM7-3	CW040	*F16*		0.33	0.17	0.8
Verrucomicrobia	Verruco-5	WCHB1-41	*RFP12*		0.33	0.18	0.66
WPS-2					0.31	0.21	0.74

Table 2. Genus contributing to the top 60% of significant dissimilarity of bacteria between FMT and CON at 35 days of age as determined by SIMPER. Overall average dissimilarity between treatments is 22%.

					Group FMT	Group CON	
Phyla	**Class**	**Order**	**Family**	**Genus**	**Average Abundance**	**Average Abundance**	**%**
Actinobacteria	Coriobacteriia	Coriobacteriales	*Coriobacteriaceae*	*Collinsella*	0.37	0.28	0.77
Bacteroidetes	Bacteroidia	Bacteroidales	*Paraprevotellaceae*	*YRC22*	0.21	0.28	0.9
				CF231	0.75	0.88	0.74
					0.7	0.55	0.69
			Porphyromonadaceae	*Parabacteroides*	0.52	0.59	0.94
			Prevotellaceae	*Prevotella*	1.19	1.3	1.12
			S24-7		1.32	1.3	0.82
		Bacteroidales			0.43	0.41	0.9
Deferribacteres	Deferribacteres	Deferribacterales	*Deferribacteraceae*	*Mucispirillum*	0.35	0.26	0.99
Elusimicrobia	Elusimicrobia	Elusimicrobiales	*Elusimicrobiaceae*		0.21	0.07	0.79
Fibrobacteres	Fibrobacteria	Fibrobacterales	*Fibrobacteraceae*	*Fibrobacter*	0.31	0.2	0.78
			Streptococcaceae	*Streptococcus*	0.45	0.32	1.01
Firmicutes	Clostridia	Clostridiales	*Clostridiaceae*	*Sarcina*	0.53	0.73	1.31
				SMB53	0.86	0.88	0.84
			Clostridiaceae		0.94	0.95	0.82
			Lachnospiraceae	*Shuttleworthia*	0.53	0.41	1.64
				Lachnobacterium	0.56	0.79	1.63
			Lachnospiraceae		1.32	1.4	1.39
				Roseburia	1.47	1.55	1.1
				Anaerostipes	0.4	0.32	0.87
				Lachnospira	0.95	1.08	0.81
				Ruminococcus	1.06	1.03	0.79
				Dorea	0.68	0.71	0.78
				Coprococcus	0.94	0.96	0.72
			Peptococcaceae	*rc4-4*	0.11	0.28	0.87
			Ruminococcaceae	*Faecalibacterium*	1.18	1.08	0.82
				Oscillospira	1.28	1.23	0.77
			Veillonellaceae	*Dialister*	0.91	0.34	2.29
			Veillonellaceae		0.71	0.66	1.43
				Acidaminococcus	0.53	0.34	1.4
				Mitsuokella	0.65	0.53	1.35
				Megasphaera	0.7	0.53	1.28
				Phascolarctobacterium	0.72	0.96	0.99
	Clostridia				0.24	0.04	0.86
	Erysipelotrichi	Erysipelotrichales	*Erysipelotrichaceae*	*Catenibacterium*	0.75	0.58	1.13
				Bulleidia	0.98	0.91	1.09
				p-75-a5	0.64	0.68	0.77
				L7A_E11	0.33	0.25	0.74

Table 2. *Cont.*

					Group FMT	Group CON	
Phyla	**Class**	**Order**	**Family**	**Genus**	**Average Abundance**	**Average Abundance**	**%**
Proteobacteria	Alphaproteobacteria	RF32			0.36	0.45	1.09
	Betaproteobacteria	Burkholderiales	*Oxalobacteraceae*	*Oxalobacter*	0.14	0.15	0.73
		Tremblayales			0.32	0.26	1.44
	Deltaproteobacteria	GMD14H09			0.52	0.45	1.36
	Epsilonproteobacteria	Campylobacterales	*Campylobacteraceae*	*Campylobacter*	0.77	0.67	1.34
	Gammaproteobacteria	Aeromonadales	*Succinivibrionaceae*	*Succinivibrio*	0.93	0.98	1.84
			Succinivibrionaceae		0.47	0.2	1.7
		Enterobacteriales	*Enterobacteriaceae*	*Escherichia*	0.34	0.27	0.81
		Pasteurellales	*Pasteurellaceae*	*Actinobacillus*	0.32	0.15	0.93
Spirochaetes	Spirochaetes	Spirochaetales	*Spirochaetaceae*	*Treponema*	0.91	1.01	1.65
Synergistetes	Synergistia	Synergistales	*Dethiosulfovibrionaceae*		0.22	0.27	0.7
TM7	TM7-3	CW040	*F16*		0.38	0.41	0.93
Unclassified Bacteria					0.26	0.14	0.81

Of those taxa which could be classified to the species level at 24 days, *Escherichia coli*, *Desulfovibrio D168*, *Ruminococcus flavefaciens*, *Mucispirillum schaedleri*, *Eubacterium cylindroides*, *Lactobacillus mucosae* and *Streptococcus luteciae* were more abundant in animals treated with the faecal transplant and *Prevotella copri*, *Prevotella stercorea*, *Faecalibacterium prausnitzii*, *Ruminococcus bromii*, *Mitsuokella multacida*, *Sharpea azabuensis*, *Collinsella aerofaciens*, *Roseburia faecis*, *Ruminococcus callidus* and *Helicobacter equorum* were more abundant in control animals, contributing significantly to the top 60% of dissimilarity between these treatment groups. Of those taxa which could be classified to the species level in 35 day old pigs, *Prevotella copri*, *Desulfovibrio D168*, *Ruminococcus bromii*, *Lactobacillus mucosae*, *Mucispirillum schaedleri*, *Eubacterium cylindroides*, *Prevotella stercorea*, *Faecalibacterium prausnitzii*, *Escherichia coli*, *Collinsella aerofaciens* and *Clostridium hathwayi* were more abundant in animals that had been treated with a faecal microbiota transplant and *Mitsuokella multacida*, *Roseburia faecis*, *Ruminococcus flavefaciens*, *Lactobacillus reureri*, *Ruminococcus callidus*, *Oxalobacter formigenes* and *Coprococcus eutactus* were more abundant in control animals, contributing to the top 60% of dissimilarity between these treatment groups.

4. Discussion

Our data support the hypothesis that weaning stress would cause enteric dysbiosis that is corrected through FMT post weaning and, to our knowledge, this is the first study investigating FMT in pigs that has demonstrated an effect after a single FMT post weaning.

It is accepted that an increase in microbial diversity is beneficial for the gut health of an individual (for reviews, see: [10,32,33]). The greater the diversity of bacteria present, the less chance pathogenic bacteria have to colonise and cause disease. It is not the presence of pathogenic bacteria that causes disease, but rather whether they proliferate to an extent that overwhelms the commensal microbial population [32]. In the present study, treating piglets with FMT post weaning resulted in a significant increase in faecal microbiota diversity at both 24 and 35 days of age. This increase in diversity likely indicates that the piglets within the FMT treatment may have an improved ability to cope with and adapt to the challenges associated with weaning. Indeed, this was corroborated by the observation of an increase in bacterial diversity and improvements in goblet cell mucin stores, and a reduced necrotising enterocolitis incidence in those pigs treated with FMT during the first few days of life [34]. Furthermore, Geng et al., [35] observed improvements in bacterial diversity and a reduced susceptibility to epithelial injury in those piglets treated with FMT for the first 14 days of life. Both studies demonstrated the positive implications increased microbial diversity can have on intestinal barrier function.

