

**Off to a Flying Start: Effects of Colostrum Oligosaccharides on
Litter Performance and Microbial Dynamics in an *In Vitro* Gut
Model**

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I confirm that the work submitted is my own, except where work which has formed part of jointly authored publications has been included. My contribution and the contribution of other authors to this work has been explicitly indicated below. I confirm that appropriate credit has been given within the thesis where reference has been made to the work of others.

The following publication has been accepted at time of thesis submission:

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Abstract

The pig production industry is under pressure to increase sustainability and efficiency. Hyper-prolific sows now birth over 16 piglets per litter. Unfortunately, increased litter size is positively associated with pre-wean mortality due to increased numbers of low-birth-weight (LBW) piglets, and lack of functional teats, often resulting in use of commercial milk replacer to supplement nutrition provided by the sow. Colostrum, critical to neonate survival, is rich in bioactive components, such as milk oligosaccharides, which have prebiotic and immunomodulatory effects. This thesis aimed to explore concentrations of porcine milk oligosaccharides (PMO) in hyper-prolific sows and their associations with litter performance, before developing an *in vitro* model to screen various PMO combinations and identify the best for supplemental use. The most abundant PMO in sow colostrum was 3'-sialyllactose (3'SL) and litter size was a significant but modest predictor of lacto-N-neotetraose (LNnT) concentration ($R^2 = -0.198$; $P = 0.0143$), with concentration decreasing as litter size increased. This suggests some degree of maternal regulation and that larger litters may benefit from supplementation. Higher concentrations of 2'-fucosyllactose (2'FL) were associated with lower pre-weaning mortality, although this relationship was attenuated following *post-hoc* adjustment ($p = 0.0466$; *post-hoc* $p = 0.435$), indicating that the potential contribution of individual PMOs to piglet survival requires further investigation. Further, methodology was developed to optimise a protocol for *in vitro* fermentation in which PMO combinations could be assessed for..... The results demonstrated that fermentation conditions influenced microbial and metabolite responses, highlighting the importance of model design when evaluating candidate prebiotics. Colostrum fermentation produced a distinct microbial profile, and no PMO treatment was able to influence the community composition beyond that which was produced through fermentation of milk replacer (*Bray-Curtis*; $F = 0.914$; $R^2 = 0.301$; $p = 0.637$). The addition of 3'SL to milk replacer, however, was associated with lower *Streptococcus* and increased *Bacteroides*, demonstrating a more 'colostrum-like' genus profile than other PMO tested. These findings suggest key PMO, namely 3'SL, LNnT and 2'FL, should be explored further and considered as additive prebiotics for piglet supplemental milk to support LBW piglets.

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Abbreviations

2'FL	2'-Fucosyllactose
3'FL	3'-Fucosyllactose
3'GL	3'-Galactosyllactose
3'GL	3'-Galactosyllatose
3'SL	3'-Sialyllactose
6'SL	6'-Sialyllactose
AHDB	Agriculture and Horticulture Development Board
ANCOM-BC	Analysis of Compositions of Microbiomes with Bias Correction
ANOVA	Analysis of Variance
BMO	Bovine Milk Oligosaccharides
BW1	Piglet Bodyweight at Day 1
BW28	Piglet Bodyweight at Day 28
BW7	Piglet Bodyweight at Day 7
CFU	Colony Forming Units
CMB	Complex Mineral Buffer
CO₂	Carbon Dioxide
DEFRA	Department for Environment, Food and Rural Affairs
DiFL	Difucosyllactose
DM	Dry Matter
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic Acid
EFSA	European Food Safety Authority
FDR	False Discovery Rate
FOS	Fructooligosaccharides
Fuc	L-fucose
Gal	D-galactose
Glc	D-glucose
GlcNAc	N-acetylglucosamine
GOS	Galactooligosaccharides

GSL 1	Galactosyllactose 1
GSL 2	Galactosyllactose 2
H₂	Hydrogen
Hex	Hexose
HMO	Human Milk Oligosaccharides
HPLC	High Performance Liquid Chromatography
IL	Interleukin
IUGR	Intra-Uterine Growth Restriction
LBW	Low Birth Weight
LFC	Log Fold Change
LNnH	Lacto-N-neohexaose
LNnT	Lacto-N-neotetraose
LNT	Lacto-N-tetraose
LSTc	Sialyllacto-N-neotetraose c
MCM	Mucin Covered Microcosm
MS	Mass Spectrometry
NeuAc	N-acetylneuraminic acid
NeuGc	N-glycolylneuraminic acid
NMDS	Non-metric Multidimensional Scaling
NMR	Nuclear Magnetic Resonance
NPC	National Pig Centre
OTU	Operational Taxonomic Unit
PBS	Phosphate Buffered Saline
PERMANOVA	Permutational Multivariate Analysis of Variance
pLNnH	Para- lacto-N-neohexaose
RFID	Radio Frequency Identification
RPA	Rural Payments Agency
rRNA	Ribosomal Ribonucleic Acid
SCFA	Short Chain Fatty Acid
SE	Standard Error
SGF	Simulated Gastric Fluid
SIF	Simulated Intestinal Fluid

SLNnH	Sialyl-lacto-N-neohexaose
S-LN-Tri	Sialyl-lacto-N-triose
SOP	Standard Operating Procedure
TLR	Toll-like Receptor
TNF	Tumor Necrosis Factor
UK	United Kingdom
UPLC	Ultra Performance Liquid Chromatography
US	United States
USA	United States of America
UV	Ultraviolet
XOS	Xylooligosaccharides

Chapter 1 General Introduction

1.1 UK Pig Production

The human population is rising at a rapid rate and is expected to continue, with projections of over 10 billion people by 2059 (UN, 2022). This increase in population estimates a global pork demand of 128.9 million metric tons in 2031, which is an 18.4 million increase from the 2019-2021 period (OECD, 2022). While the increase in global demand can be attributed in large to Asia, Europe continues to be the second largest pork market (OECD, 2022) and with pressure rising to meet these increasing demands for pork, it is more important than ever to adopt the most efficient, effective and profitable production styles.

There is reduced pig production forecast in the UK for 2026 (AHDB, 2026a) and while the ideal solution in this period is to increase product price, it is not a likely resolution as increased cost would reduce demand. Instead, imports may increase to cover the absence of UK products and could undercut UK pork prices by a significant margin, due to differences in husbandry and management standards (Batters, 2023). Long-term reliance on imported product might drive UK production down further, or shift home-grown products into exports if there is little incentive to continue producing with costly high welfare standards.

Many agreements, both trade and climate conscious, can be based on ideals but lack input from key stake holders. The Government's Climate Change Committee proposed a reduction in meat consumption of 20–50% in order for the UK to reach net zero by 2050 which is backed by the National Food Strategy which recommends a 30% reduction over a 10 year period (Climate Change Committee, 2020; National Food Strategy, 2021). Recommendations to consumers to reduce meat consumption, paired with pressures on farmers to increase production to avoid being forced into import/export, are confusing at best and could lead to compromised production and welfare. Although often thought of as poor-welfare systems, intensive farming strategies can sometimes be the most carbon efficient option (Gupta, 2014). For example, the policy released by China's Ministry of Agriculture and Rural Affairs allowing high-rise breeding facilitates to be constructed. This was shortly followed by the building of Zhongxin Kaiwei Modern Farming's 26 storey pig facility in Hubei which has the capacity to slaughter 1.2 million pigs per year (Anon, 2022; Wakabayashi and Fu, 2023) with

a bio-gas waste system, converting manure into clean energy. Vertical farming systems could offer an innovative solution to concerns over large area land use while also reducing greenhouse gas emissions (as reviewed by Zhi-cong et al.(2022) and Akintuyi (2024)).

1.1.1 Indoor Production

Approximately 50% of breeding sows in the UK are farmed indoors (NPA, 2024). Indoor pig production systems are often perceived as poorer welfare systems by the consumer, comparative to outdoor systems (Lau et al., 2015), due to lack of environmental stimuli. Indoor systems use farrowing crates to restrict movement of the sow, which can result in inability to express natural farrowing behaviours such as nest building (Swan et al., 2018). This has resulted in much concern for the welfare of the sow when using crates, culminating in some countries, including Norway and Sweden, banning sow confinement (Baxter et al., 2022) and others, such as Austria and Germany, moving to phase out crates over the next 10 years (Goumon et al., 2022).

Farrowing crates are used to minimise sow movement due to the high incidence of crushing injuries and fatalities that occur in piglets during early-life (KilBride et al., 2012; Edwards and Baxter, 2015). KilBride et al (2012) reported that 55% of UK piglet mortality was due to crushing, suggesting the crating systems are necessary to prevent increased piglet mortality.

It is difficult to balance the welfare needs of the sow in addition to those of her piglets. Temporary crating allows for adjustments to be made on an individual level, with stock managers able to lock a sow in, or release her, at any point while housed in the farrowing pens. Temporary crates offer a compromise between restricting sow movement for the entirety of the farrowing and lactating period, as done with traditional crates, and free/loose farrowing, which increases risk to piglets (Glencorse et al., 2019). Over 50% of piglet mortality has been reported to occur in the first four days of life (Marchant et al., 2000) which suggests this is the most critical time for sow movement to be restricted. Work by Radtke et al. (2026) confirmed this when testing optimal crating conditions using temporary crates and loose farrowing. It was concluded that crating sows 24 hours prior to parturition and restricting the sow for four days postpartum was optimal for lowering piglet mortality without compromising sow stress (assessed via faecal

cortisol) or physical health of the sow (assessed by body condition, lameness, lesion and faecal scoring).

1.2 Although temporary crating represents a management strategy to reduce piglet crushing, it does not address further mortality risk factors such as low viability or starvation. Large Litters: Consequences and Interventions

Sows have been selectively bred for reproductive performance (Marantidis et al., 2013), with the average litter size having grown from 10-12 to 14-16 piglets born alive over the past two decades (Devillers et al., 2011; Zhang et al., 2018; AHDB, 2026b). Although this appears to be beneficial from a profit-per-sow perspective, litter size is positively associated with piglet mortality (Canario, Cantoni, et al., 2006; Canario, Roy, et al., 2006; Rosendo et al., 2007).

The mortality rate for pre-wean piglets ranges from 10-20% (KilBride et al., 2012; Edwards and Baxter, 2015; Koketsu et al., 2021), with the Agriculture and Horticulture Development Board (AHDB) reporting average pre-weaning mortality for indoor UK herds to be around 12.5-13% over the last 3 years (AHDB, 2026b). This implies that improvements can be made, to not only maximise profit margins per litter and increase production efficiency, but to address the welfare concerns arising from these high piglet losses.

1.2.1 Consequence: Low Birth Weight Piglets

As number of fetuses *in utero* increases, it is only logical that the average size/weight of each fetus reduces. This effect has been evidenced in multiple reports characterising an increase in litter size, with higher variation in birth weights and increased numbers of low birth weight (LBW) piglets (Quiniou et al., 2002; Mallmann et al., 2019; Peltoniemi et al., 2021). Higher numbers of LBW piglets occur due to factors including intra-uterine crowding and compromised placental efficiency (Rutherford et al., 2013; Ward et al., 2020). Excessively high fetus numbers, often characterised as over 16 (Andersson et al., 2016; Nowak et al., 2024), can also lead to more serious conditions such as intra-uterine growth restriction (IUGR). Piglets suffering from IUGR are characterised by a 'dolphin-like' head shape (Chevaux et al., 2010) and are not likely to survive due to their exceptionally compromised immune and metabolic functions (Hales et al., 2013).

It is recommended that management interventions exclude IUGR piglets and instead focus on non-IUGR, low birth weight (LBW) piglets if trying to efficiently reduce litter mortality rates (Ward et al., 2020).

While there is a consensus that crushing is the leading cause of death in neonatal piglets (Edwards, 2002; Andersen et al., 2011; KilBride et al., 2012), LBW piglets are at a higher risk of suffering from low viability which accounts for 14% of mortalities (KilBride et al., 2012). LBW piglets have poorer thermoregulation (Herpin et al., 2002), therefore struggle greatly with chilling, which can often result in these piglets staying closer to the sow for body heat and thus increasing their chances of being crushed. The LBW piglets also tend to perform poorly in competition for teat access and consequently succumb to a cycle of increased weakness due to lack of nutrition, and lack of nutrition due to reduced fitness and poor performance in teat competition. Furthermore, LBW piglets have compromised growth of the gastrointestinal tract, vital organs, muscle and skeleton (Wang et al., 2005; Morise et al., 2008; Cromi et al., 2009), predisposing them to physical fragility and subsequently increasing the likelihood of fatal crushing incidents. Frequency of scenarios where crushing is likely could also increase, as LBW piglets may struggle to move out of range of the sow with sufficient agility, compared to higher vitality littermates.

Causes of LBW, such as increased litter size and subsequent reduced uterine space, can be exacerbated further by reduced gestation periods (Vanderhaeghe, Dewulf, Ribbens, et al., 2010; Vanderhaeghe, Dewulf, De Vlieghe, et al., 2010; Vanderhaeghe et al., 2011). This will drastically impact the piglets' already lower capacity to achieve maximal development *in utero*. Earlier farrowing with larger litters may be due to increased foetal cortisol levels feeding back to the sow and triggering an early parturition (van Dijk et al., 2005). This is far from ideal as there are reports suggesting the final days of gestation are key for healthy development of the piglets (Leenhouders et al., 2002) and will certainly have a negative impact on the weight at which the piglet is born. There is evidence suggesting use of progestogens such as 'Altrenogest' can prolong the gestation and subsequently ensure maximal development prior to birth (Foisnet et al., 2010; Vanderhaeghe et al., 2011), however this is not yet common practice in industry. The literature surrounding efficacy of Altrenogest is conflicting, with recent reports suggesting positive results when delaying

parturition but some negative side effects including increased still births, reduced colostrum intake, and reduced colostrum IgG concentrations (Taechamaeteekul, Dumniem, Sang-Gassanee, et al., 2022; Taechamaeteekul, Dumniem, Pramul, et al., 2022); the importance of these will be discussed in more detail in later sections. These side effects indicate that dependency on progestogens may not be wise. Furthermore, products licenced for use in the UK that contain Altrenogest are to be used for the synchronisation, suppression, or postponement of oestrus, with guidelines dictating the medicine not be administered to pregnant gilts or sows (DEFRA, 2026). Therefore, reliance on progestogens is not a suitable strategy for increasing birth weights by prolonging gestation, in the UK, and there is still a need for post-natal support, particularly in larger litters. In addition to litter size, parity has also been shown to effect number of LBW piglets born with primiparous sows as well as those at a high parity (> 5) most likely to give birth to a higher proportion of LBW piglets (Milligan et al., 2002; Wijesiriwardana et al., 2019). These sows also have a generally increased variation of birth weights within their litters which has been linked to poorer piglet performance (Moreira et al., 2020) as a large range in body weights can exacerbate competition within the litter, which disproportionally affects LBW piglets (for expansion on this point, see Section 1.2.3).

Furthermore, LBW piglets will find the weaning process more challenging, which could result in increased reliance on antibiotics and increased loss of piglets at this stage if they are not sufficiently robust. Current industry practice in the UK is to abruptly wean piglets at approximately 4 weeks of age but at a minimum of 3 weeks, depending on facility (DEFRA, 2020; RPA, 2020). This abrupt style is considered the most practical for production facilities but does cause piglets to make rapid social and behavioural adjustments, as well as coping with a drastic dietary shift from milk to grains. Weaning does not occur naturally until approximately 10-12 weeks (Moeser et al., 2017), implying that piglets may not be sufficiently developed physiologically at the 3-4 week mark. This is confirmed in numerous reports referring to weaning as 'the largest challenge' in a commercial pig's life, and its negative impact on piglet health and welfare is well documented (Colson et al., 2006; Zhu et al., 2012; Frese et al., 2015; Bian et al., 2016; Yang et al., 2016; Guevarra et al., 2018; Y. Li et al., 2018). Pre-weaning

piglet health, particularly of those with LBW, is therefore of critical importance to increase their robustness and vitality in time to successfully wean.

If LBW piglets survive the challenging first month of life, there are still identifiable markers post-wean and even after slaughter that can identify a LBW piglet (Quiniou et al., 2002; Zeng et al., 2018), including poor reproductive performance when mature (Magnabosco et al., 2016). At best, LBW piglets will require increased days before reaching market weight, with reports of 3-6 extra days required, depending on birth weight (Beaulieu et al., 2010; Jourquin et al., 2016; Lanferdini et al., 2018), and therefore cause increased expense to the farm, requiring extra feed input and taking up valuable finishing space. It is therefore important to intervene at early stages and provide additional support to LBW piglets to increase their chances of thriving and reduce any financial burden they may induce on farm.

Identifying which piglets require intervention could be difficult, with some studies relying on visual assessment to define “non-thriving” piglets (Lee et al., 2023). However, Feldpausch et al. (2019) conducted a pooled, multi-study analysis to propose an objective LBW threshold of 1.11kg (see Figure 1.1).

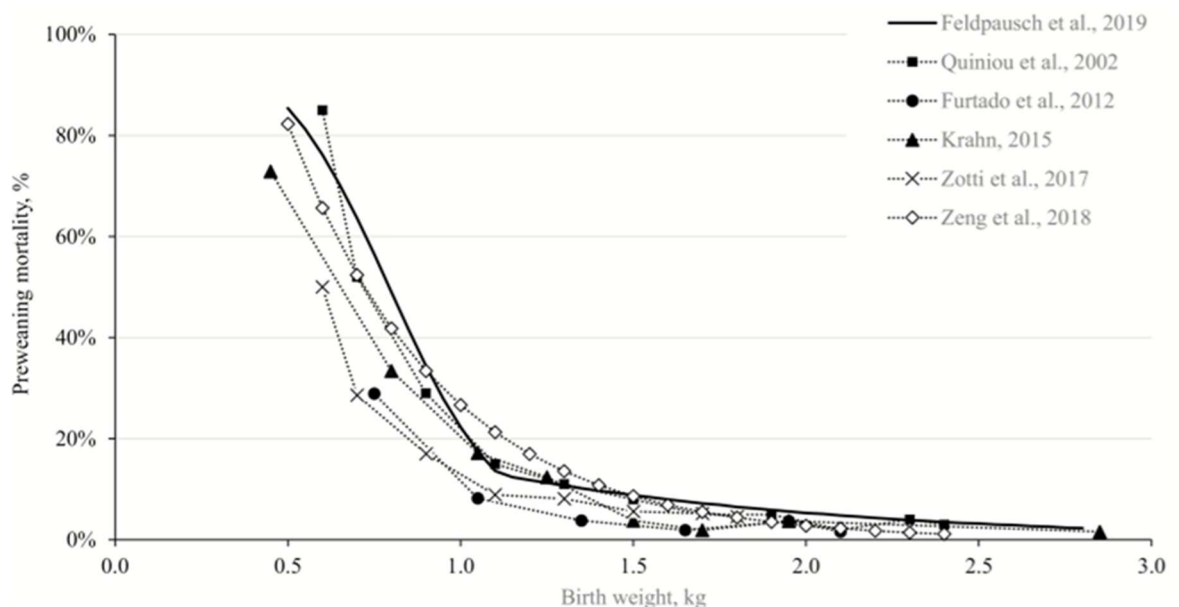


Figure 1.1 Relationship between pre-weaning mortality and piglet birth weight as presented by Feldpausch et al. (2019) where each dotted line denotes results of various studies indicated in the top right key, and the solid line based on predicted values from Feldpausch et al. (2019)

Below this proposed cut-off of 1.11 kg, piglets have a much lower chance of surviving the pre-weaning period (Feldpausch et al., 2019) and it is these

piglets which will most benefit from any management intervention. This threshold supports visual assessment as work by Lee et al. (2023) reported mean weights of “non-thriving” piglets to be between 0.9-1.34kg.

1.2.2 Consequences: Mismatched Functioning Teats

In hyper-prolific sow lines, it is not uncommon for litter sizes to be so large that number of live born piglets exceed the number of functioning teats on the sow (Rohrer and Nonneman, 2017; Icely et al., 2026). This increases the risk of starvation, which has been reported as responsible for 56% of pre-wean mortality in large litters (Kobek-Kjeldager et al., 2020).

Furthermore, sows can undergo a reduction in number of functioning teats when these teats are not being suckled (Theil et al., 2006; Ocepek et al., 2016), due to regression of the unused gland (Hurley, 2019). Gilts could be considered high-risk of teat regression as gilts have been shown to produce smaller litters (Lavery et al., 2019). This was confirmed by the author with internal data showing highly significant difference in both total number of piglets born and number of piglets born alive in gilts, compared to sows of parities 2-6 (Appendix A). When a gilt is supporting a smaller litter, there may be unused teats, potentially increasing risk of supernumerary piglets in future litters due to regression of the non-suckled teats.

Lack of sufficient functional teats is especially important to consider regarding adequate intake of colostrum. Colostrum is the first mammary secretion, highly nutrient dense, and consumption for piglets within the first day of life is critical to neonate survival (Le Dividich et al., 2005; Bailey et al., 2005; Devillers et al., 2011). This is largely because pigs have an epitheliochorial placenta, and so key immune proteins cannot be passed *in utero* (Macdonald and Bosma, 1985). Consequently, piglets must consume colostrum urgently to acquire passive immunity from the sow before development of their own active systems can occur. Additionally, colostrum quantity produced by the sow is not linear to litter size or weight (Devillers et al., 2005; Devillers et al., 2007; Quesnel, 2011), resulting in higher competition for the same limited supply in large litters (Gourley et al., 2020), which is exacerbated further if there are too few functioning teats.

It has been proposed that the reduced fitness seen in LBW piglets may be due, in part, to deficiency in immunoregulatory proteins (Wang et al., 2008) which cements the importance of timely and quality colostrum intake. Concentrations of IgG, seen as the most critical immunoglobulin, decline within the first 24 hours of lactation. This can result in a birth order bias favouring first born piglets who can hypothetically consume the best quality secretions before arrival of competitors. Furthermore, IgG concentration varies across each teat, with reports that anterior teats contain higher concentrations than posterior teats (Passillé et al., 1988; Ogawa et al., 2014). This is particularly relevant because piglets have displayed teat fidelity in previous studies (Rosillon-Warnier and Paquay, 1984; De Passillé et al., 1988), supporting the importance of birth order in accessing sufficient colostrum. Beyond colostrum intake, teat fidelity remains important throughout the suckling period as there is evidence of anterior teats being more productive throughout lactation (Kim et al., 2000). During lactation of mature milk, Devillers et al. (2016) found that piglets were able to identify the more productive teats and competition for these was more fierce. Teat fidelity and preference for anterior teats could also contribute to recession of posterior teats if not suckled from, worsening teat inequality.

1.2.3 Intervention: Cross-fostering

Cross-fostering is the process of moving piglets from the birth sow to another lactating sow to provide milk, and this can often be necessary in very large litters due to piglet number exceeding the number of functioning teats on a sow. Hyperprolific sows are defined as birthing more than 16 piglets due to teat numbers often being maximised at 16 teats (Andersson et al., 2016; Nowak et al., 2024). Select piglets will be moved across to a sow with a smaller birth litter and therefore spare functioning teats. Vande Pol et al. (2021) demonstrated the benefits of this principle, finding that piglet mortality was reduced when litter size was lower than the number of functioning teats, compared to litter size being greater than number of functioning teats.

This is, however, becoming more difficult when the majority of sows are farrowing supernumerary piglets and sometimes, if there are no such sows available in the same batch to take excess piglets, a sow from the previous batch who has just been weaned from will be brought back in as a nurse to suckle the

necessary piglets. This negates some of the benefits of the batch system, which aims to limit disease transfer between batches (Icely, 2024).

It is a significant undertaking of energy for nurse sows, who have been suckled by their birth litters already, to be required to lactate for a further four weeks. This has been demonstrated by Sørensen et al. (2016) who reported these sows exhibited more injury on their legs and udders, in addition to poorer litter hygiene, and increased tendency toward lameness in the piglets (Sørensen et al., 2016). Additionally, the act of cross-fostering can be labour intensive for the farmer and is not always a viable solution depending on ratio of farm staff to livestock. Particularly when this intervention is not as simple as matching number of bodies with number of teats, as success of the strategy has been reported as dependant on weight matching litters (Huting et al., 2017; Vande Pol et al., 2021).

A large standard deviation of weights within a litter has previously been associated with poorer piglet performance and this variation often increases with litter size (Moreira et al., 2020). It can be postulated that an increased variation in birth weights negatively impacts the piglets due to increased competition and results in a more challenging environment, particularly for LBW piglets who are already struggling. For these reasons, it is common practice to match for birth weight when cross-fostering piglets to create litters with more similar weighted piglets. This is of particular importance when dealing with very LBW piglets who are the most at risk as discussed earlier.

It is recommended to select the lowest birth weight piglets (Huting et al., 2017; Vande Pol et al., 2021), from various litters to foster onto a new sow (often a sow that was weaned early from the previous batch) to give the piglets the best chance of survival. Studies that focus on cross-fostering strategies sometimes conclude that mixed birth weight groups can actually be beneficial to some piglets, or that non-uniform litters have no significant impact on performance (Vande Pol et al., 2021). However, it must be considered that piglets of a heavier starting weight will logically thrive more in mixed litters due to the easier competition with lighter littermates. Conversely, while a LBW piglet may not show statistically significant improvement in growth, if the outcome measure is, instead, survival, when it previously would not have been, this becomes a substantial gain for the farm. Huting et al. (2017) have demonstrated this effect where they reported LBW piglets achieved a higher weaning weight when in uniform-low-

weight litters, but heavy piglets perform better in the mixed litter group than the uniform-heavy-weight litters.

It is not necessarily practical to start selectively breeding for smaller litters to increase birth weights and reduce mortality rates, as this requires long term commitment from all stakeholders and likely a complex action plan pushing through new policy in order to be sustained. There are, however, other areas of pig management to be explored as potential strategies for supporting LBW piglets.

1.2.4 Intervention: Supplementary Milk

As has been discussed in previous sections, pressure to increase pig production has led to litter sizes that are too large for the sow to comfortably support herself. The two main forks of this argument are; (1) piglets are being born physically smaller, predisposing them to a more challenging first few weeks of life, and; (2) piglet numbers can exceed the number of functioning teats on the sow, therefore compromising access to critical nutritional support. One strategy to reduce these limitations is the provision of supplemental milk (hereafter, milk replacer) throughout the suckling period to reduce the pressure and energy requirements of the sow and ensure all piglets have access to appropriate nutrition. This can be targeted, for example, to LBW piglets, or can be provided as standard to every litter.

There are multiple successful reports of milk replacer being deployed. Specifically, provision of milk replacer to piglets has been evidenced to reduce pre-weaning mortality rate (Park et al., 2014; Kobek-Kjeldager et al., 2020; Correa et al., 2023) and increase piglet body weights (Volter et al., 2002; Vergauwen et al., 2017), in comparisons to piglets not provided with the milk replacer. Conversely, milk replacer does not always generate enhanced performance. Studies from Miller et al. (2012) and Pustal et al. (2015) reported no effect of milk replacer on piglet mortality rate. Additionally, work by Correa et al. (2023) and Luo et al. (2022) found that the weaning weight of piglets was unaffected by provision of milk replacer.

The inconsistent narrative of milk replacer efficacy could stem from formulation issues. Milk replacer compositions have historically been formulated based on bovine milk (Pieper et al., 2016), with some manufacturers (including

Provimi™, Sav-A-Caf®, and Purina®) marketing milk replacer for use in a wide range of animals, despite compositional differences in milk between species (Hurley, 2015; Roy et al., 2020). Products specifically marketed for neonatal and/or suckling pigs, still often lack an accurate reflection of the composition of sow milk (see Table 1.1), with higher lactose content and low protein and fat. Human breast milk and infant formula are compared in the table below to effectively demonstrate the disparity in accuracy of formula composition.

Table 1.1 Comparative key macronutrient content from example pig and human milks and formula; values given as fed when prepared according to instructions

	Sow milk ¹	Piglet formula ²	Human milk ³	Infant formula ⁴
Protein (%)	4.7-7.1	2.2-3.4	0.9-1.9	1.2-1.3
Fat (%)	5.3-10.8	1.8-3.0	2.1-4.0	3.4-3.7
Lactose (%)	4.0-5.8	6.1-6.9	6.3-7.0	7.0-7.3

¹As taken from 12-15 days lactation (Hurley, 2015)

²DanMilk™ Pro, AB-Neo, Huygelen *et al.* (2012), and Pieper *et al.* (2016)

³As per Roy *et al.* (2020)

⁴Aptamil®, SMA®, and Cow & Gate™ (all First Infant Milks)

Despite some evidence that lower lactose in colostrum increases piglet growth (Szyndler-Nędza et al., 2020), data from a meta-analysis recommended that lactose be dropped from 20% immediately-post-wean to 0% from day 14 post-wean due to the lack of response as lactase activity decreases with age (Zhao et al., 2021). The higher capacity for lactose digestion when younger (particularly when suckling age) may explain why milk replacer is a little higher in lactose compared to what is seen in sow milk (Table 1.1). This may be a strategy to increase energy density when the fat content in formula is so low. It is challenging to achieve such a high fat content in powdered formula, to reflect what is seen naturally, due to the hydrophobic nature of the molecules and the requirement of the powder to be homogenised with water prior to feeding.

High proportions of crude protein and fat, as seen naturally in sow milk (Table 1.1), will promote growth and repair of tissues, support transport of hormones and micronutrients, and promote weight gain. However, sow milk will also provide immune peptides, milk fat globule membranes, and many more bioactive components (Zhang et al., 2018), which are instrumental in supporting

piglet immunity. A summary of some bioactive components found in human and sow milks and their respective functions are displayed in Table 1.2.

Table 1.2 Bioactive components found in human and porcine milks (information summarised from Zhang et al. (2018), Bardanzellu et al. (2020), and Gallo et al. (2024))

Main category	Examples	Main functions
Proteins and bioactive peptides	Lactoferrin, lactalbumin, lactoglobulins, casein-derived peptides	Antimicrobial activity, modulation of immunity, intestinal barrier function, mineral binding; some peptides may influence gut physiology
Immunoglobulins	IgG, IgA, IgM	Passive immune protection; interaction with intestinal pathogens and mucosal immunity
Milk oligosaccharides	Lactose-derived oligosaccharides such as 6'-sialyllactose, 2'-fucosyllactose, lacto-N-neotetraose	Fermentable substrate for intestinal microbiota; potential prebiotic effects; energy supply
Lipids	Phospholipids, short/medium-chain fatty acids, CLA	Energy, membrane structure, signalling, possible effects on inflammation and gut integrity
Milk-derived minerals	Calcium, phosphorus, magnesium, zinc	Bone development, skeletal mineralisation, enzyme/cofactor functions
Vitamins and vitamin-like compounds	Vitamins A, D, B-group vitamins; choline	Growth, metabolism, immune and physiological functions
Growth factors and signalling proteins	IGF-related factors, EGF, TGF- β and others	Intestinal development, epithelial repair and cellular signalling
Enzymes	Lactoperoxidase, lysozyme and other endogenous enzymes	Antimicrobial activity and possible effects on digestion/microbial ecology
Microorganisms	Potentially probiotic organisms associated with milk	Modulation of gut microbiota, competitive exclusion, digestive and immune effects

Common bioactive components added to increase health effects are probiotics and prebiotics (microorganisms and milk oligosaccharides, respectively, as per). As probiotics are microorganisms intentionally added to feed to help stabilise or modify the gut microbiota, they are categorised as feed additives for regulatory purposes by European Food Safety Authority (Anon, 2003). Regulation 1831/2003 specifically includes microorganisms within the

definition of feed additives and requires authorisation before an additive can be placed on the market. This includes species added to piglet milk replacers (see Table 3.2). On the other hand, prebiotics are a substrate used by microorganisms and are classified, instead, as a feed material (EU Regulation 767/2009 (Anon, 2009)). However, the classifications and regulatory status are dependent on intended use of the substrate. Despite pre- and pro- biotics having the same general function (modulation of gut microbiota), their regulatory status differs, leading to probiotics generally requiring consideration as feed additives, whereas prebiotic substrates may be marketed as supplementary feed materials depending on their composition, principal nutritional purpose and intended claims.

The consequences of the lack of these components in piglet milk replacer is evidenced by studies highlighting poorer intestinal morphology (Vergauwen et al., 2017) and reduced immune function (Tang et al., 2025) when rearing piglets on a milk replacer. Van den Borne et al. (2007) speculated that the increased pancreatic secretions they reported in piglets fed a milk replacer diet were likely a compensatory antimicrobial mechanism in response to the low availability of such bioactive factors in milk replacer. Even the report that low lactose content increases growth by Szyndler-Nędza et al. (2020) could be hypothesised as the lower lactose making space for higher concentrations of other beneficial components like immunoproteins, and that increase in bioactives is why they reported better growth.

Addition of bioactive components, more specifically of prebiotics, has been explored in more depth in human formula with the addition of galacto-, fructo-, and human milk- oligosaccharides (GOS, FOS, and HMO), all of which boast prebiotic effects in the neonatal gut. Human milk contains higher concentrations of milk oligosaccharides than most other mammals (Bode, 2012; Albrecht et al., 2014), somewhat justifying the rapid progression of HMO inclusion in infant formula, however, there is evidence that supplementation of HMO can improve neonatal and post-wean health of piglets (Wang et al., 2007; Berding et al., 2016; Yin et al., 2019; Luo et al., 2022). These benefits are discussed in greater detail in Section 1.4.

The success of adding bioactive components to human infant formula can be attributed to the beneficial effects on the infant gut microbiome and

subsequent cascade of positive immunoregulatory and physiological developments. It is therefore important to understand the role of the piglet gut microbiome and how this could be modelled and manipulated for improving pre-wean health and performance.

1.3 The Gut Microbiome

The distal third of the transverse colon, descending and sigmoid colon, and upper two-thirds of the anorectal canal are collectively termed as the hindgut (Figure 1.2) in monogastric mammals (Yang et al., 2018).

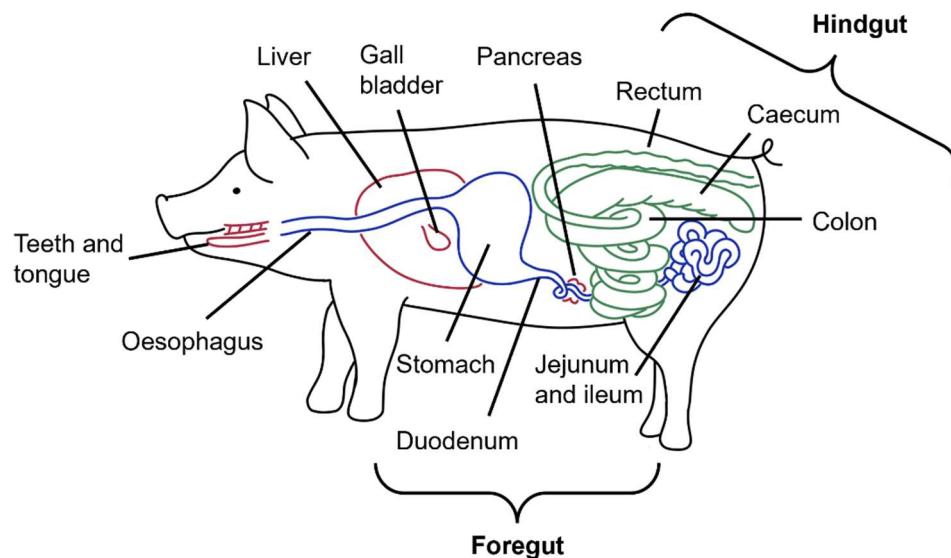


Figure 1.2 Division of the pig foregut (blue) and hindgut (green) regions with accessory digestive organs (red) (Author's own)

The foregut, considered to be the stomach and duodenum, ileum and jejunum (Thomson and Friendship, 2019), is the primary site for digestion of protein, simple carbohydrates, and fat, via enzymatic hydrolysis (Blachier et al., 2013; Thomson and Friendship, 2019). More complex structures such as cellulose or milk oligosaccharides are resistant to foregut enzymes and are instead broken down via microbial fermentation in the colon and caecum (Engfer et al., 2000; Gnoth et al., 2000).

Despite some microbial colonisation in the foregut region, the monogastric hindgut is the most densely populated microbial environment in the body, with estimations in humans of bacterial cells at 10^{11} to 10^{12} cells per millilitre, encoding for over three million genes (Rinninella et al., 2019). In the context of digestion, the microbiota ferment complex carbohydrates to facilitate the breakdown and

absorption of these and any residual nutrients from the foregut. What shapes and influences this microbial population has been extensively researched across multiple species in recent decades, due to reports of significant associations between the hindgut microbiome and health status of the individual (Fishbein et al., 2023; Lachance et al., 2024; Zhou et al., 2024).

Twin studies have suggested some degree of heritability which may influence the human gut microbiome *in utero* (Goodrich et al., 2014; Goodrich et al., 2016), and there have been published reports of microbial DNA present in meconium of premature infants (Mshvildadze et al., 2010), in amniotic fluids (DiGiulio et al., 2010; DiGiulio, 2012), and in the placenta (Satokari et al., 2009), suggesting an *in utero* transfer of the microbiome is possible. Despite being a contentious theory in the human field, there are also reports suggesting similar transmission in pigs.

Nowland et al. (2021) determined that stillborn piglets present with microbial colonisation of the spiral colon, and concluded passive transfer of microbes in the developing foetus. Despite sampling from the spiral colon to reduce chances of fluid ingestion before death having an effect, it is still difficult to believe this colonisation is happening *in utero* without studying the gut of piglets delivered by caesarean section, which is highly irregular in the pig industry. Regardless, the hypothesis of *in utero* transmission of microbes is more strongly substantiated in the human, where the placenta is haemochorial, meaning the foetus is in direct contact with maternal blood, unlike the pig where several maternal tissue layers remain (Macdonald and Bosma, 1985). Consequently, the piglet is considered born with a “sterile gut”, with postnatal vertical transmission of the microbiome in the same manner as humans; from exposure to the vaginal canal (Orrhage and Nord, 1999; Biasucci et al., 2010) and ingestion of breast milk (Martín et al., 2012; Jost et al., 2014).

Later in life, diet continues to be a main driver for microbial community composition across mammalian species (reviewed by Ross et al. (2024)). Importantly, the bacterial genera gained from horizontal transmission (the environment) in mammals, are more likely to exhibit virulence than those that are transmitted vertically (Moeller et al., 2018), cementing the importance of establishing an optimal microbiome in the neonatal and pre-weaning periods.

Colostrum represents the first source of dietary substrates encountered by the neonatal piglet and therefore coincides with a critical period of intestinal microbial establishment (Le Dividich et al., 2005; Bailey et al., 2005; Devillers et al., 2011). The neonatal piglet gastrointestinal tract undergoes rapid microbial colonisation following birth, with the composition of the microbial community changing considerably during the first days and weeks of life (Everaert et al., 2017; Liu et al., 2019; Trevisi et al., 2021). Studies using longitudinal sampling have demonstrated distinct phases of microbial development during the suckling period. Recent work identified an initial colonisation phase during approximately days 1-7, followed by a period of rapid microbial transition around days 14-21 and subsequent stabilisation towards days 24-28 (Song et al., 2026). Other longitudinal studies similarly demonstrate marked microbial succession between birth, the pre-weaning period and the post-weaning period (Everaert et al., 2017; Liu et al., 2019; Trevisi et al., 2021). Therefore, the microbiome observed following colostrum ingestion should be regarded as an early stage of a continuing developmental trajectory.

Early life gut health can have life-long implications as demonstrated by gut-barrier breakdown due to weaning stress (Moeser et al., 2017) and prediction of poor performing pigs through characterisation of pre-wean microbiota (Li et al., 2018a; Li et al., 2019). So despite colostrum only being ingested in the first 1-2 days, there is potential for long-term impact. During the nursing phase, colostrum and milk provide important dietary substrates to the developing microbiota, with milk oligosaccharides (discussed in more detail later) b representing an important source of fermentable carbohydrate available to intestinal microorganisms (Tao et al., 2010; Zhang et al., 2018). Bacteria capable of utilising these glycans may therefore gain a competitive advantage during early colonisation, while fermentation of these substrates can generate metabolites such as short-chain fatty acids that influence the intestinal environment and host physiology (Ward et al., 2006; Perdijk et al., 2019; Piotrowski et al., 2022). More broadly, work in mammals has demonstrated that milk oligosaccharides can shape the developing microbiome by selectively supporting microorganisms capable of metabolising them (Garrido et al., 2011; Moon, Joo, et al., 2016; Cheng, Kong, et al., 2020), which is discussed in more detail in the following section.

The importance of continued milk feeding is also illustrated by the rapid restructuring of the microbial community that occurs at weaning due to the replacement of milk-derived substrates with plant-derived pelleted feed (Frese et al., 2015; Guevarra et al., 2018). In longitudinal studies, microbial diversity and community composition change markedly within days following weaning, with increases in bacteria associated with the utilisation of plant-derived carbohydrates, such as *Prevotella*, and decreased milk-associated genera, such as *Bacteroides* (Frese et al., 2015; Guevarra et al., 2018).

It is therefore unlikely that colostrum alone determines the longer-term composition of the piglet microbiome. Rather, colostrum should be viewed as an important early component of a succession of maternal and environmental influences. Colostrum provides an exceptionally concentrated source of immunological and bioactive components (Hurley, 2015; Craig et al., 2019) at a time when the neonatal intestine and immune system are immature, while subsequent milk maintains exposure to maternal nutrients and bioactive factors during the period of rapid microbial development.

Previous literature has defined what a healthy and unhealthy gut microbiome may look like in the piglet (Guevarra et al., 2018; Gresse et al., 2019) with commensal phyla such as Bacteroidota and Bacillota dominating the environment. Generally, beneficial genera such as *Bifidobacteria* and *Lactobacillus* provide fermentation products and energy to colonocytes, while the likes of *Clostridia* and *Escherichia* are associated with virulence and disease (Guevarra et al., 2018; Gresse et al., 2019). Weaning stress can significantly impact the gut microbiome in a negative manner, characterised by post-weaning diarrhoea (Campbell et al., 2013) and, with weaning being an unavoidable stage in pig rearing, optimising microbiota health in the pre-wean piglet is a logical step to minimise any negative impacts of the weaning transition. The piglet gut microbiome is linked to metabolism of nutrients and subsequently effects weight gain, as well as susceptibility to post-weaning diarrhoea (Dou et al., 2017); all factors of economic importance in the pig industry. *Prevotella* has been highlighted as an important genus offering protection from post-weaning diarrhoea (Duncan et al., 2004; Dou et al., 2017), perhaps due to their adaptability in metabolism of many nutrients (Ivarsson et al., 2014). A further study suggests that high abundance of *Prevotella* and *Lactobacillus* may better allow piglets to adapt to the new dietary conditions post-

weaning (Guevarra et al., 2018), but this is largely due to their associations with fermenting plant-matter (Kim et al., 2011; Frese et al., 2015) and may not be achievable without creep feed. *Prevotella* abundance has also been positively associated with body weight, and *Clostridia* being negatively so (Mach et al., 2015), suggesting that differences in microbiome composition may be related to phenotypic variation. Furthermore, a higher Bacillota-to-Bacteroidota ratio has been reported in heavier piglets (Han et al., 2017), supporting the findings of Mach et al. (2015) that a higher proportion of Bacteroidota is negatively associated with body weight. With reports highlighting that low-birth-weight piglets can retain poor growth performance into adulthood (Gaukroger et al., 2020; Xiong et al., 2020), establishing an optimal microbiome at the start of life may have lifelong benefits to the pig. The functional effects of the gut microbiome have been associated with a wide range of diseases in humans including multiple cancers (Yang et al., 2021; Mishra et al., 2024; Lachance et al., 2024), antibiotic resistant infections (Langdon et al., 2021; Fishbein et al., 2023), and mental health disorders (Li et al., 2022; Radjabzadeh et al., 2022; Zhou et al., 2024). As research has advanced, the transition from culture-dependant methods to innovative molecular analyses has increased efficiency and depth of microbiome characterisation.