The gastrointestinal tract (GIT) microbiota undergoes dysbiosis post weaning, which is a leading cause of increased GIT permeability and PWD, both of which are associated with an increase in mortality and a reduction in feed intake and growth in pigs, known as a post-weaning growth check [1]. In common with the prior literature, the pigs within this study also showed a post-weaning growth check irrespective of treatment. Although the treatment did not improve body weight in the present study, it provided no hinderance either. It is possible that the increase in diversity caused by the FMT provided benefits to the piglets that were not observable with the current animal numbers. Average daily gain was reduced in FMT-treated animals at 24 days of age (four days post FMT); however, this was somewhat expected as the piglets receiving the FMT would have had to undergo some adjustment to the rapidly changing GIT microbiota. Incidence of PWD did not differ between treatments at this time either, while scratch score was reduced in FMT-treated pigs, indicating no negative effect on the GIT and a reduction in piglet fighting. Fighting at weaning is common and can add to the stress associated with the weaning event; therefore, the fact that the treatment reduced this may provide additional benefits. Studies have indicated that behaviour is influenced via the gastrointestinal microbiota, so its alteration via FMT may partially explain the outcome observed [36]. By 35 days of age, there was no significant difference in ADG between treatments. As no differences in weight were observed at any time-point, it indicates no long-lasting negative effect of FMT on piglet growth. The present study differs from previous studies in that FMT was conducted post weaning and

piglets were only dosed once. Earlier works [17,37] observed positive implications for piglet health by demonstrating both an improvement in average daily gain and a reduction in diarrhoea incidence during lactation when multiple FMTs were administered to piglets in early life. It is likely that no effect on the incidence of diarrhoea was observed in the present study because the animals were weaned into pens of 21 piglets rather than the large numbers often seen in commercial production and as such, the incidence of diarrhoea was relatively low in our study.

Escherichia coli is the primary infectious agent of PWD in piglets [1] and, although the piglets treated with FMT in the present study had a higher abundance of *E. coli* in their faeces at 24 and 35 days of age, no difference in diarrhoea incidence was observed. Not all strains of *E. coli* are pathogenic, with some having probiotic qualities [38,39], and as 16S rRNA amplicon analysis does not distinguish between strains, it was not possible to determine whether the *E. coli* observed in the present study were beneficial or potentially pathogenic. The presence of *E. coli* in this instance may have provided benefits to the microbiota stability, as a reduction in serotype diversity may allow for a pathogenic monoculture to develop rather than a beneficial one. Additionally, as previously mentioned, infection is not only due to the presence of a pathogenic bacteria but occurs when numbers proliferate to an extent that overwhelms the commensal microbial population. Therefore, it is likely that if the *E. coli* present included a pathogenic strain, the potential pathogenic nature of the bacteria may have been negated by the presence of other bacteria. For example, *Lactobacillus mucosae* was one bacterium that was more abundant in FMT-treated animals at 24 and 35 days of age. *Lactobacillus mucosae* is known for its ability to competitively attach to the epithelium of the intestine, produce antimicrobials and inhibit pathogenic bacteria and, therefore, could have provided additional benefit in preventing the potential pathogenic properties of *E. coli* [40].

Those animals receiving FMT also had an increase in beneficial Fibrobacteres and Bacteroidetes at 35 days of age. Fibrobacteres are known as adept fibre degraders which could provide great benefit to the weaned piglet as weaning introduces a large dietary change from primarily milk to solid food [41]. Therefore, it is not surprising that FMT-treated animals had a higher abundance of Fibrobacteres as it was in the donor faeces given and would have provided these animals with a microbiota more developed for the digestion of solid feed. Additionally, the increased presence of Bacteriodetes in FMT-treated piglets at 35 days of age may have also provided benefits to piglets; a higher relative abundance of Bacteroidetes in healthy pigs after weaning compared with those that developed diarrhoea has been observed [42]. Additionally, control animals had a higher relative abundance of *Prevotella* at 24 days of age, which may explain the improvements in average daily gain observed at this age. Previous studies have documented *Prevotella* to have a positive correlation with body weight [43]. However, by 35 days of age, no difference in average daily gain was observed between treatments, while *Prevotella* remained more abundant in control animals. Interestingly, the donor faeces contained large amounts of *Prevotella,* therefore the opposite would have been expected. *Treponema*, a potentially pathogenic bacteria which is associated with swine dysentery [44] was present in higher amounts in control piglets at 35 days of age, which may have reduced the benefits of *Prevotella*. These results demonstrate the importance of community composition rather than the presence or absence of any particular bacteria.

Recently, studies investigating the use of FMT in pigs for the improvement of health and production outcomes have increased in number. However, current techniques can be quite stressful for the pig as they involve administration of multiple faecal doses over a number of days and use interventions to reduce gastric acid in order to improve post-gastric bacterial survival. The results from this study are the first to indicate that FMT is successful if done post weaning and that minimal intervention is needed in order to influence the microbiota of piglets. Only two previous studies administered a single dose, as opposed to the multi-dose approach usually implemented, and these studies were conducted during lactation. McCormack et al., [45] administered a dose at birth and observed a negative impact on growth and an altered faecal microbiota, while our research group administered a single dose at 7 days of age and saw no effect on the faecal microbiota [46]. This is likely because the GIT of a newly born piglet is relatively immature and hence would be susceptible to colonisation, while a 7-day-old piglet would have bacteria already colonised which would compete directly with the FMT. Our data indicate that weaning caused a large enough disruption

of the microbiota to enable the colonisation of new bacteria, as is likely the cause of PWD. While it makes for an optimal time for pathogenic bacterial colonisation, it also offers an appropriate time to implement a treatment targeted at influencing positive microbiota development, such as FMT.

When assessing the effect of weaning on the microbiota of piglets, it is evident that diversity does not increase linearly with age. In the present study, Shannon's diversity and the number of taxa increased from weaning to 2 days post weaning and, thereafter, by 35 days decreasing to levels similar to those observed on day 18, while evenness increased steadily from 18 to 35 days of age. The reduction in bacterial diversity observed post weaning is likely the result of dietary and environmental changes induced by weaning. Similarly, an earlier review documented weaning's negative impact on the GIT microbiota and its impact on alterations to the resident bacteria [47]. Therefore, the change in microbiota we observed may be due to GIT stabilisation during the post-weaning period. Hence, it is probable that the increase in diversity observed at 20 days of age are when the bacteria needed for milk and solid feed digestion coexist. Thereafter, the microbiota required for the digestion of solid feed remain while those bacteria required for milk digestion decline, resulting in a drop in diversity. This GIT stabilisation can also be observed when looking at the variability between piglets at each age stage. It is evident that as age increases, the variability between piglets decreases. This is to be expected as the influence of the sow is removed at weaning and all piglets are housed in the same environment on the same feed.

5. Conclusions

Our findings document that FMT significantly affected the piglet's microbiota post weaning and reduced the scratch scores observed at 24 days of age. As such, our data suggest that administering FMT after weaning from a healthy donor piglet that is 4 weeks older is an effective tool in altering the microbiota in piglets. Whether this change is transient or stable cannot be determined within the current study design; however, the aim of the study was to identify whether FMT could cause a change that would positively impact the pig within the period where they are most at risk to enteric dysbiosis post weaning. Hence, further studies are required to elucidate the durability of the effect of FMT beyond 14 days and to determine whether FMT has any long-lasting production outcomes. To our knowledge, this is the first study to document a change in the microbiota of piglets after a single FMT post weaning. The findings from this study provide valuable information for the development of future work investigating FMT in the post weaning period.

Author Contributions: T.L.N., R.N.K., M.D.B., K.J.P., V.A.T. and W.Y.L. conceptualised the experiment. T.L.N. organised and ran the experiment. T.L.N. and R.N.K. collected the faecal samples and performed the faecal transplant. V.A.T. and T.L.N. undertook pre-nucleic acid sample preparation. T.L.N., V.A.T., K.J.P. and W.Y.L. conducted the statistical analysis. T.L.N. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This work received no specific grant from any funding agency in the public, commercial or not-for-profit sectors, however T. Nowland was supported by Australian Pork Limited and the University of Adelaide conducted the study.

Acknowledgments: The authors wish to thank Sophia Ward, Justin Ayris, Jessica Zemitis and Bryony Tucker for their expert technical assistance.

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

References

1. Gresse, R.; Chaucheyras-Durand, F.; Fleury, M.A.; Van de Wiele, T.; Forano, E.; Blanquet-Diot, S. Gut microbiota dysbiosis in postweaning piglets: Understanding the keys to health. *Trends Microbiol.* **2017**, *25*, 851–873. [CrossRef]
2. Boudry, G.; Péron, V.; Huërou-Luron, I.L.; Lallès, J.P.; Sève, B. Weaning induces both transient and long-lasting modifcations of absorptive, secretory, and barrier properties of piglet intestine. *Nutr. Metab.* **2004**, *134*, 2256–2262.

3. Pluske, J.R.; Kim, J.C.; Hansen, C.F.; Mullan, B.P.; Payne, H.G.; Hampson, D.J.; Callesen, J.; Wilson, R.H. Piglet growth before and after weaning in relation to a qualitative estimate of solid (creep) feed intake during lactation: A pilot study. *Arch. Anim. Nutr.* **2007**, *61*, 469–480. [CrossRef]
4. Dong, X.; Xu, Q.; Wang, C.; Zou, X.; Lu, J. Supplemental-coated zinc oxide relieves diarrhoea by decreasing intestinal permeability in weanling pigs. *J. Appl. Anim. Res.* **2019**, *47*, 362–368. [CrossRef]
5. Reid, G.; Friendship, R. Alternatives to antibiotic use: Probiotics for the gut. *Anim. Biotechnol.* **2002**, *13*, 97–112. [CrossRef]
6. Jensen, J.; Larsen, M.M.; Bak, J. National monitoring study in denmark finds increased and critical levels of copper and zinc in arable soils fertilized with pig slurry. *Environ. Pollut.* **2016**, *214*, 334–340. [CrossRef] [PubMed]
7. Slifierz, M.J.; Friendship, R.; Weese, J.S. Zinc oxide therapy increases prevalence and persistence of methicillin-resistant staphylococcus aureus in pigs: A randomized controlled trial. *Zoonoses Public Health* **2015**, *62*, 301–308. [CrossRef]
8. Abeles, S.R.; Pride, D.T. Molecular bases and role of viruses in the human microbiome. *J. Mol. Biol.* **2014**, *426*, 3892–3906. [CrossRef]
9. Hallen-Adams, H.E.; Suhr, M.J. Fungi in the healthy human gastrointestinal tract. *Virulence* **2017**, *8*, 352–358. [CrossRef]
10. Gensollen, T.; Iyer, S.S.; Kasper, D.L.; Blumberg, R.S. How colonization by microbiota in early life shapes the immune system. *Science* **2016**, *352*, 539–544. [CrossRef]
11. Ruczizka, U.; Metzler-Zebeli, B.; Unterweger, C.; Mann, E.; Schwarz, L.; Knecht, C.; Hennig-Pauka, I. Early parenteral administration of ceftiofur has gender-specific short and long-term effects on the fecal microbiota and growth in pigs from the suckling to growing phase. *Animals* **2019**, *10*, 17. [CrossRef]
12. Niederwerder, M.C.; Constance, L.A.; Rowland, R.R.R.; Abbas, W.; Fernando, S.C.; Potter, M.L.; Sheahan, M.A.; Burkey, T.E.; Hesse, R.A.; Cino-Ozuna, A.G. Fecal microbiota transplantation is associated with reduced morbidity and mortality in porcine circovirus associated disease. *Front. Microbiol.* **2018**, *9*, 1631. [CrossRef] [PubMed]
13. Sangild, P.T.; Siggers, R.H.; Schmidt, M.; Elnif, J.; Bjornvad, C.R.; Thymann, T.; Grondahl, M.L.; Hansen, A.K.; Jensen, S.K.; Boye, M.; et al. Diet- and colonization-dependent intestinal dysfunction predisposes to necrotizing enterocolitis in preterm pigs. *Gastroenterology* **2006**, *130*, 1776–1792. [CrossRef] [PubMed]
14. Cahenzli, J.; Köller, Y.; Wyss, M.; Geuking, M.B.; McCoy, K.D. Intestinal microbial diversity during early-life colonization shapes long-term ige levels. *Cell Host Microbe* **2013**, *14*, 559–570. [CrossRef]
15. Bakken, J.S.; Borody, T.; Brandt, L.J.; Brill, J.V.; Demarco, D.C.; Franzos, M.A.; Kelly, C.; Khoruts, A.; Louie, T.; Martinelli, L.P.; et al. Treating clostridium difficile infection with fecal microbiota transplantation. *Clin. Gastroenterol. Hepatol.* **2011**, *9*, 1044–1049. [CrossRef] [PubMed]
16. Brandt, L.J.; Aroniadis, O.C. An overview of fecal microbiota transplantation: Techniques, indications, and outcomes. *Gastrointest. Endosc.* **2013**, *78*, 240–249. [CrossRef]
17. Hu, L.S.; Geng, S.J.; Li, Y.; Cheng, S.S.; Fu, X.F.; Yue, X.J.; Han, X.Y. Exogenous fecal microbiota transplantation from local adult pigs to crossbred newborn piglets. *Front. Microbiol.* **2018**, *8*, 2663. [CrossRef]
18. Seyfang, J.; Plush, K.J.; Kirkwood, R.N.; Tilbrook, A.J.; Ralph, C.R. The sex ratio of a litter affects the behaviour of its female pigs until at least 16 weeks of age. *Appl. Anim. Behav. Sci.* **2018**, *200*, 45–50. [CrossRef]
19. Stirling, G.R.; Griffin, D.; Ophel-Keller, K.; McKay, A.; Hartley, A.; Curran, J.; Stirling, A.M.; Monsour, C.; Winch, J.; Hardie, B. Combining an initial risk assessment process with DNA assays to improve prediction of soilborne diseases caused by root-knot nematode (meloidogyne spp.) and fusarium oxysporum f. Sp. Lycopersici in the queensland tomato industry. *Australas. Plant Path.* **2004**, *33*, 285–293. [CrossRef]
20. Torok, V.A.; Ophel-Keller, K.; Loo, M.; Hughes, R.J. Application of methods for identifying broiler chicken gut bacterial species linked with increased energy metabolism. *Appl. Environ. Microbiol.* **2008**, *74*, 783–791. [CrossRef]
21. Torok, V.A.; Percy, N.J.; Moate, P.J.; Ophel-Keller, K. Influence of dietary docosahexaenoic acid supplementation on the overall rumen microbiota of dairy cows and linkages with production parameters. *Can. J. Microbiol.* **2014**, *60*, 267–275. [CrossRef]
22. Zhang, J.; Kobert, K.; Flouri, T.; Stamatakis, A. Pear: A fast and accurate illumina paired-end read merger. *Bioinformatics* **2014**, *30*, 614–620. [CrossRef] [PubMed]