To address the experimental and ethical challenges of studying the gut microbiome in humans, animal models are often used to provide pre-clinical insight. Some commonly used species, such as rodents, can have large physiological variance to humans as well as compositional and functional microbiome differences (Treuting et al., 2017; Hugenholtz and de Vos, 2018). To negate these concerns, porcine models have become popular due to better similarity across a range of factors (summarised by Guilloteau et al. (2010) and Odel, et al. (2014)). Porcine and human modelling will be referenced interchangeably throughout this thesis due to the many published porcine models for extrapolation to human medicine, where the focus of this thesis is the porcine models for porcine knowledge expansion. Though used historically, animal models are ethically demanding, requiring licensing and low throughput to remain acceptable. *In vitro* models eliminate these challenges and offer opportunities to test more dangerous or toxic compounds.

1.3.1 *In Vitro* Digestive Modelling

There are many different adaptations of *in vitro* digestion models that have been extensively reviewed. Examples of such reviews include Coles et al. (2005), Dupont et al. (2019), Fournier et al. (2022), and Isenring et al. (2023). Unfortunately this diversity in modelling brings with it challenges such as between-model comparisons and reproducibility of data. In recognition of these issues, Minekus et al. (2014) published the INFOGEST model. This is an *in vitro* foregut digestion protocol, developed by an international network of scientists with the aim to standardise the method for simulated digestion. A later update (Brodkorb et al., 2019) confirmed validity and reliability, suggesting this was a successful feat, and many others have since published results using the INFOGEST protocol (as summarised by Zhou et al. (2023)).

Although recommendations for adapting samples from INFOGEST to go into hindgut fermentation systems are included in the publication, there remains to be seen anything of this nature for standardising a hindgut model. This is surprising given the physiological relevance and biological impact of the hindgut microbiota. As mentioned earlier, the colonic environment harbours the most dense population of microbes in the body of monogastric species (Aggarwal et al., 2022) and is considered the most significant microbiota for affecting health outcomes across a range of mammals (Hou et al., 2022). It is therefore of great scientific importance that suitable *in vitro* systems exist to drive research progression. Alas, there is a great number of independent hindgut models published, ranging from traditional batch fermentation (mostly developed from work by Menke and Steingass (1988)) to more advanced continuous systems (Tanner et al., 2014; Dufourny et al., 2019). The lack of standardisation for hindgut systems likely stems from this large difference in systems available. The optimal protocol for one continuous bioreactor, may not provide applicable results in a batch fermentation protocol (Coles et al., 2005; Moon, Li, et al., 2016). These methods and their differences will be discussed in further detail in Section 3.1.

Regardless of how it is modelled, the microbiome has significant influence over piglet health and performance. As mentioned briefly in Section 1.2.4, particularly effective compounds that can positively modulate the microbiome, and have been used with great success for improving human formula-fed infant mortality (Nützenadel, 2010; Bode, 2012), are complex glycans naturally abundant in sow milk called milk oligosaccharides.

1.4 Milk Oligosaccharides

Milk oligosaccharides can be categorised by various structural components, all with a lactose reducing end, and usually based on sialylation (acidic) or lack thereof (neutral). Neutral structures are further defined by fucosylation, which is the presence or absence of the L-fucose monomer. The basic structure of milk oligosaccharides uses five key constituents: D-glucose (Glc), D-galactose (Gal), N-acetylglucosamine (GlcNAc), L-fucose (Fuc), and N-acetylneuraminic acid (NeuAc) or N-glycolylneuraminic acid (NeuGc); both types of sialic acid, which are used to describe structure using an ordered Hex-HexNAc-Fuc-NeuAc-NeuGc system (where Hex = Hexose (Glc or Gal)), with linkage type and carbon numbers specified where possible.

The relevance of these complex and structurally diverse glycans to neonatal nutrition stems from their resistance to digestion in the foregut, allowing them to reach the hindgut intact, where they act as a prebiotic (Gibson and Roberfroid, 1995; Gibson et al., 2017). The fermentation of these structures by resident microbes alters the composition and activity of the hindgut microbiota, triggering downstream effects that elicit benefits including regulation of intestinal epithelial cells (Perdijk et al., 2019), blocking adhesion of pathogens (Piotrowski et al., 2022) and promoting proliferation of beneficial gut bacteria (Ward et al., 2006; Shen et al., 2011; Yu et al., 2013).

Milk oligosaccharides were first identified *circa* 1930 (Polonowski and Lespagnol, 1929; Polonowski and Lespagnol, 1931; Kunz, 2012), with their anti-pathogenic properties recognised since the 1980s (Evans et al., 1988; Simon et al., 1997). Reduction in pathogens can be enhanced by various characteristics of milk oligosaccharides; the binding of the oligosaccharide to the pathogens directly, thus rendering the binding site unavailable to the epithelium (Newburg et al., 2005); through the upregulation of mucus to increase mucin binding (Baurhoo et al., 2007; Baurhoo et al., 2009); both followed by subsequent elimination via peristalsis; or by increasing proliferation of beneficial bacteria and inducing competitive exclusion (Ward et al., 2006; Shen et al., 2011; Yu et al., 2013).

Beneficial effects of HMO have been well-researched over the last couple of decades (Bode, 2012; Triantis et al., 2018; Dinleyici et al., 2023). Since the start of the 20th century, it was recognised that breast milk was important for infant survival, with bottle-fed infants having a seven-fold higher mortality rate

(Nützenadel, 2010) but it was not until 2016 that HMO were first added to infant formula by a US company (Abbott, n.d.). Few brands have since followed in adding HMO, creating “gold standard” infant nutrition (Cheng and Yeung, 2021; Zhu, Zhang, et al., 2022) and many more opt for supplementing FOS and/or GOS as a more cost-effective prebiotic alternative. As of writing, there are seven milk oligosaccharide structures that have been approved as safe for human consumption and these include a variety of structure types: Acidic (3'-sialyllactose (3'SL) and 6'-sialyllactose (6'SL)); fucosylated (2'-fucosyllactose (2'FL), 3-fucosyllactose (3'FL), and difucosyllactose (DiFL)); and non-fucosylated neutral (lacto-N-neotetraose (LNnT) and lacto-N-tetraose (LNT)) (Zhu, Luo, et al., 2022). In comparison, the livestock milk formula market is behind; despite a growing demand for supplementary milk to support the large litters of modern hyper-prolific sows (Kobek-Kjeldager et al., 2020; Peltoniemi et al., 2021; Kobek-Kjeldager et al., 2021).

Exploration of HMO functional effects is a broad field of study due to reports suggesting responses are structure-specific and synergistic between HMO compounds (Kavanaugh et al., 2013; Kong et al., 2019; Cheng, Kong, et al., 2020). Though many benefits seen are tested across HMO compounds, there is much cross-over between key oligosaccharides that appear in both human and porcine milk including, but not limited to, 3'SL, 6'SL, 2'FL, and LnNT (Tao et al., 2010; Salcedo et al., 2013; Mudd et al., 2016; Wei et al., 2018; Trevisi et al., 2020; Smith et al., 2026). The prebiotic and immunomodulatory effects of these shared structures can therefore be inferred as applicable to piglets too, given the similar physiology and microbiome between species (Odle et al., 2014; Ferenc et al., 2014; Yin et al., 2017).

1.4.1 Reduction of Pathogen Adhesion

Several pathogens have been tested to identify strengths and limitations in the adhesion characteristics of milk oligosaccharides, including *Escherichia coli* (Facinelli et al., 2019; Wang et al., 2020; Faria et al., 2022) and *Clostridioides difficile* (Piotrowski et al., 2022). Coppa et al. (2006) identified that acidic fractions were the most suited to reducing adhesion of a range of pathogens and, while neutral fractions were moderately effective, they did not reduce all strains tested (Coppa et al., 2006), confirming the structure-dependant nature of functional HMO effects.

Fucosylated neutral oligosaccharides, such as 2'FL, mimic host epithelial cell surface glycans, protecting the gut from pathogens and viruses by either binding to the epithelial surface (Morrow et al., 2004), thus rendering the site unavailable to pathogens, or through direct pathogen binding, followed by wash out (Craft and Townsend, 2018). This has been evidenced in specific strains such as *Campylobacter jejuni* (Yu et al., 2016) and *Escherichia coli* (Facinelli et al., 2019). However, other groups of oligosaccharides, namely sialylated structures, can also elicit this response by acting as decoy receptors (ten Bruggencate et al., 2014). Compounds containing sialic acid, such as 3'SL and 6'SL, have shown ability to reduce adhesion of several pathogens *in vitro* including *Salmonella* (Coppa et al., 2006; Salcedo et al., 2013) and *Helicobacter pylori* (Simon et al., 1997; Salcedo et al., 2013).

There is little evidence that nonfucosylated neutral structures, such as LNnT, bear resemblance to host glycans and therefore may not be able to reduce pathogen adhesion in the same way (Hester et al., 2013). However, Terrazas et al. (2001) demonstrated that LNnT increases anti-inflammatory mediators when injecting into a mouse model, so could still possess some antipathogenic properties. Additionally, fermentation of LNnT results in increased short chain fatty acid (SCFA) production (Li et al., 2012), which have been shown to reduce pathogen virulence and survival (Peng and Biswas, 2017; Zhan et al., 2022). GOS and FOS, which are often used as substitutes to HMO, do not possess the key structural features like fucosylation or sialic acid groups, limiting their ability to mimic host glycans in the same way as HMO.

Due to the structural diversity of HMO and the multiple mechanisms available to inhibit pathogens, it can be inferred that a broad spectrum of oligosaccharides would offer the greatest benefit.

1.4.2 Increasing Mucin Production

Within the intestine, goblet cells are responsible for secretion of mucus and these are found in a higher proportion towards the distal colon, much like the proportion of bacteria (Karam, 1999). This correlation suggests that there is microbial modulation of goblet cells (Deplancke and Gaskins, 2001). This is further supported by findings showing fewer and smaller goblet cells in intestines of germ free rodents when compared to conventional ones (Kandori et al., 1996).

Mucus itself is comprised of major glycoproteins called mucins which are secreted either continuously at low-levels or, if stimulated, through a compound exocytosis of the mucin granules. The primary role of mucins is to provide the epithelial cells with protection, which is achieved through a dual layer; one anchored to the goblet cells, and one detachable (Hansson, 2019; Paone and Cani, 2020). This enables the mobile mucins to reduce adhesion of pathogens through attachment and release from the epithelial wall, followed by elimination by peristaltic wash-out, as well as using a less permeable wall, inhibiting transport of pathogens through to epithelial cells.

Modulation of goblet cells, and subsequent increased mucin production would be beneficial by enhancing gut wall protection. Cheng et al. (2019) has demonstrated modulation of goblet cells by 2'FL and 3'FL through increased expression of MUC2, the most notable mucin gene. This study investigated homeostatic conditions as well as more challenging conditions when introducing inflammatory cytokines and found that 2'FL upregulated MUC2 in an IL-13 challenge but not in the TNF- α challenge, nor in homeostatic conditions, suggesting that in addition to structure-dependant effects, some HMO effects are triggered or altered in the presence of inflammation (Cheng, Kong, et al., 2020). Zehra et al. (2018) also found that 2'FL had no effect on TNF α induced cytokine release, where 6'SL did. They proposed that there are distinct mechanisms of action, unique to different HMO structures (Zehra et al., 2018). In further support of this theory, not all HMO are able to modulate epithelial cells, as evaluation of 3'SL and 6'SL did not show any impact on goblet cell numbers (Monaco et al., 2018).

Instead of only considering direct effects of HMO on mucin regulation, many metabolites can also induce or influence secretion of mucins, including SCFA (Burger-van Paassen et al., 2009), which are produced as a biproduct when gut microbes are fermenting HMO. Specifically, butyrate has been reported as a key SCFA in upregulation of colonic mucins (Gaudier et al., 2004; Hatayama et al., 2007). This cements the importance of a healthy microbiota, as metabolite production from the microbial community drives key gut health markers.

1.4.3 Proliferation of Beneficial Microbes

HMO cause selective proliferation of specific microbes as only certain strains are able to ferment each type of structure. For example, some bacteria may secrete alpha-fucosidases which are able to cleave the fucose sugar, while others will express sialases to metabolise sialic acids. This varies at the strain level and means that these strains are at an advantage in their ability to utilise that specific oligosaccharide, where other strains (often pathogens) are not, and a consequential decrease in pathogen abundance by competitive elimination will occur.

Sialyllactose is broken down into sialic acid and lactose extracellularly (Kiyohara et al., 2011; Nishiyama et al., 2018), allowing the free sialic acid to be used as an energy source by *Bifidobacteria*, thus promoting proliferation of these species. *Bifidobacteria* are considered a key and beneficial commensal of the infant gut due to strong associations between prevalence and health status (Tojo et al., 2014; Arbolea et al., 2016; Lewis and Mills, 2017; Milani et al., 2017). Consequently, human research focusses on how to increase proliferation of this taxa; successful supplements being termed as having a “bifidogenic effect”. Although less prevalent commensally in piglets, there is still evidence that *Bifidobacteria* are beneficial to piglet health and performance (Hansen et al., 2022; Hansen et al., 2025). *Bifidobacteria* produce beneficial SCFA, such as butyrate, as a biproduct of fermentation (Fleming et al., 1991) which can then be used as fuel for colonocytes, or, as described above, to decrease intestinal permeability, both benefiting the health of the host.

Multiple studies have demonstrated the bifidogenic effect of HMO but many also point out the strain-specific effects seen. For example, Moon *et al.* (2016) reported that *Bifidobacterium longum* subsp. *infantis* and *B. bifidum* are both able to effectively metabolise 3'SL and 6'SL, where other *Bifidobacteria* such as *B. breve* and *B. adolescentis*, although able to utilise the HMO, cannot do so as effectively (Moon, Joo, et al., 2016). This finding is consistent with other studies boasting *B. longum* subsp. *infantis* as able to metabolise a wide range of HMO, but other strains of *Bifidobacteria* falling short (LoCascio et al., 2007; Sela et al., 2008). Interestingly, *B. longum* has no fucosidases or sialidases and is unable to utilise HMO effectively, despite the *B. longum* subsp. *infantis* strain being able to do so (Marcobal et al., 2010).

The bifidogenic effect in the gut induces competitive exclusion whereby disadvantageous species are reduced due to diminishing resources as the beneficial species take over. This can occur simply as the beneficial strains become more competitive but, additionally, Moon *et al.* (2016) showed that harmful bacteria were unable to metabolise the HMO tested (3'SL and 6'SL) even when cultured individually. This supports use of HMO as a safe prebiotic as even when proliferation of beneficial bacteria is not demonstrated, the HMO will not be used to enhance pathogenic species.

Other beneficial gut strains would include *Lactobacillus*, however it seems sialyllactoses cannot be used in the same way by *Lactobacillus* due to lack of extracellular sialidase activity (Thongaram *et al.*, 2017; Perdijk *et al.*, 2019), which is necessary to cleave the sialic acid. Research investigating effects of non-milk oligosaccharides are conflicting in impact on *Lactobacillus* with Finegold *et al.* (2014) suggesting xylooligosaccharides (XOS) have no significant effect on abundance of *Lactobacillus* in humans (Finegold *et al.*, 2014). Conversely, Pan *et al.* (2019) and Stanley *et al.* (2025a) both reported enhanced abundance in swine when fed a XOS supplement. The metabolism pathway of HMO by *Lactobacillus* is complex but evidence summarised by Zúñiga *et al.* (2021) suggests some species of *Lactobacillus* have genes encoding for fucosylated oligosaccharide hydrolysis (Zúñiga *et al.*, 2021) but that the pathway is likely intracellular due to lack of secretion signalling unlike use of sialylactose in *Bifidobacterium* species (Rodríguez-Díaz *et al.*, 2011). Additional beneficial species, *Bacteroides fragilis* and *Bacteroides vulgatus*, have an increased number of genes that encode for glycoside hydrolases, meaning they can also metabolise a wide range of HMO (Marcobal *et al.*, 2010).

Due to costly synthesis of HMO compounds, similar compounds are often included in infant milk formulations, such as GOS and FOS, and despite being less structurally complex than HMO, they retain some prebiotic benefits such as epithelial wound repair (Perdijk *et al.*, 2019), and, most notably, stimulation of *Bifidobacteria* (Fanaro *et al.*, 2005; Sierra *et al.*, 2015; Matsuki *et al.*, 2016). This makes them a popular substitute for HMO, given how beneficial *Bifidobacteria* are to infants, however the stimulatory effects are less specific for GOS/FOS because of the structural simplicity. Hoeflinger *et al.* (2015) showed that several *Enterobacteriaceae* strains would not grow on 2'FL, 6'SL, or LNnT, but did on

GOS. This included pathogens such as *Klebsiella pneumoniae* subsp. *pneumoniae* and *Escherichia coli* (Hoeflinger et al., 2015). This suggests that a structurally diverse HMO combination be optimal for increasing proliferation in multiple beneficial genera.

1.5 Thesis Structure

Current consensus from the literature outlines the prebiotic potential of milk oligosaccharides (reviewed by Bode (2012)), particularly for human infants (Kuhn et al., 2015; Bering, 2018; Cheng and Yeung, 2021). Studies supplementing similar oligosaccharide structures such as GOS and XOS in piglets have highlighted similar prebiotic benefits (Lee et al., 2023; Stanley et al., 2025a), suggesting that PMO may be a good target for enhancing piglet gut health, creating more robust piglets and lowering pre-weaning mortality rates. Current gaps in the literature include associations between key PMO in sow colostrum and litter performance, and appropriate *in vitro* methods to model a pre-wean piglet gut, necessary for efficient analysis of these compounds. Therefore, this thesis will explore the potential of milk oligosaccharides (shared HMO/PMO structures) as a prebiotic to be considered for addition to piglet milk replacer to enhance pre-wean piglet gut health and performance in UK indoor production systems.

Steps to determine the role of PMO in the performance of the pre-weaning piglet begin in Chapter 2: The Colostrum Chronicles. This chapter employed quantification of PMO in colostrum of hyper-prolific sows using HPLC-MS/MS (performed by associates at DSM-Firmenich) and explored associations with litter performance in the pre-wean period. It was hypothesised that sialylated structures would dominate the samples, based on previous studies, and that the PMO profile may be associated with pre-weaning mortality. This provided preliminary understanding of highly abundant compounds, which may be good targets for supplementation, as well as identification of particularly critical structures which impacted key performance metrics, such as mortality rate.

Following this, method development was undertaken in Chapter 3: Bottled Piglets. This built on previous batch fermentation literature, and aimed to optimise an *in vitro* model of the pre-wean piglet colon. Several variable parameters were tested in consecutive experiments to determine the most efficient protocol, able

to provide a high-throughput and ethical way to screen and test any milk oligosaccharide supplementation. There were minimal hypotheses made due to conflicting literature regarding best practice.

The final data chapter, Chapter 4: A Spoon Full of Sugar, combines results from both previous chapters to test a range of milk oligosaccharide combinations in the *in vitro* fermentation model. This was performed to explore the fermentation kinetics, metabolite production and microbial community composition of various PMO combinations, in comparison to piglet milk replacer, and colostrum, and determine if any PMO combination could shift the measured parameters to be more similar to the 'gold standard' of colostrum. It was hypothesised that combining LNnT, 2'FL and one of either 3'SL or 6'SL would provide the most colostrum-like microbial profile, due to different structures eliciting different functional outcomes.

Chapter 2 The Colostrum Chronicles: Identifying Porcine Milk Oligosaccharides in Colostrum and Investigating Their Role in Litter Performance

2.1 Introduction

Colostrum, the first secretion from the mammary gland, plays a critical role in health and survival of mammalian neonates, including humans, due to the provision of fundamental nutrients and bioactive compounds (Baumrucker et al., 2023). Colostrum is responsible for the passive transfer of immunity from sow to piglet and therefore is essential for piglets to consume within 36 hours of birth (Le Dividich et al., 2005; Bailey et al., 2005). Early colostrum consumption by the neonate is critical due to containing many bioactive components including immunoglobulins and milk oligosaccharides which trigger and support immune development in the piglet (Tao et al., 2010; Zhang et al., 2018). Due to colostrum being vital for piglet survival, anything that restricts intake can lead to major health disturbances (Baumrucker et al., 2023). This challenge is particularly pronounced in modern hyper-prolific sows, where large litter sizes increase competition for colostrum.

Colostrum production is highly variable between sows and volume produced is not associated with the size or weight of the litter (Devillers et al., 2005; Devillers et al., 2007; Quesnel, 2011), resulting in piglets from larger litters having reduced access to colostrum. Through selective breeding for reproductive performance, the modern sow has become hyper-prolific, giving birth to 15-17 live piglets per litter in Europe (Hansen, 2018; AHDB, 2023; Sanz-Fernández et al., 2024). Although this appears to be beneficial from a profit-per-sow perspective, litter size is positively associated with pre-weaning mortality due to factors such as inter-uterine growth restriction (Santos et al., 2022), compromised placental efficiency (Wu et al., 2006), and deficiency in functional teats on the sow (Rohrer and Nonneman, 2017; Obermier et al., 2023).

Management strategies to reduce mortality often focus on cross-fostering or supplemental milk replacer, however, the former is labour intensive and time sensitive (Zhang et al., 2021) and the latter usually lacks critical bioactive components as evidenced in ingredients of commercial products (AB Neo, personal communication, March 2023; Buitelaar Agriculture, personal

communication, Feb 2026; sav-A-caf, 2026). Whilst the importance of immunoglobulins in porcine colostrum has been well studied (summarised by Inoue and Tsukahara (2021)), porcine milk oligosaccharides (PMO) have received less attention.

Milk oligosaccharides are structurally diverse glycans found in abundance in colostrum and mature milk of most mammals (Albrecht et al., 2014). They are prebiotic in nature due to the lack of hydrolysis in the foregut (Gibson and Roberfroid, 1995), allowing their passage, in-tact, into the hindgut where they are fermented by resident microbes (Bode, 2012). As discussed in the previous chapter, effects of human milk oligosaccharides (HMO) have been widely researched since discovering the importance of them to infant health and immunity; the most notable benefits being pathogen reduction and immune modulation.

Reduced pathogen adhesion through use of HMO in murine and *in vitro* models has been evidenced in many prominent virulent species including *E. coli*, *C. difficile* and *H. pylori* (Evans et al., 1988; Wang et al., 2020; Piotrowski et al., 2022), and similar effects are seen *in vivo* with HMO reducing diarrhoea in infants caused by *E. coli* infection (Newburg et al., 2004), *Campylobacter spp.* infection and norovirus (Morrow et al., 2004), and reducing general enteric disease risk (Stepans et al., 2006). The immune modulation capacity of HMO is likely through many mechanisms including TLR4 inhibition (Sugiharto et al., 2015; Sodhi et al., 2021), which impacts inflammatory responses, and mucin upregulation (Wu et al., 2019; Wang et al., 2020), which facilitates a better physical barrier to pathogens.

There is a breadth of literature boasting the numerous benefits of HMO on infant gut health and immunity (as summarised by Bode (2012) and Dinleyici et al. (2023)) and as piglets are often used as a model for the human infant due to physiological similarities (Odle et al., 2014), these benefits can be considered likely to occur in piglets also. One critical consideration when extrapolating findings across species is that humans rarely birth more than two babies, whereas sows are producing 14-16 live piglets per litter. This could potentially impact the applied effects where benefits at the gut level are not co-linear with performance gains valuable to the producer such as increased weight gain and

reduced litter mortality, and if this is the case, expensive synthesised HMO may not be an attractive mechanism for supporting vulnerable piglets on farm.

Bovine milk-based supplemental milk replacer often also offer oligosaccharide content as some structures are retained through conjugation to proteins and phospholipids (Barile et al., 2009; Badman et al., 2019). The benefits of bovine colostrum supplementation in place of standard milk replacer to piglets have been reported previously and include improved growth performance (Huguet et al., 2012), faster recovery of intestinal function post inflammatory episode (Støy et al., 2014) and improved immune response (Sugiharto et al., 2015). Furthermore, bovine colostrum is more readily available than HMO due to being a by-product of the dairy industry (Lallès et al., 2009; Poulsen et al., 2017), therefore offers a more cost-effective avenue to pursue for increasing milk oligosaccharide provision to piglets. Although some oligosaccharide structures are shared between bovine and porcine colostrum, like HMO, these do not always occur in comparative concentrations (Albrecht et al., 2014). It would therefore be more effective to develop targeted supplements specifically for piglets, that boast PMO compositions reflective of what is seen naturally in the sow, as these are likely to offer greater benefits to piglets than human- or bovine- derived components.

However, research identifying and quantifying PMO remains comparatively sparse. So far 55 unique PMO have been reported, compared to over 200 HMO (Wei et al., 2018), and sialylated compounds appear to be the dominant fraction in sow samples (Tao et al., 2010; Cheng et al., 2016; Difilippo, Pan, Logtenberg, Willems, et al., 2016; Wei et al., 2018) compared to the majority fucosylated compounds seen in humans. There are reports of significant effects of breed and lactation period on PMO concentrations (Tao et al., 2010; Cheng et al., 2016; Mudd et al., 2016; Trevisi et al., 2020), but understanding of the impact on litter performance is limited.

This study aimed to identify and quantify PMO in colostrum of highly productive sows on the same farm, under identical commercial conditions and explore if PMO profile is correlated with litter performance parameters. It was hypothesised that sialylated structures would dominate across samples and highly abundant structures such as 3'-sialyllactose (3'SL), 6'-sialyllactose (6'SL), and lacto-N-neotetraose (LNnT) were expected, as reported by previous studies

(Tao et al., 2010; Mudd et al., 2016; Salcedo et al., 2016; Wei et al., 2018; Trevisi et al., 2020). If naturally abundant PMO are associated with improved litter performance, this would strengthen the case for incorporating them as prebiotic additives in piglet milk replacer or creep feed to support neonatal growth, survival and the weaning transition on farms.

2.2 Methods

All animal practices abided by the DEFRA Code of Practice for the Welfare of Pigs (DEFRA, 2020) and Welfare of Farmed Animals (England) Regulations (2007). No housing or husbandry practice for this study differed from usual management of farrowing sows at the National Pig Centre (NPC; University of Leeds, Leeds, UK) according to the Council Directive 2008/120/EC. All procedures were reviewed and approved by the NPC named veterinary surgeon and ethical approval was received from the animal welfare and ethical review board prior to study commencement (Appendix C). No procedures in this study came under the Animals (Scientific Procedures) Act 1986.

2.2.1 Sow and Piglet Management

Sows were multiparous Large White x Landrace housed at the NPC on an indoor production system. Sows at the NPC are kept on a 3-week batch farrowing system, rotating at 50-60 sows per batch. Dry and gestating sows were kept on straw in a dynamic herd until being moved into individual farrowing pens with temporary crating (JLF15, Jyden, 5.76 m²) approximately one week prior to their due date. Sows were secured into crates around 24 hours prior to parturition (varying on an individual basis, at the discretion of the stock manager) and released at four days post-partum (Radtke et al., 2026). Sows were all fed the same commercial gestating and lactating sow diet following standard commercial practice at the NPC, according to stage of gestation and body condition. Shredded paper (topped up daily) and a wooden block on a chain were offered as enrichment within the farrowing house.

Approximately 24 hours post-parturition, piglets were individually ear tagged using RFID tags and body weight and sex were recorded. An intramuscular injection of combined 45 mg toltrazuril and 200 mg iron was administered within 48 hours of birth for the prevention of iron deficiency and swine coccidiosis. Individual body weights were recorded again at average day 7

and at average day 28. These body weights were corrected to actual day 7 and actual day 28 weights using day of age at weighing and average daily gain data. Litter size at birth was recorded. All fostering history was recorded along with piglet mortality. Piglets had *ad-libitum* creep feed (A-One FlatDeck XL Blue: crude protein 20.5%, crude oils and fats 8.5%, crude ash 6%, crude fibre 2.2%, lysine 1.75%, calcium 0.75%, phosphorus 0.73%, methionine 0.68%, sodium 0.3%) provided from day seven in addition to sow milk and access to fresh water.

2.2.2 Colostrum Sampling

Samples of colostrum were opportunistically obtained from 45 sows (parity 2-8) by manual manipulation of the teat during parturition, when sows were already crated. Samples were taken as close to onset of parturition as possible. A maximum volume of 10 mL colostrum was taken per sow and pooled from a variety of functioning teats, to reduce teat-quality-bias (Ogawa et al., 2014). Colostrum was stored on ice immediately after collection and frozen at -80°C within approximately 6 hours after collection, until use.

2.2.3 Chemicals and Reagents

Acetonitrile (HPLC-MS grade) used as an eluent was purchased from TH. Geyer GmbH & Co. KG (Renningen, Germany). High-purity deionized water for eluent preparation and sample treatment (Milli-Q, 18.2 MΩ cm) was obtained from a Nanopure Diamond water purification system (APS Water Services Corporation, Lake Balboa, California, USA). Ammonium formate (HPLC-grade) and HPLC-MS grade 50% formic acid solution in water, used for eluent preparation, were purchased from Honeywell Fluka International Inc. (Charlotte, North Carolina, USA). Anhydrous dimethyl sulfoxide, glacial acetic acid, benzocaine, and picoline borane, for the labeling reaction, were purchased from Merck KGaA (Darmstadt, Germany).

Oligosaccharide standards 2'-fucosyllactose (2'FL), 3'SL, 6'SL, LNnT, and para-lacto-N-neohexaose (pLNnH) (all analytical grade) were used for quantitation, produced by DSM-Firmenich. Their purity was assessed using quantitative NMR with certified reference materials. Panose was used as an internal standard and purchased from Megazyme Ltd. (Wicklow, Ireland). Additional oligosaccharide standards 3'-galactosyllactose (3'GL), lacto-N-neohexaose (LNnH), and

sialyllacto-N-neotetraose c (LST-c) were used for peak identification, produced by DSM-Firmenich.

2.2.4 Oligosaccharide Identification and Quantification

Oligosaccharide identification and quantification was performed by DSM-Firmenich.

Colostrum was defrosted at ambient temperature and 200 μ L colostrum and 200 μ L acetonitrile were vortexed and centrifuged at 17,000 x g in a Thermo Scientific Pico 17 microcentrifuge (Thermo Scientific, Waltham, Massachusetts, USA) for three minutes. The supernatant was decanted into a separate tube and centrifuged again under the same conditions. Samples were then labelled by benzocaine and analysed by HPLC-UV as described previously (Molnár-Gábor et al., 2024). Briefly, 50 μ L of calibration standard, or the colostrum supernatant (as obtained previously) was added to 50 μ L internal standard solution (1 g/L panose) and 250 μ L of derivatization reagent (prepared from 2 g benzocaine and 5 g picoline borane in 20 mL 70:30 DMSO-acetic acid) added. The tube was then vortexed and kept in a heating block at 60 °C for three hours with shaking at 600 rpm (Eppendorf Thermomixer C, Eppendorf AG, Hamberg, Germany). Once finished, tubes were removed and cooled to ambient temperature, 500 μ L 9:1 acetonitrile-water was added before vortexing and centrifuging at 17,000 x g for three minutes. The supernatant was analysed using HPLC (UltiMate 3000 with Accucore Amide HILIC column (150 $\times$ 2.1 mm, 2.6 μ m), Thermo Fisher Scientific, Waltham, USA) using a gradient of acetonitrile (A) and 10 mM ammonium formate (B) pH adjusted to 3.0 with formic acid). The gradient profile started at 94% A and 6% B, which was held for 0.1 minutes, and then changed to 88% A and 12% B over 0.1 minutes. Then it was changed to 80% A and 20% B over 5.8 minutes, and afterwards to 60% A and 40% B over 2 minutes. This was maintained for 3 minutes, then changed back to the initial eluent composition over 0.1 minutes. The system was reequilibrated with this composition for 2.9 minutes. Detection was by a diode array detector at a wavelength of 300 nm.

Oligosaccharides present in the samples were identified by using reference materials as well as HPLC-MS measurements using conditions identical to the method described above. Underivatized molecular masses were calculated. Where reference materials were not available, exact structures could

not be confirmed, therefore constituent monosaccharides were estimated from the mass (referred to in results as ‘unconfirmed’ structures). A five-point calibration was prepared from 2’FL, 3’S_L, 6’S_L, LNnT, and pLNnH for the quantification, and unknown compounds were quantified with the closest calibration peaks. Detector response was taken to be equimolar, as would be expected based on the principle of detection (Bigge et al., 1995; Austin et al., 2019). Total concentration was calculated by adding concentrations of identified PMO together for each sow.

2.2.5 Statistical Analysis

Statistical analysis was conducted using R version 4.5.1 (R Core Team, 2025) within RStudio 2025.05.1-513 (Posit Software, 2025). Data consisted of concentrations of PMO identified (g/L) and total concentration per sow, and performance variables as follows: pre-weaning mortality (%); birth litter size; parity; piglet bodyweight at day one (BW1); piglet bodyweight at day seven (BW7); and piglet bodyweight at day 28 (BW28). Performance variables were analysed at either sow-level (pre-weaning mortality (%); litter size; parity) or piglet-level (bodyweight at days 1, 7 and 28 (kg)), with birth sow controlled for when conducting analyses at piglet-level. There was only one sow of parity eight, therefore this was grouped into parity seven to limit skewing.

Throughout this study, the term ‘colostrum composition’ is used to denote the overall oligosaccharide profile of a colostrum sample, defined by the joint distribution of all quantified oligosaccharides measured in that sample, rather than the concentration of any single oligosaccharide. To investigate colostrum composition effects, a multivariate analysis was used as follows. Concentrations were transformed into relative abundance values and visualised using non-metric multidimensional scaling (NMDS) based on Bray-Curtis distance. Performance variables listed above were fitted to the ordination using the *envfit* function in the “vegan” package v2.7-1 (Oksanen et al., 2025), which tests for associations between metadata variables and the ordination space via permutation. To formally test the influence of performance variables on overall colostrum composition, a permutational multivariate analysis of variance (PERMANOVA) was conducted with the *adonis2* function in “vegan”. This enabled better control over nested data which was necessary when looking at the piglet level performance variables.

For univariate analysis, concentrations of PMO were transformed using the “bestNormalize” package v1.9.1 (Peterson and Cavanaugh, 2020; Peterson, 2021). Body weight measurements were normally distributed and were used as response variables in mixed-effects models, with the PMO as predictors. In these models, litter size and BW1 were included as covariates, and birth sow was incorporated as a random effect. Models were performed using packages “lme4” (Bates et al., 2015) and “lmerTest” (Kuznetsova et al., 2017).

Pre-weaning mortality exhibited characteristics of a Tweedie distribution, therefore was modelled using a generalized mixed model with a Tweedie family and a log link function with the package “glmmTMB” v1.1.11 (Brooks et al., 2017; McGillicuddy et al., 2025). In this model, PMO were used as predictors, litter size as a covariate, and parity was treated as a random effect. Pre-weaning mortality was analysed at the sow-level thus birth sow as a random effect was unnecessary. Traditional R^2 values for the purpose of understanding how much variation was explained by each PMO are not defined for this model, therefore the conditional pseudo- R^2 value is used following Nakagawa and Schielzeth (2013) using the package “performance” v0.16.0 (Lüdtke et al., 2021) which gives an appropriate substitution for generalized mixed models reflecting variance explained by both fixed and random factors.

To explore associations between PMO and sow factors, litter size and parity were used as predictors in linear models with the PMO as response variables. Multiple testing correction was performed using a false discovery rate (FDR) approach. Specifically, the Benjamini-Hochberg step-up procedure was used to control the expected proportion of false positives among significant results. Significance was reported at $P < 0.05$ and trending at $P < 0.1$. Regression coefficients are reported as ‘ β ’.

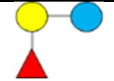
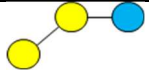
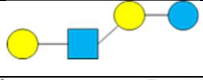
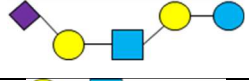
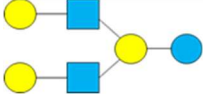
2.3 Results

2.3.1 Oligosaccharide Identification and Concentration

A total of 11 PMO were identified in porcine colostrum; five sialylated, five neutral and one fucosylated compound. Of the 11 found, seven were confirmed by retention times and mass spectrometry of reference compounds. Confirmed structures were 2'-fucosyllactose (2'FL); 3'-galactosyllactose (3'GL); 3'-sialyllactose (3'SL); 6'-sialyllactose (6'SL); lacto-N-neohexaose (LNnH); lacto-N-

neotetraose (LNnT); sialyllacto-N-neotetraose c (LSTc). The remaining four were identified based on mass alone and these unconfirmed structures are reported here as galactosyllactose 1 and 2 (GL1 and GL2), sialyl-lacto-N-triose (S-LN-Tri), and sialyl-lacto-N-neohexaose (SLNnH). See Table 2.1 for spectroscopy results.

Table 2.1 Porcine milk oligosaccharide structures identified in colostrum of Large White x Landrace sows

Compound name	Compound abbreviation	Retention time, min	Monoisotopic mass	Molecular weight	Structure (where known) ¹
2'-fucosyllactose	2'FL	5.63	488.17	488.44	
3'-galactosyllactose	3'GL	7.42	504.17	504.44	
3'-sialyllactose	3'SL	7.61	633.21	633.55	
6'-sialyllactose	6'SL	8.81	633.21	633.55	
Galactosyllactose 1	GL1	7.14	504.17	504.44	
Galactosyllactose 2	GL2	7.88	504.17	504.44	
Sialyl-lacto-N-triose	S-LN-Tri	8.99	836.29	836.75	
Lacto-N-neotetraose	LNnT	9.35	707.25	707.63	
Sialyllacto-N-neotetraose c	LSTc	10.40	998.34	998.89	
Lacto-N-neohexaose	LNnH	10.76	1072.38	1072.97	
Sialyl-lacto-N-neohexaose	SLNnH	11.10	1363.47	1364.22	

¹Diagrams as per Varki et al. (2015) as follows: yellow circle is Gal; blue circle is Glc; red triangle is Fuc; purple diamond is Neu5Ac; blue square is GlcNAc

Total oligosaccharide concentrations ranged from 0.827 g/L to 4.32 g/L with a mean value of 2.17 ± 0.764 g/L. The most abundant compound across all sows was 3'SL with mean concentration of 1.14 ± 0.498 g/L. The next three most abundant were all unconfirmed compounds; S-LN-Tri (0.282 ± 0.310 g/L), GL1 (0.268 ± 0.197 g/L), and GL2 (0.217 ± 0.0770 g/L). Six of the 11 identified compounds were present in particularly low concentrations (< 0.1 g/L); these were 6'SL, 2'FL, LNnT, LNnH, LSTc, and SLNnH. The concentrations of the identified PMO are presented in Figure 2.1. Exact concentrations of each PMO for each sow can be seen in Appendix B (Table 5.2).

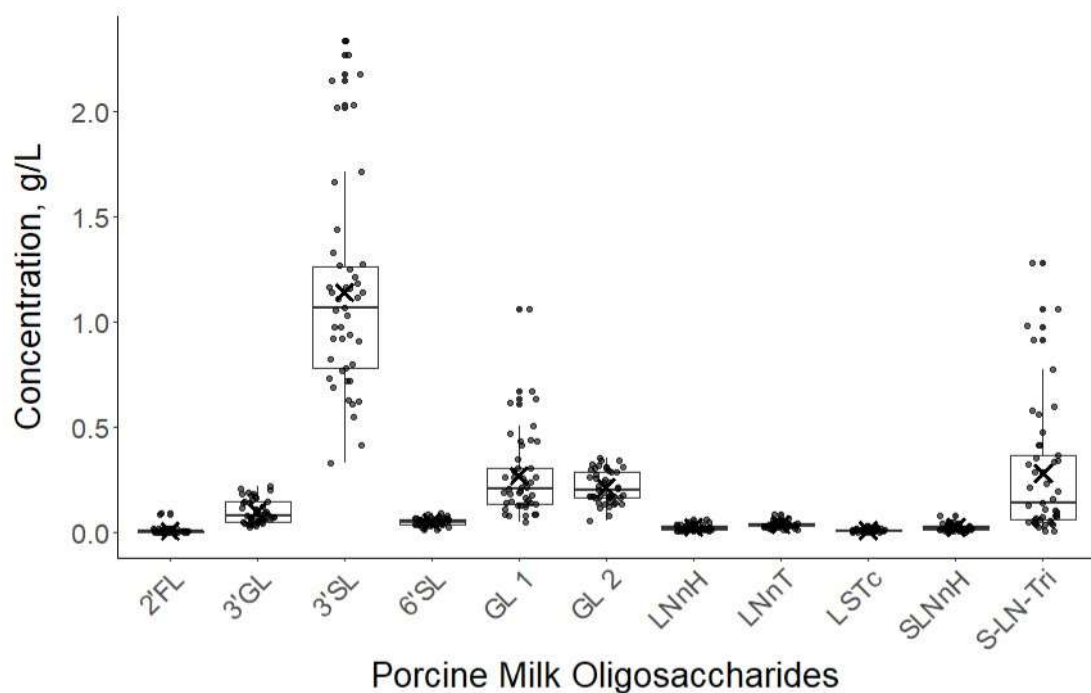


Figure 2.1 Variation in concentration (g/L) of each identified porcine milk oligosaccharide. Boxes display interquartile range, 'whiskers' show range, with line at the median and cross for mean. Abbreviations from left to right: 2'-fucosyllactose (2'FL); 3'-galactosyllatose (3'GL); 3'-siallylactose (3'SL); 6'-siallylactose (6'SL); galactosyllactose 1 (GL 1); galactosyllactose 2 (GL 2); lacto-N-neohexaose (LNnH); lacto-N-neotetraose (LNnT); sialyllacto-N-neotetraose c (LSTc); sialyl-lacto-N-neohexaose (SLNnH); sialyl-lacto-N-triose (S-LN-Tri). Concentrations calculated by DSM-Firmenich, statistics and figure by main author.

2.3.2 Associations Between Oligosaccharides and Performance

Performance variable averages are displayed in Table 4.

Table 2.2 Performance variable averages and spreads of data where average is mean and spread is standard deviation unless otherwise indicated

Variable	Average	Spread
BW1	1.46	0.37
BW7	2.59	0.67
BW28	7.96	1.97
Pre-weaning mortality	11.59	0.98
Litter size	19 ¹	16-21 ²
Parity	4 ¹	2-5 ²

¹Median value

²Interquartile range

At the multivariate level, colostrum composition was generally not associated with performance variables ($P > 0.1$), although pre-weaning mortality showed a marginal association ($F_{1, 43} = 2.87$; $P = 0.0560$); the relationships between colostrum composition and performance variables are illustrated in Figure 2.2.