23. Caporaso, J.G.; Kucztnski, J.; Stombaugh, J.; Bittinger, K.; Bushman, F.D.; Costello, E.K.; Fierer, N.; Peña, A.G.; Goodrich, J.K.; Gordon, J.I.; et al. Qiime allows analysis of high-throughput community sequencing data. *Nat. Methods* **2010**, *7*, 335–336. [CrossRef] [PubMed]
24. Edgar, R.C. Search and clustering orders of magnitude faster than blast. *Bioinformatics* **2010**, *26*, 2460–2461. [CrossRef] [PubMed]
25. Edgar, R.C.; Haas, B.J.; Clemente, J.C.; Quince, C.; Knight, R. Uchime improves sensitivity and speed of chimera detection. *Bioinformatics* **2011**, *27*, 2194–2200. [CrossRef] [PubMed]
26. Edgar, R.C. Uparse: Highly accurate otu sequences from microbial amplicon reads. *Nat. Methods* **2013**, *10*, 996–998. [CrossRef]
27. DeSantis, T.Z.; Hugenholtz, P.; Larsen, N.; Rojas, M.; Brodie, E.L.; Keller, K.; Huber, T.; Dalevi, D.; Hu, P.; Andersen, G.L. Greengenes, a chimera-checked 16s rrna gene database and workbench compatible with arb. *Appl. Environ. Microbiol.* **2006**, *72*, 5069–5072. [CrossRef]
28. Bray, J.R.; Curtis, J.T. An ordination of the upland forest communities of southern wisconsin. *Ecol. Monogr.* **1957**, *27*, 325–349. [CrossRef]
29. Clarke, K.R. Non-parametric multivariate analyses of changes in community structure. *J. Ecol.* **1993**, *18*, 117–143. [CrossRef]
30. Kruskal, J.B. Multidimensional scaling by optimizing a goodness of fit to a nonmetric hypothesis. *Phsychometrics* **1964**, *29*, 1–28. [CrossRef]
31. Shepard, R.N. The analysis of proximities: Multidimensional scaling with an unknown distance function. I. *Psychometrika* **1962**, *27*, 125–140. [CrossRef]
32. Pluske, J.R.; Turpin, D.L.; Kim, J.-C. Gastrointestinal tract (gut) health in the young pig. *Anim. Nutr.* **2018**, *4*, 187–196. [CrossRef] [PubMed]
33. Nowland, T.L.; Plush, K.J.; Barton, M.; Kirkwood, R.N. Development and function of the intestinal microbiome and potential implications for pig production. *Animals* **2019**, *9*, 76. [CrossRef] [PubMed]
34. Brunse, A.; Martin, L.; Rasmussen, T.S.; Christensen, L.; Skovsted Cilieborg, M.; Wiese, M.; Khakimov, B.; Pieper, R.; Nielsen, D.S.; Sangild, P.T.; et al. Effect of fecal microbiota transplantation route of administration on gut colonization and host response in preterm pigs. *ISME J.* **2019**, *13*, 720–733. [CrossRef] [PubMed]
35. Geng, S.; Cheng, S.; Li, Y.; Wen, Z.; Ma, X.; Jiang, X.; Wang, Y.; Han, X. Faecal microbiota transplantation reduces susceptibility to epithelial injury and modulates tryptophan metabolism of the microbial community in a piglet model. *J. Crohns. Colitis* **2018**, *12*, 1359–1374. [CrossRef]
36. Sylvia, K.E.; Demas, G.E. A gut feeling: Microbiome-brain-immune interactions modulate social and affective behaviors. *Horm. Behav.* **2018**, *99*, 41–49. [CrossRef]
37. Cheng, C.S.; Wei, H.K.; Wang, P.; Yu, H.C.; Zhang, X.M.; Jiang, S.W.; Peng, J. Early intervention with faecal microbiota transplantation: An effective means to improve growth performance and the intestinal development of suckling piglets. *Animal* **2018**, *13*, 533–541. [CrossRef]
38. Grabig, A.; Paclik, D.; Guzy, C.; Dankof, A.; Baumgart, D.C.; Erckenbrecht, J.; Raupach, B.; Sonnenborn, U.; Eckert, J.; Schumann, R.R.; et al. Escherichia coli strain nissle 1917 ameliorates experimental colitis via toll-like receptor 2- and toll-like receptor 4-dependent pathways. *Infect. Immunol.* **2006**, *74*, 4075–4082. [CrossRef] [PubMed]
39. Grozdanov, L.; Raasch, C.; Schulze, J.; Sonnenborn, U.; Gottschalk, G.; Hacker, J.; Dobrindt, U. Analysis of the genome structure of the nonpathogenic probiotic escherichia coli strain nissle 1917. *J. Bacteriol.* **2004**, *186*, 5432–5441. [CrossRef]
40. Lee, J.H.; Valeriano, V.D.; Shin, Y.R.; Chae, J.P.; Kim, G.B.; Ham, J.S.; Chun, J.; Kang, D.K. Genome sequence of lactobacillus mucosae lm1, isolated from piglet feces. *J. Bacteriol.* **2012**, *194*, 4766. [CrossRef]
41. McCormack, U.M.; Curiao, T.; Metzler-Zebeli, B.U.; Wilkinson, T.; Reyer, H.; Crispie, F.; Cotter, P.D.; Creevey, C.J.; Gardiner, G.E.; Lawlor, P.G. Improvement of feed efficiency in pigs through microbial modulation via fecal microbiota transplantation in sows and dietary supplementation of inulin in offspring. *Appl. Environ. Microbiol.* **2019**, *85*, 1–18. [CrossRef] [PubMed]
42. Dou, S.; Gadonna-Widehem, P.; Rome, V.; Hamoudi, D.; Rhazi, L.; Lakhal, L.; Larcher, T.; Bahi-Jaber, N.; Pinon-Quintana, A.; Guyonvarch, A.; et al. Characterisation of early-life fecal microbiota in susceptible and healthy pigs to post-weaning diarrhoea. *PLoS ONE* **2017**, *12*, e0169851. [CrossRef] [PubMed]

43. McCormack, U.M.; Curiao, T.; Buzoianu, S.G.; Prieto, M.L.; Ryan, T.; Varley, P.; Crispie, F.; Magowan, E.; Metzler-Zebeli, B.U.; Berry, D.; et al. Exploring a possible link between the intestinal microbiota and feed efficiency in pigs. *Appl. Environ. Microbiol.* **2017**, *83*, 1–16. [CrossRef]
44. Rees, A.S.; Lysons, R.J.; Stokes, C.R.; Bourne, F.J. Antibody production by the pig colon during infection with treponema hyodyseneriae. *Res. Vet. Sci.* **1989**, *47*, 263–269. [CrossRef]
45. McCormack, U.M.; Curiao, T.; Wilkinson, T.; Metzler-Zebeli, B.U.; Reyer, H.; Ryan, T.; Calderon-Diaz, J.A.; Crispie, F.; Cotter, P.D.; Creevey, C.J.; et al. Fecal microbiota transplantation in gestating sows and neonatal offspring alters lifetime intestinal microbiota and growth in offspring. *Am. Soc. Microbiol.* **2018**, *3*. [CrossRef]
46. Nowland, T.L.; Torok, V.A.; Low, W.Y.; Barton, M.D.; Plush, K.J.; Kirkwood, R.N. Faecal microbiota analysis of piglets during lactation. *Animals* **2020**, *10*, 762. [CrossRef]
47. Lallès, J.-P.; Bosi, P.; Smidt, H.; Stokes, C.R. Weaning—Achallenge to gut physiologists. *Livest. Sci.* **2007**, *108*, 82–93. [CrossRef]