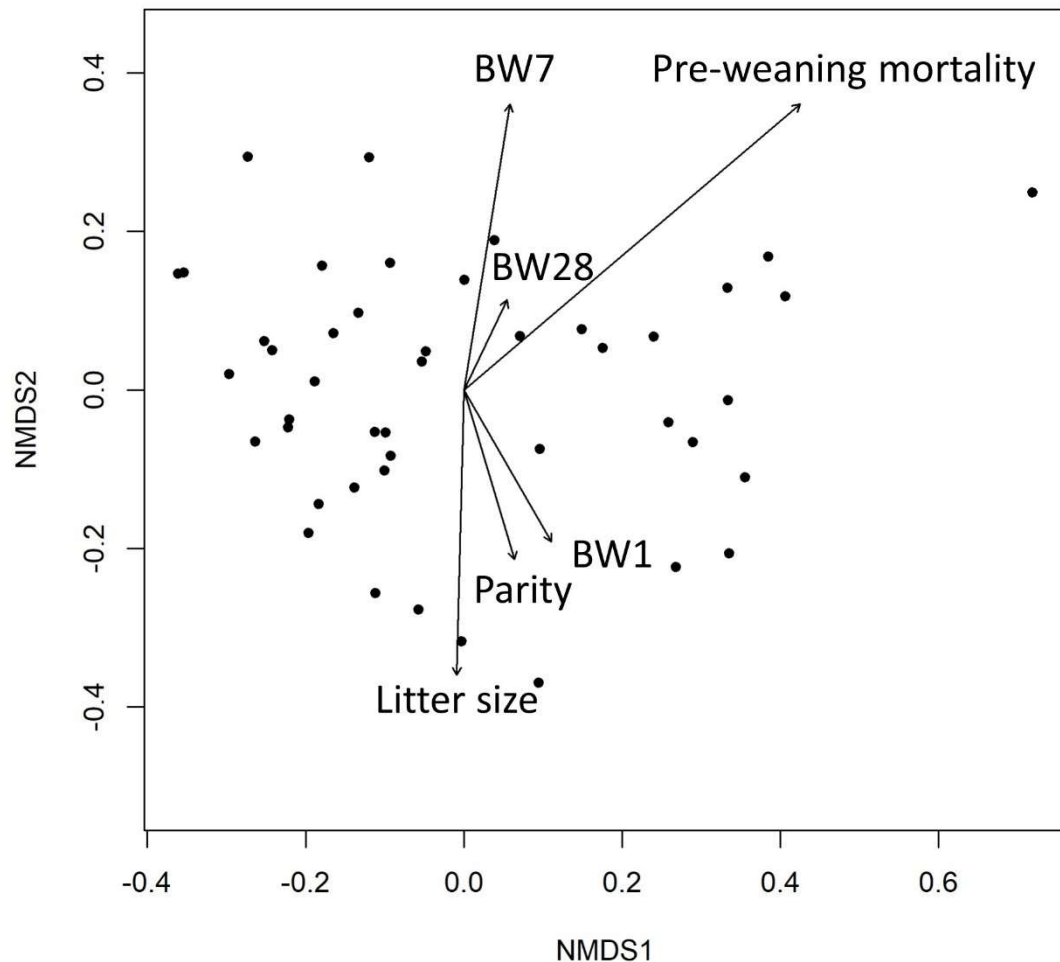


Figure 2.2 Non-metric multidimensional scaling (NMDS) plot of oligosaccharide profiles based on Bray-Curtis dissimilarities. Each point represents one sow's colostrum composition. Arrows indicate the direction and strength of associations between the variable and the NMDS axis. No variables were significant ($P > 0.05$); pre-weaning mortality showed a trend ($P = 0.0560$). Variable abbreviations: piglet bodyweight at day one (BW1); piglet bodyweight at day seven (BW7); and piglet bodyweight at day 28 (BW28)

Univariate analysis for individual oligosaccharide concentrations found no PMO to be significant predictors of BW1, BW7 or BW28 ($P > 0.1$), using mixed effect linear modelling (all values available in Appendix B; Table 5.3).

No PMO were significant predictors of pre-weaning mortality rate, although 2'FL concentration was nominally significant prior to FDR correction ($P = 0.0466$), with increase in 2'FL concentration associated with a decrease in pre-weaning mortality rate ($R^2 = 0.332$; $\beta = -0.510$).

Parity did not significantly predict any PMO concentration. Litter size was a significant predictor of LNnT concentration ($F_{1,43} = 11.85$; $t = -3.44$; $P = 0.0143$), with increased litter size predicting lower LNnT ($R^2 = -0.198$). All statistical results for sow-level parameters can be seen in Appendix B (Table 5.4).

Further testing on sow-level parameters showed significant effect of parity ($z = 2.62$; $P = 0.00869$) and litter size ($z = 2.95$; $P = 0.00323$) on prediction of pre-weaning mortality. Although both parity and litter size had positive main effects ($\beta = 1.29$ and 0.343 , respectively), their interaction term was negative ($\beta = -0.0600$; $P = 0.0208$), indicating that the increase in mortality rate associated with larger litter sizes diminishes at higher parities (see Table 2.2).

Table 2.3 Coefficients of tweedie model using sow factors ('effect') to predict pre-weaning mortality

Effect	Estimate	Standard Error	Z-value	P-value
Parity	1.29	0.492	2.62	0.00869
Litter Size	0.343	0.116	2.95	0.00323
Parity:Litter Size	-0.0600	0.0259	-2.31	0.0208

2.4 Discussion

Colostrum plays a vital role in neonatal health. The bioactive components of which (summarised in Table 1.2), serve as the first line of immune defence in the first few days after birth. Notably, colostrum yield is not influenced by litter size (Devillers et al., 2005; Devillers et al., 2007; Quesnel, 2011) which may result in inadequate intake for individual piglets in larger litters. Given the increased mortality rates observed in large litters (Canario, Cantoni, et al., 2006; Canario, Roy, et al., 2006; Rosendo et al., 2007), nutritional supplementation with milk replacers is often considered. However, current piglet milk replacers are suboptimal because they typically lack bioactive components such as prebiotics, which are routinely incorporated into human infant formulas and are important for neonatal gut health. To better inform the development of more optimal milk replacer, the present study aimed to quantify porcine milk oligosaccharides (PMO) in sow colostrum and investigate their associations with litter performance metrics.

Three of the four most abundant PMO identified were unconfirmed structures (GL1, GL2 and S-LN-Tri), reference to which has been identified in similar abundance in previous porcine literature (Mudd et al., 2016; Wei et al., 2018). The two isomers deemed 'galactosyllactose' (GL1 and GL2) are believed to have been identified in porcine samples across other studies (Tao et al., 2010; Salcedo et al., 2016; Wei et al., 2018) based on reported masses and abundances. These tri-saccharides are never discussed in depth due to lack of published knowledge on the structures. This may be due to humans lacking milk oligosaccharides with an α 1-3 linked galactose (which is assumed in the GL1 and GL2 here, based on the other porcine studies cited above) and it could be an allergen for human infants (Nguyen et al., 2005; Wilson et al., 2017). Diversity and inconsistency in analytical techniques and nomenclature contribute to this lack of functional knowledge of lesser known PMO, despite the obvious abundance and frequency in which they are identified. Consequently, comparison between studies is challenging as many rely on describing general numerical glycan structure (Hex-HexNAc-Fuc-NeuAc-NeuGc) with limited acknowledgement of isomers. Development of techniques and methodologies such as UPLC-MS/MS or multi-faceted approaches such as described in Hsu et al. (2018) will provide further identification that might enable better discussion regarding their importance in sow colostrum and milk. A recent advance has been made in creating a database for comparison of milk oligosaccharides between mammal species (Durham et al., 2023), but more than anything, this highlights the diversity and scope of unique structures that we need to understand the functionality of.

Prior to false discovery rate adjustment, 2'FL was a statistically significant predictor of pre-weaning mortality. After correction this was not statistically significant, likely due to lack of power and low average concentration (< 0.1 g/L), however this was a pilot study and it can be suggested that a larger sample size would yield significant results here, particularly with the biological relevance of a negative regression coefficient (-0.510), which would result in a decrease in mortality rate by roughly 40% (based on the Tweedie log link model) per unit (in this study g/L) increase of 2'FL. The low abundance is corroborated in other porcine studies (Mudd et al., 2016; Wei et al., 2018), and the negative association with pre-weaning mortality may be related to the reported benefits of 2'FL such

as increased proliferation of beneficial gut bacteria (Yu et al., 2013), improved cognitive ability (Oliveros et al., 2016), and reduced inflammation (He et al., 2016). These notable benefits have led to the commercial synthesis and inclusion of 2'FL as a prebiotic in human infant formula with much success (Vandenplas et al., 2018; Reverri et al., 2018). The economic value of PMO intervention in commercial pig production should be considered in relation to the magnitude of the production benefit obtained. Pre-weaning mortality represents a substantial economic loss to commercial producers, but the economic value of reducing mortality is dependent on production costs, litter size, piglet value and the magnitude of the mortality reduction achieved. For example, an economic analysis of interventions targeting piglet mortality estimated that a one-percentage-point reduction in pre-weaning mortality corresponded to an intervention cost of approximately €0.5 per piglet, although this varied according to production conditions (Stygar et al., 2022). If supplemental use of 2'FL was able to mimic a unit increase as per the results from this chapter (increase of 1 g/L associated with 40% reduction in mortality), it would support economic feasibility of 2'FL on commercial farms.

In humans, the abundance of 2'FL is linked to secretor and Lewis genes for blood typing (Bode, 2012). Briefly, those expressing secretor genes at the mammary gland can produce fucosylated milk oligosaccharides and although no blood testing in these sows is available yet to confirm this, it is a likely explanation for the low concentrations seen. Based on how vast and positive the literature is surrounding 2'FL, and the present preliminary results suggesting a possible effect on pre-weaning mortality, 2'FL may be an obvious compound to target for inclusion in milk replacer formula for piglets to support gut health through its prebiotic activity, particularly when sows generally appear to produce such low concentrations naturally (Mudd et al., 2016; Wei et al., 2018). Work assessing 2'FL as a prebiotic supplement has begun using a continuous *in vitro* model based on work by Davis Birch et al. (2023), adapted for modelling the piglet weaning transition (Moura et al. *in prep*). The aim of this preliminary study is to determine if 2'FL supplementation in the model facilitates better recovery from a carbapenem-resistant *Klebsiella pneumoniae* infection. Studying the effects of 2'FL using a pathogen challenge provides greater insight into its biological efficacy than assessment of changes to the baseline microbiome alone. In an

undisturbed system, the existing microbial community may already be relatively stable and functionally competent, limiting the extent to which a treatment can induce detectable changes. A pathogen challenge provides an opportunity to assess whether the treatment alters the susceptibility of the microbiome to disruption, its ability to resist pathogen establishment, and/or its capacity to recover. Conducting this work *in vitro* to establish preliminary efficacy, reduces the need for pathogen challenge studies in healthy piglets, thereby addressing important ethical considerations associated with experimental infection and the potential for morbidity or mortality.

Modelling parity as a predictor for the PMO in colostrum did not produce significant results. Parity may not have been a strong enough predictor because it was not a balanced factor in this study due to the opportunistic sampling method, however, other studies concur that parity is not a significant effect regarding PMO (Wei et al., 2018; Trevisi et al., 2020). Wei et al., (2018) do report differences in transitional and mature milk, but much less so in the colostrum of gilts compared to sows. Trevisi et al., (2020) used a mixed breed population and saw no influence of parity, despite inclusion of gilt samples. Gilts were excluded from the present study due to concerns they produce less colostrum than multiparous sows (Beyer et al., 2007). Gilts have less developed mammary tissue and mammary DNA (Nielsen et al., 2001), and there is evidence of differences in colostrum composition between primiparous and multiparous sows, such as fat content (Luise et al., 2018; Amatucci et al., 2022). Supplementation of multiparous colostrum to gilt progeny has been shown to reduce the high variability in bodyweights seen in gilt litters and reduce the need for antibiotics (Miguel et al., 2021). However, the majority of literature confirms the main difference to be regarding yield rather than composition (Hansen et al., 2006; Craig et al., 2019; Nuntapaitoon et al., 2020). Yield differences may still explain the benefits observed by Miguel et al. (2021), as the effect appeared to stem from the provision of additional colostrum itself, rather than it being specifically derived from multiparous sows. Lack of parity effect seen in the present study is therefore expected.

Litter size was found to be a significant predictor of LNnT concentration with the more prolific sows producing less LNnT in their colostrum. Though this suggests some degree of maternal control, the negative association does not

support a simple adaptive model. Further possibilities include outpaced physiological optimisation, and energy cost trade-offs. The former implies that selection for larger litters has accelerated beyond the mammary gland's adaptive capacity, resulting in suboptimal colostrum composition relative to litter sizes. The latter is logical given the increased energy costs associated with gestating a larger than average litter, as this may lead to prioritisation of lactose and macronutrient synthesis over PMO such as LNnT. While speculative, these possibilities suggest that litter size-associated variation in LNnT may arise from systemic maternal regulation.

The reduction in LNnT concentration seen in larger litters could be a substantial finding due to evidence suggesting how beneficial LNnT is to the immature gut and concerns regarding mortality rates in large litters; also evidenced in this study where every additional piglet resulted in a 0.343% increase in pre-weaning mortality, regardless of parity. The benefits of supplementing LNnT to piglets have been shown through reduction in porcine rotavirus replication *in situ* (Hester et al., 2013) as well as reduction in duration of diarrhoea in rotavirus infected piglets (Li et al., 2014). Recently, the structural isomer of LNnT, Lacto-N-tetraose (LNT), has been added to UK human infant formula (Anon, n.d.) following the approval of it in Europe as a novel foodstuff (EFSA et al., 2019). The inclusion of LNT rather than LNnT may stem from reports of LNT being a significant human milk component going back to the 1950s (Kuhn, 1958). Human milk contains both isomers (Soyyılmaz et al., 2021), while it is hypothesised here that sow colostrum contains only LNnT as previous porcine studies either confirm absence of LNT or cannot distinguish between isomers due to methodology limitations (Mudd et al., 2016; Salcedo et al., 2016; Wei et al., 2018).

The difference in structure between the isomers is the linkage type on the terminal galactose (Gal) being β 1-3 or β 1-4 for LNT and LNnT, respectively. Importantly, both contain the N-acetylglucosamine sugar linked to this terminal Gal which is valued due to having the 'bifidogenic effect'; meaning this encourages growth of *Bifidobacterium spp.* (Cordeiro et al., 2023; Li et al., 2023), many of which are beneficial to the human and piglet gut alike (Pang et al., 2022; He et al., 2023). In human breastfed infants, *Bifidobacterium spp.* typically dominate the gut microbiota and are strongly associated with HMO utilisation

(Sela and Mills, 2010; Zhang et al., 2021). Piglets generally have lower baseline *Bifidobacterium spp.* abundance than human infants (Wang and Donovan, 2015) and it is important to acknowledge that the ecological relevance of bifidogenic effects will therefore differ between the species. Although the functional consequences of LNT on *Bifidobacterium spp.* proliferation in piglets may not be parallel to those observed in humans, benefits reported following probiotic supplementation of *Bifidobacterium spp.* suggest that promoting these species may nonetheless contribute positively to gut health in the piglet (Hansen et al., 2022; Hansen et al., 2025).

Additionally, N-acetylglucosamine has been shown to enhance multiple gut barrier functions in a mouse model, such as increasing expression of tight junction proteins and reducing loss of mucin-secreting areas (Choi et al., 2023). The immunomodulatory and prebiotic effects of LNT could be harnessed through addition of LNT to a supplemental milk formula to reduce pre-weaning mortality rates via improved piglet gut health and immunity. Litter size was not a predictor of any other individual PMO, nor was it significantly associated with colostrum composition, which aligns with other findings suggesting colostrum quantity is not impacted by number of piglets (Devillers et al., 2005; Devillers et al., 2007; Quesnel, 2011). It is an interesting phenomenon and affirms the need for supplemental milk in large litters. Therefore, LNT is a PMO of interest for developing supplemental milk for piglets which may improve mortality outcomes on farms.

Lack of effects for longer term performance parameters such as BW7 and BW28 was expected as mature milk is the largest provision of nutrients after the first day of lactation and so lasting performance effects from colostrum would be exceptional. Colostrum was analysed in this study due to the pilot nature of this work and the practical and ethical simplicity relative to collecting mature milk, which often requires injection of oxytocin (Tao et al., 2010; Mudd et al., 2016). Further research should certainly explore litter performance traits associated with PMO concentrations in mature milk at various points in lactation to fully understand how these structures affect piglet health and performance longitudinally. This work could additionally incorporate the study of teat fidelity, as samples in the present study were pooled to reduce teat influence. Quality differs between teats (Ogawa et al., 2014) and there is already evidence

suggesting anterior teat sucklers weigh more at weaning than piglets suckling posterior teats (Dierking et al., 2025; Mata et al., 2025). Furthermore, piglets show loyalty to a preferred teat throughout the suckling period (De Passillé et al., 1988), as well as awareness regarding teat productivity (Devillers et al., 2016), therefore links between teat selection and piglet performance would be interesting to explore.

The quantification method used in the present study was based on work by Molnár-Gábor et al. (2024) with modifications made to adapt the protocol to use of colostrum. Total solids, fat and lactose values can be similar between milk and colostrum, depending on lactation period (Hurley, 2015), but one of the largest dissimilarities between the matrices is the immunoglobulin content, which is significantly higher in colostrum (Hurley and Theil, 2013). During sample preparation, proteins are precipitated out of the matrix using acetonitrile, with samples being processed once the supernatant is clear enough, thus minimising risk of any contamination. It is generally accepted to prepare samples in a similar manner as long as the matrices are broadly similar, for example, colostrum, mature milk, or other liquid feeds (Tao et al., 2009; Fischer-Tlustos et al., 2020; Bunyatratchata et al., 2021). Furthermore, identically prepared cross-species milk samples are often compared without performing methodological changes for each matrix (Albrecht et al., 2014; Urrutia-Baca et al., 2023), despite milk compositional differences having been reported between species (Roy et al., 2020). The derivatisation procedure in the present study relies on the reaction between the labelling agent (benzocaine) and the formyl group of each PMO. Labelled PMO are then separated via HPLC using a technique that favours hydrophilic compounds. Interference using this method is low, as compounds would need to meet all the following criteria to pose a risk: remain in the aqueous supernatant during sample preparation; contain a formyl group or coincidentally absorb at the wavelength used; and be sufficiently hydrophilic to be retained on the chromatographic column to an extent comparable to PMO. Therefore, the analytical method should be broadly applicable across various milk-adjacent substrates.

Beyond agricultural relevance, the neonatal pig is widely recognised as a translational model for human infant nutrition and development. Pre-clinical studies using piglets have demonstrated that supplementation with milk

oligosaccharides, including 2'FL and LNnT, can modulate gut microbial composition (Li et al., 2014; Wang et al., 2021), enhance intestinal barrier function (Berding et al., 2016; Yin et al., 2019), and impact aspects of neurodevelopment (Wang et al., 2007). These findings contribute to the evidence base supporting the inclusion of these compounds in milk formula. In this context, characterising the natural variation of PMO and their associations with piglet outcomes not only informs swine production but also strengthens interpretation of the pig as a biomedical model for early life nutritional programming.

In conclusion, porcine milk oligosaccharide (PMO) concentrations are highly individual to each sow, regardless of parity, despite controlled breeding, feeding and environment. No PMO significantly impacted bodyweights at any time point. Higher 2'FL concentration may reduce pre-weaning mortality, but a larger powered study is necessary to confirm this. Concentration of LNnT was negatively associated with litter size, suggesting this may be a good target for supplementation given the beneficial effects reported in literature and the increase of pre-weaning mortality with litter size. Both 2'FL and a structural isomer of LNnT have been included in human infant formula with success therefore consideration should be made for the pig industry to adopt similar advances.

Chapter 3 Bottled Piglets: Developing an *In Vitro* Model of the Pre-weaning Piglet Colon Using a Batch Fermentation Protocol

3.1 Introduction

The human gut microbiome has become a popular area of interest due to its association with a wide range of illnesses including cancers (Yang et al., 2021; Mishra et al., 2024; Lachance et al., 2024), antibiotic resistant infections (Langdon et al., 2021; Fishbein et al., 2023), and mental health disorders (Li et al., 2022; Radjabzadeh et al., 2022; Zhou et al., 2024). Optimising ‘gut health’ to reduce these pathologies is an ambiguous aim due to historic lack of definition regarding what gut health means. The ISAPP has defined gut health as “a state of normal gastrointestinal function without active gastrointestinal disease and gut-related symptoms that affect quality of life” (Marco et al., 2026; ISAPP, 2026). The gut microbiome is one of six proposed metrics indicating gut health status as assessed through microbiome analysis and short-chain fatty acid production, however, the authors acknowledge the lack of consensus on what is ‘normal’ (Marco et al., 2026). In piglets, the colonisation of the gut during the pre-wean period can have life-long health implications (Li et al., 2018a; Li et al., 2019). This, in conjunction with high pre-wean mortality rates, has led to investigations into additional supplementation to support piglet gut health, such as isotonic protein solution (Gong et al., 2025), prebiotics (Herfel et al., 2011; Yin et al., 2019), and probiotics (Hansen et al., 2022; Hansen et al., 2025).

Health and activity of the gut microbiome is an invasive and ethically challenging area to explore, due to the internal location and requirement for dissection or fistulation to collect samples, which has led to the development of *in vitro* models. These models aim to simulate the luminal environment to enable experimental testing while addressing the principles of the 3Rs (Russell and Burch, 1959; NC3Rs, 2026) for ethical animal research. Use of *in vitro* systems can reduce the number of animals being used when deployed in conjunction with animal trials, offers a way to refine protocols prior to testing in animals, and aims to replace the need for animal models entirely in some contexts. The published protocols for *in vitro* hindgut systems are broad and varied, often lacking in justification of methodology, leading to a gap in knowledge regarding the optimal protocol for an *in vitro* pre-wean piglet colon model.

3.1.1 Dynamic and Continuous *In Vitro* Fermentation

With technological advancement, models have become dynamic to better replicate the spatial and longitudinal capacity achievable in *in vivo* studies. There are many different dynamic models published (reviewed by Dupont et al. (2019)), some of which are single-stage, encompassing one bioreactor, such as PigutIVM (Fleury et al., 2017), and others are multi-stage models including the SHIME (Molly et al., 1993), Baby-SPIME (Dufourny et al., 2019), and PolyFermS (Tanner et al., 2014). Although more accurate at replicating the hindgut environment, dynamic models can be costly and inaccessible, requiring large and unique equipment with high labour requirements and specialist training. A model that aimed to reduce these considerations was the MiGut platform (Davis Birch et al., 2023). While MiGut addresses scalability, aiming to match accuracy of multi-stage models with the throughput capacity of batch fermentation, each run still requires several weeks of input. Reaching the number of biological replicates that are possible using batch systems necessitates more space and more investment in vessels, ultimately making it remain a costly investment, relative to batch systems.

The multi-stage dynamic models are most accurate when it comes to simulating *in vivo* data, however these will never achieve the throughput capacity of batch models, and both have their place. Justification for using batch fermentation protocols include the shorter run times and higher throughput, both of which are priorities for screening. Screening is a necessary and time-saving aspect of developing nutritional or pharmacological interventions. Using a complex model requiring weeks of stabilisation to screen would be inefficient, and perfect accuracy is not always a priority for the screening process. This allows for batch fermentation models to excel as a strategy to efficiently test varying concentrations or combinations of treatment to determine optimal methods before running the more time-consuming, costly, and labour intensive dynamic model (or animal model).

3.1.2 Batch Fermentation

Batch fermentation models are briefly designed to mimic static microbial fermentation of a particular substrate (often fibre-based), by providing a starting population of microbes from faecal samples, or, in rarer cases, fresh colonic

contents. As they are a static model, typical incubation time for batch systems is 24 hours (Coles et al., 2005). Despite 24 hours not always being an accurate representation of transit time, depending on the focal species, longer incubation periods can result in build-up of metabolites and subsequent pH drop due to it being a closed system. These effects can alter the fermentation pattern and microbial composition, leading to low longitudinal accuracy (Moon, Li, et al., 2016).

The faecal samples collected are usually pooled to eliminate individual variation and homogenised into a slurry using a buffer for ease and consistency when inoculating the model. Faecal samples are most often used to inoculate models due to the practical and ethical challenges of sourcing fresh gut contents, but despite subtle differences in the microflora, it is generally accepted that faecal samples are a suitable alternative to colonic contents for monogastric research (Dung and Udén, 2002; Bindelle et al., 2007).

The fermentation of the substrate can be measured by various aspects including dry matter degradation, gas production, or output of bi-products such as short chain fatty acids (SCFA). Gas production techniques are not mutually exclusive to batch fermentation, but do offer the ability to analyse fermentation kinetics at various stages of the process (Theodorou et al., 1994). Monitoring gas production is cost effective, non-destructive and gives a direct indication of microbial activity and substrate digestibility.

3.1.3 Porcine Modelling

Once in the porcine-specific sphere of models, there is still limited research focussing on pre-wean piglets, with many studies investigating weaned nursery piglets or growing pigs (Williams et al., 2011; Mikkelsen et al., 2011; Bai et al., 2020; Bai, Zhou, et al., 2021). However, the majority of piglet losses occur in the first few weeks of life (Edwards and Baxter, 2015), when piglets have immature immune systems and are at their most vulnerable. Studies *in vivo* highlight how rapidly and significantly the gut environment changes in the first few weeks of life (Liu et al., 2019), creating barriers to developing an adequate static *in vitro* model. At this point it must be recognised that continuous *in vitro* systems have been published that have been geared towards the younger pig but again, these study

post-wean pigs (Fleury et al., 2017) or model the weaning transition (Dufourny et al., 2019; Gresse et al., 2021), rather than early-life.

3.1.4 Inconsistencies Across Published Protocols

There are many batch fermentation protocols published that aim to mimic the pig hindgut (Smiricky-Tjardes et al., 2003; Vester Boler et al., 2009a; Li et al., 2012; Tanner et al., 2014; Fleury et al., 2017; Uerlings et al., 2019; Gresse et al., 2019; Palowski et al., 2021; Bai, Wang, et al., 2021; Nissen et al., 2022), however, each study varies in methodology, with many based on historical rumen studies (Menke and Steingass, 1988), which are not always translational. As outlined by Coles et al. (2005), there are several shortcomings consistent across most studies including limited or no justifications for parameters and conditions used, absence of method standardisation, no validation with *in vivo* data, and lack of variety in substrates tested.

3.1.4.1 Use of Redox Agents

In addition to the basic formula of substrate, buffer and inoculum, extra nutrients or redox agents can be added but this is at the discretion of each research group and not consistent across studies. Some of the more frequently added reagents are L-cysteine hydrochloride (hereafter L-cysteine) or sodium sulphide (Menke and Steingass, 1988; Theodorou et al., 1994), both of which are added for their reductive capabilities, as maintaining an anaerobic solution for hindgut models is imperative. Reducing agents can, however, interfere with a number of factors either directly; for example, microbial activity (Fukushima et al., 2003); or indirectly through production of metabolites, which then effects accurate quantification of metabolites of interest (Poelaert et al., 2018), as well as disrupting cell function (Andriamihaja et al., 2010; Medani et al., 2011); relevant if using tissue culture techniques downstream. In light of these drawbacks, Poelaert et al. (2018) evaluated whether reducing agents enhanced model performance using an *in vitro* gas production method and concluded that they conferred no measurable benefit. Regardless, reducing agents are still a popular addition to batch and continuous fermentation models (Palowski et al., 2021; Pérez-Burillo et al., 2021; Nissen et al., 2022).

3.1.4.2 Stabilisation Periods

In vitro fermentation systems which run for over 48 hours (mostly the continuous systems) often have a period of stabilisation prior to any experimental testing taking place (Tanner et al., 2014; Fleury et al., 2017; Dufourny et al., 2019; Davis Birch et al., 2023). These periods allow the microbiota to adapt to the artificial conditions and reach a pseudo-steady-state prior to experimental intervention, which is likely to shift the community. The duration of these adjustment periods vary across model types and study aims, and justification for the time period chosen is usually lacking. Typically a stabilisation period ranges from as little as 72 hours for specific colonisation (Tanner et al., 2014), to 7-14 days. Shorter periods have been shown to be sufficient in some continuous models to minimise variation in microbial composition and metabolite production, representing a practical compromise between experimental time and system stability (Fleury et al., 2017). However, 14 days is most commonly used to ensure stable and dense community structure and metabolic outputs (Van de Wiele et al., 2015; Dufourny et al., 2019; Davis Birch et al., 2023). Extending stabilisation beyond 14 days could hypothetically enhance ecological stability and reproducibility further, but will also increase experimental cost and time which is likely why studies do not exceed 14 days. Conversely, insufficient stabilisation may result in ongoing microbial succession and community instability, as *in vitro* communities can undergo significant compositional shifts and emergence of previously low abundance taxa during early adaptation phases (Van de Wiele et al., 2015; Dufourny et al., 2019; Davis Birch et al., 2023). Importantly, evidence from dietary adaptation in sows suggests that microbial functional adaptation can exceed 19 days, particularly for complex substrates, highlighting that shorter periods may not fully capture steady-state functionality (Sappok et al., 2015). Furthermore, in an *in vitro* rumen system, microbial responsiveness to feed additives was improved when cows were adapted to the same compound prior to rumen fluid collection (Rossi et al., 2025). Therefore, choice of stabilisation length represents a trade-off between achieving ecological realism and maintaining experimental feasibility, with longer periods improving stability and representation while shorter periods favour throughput and cost-effectiveness.

In batch fermentation systems (short-term, often 24 hours), the concept of a stabilisation period is fundamentally different from that used in continuous models because the system does not reach a true steady-state, nor is that the

aim of these methods (Coles et al., 2005; Moon, Li, et al., 2016). Batch fermentation relies on tightly controlled inoculum preparation to minimise variability. It may be of use to include a brief equilibrating period after inoculation to allow microbial recovery after handling and adaptation to the anaerobic conditions before measurements begin, particularly if inoculum has been frozen prior to use (Poppe et al., 2023). This could reduce artefacts associated with lag-phase metabolism, and reduce variability caused by oxygen exposure or shock. It could, therefore, enhance standardisation of conditions by pre-incubating faecal slurries under anaerobic conditions prior to substrate addition, effectively separating microbial recovery from substrate fermentation.

Overall, in batch models, stabilisation is best viewed as a brief microbial recovery period, rather than true ecological stabilisation of the community structure, with a trade-off of improving reproducibility and reducing artefacts but compromising ability to capture early fermentation kinetics and rapid microbial response.

3.1.4.3 Buffer Composition

In batch fermentation models, buffer composition plays a pivotal role in shaping microbial activity, metabolite production, and the physiological relevance of the system. Simple phosphate buffers have been used historically due to their effective pH control and experimental simplicity (Barry et al., 1995; Edwards et al., 1996); however, their function is largely restricted to maintaining pH stability, offering little in the way of essential nutrients required by the diverse and metabolically demanding colonic microbiota. As a result, such simplified buffers may limit microbial growth, reduce activity and fail to accurately reproduce *in vivo* fermentation patterns. In contrast, nutrient-rich media is often used for continuous systems to avoid substrate limitation, sustain anaerobic conditions, and support microbial metabolism (Fleury et al., 2017; Dufourny et al., 2019; Davis Birch et al., 2023), but this is more laborious, costly, and arguably not necessary for short-term models such as batch fermentation systems (Moon, Li, et al., 2016).

A compromise may be a more complex mineral-salt composition than phosphate buffer, but a composition that omits the additives used for continuous systems. One example of this was brought forth by Mould et al. (2005), who concluded that additional nutrients were unnecessary as long as the main

components of carbonate and phosphate salts were appropriate. Despite being developed for rumen batch fermentation, buffers of similar composition have been used successfully in porcine models (Palowski et al., 2021). The most critical development of this from the historic phosphate buffer is the addition of carbonates (bicarbonate). While phosphate buffers provide stable pH control, they do not reflect the dominant buffering system needed in the colon, where the bicarbonate-carbon dioxide equilibrium plays a central role in maintaining luminal pH under anaerobic conditions (Buck and Levin, 2011). Carbonate-based buffers more closely replicate this by establishing a dynamic equilibrium with dissolved CO₂, thereby enabling continuous pH regulation in response to microbial metabolite production. This is particularly important in a closed system, such as batch fermentations, where accumulation of SCFA would otherwise rapidly decrease pH and inhibit microbial activity. Evidence from *in vitro* rumen fermentation demonstrates that bicarbonate supplementation increases total SCFA production and alters microbial composition, reflecting improved metabolic activity and ecological stability (Mao et al., 2017). Mechanistically, this is linked to the role of bicarbonate as both a buffering agent and a substrate in microbial metabolic pathways, including carboxylation reactions and cross-feeding interactions that underpin SCFA production (Erb, 2011; Venkata Mohan et al., 2017).

3.1.4.4 Incubation Period

As mentioned earlier, batch fermentation systems are limited in their long-term capacity due to build up of metabolites and reduction of nutrients which can cause pH shifts that compromise the system and influence the microbial communities. Incubation periods up to 72 hours have been reported (Palowski et al., 2021), however it should be acknowledged that this study was focussing on digestibility and macronutrient degradation, and did not perform microbial analysis. Most studies confine incubation up to 24 hours, but there is still much variation between methods with some terminating at 12 hours (Smiricky-Tjardes et al., 2003; Vester Boler et al., 2009a; Li et al., 2012), at 14 hours (Dufourny et al., 2019), or at 24 hours (Fleury et al., 2017; Nissen et al., 2022).

Given the lack of consensus in the literature regarding the optimal incubation period, systematic assessment of multiple timepoints is needed. Due to the closed system of batch fermentations, it is not advised to incubate far

beyond substrate depletion, however, to determine full impact of experimental conditions on fermentation kinetics, achieving the plateau phase could be optimal. Both fermentation kinetics and microbial analysis would therefore be needed to help identify the balance between complete fermentation and compromising the microbiota.

3.1.4.5 Addition of Mucin

Another contentious and inconsistently added nutrient are mucins; complex glycoproteins found throughout the digestive system. The primary role of mucins in the hindgut is physical protection of intestinal cells from a range of hazards including pathogenic bacteria, toxins and enzymes. This is done through the laminated inner mucus layer which is anchored to goblet cells and acts as a filter to block pathogens from penetrating; and a more mobile outer mucus layer, able to detach from the epithelium and remove pathogens and debris through peristalsis (Hansson, 2019; Paone and Cani, 2020). While significant *in vivo*, these functions are not valuable in *in vitro* batch models due to the absence of intestinal cells present and closed configuration that lacks intestinal flow. Conversely, the outer mucus layer does also provide an attachment site and source of nutrients for the gut microbiota (Van den Abbeele et al., 2012; Paone and Cani, 2020); roles which may have translational benefits in a batch fermentation and therefore warrant consideration.

A distinct mucosa-associated microbial community has been reported (Zoetendal et al., 2002; Macfarlane, 2008), with speculation that this specific community plays an integral part in pathogen prevention and immune modulation (Van den Abbeele et al., 2012). Several methods to include a mucosal surface have been deployed previously, often based on various bead-like structures: addition of mucin covered microcosms to the vessel (Van den Abbeele et al., 2012; Tran et al., 2016) and immobilisation of the faecal microbiota through gel beads (Cinquin et al., 2004; Cinquin et al., 2006). Additionally, some simply add mucin directly to enrich the media (A. Buckley, *personal comms.*, Oct 2023; Davis Birch et al., 2023). Regardless of method of application, mucin is not included as standard across *in vitro* batch fermentation models (Moon, Li, et al., 2016; Pérez-Burillo et al., 2021) and requires further investigation.

3.1.4.6 Foregut Digestion Prior to Fermentation

Many published studies using an *in vitro* fermentation system do not incorporate any methodology that would mimic foregut digestion (Smiricky-Tjardes et al., 2003; Vester Boler et al., 2009a; Li et al., 2012; Nissen et al., 2022). By eliminating this phase, those studies by default do not discuss or justify this choice, allowing for speculation by the reader. One justification for adding substrate into a hindgut model without a foregut phase is use of very fibrous feedstuffs, such as high lignin content, which would not be affected by foregut hydrolysis. Another reason to discount the foregut phase could be when modelling a specific environment with limited *in vivo* foregut activity, such as a neonate or agastric species.

As well as the INFOGEST method already discussed (Minekus et al., 2014; Brodkorb et al., 2019), commonly cited methods to simulate foregut hydrolysis include the works of Boisen and Fernandez (1997) or Bindelle et al. (2007); the latter of which used the former's methods. Briefly, this involves dilution of the substrate with a phosphate buffer, pH adjustment to 2.0, and addition of pepsin before incubation while stirring for two hours. After the gastric phase, further phosphate buffer is added, followed by a pH adjustment to 6.8, addition of pancreatin, and further agitated incubation for four more hours. Several variations of this basic method exist including where the phosphate buffer is supplemented, increasing complexity and better mimicking electrolyte solutions in the body (as seen in INFOGEST); further components are added to assist substrate degradation such as salivary amylase, gastric lipase and bile; incubation periods are altered to reflect the transit time of the chosen species.

One key example of altering the foregut protocol is Ménard et al. (2018) who aimed to create a static infant digestion model. This study was inspired by the consensus published for the adult stage (Minekus et al., 2014) and was heavily informed by reviews which compiled infant digestive physiology data (Abrahamse et al., 2012; Bourlieu et al., 2014). To summarise, no oral phase was proposed due to liquid feed spending minimal time in the mouth, simulated fluids (buffers) were adjusted to reduce complexity and strength, enzymes were added in reduced activity/concentration, pH was increased, and incubation time was reduced. To the authors knowledge, nothing like this exists for the piglet, the closest models being the dynamic PigutIVM (Fleury et al., 2017) or baby-SPIME (Dufourny et al., 2019), neither of which look at piglets younger than weaning.

It should be acknowledged that provision of creep feed during the pre-weaning period complicates methodology design. The work by Ménard et al. (2018) proposed no oral phase for the human infant, however, in the case of the piglet, although much of the diet is liquid, often a plant-based feed is provided towards the end of the suckling period. This additional feed is given with the aim to increase feed intake post-weaning (Muns and Magowan, 2018), stimulate physiological adaptation to this type of diet, and generally boost piglet growth prior to abrupt weaning (reviewed by Muro et al. (2023)). The addition of plant-based materials in the pre-wean period suggests that a foregut simulation may be an important addition to an *in vitro* piglet fermentation model, particularly if exploring the effects of creep feed on the gut microbiota.

3.1.5 Aims

This study aimed to test variable parameters in existing batch fermentation methods to optimise an efficient protocol for modelling the pre-wean piglet colon *in vitro*. Each parameter was tested through consecutive experiments laid out in this Chapter. The benefits of developing a batch *in vitro* model include high-throughput, increasing efficiency for screening, and accessibility, reducing cost and labour intensity associated with continuous models (Davis Birch et al., 2023). Furthermore, the pre-wean period is across 21-28 days in commercial farms and encompasses temporal shifts in microbiota (Everaert et al., 2017; Liu et al., 2019; Trevisi et al., 2021). However a model of this age group is currently lacking in the literature. Therefore the goal for this model development was not an exact simulation of a pre-wean piglet colon, but a standardised protocol that upheld general indicators of good gut health, as previously documented in pre-wean piglets, such as, enhanced butyrate production (Gaudier et al., 2004; Hatayama et al., 2007), stable pH around 6.5 (Li et al., 2012; Gresse et al., 2021), and high prevalence of commensal bacteria such as *Bacteroides* (Kubasova et al., 2017; Chen et al., 2018).

Experiment 1 tested the addition of L-cysteine, inclusion of a boiling step, and timing of slurry inoculation in a 2 x 2 x 2 multifactorial design to assess each main effect and any interactions. It was hypothesised that there may be an interaction between L-cysteine and boiling due to previous reports that L-cysteine is heat dependant (Pokorný et al., 2023), and interactions were also expected between L-cysteine and inoculation timing due to denaturing of the compound

overnight. It was expected that L-cysteine and boiling when used together would enhance anaerobiosis of the media and therefore be optimal, with the inoculation timing being optimal immediately before incubation. Inoculating the media overnight to induce a pseudo-adaptation period is hypothesised to result in reduced fermentation kinetics and increase in abundance of opportunistic pathogens due to the lack of nutrients provided in the media.

Experiment 2 tested buffer composition and incubation period in a 2 x 4 multifactorial design. Two buffer compositions, a simple phosphate buffer, and a more complex phosphate-carbonate buffer, were used across 8, 10, 12, and 24 hour incubation periods. Using a simple phosphate buffer was popular historically (Barry et al., 1995; Edwards et al., 1996) and is still used in slurry preparation for *in vitro* fermentation (Moon, Joo, et al., 2016; Dufourny et al., 2019; Loo et al., 2023). Incubation period can also heavily impact labour intensity, as well as being critical in a closed system to avoid build-up of metabolites and substrate depletion, however, literature ranges from 8-24 hours (Li et al., 2012; Dufourny et al., 2029; Nissen et al., 2022). It was hypothesised that the carbonate buffer may provide better buffering capacity than a simple phosphate buffer but that this would not significantly impact microbial populations, whereas the community composition would shift significantly between 12 and 24 hours for both buffer conditions.

Experiment 3 tested the addition of porcine mucin to the media. It was hypothesised that the mucin would not impact fermentation parameters, but would capture microbe populations previously unseen in other experimental results due to mucin specific communities existing (Zoetendal et al., 2002; Macfarlane, 2008).

Experiment 4 assessed whether simulated foregut digestion was necessary when modelling the pre-wean piglet due to the immaturity of the foregut in such a young mammal (Abrahamse et al., 2012; Bourlieu et al., 2014). This experiment used piglet milk replacer and colostrum as substrates but no interactions between main effects were modelled. It was hypothesised that the foregut digestion would not impact measured parameters in either substrate significantly, but may dampen fermentation activity of milk replacer as this substrate is more readily fermentable.

Experiment 5 aimed to validate the protocol developed thus far by analysing colonic digesta and fresh faecal samples from sacrificed piglets of varying ages and identifying conserved differences when passed through the batch fermentation model. It was hypothesised that fermentation of the substrate would shift microbial communities, but some pre-fermentation differences would be conserved.

3.2 General Chapter Methods

3.2.1 Faecal Sampling

All animal practices abided by regulatory bodies detailed in Section 2.2. All procedures reviewed and approved by the named veterinary surgeon for the National Pig Centre, University of Leeds (Appendix C). All animal husbandry was as described in Section 2.2.1.

Faecal samples were obtained from Large White x Landrace x Duroc piglets at the National Pig Centre between 5 and 27 days old, still nursing and housed with the sow. Faecal samples were freely collected upon defecation into 50 mL screw top centrifuge tubes (Fisher Scientific, Leicestershire, UK) and stored on ice at location before being transported to the lab where they were stored at -80°C until use. Samples were collected as fresh as possible but contamination with the pen floor was not avoided. Sampling occurred over numerous dates from March to May 2023 in order to provide sufficient material for all experiments, thus reducing batch variation.

3.2.2 Slurry preparation

The faecal samples were pooled to minimise individual variation and capture as much diversity of the pre-weaning period as possible. Samples were collectively defrosted at ambient temperature and a slurry prepared by homogenising all samples with a mineral buffer (see Table 3.1) at 70% wt/vol. The dilution to 70% wt/vol resulted in the most practical consistency for handling and straining. Slurry was divided into approximate 25 g aliquots and frozen at -80°C until required.

Table 3.1 Composition of ‘complex mineral buffer’ (Palowski et al., 2021)

Compound	Concentration (g/L)
Sodium hydrogen carbonate	8.295
Potassium dihydrogen phosphate	1.469
Disodium hydrogen phosphate	1.351
Ammonium hydrogen carbonate	0.948
Sodium chloride	0.526
Magnesium sulphate	0.138
Calcium chloride	0.0158
Cobalt chloride	0.0012
Manganese chloride	0.012
Ferric chloride	0.0096

3.2.3 Standard Fermentation Protocol

As this chapter aims to develop and refine a novel protocol, multiple methodological factors were systematically varied across experiments and these are detailed in each following experimental section. Although specific parameters differed between experimental conditions, the overall workflow remained consistent throughout. Broadly, each experiment involved a two-day process with the first day being preparatory and the second day being when the fermentation began. Generally each first day consisted of (1) preparation of the media, (2) weighing the substrate into serum bottles, (3) laying out equipment to streamline next day. The following morning, each experiment involved (1) reviewing pH of media and adjusting if necessary, (2) adding media to each serum bottle, (3) bubbling each replicate bottle with CO₂, (4) sealing bottle and incubating at 39°C (Palowski et al., 2021; Gresse et al., 2021), (5) gas release and recording every 2 hours, (6) termination in an ice bath, (7) final pH recording and taking samples for downstream analysis. The workflow in chronological order and how each experiment varied this workflow is detailed in Figure 3.1.

3.2.3.1 Day One: Preparation of Media and Substrates

Media preparation throughout all experiments included addition of resazurin (1.25 mg/L) as a redox indicator, and pH adjustment to 6.8 (Uerlings et al., 2019; Palowski et al., 2021) using 5M hydrochloric acid. Inducing anaerobiosis by bubbling CO₂ through the media for approximately 40 minutes was done in every media preparation, regardless of any additional experiment-specific reducing factors used. At the point of media inoculation, this was done with the pre-prepared slurry (see Section 3.2.2) to make a final concentration of 50 g/L faecal matter (approximately 71.43 g/L slurry) and strained through four layers of cheesecloth before adjusting pH if necessary and bubbling with CO₂ for an additional 30 minutes to ensure colour change from redox indicator remained. Media was sealed and stored at 39°C overnight.

Experiments generally used 0.5 ± 0.001 g per replicate of milk replacer powder (DanMilk™ Pro, AB Neo; see Table 3.2 for composition) which was added in triplicate to 125 mL serum bottles. Bottles were covered and left at room temperature overnight. Any divergence from this substrate preparation is specified in the relevant experiment-specific method section.

Table 3.2 Nutritional information as advertised for DanMilk™ Pro piglet milk replacer

Typical values on a dry matter basis	Crude protein	16%
	Crude fat	13.60%
	Crude ash	7.30%
	Crude fibre	0%
	Lactose	45%
	Lysine	16 g/kg
	Methionine	4.7 g/kg
	Calcium	10.2 g/kg
	Phosphorus	5.7 g/kg
Nutrients added per kg	Sodium	4.3 g/kg
	Vitamin A	25,500 IE
	Vitamin D3	2550 IE
	DL-Alpha-tocopherol	130 mg
	Zinc	100 mg
	Copper	130 mg
	Iodine	2.0 mg
Digestibility enhancers	Iron	150 mg
	Bacillus Subtilis	0.6 bn CFU
	Bacillus Licheniformis	0.6 bn CFU

3.2.3.2 Day Two: Fermentation

Media was removed from the incubator and pH adjusted to 6.8 using concentrated hydrochloric acid or sodium hydroxide. Following this, 50 mL of inoculated media was added to each serum bottle containing pre-weighed substrate and bubbled through with CO₂ for five minutes on a plate warmer before capping with a rubber stopper and aluminium crimp cap. Bottles were incubated at 39°C for 8-24 hours (specified for each Experiment) with gas pressure released and recorded at two hour intervals. Bottles were agitated gently at these times also. To terminate the fermentation process, bottles were submerged in an ice bath after final gas recording. End pH was recorded at this point and fermentation fluid samples removed for analysis. For SCFA analysis, 1.5 mL fermentation fluid was placed into a 1.5 mL tube (Eppendorf, Hamburg, Germany) and frozen at -20°C. For microbial 16S rRNA sequencing, 2 mL was removed and centrifuged at 16,000 x g for 10 minutes (1-14 mini, Sigma, Osterode, Germany) at room temperature. Supernatant was removed and pellets were frozen at -80°C until required for DNA extraction.

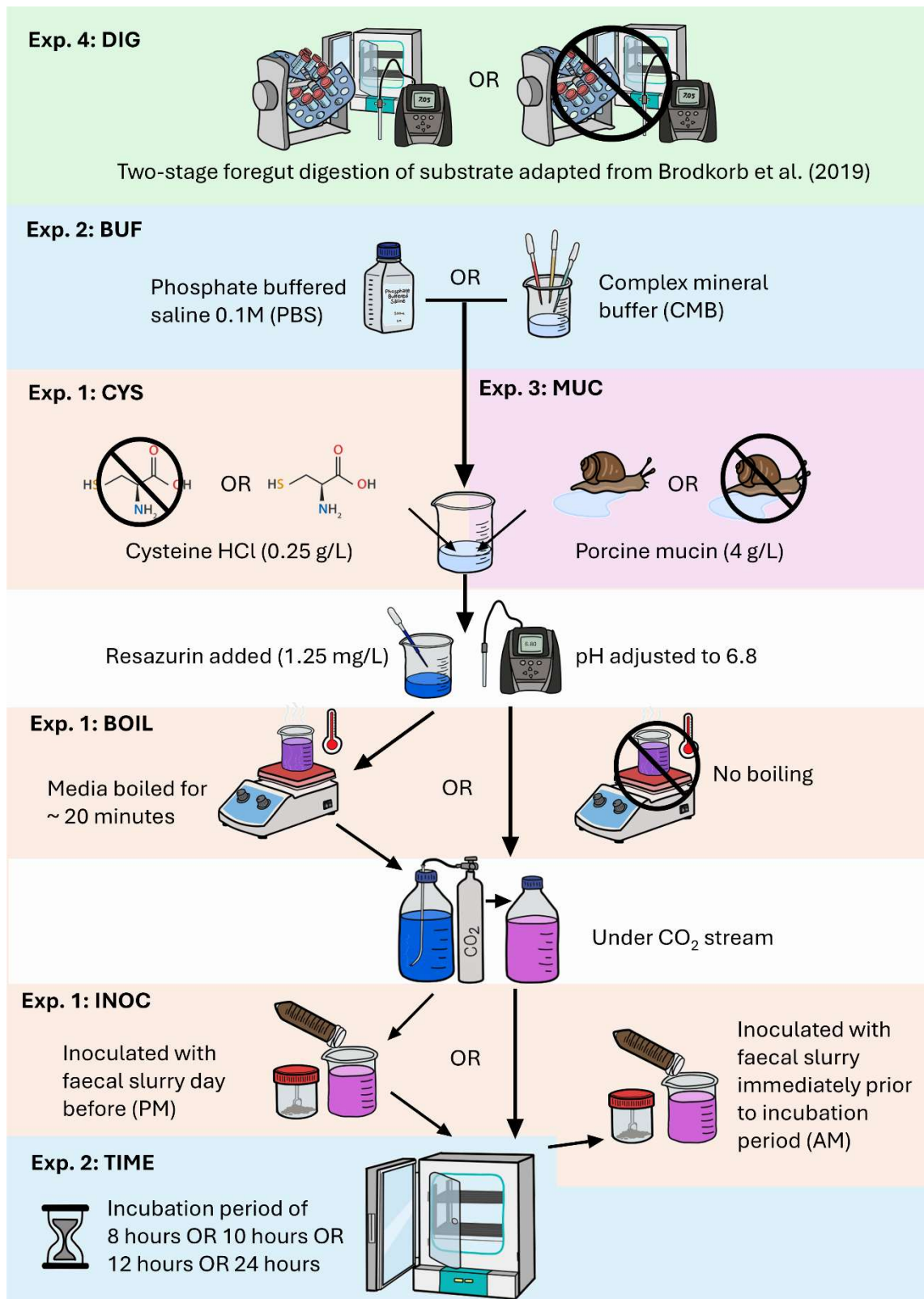


Figure 3.1 Chronological protocol for *in vitro* fermentation experiments. Each experiment tested the effects of changing a certain condition, stated in shorthand. Experimental shorthand from top to bottom: DIG, foregut digestion phase; BUF, buffer composition; CYS, addition of L-cysteine; MUC, addition of mucin; BOIL, boiling the media; INOC, inoculation timing; TIME, incubation period.

3.2.4 Gas Chromatography

Samples were thawed at room temperature in a tube rack and prepped for gas chromatography according to Jouany (1982). Once thawed, samples were centrifuged at 8000 x g for 2 minutes to reduce solid particle transfer downstream. To a centrifuge tube (Nalgene™ Oak Ridge, Thermo Fisher, MA, USA), 1 mL supernatant of sample or 1 mL calibration standard (5 mM of each; acetate, propionate and butyrate) was added, followed by 250 µL internal standard solution (mercuric chloride (2 g/L), orthophosphoric acid (85% aq. solution; 20 mL/L), 4-methylvaleric acid (2 g/L)). Tubes were capped immediately following addition of internal standard solution and vortexed for approximately 10 seconds. Samples were then centrifuged at 20,000 x g for 20 minutes at 10°C (L8-70M Ultracentrifuge with 70.1 Ti rotor, Beckman Coulter, California, USA). Approximately 600 µL of supernatant was then transferred to a 2 mL glass vial (Virtuoso™ 11mm, Thermo Fisher) and stored at 4°C until analysis. The gas chromatograph (Varian 3400 with 8200 CX autosampler, Varian Inc., CA, USA) was fitted with a polyethylene glycol nitroterephthalic acid-treated capillary column (15 m x 0.53 mm, 0.5 µm film thickness; BP21, SGE, Europe Ltd., Bucks, UK) and 0.5 µL sample was injected to the column at 240°C using helium as the carrier gas (flow rate of 5 mL/min). The detector was a flame ionisation detector at 280°C. Concentrations of acetate, propionate and butyrate were calculated using the area of their peaks as a ratio comparative to the internal standard peak area. Where necessary, blank fermentation samples (no substrate added) were run alongside and concentrations corrected by removing the blank sample values. Total SCFA concentration was calculated by the sum of each individual measure (acetate, butyrate, and propionate concentrations).

3.2.5 Microbial DNA Extraction

Microbial DNA was extracted using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) which has been validated for use in ecological studies of gut microbiota (Li et al., 2003). The kit was used following the manufacturer's instructions with minor adjustments. Samples were heated at 95°C for 5 minutes, vortexed for 30 seconds and heated a further 5 minutes following addition of InhibitEX buffer. Proteinase K was added at 15 µL. Other reagent volumes were doubled, and DNA was eluted using 50 µL buffer ATE. Quality of DNA was confirmed using a microvolume UV-Visible spectrophotometer (NanoDrop™

2000, Thermo Fisher) at 260/280 and 230/280 wavelengths. Samples were then frozen at -80°C until transport for analysis. Samples were shipped on dry ice to Novogene Company Ltd (Cambridge, UK) for library preparation and 16S rRNA sequencing using Illumina NovaSeq platform (Illumina, San Diego, CA, USA). The V4 region was amplified using 515F (GTGCCAGCMGCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT) primers (Novogene, 2026).

Returned reads with primer sequences removed were analysed using Mothur v1.48.0 following standard operating procedures developed by Schloss et al. (2009) and Kozich et al. (2013) for 16S processing. Briefly, raw forward and reverse reads for each sample were combined to form contigs which were filtered out if any ambiguous bases were present, anything longer than 275 base pairs, or with homopolymers higher than eight. Unique sequences were brought forward and aligned to the V4 coordinates for the SILVA database (v138.2) before pre-clustering allowing for two differences per 100 base pairs. Following this, chimeras (Haas et al., 2011) and undesirable taxa were removed (Chloroplast, Mitochondria, unknown, Archaea and Eukaryota). All datasets used were considered 'small' therefore clustering sequences into operational taxonomic units (OTUs) was approached using a final cutoff distance of 0.03 (97% similarity) before classifying OTU taxonomy. Use of OTU over ASV was cohesive with the Mothur pipeline and is a well-established approach for characterising bacterial community composition with 16S rRNA data (Schloss et al., 2009; Kozich et al., 2013). Use of OTUs enabled comparison to previous literature (Mach et al., 2015; Chen et al., 2018) and for the present research objectives, provided adequate depth. Final files containing number of sequences in each OTU from each group and taxonomy for each OTU were saved and further analyses conducted in RStudio (see Section 3.2.6.3).

3.2.6 Statistical Analysis

Statistical analysis varied on an experimental basis therefore any deviations from the following are clarified in the experiment-specific methodology sections. Outside of differences in data distribution and number of factors tested, the following workflow was performed for every experiment.

All statistical analysis was conducted using R v4.5.1 (R Core Team, 2025) within RStudio 2025.05.1-513 (Posit Software, 2025) with the following packages

consistently loaded for data curation and routine testing: “tidyverse” v2.0.0 (Wickham et al., 2019); “emmeans” v2.0.1 (Lenth and Piaskowski, 2025); “glmmTMB” v1.1.14 (McGillucuddy et al., 2025); “lme4” v1.1.38 (Bates et al., 2015) always in conjunction with reduction using “lmerTest” v3.2.0 (Kuznetsova et al., 2017).

3.2.6.1 Fermentation Kinetics and pH

Accumulated gas pressure (kPa) was measured using a digital manometer (Digitron 2023P, Sifam Instruments Ltd., Torquay, UK) and recorded approximately every two hours during incubation. After pressure was recorded, each bottle was returned to atmospheric pressure by needle insertion until silent (approximately 3 seconds). Each bottle was also gently agitated at this time. Gas pressure was corrected for the mean pressure of gas produced in blank bottles (containing inoculated media and no substrate). Gas pressure was converted into gas volume (mL) using the following equation (López et al., 2007; McDermott, 2018):

$$V = \frac{V_h}{P_a} \times P_m$$

Where V = gas volume (ml), V_h = total headspace volume in serum bottle (110.4 ml), P_a = atmospheric pressure (100.52 kPa; altitude of laboratory 71 m) and P_m = gas pressure recorded (kPa).

Frequency of gas sampling allowed for cumulative gas production curves to be fitted. For this, a three-parameter Gompertz model (Winsor, 1932) using nonlinear least squares was implemented with the *nlsLM* function from package “minpack.lm” v1.2-4 (Elzhov et al., 2023). The model was defined as:

$$Y = a \times \exp(-b \times \exp(-c \times H))$$

Where Y = cumulative gas volume, a = maximum gas production, b = scaling parameter related to the lag phase (hereafter, lag phase), c = fractional rate constant of gas production (hereafter, growth rate), and H = incubation time (hours).

The resulting asymptotic parameters (a , b and c) were extracted as they represent biologically meaningful estimates of the measured fermentation

kinetics. These were treated as response variables and compared among treatments using linear mixed-effects models.

Final pH was analysed with one of T-tests, Wilcoxon signed rank tests, or linear mixed-models, depending on experimental design and data distribution (specified in relevant sections).

3.2.6.2 Short Chain Fatty Acid Concentrations

Concentrations of independent SCFA (acetate, butyrate and propionate) and total SCFA concentration (sum of acetate, butyrate and propionate values) were generally analysed using a linear mixed-effects model followed by an ANOVA reduced using *lmerTest* to determine which factors significantly influenced each SCFA measure, with batch included as a random factor. Confirmation of suitability for this testing was done by assessing normality of model residuals and homogeneity. Differentiation from this is specified in the relevant section.

3.2.6.3 Microbial Community Composition

Statistical analysis of the microbial data was generally consistent across experiments and deviations from the following will be specified in experiment-specific sections. Consistent packages used in R for microbiota analysis are as follows: “BiocManager” v1.30.27 (Morgan and Ramos, 2025) for accessing bioinformatics packages from the Bioconductor project; “phyloseq” v1.54.0 (McMurdie and Holmes, 2013) for constructing objects; “vegan” v2.7.2 (Oksanen et al., 2025) and “pairwiseAdonis” v0.4.1 (Martinez-Arbizu, 2017) for beta diversity analysis; “ANCOM-BC” v2.12.0 (Lin and Peddada, 2020; Lin et al., 2022; Lin and Peddada, 2024) for differential abundance of taxa; “pheatmap” 1.0.13 (Kolde, 2025) for graphics.

Alpha diversity was estimated using the Chao1 index (Chao, 1984) for species richness, indicating how many taxa were present, the Shannon-Wiener index (Shannon, 1948) was used to indicate richness and evenness, as the Chao1 value can be influenced by rare taxa, this provides additional information about community composition, and the Simpson index (Simpson, 1949) was used which also reflects richness and evenness but is weighted towards dominant taxa. Using all three measures provides information on richness, evenness and dominance to get a holistic view of the community structure and using the indices together can provide relative information, for example, low Shannon with low

Simpson values indicates more dominance of a taxon. Differences between indices per treatment were assessed using T-tests, Wilcoxon signed rank tests, or linear mixed-models depending on experimental design and data distribution (specified in relevant sections).

Community composition dissimilarity was analysed with a Permutational Multivariate Analysis of Variance Using Distance Matrices (PERMANOVA) using the *adonis2* function in the package “vegan” with the Bray-Curtis distance to identify significant factor effects and interactions (Oksanen et al., 2025). Pairwise *post-hoc* comparisons were performed where necessary (Martinez-Arbizu, 2017) and are specified in the relevant experimental methods. Beta diversity was visualised using non-metric multidimensional scaling (NMDS) using the Bray-Curtis distance.

Differentially abundant OTUs were identified using the analysis of compositions of microbiomes with bias correction (ANCOM-BC), where log fold change (LFC) values and adjusted p values were extracted (Lin and Peddada, 2020; Lin et al., 2022; Lin and Peddada, 2024). Taxa were considered notable (and reported in results) when statistically significant to the $p < 0.05$ threshold, alongside a LFC value greater than one or less than minus one. Heatmaps were created using the package “pheatmap” to visualise the differential genera which appeared at >1% mean relative abundance in at least one group and dendrograms were created using Bray-Curtis dissimilarity to cluster heatmap columns. In some instances the relative abundance threshold was increased for genera presented in figures; this was done in cases where too many genera were present at >1% across various groups and it was impractical to present in one figure.

3.3 Experiment 1 Exploring L-cysteine, Boiling, and Inoculation Timing

3.3.1 Methodology

This experiment was a multifactorial 2 x 2 x 2 design testing the addition of L-cysteine, the inclusion of a boiling step, and the timing of slurry inoculation. The use of L-cysteine is common as a redox agent, added to reduce the buffer and encourage a more stable anaerobic environment. Boiling the buffer is also

deployed as a reducing technique. Inoculating the model at different times was explored both to reduce labour intensity and explore a pseudo-stabilisation period for the microbiota, as done in continuous models. As the purpose was to optimise a complex *in vitro* fermentation system, testing combinations was preferable to optimising each component independently, to determine any interdependency. Use of any preparation that induces anaerobiosis (L-cysteine and boiling), may only be short-lived (Fukushima et al., 2003), therefore, testing these parameters with inoculation timing allowed for that to be assessed. Furthermore, activity of L-cysteine is heat-dependant (Pokorný et al., 2023), so determining any interaction between addition of L-cysteine and boiling the buffer was also important.

As a multifactorial experiment, each group of conditions will be referred to using a code (see Table 3.3), with each letter denoting the condition (C/c, L-cysteine addition; B/b, boiling the media; I/i, inoculation timing) and the capitalisation of each letter representing whether that factor was implemented in the media preparation (capital), or not (lowercase), resulting in a total of eight groups.

Table 3.3 Experiment 1 treatment codes

Code	L-cysteine ¹	Boiled ¹	Inoculation ²
CBI	Y	Y	PM
CBi	Y	Y	AM
CbI	Y	N	PM
Cbi	Y	N	AM
cBI	N	Y	PM
cBi	N	Y	AM
cbI	N	N	PM
cbi	N	N	AM

¹Where 'Y' indicates addition of this condition and 'N' indicates absence

²Where 'PM' indicates evening and 'AM' indicates following morning

Inoculation timing is capitalised for the PM groups due to the fact that media is prepared the day before the fermentation is initiated, thus if media is inoculated as part of the preparation phase (i.e in the evening), then it was an addition. Whereas, holding off on the addition of the slurry while preparing the media the day before can be seen as an absence of this condition and lack of any pseudo-stabilisation period.

3.3.1.1 Fermentation Protocol

The protocol as described in Section 3.2.3.1 was followed with the following amendments and was repeated on three separate occasions, hereafter referred to as each 'batch'.

Complex mineral buffer was made up as per Table 3.1 and pH adjusted to 6.8 with 5M hydrochloric acid. The buffer was then divided into two equal portions; one receiving an addition of L-cysteine (0.25 g/L) simultaneously with resazurin; and the other portion only receiving resazurin. Both portions were then divided again, resulting in four buffer portions, two with added L-cysteine and two without. One of each pair were subjected to boiling for approximately 15 minutes before CO₂ transfusion, and the remaining two were infused with CO₂ without boiling. Finally, each resulting preparation was subsequently split once more into two equal portions, with one portion of each condition having faecal slurry added to a final faecal content of 50 g/L, and the remaining bottles being sealed and stored at 39°C overnight without being inoculated. The protocol then resumes from the inoculation step the morning of the fermentation commencement for the portions lacking slurry. Substrate preparation is as described in Section 3.2.3.1 for all eight conditions, in triplicate, resulting in a total of 48 serum bottles per batch, including triplicate blanks (bottles containing only media, no substrate) for each media condition.

Inoculated media was added at 50 mL per serum bottle and each bottle bubbled with CO₂ for 5 minutes on a plate warmer before sealing and incubating for 8 hours at 39°C. Gas pressure was recorded and released at 2 hour intervals and the fermentation terminated in an ice bath after the final reading at 8 hours. A final pH was recorded at this point and samples removed for further analysis as described in Section 3.2.3.2.

3.3.1.2 Sample Processing

As per Section 3.2.4, 1.5 mL fermentation fluid from each bottle was removed after termination of the experiment and frozen at -20°C until processing for gas chromatography as described by Jouany (1982). Concentrations of acetate, propionate and butyrate were calculated using the area of their peaks and total SCFA concentration calculated from the sum of these measures. Values were corrected by subtracting data from blank bottles.

As per Section 3.2.5, 2 mL fermentation fluid from each bottle was removed after termination of the experiment and centrifuged at $16,000 \times g$ for 10 minutes, supernatant discarded, and pellets frozen at -80°C until processed for DNA extraction. Extraction was performed using the QIAamp DNA Stool Mini Kit and the DNA sent on dry ice to Novogene Company Ltd. for 16S rRNA sequencing of the V4 hypervariable region. Returned raw reads were filtered, aligned and classified using Mothur v1.48.0 (Schloss et al., 2009; Kozich et al., 2013), as previously described.

3.3.1.3 Statistical Analysis

Fermentation kinetics were analysed as described in Section 3.2.6.1. Briefly, gas pressure (kPa) was converted to gas volume (mL) and the continuous mean gas volumes for each media condition were modelled using a Gompertz growth curve. Key parameters (maximum gas volume, lag phase, and growth rate) were extracted per condition combination and compared using a linear mixed-effects model with all factors (L-cysteine, boiling, inoculation timing) included as fixed effects including interactions, and batch as a random factor. Significance of fixed effects was assessed using a reduced ANOVA. Final pH, SCFA measurements, and alpha diversity indices were all analysed using an identical linear mixed-effect model to the Gompertz parameters, replacing the response variable with the relevant measure. The remaining microbial community analysis was as described in Section 3.2.6.3.

3.3.2 Results

See Table 3.3 for treatment code descriptors.

3.3.2.1 Fermentation Parameters

Key parameters were extracted from Gompertz growth curves and inoculation timing significantly impacted maximum gas volume ($F_{1, 14} = 4.6484$; $p = 0.0490$), where inoculation immediately before incubation (AM), increased gas production (Figure 3.2).

The lag parameter was affected by a three-way interaction between boiling, L-cysteine addition, and inoculation timing ($F_{1, 16} = 6.20$; $p = 0.0241$) but the strongest main effect was inoculation timing, which increased the lag

parameter in the AM conditions ($F_{1, 16} = 45.3$; $p < 0.0001$). Boiling the buffer significantly increased the lag parameter also ($F_{1, 16} = 9.86$; $p = 0.00632$).

Additionally, the growth rate highlighted an interaction between boiling and inoculation timing ($F_{1, 14} = 6.88$; $p = 0.0201$), where boiling increased growth rate when also AM inoculated but reduced growth rate in the PM inoculated groups (Figure 3.2). Again, inoculation timing independently increased growth rate in the AM condition ($F_{1, 14} = 99.2$; $p < 0.001$).

There was a significant interaction between addition of L-cysteine and boiling the buffer ($F_{1, 62} = 5.96$, $p = 0.0175$), and additional interactions between boiling and inoculation timing ($F_{1, 62} = 13.0$, $p < 0.001$), as well as a three-way interaction ($F_{1, 62} = 7.61$, $p = 0.00762$). This resulted in the end pH being affected in different ways, depending on the combination of all three conditions tested. Fixed effects that significantly impacted final pH were addition of L-cysteine ($F_{1, 62} = 10.3$, $p = 0.00209$) which appeared to reduce pH, and boiling the buffer ($F_{1, 62} = 26.0$, $p < 0.001$) which appeared to increase pH. The maximum mean pH was in cBI (6.56 ± 0.314) and minimum mean pH was in cbl (6.22 ± 0.111), showing that the greatest difference was between groups with the same cysteine and inoculation conditions (L-cysteine added, and inoculated in the PM) but cBI being boiled and cbl not being boiled.

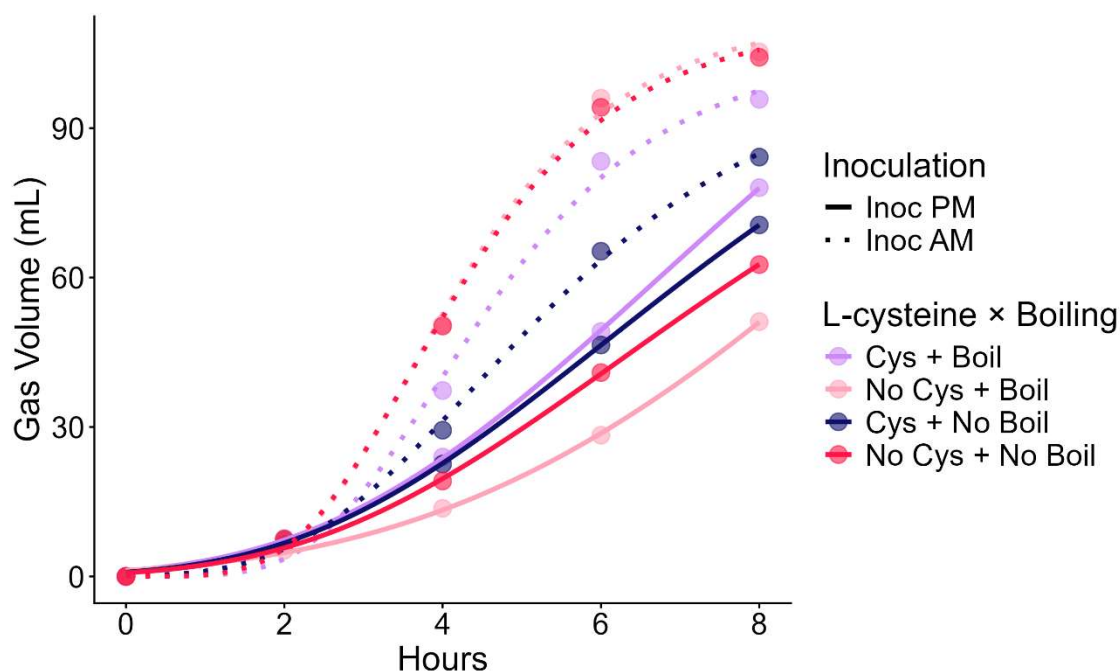


Figure 3.2 Gompertz growth curves fitted to cumulative gas production during fermentation. Points represent measured gas volumes and curves represent predicted growth. Line type indicates inoculation timing (AM, dotted; PM, solid), colour indicates L-cysteine addition (purple, present; pink, absent), and colour intensity indicates boiling treatment (pastel, boiled; dark, not boiled).

3.3.2.2 Short Chain Fatty Acid Concentrations

Acetate concentration was significantly influenced by interaction between boiling and inoculation timing ($F_{59.3} = 16.1$; $p < 0.001$), where lack of boiling the PM inoculated condition produced high acetate concentrations and lack of boiling in the AM inoculated group induced low acetate. Independently these factors were also significant ($F_{59.3} = 7.13$; $p = 0.00975$, boiling; $F_{59.3} = 50.4$; $p < 0.001$, inoculation timing). The lowest concentration of acetate was produced by Cbi at 9.73 ± 1.13 mM, and the highest concentration by cbl at 27.2 ± 5.33 mM.

Propionate concentration followed a similar pattern with significant interaction between boiling and inoculation timing ($F_{59.5} = 38.2$; $p < 0.001$), and additionally a significant interaction between inoculation timing and addition of L-cysteine ($F_{59.5} = 4.23$; $p = 0.0441$). Propionate was independently effected by boiling ($F_{59.5} = 41.6$; $p < 0.001$) and inoculation timing ($F_{59.5} = 76.4$; $p < 0.001$). The lowest concentration of propionate was produced by cBi at 1.96 ± 0.22 mM and the highest produced by cbl with 15.02 ± 2.74 mM.

Butyrate concentration reflected significant interactions between addition of L-cysteine and boiling ($F_{61} = 16.4$; $p < 0.001$), addition of L-cysteine and inoculation timing ($F_{61} = 25.1$; $p < 0.001$), and interaction between all three conditions ($F_{61} = 5.30$; $p = 0.0247$). It was also significantly impacted by inoculation timing ($F_{61} = 5.03$; $p = 0.0286$) but no other independent conditions. The lowest concentration of butyrate was produced by Cbi at 3.26 ± 0.36 mM and the highest produced by CBI with 6.70 ± 0.47 mM.

Total SCFA concentration (sum of concentrations of acetate, propionate and butyrate) produced a significant interaction between boiling and inoculation timing ($F_{59.7} = 22.3$; $p < 0.001$), as well as an interaction between boiling and addition of L-cysteine ($F_{59.7} = 5.63$; $p = 0.0209$). Total SCFA was significantly influenced by boiling ($F_{59.7} = 13.8$; $p < 0.001$) and inoculation timing ($F_{59.7} = 59.6$; $p < 0.001$), as with other measures. for the detected concentrations of SFCA's are provided in Table 3.4.

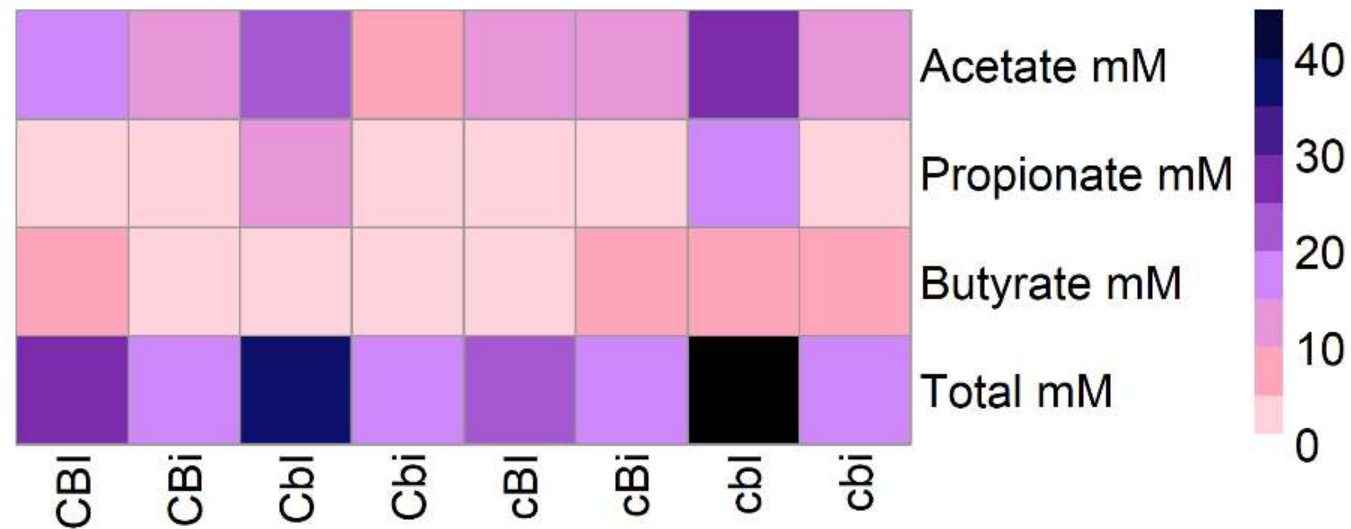


Figure 3.3 Heatmap of short chain fatty acid concentrations (mM) of each treatment (Table 3.3). Letter denotes effect; C/c, addition of L-cysteine; B/b, boiling of media; I/i, inoculation timing. Capitalisation denotes inclusion (capital), or not (lowercase). Colour denotes strength of production where pale boxes are lower production and dark boxes are higher production, as per right-hand key.

Table 3.4 Short chain fatty acid (SCFA) concentrations produced and p-values for each fixed condition and interaction as per linear mixed-effects model and reduced ANOVA

										p-values ²						
										Conditions			Interactions			
Treatment code ¹		CBI	CBi	Cbl	Cbi	cBI	cBi	cbl	cbi	L-Cysteine	Boiling	Inoculation	Cys*Boil	Cys*Inoc	Boil*Inoc	Cys*Boil*Inoc
Acetate (mM)	Mean	17.64	12.58	22.40	9.73	14.29	11.41	27.21	10.80	0.865	0.00975	<0.001	0.059	0.834	<0.001	0.294
	SE	1.32	1.09	1.97	1.13	1.79	0.48	5.33	1.06							
Propionate (mM)	Mean	3.95	2.83	10.67	2.63	4.35	1.96	15.02	2.53	0.181	<0.001	<0.001	0.096	0.044	<0.001	0.261
	SE	0.85	0.19	0.93	0.30	1.47	0.22	2.74	0.15							
Butyrate (mM)	Mean	6.70	4.28	4.79	3.26	3.54	5.10	5.09	5.03	0.819	0.190	0.0286	<0.001	<0.001	0.506	0.0247
	SE	0.47	0.26	0.23	0.36	0.34	0.12	1.01	0.20							
Total SCFA (mM)	Mean	28.29	19.68	37.86	15.62	22.18	18.48	47.32	18.36	0.556	<0.001	<0.001	0.021	0.827	<0.001	0.163
	SE	1.35	1.35	2.86	1.11	3.41	0.77	9.05	1.17							

¹Specified in Table 3.3: Briefly, capitalisation of the letter indicates C, addition of L-cysteine; B, media was boiled; I, media was inoculated on the preparatory day (PM); lower case indicates c, no L-cysteine; b, media not boiled; l, media inoculated immediately prior to incubation (AM).

²Displayed to three significant figures unless $p < 0.001$

3.3.2.3 Microbiota

A total of 72 samples were processed to extract microbial DNA. Three samples did not yield sufficient DNA concentration ($< 10 \text{ ng/uL}$) to be sequenced and one additional sample failed quality checks during library preparation. A final 68 samples were sequenced using 16S rRNA amplification of the hypervariable V4 region. A total of 4,462,930 reads were generated and after screening and filtering 23,452 high-quality unique reads were aligned.

3.3.2.3.1 Alpha Diversity

Alpha diversity was assessed using the Chao1, Shannon and Simpson indices. Chao1 values were not significantly impacted by any individual conditions but did highlight interactions between boiling and inoculation timing ($F_{58.5} = 6.14$; $p = 0.0162$) as well as a three-way interaction ($F_{58.5} = 5.58$; $p = 0.0215$), between addition of L-cysteine, boiling, and inoculation timing. This was characterised by a lower Chao1 value in groups that were both boiled and inoculated in advance (PM) (452 ± 25.9 and 511 ± 34.7 for cBI and CBI, respectively) when compared to conditions with a divergence of boiling and inoculation timing (557 ± 12.3 and 599 ± 39.4 for cbl and cBi, respectively). Shannon diversity was lower in conditions that were boiled ($F_{58.2} = 28.7$; $p < 0.001$) and higher in conditions that were inoculated in advance (PM) ($F_{58.2} = 132$; $p < 0.001$). Shannon diversity also highlighted the same interactions as shown for Chao1; boiling and inoculation timing ($F_{58.2} = 17.2$; $p < 0.001$) and the three-way interaction ($F_{58.2} = 4.35$; $p = 0.0415$). The Simpson indices were impacted by boiling ($F_{58.2} = 11.5$; $p = 0.00125$) and inoculation timing ($F_{58.2} = 72.5$; $p < 0.001$) only, and in the same way as Shannon diversity; lower values when boiled and higher values for PM inoculation.

3.3.2.3.2 Beta Diversity

A factorial PERMANOVA revealed a significant but weak interaction between boiling and inoculation ($F_{1, 60} = 14.7$, $R^2 = 0.09$, $p = 0.001$). Effect of experimental conditions on microbial community composition demonstrated that the combination of L-cysteine addition, boiling, and inoculation timing explained 62.9% of the observed compositional variation (*Bray-Curtis*; $F_{7, 60} = 14.5$; $R^2 = 0.629$; $p = 0.001$). Upon splitting by terms, although addition of L-cysteine and boiling were both statistically significant ($F_{1, 60} = 3.45$; $R^2 = 0.0213$; $p = 0.022$

and $F_{1, 60} = 16.7$; $R^2 = 0.103$; $p = 0.001$, respectively), it was inoculation timing that explained 38.7% of the compositional variation ($F_{1, 60} = 62.5$; $R^2 = 0.387$; $p = 0.001$), which is highlighted in Figure 3.4.

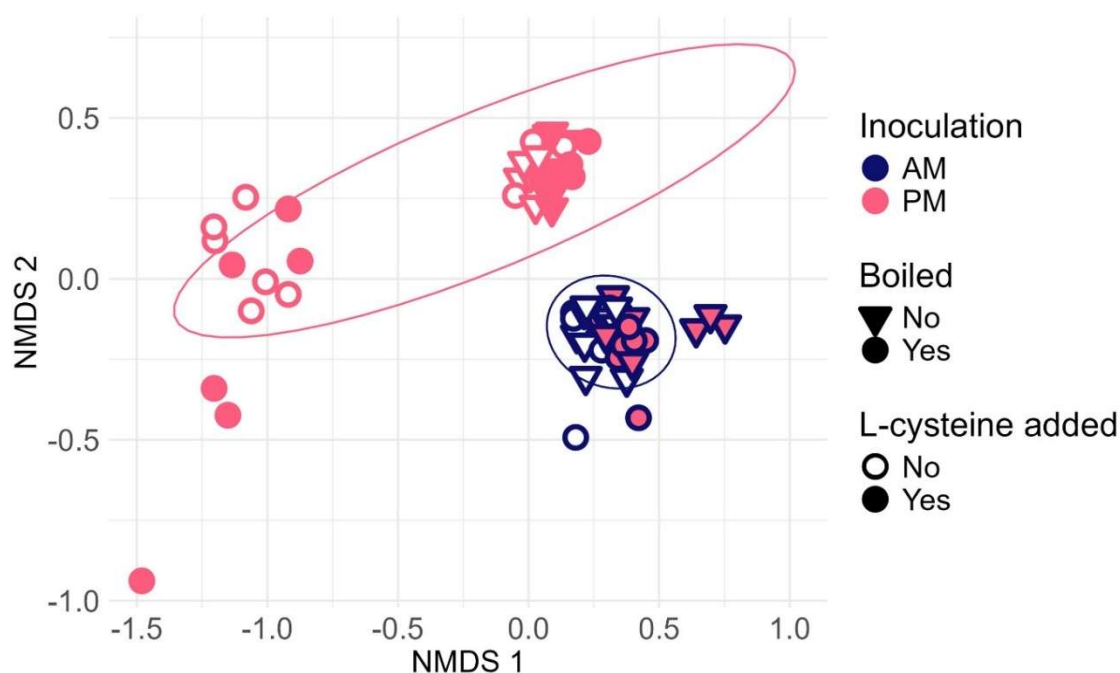


Figure 3.4 Non-metric multidimensional scaling (NMDS) ordination of microbial community composition based on Bray-Curtis dissimilarity. Each point represents a sample, with colours indicating inoculation timing; purple, morning inoculation before bottles were incubated (AM); pink, evening inoculation the day before incubation (PM). Shape denotes whether the buffer was boiled (circle) or not (triangle) and fill denotes the addition of L-cysteine; no fill, no L-cysteine; filled in pink, addition of L-cysteine (0.25 g/L). Ellipses show 95% confidence intervals around group centroid based on inoculation timing as the largest effect on variation in communities (38.7%).

3.3.2.3.3 Relative Abundance: Phylum Level

Bacillota dominated most groups (CBI, Cbl, cBI, cBi, cbl, cbi) with Pseudomonadota the second most abundant in these groups. Bacillota was most abundant in cBI at $78.2 \pm 8.51\%$ and least abundant in Cbl at $39.2 \pm 1.56\%$. Groups that had more Pseudomonadota than Bacillota were CBI and Cbi with $54.1 \pm 2.95\%$ Pseudomonadota and $43.7 \pm 2.80\%$ Bacillota, and $50.1 \pm 1.18\%$ Pseudomonadota and $44.4 \pm 1.86\%$ Bacillota, respectively. Third most abundant phylum was always Bacteroidota which ranged from $1.85 \pm 0.209\%$ in CBI to $18.4 \pm 1.15\%$ in Cbl and fourth most abundant was always Actinomycetota which ranged from $0.281 \pm 0.065\%$ in cBi to $10.8 \pm 2.44\%$ in Cbl.

Phyla changes were considered notable when both statistically significant ($p < 0.05$) and with a log fold change (LFC) of more than 1 in a positive or negative direction. All reported changes were associated with the inoculation timing effect. Actinomycetota increased in groups that were inoculated the day before (PM) in comparison to groups that were inoculated the day of incubation (AM) (LFC = 2.31 ± 0.127 ; $p < 0.001$). However, Actinomycetota decreased when inoculated the day before in combination with being boiled (LFC = 1.67 ± 0.377 ; $p < 0.001$). The same pattern was seen with Bacteroidota where inoculation the day before increased abundance (LFC = 1.88 ± 0.145 ; $p < 0.001$), yet PM inoculation following the boiling step reduced Bacteroidota (LFC = 1.92 ± 0.401 ; $p < 0.001$). Pseudomonadota always decreased in the PM inoculation groups (LFC = 1.05 ± 0.222 ; $p < 0.001$), regardless of other conditions (Figure 3.5).

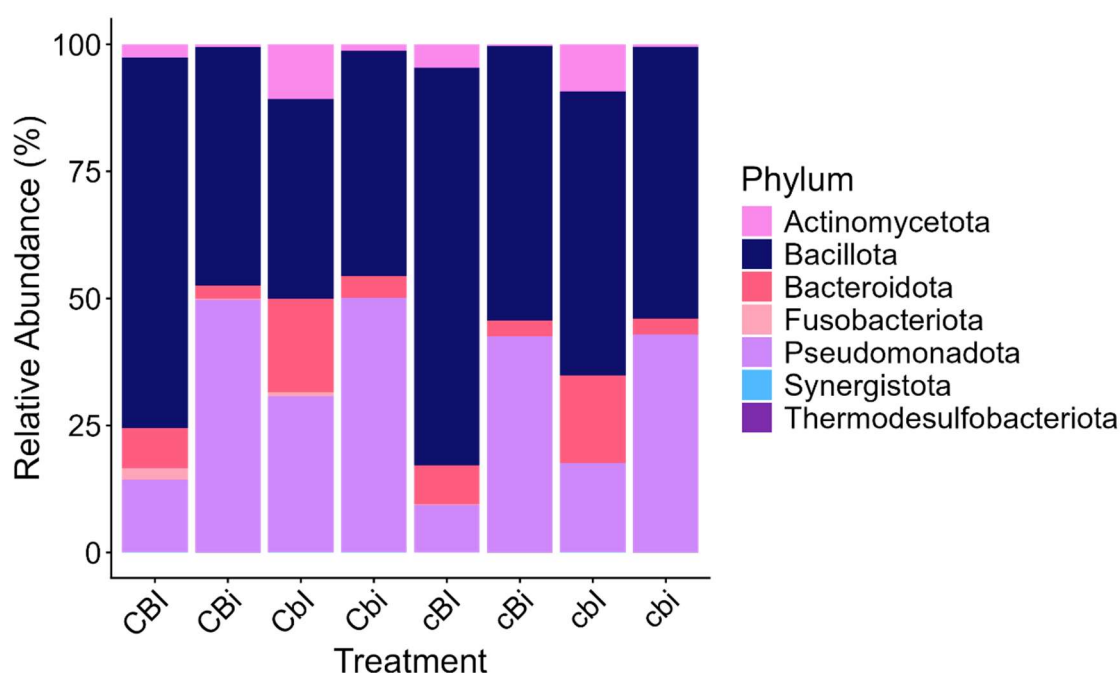


Figure 3.5 Percentage relative abundance of phyla present in each treatment where letter denotes main effect; C/c, addition of L-cysteine; B/b, boiling of media; I/i, inoculation timing and capitalisation of each letter denotes inclusion (capital), or not (lowercase)

3.3.2.3.4 Relative Abundance: Genus Level

Numerous genera had notable changes and this followed a similar trend to changes seen for phyla where most were influenced by inoculation timing (including *E. Shigella*, *Limosilactobacillus*, *Streptococcus*, *Lactobacillus*, *Clostridium* and *Bacteroides*), and some of the same and others being influenced by the combination of inoculation timing and boiling (including

Ruminococcus_gnavus_group, *Enterocloster*, *Erysipelotrichaceae_insertae_sedis_genus*, and *Collinsella*). There was no consistency with a dominant genus and several changes in genera profiles across different treatments (Figure 3.6).