Chapter 8: Discussion

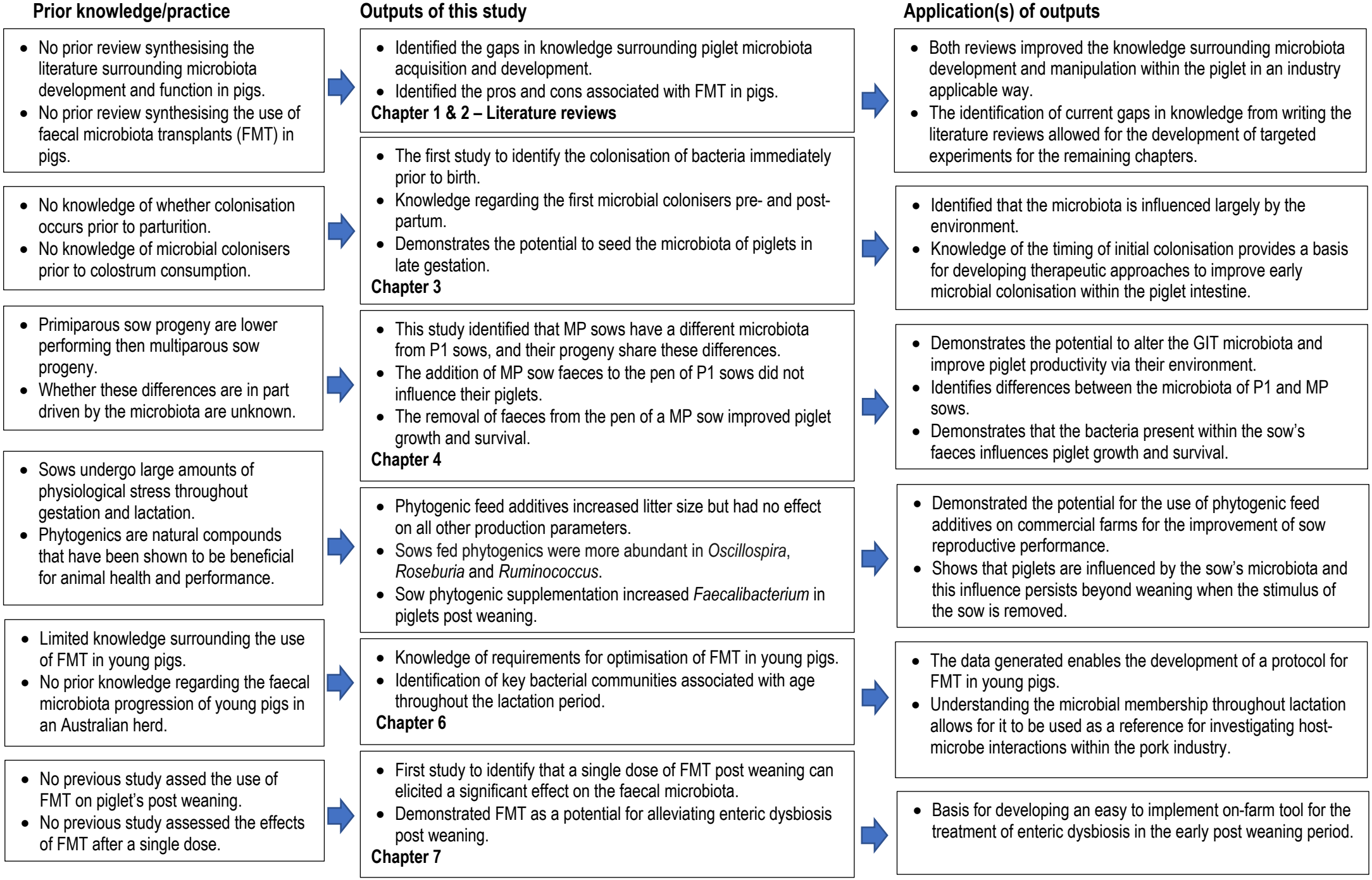
These studies addressed factors influencing the colonisation and development of the gastrointestinal microbiota in piglets, primarily within the lactation period. The overall hypothesis for this thesis was that enteric microbiota acquisition in piglets occurs in late gestation and during the immediate post-natal period from seeding by the sow. This, then, would allow for the piglet's gastrointestinal tract (GIT) microbiota to be influenced via the addition of a treatment either through the environment or the sow. The approaches used to address this hypothesis included: (1) investigating the GIT microbiota of piglets at different life stages, (2) investigating the impact of environmental manipulation and the sow on the piglets GIT microbiota, and (3) investigating different methods for altering the GIT microbiota of the piglet directly, to ensure optimal microbial colonisation. The major outputs of this thesis and their potential applications are summarised in Figure 1 and described below.

It is well established that initial microbial colonisers play a determinant role in the health of their host, with studies demonstrating that dysbiosis during early life can have negative health consequences (Rautava *et al.*, 2012, Nowland *et al.*, 2019). As a result, research into identifying and understanding when initial microbial colonisation occurs and what the first colonisers are has expanded, with the idea that this information can assist in the development of methods that ensure colonisation is beneficial. Studies in mice (Younge *et al.*, 2019), rhesus macaques (Chu *et al.*, 2017) and humans (Collado *et al.*, 2016) indicate possible colonisation of the GIT tract *in utero*. Even with these findings, this is still a topic of debate with some studies refuting these claims (Perez-Munoz *et al.*, 2017, De Goffau *et al.*, 2019). However, no research to date has investigated this in pigs and none exists surrounding the initial microbial colonisers prior to colostrum ingestion (see literature review, Chapter 1.2). Therefore, the aim of Chapter 3 was to determine the GIT colonisation before and after birth in piglets that had or had not sucked. Chapter 3 reports on microbial colonisation in the spiral colon of stillborn piglets suggesting that colonisation occurs in the piglet at least immediately prior to parturition. Additionally, Chapter 3 identified the main microorganisms colonising the spiral colon within the hours proceeding birth, demonstrating rapid and diverse microbiota colonisation with exposure to the environment and sow colostrum. These

findings identify that the GIT microbiota of piglets is largely influenced by the microorganisms present in the environment, not just the ingestion of colostrum. Therefore, providing the basis for developing approaches to improving early microbial colonisation within the piglet that involved both manipulation of the environment (Chapter 4) and through the sow (Chapter 5).

Recent research into environmental microbial exposure in the days after birth revealed that exposure to sow faeces in the first 10 days of life improves white blood cell counts by 25% and increases feed intake and weight gain (Aviles-Rosa *et al.*, 2019). Unfortunately, this study did not explore the GIT microbiota of the piglets so a connection with GIT microbiota can only be speculated. The findings of Chapter 3 further corroborate those of Aviles-Rosa *et al.* (2019) by confirming the importance of the sow and environment on microbial colonisation of the GIT in piglets. Additionally, given that primiparous (P1) sow progeny are born lighter, remain lighter throughout each phase of production and have a higher mortality rate than multiparous (MP) sow progeny (Craig *et al.*, 2017), it was pertinent to investigate the cause of these differences and whether microbial exposure from the environment (e.g. sow faeces) was a driver for superior performance in MP sow progeny. Additionally, as piglets are housed within a pen exclusively with their sow, environmental manipulation would be an easy to implement strategy for the pork industry. Hence, Chapter 4 investigated the effect of sow faeces present within the environment, however, given the previous knowledge surrounding the differences between MP and P1 sows, treatments were applied that involved the movement of faecal material from MP sows to P1 sow pens. This was done with the aim of determining whether contact with faeces from an older parity sow could improve growth and survival of piglets reared on P1 sows and if so, whether these differences were associated with GIT microbiota. The findings of this study demonstrated that faecal microbiota populations differed between sows of different parities, supporting the findings of Gaukroger *et al.* (2020b) who observed sow microbiota differences between parities throughout gestation and lactation. Additionally, faecal transfer from MP sows provided no benefit or hinderance to piglets born and reared on P1 sows, while the removal of faeces from the pen for the first 10 days was beneficial. Piglets housed in a pen that was cleaned daily for the first 10 days of life exhibited better growth, survival and an altered faecal microbiota then those exposed to faeces.

Figure 1. Major outputs from this study and their potential applications.



Additionally, this study demonstrated that MP and P1 sows not only had differing faecal microbiota, but MP sows had a higher abundance of potentially pathogenic bacteria, *Escherichia, Clostridium, Campylobacter* and *Treponema*, while P1 sows had a higher abundance of potentially beneficial bacteria *Lactobacillus* and *Prevotella*. However, despite this and similar to other studies, the growth and survival of P1 progeny was consistently lower than MP sow progeny. Chapter 4 demonstrates the potential to alter a piglet's microbiota and improve piglet productivity through the environment while also highlighting the importance of the sow's microbiota.

After the findings of Chapters 3 and 4, the next step was to address whether feeding a sow a dietary intervention that has microbiota modulating components could influence the sows' GIT microbiota and whether this change could be transferred to their piglets. The overall aim was to see if sow and piglet performance could be influenced via a dietary additive through the colonisation of beneficial bacteria. Chapter 5 builds on the findings of Chapters 3 and 4 and assesses the inclusion of a dietary additive during gestation and lactation in order to measure the effect on the piglet's faecal microbiota. To assess its commercial relevance this was tested in partnership with industry on a large commercial pig farm.

Sows experience physiological stress throughout gestation and lactation which often leads to reduced feed intake, metabolic stress and enteric dysbiosis, hence impairing sow health and reducing their reproductive potential (Thaker and Bilkei, 2005, Gresse *et al.*, 2017, Cheng *et al.*, 2018). Phytogenic additives (PAs) are natural flavour and sensory compounds derived from plants which have potent antioxidant and anti-inflammatory properties and have been shown to improve animal performance when added to feed (Windisch *et al.*, 2008). However, there is little published evidence on the efficacy of PAs in reproductive performance of the sow and its role in GIT microbiota modulation. Therefore, Chapter 5 aimed to determine whether the provision of a gestation and/or lactation diet containing PAs would alter the GIT microbiota of sows, and thus that of their piglets, and so improve performance. The major finding of Chapter 5 was that the inclusion of a PA throughout gestation increased the number of piglets born and tended to reduce the wean to service interval in sows. These results have major economic

implications for farmers as this could potentially increase the number of piglets born per sow per year and reduce the number of non-productive days per sow.

Other results from Chapter 5 indicate that further research into the use of PAs is required before it can be recommended as a commercial treatment. As although litter size was increased, this did not translate to an increase in the number of piglets born alive in the current study, therefore further investigation is essential. The reduction in piglets born alive was the result of an increase in stillbirth in PA treated sows, however, the reasoning behind why this may have occurred is unclear. Additionally, when investigating the dietary effect on the sow's faecal microbiota it was evident that the addition of a PA to a gestation diet increased the number of potentially beneficial bacteria present. While a combination of potentially pathogenic and beneficial bacteria were observed in the faecal microbiota of piglets reared on sows fed PAs across ages (21 and 35 days). This seemed to cause neither hinderance nor advantage to piglets during lactation, but whether this is the case post weaning could not be determined as weights were not collected beyond weaning. Hence, further work should be conducted investigating the effect of sow dietary PA supplementation on piglet performance post weaning. Additionally, the study finding that piglets are influenced by the sow's microbiota, and that this influence persists beyond weaning when the presence of the sow is removed, is interesting and not widely documented. Overall, Chapter 5 demonstrates the potential for dietary interventions during gestation and lactation to influence both sow and piglet microbiota development and performance, therefore, identifying a potential industry appropriate way of positively influencing both sow and piglet microbiota and subsequent production performance.

Collectively, Chapters 3, 4 and 5 indicate that microbiota establishment in piglets potentially begins prior to parturition and its development is largely influenced by the environment and the sow's microbiota. Although influencing the piglet's microbiota through supplementation of the sow was demonstrated, it is evident that this may not be an ideal method for many farmers, primarily due to the costs associated with a dietary inclusion. Therefore, Chapters 6 and 7 went on to investigate a method for altering the microbiota

of a piglet directly both pre- and post-weaning, with the idea that it could be applied to an “at risk” piglet or those with enteric dysbiosis as an alternative to antimicrobials.

Faecal microbiota transplantation (FMT) has been successfully used for the treatment of *Clostridium difficile* infections in humans and has demonstrated a success rate of >90% in patients with re-occurring *C. difficile* (Bakken *et al.*, 2011). As a result, research in production animals is increasing. While not all results have been positive (McCormack *et al.*, 2018), some success has been demonstrated and FMT has been associated with a reduction in diarrhoea and improvements in average daily gain, intestinal barrier and immune system function in pigs (Xiao *et al.*, 2017, Hu *et al.*, 2018a, Hu *et al.*, 2018b, Cheng *et al.*, 2019). Although these studies demonstrated opportunities for FMT in pigs, they did not address its use in an industry applicable way, with most studies involving the reduction of stomach acid via proton pump inhibitors and administering multiple FMT doses over a prolonged period. Therefore, Chapter 6 aimed to provide a proof of concept for the use of FMT on farm for the treatment of enteric dysbiosis in pigs. This was assessed by the administration of either a fresh or previously frozen dose of FMT to antibiotic treated piglets at 13 days of age. Results reported in Chapter 6 did not demonstrate any potential for therapeutic application of FMT in piglets, however, relevant information regarding its potential application on farm and information relating to microbial membership during the pre-weaning period was gained. Additionally, while Chapter 3 did not go into detail regarding microbial colonisation beyond 1 day of life, Chapter 6 outlined microbiota development up to 18 days. The findings of this study demonstrate the plasticity of the GIT microbiota within the first two weeks of life, suggesting that GIT microbiota stabilisation occurs pre-weaning in the piglet. This somewhat mimics the existing literature in humans which identifies GIT stabilisation to occur around the onset of ingestion of solid food (Palmer *et al.*, 2007, Koenig *et al.*, 2011). Furthermore, piglets are known to exhibit coprophagy (Chapter 4; Horrell and Hodgson, 1992, Aviles-Rosa *et al.*, 2019) and begin consuming creep feed as early as two weeks of age (Wattanakul *et al.*, 2005).

The findings of Chapter 6 also identified other considerations that should be taken into account when performing FMT in pigs. Some reasons why treatment with FMT was unsuccessful in Chapter 6 include the possibility that the piglets were too young at the time of treatment administration, the donor used was a similar age so may not have had a faecal microbiota that was different enough from the piglets to observe a difference post FMT, and the antibiotic used was ineffective at disrupting the GIT microbiota, so no dysbiosis was present to correct. As a result of this, Chapter 7 went on to identify the application of FMT in older pigs, during the immediate post weaning period, when piglets are naturally at the greatest risk for enteric dysbiosis (Pluske *et al.*, 2018).

Chapter 7 demonstrated that a single FMT dose post weaning was sufficient to cause a change within the GIT microbiota of piglets. This is the first study to show that a single FMT dose can cause an effect in pigs. Additionally, FMT post weaning facilitated the colonisation of potentially pathogenic *Escherichia coli* and potentially beneficial bacteria *Lactobacillus mucosae* and genera *Fibrobacteres* and *Bacteroidetes*. Even though the donor chosen would influence this, further studies should investigate its use on larger animal numbers to evaluate the inferences on production characteristics, such as growth and survival, to identify potential advantages on a commercial scale.