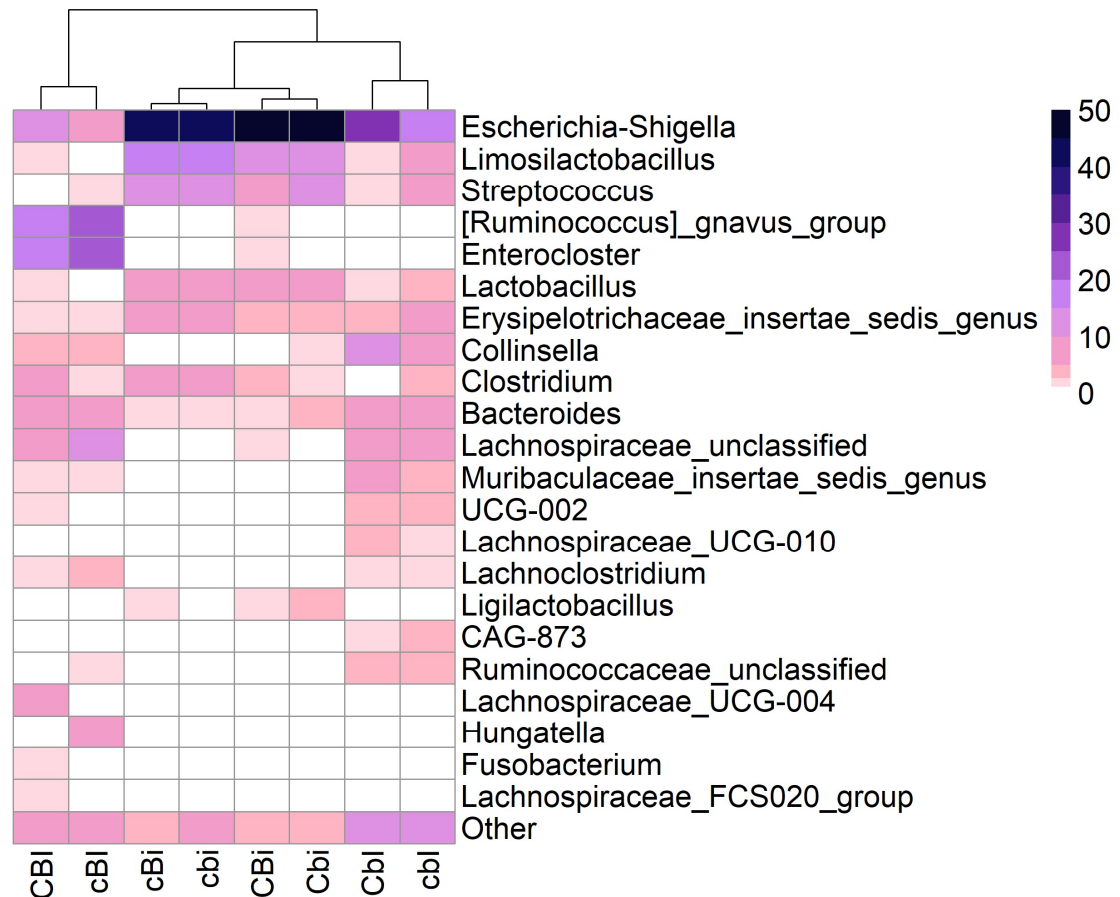


Figure 3.6 Heatmap indicating percentage relative abundance of genera that were > 2% in at least one treatment (x-axis). On x-axis letter denotes effect; C/c, addition of L-cysteine; B/b, boiling of media; I/i, inoculation timing. Capitalisation denotes inclusion (capital), or not (lowercase). Colour denotes magnitude of abundance where pale boxes are lesser and dark boxes are increased, as per right-hand key. Dendrogram clusters columns based on Bray-Curtis dissimilarity

3.3.3 Discussion

This experiment aimed to identify differences across fermentation characteristics, microbial communities and metabolite production when fermentation media was prepared in different ways. The 2 x 2 x 2 factorial design allowed for three aspects of media preparation to be assessed including interactions between each main effect. The main effects investigated were: adding L-cysteine (0.25 g/L) as a

reducing aid; boiling the buffer, also to aid reduction; adding the faecal inoculum at different time points to either facilitate a pseudo-stabilisation period (PM inoculation), or adding fresh prior to incubation (AM inoculation).

It was hypothesised that use of L-cysteine and boiling together would provide the optimal anaerobic environment, and that a pseudo-stabilisation period may increase abundance of undesirable bacteria. These hypotheses are rejected in favour of concluding L-cysteine elicits minimal measurable benefits, and fresh inoculation is associated with increased abundance of undesirable bacteria, such as *Escherichia-Shigella*, which is linked to poor health when present in piglets (Sun et al., 2019; Gryaznova et al., 2022).

3.3.3.1 Effects of Boiling the Fermentation Media

A common trend throughout the results of this experiment is the interaction between the boiling and the inoculation timing effects (B/b and I/i). This often pushed groups CBI and cBI into similar areas, and usually further from all other condition combinations (seen in Figure 3.4, and clustering shown in dendrogram on Figure 3.6). This may be due to suboptimal methodology, as the temperature of the media after boiling was not assessed prior to the addition of the faecal slurry, potentially resulting in the solution not being sufficiently cool and therefore causing heat-shock to the faecal bacterial populations. This would explain the consistently significant interaction as only the PM inoculation groups would be affected when media was boiled (CBI and cBI).

Adding the slurry while the buffer was a high temperature could compromise the microbiota as some are more heat tolerant than others. Chao1 and Shannon diversity indices were significantly lower in the CBI and cBI groups compared to their non-boiled counterparts (Cbl and cbl). This could suggest if there was a heat shock, this reduced overall richness and evenness across the samples. If this is considered to be the case, it is logical to assume the temperature of the media was considerably higher than the incubator (39°C) to effect the population dynamics so significantly. It is therefore difficult to locate comparable studies as it is not ethical to expose animals to such extreme temperatures, and *in vitro* there is little justification for exploring such temperatures if not ecologically relevant.

Adult pigs when subjected to 34°C at a high relative humidity (75-85%), showed increased opportunistic pathogens by day 14 and significantly diminished *Bacteroides* (Hu et al., 2022). Xia et al. (2022) reported reduced propionate production in the caecum of heat stressed pigs as well as distinct clustering of the microbiota based on heat treatment exposure, which is also reflected in this experiment (see Table 3.4 and Figure 3.4). Higher abundance of *Lachnospiraceae_UCG_004* was also apparent under heat stress (Xia et al., 2022) which is reflected in group CBI but not consistent in group cBI. *Bacteroides* were also higher in heat stressed pigs and *Lactobacillus* lower (Xia et al., 2022), both of which are seen in the CBI and cBI groups, however the consistency of the opposite effect appearing in groups CBI, Cbi, cBI and cbi (all inoculated in AM) suggests this may be an inoculation timing effect, rather than heat shock. Lactic-acid bacteria can offer cross-protection from environmental stressors (van de Guchte et al., 2002), making it challenging to predict which taxa may be affected. Despite this, *Lactobacillus* and *Streptococcus* were both less abundant in CBI and cBI groups compared to other groups. This may be because any protective mechanisms did not have time to activate given how sudden the temperature change would have been.

Environmental studies may also offer a useful comparison here through exploration of extreme temperatures. Ji et al. (2024) found structural compositional changes when analysing the bacterial community in sediment of mountain streams based on hot or cold seasons. Although the average summer temperature reported was low relative to the incubator used in the present experiment (19°C), the temperature differential between summer and winter months was high (31°C). There was no temperature effect on functional composition reported, only on structural composition, with temperature critically influencing bacterial metabolic processes. This is also reflected in our experimental results where there are many differences at the genus level, but this diminishes at the phylum level, implying overall function of the community remains intact.

3.3.3.2 Effects of Inoculation Timing

Inoculating the media immediately before incubation (AM) increased all extracted fermentation curve parameters. The longer lag phase was unexpected with AM inoculation as it was hypothesised that inoculating the media ~12 hours prior to

use, without substrate available, could compromise the microbiota health and increase lag phase in the PM group. From Figure 3.2 it is clear that 8 hours is almost sufficient time for the AM groups to reach a plateau, but conversely does not appear sufficient time for the PM group, which may still be in active fermentation of the substrate at the point of experiment termination.

The rapid growth rate and increased maximum gas volumes of the AM inoculated groups at first suggested a more complete fermentation and prior to further analysis it was expected that these groups would yield increased SCFA measures as a result. However, the opposite is true, with mostly reduced SCFA concentrations (with the exception of one butyrate pairwise comparison when media lacked L-cysteine and was boiled (cBI/cBi)). Such an active fermentation without correspondingly high SCFA production was unexpected as metabolic activity and gas production has historically been considered strongly correlated (Blümmel et al., 1997; Rymer and Givens, 2002). Cone and Becker (2012) found that when comparing fermentation of starchy feedstuffs, highest gas production was indeed associated with high total SCFA production, but lowered the proportion of acetate and butyrate, relative to the other SCFA measured.

Rapid fermentation by the AM inoculum may have resulted in the production of metabolites not quantified within the present SCFA analysis, such as lactate or formate, or in the production and subsequent utilisation of intermediate metabolites through microbial cross-feeding (Erb, 2011; Venkata Mohan et al., 2017). Differences in the allocation of degraded substrate towards microbial biomass could also contribute, since gas production and microbial biomass yield are not necessarily positively related (Blümmel et al., 1997).

These possibilities could be investigated through measurement of SCFAs at multiple time points corresponding to different phases of the gas-production curve. This would determine whether the lower endpoint concentrations reflected reduced production or subsequent utilisation by microbes. Quantification of additional metabolites, together with residual substrate and microbial biomass, would establish whether substrate carbon was redirected towards products not measured in the present study. Analysis of headspace gas composition would further distinguish differences in total gas volume from differences in the metabolic origin of that gas, however, this would require alternative fermentation equipment to be used to avoid loss of headspace gas. Finally, coupling these

measurements with microbial gene profiling could identify taxa and metabolic pathways responsible for the contrasting gas and metabolite phenotypes. Such analyses would therefore help determine whether the greater gas production observed following AM inoculation reflected greater overall substrate utilisation, altered fermentation kinetics, or differences in microbial carbon partitioning.

When exploring the microbiota results, Chao1 and Shannon diversity indices are lower when AM inoculated, highlighting reduced richness and evenness. The lack of evenness is visualised in the domination of *Escherichia-Shigella* in Figure 3.6. The high relative abundance of *Escherichia-Shigella* in these groups may explain the heightened fermentation kinetics and low SCFA output as this genus is not associated with strong SCFA production (Clark, 1989; Baltazar-Díaz et al., 2022). *Escherichia spp.* tend to produce biproducts such as CO₂ and H₂ (Roger et al., 2021), explaining the increased gas production, and if outcompeting other genera (as suggested by these results), could shift carbon flux toward formation of lactate and ethanol, thus diverting it away from SCFA formation. Even at the genus level we see the AM inoculation significantly increased Pseudomonadota causing subsequent significant reduction of Actinomycetota, Bacteroidota and, although not statistically significant, Bacillota reduction. These reduced phyla are the ones containing SCFA producers and cross-feeders, further explaining the reduction in SCFAs despite the increased gas production.

It is not clear why the conditions inoculated immediately before incubation (AM) contained such high abundance of *Escherichia-Shigella* as, although this genus has previously been reported in the gut of suckling pigs, it is highest in the first day or two of life and then declines as more metabolically efficient microbes proliferate (Mach et al., 2015; Bian et al., 2016; Chen et al., 2018). Prolonged enrichment of *Escherichia-Shigella* is commonly associated with incidence of diarrhoea in piglets (Sun et al., 2019; Gryaznova et al., 2022), therefore conditions exhibiting these extreme profiles are not useful for simulating a normal and healthy pre-wean gut microbial community. Absolute abundance of *Escherichia-Shigella* is positively correlated with high redox potential, indicative of an oxygenic environment (Feng et al., 2024) which, by absence of this genus in the PM inoculated groups, suggests that those conditions were better maintained as anaerobic. It is challenging to be certain the media is maintaining

anaerobiosis once inoculated due to the slurry masking the redox indicator colour so it is possible that the AM groups were not sufficiently purged with CO₂ prior to incubation.

3.3.3.3 Effects of L-cysteine Addition

L-cysteine had limited impact on any parameters tested despite many studies adding it as a reducing agent (Ricca et al., 2010; Tanner et al., 2014; Dufourny et al., 2019). The lack of effect seen could have been due to boiling (when used) and thorough CO₂ purging sufficiently establishing anaerobiosis, rendering the L-cysteine addition unnecessary. Resazurin was used in this study as a redox indicator and there was a small difference in time till colour change when L-cysteine was used in conjunction with not boiling (data not presented). Boiling the media was an efficient way to induce the redox colour change within 15 minutes, however, conditions that were not boiled did change colour faster under CO₂ when L-cysteine had been added. This agrees with work by Poelaert et al. (2018) who also found reducing agents could be omitted in favour of fully saturating the fermentation model with CO₂ before incubation. Most justification for not using L-cysteine stems from the toxicity of metabolites released (Attene-Ramos et al., 2006; Andriamihaja et al., 2010) which, though they could affect microbe populations, largely impact cell cultures and no cell-based downstream assays were used in the present study.

3.3.3.4 Recommendations for Protocol

For subsequent experiments it is recommended that boiling be used as an efficient way to induce anaerobiosis, however, the protocol should be modified to include an ice and water bath while bubbling the media with CO₂ in order to cool the buffer efficiently prior to inoculation. To confirm this, the temperature can be checked with a probe to ensure the media is below 35°C before adding the faecal slurry. It is concluded that L-cysteine did not impact any tested parameters sufficiently enough to warrant inclusion in the media composition, particularly considering the efficiency of boiling to purge oxygen and induce redox indicator colour change. The excessive proliferation of *Escherichia-Shigella*, related concerns regarding maintenance of the anaerobic environment, and desire to reduce labour intensity has determined that a pseudo-stabilisation period is

optimal to allow microbes to recover from handling stress and freezing prior to inoculating the model.

3.4 Experiment 2 Exploring Buffer Composition and Incubation Period

3.4.1 Methodology

This experiment was a factorial 2 x 4 design assessing two buffer compositions and four termination timepoints. The buffers that were used were 0.1 M phosphate buffered saline (PBS), and a complex mineral buffer (CMB) as described in detail in Table 3.1. Historical use of PBS as a main fermentative buffer (Barry et al., 1995; Edwards et al., 1996) has been replaced by a carbonate/phosphate buffer (Mould et al., 2005; Palowski et al., 2021). However, PBS remains most commonly used for preparation and dilution of faecal material (Moon, Joo, et al., 2016; Dufourny et al., 2019; Loo et al., 2023). The inclusion of PBS provided a useful comparator as a minimal/non-nutritive condition to determine how much fermentation activity was attributable to the media. The incubation periods that were tested were termination at 8 hours (as in previous experiment, Section 3.3), 10 hours, 12 hours, or 24 hours, to determine the most optimal from the range reported in the literature (Li et al., 2012; Dufourny et al., 2029; Nissen et al., 2022).

3.4.1.1 Fermentation Protocol

Each buffer was made up at room temperature by dissolving components in purified water using a magnetic stirrer before adjusting pH to 6.8 with hydrochloric acid (0.1M for PBS, 5M for CMB), and adding resazurin as a redox indicator (1.25 mg/L). As per findings from the previous experiment (see Section 3.3), both buffers were then boiled until the redox indicator changed (approximately 15 minutes), cooled in an ice and water bath under a stream of CO₂ (approximately 40 minutes), and inoculated with defrosted faecal slurry (prepared as per Section 3.2.2) once the buffer was at a maximum temperature of 35°C before second pH adjustment to 6.8; this was not required for the CMB condition. Inoculated media was then sealed and stored at 39°C overnight.

Substrate used for this experiment was milk replacer powder (DanMilk Pro, AB Neo) which was added at 0.5 ± 0.001 g to each serum bottle. There was a

total of 8 conditions being assessed with each buffer composition fermented at each timepoint. Each condition had triplicate replications per batch and the experiment repeated twice, resulting in six replicates per condition. One blank bottle was prepared with only inoculated media (no substrate) for each condition, each batch.

Inoculated media was added at 50 mL per bottle and bottles processed as described in Section 3.2.3.2. Regardless of incubation period, gas pressure was recorded and released every 2 hours, and termination and following procedures were all as described previously (Section 3.2.3.2).

3.4.1.2 Sample Processing

Gas chromatography for SCFA quantification was as detailed in Section 3.2.4 and DNA extraction as per Section 3.2.5.

3.4.1.3 Statistical Analysis

As per previous experiment for all measures (see Section 3.3.1.3), replacing main effects with buffer type x incubation period.

3.4.2 Results

3.4.2.1 Fermentation Parameters

Maximum gas volume was significantly increased in the complex mineral buffer (CMB) condition ($F_{1,10} = 26.2$; $p < 0.001$), regardless of time and there was no interaction between buffer and time. No conditions significantly impacted lag parameter. Buffer type significantly impacted growth rate ($F_{1,10} = 12.2$; $p = 0.005$), with increased growth rates seen in the CMB group (Figure 3.7).

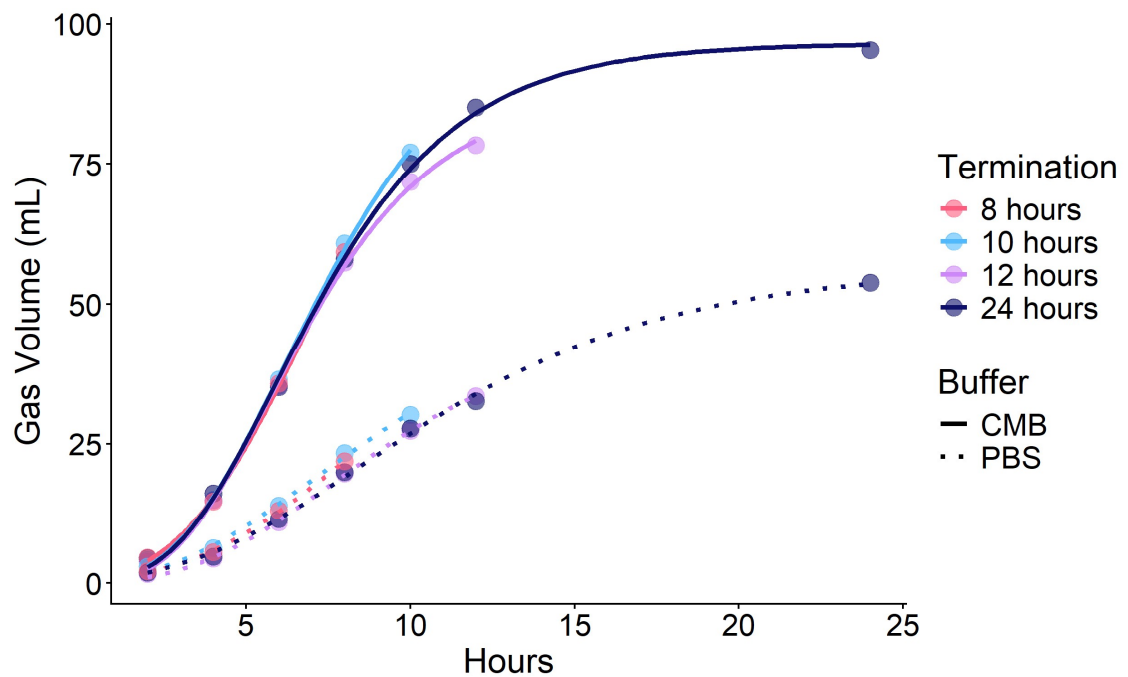


Figure 3.7 Gompertz growth curves fitted to cumulative gas production recorded during fermentation. Points represent measured gas volumes and curves represent predicted growth. Line type indicates buffer type (PBS (phosphate buffered saline 0.1 M), dotted; CMB (complex mineral buffer), solid), colour indicates incubation time before termination (pink, 8 hours; blue, 10 hours; lilac, 12 hours; dark purple, 24 hours).

The final pH was impacted by an interaction ($F_{1, 39} = 18.8$; $p < 0.001$) and differed significantly for buffer ($F_{1, 39} = 2373$; $p < 0.001$) and time ($F_{1, 39} = 15.0$; $p < 0.001$) with a drop in pH associated with the PBS condition (mean CMB 6.35 ± 0.0867 vs mean PBS 4.69 ± 0.867). *Post-hoc* testing revealed that within the CMB condition there is no effect of time but within the PBS condition there are significant differences between 8 and 12 hours ($p = 0.0259$) and additionally the 24 hour point is different to every other timepoint (all $p < 0.001$). The lowest recorded pH was the PBS condition at 12 hours (4.46 units) and the highest was the CMB condition at 12 hours (6.41 units).

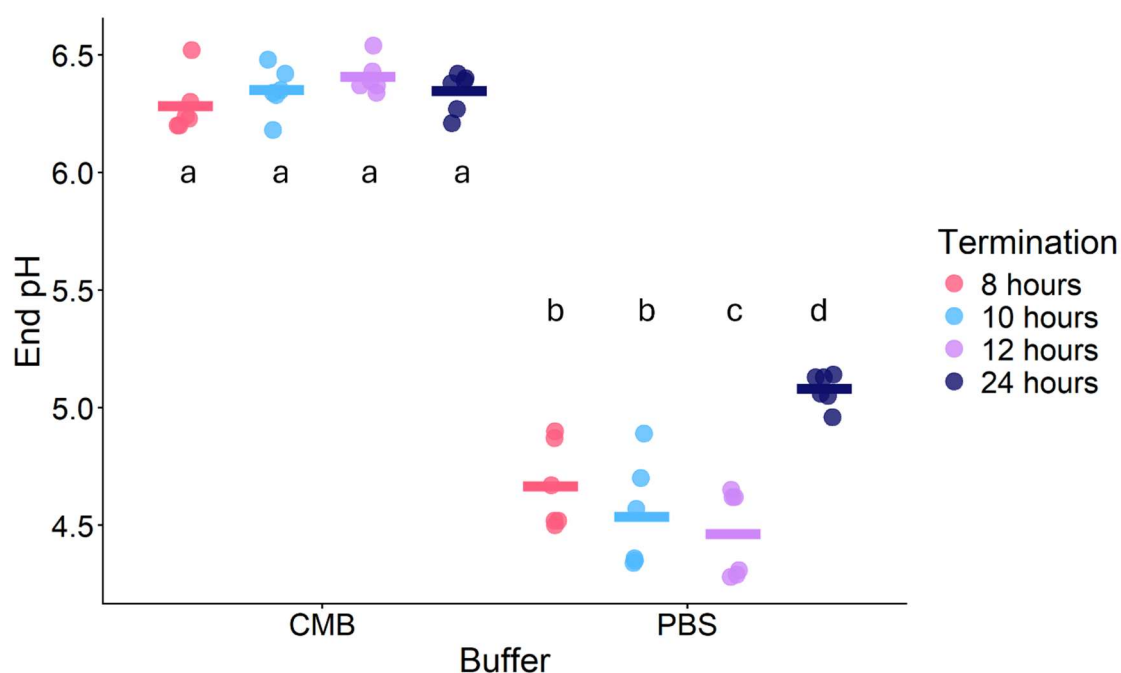


Figure 3.8 Recorded pH after fermentation was terminated (CMB = complex mineral buffer; PBS = phosphate buffered saline). Colour denotes incubation time (pink, 8 hours; blue, 10 hours; lilac, 12 hours; dark purple, 24 hours). Different letters indicate a significant difference (*Tukey-adjusted*; $p < 0.05$)

3.4.2.2 Short Chain Fatty Acid Concentrations

Acetate, butyrate and propionate concentrations were all impacted by buffer ($F_{1, 39} = 368$; $p < 0.001$, $F_{1, 39} = 138$; $p < 0.001$, $F_{1, 38} = 425$; $p < 0.001$, respectively), as was the total SCFA concentration ($F_{1, 38} = 374$; $p < 0.001$). Generally, the concentration values decreased in the PBS group when compared with CMB, however, where the time condition was significant, there was also a significant interaction (see Table 3.5) due to time having no effect on the CMB group, but eliciting an effect on the PBS group. This resulted in concentrations being generally higher at all time points in the CMB group, apart from butyrate at 24 hours which was higher in the PBS group (5.90 mM vs 4.18 mM).

Table 3.5 Short chain fatty acid (SCFA) concentrations produced for conditions of different buffers and incubation periods ('time'). Different superscript letters across rows indicate significant difference (linear mixed-effects model with reduced ANOVA; $p < 0.05$)

	Buffer	CMB ¹					PBS ²				p-values ³		
	Time	8	10	12	24	8	10	12	24	Buffer	Time	Interaction	
Acetate (mM)	Mean	24.59 ^a	27.63 ^a	27.57 ^a	26.58 ^a	6.76 ^b	7.68 ^b	9.10 ^b	8.93 ^b	<0.001	0.235	0.827	
	SE	1.38	1.03	0.9	3.78	0.48	0.44	0.34	0.51				
Propionate (mM)	Mean	13.5 ^a	15.33 ^a	15.18 ^a	14.12 ^a	3.12 ^b	3.90 ^b	4.73 ^b	8.80 ^c	<0.001	<0.001	<0.001	
	SE	0.54	0.36	0.44	1.93	0.22	0.19	0.17	0.39				
Butyrate (mM)	Mean	3.67 ^a	3.79 ^a	3.89 ^a	4.18 ^a	0.49 ^b	0.67 ^b	0.68 ^b	5.90 ^b	<0.001	<0.001	<0.001	
	SE	0.08	0.11	0.21	0.54	0.22	0.22	0.22	0.11				
Total SCFA (mM)	Mean	41.76 ^a	46.75 ^a	46.63 ^a	44.88 ^a	10.38 ^b	12.25 ^b	14.51 ^b	23.63 ^c	<0.001	0.00709	0.0255	
	SE	1.94	1.30	1.51	6.25	0.83	0.73	0.60	0.90				

¹Complex mineral buffer, composition detailed in Table 3.1

²Phosphate buffered saline (0.1 M)

³Displayed to three significant figures unless < 0.001

3.4.2.3 Microbiota

A total of 48 samples were processed to extract microbial DNA and a total of 3,075,401 reads were generated through 16S rRNA sequencing of the hypervariable V4 region. After screening and filtering 19,056 high-quality unique reads remained.

3.4.2.3.1 Alpha Diversity

Chao1, Shannon and Simpson indices were used to assess richness and evenness between and within conditions tested (see Table 3.6). Chao1 richness did not differ between buffers but did change at different incubation periods ($F_{1, 39} = 6.60$; $p = 0.00102$) with the greatest richness apparent at 8 hours for both buffer types (CMB, 624 ± 35.2 ; PBS, 616 ± 21.7). There was an interaction between buffer type and incubation period ($F_{1, 39} = 3.72$; $p = 0.0192$), as the richness was more consistent across incubation periods for the CMB group, whereas the PBS group experienced a large drop at 24 hours (477 ± 14.3 compared to 616 ± 32.5 at 12 hours).

Shannon and Simpson diversity only differed between buffer conditions (Shannon, $F_{1, 39} = 125$; $p < 0.001$. Simpson, $F_{1, 39} = 70.0$; $p < 0.001$), where the CMB groups maintained higher diversity than PBS across all time points, indicating less dominance and more evenness.

Table 3.6 Alpha diversity indices (Chao1, Shannon, Simpson) of microbial samples from fermentation fluid of different buffers and incubation periods ('time'). Different superscript letters across rows indicate significant difference (linear mixed-effects model with reduced ANOVA; $p < 0.05$)

	Buffer	CMB ¹				PBS ²				p-values ³		
	Time ⁴	8	10	12	24	8	10	12	24	Buffer	Time	Interaction
Chao1	Mean	624.74 ^a	536.39 ^a	548.49 ^a	560.01 ^a	616.21 ^a	570.27 ^a	616.02 ^a	476.96 ^b	0.885	0.00102	0.0192
	SE	35.20	25.20	7.85	21.05	21.68	21.55	32.52	14.29			
Shannon	Mean	3.68 ^a	3.67 ^a	3.71 ^a	3.74 ^a	3.26 ^b	3.22 ^b	3.40 ^b	3.24 ^b	<0.001	0.234	0.315
	SE	0.03	0.02	0.05	0.07	0.08	0.04	0.06	0.07			
Simpson	Mean	0.95 ^a	0.94 ^a	0.95 ^a	0.95 ^a	0.91 ^b	0.91 ^b	0.92 ^b	0.92 ^b	<0.001	0.239	0.410
	SE	0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.01			

¹Complex mineral buffer

²Phosphate buffered saline (0.1 M)

³Displayed to three significant figures unless < 0.001

⁴Incubation period in hours

3.4.2.3.2 Beta Diversity

Microbial community composition was dependant a significant interaction between the two conditions ($F_{1, 47} = 6.10$; $R^2 = 0.0656$; $p = 0.002$), with buffer independently explaining the most variation in composition (*Bray-Curtis*; $F_{1, 47} = 200$; $R^2 = 0.720$; $p = 0.001$), and incubation period being significant but weak ($F_{1, 47} = 6.60$; $R^2 = 0.0712$; $p = 0.002$). *Post-hoc* analyses using the *pairwiseAdonis* function revealed differences between CMB and PBS conditions at all time points (all $p < 0.001$; Figure 3.9), no differences between different incubation periods within the CMB group, and significant community shifts within the PBS group at various time points: 8 vs 12 hours ($R^2 = 0.594$; $p = 0.018$); 10 vs 12 hours ($R^2 = 0.527$; $p = 0.024$); 12 vs 24 hours ($R^2 = 0.639$; $p = 0.012$).

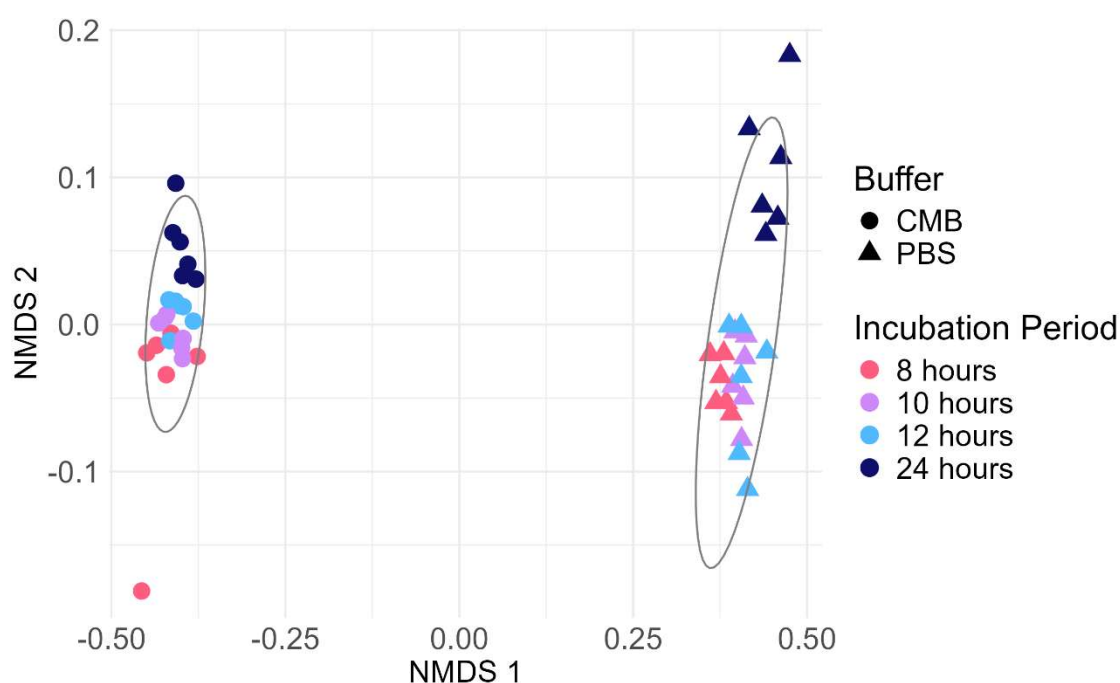


Figure 3.9 Non-metric multidimensional scaling (NMDS) ordination of microbial community composition based on Bray-Curtis dissimilarity. Each point represents a sample with colour denoting incubation period (pink, 8 hours; blue, 10 hours; lilac, 12 hours; dark purple, 24 hours) and shape denoting buffer used (round, CMB (complex mineral buffer); triangle, PBS (phosphate buffered saline)). Ellipses show 95% confidence intervals around buffer group centroids as buffer type explained the most variation seen (71.9%)

3.4.2.3.3 Relative Abundance: Phylum Level

All groups of the CMB condition were dominated by Bacteroidota (average across incubation periods being $52.2 \pm 1.80\%$), whereas Bacteroidota were low in abundance across all PBS groups (average being $2.71 \pm 0.298\%$). The PBS

groups were instead dominated by Bacillota (average of $80.6 \pm 2.43\%$) which was second most abundant for all CMB groups (average of $24.8 \pm 1.14\%$) and similar in abundance in CMB groups to Pseudomonadota ($19.7 \pm 1.63\%$), which was a little lower in PBS groups ($14.6 \pm 2.40\%$).

Comparison using ANCOM-BC between buffer conditions highlighted significant and high LFC at all time points for Bacillota (LFC > 1; $p < 0.001$) and Bacteroidota (LFC > 2; $p < 0.001$) where across all incubation periods, the CMB group had increased abundance of Bacteroidota and the PBS group had increased Bacillota.

Time differences within each buffer condition showed only one phylum altered by more than 1 LFC which was Actinomycetota abundance between 8 hours and 24 hours in the CMB group (LFC = 1.06 ± 0.189 ; $p < 0.001$). As seen in Figure 3.10, this is due to a small increase in Actinomycetota at the 12 hour mark, followed by a larger increase at 24 hours.

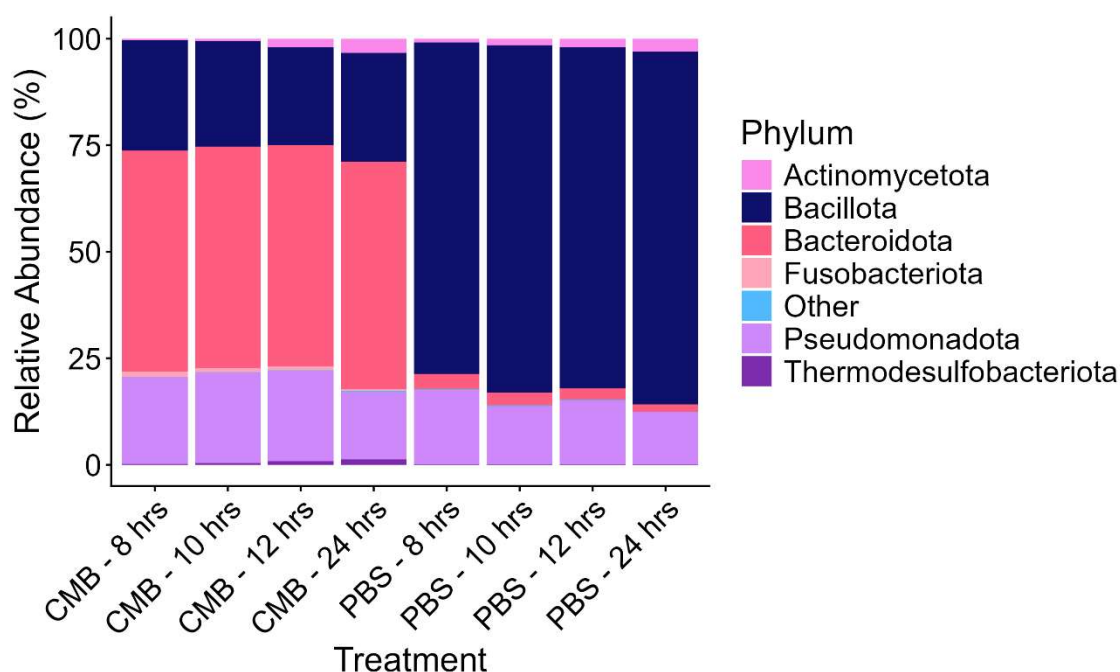


Figure 3.10 Percentage relative abundance of phyla present at above 1% in at least one group where treatment is a combination of different buffer compositions (CMB, complex mineral buffer; PBS, phosphate buffered saline (0.1 M)) and incubation periods. 'Other' denotes the sum of phyla present at <1% relative abundance

3.4.2.3.4 Relative Abundance: Genus Level

Five genera were significantly different and had high log fold change between buffer conditions at all time points measured. These were *Lactobacillus*,

Limosilactobacillus, *Bacteroides*, *Sharpea*, and *Parabacteroides* (all LFC > 1 and all $p < 0.001$). Additionally, there were notable differences in *[Ruminococcus]_gnavus_group* between buffers at 8 hours (LFC = 1.55 ± 0.138 ; $p < 0.001$) and 10 hours (LFC = 1.35 ± 0.105 ; $p < 0.001$) but not at other timepoints.

The differences between time points within each buffer condition included some difference in *[Ruminococcus]_gnavus_group* abundance within the CMB condition between 8-12 hours (LFC = 1.21 ± 0.137 ; $p < 0.001$), 8-24 hours (LFC = 1.69 ± 0.130 ; $p < 0.001$), and 10-24 hours (LFC = 1.41 ± 0.0586 ; $p < 0.001$), where the abundance decreases at and after 12 hours. No other genera were changed to a notable level within the CMB group. Within the PBS group, changes were focussed around the 24 hour group where there was increase in *Enterococcus* between 8-24 hours (LFC = 1.20 ± 0.300 ; $p = 0.00241$), and increased *Megasphaera* between 10-24 hours (LFC = 2.51 ± 0.156 ; $p < 0.001$) as well as 12-24 hours (LFC = 2.44 ± 0.157 ; $p < 0.001$), as shown in Figure 3.11.

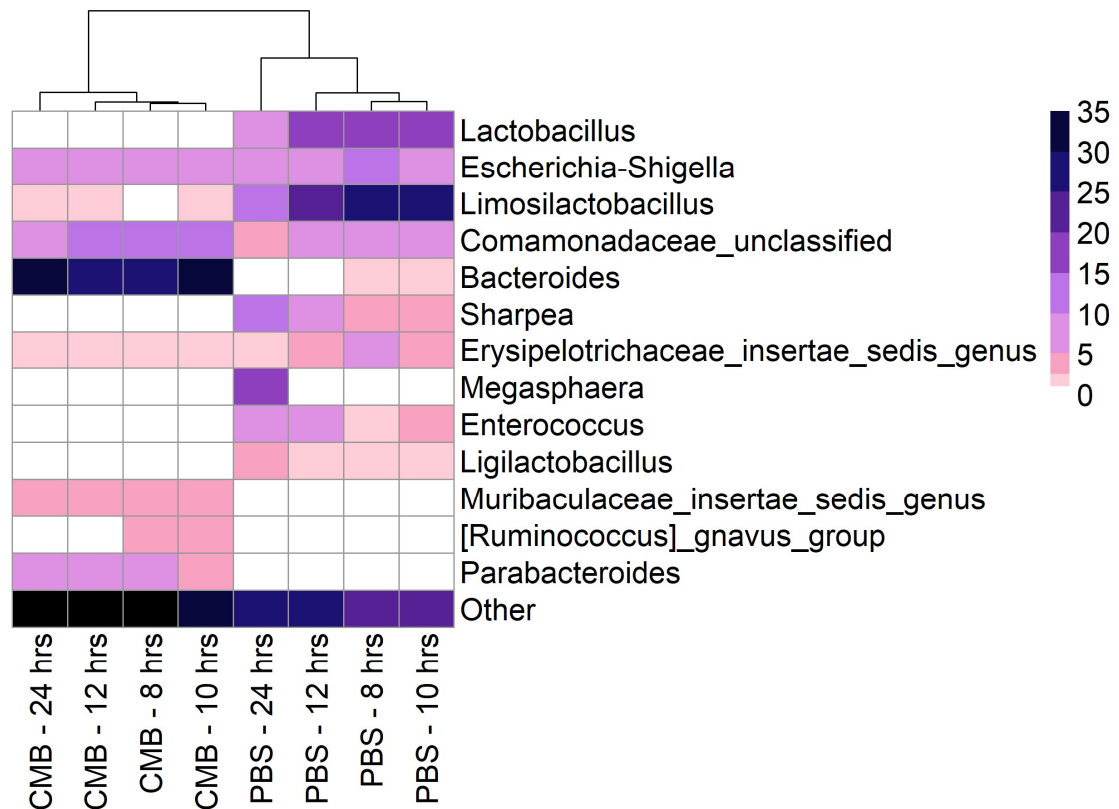


Figure 3.11 Heatmap indicating relative abundance of genera that were above 4% in at least one group where treatment is a combination of different buffer compositions (CMB, complex mineral buffer; PBS, phosphate buffered saline (0.1 M)) and incubation periods. ‘Other’ denotes the sum of genera present at <4% relative abundance. Colour denotes magnitude of abundance where pale boxes are lesser and dark boxes are increased, as per right-hand key. Dendrogram clusters columns based on Bray-Curtis dissimilarity

3.4.3 Discussion

This experiment aimed to identify differences across fermentation characteristics and microbial communities when using two fermentation buffer compositions, and four different incubation periods in a fully factorial design. This experiment investigated use of phosphate buffered saline (PBS) at 0.1M as the media base, or using a complex mineral buffer (CMB) as described by Palowski et al. (2021). Both buffer compositions were tested across four incubation periods (8, 10, 12 and 24 hours) to identify an optimal buffer and fermentation termination point for the development of a batch *in vitro* pre-weaning piglet colon model.

The hypothesis for optimal buffer composition is supported whereby the carbonate-phosphate buffer (CMB) had higher buffering capacity, however, there was minimal microbiota shift between 12 and 24 hours incubation within the CMB

group, rejecting the hypothesis that the microbiota would be compromised in this period.

3.4.3.1 Buffer Composition Effects

Fermentation kinetics were significantly different between buffers, with lower growth rate and lower maximum gas volume produced by the PBS condition. In conjunction with the significantly lower alpha diversity across the PBS condition, as well as reduced SCFA production and low final pH, these results suggest PBS may not be a sufficiently supportive media for bacterial fermentation. As batch fermentations are closed systems, the depletion of substrate and accumulation of acidic metabolites can reduce the pH heavily, which is demonstrated here. The CMB condition, despite not containing additional nutrients or nitrogen sources as included in continuous systems (Tanner et al., 2014; Fleury et al., 2017), was better able to maintain the appropriate pH up to and including 24 hours incubation time.

Sequencing data revealed that the PBS conditions were dominated by Bacillota, to the point of considerably low Bacteroidota, while the CMB conditions had increased Bacteroidota while able to retain a strong proportion of Bacillota (~25%). While these two phyla were expected to be the top two OTUs, as seen previously, the extreme proportion of Bacillota, as per PBS, is more commonly seen either at 1 day of age (Bian et al., 2016; Chen et al., 2018), or in weaned pigs (Kim et al., 2011; Schmidt et al., 2011) with Bacteroidota being the dominant phyla during the suckling period (Chen et al., 2018).

Enrichment of Bacteroidota, particularly *Bacteroides* (seen in the CMB conditions, Figure 3.11), is also reported in breastfeeding infants (Palmer et al., 2007; Marcobal et al., 2011) and associated with their ability to efficiently metabolise complex glycans, rich in breastmilk and sow milk. The decline of *Bacteroides* with increasing age (Mach et al., 2015) is therefore likely due to the dietary shift from milk to plant-based feedstuffs, more suited to genera such as *Lactobacillus* and *Prevotella* (Kim et al., 2011; Frese et al., 2015). Furthermore, Bacteroidota is positively correlated with pre-wean weight gain, where Bacillota is negatively correlated (Ding et al., 2019). Low abundance of *Bacteroides* is associated with diarrhoea (Gryaznova et al., 2022), further supporting the theory that the PBS condition has more similarity to an unhealthy microbiota, and the

CMB condition producing a microbial community more reflective of the healthy pre-wean piglet.

Considering the final pH of the PBS fermentation was significantly lower than the CMB group at all incubation time points, it is logical that genera such as *Lactobacillus* and *Limosilactobacillus* thrived as they are lactic acid bacteria. The prevalence of lactic acid bacteria explains the decreased pH, without the usually associated increase in SCFA production, as the accumulation of lactic acid will lower the environmental pH. Usually lower pH is associated with increased SCFA production, and as lactate can be converted to SCFA such as butyrate via lactate utilising bacteria (Pryde et al., 2002; Duncan et al., 2004), it is surprising that the PBS group had such low SCFA output. Additionally, *Lactobacillus* is often used as a probiotic due to associations with enhanced neonatal gut health (Rinne et al., 2005; Berrington et al., 2013) and is commonly reported as abundant in the piglet colonic microbiota (Mach et al., 2015; Gaukroger et al., 2020). It is therefore concerning that *Lactobacillus* was so lacking in the CMB condition, however, this is one of the only contradictions to other data which, when looked at holistically, suggests CMB as the optimal buffer composition.