By combining the results from all experiments, we can deduce that several factors influence piglet microbiota colonisation and development throughout the lactation period. These studies suggest that microbial colonisation of the piglet occurs at least immediately prior to birth and rapidly evolves in the hours proceeding birth (Chapter 3), while GIT stabilisation occurs around day 14 (Chapter 6) and manipulation via FMT prior to weaning is unsuccessful (Chapter 6) unless the stimulus is continuous for at least the first 10 days and present in early life (Chapter 4 and 5). Additionally, these changes can persist to at least two weeks post weaning (Chapters 5), however, if a treatment is applied to the piglet during the immediate post weaning period, prolonged exposure to the stimulus does not have to occur to cause a change (Chapter 6). This supports the overall hypothesis for this thesis, that enteric microbiota acquisition in piglets is initiated in late gestation and evolves rapidly during the immediate post-natal

period from seeding by the sow. This, then, would allow for the piglet's GIT microbiota to be influenced via the addition of a treatment either through the environment or the sow. This information allows for further development of industry applicable practices and interventions that enable optimal microbial colonisation in sows and piglets for the improvement of pig health and productivity.

Further research

This study has identified a number of potential topics for follow-on and future research, they include:

Increasing our understanding surrounding when initial colonisation begins is required as although Chapter 3 indicates the presence of bacteria immediately prior to birth, the timing surrounding when this colonisation begins and how it develops needs to be described. Additionally, the role the vaginal and environmental microbiota have on the intestinal colonisers observed is needed in order to understand the presence of the pathogenic bacteria noted in Chapter 3. This can be done by conducting experiments that sample the amniotic fluid of piglets during gestation and immediately post parturition, along with microbiota analysis of the sow's vagina, faeces, udder, colostrum and environment. Allowing for an in-depth identification of the initial microbial colonisers and their origin. This information is important as a more detailed understanding surrounding initial colonisation would allow for the application of more precisely timed interventions that are aimed at ensuring optimal colonisation occurs.

Research documented in Chapters 4-6 identifies practical ways of influencing GIT microbiota development in piglets. However, what impact these environmental and dietary changes implemented during lactation have on piglets post weaning was not evaluated. This is something that should be investigated further as it is well established within the human literature that early life microbial colonisation has implications for long-term health (Cahenzli *et al.*, 2013, Wang *et al.*, 2016, Carlson *et al.*, 2018). Similarly, FMT was only assessed in a preliminary manner within the current thesis and although success was observed in regard to changes in the faecal microbiota, further investigation into its use at a commercial level and its impact on production characteristics such as growth and survival are warranted.

Additionally, research surrounding encapsulation technologies for the FMT should also be conducted to reduce the dosage required and to protect the faecal microbial solution from stomach acid. Ideally, studies should evaluate its efficacy when released within the small intestine not the stomach or via colonic enema in order to determine the correct dose required. The potential for the use of FMT on farm has been highlighted within this thesis (Chapters 6 and 7) and by others (Xiao *et al.*, 2017, Hu *et al.*, 2018a, Hu *et al.*, 2018b, Cheng *et al.*, 2019, McCormack *et al.*, 2019). However, it is clear that more research targeted at refining the technology and assessing its application at a commercial level are now needed.

Conclusions

The experiments conducted as part of this thesis provide knowledge surrounding the colonisation of bacteria in piglets immediately prior to birth through to two weeks post weaning. The impact of environment and sow diet on a piglet's GIT microbiota, and information surrounding FMT in piglets pre- and post-weaning, support my unifying hypothesis. These studies are the first step and provide evidence that will aid the development of a suite of industry applicable practices that enable optimal microbiota development within the piglet through further research at a commercial scale.

Collated references

Abeles SR and Pride DT 2014. Molecular bases and role of viruses in the human microbiome. J Mol Biol 426, 3892-3906.

Arrieta MC, Stiemsma LT, Amenyogbe N, Brown EM and Finlay B 2014. The intestinal microbiome in early life: health and disease. Front Immunol 5, 1-18.

Arrieta MC, Stiemsma LT, Dimitriu PA, Thorson L, Russell S, Yurist-Doutsch S, Kuzeljevic B, Gold MJ, Britton HM and Lefebvre DL 2015. Early infancy microbial and metabolic alterations affect risk of childhood asthma. Sci transl med 7, 1-14.

Aviles-Rosa EO, Rakhshandeh A and McGlone JJ 2019. Preliminary study: Depriving piglets of maternal feces for the first seven days post-partum changes piglet physiology and performance before and after weaning. Animals 9, 1-12.

Bakken JS, Borody T, Brandt LJ, Brill JV, Demarco DC, Franzos MA, Kelly C, Khoruts A, Louie T, Martinelli LP, Moore TA, Russell G and Surawicz C 2011. Treating *Clostridium difficile* infection with fecal microbiota transplantation. Clin Gastroenterol Hepatol 9, 1044-1049.

Bauer E, Williams BA, Smidt H, Verstengen MW and Mosenthin R 2006. Infuence of the gastrointestinal microbiota on development of the immune system in young Animals. Curr issues intestinal microbiol 7, 35-52.

Cahenzli J, Köller Y, Wyss M, Geuking MB and McCoy KD 2013. Intestinal microbial diversity during early-life colonization shapes long-term IgE levels. Cell Host Microbe 14, 559-570.

Carlson AL, Xia K, Azcarate-Peril MA, Goldman BD, Ahn M, Styner MA, Thompson AL, Geng XJ, Gilmore JH and Knickmeyer RC 2018. Infant gut microbiome associated with cognitive development. Biol Psychiatry 83, 148-159.

Cheng C, Wei H, Yu H, Xu C, Jiang S and Peng J 2018. Metabolic syndrome during perinatal period in sows and the link with gut microbiota and metabolites. Front Microbiol 9, 1-13.

Cheng CS, Wei HK, Wang P, Yu HC, Zhang XM, Jiang SW and Peng J 2019. Early intervention with faecal microbiota transplantation: an effective means to improve growth performance and the intestinal development of suckling piglets. Animal 13, 533-541.

Chu D, Prince A, Ma J, Baquero K, Blundell P, Takahashi D, Dean T, Grove K and Aagaard K 2017. Evidence of fetal microbiota and its maternal origins in a non-human primate model. Am J Obstet Gynecol 216, S80.

Collado MC, Rautava S, Aakko J, Isolauri E and Salminen S 2016. Human gut colonisation may be initiated in utero by distinct microbial communities in the placenta and amniotic fluid. Sci Rep 6, 23129.

Craig JR, Collins CL, Bunter KL, Cottrell JJ, Dunshea FR and Pluske JR 2017. Poorer lifetime growth performance of gilt progeny compared with sow progeny is largely due to weight differences at birth and reduced growth in the preweaning period, and is not improved by progeny segregation after weaning. J Anim Sci 95, 4904-4916.

Daigle C 2018. Parallels between postpartum disorders in humans and preweaning piglet mortality in sows. Animals 8, 1-11.

De Goffau MC, Lager S, Sovio U, Gaccioli F, Cook E, Peacock SJ, Parkhill J, Charnock-Jones DS and Smith GCS 2019. Human placenta has no microbiome but can contain potential pathogens. Nature 572, 329-334.

Dominguez-Bello MG, Godoy-Vitorino F, Knight R and Blaser MJ 2019. Role of the microbiome in human development. Gut 68, 1108-1114.

Gaukroger CH, Stewart CJ, Edwards SA, Walshaw J, Adams IP and Kyriazakis I 2020a. Changes in faecal microbiota profiles associated with performance and birthweight of piglets. Front Microbiol 11, 917.

Gaukroger CH, Edwards SA, Walshaw J, Nelson A, Adams IP, Stewart CJ and Kyriazakis I 2020b. Shifting sows: longitudinal changes in the periparturient faecal microbiota of primiparous and multiparous sows. Animal, 1-8.