It is acknowledged that *Escherichia-shigella* is prominent in both buffer conditions, but, as discussed earlier (see Section 3.3.3.2), this genus is found in suckling piglets commensally (Mach et al., 2015; Bian et al., 2016; Chen et al., 2018) and is not present at such an increased proportion as was found in the previous experiment, thus is less concerning when seen here. The proliferation of this genus in the previous experiment was extreme and compromised overall richness and evenness, which is not currently demonstrated. Both conditions also show a substantial population of *Other* genera (those prevalent at < 1%), highlighting the diversity maintained during the fermentation.

3.4.3.2 Incubation Time Effects

Time had no effect on fermentation kinetics, which was expected. Figure 3.7 shows the truncated nature of the 8, 10 and 12 hour incubations, highlighting that they did not include a plateau phase and followed the same predicted curve as 24 hours. Consequently, the Gompertz model predicted maximum gas production potential of these groups to be no different to the 24 hour group, explaining the lack of significance between asymptote maximum gas volume values. After 24

hours of incubation, a more distinct plateau is visible in both buffer conditions, suggesting this period may give a fuller fermentation profile than other incubation periods tested, therefore providing better clarity for comparisons in future investigations. Conversely, by having such an extended plateau in a closed system, it would be expected that the lack of nutrients available may compromise the microbiota. If this were the case, the argument could be made for terminating at 15 hours, for example, as this appears to be an approximation of substrate depletion according to Figure 3.7. This, however, would be highly impractical to conduct due to labour intensity and subsequent fatigue increasing the risk of errors. Nonetheless, the microbial community composition did not significantly change (to be discussed shortly) in the CMB group between 12 and 24 hours, confirming this to be a suitable termination point.

Incubation period had a minimal effect on CMB conditions throughout but had many effects on the PBS group, resulting in frequent significant interactions between main effects across various recorded parameters. The first example of this is seen in final pH where the CMB group stays consistent across incubation periods, but the PBS group drops significantly at 24 hours in comparison to all other timepoints within that buffer condition. This distinction continued with a sharp increase propionate and butyrate production at 24 hours, as well as a steep drop in the Chao1 index value, indicating more dominance and less evenness in the microbial community. There was no indication of this at the phylum level, however, at the genus level, previously abundant genera at the 8-12 hour periods, such as *Lactobacillus* and *Limosilactobacillus*, were reduced in favour of *Megasphaera* and *Sharpea* at 24 hours. Neither of which are seen at this level of abundance in the *in vivo* piglet gut (Mach et al., 2015; Bian et al., 2016; Chen et al., 2018), further cementing previous conclusions that PBS is not a suitable buffer for this model, and specifically not for fermentations of 24 hours.

3.4.3.3 Recommendations for Protocol

Phosphate buffered saline is not recommended for use as a buffer for fermentation. Instead, a more complex composition should be used to provide optimal buffering capacity such as detailed in this experiment as 'complex mineral buffer' (Palowski et al., 2021), the composition of which is detailed in Table 3.1. An incubation period of 24 hours should be used moving forward as this best

balances practicality and accessibility of the protocol without omitting valuable fermentation kinetics and does not compromise the microbiota significantly.

3.5 Experiment 3 Exploring the Addition of Mucin

3.5.1 Method

This experiment was a simple parallel comparison, exploring the effect of adding porcine mucin (2 g/L) to the fermentation media as mucin-specific microbial communities have been reported (Zoetendal et al., 2002; Macfarlane, 2008) and addition of mucin directly to fermentation media has been previously advised (A. Buckley, *personal comms.*, Oct 2023; Davis Birch et al., 2023).

3.5.1.1 Fermentation Protocol

As per findings from previous experiments, the base buffer used was CMB (see Table 3.1 for composition), and this was prepared identically to Section 3.4.1.1. Briefly, the component buffer salts were added to purified water and pH adjusted to 6.8 with 5M hydrochloric acid. At this point, for the mucin condition, 2 g/L porcine mucin was mixed with a small sample of removed media (approximately 10 mL) and homogenised on a heated magnetic stirrer for roughly 10 minutes. This was necessary due to the hydrophobic nature of the mucin. Protocol then continues identically for both conditions. Resazurin was added (1.25 mg/L) and the solution boiled until colour change. The media was then cooled in a water and ice bath under a stream of CO₂ until at a maximum temperature of 35°C before slurry was added and the media filtered through four layers of cheesecloth. Media was bubbled with CO₂ for a further 30 minutes to ensure anaerobiosis and sealed and stored at 39°C overnight.

Substrate and serum bottle preparation was identical to that described in previous experiments (see Section 3.2.3.1), using 0.5 g piglet milk replacer powder, and incubation period was 24 hours as per findings from Section 3.4. Termination in an ice bath followed final gas release and recording which was otherwise done at 2 hour intervals until 10 hours.

3.5.1.2 Sample Processing

Fermentation fluid was removed from each bottle after final pH was recorded and samples processed for gas chromatography and DNA extraction as described in Sections 3.2.4 and 3.2.5, respectively.

3.5.1.3 Statistical Analysis

Extracted Gompertz parameters, final pH and SCFA data were analysed using a linear mixed model with batch as a random factor. ANOVA was applied to the models followed by reduction using *lmerTest*. Alpha diversity indices were analysed using an independent samples *t*-test after confirming appropriate distribution using Shapiro-Wilk. The remaining microbiota analysis was performed as described in 3.2.6.3.

3.5.2 Results

3.5.2.1 Fermentation Parameters

Addition of mucin yielded no significant difference in maximum gas volume. Lag parameter and growth rate were significantly different ($F_{1,4} = 26.2$; $p = 0.00690$ and $F_{1,4} = 16.5$; $p = 0.0153$, respectively), with the mucin eliciting a reduced lag asymptote and increase growth parameter. These results are visualised in Figure 3.12.

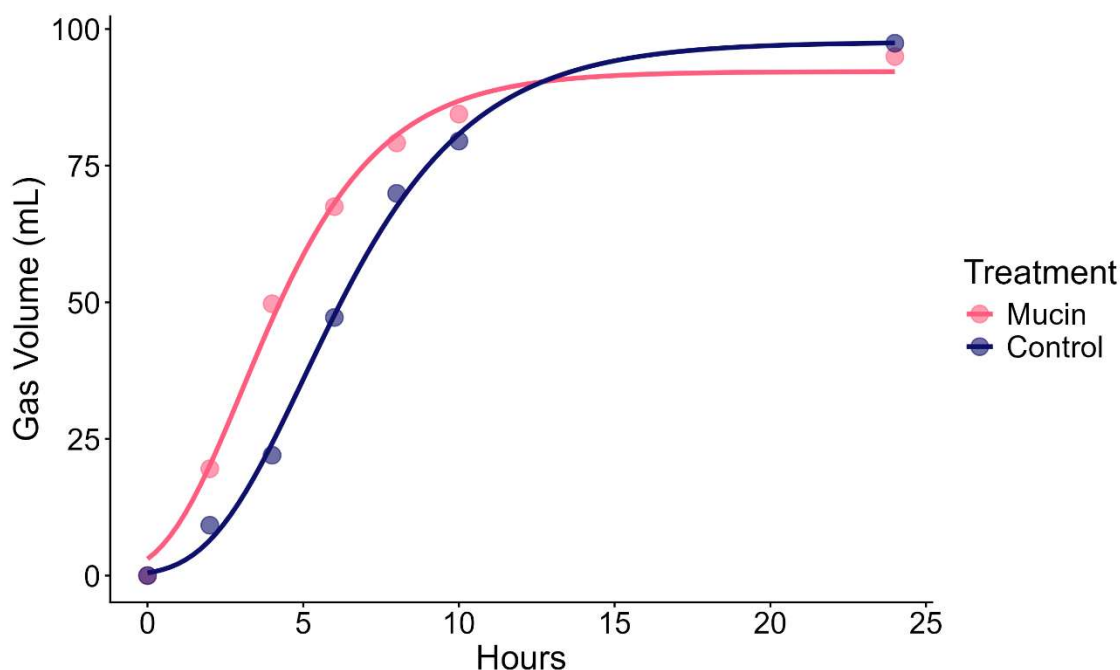


Figure 3.12 Gompertz growth curves fitted to cumulative gas production recorded during fermentation. Points represent measured gas volumes and curves represent predicted growth. Colour denotes treatment (pink, mucin added (2 g/L); purple, no mucin)

The end pH for each group was 6.28 ± 0.0142 for the mucin condition and 6.38 ± 0.0152 for the control group which was significantly different ($t = 13.74$; $p < 0.001$).

3.5.2.2 Short Chain Fatty Acid Concentrations

There was no significant difference in acetate concentration between groups ($F_{1, 14} = 2.07$; $p = 0.172$). There was a difference in butyrate ($F_{1, 14} = 23.0$; $p < 0.001$), propionate ($F_{1, 14} = 401$; $p < 0.001$), and total SCFA concentration ($F_{1, 14} = 53.3$; $p < 0.001$), where the mucin condition increased concentrations for each, but the effect size was minimal for butyrate (Table 3.7).

Table 3.7 Short chain fatty acid (SCFA) concentrations produced after fermentation when adding porcine mucin at 2 g/L (mucin) or not (control) to the media. Different superscript letters across rows indicate significant difference (linear mixed-model with reduced ANOVA; $p < 0.001$)

		Control	Mucin	p-value
Acetate (mM)	Mean	24.36 ^a	25.99 ^a	0.172
	SE	0.95	0.67	
Propionate (mM)	Mean	15.37 ^a	25.6 ^b	<0.001
	SE	0.22	0.55	
Butyrate (mM)	Mean	7.15 ^a	8.03 ^b	<0.001
	SE	0.39	0.22	
Total SCFA (mM)	Mean	46.88 ^a	59.62 ^b	<0.001
	SE	1.38	1.07	

3.5.2.3 Microbiota

A total of 18 samples were processed to extract microbial DNA and a total of 1,175,727 reads were generated through 16S rRNA sequencing of the hypervariable V4 region. After screening and filtering 7562 high-quality unique reads remained.

3.5.2.3.1 Alpha Diversity

There was no difference in Chao1 values between conditions ($t = 1.66$; $df = 15.9$; $p = 0.116$), despite mucin having a lower mean Chao1 value (584 ± 20.1 vs 630 ± 18.4) but Shannon and Simpson diversity were significantly lower with the

addition of mucin (Shannon, $t = 2.94$; $df = 15.4$; $p = 0.0100$; Simpson, $t = 2.67$; $df = 14.4$; $p = 0.0181$). Shannon diversity was 3.08 ± 0.0839 for the mucin group and 3.47 ± 0.103 for the control group, and mucin produced a Simpson index of 0.866 ± 0.0100 and control was 0.910 ± 0.0135 .

3.5.2.3.2 Beta Diversity

Using the PERMANOVA to assess microbial community composition pointed to a significant shift in composition between mucin and control groups (*Bray-Curtis*; $F_{1, 17} = 59.3$; $R^2 = 0.455$; $p = 0.001$) but batch was also a significant explainer of the community shift ($F_{1, 17} = 18.3$; $R^2 = 0.281$; $p = 0.001$) and this caused a significant interaction ($F_{1, 17} = 11.2$; $R^2 = 0.172$; $p = 0.001$), which can be seen in Figure 3.13.

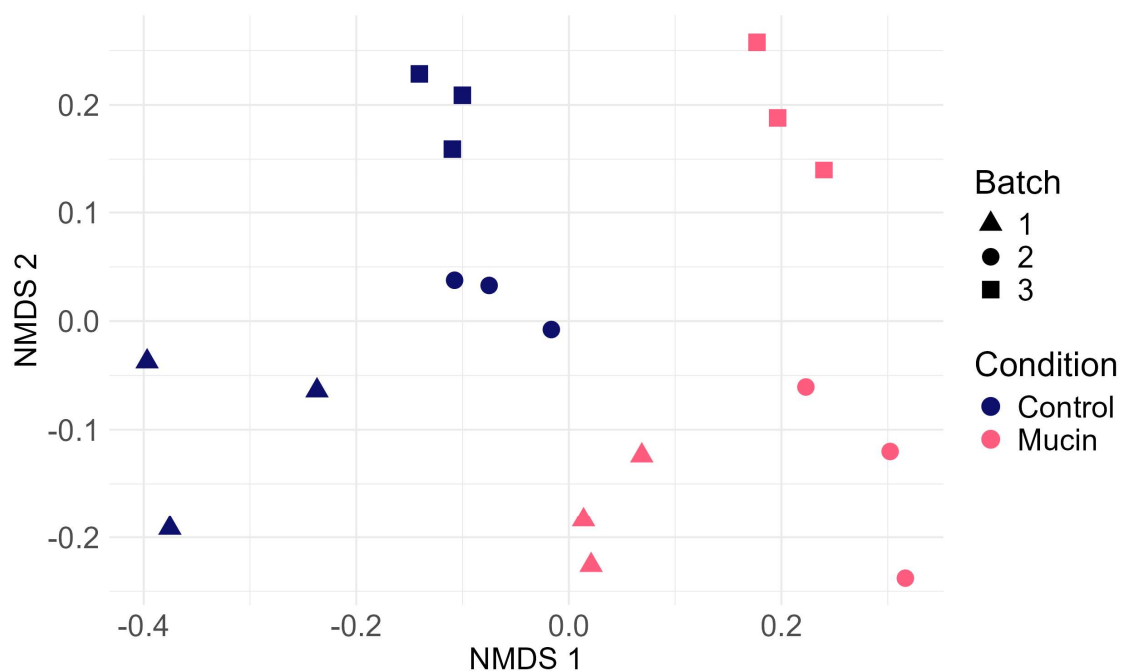


Figure 3.13 Non-metric multidimensional scaling (NMDS) ordination of microbial community composition based on Bray-Curtis dissimilarity. Each point represents a sample with colour denoting treatment (purple, control; pink, mucin added) and shape denoting batch (triangle, batch one; round, batch two; square, batch three)

3.5.2.3.3 Relative Abundance: Phylum Level

The only notable phylum shift was Fusobacteriota (LFC = 1.10 ± 0.311 ; $p = 0.00209$) which decreased with the addition of mucin (from 5.08% to 0.221%). There were other statistically significant differences between mucin and control conditions but not to a notable LFC threshold (Figure 3.14).

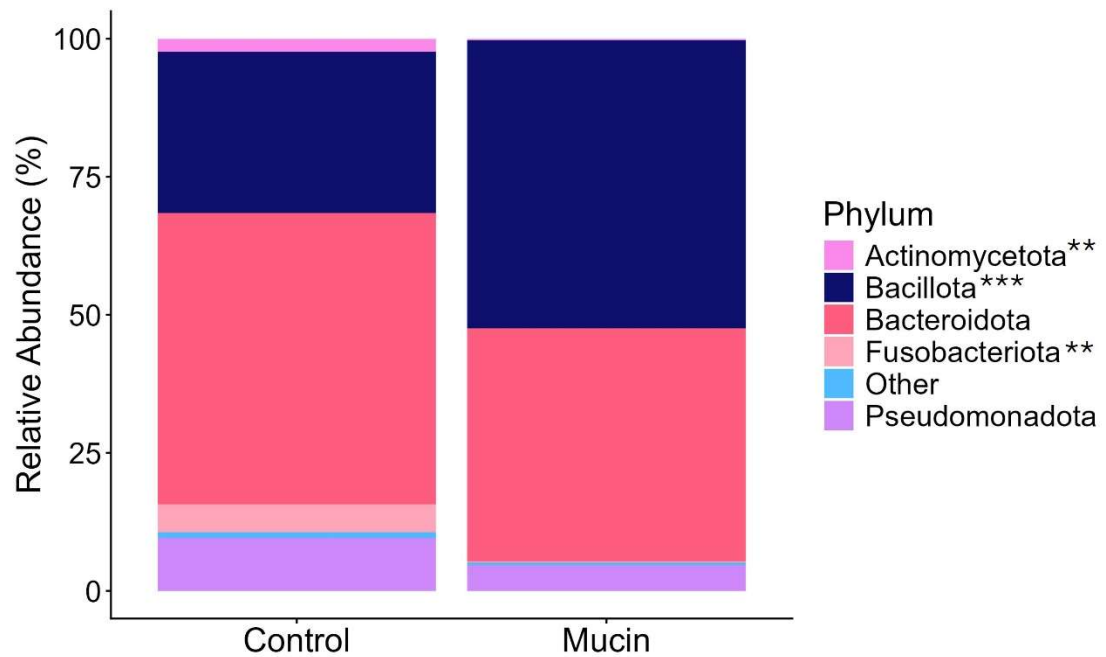


Figure 3.14 Percentage relative abundance of phyla present at above 1% in at least one group. 'Other' denotes sum of phyla present at <1% relative abundance. Significant differences between control and mucin treatments are indicated (ANCOM-BC: *, $p < 0.05$; **, $p < 0.01$; *, $p < 0.001$)**

3.5.2.3.4 Relative Abundance: Genus Level

With the addition of mucin, *Streptococcus* abundance increased from 3.78% in the control to 23.9% (LFC = 1.87 ± 0.180 ; $p < 0.001$), *Holdemanella* increased from 0.296% to 4.39% (LFC = 1.33 ± 0.224 ; $p < 0.001$) and *Fusobacterium* decreased from 5.08% to 0.221% (LFC = 1.24 ± 0.288 ; $p < 0.001$). As with the phylum level analysis, there were other genera that were significantly different but not to a notable LFC threshold (Figure 3.15).

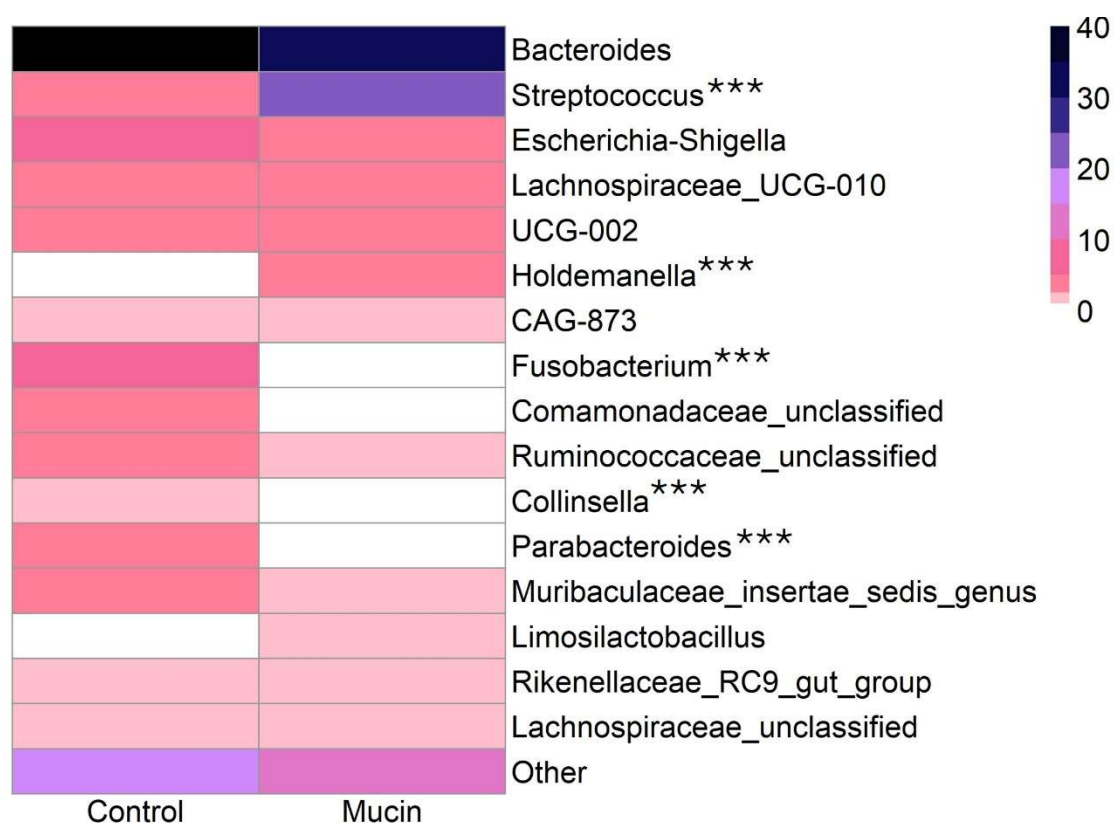


Figure 3.15 Heatmap indicating relative abundance of genera that were above 1% in at least one group. ‘Other’ denotes sum of genera at <1% relative abundance. Colour denotes magnitude of abundance where pale boxes are lesser and dark boxes are increased, as per right-hand key. Dendrogram clusters columns based on Bray-Curtis dissimilarity. Significant differences between control and mucin treatments are indicated (ANCOM-BC: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$)

3.5.3 Discussion

This experiment explored the addition of porcine mucin to the fermentation media with the hypothesis that this would capture previously hidden mucin-associated microbes. The hypothesis was supported, evidenced by changes in microbial populations captured, with additional increased fermentation shown also.

3.5.3.1 Increased Fermentation with Mucin

Despite significantly different lag and growth asymptote parameters, both conditions showed comparable fermentation profiles regarding overall shape, reaching the plateaux phase at similar points. The difference in kinetics, although statistically significant, is much less stark than seen in main effects of previous experiments (see Section 3.3.2.1 for inoculation effect and 3.4.2.1 for buffer

effect), but nevertheless, it can be concluded that the addition of mucin encourages a more active fermentation. Mucin supporting a more active fermentation is further evidenced by the increased production of SCFA, most substantially, propionate. This is reflected in the literature with introduction of mucin covered microcosms (MCM) increasing gas production, even in the absence of a substrate, and increasing propionate production after a 24 hour incubation (Tran et al., 2016). This is also confirmed by Fox et al. (2009) who reported significant increase in acetate, butyrate and propionate production when adding mucin to an *in vitro* fermentation in comparison with the control. Interestingly, the mucin condition in this study increased or produced similar concentration of SCFA in comparison to highly fermentable carbohydrate components such as mannose and galactose, which were hypothesised to ferment more readily (Fox et al., 2009). Increased fermentative activity with mucin could be due to consumption as an energy source by some bacteria. Crost et al. (2013) reported certain *Ruminococcus gnavus* strains belonging to the *Lachnospiraceae* family use mucin as a carbon source and produce propionate, however, there are no clear differences in abundance of any *Lachnospiraceae* between the control and mucin conditions in the present study. Other bacteria do use mucin as an energy source, particularly when low on alternatives (Rabiu and Gibson, 2002), which could be happening here when the substrate is being degraded and not replaced. An additional source of energy compared with the control would explain the increased fermentation activity.

The final pH was also statistically significant, with the mucin eliciting a drop of 0.1 units, however, this is not considered biologically relevant for the following reasons. The colonic environment has been reported to drop by approximately 1.2 units naturally over a 12 hour period (Li et al., 2012) due to the normal production of acidic biproducts through microbial fermentation. Furthermore, other studies modelling the piglet gut *in vitro* have used lower starting pH values of 6.5 (Li et al., 2012) or 6.0 (Gresse et al., 2021), highlighting the relative nature of pH comparisons. Finally, the main justification for recording final pH across all experiments was to monitor more extreme examples of acidosis as seen in Section 3.4.2.1 with the use of a PBS based media. The statistical significance likely stems from exceptionally low variation across replicates within each condition. A variation of more than 0.1 pH units between replicates would be

unsurprising, therefore it can be concluded that this difference between treatments is unremarkable.

3.5.3.2 Microbial Population Shifts

Based on previous studies exploring which bacterial populations thrive in a mucin environment, it was hypothesised that addition of mucin may increase populations such as *Lactobacillus* (Van den Abbeele et al., 2012) and *Bacteroides fragilis* (Macfarlane et al., 2005). This was not the case and although the genus *Bacteroides* was prevalent in the mucin condition, it was no significantly different to the control. No *Lactobacillus* was identified at >1% relative abundance which is concurrent with Tran et al (2016) who postulated that batch fermentation models are not as well suited to nurturing mucosal communities. Continuous models offer stabilisation periods allowing the mucus-binding bacteria to adhere and thus avoid wash-out better than non-adherent populations. Conversely, the closed batch system, with no wash-out risk, cultivates a community which is strongly driven by early colonisers.

Van den Abbeele et al. (2012) proposed that faecal samples do not provide appropriate mucus-associated microbe populations in the same way as gut samples which will have limited the opportunity to capture these in the current study. Although not *Lactobacillus*, a member of the order Lactobacillales was significantly increased in the mucin condition. *Streptococcus* elicited a positive 1.87 log fold change when inoculum was fermented with mucin. As a fellow lactic acid bacterial genus, *Streptococcus* is an expected find when fermenting a lactose-based substrate, as well as *in vivo* in suckling pigs (Li et al., 2018b; Mu et al., 2019). On the other hand, the relative abundance of *Streptococcus* found previously is considerably lower than the present results (Li et al., 2018b; Mu et al., 2019), so it is recommended that mucin concentration is decreased to discourage such competitive proliferation.

Increased *Streptococcus* may be due to similar utilisation of mucin but without sufficient *Lactobacillus* present in the faecal community. It could be argued that the buffering capacity of the fermentation media and high starting pH will deter *Lactobacillus* as they thrive in more acidic environments (Chen, 2021). Tran et al. (2016) recommended early sampling time than they used (24 hours), however, sampling as early as 8 hours in a previous experiment did not identify

any lactobacillus using the optimal buffer, but did highlight a strong population using PBS, likely due to the reduced buffering capacity and lower pH (see Section 3.4.2.3.4). Rapid and sustained pH decrease is not a beneficial aspect to mimic *in vitro*, hence why other options to enhance *Lactobacillus* prevalence were explored, such as mucin addition, because it has been previously reported in piglets *in vivo* (Mach et al., 2015; Gaukroger et al., 2020).

3.5.3.3 Recommendations for Protocol

To conclude, porcine mucin is recommended as an addition to the fermentation media composition but at a reduced concentration of 1 g/L with the view that this allows for proliferation of microbial populations previously not captured, but without overgrowth of these.

3.6 Experiment 4 Exploring Different Substrates and Use of a Foregut Digestion Simulation Prior to Fermentation

3.6.1 Methodology

This experiment simultaneously manipulated two factors (substrate type and digestion status), allowing the estimation of the main effect on two substrate types within a single experiment, with no interaction hypothesised. The two substrates were piglet milk replacer (as used in previous experiments) and sow colostrum. Both of these substrates were used either in their standard form, or having undergone a foregut digestion phase prior to use in the fermentation (hereafter referred to as 'pre-digested' substrates), and here digestion is the effect investigated. Many *in vitro* fermentation models do not incorporate a foregut phase (Smiricky-Tjardes et al., 2003; Vester Boler et al., 2009a; Li et al., 2012; Nissen et al., 2022), however, there is evidence to suggest foregut hydrolysis significantly alters the substrate, and subsequent microbial fermentation activity (Boisen and Fernandez, 1997; Bindelle et al., 2007; Fleury et al., 2017; Dufourny et al., 2019). Due to the contradicting literature and premature nature of the pre-wean piglet foregut (Abrahamse et al., 2012; Bourlieu et al., 2014), this experiment was necessary to determine the efficacy of this additional phase. Difference between substrates was not explored at this time, due to high variance in composition, and existing reports of significant substrate-driven fermentation (reviewed by Coles et al. (2005)).

3.6.1.1 Colostrum Sampling

Colostrum sampling was as described in Chapter 2 (Section 2.2.2). Briefly, the samples were collected from Large White X Landrace sows (parity 2-8) housed at the National Pig Centre, University of Leeds. Colostrum was extracted by manual manipulation of the teats at onset of parturition. Samples per sow were pooled from a range of teats to reduce teat quality bias. Samples were also pooled from all sows to capture an average composition and reduce individual sow variation. Samples were frozen in aliquots at -80°C until use.

3.6.1.2 Foregut Digestion Simulation

The foregut digestion phase took place the day prior to the standard preparatory day, making the experimental protocol across a 4-day period: (1) foregut digestion, (2) media preparation, (3) fermentation commencement, (4) termination of fermentation and sample processing. The foregut digestion simulation was based on studies discussed in Sections 1.3.1 and 3.1, largely following the work of Ménard et al. (2018) but also influenced by the INFOGEST protocol (Minekus et al., 2014; Brodkorb et al., 2019) and personal communication with an experienced technician in the field (Rigby, 2024). Adjustments were made to better simulate conditions of the young piglet foregut. This included bypassing the oral phase, reducing complexity of simulated fluid composition, decreasing concentration of enzymes, increasing pH, and reducing incubation periods. It should be acknowledged that a lot of the referenced work is from the human infant field, however, considering the lack of comparable piglet studies and similarities between infant and piglet foregut function and physiology (Odle et al., 2014; Ferenc et al., 2014; Yin et al., 2017), this is seen as an acceptable basis for the method.

3.6.1.2.1 Simulated Fluid Preparation

In advance of the foregut digestion protocol, electrolyte stock solutions (sodium chloride, potassium chloride, and sodium bicarbonate; see Table 3.8 for concentrations) were made up to provide the base for the simulated gastric fluid (SGF) and simulated intestinal fluid (SIF). These were frozen in aliquots at -20°C until use. The foregut digestion phase began with defrosting the stock solutions at room temperature before warming up to 39°C in a water bath. Enzymes were prepared fresh when required, allowing containers to warm to room temperature

before opening (Rigby, 2024) and solutions kept on ice to minimise activity loss. Final concentration of each component in SGF and SIF once all components are added can be seen in Table 3.8.

Table 3.8 Final compositions of simulated fluids

Simulated gastric fluid		Simulated intestinal fluid	
Sodium chloride	94 mM	Sodium chloride	164 mM
Potassium chloride	13 mM	Potassium chloride	10 mM
Pepsin ¹	328 U/mL	Sodium bicarbonate	85 mM
Gastric lipase ¹	23 U/mL	Calcium chloride ²	3 mM
		Porcine bile extract	3.1 mM
		Pancreatin	90 U/mL lipase 16 U/mL trypsin

¹Provided through use of rabbit gastric extract (Lipolytech, Marseille, France) with supplemented porcine pepsin to reach necessary activity (Sigma-Aldrich, Darmstadt, Germany)

²Added fresh to avoid precipitation in stock solution

Pepsin and gastric lipase activity were calculated by 63 U pepsin and 4.5 U lipase per mL gastric content per kg bodyweight, as previously reported for infants (Armand et al., 1996; Roman et al., 2007). A pseudo pre-weaning piglet weight of 5.2 kg was used based on collected piglet data from the NPC (unpublished data, collected 2023). Chloride salts were used to make the electrolyte solutions as carbonates can release CO₂ and may cause pH shifts. To minimise this when adding the sodium bicarbonate for the SIF, limited headspace was given in storage.

3.6.1.2.2 Substrate Preparation

For substrate preparation, the milk replacer powder was mixed with water according to manufacturer's instructions (135 g/L) to better compare with processing of the liquid colostrum and more accurately represent how it would enter the stomach *in vivo*. Using a liquid feed also more strongly justified the removal of the oral phase as the feed does not undergo mastication and does not spend sufficient time in the oral cavity to be affected by any salivary enzymes (Ménard et al., 2018; Brodkorb et al., 2019), which are in very low concentrations in the piglet regardless (Bourlieu et al., 2014). Both liquid substrates were measured into 50 mL screw top tubes at 10 mL in triplicate and warmed in a water bath at 39°C.

3.6.1.2.3 Gastric Digestion Simulation

Pre-warmed SGF was added to each tube containing substrate, and pH adjusted to 5.3 using 1M hydrochloric acid before freshly prepared enzyme solutions were added and pH verified and adjusted again if necessary (rarely). Complete SGF including enzymes was added at a 1:1 ratio with the substrate, giving a total volume of 20 mL at this stage. Tubes were placed in a rotating rack in an incubator at 39°C for 60 minutes. Every 15 minutes, pH was checked and adjusted if necessary which usually took 5-10 uL of 0.5-1 M hydrochloric acid to maintain pH within 0.2 units.

3.6.1.2.4 Small Intestine Digestion Simulation

After gastric incubation, the tubes were removed and pH increased to 7.0 using 1M sodium hydroxide to stop pepsin activity (Brodkorb et al., 2019) before beginning the intestinal phase. During gastric incubation, the SIF was defrosted and warmed to 39°C and the bile solution prepared as it was hydrophobic and required a long period of mixing to fully homogenise. The SIF and bile were then added to the sample tubes containing the gastric chyme and placed back into the rotating rack in the incubator for 30 minutes to complete bile solubilisation. The calcium chloride and enzymes were then added and the pH adjusted to 6.6 using 1M hydrochloric acid. Total SIF volume (stock solution, enzymes and bile) was added in a 1:1 ratio with the gastric chyme, resulting in total volume of 40 mL. Tubes were placed back in the rotator at 39°C for 60 minutes and pH was verified and adjusted if needed every 15 minutes.

After intestinal incubation, tubes were placed in an ice bath to terminate enzyme activity and centrifuged at 5000 rpm for 15 minutes at 4°C to obtain the pellet carried forward for *in vitro* fermentation (Loo et al., 2022). Pellets were approximately 0.5 g per 40 mL of intestinal chyme and kept refrigerated at 4°C until use (maximum 24 hours).

3.6.1.3 Fermentation Protocol

The fermentation media composition, preparation and inoculation was exactly as described in Section 3.4.1, with the addition of mucin at 1 g/L as per findings from the previous experiment. Substrate preparation differed for the current experiment as milk replacer was in liquid form (at 135 g/L, as per manufacturer's instructions) and substrates were added to serum bottles on a dry matter basis

to correct for the difference in dry matter between volumes or weights. All treatments were added in triplicate to the 125 mL serum bottles at approximately 0.5 g dry matter resulting in 3.70 mL undigested milk replacer, 2.08 mL undigested colostrum (based on approximately 24% dry matter content (Csapó et al., 1996)), and 0.5 g sediment of each pre-digested substrate (Sirisena et al., 2018). To the measured substrates, 50 mL of inoculated fermentation media was added. Bottles were sealed and incubated for 24 hours, with gas pressure recorded and released at 2 hour intervals. Termination and final pH recording was as previous experiments.

3.6.1.4 Sample Processing

Fermentation fluid was removed from each bottle after fermentation termination in an ice bath and samples processed for gas chromatography and DNA extraction as described in Sections 3.2.4 and 3.2.5, respectively.

3.6.1.5 Statistical Analysis

For this experiment the main effects were analysed independently, resulting in separation of data to determine effect of digestion only, on each substrate. Extracted Gompertz asymptotes were analysed as previously described in Section 3.2.6.1. Concentrations of SCFA were analysed using an independent samples *t*-test following confirmation of normality (Shapiro-Wilk). Difference in alpha diversity indices between digestion conditions was assessed by either *t*-test or Wilcoxon signed-rank test as determined by distribution and homogeneity of data. Further microbiota analysis was as described previously (see Section 3.2.6.3). During microbiota object curation (the “Biom”) in R version 4.5.1 (R Core Team, 2025) within RStudio 2025.05.1-513 (Posit Software, 2025), 6 of the sequenced samples were removed from consideration as mistakenly sequenced blanks. This resulted in 42 samples being processed in Mothur and presented data consisting of 36 samples.

3.6.2 Results

3.6.2.1 Fermentation Parameters

Digestion of colostrum did not significantly impact any extracted Gompertz parameters, but was close to the threshold of significance for maximum gas volume ($F_{1,2} = 16.77$; $p = 0.0548$). Digestion of milk replacer did impact maximum

gas volume ($F_{1,2} = 50.1$; $p = 0.0194$), and lag parameter ($F_{1,4} = 9.02$; $p = 0.0398$) but not growth rate ($F_{1,2} = 13.08$; $p = 0.0687$). This can be seen in Figure 3.16 where digested conditions yielded lower, flatter curves.

Digestion did not affect end pH for either substrate ($p = 0.206$). Mean end pH for pre-digested colostrum was 6.98 ± 0.0124 compared to the colostrum control of 6.99 ± 0.0287 with pre-digested milk replacer at 6.86 ± 0.0169 vs control at 6.75 ± 0.0769 .

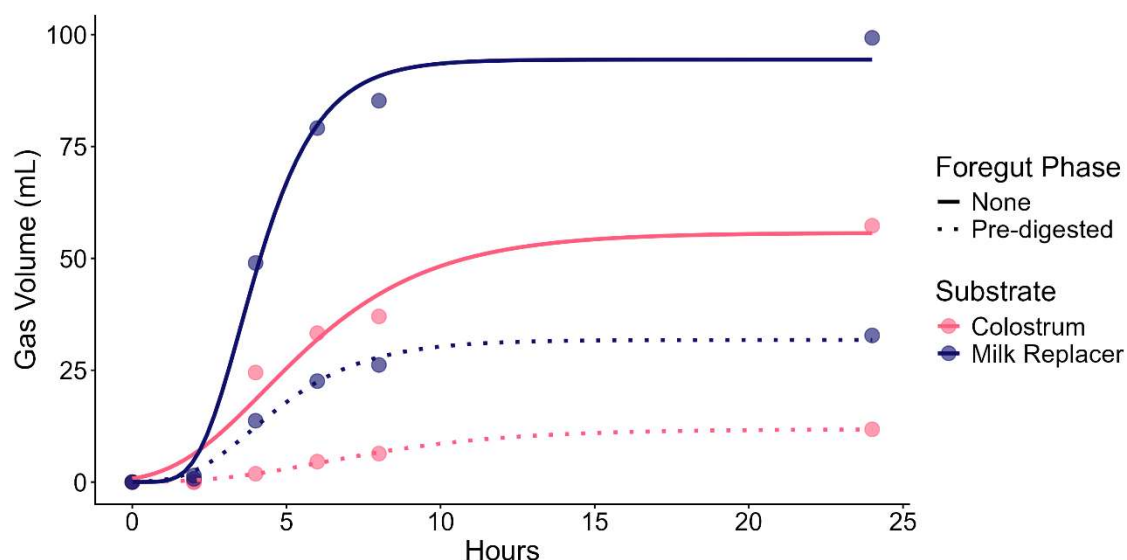


Figure 3.16 Gompertz growth curves fitted to cumulative gas production recorded during fermentation. Points represent measured gas volumes and curves represent predicted growth.

3.6.2.2 Short Chain Fatty Acid Concentrations

Within the colostrum group, digestion had a significant impact on all tested SCFA parameters (all $p < 0.001$), whereby the acetate, butyrate, propionate and total SCFA concentrations were all significantly lower when digested prior to fermentation.

There was a significant difference in concentration across acetate, butyrate, propionate, and total SCFA concentration when milk replacer was digested or not before fermentation (all $p < 0.001$) and in all cases, the digested milk replacer condition exhibited lower values.

Exact values for each SCFA measured can be seen in Table 3.9.

Table 3.9 Short chain fatty acid (SCFA) concentrations produced by fermentation of different substrates, having undergone foregut digestion simulation (digested), or not (control). Different superscript letters across a row, within each substrate condition, denotes significant effect of digestion

Substrate		Milk replacer		p-value ¹	Colostrum		p-value ¹
Digestion		Control	Digested		Control	Digested	
Acetate (mM)	Mean	18.13 ^a	9.68 ^b	<0.001	14.03 ^a	6.53 ^b	<0.001
	SE	0.53	0.92		0.95	0.34	
Propionate (mM)	Mean	11.35 ^a	6.49 ^b	<0.001	10.54 ^a	4.12 ^b	<0.001
	SE	0.39	0.41		0.92	0.41	
Butyrate (mM)	Mean	8.42 ^a	3.03 ^b	<0.001	5.81 ^a	2.63 ^b	<0.001
	SE	0.81	0.49		0.46	0.16	
Total SCFA (mM)	Mean	37.90 ^a	19.20 ^b	<0.001	30.39 ^a	13.27 ^b	<0.001
	SE	1.67	1.41		2.31	0.84	

¹Displayed to three significant figures unless < 0.001

3.6.2.3 Microbiota

A total of 42 samples were processed to extract microbial DNA and a total of 2,672,447 reads were generated through 16S rRNA sequencing of the hypervariable V4 region. After screening and filtering 15,507 high-quality unique reads remained.

3.6.2.3.1 Alpha Diversity

Parameters used to assess richness and evenness (Chao1, Shannon, Simpson indices) were not impacted by digestion prior to fermentation for the colostrum substrate. Digested colostrum had a numerically higher diversity indices for all measures (Figure 3.17).

For the milk replacer, all alpha diversity measures were significantly affected by digestion of the substrate prior to fermentation. The Chao1 measure of richness was higher when milk replacer was digested at 730 ± 48.3 , compared to the control value of 539 ± 22.4 ($t(11.3) = -3.59$; $p = 0.00408$). The Shannon diversity was also higher for the digested condition at 3.49 ± 0.0929 compared to 3.107 ± 0.0823 for the control ($W = 12$; $p = 0.0106$). The Simpson index was also higher for digested compared to control with values of 0.929 ± 0.0100 and 0.890 ± 0.0101 , respectively ($W = 11$; $p = 0.00778$).

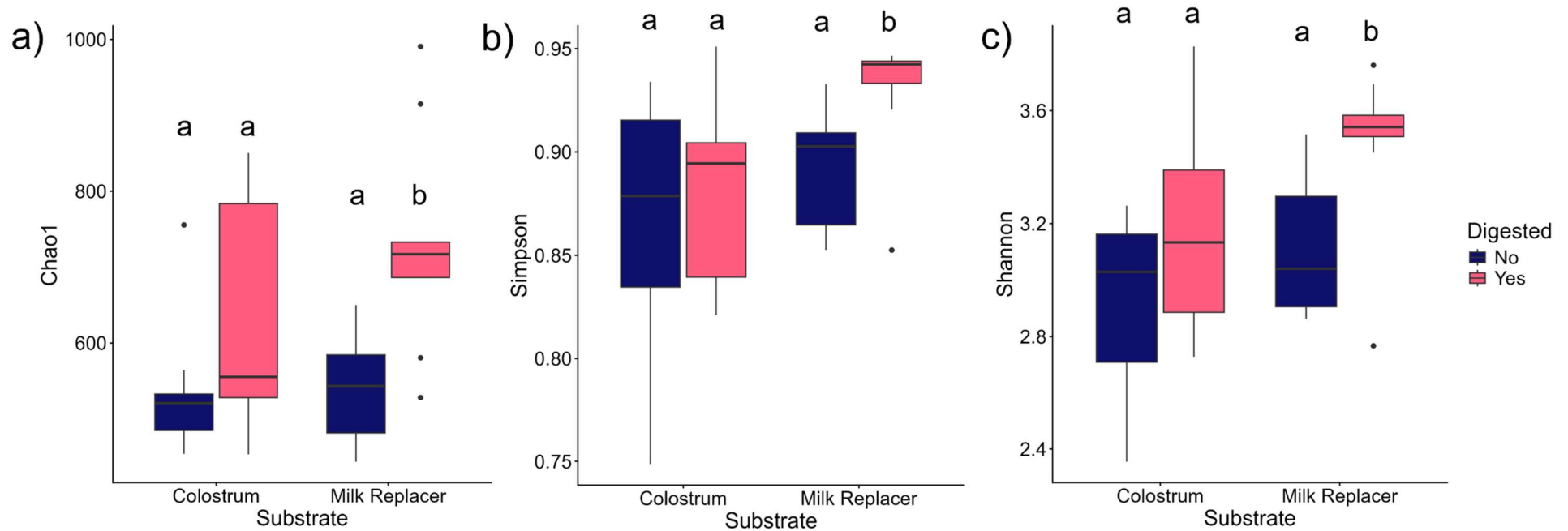


Figure 3.17 Alpha diversity indices: a) Chao1; b) Simpson; c) Shannon. Where colour denotes digestion status (pink, pre-digested; purple, not digested). Different letters denote significant effect of digestion, within each substrate

3.6.2.3.2 Beta Diversity

Both the colostrum samples and milk replacer samples showed significant community shifts when digested (*Bray-Curtis*: colostrum: $F_{1, 17} = 6.01$; $R^2 = 0.273$; $p = 0.001$; Milk replacer: $F_{1, 17} = 10.5$; $R^2 = 0.396$; $p = 0.001$). Digestion explained more of the shift in the milk replacer group than it did for colostrum (39.6% vs 27.3%). Clustering of each group can be seen in Figure 3.18.

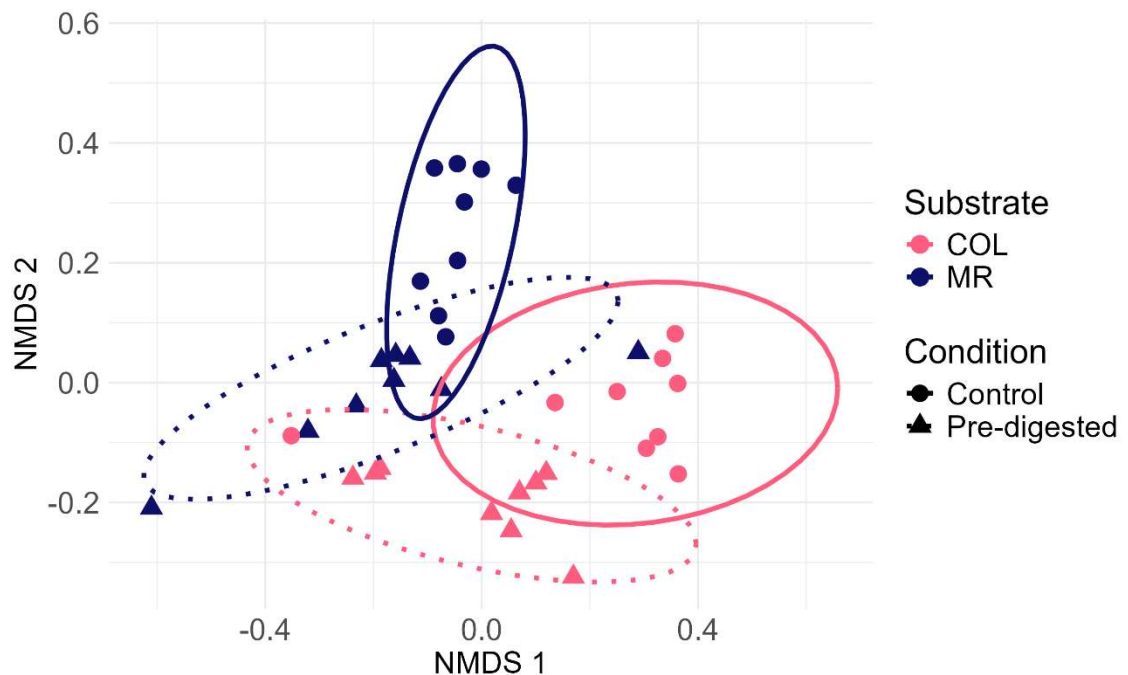


Figure 3.18 Non-metric multidimensional scaling (NMDS) ordination of microbial community composition based on Bray-Curtis dissimilarity. Each point represents a sample with colour denoting type of substrate (Pink, COL, colostrum; Purple, MR, milk replacer) and shape denoting digestion status (round, undigested control; triangle, pre-digested). Ellipses show 95% confidence intervals around group centroids with colour denoting substrate (pink, colostrum; purple, milk replacer) and line type denoting digestion (solid, control; dotted, pre-digested)

3.6.2.3.3 Relative Abundance: Phylum Level

For the colostrum substrate, both digestion conditions were dominated by Bacteroidota ($66.5 \pm 4.80\%$, control group; $59.1 \pm 4.02\%$, digested group), with Bacillota as second most abundant for both conditions ($21.5 \pm 2.99\%$, control; $19.6 \pm 2.73\%$, digested group), however neither of these phyla were significantly affected by digestion. No phyla were both statistically significant and with a log fold change of more than one in the colostrum group. Digestion of colostrum did have an impact on abundance of Pseudomonadota which had a relative

abundance of $4.56 \pm 1.28\%$ in the control condition, which rose to $10.1 \pm 1.11\%$ when digested prior to fermentation ($\text{LFC} = 0.777 \pm 0.209$; $p = 0.00126$).

The milk replacer community had the same top two most abundant phyla as colostrum, however these were flipped with Bacillota dominating regardless of digestion status ($63.4 \pm 4.10\%$, control; $40.9 \pm 3.61\%$ digested) and Bacteroidota second most abundant ($28.9 \pm 2.61\%$, control; $35.8 \pm 4.05\%$ digested). The increase seen in Fusobacteriota abundance when substrate was digested was significant ($\text{LFC} = 1.32 \pm 0.225$; $p < 0.001$), but other changes were not at the notable LFC threshold. More differences were apparent from the digestion of milk replacer than digestion of colostrum (Figure 3.19).

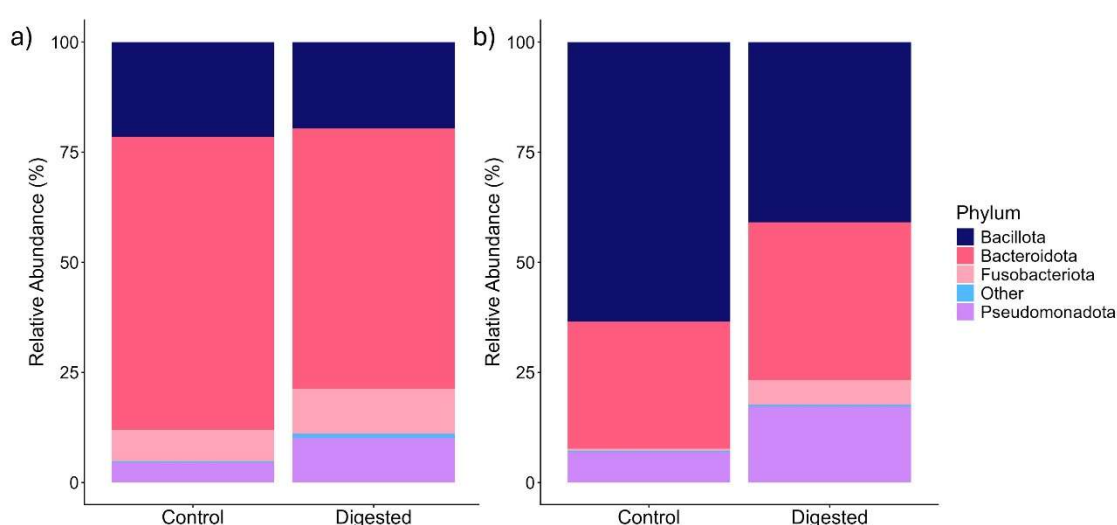


Figure 3.19 Percentage relative abundance of phyla present at above 1% in at least one group when fermenting different substrates: a) colostrum; b) milk replacer; that are either digested or not (control). ‘Other’ denotes sum of phyla at <1% relative abundance

3.6.2.3.4 Relative Abundance: Genus Level

The dominant genus in both conditions of the colostrum group was *Bacteroides* with $23.4 \pm 1.94\%$ in the control condition and $32.7 \pm 2.43\%$ in the digested condition. As with analysis at the phylum level, no genera were both statistically significant and with a large log fold change, however some notable differences were abundance of *Prevotellaceae_NK3B31_group*, which dropped from $24.3 \pm 4.66\%$ to $7.91 \pm 2.42\%$ when digested prior to fermentation ($\text{LFC} = 1.31 \pm 0.560$; $p = 0.554$), *Lachnospiraceae_unclassified*, which approximately halved when digested from $6.88 \pm 0.732\%$ to $3.57 \pm 0.417\%$ ($\text{LFC} = 0.643 \pm 0.113$; $p < 0.001$), and *Escherichia-Shigella*, which increased in

the digested group from $3.13 \pm 1.11\%$ to $7.50 \pm 0.927\%$ (LFC = 0.788 ± 0.249 ; $p = 0.0548$).

Both milk replacer conditions differed in community to the colostrum conditions, with the top four genera in the control milk replacer (non-digested) group being *Streptococcus* ($26.0 \pm 2.14\%$), *Limosilactobacillus* ($11.7 \pm 1.74\%$), *Bacteroides* ($10.6 \pm 2.06\%$), *Lachnospiraceae_unclassified* ($9.95 \pm 1.44\%$), whereas the digested milk replacer consisted mainly of *Escherichia-Shigella* ($13.8 \pm 2.17\%$), *Bacteroides* ($13.5 \pm 1.83\%$), *Limosilactobacillus* ($10.4 \pm 1.62\%$), and *Muribaculaceae_insertae_sedis_genus* ($6.34 \pm 0.829\%$). The difference in *Streptococcus* abundance between control and digested milk replacer conditions is stark (LFC = 2.14 ± 0.219 ; $p < 0.001$), with a loss of 23.1% when digested (Figure 3.20). *Fusobacterium* and *Lachnospiraceae_UCG-004* are also affected by the digestion condition with the former increasing in abundance in the digested group (LFC = 1.28 ± 0.244 ; $p < 0.001$), while the latter decreases (LFC = 1.01 ± 0.220 ; $p < 0.001$).

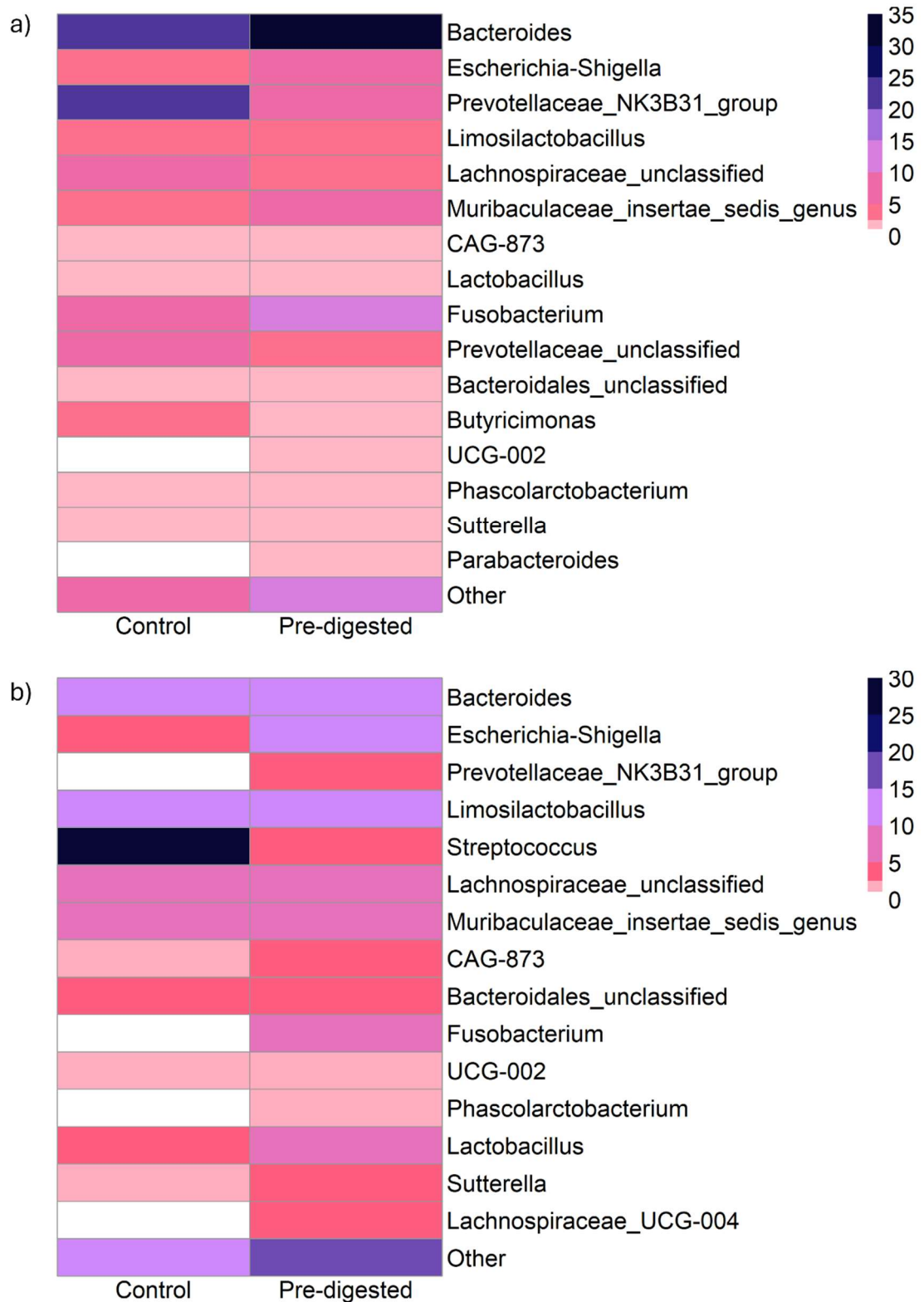


Figure 3.20 Heatmap indicating relative abundance of genera present after fermenting different substrates: a) colostrum (showing genera above 1%); b) milk replacer (showing genera above 2%). 'Other' includes the sum of genera at <1% (colostrum) or <2% (milk replacer) relative abundance. Colour denotes magnitude of abundance where pale boxes are lesser and dark boxes are increased, as per right-hand key

3.6.3 Discussion

This experiment aimed to assess the efficacy of simulating foregut hydrolysis on substrates used in the batch fermentation model. Two substrates (colostrum and piglet milk replacer) were assessed independently to determine differences in fermentation kinetics, metabolite production and microbial community composition when fermented; either as fed, or following a digestion simulation based on works by of Ménard et al. (2018), Brodkorb et al. (2019) and Rigby (2024), adjusted to reflect parameters seen in the pre-wean piglet foregut. It was hypothesised that the digestion would not significantly impact results due to the immaturity of the piglet foregut, however, this hypothesis has been rejected.

3.6.3.1 Colostrum Digestion

Colostrum, though more complex in composition than milk replacer, was still altered by the inclusion of the simulated digestion. When pre-digested, colostrum produced lower concentrations across all measured SCFA in comparison to the non-digested colostrum. One genus with strong positive correlations to production of acetate, propionate and total SCFA measures is *Prevotellaceae_NK3B31_group* (Jiang et al., 2020) which, though not statistically significant, dropped a substantial amount when colostrum was pre-digested and could be responsible for the low SCFA concentrations reported in that group. Interestingly, Jiang et al. (2020) reported *Prevotellaceae_NK3B31_group* as the predominant genus in the colon of weaned pigs, where *Bacteroides* dominated in suckling piglets. It would be expected that *Prevotellaceae_NK3B31_group* not be so abundant when colostrum is fermented if more associated with the microbiota of weaned piglets as the diet at this stage is highly contrasting. Decrease of *Bacteroides* and increase of *Prevotella* is well-established as a characterisation of weaning, when the diet shifts from milk-based to plant-based (Frese et al., 2015; Guevarra et al., 2018). *Prevotella* is a fibre-degrading taxa (Ivarsson et al., 2014) so it could be speculated that the high content of structurally complex glycans in colostrum, such as milk oligosaccharides, are sufficiently fibre-like to encourage growth of this genus.

Alpha diversity indices were numerically higher when colostrum had been digested prior to fermentation, though this was not statistically significant. This could be due to a range of factors including increased substrate availability, or

enhanced cross-feeding networks. By digesting the colostrum, compounds are more readily available for fermentation, creating more niches for a wider range of metabolic preferences rather than inducing competitive exclusion as may happen when strong fermenters of one particular nutrient dominate. This would also support more trophic layers whereby increased diversity of primary degraders consequently produce more diverse metabolites, thus encouraging diverse secondary consumers and ultimately driving the coexistence of more taxa for a richer and more complex community structure.

3.6.3.2 Milk Replacer Digestion

As expected, exposing the milk replacer powder to the simulated digestion protocol decreased the fermentation activity, as demonstrated by reduced kinetic parameters and reduced SCFA production, in comparison to the undigested samples. This was expected due to the formulation being primarily macronutrients that are broken down and absorbed in the small intestine, such as protein, fat and lactose. The difference being so stark between the two conditions was a little surprising because the pre-wean piglet gut is immature (Pluske, 2016; Amdi et al., 2022) and lacks the extreme pH and high enzyme concentrations seen in adult pigs, so it was hypothesised that the fermentation activity be reduced, but not to the extent seen here.

All alpha diversity indices were significantly higher when the milk replacer had been digested prior to inclusion in the fermentation model, in comparison with milk replacer being fermented as fed. As discussed in the above section with reference to colostrum, this may be due to better availability of the substrate to a wider range of taxa, reducing the dominance of particular bacteria, for example, *Streptococcus*, and allowing a more metabolically diverse community to grow.

When piglet milk replacer was digested prior to fermentation, the abundance of Bacillota reduced greatly, likely driven by changes in abundance of *Lachnospiraceae_UCG-004* and *Streptococcus* which both reduced significantly in the digested group. As lactic acid bacteria, *Streptococcus* are known for metabolisation of simple carbohydrates such as glucose and lactose (Stafford et al., 2025), fermenting them into lactate. The reduction of this genus when fermenting a pre-digested substrate is logical because simpler sugars will be the easiest for the immature piglet foregut to hydrolyse. With less of these

structures still available in the hindgut fermentation phase, *Streptococcus* will not proliferate as well. Reduction of *Lachnospiraceae_UCG-004* may potentially be due to a similar mechanism as they are also carbohydrate-degrading. However, this genus is associated with fibre fermentation rather than simple sugars and it would be surprising if the protocol used to simulate the piglet foregut was strong enough to break down fibrous structures when this is unusual even in adults. *Lachnospiraceae_UCG-004* is a key butyrate producer in the colon, therefore the reduction in abundance within the pre-digested condition could partially explain the reduced butyrate concentration reported.

The reduction in Bacillota in the digested group was compensated by increased Fusobacteriota and Pseudomonadota, characterised at the genus level by increased *Fusobacterium*, *Sutterella* and *Escherichia-Shigella*. *Fusobacterium* are highly adaptable bacteria, able to thrive in diverse environments shifting from anaerobic to oxygen rich (Li et al., 2025). The marked increase in *Fusobacterium* when the milk replacer was pre-digested may be attributable to the digestion phase, which likely shifted substrate availability by depleting the rapidly fermentable carbohydrates. Specifically, this depletion could suppress growth of primary saccharolytic taxa that typically dominate early fermentation, thereby reducing competitive pressure and creating a niche that favours the proliferation of metabolically versatile microbes such as *Fusobacterium*. The parameters used for simulating the foregut digestion were weak, as per *in vivo* evidence, and it is questionable whether the enzyme concentrations would be sufficient to degrade proteins fully. If not, this also supports the proliferation of *Fusobacterium* as perhaps there was enough action to make the proteins more accessible than the non-digested substrate, and thus more freely metabolised by *Fusobacterium* which is adaptable to fermentation of proteins and carbohydrates (Li et al., 2025). There has been very little recovery of dietary proteins and amino acids reported *in vivo*, suggesting high absorption capacity for all (Lin et al., 2009). Without an absorptive mechanism in the present model, it is difficult to identify exactly what effects are seen when these components are pulled through into the colonic microbiota. Performing proximate analysis on the substrates pre- and post-foregut digestion would provide quantitative information as to which nutrients were being broken down in this phase and by how much. Unfortunately this was beyond the financial scope of this project but is recommended for future work.

3.6.3.3 Recommendations for Protocol

There were sufficient significant differences associated with the digestion simulation to warrant inclusion of this phase in the protocol going forward when fermenting sow colostrum or piglet milk replacer. It remains unclear if foregut simulation would be necessary prior to fermentation of particularly resistant substrates due to the immature nature of the pre-wean piglet foregut.

3.7 Experiment 5 Validation Against Post-mortem Samples

3.7.1 Methodology

This experiment tested the inoculum source to confirm that the protocol developed through experiments 1-4 was able to conserve sufficient microbial community composition post-fermentation. This was done through comparison to samples freshly collected from piglet large intestines *post mortem*. This experiment contains both a 2 x 3 factorial design (for fermentation data) and a 2 x 3 x 2 design for microbial community analysis (see Table 3.10 for details). This was done by using two inoculum sources (colonic digesta and faeces) and three piglet ages (1, 2 and 3 weeks old), with an additional 'status' factor of two levels for the microbiota comparison ('fresh' vs 'fermented' samples). The 'control' used was the faecal slurry as used in previous experiments.

3.7.1.1 Dissection of the Large Intestine

Three female Large White x Landrace x Duroc piglets from the NPC were selected at random when meeting the following criteria; not on any existing research trials, no non-standard medication administered since birth, within normal weight boundaries for age, no current signs or history of disease. Piglets were kept as described in Section 3.2.1. One piglet was 1 week of age, one piglet was 2 weeks of age and one piglet was 3 weeks of age. Piglets were removed from the pen and transported to the on-site lab facilities according to the standard operating procedures (7.03: Dissections, National Pig Centre, 2023). Piglets were stunned using a captive-bolt gun and exsanguinated according to Schedule 1 Animals (Scientific Procedure) Act 1986.

A ventral incision allowed for the gastrointestinal tract to be isolated and removed from the piglet for sample collections. The colon was identified and clamped from colocaecal junction to rectum, as identified by solidification of

faecal material. Samples of luminal digesta were collected from the entire colon, and placed into screw top centrifuge tubes on ice and transported to the lab within 60 minutes. For each sample, 0.2 g was removed into a sterile 1.5 mL centrifuge tube and the remaining samples stored at -80°C until use.

The removed subsamples immediately underwent microbial DNA extraction. To each 0.2 g sample, 0.2 g of 0.1 mm silica beads were added with the 1 mL InhibitEX lysis buffer (QIAamp DNA Stool Mini Kit) and tubes placed in a bead beater for 2 minutes at maximum speed (TissueLyser LT, Qiagen). The rest of the extraction was as described in 3.2.5, following manufacturer's instructions. These samples were referred to as "fresh", as opposed to the "fermented" samples which underwent fermentation prior to analysis, as detailed below.

3.7.1.2 Fermentation Protocol

Fermentation media was prepared as described in the previous experiment, up to point of inoculation. Media was divided into 13 equal parts and inoculated with either defrosted colonic contents from each piglet ('colonic digesta'), defrosted faecal sample sourced directly from the rectum ('faeces'), or with the pooled pre-prepared slurry ('control') as used in previous experiments. This resulted in 13 final inoculated media conditions (see Table 3.10) which were sealed and stored at 39°C overnight.

Table 3.10 Experimental inoculum conditions

Code	Age (weeks)	Inoculum source	Status
1C Fermented	1	Colonic digesta	Fermented
1F Fermented	1	Faeces	Fermented
1C Fresh	1	Colonic digesta	Not fermented
1F Fresh	1	Faeces	Not fermented
2C Fermented	2	Colonic digesta	Fermented
2F Fermented	2	Faeces	Fermented
2C Fresh	2	Colonic digesta	Not fermented
2F Fresh	2	Faeces	Not fermented
3C Fermented	3	Colonic digesta	Fermented
3F Fermented	3	Faeces	Fermented
3C Fresh	3	Colonic digesta	Not fermented
3F Fresh	3	Faeces	Not fermented
Control	N/A	Slurry	Fermented

Substrate used was 0.5 g pre-digested milk replacer residue (see Section 3.7.1.2 for detail regarding the substrate digestion method) which was added to 125 mL serum bottles in triplicate, per inoculation source, resulting in a total of 52 bottles including one blank bottle per treatment. One blank was used per treatment as there was not sufficient colonic material for triplicate blanks. The fermentation was conducted in three batches.

To each serum bottle, 50 mL inoculated media was added and bottles were infused with CO₂ on a plate warmer for 5 minutes before sealing with a rubber stopper and crimp cap, as previously described. Bottles were incubated at 39°C for 24 hours with gas pressure recorded and released at roughly equal intervals up to 9 hours, with a final gas release and recording at termination. Bottles were submerged in an ice bath to terminate fermentation and a final pH recorded.

3.7.1.3 Sample Processing

Samples were taken for further analysis as described previously in Sections 3.2.4 and 3.2.5, for gas chromatography and DNA extraction, respectively.

3.7.1.4 Statistical Analysis

Due to differences between number of control repetitions and experimental condition repetitions, as well as co-linearity between main effects within the control group, statistical analysis was performed only on *post mortem* piglet samples using age and inoculum source as main effects; the control group was not tested for significant differences. Where the control group appears in figures, this is for visual comparison only.

Fermentation profiles were analysed using a Gompertz growth curve as described in Section 3.2.6.1 with key parameters extracted and used in linear models with age and inoculum source as main effects. The model was passed through the *anova* function and reduced using *lmerTest* to determine significance of effects and any interactions. Batch was excluded as a random effect in this experiment due to causing singularity in a mixed-model approach. Pairwise comparisons within main effects was conducted with the *emmeans* function and this also provided mean and SE values for each condition. Final pH and each SCFA measurement were analysed with identical linear models followed by ANOVA, as above.

Microbiome analysis incorporated an additional main effect of 'status' (see Table 3.10) to enable analysis of conserved differences between samples that were used to inoculate the *in vitro* batch model ('fermented'), and samples that were DNA extracted immediately *post mortem* ('fresh'). Alpha diversity was assessed using linear modelling of Chao1, Shannon, or Simpson indices as response variables and age, inoculum source and status as main effects with interactions, followed by ANOVA (reduced) to determine significance of effects. Beta diversity was analysed using *adonis2* function with the same main effects and interaction model. To gain better understanding of any conserved differences after samples passed through the *in vitro* model, the *pairwise.adonis* function was used following the PERMANOVA (*adonis2*), only on fermented groups.

Statistical comparison across phyla and genera abundance using ANCOM-BC was not conducted for this experiment due to the complexity of main effects and low sample numbers. As a 3 x 2 x 2 design, with limited biological replicates, it was considered more suitable to compare relative abundances descriptively. Therefore only mean and SE relative abundances are presented here.

3.7.2 Results

3.7.2.1 Fermentation Parameters

For maximum gas volume asymptote, there was a significant interaction ($F_{12} = 27.7$; $p < 0.001$) and age had a significant effect ($F_{12} = 22.8$; $p < 0.001$), but not source ($p = 0.0650$). Maximum gas volume increased with age when inoculated with colonic digesta (90.9 ± 1.34 mL, 1w/o; 96.4 ± 1.34 mL, 2w/o; 96.5 ± 1.34 mL, 3w/o) but no linear trend appeared within the faecal inoculate ages (98.1 ± 1.34 mL, 1w/o; 105.1 ± 1.34 mL, 2w/o; 87.3 ± 1.34 mL, 3w/o). This led to significant pairwise differences between all age groups with inoculated with faeces (all $p < 0.01$) and significant differences between ages 1 and 2 ($t = -2.86$; $df = 12$; $p = 0.0357$) and 1 and 3 ($t = -2.95$; $df = 12$; $p = 0.0305$) within the colonic digesta inoculated condition, but no difference between ages 2 and 3 ($p = 0.996$).

Lag parameter estimate was impacted by interaction ($F_{12} = 11.6$; $p = 0.00156$) as well as independent effects of age ($F_{12} = 76.1$; $p < 0.001$) and source ($F_{12} = 15.7$; $p = 0.00187$). There were no numerical trends seen where values increased or decreased with either main effect, however similar results

where reflected by each main effect. Samples inoculated with colonic digesta exhibited the largest lag value at 2w/o (3.23 ± 0.371), which was significantly different to that at 1 w/o (2.93 ± 0.371 ; $t = -5.72$; $p < 0.001$) and 3w/o (2.99 ± 0.371 ; $t = 4.66$; $p = 0.0014$). Faecal inoculated samples also saw the largest lag at 2w/o (3.52 ± 0.371), reflecting the differences in the colonic samples between 1w/o (3.09 ± 0.371 ; $t = -8.18$; $p < 0.001$) and 3w/o (2.92 ± 0.371 ; $t = 11.4$; $p < 0.001$), but also showing statistical significance between ages 1 and 3 ($t = 3.23$; $df = 12$; $p = 0.0183$), which was not seen in colonic samples. Comparing source of inoculum pairwise within age groups highlighted significance between inocula at 1w/o ($t = -2.90$; $df = 12$; $p = 0.0135$) and 2w/o ($t = -5.36$; $df = 12$; $p < 0.001$), with the colonic inoculum eliciting the lower lag in both groups, but no difference at 3w/o ($p = 0.193$).

Only inoculum source significantly impacted the growth rate parameter ($F_{12} = 15.5$; $p = 0.00198$) and pairwise comparison revealed no impact at 2w/o ($p = 0.958$), and higher growth rate asymptote in colonic digesta for 1w/o ($t = 3.76$; $df = 12$; $p = 0.0027$) and 3w/o ($t = 3.01$; $df = 12$; $p = 0.0109$).

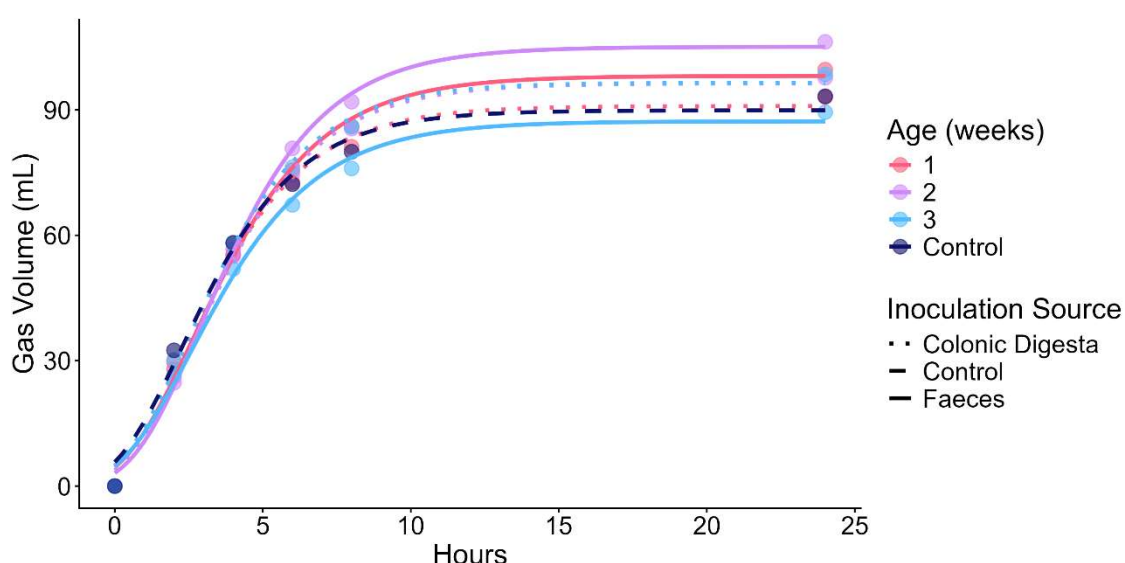


Figure 3.21 Gompertz growth curves fitted to cumulative gas production recorded during fermentation. Points represent measured gas volumes and curves represent predicted growth. Colours denote age of piglet (pink, 1 week; lilac, 2 weeks; blue, 3 weeks; dark purple, control); line type denotes source of inoculum (dotted, colonic digesta; solid, faeces; dashed, control)

No main effects affected final pH ($p = 0.133$), with all groups being approximately pH 6.5 at fermentation termination. The minimum pH was 2 week old piglet colonic digesta (6.41 ± 0.0523) and the maximum pH was 1 week old

piglet colonic digesta (6.61 ± 0.00985). For reference, the slurry control final pH was 6.51 ± 0.00985 .

3.7.2.2 Short Chain Fatty Acid Concentrations

Acetate, propionate and total SCFA concentrations were not significantly affected by either age or inoculum source, nor was any significant interaction highlighted. Inoculum source did, however, have a significant effect on butyrate concentration ($F_{1,48} = 5.92$; $p = 0.0187$), where the colonic digesta consistently produced lower concentrations than faecal inoculum, regardless of age. Interestingly, the magnitude of this effect was minimal ($R^2 = 0.0538$). The consistency in SCFA production across groups can be seen in Table 3.11 where the control slurry appears for comparison purposes but was not included in statistical analysis, as explained earlier.

Table 3.11 Short chain fatty acid (SCFA) concentrations produced after fermentation of inoculum from different sources and ages of piglet donor. Control not included in statistical analysis; values for reference only. Different superscript letters across a row denote significant difference (*ANOVA*: $p < 0.05$)

	Source	Colonic digesta			Fresh faeces			<i>Control</i>	p-values		
	Age	1	2	3	1	2	3		Source	Age	Interaction
Acetate (mM)	Mean	26.30	28.83	29.27	28.67	33.60	33.30	28.01	0.164	0.411	0.93
	SE	0.48	0.88	1.73	0.97	4.62	6.02	1.63			
Propionate (mM)	Mean	18.28	20.86	23.37	20.64	22.77	26.75	27.17	0.228	0.100	0.957
	SE	0.92	0.85	0.57	1.03	3.73	4.72	1.07			
Butyrate (mM)	Mean	7.02 ^a	7.16 ^a	6.98 ^a	8.01 ^b	8.44 ^b	9.62 ^b	7.35	0.0187	0.629	0.566
	SE	0.12	0.09	0.13	0.27	1.26	1.53	0.09			
Total SCFA (mM)	Mean	51.61	56.85	59.62	57.31	64.81	69.67	62.53	0.135	0.28	0.944
	SE	1.29	1.65	2.26	2.05	9.55	11.76	2.38			

3.7.2.3 Microbiota

A total of 90 samples were processed to extract microbial DNA and information from the V4 region was amplified using 16S rRNA sequencing. A total of 6,751,357 reads were generated and after screening and filtering, 46,432 reads were successfully classified.

3.7.2.3.1 Alpha Diversity

Prediction of the Chao1 diversity index was impacted by interaction between age and inoculum source ($F_{1, 60} = 3.76$; $p = 0.0289$), with independent effects significant for age ($F_{1, 60} = 18.1$; $p < 0.001$), inoculum source ($F_{1, 60} = 4.74$; $p = 0.0333$), and fermentation status ($F_{1, 60} = 57.2$; $p < 0.001$). There was a consistent pattern with fermentation status whereby fresh samples always resulted in lower Chao1 diversity, regardless of other main effects. Inoculum source was somewhat consistent with lower Chao1 values for colonic digesta, with the exception of 3w/o where the values are higher for colonic digesta in both fermented and fresh samples. No consistent pattern was seen across ages, with Chao1 index too dependent on other main effects.

Shannon index analysis highlighted interactions between age and inoculum source ($F_{1, 60} = 9.82$; $p < 0.001$) and between age and fermentation status ($F_{1, 60} = 20.2$; $p < 0.001$). Fixed effects were significant in age ($F_{1, 60} = 34.5$; $p < 0.001$) and status ($F_{1, 60} = 22.0$; $p < 0.001$), but not inoculum source ($p = 0.162$). A significant three-way interaction between age, inoculum source, and fermentation status ($F_{1, 60} = 5.21$; $p = 0.00823$), meant that Shannon values were unpredictable between effects and no clear pattern emerged.

The Simpson index was significantly impacted by age ($F_{1, 60} = 11.9$; $p < 0.001$), inoculum source ($F_{1, 60} = 6.98$; $p = 0.0105$), but not fermentation status ($p = 0.115$), with significant interactions between age and inoculum source ($F_{1, 60} = 6.67$; $p = 0.00243$), age and status ($F_{1, 60} = 13.4$; $p < 0.001$), and a three way interaction ($F_{1, 60} = 3.45$; $p = 0.0380$). As above, the Simpson index was too dependent on multiple effects for any clear pattern to emerge.

3.7.2.3.2 Beta Diversity

The initial PERMANOVA revealed significance of all modelled interactions and each fixed effect (all $p = 0.001$). Splitting by terms highlighted that despite

seemingly equal statistical significance, inoculum source explained minimal community differences ($F_{1, 60} = 17.9$; $R^2 = 0.0357$; $p = 0.001$), and most was explained by age ($F_{1, 60} = 54.0$; $R^2 = 0.215$; $p = 0.001$), fermentation status ($F_{1, 60} = 180$; $R^2 = 0.359$; $p = 0.001$), or the interaction between the two ($F_{1, 60} = 37.1$; $R^2 = 0.148$; $p = 0.001$). Figure 3.22 shows the clustering per age group and status with lesser distance between age groups once fermented in the batch model.

A secondary PERMANOVA with pairwise comparisons focussed on the fermented samples only highlighted conserved differences between ages ($F_{1, 48} = 38.4$; $R^2 = 0.421$; $p = 0.001$) and to a lesser extent, inoculum source ($F_{1, 48} = 21.8$; $R^2 = 0.120$; $p = 0.001$), where similar distribution was shared across ages: 1-2 ($R^2 = 0.354$; $p = 0.003$); 1-3 ($R^2 = 0.381$; $p = 0.003$); 2-3 ($R^2 = 0.318$; $p = 0.003$).

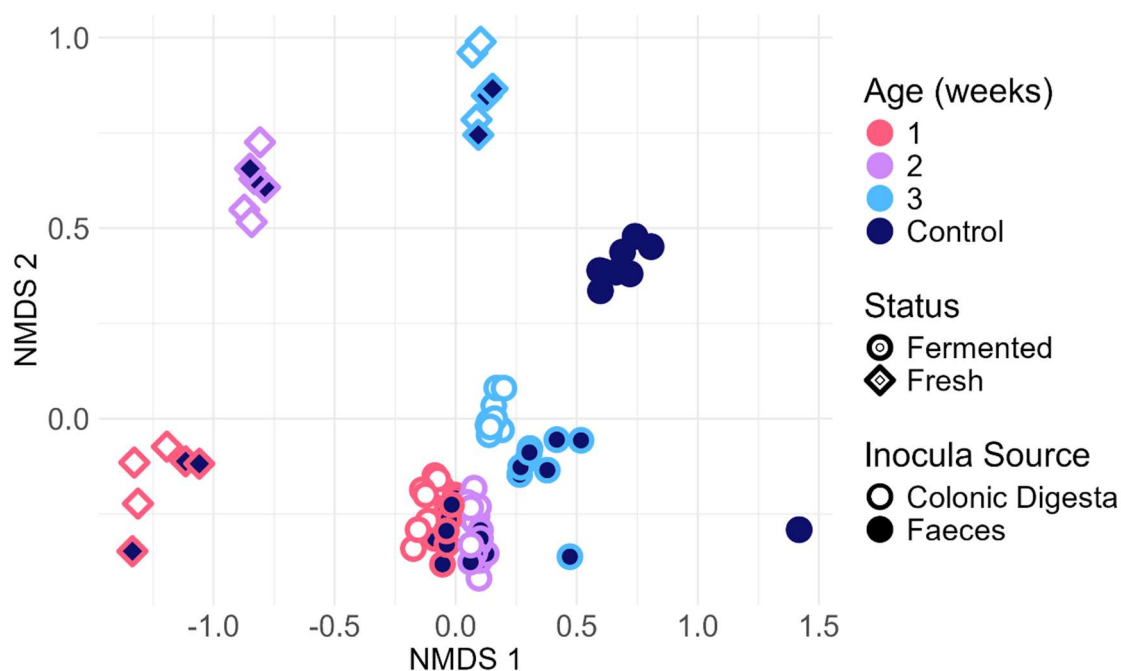


Figure 3.22 Non-metric multidimensional scaling (NMDS) ordination of microbial community composition based on Bray-Curtis dissimilarity. Each point represents a sample with outline colour denoting age (pink, 1w/o; lilac, 2w/o; blue, 3w/o; dark purple, control slurry), shape denoting fermentation status (circle, fermented; diamond, fresh), and fill denoting inoculum source (no fill, colonic digesta; dark purple fill, faeces).

3.7.2.3.3 Relative Abundance: Phylum Level

The top phyla across all conditions were Bacteroidota or Bacillota, followed by one of Pseudomonadota, Fusobacteriota, or Spirochaetota. As seen previously, the two most abundant phyla were Bacillota and Bacteroidota with the dominant

phyla changing depending on conditions. Fermented samples tended to have more Bacteroidota than Bacillota (means: $50.2 \pm 9.05\%$, Bacteroidota; $41.5 \pm 7.71\%$, Bacillota), and fresh samples more Bacillota than Bacteroidota (means: $38.5 \pm 6.21\%$, Bacillota; $37.7 \pm 6.86\%$, Bacteroidota). The highest prevalence of Bacillota was in the 2 week old fermented faecal samples ($53.5 \pm 0.778\%$) and the lowest in the 1 week old fresh colonic digesta samples ($31.2 \pm 2.88\%$). The highest prevalence of Bacteroidota was in the 1 week old fermented colonic digesta samples ($62.3 \pm 2.43\%$) and the lowest in the 1 week old fresh colonic digesta samples ($29.6 \pm 0.606\%$). Only one group produced Fusobacteriota in the top three phyla which was 2 week old piglet fermented faecal samples ($4.05 \pm 0.164\%$). All other fermented groups had Pseudomonadota as third most abundant phylum (mean $3.95 \pm 1.59\%$) and fresh groups favoured Spirochaetota instead (mean $13.4 \pm 6.29\%$), with the exception of the 3 week old piglet samples (see Figure 3.23) which contained Pseudomonadota as per fermented samples (mean $6.45 \pm 1.42\%$).

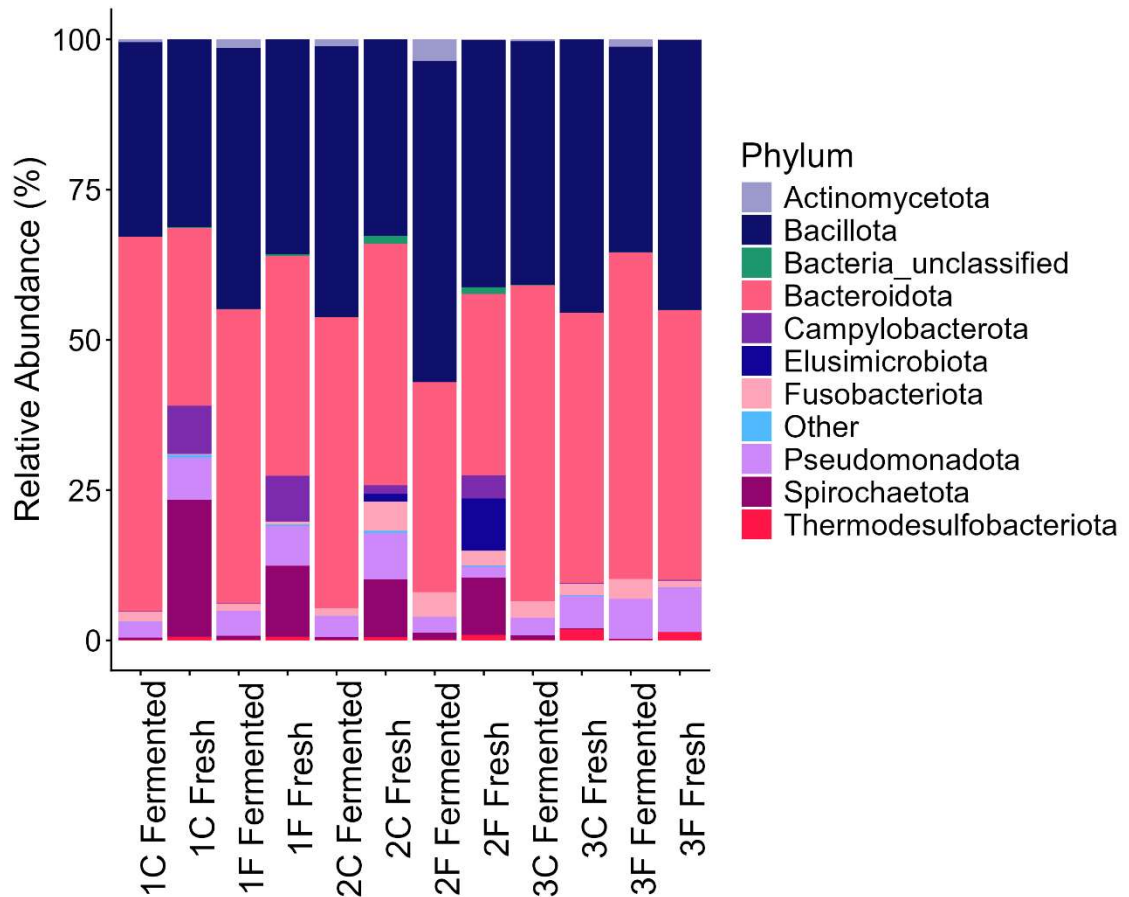


Figure 3.23 Percentage relative abundance of phyla present at >1% abundance in various inoculants (x-axis) where number indicates age of piglet (1, one week old; 2, two weeks old; 3, three weeks old) and letter indicates source (C, colonic digesta; F, fresh faeces). Of each combination, inoculants were analysed fresh *post-mortem* (fresh), or fermented *in vitro* (fermented). 'Other' includes the sum of phyla present at <1% relative abundance

3.7.2.3.4 Relative Abundance: Genus Level

No groups shared a top 3 genera and diversity across genus profiles can be seen in Figure 3.24. All fermented samples shared *Bacteroides* as the most abundant genus (mean $27.3 \pm 9.39\%$), whereas fresh samples were more diverse, each with a different top genus including *Spirochaetaceae_unclassified*, *Prevotellaceae_insertae_sedis_genus*, *Elusimicrobium*, *Lactobacillus*, CAG-873, and *Bacteroides*. Other consistencies within main effects included higher abundance of *Bacteroidales_unclassified*, *Streptococcus*, *Veillonella*, and *Holdemanella* across fermented samples (means $4.07 \pm 0.992\%$, $5.96 \pm 3.17\%$, $3.69 \pm 1.43\%$ and $5.90 \pm 3.00\%$, respectively), in comparison with fresh samples (means $0.55 \pm 0.451\%$, $0.171 \pm 0.084\%$, $0.120 \pm 0.160\%$ and $0.022 \pm 0.043\%$, respectively).