Gensollen T, Iyer SS, Kasper DL and Blumberg RS 2016. How colonization by microbiota in early life shapes the immune system. Science 352, 539-544.

Gomez-Gallego C, Garcia-Mantrana I, Salminen S and Collado MC 2016. The human milk microbiome and factors influencing its composition and activity. In Semin Fetal Neonat Med 21, 400-405.

Gresse R, Chaucheyras-Durand F, Fleury MA, Van de Wiele T, Forano E and Blanquet-Diot S 2017. Gut microbiota dysbiosis in postweaning piglets: Understanding the keys to health. Trends Microbiol 25, 851-873.

Grice EA and Segre JA 2011. The skin microbiome. Nat Rev Microbiol 9, 244-253.

Hallen-Adams HE and Suhr MJ 2017. Fungi in the healthy human gastrointestinal tract. Virulence 8, 352-358.

Horrell I and Hodgson J 1992. The bases of sow-piglet identification. 2. Cues used by piglets to identify their dam and home pen. App Anim Behav Sci 33, 329-343.

Hu J, Ma L, Nie Y, Chen J, Zheng W, Wang X, Xie C, Zheng Z, Wang Z, Yang T, Shi M, Chen L, Hou Q, Niu Y, Xu X, Zhu Y, Zhang Y, Wei H and Yan X 2018a. A microbiota-derived bacteriocin targets the host to confer diarrhea resistance in early-weaned piglets. Cell Host Microbe 24, 817-832.

Hu LS, Geng SJ, Li Y, Cheng SS, Fu XF, Yue XJ and Han XY 2018b. Exogenous fecal microbiota transplantation from local adult pigs to crossbred newborn piglets. Front Microbiol 8, 1-16.

Koenig JE, Spor A, Scalfone N, Fricker AD, Stombaugh J, Knight R, Angenent LT and Ley RE 2011. Succession of microbial consortia in the developing infant gut microbiome. PNAS USA 108, 4578-4585.

Kuitunen M, Kukkonen K, Juntunen-Backman K, Korpela R, Poussa T, Tuure T, Haahtela T and Savilahti E 2009. Probiotics prevent IgE-associated allergy until age 5 years in cesarean-delivered children but not in the total cohort. J Allergy Clin Immunol 123, 335-341.

Le Doare K, Holder B, Bassett A and Pannaraj PS 2018. Mother's milk: A purposeful contribution to the development of the infant microbiota and immunity. Front Immunol 9, 1-10.

Mach N, Berri M, Estelle J, Levenez F, Lemonnier G, Denis C, Leplat JJ, Chevaleyre C, Billon Y, Dore J, Rogel-Gaillard C and Lepage P 2015. Early-life establishment of the swine gut microbiome and impact on host phenotypes. Environm Microbiol Rep 7, 554-569.

McCormack UM, Curiao T, Metzler-Zebeli BU, Wilkinson T, Reyer H, Crispie F, Cotter PD, Creevey CJ, Gardiner GE and Lawlor PG 2019. Improvement of feed efficiency in pigs through microbial modulation via fecal microbiota transplantation in sows and dietary supplementation of inulin in offspring. App Environ Microbiol 85, 1-18.

McCormack UM, Curiao T, Wilkinson T, Metzler-Zebeli BU, Reyer H, Ryan T, Calderon-Diaz JA, Crispie F, Cotter PD, Creevey CJ, Gardiner GE and Lawlor PG 2018. Fecal microbiota transplantation in gestating sows and neonatal offspring alters lifetime intestinal microbiota and growth in offspring. Am Soc Microbiol 3, 1-20.

Mota-Rojas D, Martinez-Burnes J, Villanueva-Garcia D, Roldan-Santiago P, Trujillo-Ortega ME, Orozco-Gregorio H, Bonilla-Jaime H and Lopez-Mayagoitia A 2012. Animal welfare in the newborn piglet: a review. Veterinarni Medicina 57, 338-349.

Murphy K, O'Shea CA, Ryan CA, Dempsey EM, O'Toole PW, Stanton C and Ross RP 2015. The gut microbiota composition in dichorionic triplet sets suggests a role for host genetic factors. PloS one 10, 1-17.

Nowland TL, Plush KJ, Barton M and Kirkwood RN 2019. Development and function of the intestinal microbiome and potential implications for pig production. Animals 9, 76.

Nuntapaitoon M, Muns R and Tummaruk P 2018. Newborn traits associated with pre-weaning growth and survival in piglets. AJAS 31, 237-244.

Palmer C, Bik EM, DiGiulio DB, Relman DA and Brown PO 2007. Development of the human infant intestinal microbiota. Plos Biology 5, 1556-1573.

Patil Y, Gooneratne R and Ju X-H 2020. Interactions between host and gut microbiota in domestic pigs: a review. Gut Microbes 11, 310-334.

Perez-Munoz ME, Arrieta MC, Ramer-Tait AE and Walter J 2017. A critical assessment of the "sterile womb" and "in utero colonization" hypotheses: implications for research on the pioneer infant microbiome. Microbiome 5, 48.

Pluske JR, Turpin DL and Kim J-C 2018. Gastrointestinal tract (gut) health in the young pig. Anim Nutr 4, 187-196.

Rabhi N, Thibodeau A, Côté J-C, Devillers N, Laplante B, Fravalo P, Larivière-Gauthier G, Thériault WP, Faucitano L, Beauchamp G and Quessy S 2020. Association between tail-biting and intestinal microbiota composition in pigs. Front Vet Sci 7, 1-13.

Rautava S, Luoto R, Salminen S and Isolauri E 2012. Microbial contact during pregnancy, intestinal colonization and human disease. Nat Rev Gastroenterol Hepatol 9, 565-576.

Rea MC SC, Clayton E, O'Connor PM, Whittal RM, Zheng J, Vederas J, Ross RP and Hill C 2010. Thuricin CD, a posttranslationally modified bacteriocin with a narrow spectrum of activity against Clostridium difficile. PNAS USA 107, 9352-9357.

Schachtschneider KM, Yeoman CJ, Isaacson RE, White BA, Schook LB and Pieters M 2013. Modulation of systemic immune responses through commensal gastrointestinal microbiota. PloS one 8, 1-16.

Tang WHW, Kitai T and Hazen SL 2017. Gut microbiota in cardiovascular health and disease. Circ Res 120, 1183-1196.

Thaker MYC and Bilkei G 2005. Lactation weight loss influences subsequent reproductive performance of sows. Anim Repro Sci 88, 309-318.

Wang M, Monaco MH and Donovan SM 2016. Impact of early gut microbiota on immune and metabolic development and function. Semin Fetal Neonat Med 21, 380-387.

Wattanakul W, Bulman CA, Edge HL and Edwards SA 2005. The effect of creep feed presentation method on feeding behaviour, intake and performance of suckling piglets. App Anim Behav Sci 92, 27-36.

Windisch W, Schedle K, Plitzner C and Kroismayr A 2008. Use of phytogenic products as feed additives for swine and poultry. J Anim Sci 86, E140-E148.

Xiao Y, Yan HL, Diao H, Yu B, He J, Yu J, Zheng P, Mao XB, Luo YH and Chen DW 2017. Early gut microbiota intervention suppresses DSS-induced inflammatory responses by deactivating TLR/NLR signalling in pigs. Sci Rep 7, 1-12.

Younge N, McCann JR, Ballard J, Plunkett C, Akhtar S, Araujo-Perez F, Murtha A, Brandon D and Seed PC 2019. Fetal exposure to the maternal microbiota in humans and mice. JCI Insight 4, 1-14.