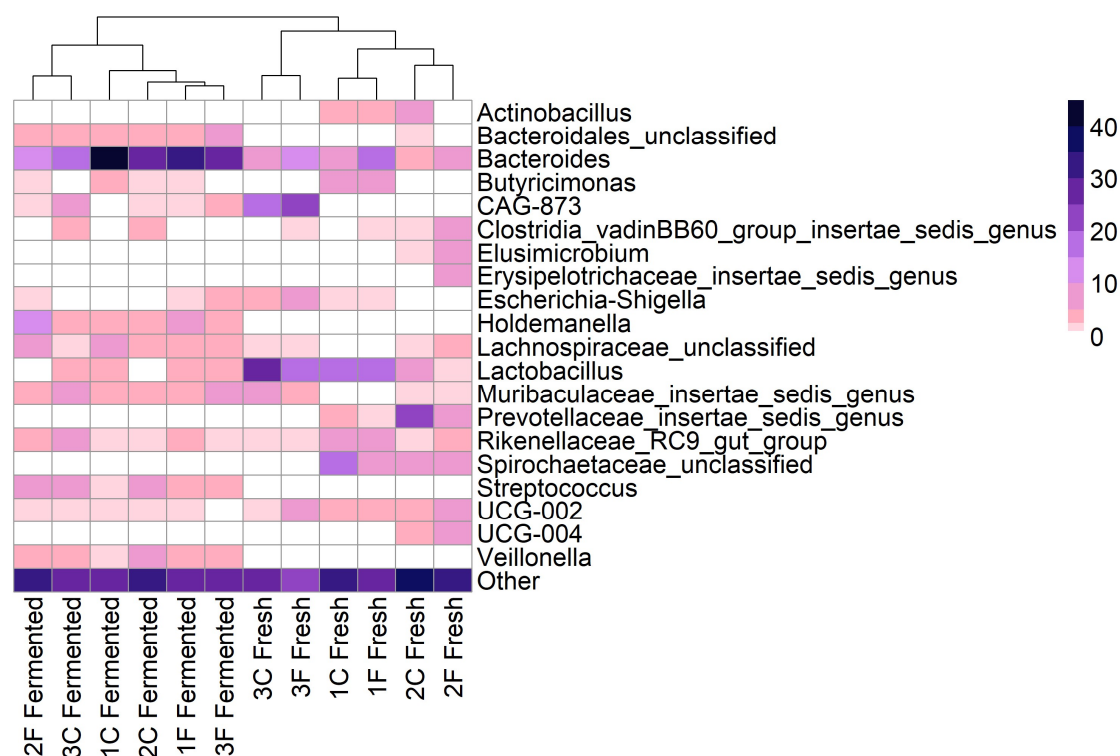


Figure 3.24 Heatmap indicating relative abundance of genera that were above 5% in at least one group (x-axis) where number indicates age of piglet (1, one week old; 2, two weeks old; 3, three weeks old) and letter indicates source (C, colonic digesta; F, fresh faeces). Of each combination, inoculants were analysed fresh *post-mortem* (fresh), or fermented *in vitro* (fermented). ‘Other’ denotes sum of genera at <5% relative abundance. Colour denotes magnitude of abundance where pale boxes are lesser and dark boxes are increased, as per right-hand key. Dendrogram clusters columns based on Bray-Curtis dissimilarity.

3.7.3 Discussion

This experiment used large intestine samples collected from pre-wean piglets *post-mortem* to inoculate the *in vitro* batch fermentation model being developed in this chapter. The experiment aimed to compare fermentation kinetics and SCFA production across inoculants from different age donors (1, 2, and 3 weeks old) as well as different sources (colonic digesta and faeces) to determine what effect inoculum source may have on fermentation activity and metabolite production. The microbial community of samples was compared to identify differences between samples collected and DNA extracted immediately *post mortem* (fresh), with those that were used to inoculate the batch fermentation model (fermented). Additionally, factors of age and inoculum source were considered here to identify differences pre-fermentation and determine if these differences were conserved after exposure to the *in vitro* model. The hypothesis

was confirmed whereby fermentation of a substrate shifted the microbial profiles closer together, but the model was able to conserve distinct differences between inoculum source.

3.7.3.1 Fermentation Parameters

There were many statistically significant differences in fermentation kinetics between and within main effects tested, however the large effect of the interaction suggests any significant differences be interpreted with caution. When interactions are very strong, it can shift main effects to be less meaningful independently. This is confirmed in the present data where there were haphazard results with little consistency in main effect significance across extracted parameters and no obvious patterns to the fermentation profiles as seen in previous experiments (for example, see Figure 3.2, where AM inoculation is consistently steeper in growth). It is therefore difficult to interpret any meaningful biological information from the statistical analysis, and, as can be seen in Figure 3.21, gas production appears relatively consistent across groups.

3.7.3.2 Short Chain Fatty Acid Concentrations

The only SCFA altered significantly by a main effect was butyrate concentration where fermentation of the colonic digesta consistently produced lower butyrate concentrations than the fresh faeces, across all piglet ages. This contradicts findings by Monsma and Marlett (1995) who reported increased rates of SCFA production, increased total SCFA production and increased butyrate production when using a caecal inoculant compared with faeces in a rat model. It is logical that the microbiota in caecal and colonic contents be better adapted to fermentation than faecal microbes due to those being the primary fermentative sites in the body. It was therefore hypothesised that if any difference were present, the colonic digesta would elicit higher SCFA production than faecal samples, as seen previously (Monsma and Marlett, 1995; Bindelle et al., 2007). Bauer et al. (2004) reported results in agreement with the present study whereby faeces elicited the most fermentation activity, followed by mid-colon, and then caecum. They found caecal inoculum was slower to ferment, with less SCFA production, including lower butyrate and hypothesised that this was due to microbiota (inoculum) in the early large intestine (caecum) growing at a constant

and maximal rate, whereas further in the tract, microbes may be in the stationary phase and therefore more ready to rapidly metabolise a new energy source.

The consistency in SCFA production across samples could be also be explained by fermentation being more strongly driven by substrate than inoculant, given that the substrate was the same in all conditions and there was no significant difference in microbial communities between fresh inoculants. It is well-established that diet is a key driver of the microbiota in humans (summarised by Ross et al. (2024)) with the same significance reflected in piglets as there are stark differences in faecal microbiota composition of sow-reared vs formula-fed piglets (Li et al., 2012; Wang et al., 2013).

3.7.3.3 Microbial Diversity

Impact of main effects on alpha diversity indices followed a similar outcome as fermentation parameters where high magnitude interactions led to difficulty interpreting meaningful results. This is reflected in the relative abundance of genera, where there are many inconsistencies in dominant genera between groups, particularly within the fresh samples which appear to have less consistent dominance than seen in fermented samples. Increased dominance often goes hand-in-hand with reduced evenness, but the Shannon and Simpson indices were too heavily impacted by strong interactions to offer clear support for this theory. We can say with relative certainty that the Chao1 index was lower for fresh samples, suggesting reduced richness.

By passing the samples through the fermentation model, the microbiota are exposed to a specific shared substrate which does not happen to the same extent *in vivo*. Although sow milk could be considered a shared substrate, the difference in ages of the piglets means that their sows were at different points in lactation, therefore producing different milk compositions (Hurley, 2015; Cheng et al., 2016; Mudd et al., 2016; Chen et al., 2018). Additionally, there is much evidence supporting the diversity of milk between individuals and even between teats on the same individual (Ogawa et al., 2014; Devillers et al., 2016; Dierking et al., 2025). Results from Chapter 2 in this thesis highlight the individual variation in milk oligosaccharide concentration in colostrum of sows with identical management and breeding (Smith et al., 2026). Such between sow variation is also reported in previous studies (Tao et al., 2010; Difilippo, Pan, Logtenberg,

Willems, et al., 2016; Trevisi et al., 2020). Given the impact milk oligosaccharides have on the gut microbiota (overview in Section 1.5), it is possible that fluctuations in certain compounds from sow to sow contribute to such individualised communities in the fresh piglet samples. Furthermore, due to the variation in ages, the piglets will have had different lengths of exposure to provided creep feed (given at day 7), however, it is reported that only approximately 45-65% of the litter consume creep feed (Pluske et al., 2018). Additionally, age has been reported to have a greater effect on the gut microbiota than consumption of creep feed (Lerch et al., 2023) so it is likely that the clustering seen (Figure 3.22) is driven by age.

Across all groups there was a substantial amount of *Other* classification at genus level due to increasing the relative abundance threshold here to 5%, as opposed to 1% in previous experiments. This was due to impractical data presentation if including all genera at >1% abundance. There were more low abundance (<1%) OTUs in this experiment in comparison to others, perhaps highlighting an increased richness and diversity from fresh samples that is not captured when using pooled faecal slurry.

3.7.3.4 Conserved Differences

Distinct clustering based on age was expected due to the breadth of literature investigating how piglet bacterial communities shift temporally in the pre-wean period (Everaert et al., 2017; Liu et al., 2019; Trevisi et al., 2021). However, it must be acknowledged that the biological replicates for this study were minimal with only one piglet representing each age group and therefore clustering seen could instead be due to individual piglet variation, supported by low variation across technical replicates, and not specifically age because age and individual were co-linear. One piglet of each age was selected to best capture microbiota dynamics which shift over the pre-wean period (Everaert et al., 2017; Liu et al., 2019; Trevisi et al., 2021), while still keeping the number of piglets sacrificed to a minimum in accordance with the 3Rs principle (Russell and Burch, 1959). Although, some studies referenced above have shown distinct age differences, others have instead defined a 'milk-associated microbiota' in suckling pigs (Kim et al., 2011; Slifierz et al., 2015; Chen et al., 2017), supporting that microbial community shifts are most strongly driven by abrupt diet change at weaning (Mach et al., 2015; Frese et al., 2015), rather than maturation.

Regardless of the cause, the clustering seen in the different ages/piglets was conserved through fermentation despite OTU differences seen. Although communities were more distinct prior to fermentation, implying that fermentation conditions and substrate availability exert stronger selective pressures than inoculum source, there was conserved dissimilarity in community composition between age groups and inoculum source after fermentation. This indicates that despite the strong effects of the substrate seen through pulling the clusters closer together, this batch fermentation protocol does allow for conservation of distinct microbial communities. Further optimisation of the model to enhance conservation of microbial communities may include one or multiple of: increasing initial population densities via more concentrated inoculation; reducing substrate dry matter; shortening the incubation period.

3.8 Conclusion

This chapter has described the development of a novel batch fermentation protocol to enable modelling of the pre-wean piglet colon *in vitro*. Through the presented series of experiments it has been shown that altering media conditions, inoculant, and substrate in batch fermentation is associated with substantial restructuring of the inoculant microbiota. The final proposed protocol is summarised in Figure 3.25.

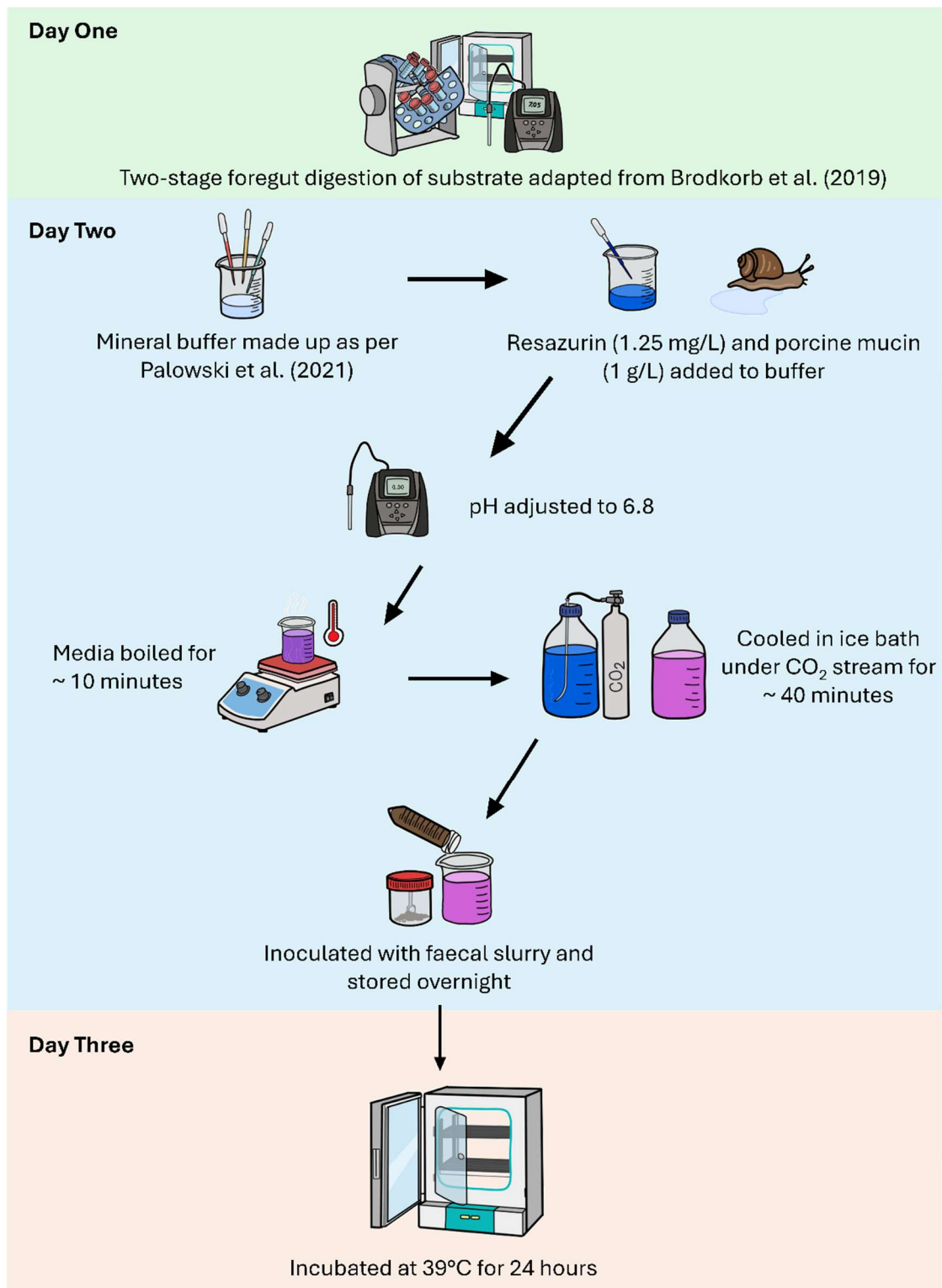


Figure 3.25 Summary of *in vitro* fermentation method

As the colon is an environment lacking in exposure to oxygen, reduction of the fermentation media and maintenance of anaerobiosis in the model is critical to avoid proliferation of aerobic or facultative anaerobic bacteria that would otherwise not be captured. Traditionally this is done by addition of reducing agents, such as L-cysteine, but here it is shown not to be necessary, provided

that oxygen is purged sufficiently by other means. It is suggested that instead, media is boiled until redox indicator colour change to initially establish reduction, and use of adequate CO₂, particularly after oxygen exposure, provides additional maintenance. To avoid heat shock to inoculant, the temperature of the media should be checked prior to inoculation with slurry.

Although reaching steady-state is not achievable in batch fermentation, therefore stabilisation periods are not required, it is proposed that media be inoculated before incubation with substrate to allow microbes to recover from handling, freezing, and to ensure appropriate anaerobiosis is maintained.

A carbonate-phosphate buffer is recommended over a simple phosphate buffer due to superior buffering capacity which is particularly important in a closed system where substrate depletion and metabolite production can reduce pH and compromise the microbiota. Furthermore, an incubation period of 24 hours allows for complete fermentation kinetics to be captured, without compromising the microbiota significantly, while still being practical to perform in the lab.

Addition of porcine mucin does capture different microbes and is recommended in the concentration of 1 g/L for the pre-wean piglet model, as increased amounts strongly drive *Streptococcus* proliferation which is not optimal for mimicking the pre-wean piglet colonic microbiota. Addition of mucin via covered beads may provide better availability of the substrate to populations such as *Lactobacillus* which have proved difficult to capture.

Despite the immaturity and low activity of the foregut in the pre-wean piglet, hydrolysis with stomach and small intestine enzymes is suggested for substrates prior to fermentation, particularly if high in rapidly digestible components.

As discussed in Section 3.1.2, batch fermentation models do not aim to perfectly recreate the *in vivo* environment. It is a controlled fermentation system to study short-term effects on microbial metabolism. Furthermore, the pre-wean period is a dynamically challenging age range to capture using pooled faecal inoculum, yet conserved differences in *post mortem* samples suggest this system could be adjusted to be more specific, if required, by altering the inoculant source.

Chapter 4 A Spoon Full of Sugar: *In Vitro* Effects of Fermenting Milk Oligosaccharides in a Pre-wean Piglet Gut Model

4.1 Introduction

Early-life gastrointestinal development is a key determinant of piglet health, survival and long-term production performance. During the pre-weaning period, the gut undergoes rapid physiological and microbial changes as the piglet matures (Everaert et al., 2017; Trevisi et al., 2021). Early microbial colonisation of the hindgut is driven by exposure to sow mucosa, delivery environment, and mammary secretions (Chen et al., 2018). This initial microbial community can have lasting implications for growth efficiency, disease resilience and post-weaning adaptation (Li et al., 2018a; Li et al., 2019). There is evidence suggesting that the early colonisers of the gut are key to establishing the mature microbiome and dysbiosis in early life may result in life-long health issues, such as reduced growth in pigs (Wang et al., 2019) and disease susceptibility in humans (Kalbermatter et al., 2021). This suggests that establishing a healthy population of microbes at an early age could help support piglet health and survival up to and through weaning.

Colostrum represents the first, and most biologically loaded, input during the neonatal period. Beyond its macronutrient provision, colostrum contains a diverse array of bioactive components including immunoglobulins and milk oligosaccharides which have immunomodulatory functions (Tao et al., 2010; Zhang et al., 2018). Immunoglobulins, particularly IgG, have attracted research interest historically because of their role providing passive immunity to the piglet (Werhahn et al., 1981; Quesnel, 2011). Piglets are born with no serum immunoglobulins and so must access provision of this through suckling as soon as possible after birth, or else their chances of survival are very poor (Devillers et al., 2011). The timely nature of this is in part due to gut closure where prolactin stimulates lactose synthesis and the gut is then unable to allow large molecules to pass through, but also because transitional and mature milk contain negligible amounts of IgG relative to colostrum (Klobasa et al., 1987; Markowska-Daniel and Pomorska-Mól, 2010).

Unlike immunoglobulins, the concentration of milk oligosaccharides in mammary secretions does not rapidly decline after 24-36 hours, with evidence

that neutral structures increase in concentration during the lactation period (Tao et al., 2010; Mudd et al., 2016; Soyyilmaz et al., 2021). This suggests that in addition to their associations with gut development and immunity, they may also provide support and prebiotic activity throughout the suckling period and could therefore be linked to litter performance and piglet/pig health long-term. Preliminary exploration indicates porcine milk oligosaccharide (PMO) concentrations have the potential to influence litter performance (Smith et al., 2026) and direct supplementation of oligosaccharides to piglets has many reported benefits including enhanced cognitive function (Wang et al., 2007), increased weight gain (Berding et al., 2016), and improved gut barrier function (Yin et al., 2019).

One of the challenges with devising suitable supplementation of milk oligosaccharides is the structural diversity of these compounds. There are over 200 structures which have been identified in human milk and over 55 in porcine milk so far (Wei et al., 2018). This variety results in varied biological effects initiated through complex mechanistic actions, most of which are poorly understood. Subtle differences such as glycosidic linkage type and monosaccharide composition can result in distinct functional outcomes when metabolised by resident microbes (Garrido et al., 2011; Kavanaugh et al., 2013; Cheng, Kong, et al., 2020).

For example, Cheng et al. (2020) found that 6'SL had a proliferative effect on *Bifidobacterium longum* subsp. *infantis* when in a co-culture with *Faecalibacterium prausnitzii* where other key HMO such as 2'FL, 3'FL and lacto-N-triose did not promote growth, confirming effects are structure specific (Cheng, Kiewiet, et al., 2020). Furthermore, when investigating adhesion of *Bifidobacterium longum* subsp. *infantis* to epithelial cells, Kavanaugh et al. (2013) reported a 4.7 fold increase in adhesion with a 6'SL treatment group but a 9.8 fold increase with a mixture of 3'SL and 6'SL, while the individual 3'SL treatment did not affect adhesion (Kavanaugh et al., 2013). This suggests not only structure dependent effects in line with Cheng et al. (2020), but also synergy when certain combinations of HMO are present. It can be hypothesised that 6'SL gives specific responses not achieved by 3'SL due to the structural difference in sialic acid location, however this would not explain the synergy seen when used together. Garrido et al. (2011) reported that when *Bifidobacterium longum* subsp. *infantis*

is grown solely on HMO, proteins are expressed that can import HMO isomers and also bind to HMO, suggesting coregulated mechanisms of transport and may more rationally explain the synergistic effects (Garrido et al., 2011).

Moreover, comparison of 2'FL and 3'FL, structures which differ by the attachment of the fucose sugar to either galactose or glucose, respectively, showed that both compounds were able to stimulate glycocalyx response, but at different capacities. Only 3'FL increased average area coverage of tested glycocalyx components, as well as the thickness of some components, where 2'FL had lesser or no effects (Kong et al., 2019).

HMO can modulate activity of epithelial cells in multiple ways, with most reports highlighting inhibition of pro-inflammatory cytokines. Inhibition of cytokine expression has been demonstrated by 3'SL (Zenhom et al., 2011), while a combination of various HMO can inhibit TNF- α induced chemokine release (Newburg et al., 2016), and 2'FL inhibits IL-8 release specifically in an *E.coli* induced response (He et al., 2016). Again, mechanisms remain elusive but it has been suggested that anti-inflammatory effects are induced via the nuclear receptor peroxisome proliferator-activated receptor gamma (Zenhom et al., 2011), yet this also seems to be a structure specific mechanism as 2'FL does not inhibit cytokine release via this pathway, whereas 6'SL does (Zehra et al., 2018).

Holscher et al. (2014) reported that LNnT, 2'FL, and 6'SL reduced cell proliferation and identified different mechanisms for each HMO action (Holscher et al., 2014). They discuss that 2'FL increased activity of sucrase, which is a marker for increased epithelial cell differentiation, and was the main influencer of differentiation out of the compounds tested. This differs to work by Kuntz et al. (2008) who did not find 2'FL as having this effect but instead found 6'SL to have the greatest effect on stimulating differentiation (Kuntz et al., 2008). The authors in Holscher et al. (2014) hypothesise this was due to differences in preconfluent vs postconfluent cell cultures. These differences in findings cement the specificity of HMO effects and suggest more research is needed to narrow down the factors that influence functionality. Furthermore, the health status of the individual in *in vivo* studies alters effects seen in 2'FL as Comstock et al. (2014) found enhanced proliferation of peripheral blood mononuclear cells when stimulated with lipopolysaccharides but the same HMO inhibited proliferation without the mitogenic stimuli (Comstock et al., 2014).

As mentioned previously, galactooligosaccharides (GOS) are often added to human infant formula as a cost effective alternative to HMO, but due to structural simplicity, lack some of the beneficial effects seen in HMO (discussed in Section 1.4.3). These could however be used in conjunction with HMO to produce complimentary effects as work by Jakobsen et al. (2019) highlighted a synergy between bovine milk oligosaccharides (BMO) and GOS in reducing pathogens *Clostridia perfringens* and *E.coli*. The GOS and BMO were less effective when used independently but provided significant reduction in tandem. Furthermore, milk oligosaccharides are strongly associated with increasing growth of the beneficial *Bifidobacteria spp.* (Sela and Mills, 2010; Zhang et al., 2021), and BMO further increased growth of *B. longum* subsp. *longum* when supplemented in addition to lactose, compared to growth on each individual carbohydrate source (Jakobsen et al., 2019).

These findings cement how complex milk oligosaccharide mechanics and functional effects are, and showcase that the intricate matrices of bioactive components in colostrum and milk cannot be easily replicated. Conversely, it is possible for significant improvements to be made regarding porcine milk replacer, as has been demonstrated in human breast milk research and subsequent improvement of infant formula (see Section 1.5). Piglet milk replacer, as discussed in Section 1.6, is rather reductionist, with focus on calorie density to increase weight gain, rather than a more holistic approach acknowledging the importance of gut health and immunity in performance. This chapter explores the potential of four key HMO/PMO (2'FL, 3'SL, 6'SL, and LNnT, shared across species) to modulate the gut microbiota in an *in vitro* pre-wean piglet colon model, with the aim to propose an optimal PMO combination for further research *in vivo*.

To address this, the present study employed an *in vitro* model of the pre-wean piglet colon, as developed through experiments detailed in Chapter 3, to compare microbial community composition and short chain fatty acid production following fermentation of colostrum, piglet milk replacer, and milk replacer supplemented with various milk oligosaccharides. It was hypothesised that oligosaccharide supplementation would better approximate the microbial and metabolic profiles observed when fermenting colostrum. Specifically, 2'FL and LNnT were considered likely to elicit microbial community shifts due to the

successful additions of these in the human infant formula market (Zhu, Zhang, et al., 2022), perhaps with addition of 3'SL due to abundance in sow samples (Smith et al., 2026). By directly contrasting whole colostrum with reductionist supplementation strategies, this work aimed to determine whether functional equivalence can be achieved through targeted formulation, or whether the properties of the colostrum matrix which drive distinct ecological outcomes are too strong to simulate in this way.

4.2 Methods

This study was a single factor between-subjects design testing a total of 17 substrate alterations (hereafter, treatments). Treatments are laid out in Table 4.1 and briefly consisted of (1) piglet milk replacer powder, as used in many Chapter 3 experiments; (2) pooled colostrum, as used in Chapter 3, Experiment 4; (3) oligosaccharide supplementation, added to piglet milk replacer powder in various ratios and combinations.

Table 4.1 Treatment compositions and codes. Abbreviations of milk oligosaccharides as follows; 2'FL, 2'-fucosyllactose; 3'SL, 3'-sialyllactose; 6'SL, 6'-sialyllactose; LNnT, lactose-N-neotetraose

Treatment	Code	Ratio
Colostrum	COL	100*
Milk replacer	MR	
2'FL	2'FL	
3'SL	3'SL	
6'SL	6'SL	
LNnT	LNnT	
2'FL + 3'SL	2-3	50:50
2'FL + 6'SL	2-6	
2'FL + LNnT	2-L	
3'SL + 6'SL	3-6	
3'SL + LNnT	3-L	
6'SL + LNnT	6-L	
2'FL + 3'SL + 6'SL	2-3-6	33:33:33
2'FL + 3'SL + LNnT	2-3-L	
2'FL + 6'SL + LNnT	2-6-L	
3'SL + 6'SL + LNnT	3-6-L	
2'FL + 3'SL + 6'SL + LNnT	All four	25:25:25:25

*Ratio refers to substrate being 100% colostrum, 100% milk replacer, and thereafter is specific to oligosaccharide combinations only, where 100 indicates individual oligosaccharides were added to fermentation media at a final concentration of 2 g/L, 50:50 indicates each oligosaccharide was added at a final concentration of 1 g/L, resulting in a total oligosaccharide presence of 2 g/L, and so on.

4.2.1 Sample Collection

Faecal samples used in this study were collected from Large White X Landrace X Duroc piglets aged between 5 and 27 days old, from National Pig Centre, University of Leeds, as previously described in Section 3.2.1. Briefly, samples were collected freely upon defecation and pooled to minimise individual and age related variation. A slurry was prepared using the pooled faecal samples and a carbonate-phosphate buffer (for composition see Table 3.1) at 70% wt/vol and frozen in aliquots at -80°C until use, as described in detail in Section 3.2.2.

Colostrum used in this study was collected from Large White X Landrace sows (parity 2-8) by manual manipulation of the teats at onset of parturition. Further details can be found in Section 2.2.2. Multiple teats were sampled from per sow and all samples were pooled to reduce individual variation before freezing at -80°C until use. Colostrum used in this chapter was the same batch as that used in Section 3.7.

4.2.2 Porcine Milk Oligosaccharides

Milk oligosaccharides used were commercially available isolates 2'-Fucosyllactose (2'FL), 3'-Sialyllactose (3'SL), 6'-Sialyllactose (6'SL), and Lacto-N-neotetraose (LNnT), which were kindly provided by DSM-Firmenich (Hørsholm, Denmark).

4.2.3 *In vitro* Fermentation

A summary of the protocol used in this chapter can be seen in Section 3.8 (Figure 3.25). The pooled colostrum and the piglet milk replacer, underwent a foregut digestion phase prior to use in the hindgut fermentation, as per conclusions from Section 3.7. This is described in detail in Section 3.7.1.2 and, briefly, consisted of agitated incubations with simulated gastric and intestinal fluids and appropriate enzymes. Upon completion of the small intestine stage, samples were placed in an ice bath to terminate enzymatic action and then centrifuged at 5000 rpm for 15 minutes at 4°C and the supernatant discarded (Loo et al., 2022). The foregut hydrolysis was performed in bulk over two days with residual pellets being pooled and refrigerated (for a maximum of 24 hours) until sufficient material was gained. Once approximately 2 g of colostrum and 9 g milk replacer residues were acquired, the pooled matter was aliquoted by weight (0.55 g) before storing at -20°C until needed. This process was repeated twice more for the production of pre-digested milk replacer, resulting in one batch of colostrum used for all repetitions of experiments, and a new batch of milk replacer for each experimental repetition.

For the fermentation phase, best practice identified through experiments in Chapter 3 was used. This included making up a mineral buffer as per Palowski et al (2021) (without reducing solution), with the addition of 1 g/L porcine mucin and 1.25 g/L resazurin. The pH was then adjusted to 6.8 before boiling for ~10 minutes until redox indicator colour change. At this stage, the solution was cooled in a water and ice bath under a stream of CO₂ until no higher than 35°C (approximately 40 minutes). The defrosted slurry was then added at final faecal content of 50 g/L (approximately 76 g/L slurry) and strained through four layers of cheesecloth. This inoculated buffer was bubbled through with CO₂ for an additional 30 minutes to purge oxygen introduced through the addition of the slurry, before sealing and storing at 39°C overnight (approximately 10-12 hours).

One 125 mL serum bottle was prepared with each treatment (see Table 4.1) to approximately 0.5 g DM each. Oligosaccharide addition was to a final concentration of 2 g/L and was achieved by making stock solutions of each combination with water to 1 g total oligosaccharide mass with 5 mL purified water and 0.5 mL of this solution was added to each appropriate serum bottle (final volume of ~50 mL). When an oligosaccharide combination was added, 0.1 g milk replacer was removed. This resulted in 0.5 g each for pre-digested colostrum and pre-digested milk replacer, and 0.4 g pre-digested milk replacer in other treatments with 0.1 g total oligosaccharide added (in 0.5 mL solution).

Serum bottles with treatments were warmed in an incubator at 39°C before use. To each serum bottle, 50 mL of inoculated buffer was added before bubbling with CO₂ for 5-6 minutes and sealing each bottle with a crimp cap. Bottles were incubated at 39°C for 24 hours with gas released and pressure readings taken at 2, 4, 6, 8 and 24 hours. To terminate the fermentation, bottles were placed in an ice bath after the final gas release and recording. Final pH was measured and samples taken for downstream analysis. Three batches of the fermentation were performed overall, with one rep (serum bottle) per treatment per batch.

4.2.4 Gas Chromatography

For gas chromatography, 1.5 mL of fermentation fluid was placed in an Eppendorf and stored at -20°C until use. Gas chromatography was performed as described in Section 3.2.4 and was used to quantify acetate, butyrate and propionate by comparing to an internal standard solution (using 4- methylvaleric acid) and calibration standards of each compound of interest (5 mM). Total SCFA concentration was calculated as the sum of each independent measure.

4.2.5 Microbial DNA Extraction

For microbial DNA extractions, 1.5 mL fermentation fluid was centrifuged at 16,000 x g for 5 minutes at room temperature. The supernatant was then discarded and a further 1 mL fermentation fluid added before centrifuging again. The final pellets were stored at -80°C until use.

Microbial DNA was extracted using the QIAamp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany), following manufacturer's instructions, with slight amendments. After heating the pellet with InhibitEX buffer at 95°C for 5 minutes,

tubes were vortexed for 10 seconds and then heated for a further 5 minutes at 95°C. Proteinase K was added at 25 µL then 400 µL supernatant was added and 400 µL Buffer AL. After heating, 400 µL ethanol was added and 600 µL lysate transferred to the spin column. After first spin, the remaining lysate was added and spun again. The wash steps remain as per manufacturer's instructions and 50 µL Buffer ATE was used to elute. These adjustments resulted in greater DNA yield and improved quality based on nanodrop assessment. Eluted DNA was quantified using a NanoDrop spectrophotometer (NanoDrop 2000, Thermo Fisher). Microbial DNA samples were sent for library preparation and 16S rRNA sequencing of the V4 region to Novogene Company Ltd (Cambridge, UK). Returned reads were analysed using Mothur as detailed in Section 3.2.5.

4.2.6 Statistical Analysis

Statistical analysis was performed using RStudio and associated packages as described in Section 3.2.6.

Briefly, gas volume was plotted continuously and key parameters of the Gompertz growth models were extracted (detailed in Section 3.2.6.1) to be used in mixed effect linear models with treatment as the main effect and batch as a random factor. An ANOVA was then conducted on the mixed model created and any relevant pair-wise comparison performed using Tukey *post-hoc* testing. Difference in pH and SCFA concentrations were tested using ANOVA, following confirmation of parametric assumptions. Microbiome analysis was conducted as described in Section 3.2.6.3 using the SOP for 16S data on the Mothur platform (Schloss et al., 2009; Kozich et al., 2013), followed by data curation, visualisation and statistics using RStudio. Use of ANCOM-BC was foregone for phylum and genus level comparisons between treatments due to imbalance in the number of replications and number of treatments. These are instead described using mean $\pm$ standard error presented in percentage relative abundance.

4.3 Results

A total of 17 treatments were tested including two standard substrates (colostrum and milk replacer) as well as 15 milk oligosaccharide formulas which were supplemented to a milk replacer base. These were '2'FL', '3'SL', '6'SL', and 'LNnT' added individually and in every pairwise, triple or quadruple formulation (see Table 4.1).

4.3.1 Fermentation Parameters

Curves were modelled from cumulative gas production (mL) data and Gompertz asymptote parameters extracted for statistical analysis. Treatment had a significant effect on maximum gas volumes produced ($F_{34} = 2.64$; $p = 0.00857$) where colostrum yielded the lowest mean maximum gas volume (60.5 ± 8.49 mL). The highest maximum gas volume produced was with the addition of LNNt to the milk replacer base (81.4 ± 4.83 mL). Treatment also had a significant effect on growth rate ($F_{34} = 2.46$; $p = 0.0134$), with colostrum generating the lowest value (0.325 ± 0.0535) and treatment 2-3-L recorded the highest (0.455 ± 0.0205). There was no significant effect of treatment on lag parameter ($p = 0.127$). Significant pairwise results for maximum gas volume and growth rate asymptotes can be seen in Table 4.2, where colostrum is the consistently different treatment.

Table 4.2 Significant pairwise comparisons (*Tukey*) of extracted maximum gas volume and growth rate parameters

	Comparison	Estimate	t - value	p value
Max. gas volume	COL LNNt	-20.89	-4.51	0.0068
	COL 2-3-L	-18.52	-3.99	0.0261
	COL 2-6	-20.44	-4.41	0.0088
	COL 2-L	-20.43	-4.41	0.0089
	COL 2'FL	-17.89	-3.86	0.0367
	COL 3-6-L	-17.62	-3.8	0.0421
	COL 3-6	-20.08	-4.33	0.0109
	COL 3-L	-18.64	-4.02	0.0244
	COL 6'SL	-19.59	-4.23	0.0144
Growth rate	COL 2-3-6	-0.113	-4.24	0.0137
	COL 2-3-L	-0.130	-4.91	0.0022
	COL 2-6-L	-0.117	-4.4	0.0091
	COL 2-L	-0.102	-3.84	0.0383
	COL 3-6-L	-0.106	-3.99	0.0262
	COL 3-6	-0.123	-4.62	0.005
	COL 3-L	-0.102	-3.83	0.039

There was similarity in curve profile across treatments with colostrum having a lower, flatter curve (Figure 4.1).

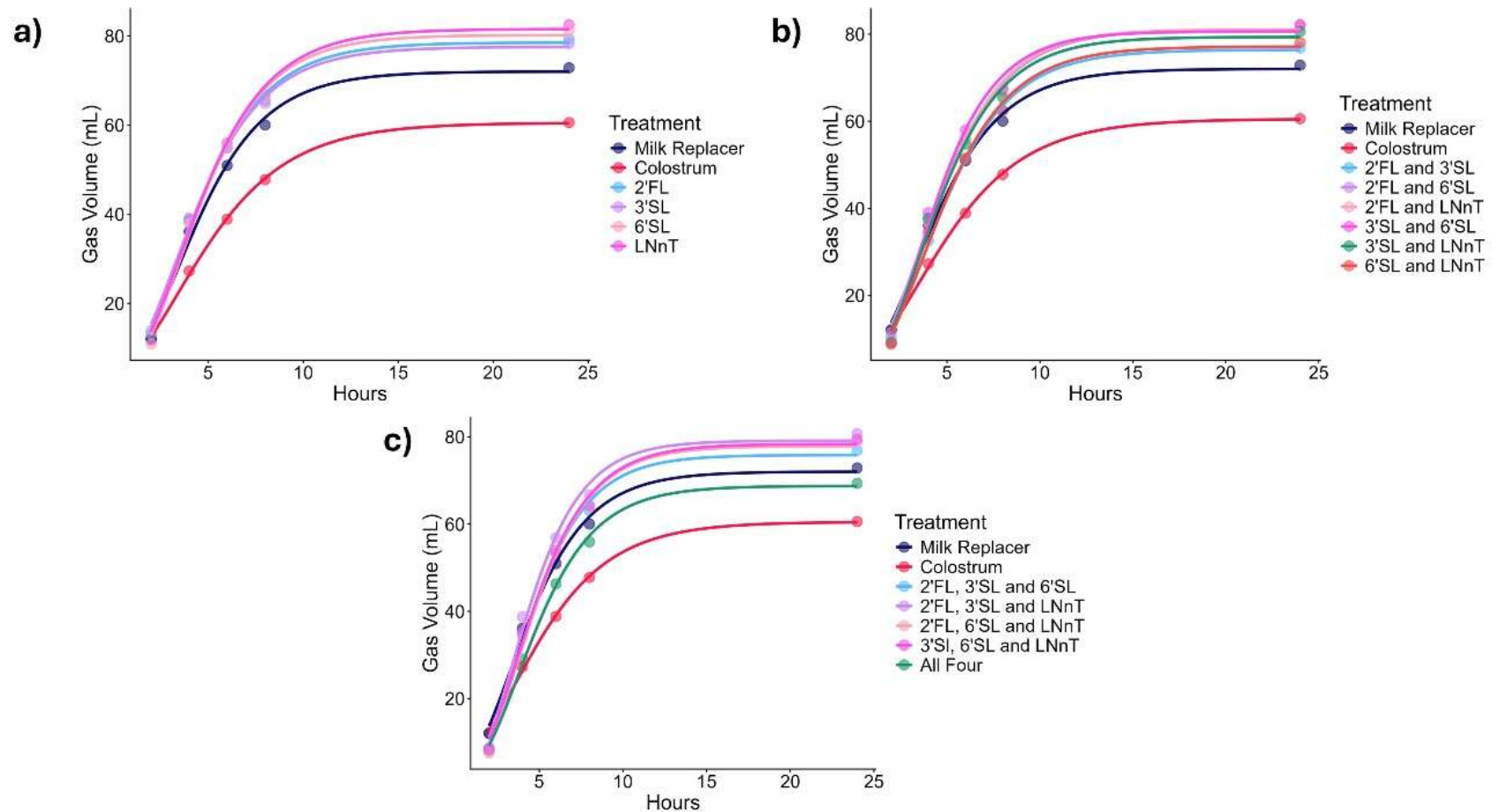


Figure 4.1 Gompertz growth curves fitted to cumulative gas production recorded during fermentation. Points represent mean measured gas volumes and curves represent predicted growth. Colour denotes treatment as specified in each figure key. Treatments are displayed based on ratio combination with inclusion of standard substrates in each figure for comparison: a) individual treatments (100); b) paired treatments (50:50); c) group treatments (33:33:33 and 25:25:25:25).

The final pH was significantly impacted by treatment ($F_{34} = 2.81$; $p = 0.00552$), with highest pH for the colostrum condition (6.9 ± 0.1) and many treatments tied for lowest final pH at $6.47 \pm 0.0577 - 0.153$ (3'SL, LNnT, 2-L, 3-L, 6-L, 2-6-L, and all four). There was a significant pairwise difference in final pH between colostrum and every other condition (all to $p < 0.01$ threshold, less 2'FL where $p = 0.0164$), with no other significant pairwise differences.

4.3.2 Short Chain Fatty Acid Concentrations

Treatment did not significantly impact acetate ($p = 0.469$), propionate ($p = 0.533$), butyrate ($p = 0.473$), or total SCFA concentration ($p = 0.498$). The highest concentration of acetate, propionate and highest total SCFA concentration was produced by the oligosaccharide treatment 6-L with mean values of 36.9 ± 13.1 mM, 39.6 ± 13.9 mM and 84.3 ± 29.0 mM, for acetate, propionate and total concentrations, respectively. Colostrum was the highest butyrate producer (10.3 ± 5.07 mM), with milk replacer producing the lowest butyrate concentration (4.06 ± 0.323 mM). Exact values for each SCFA per treatment can be seen in Table 4.3.

Table 4.3 Short chain fatty acid (SCFA) concentrations produced after fermentation of various substrates (treatment)

		Acetate (mM)		Propionate (mM)		Butyrate (mM)		Total SCFAs (mM)	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE
Treatment	Colostrum	16.97	11.87	20.18	6.21	10.34	5.07	47.49	23.07
	MR	23.96	2.34	22.18	4.98	4.06	0.32	50.20	7.64
	2'FL	20.61	1.36	23.19	1.55	4.82	0.44	48.62	2.72
	3'SL	20.93	0.40	22.48	2.16	4.83	0.45	48.24	2.80
	6'SL	19.01	0.52	23.46	1.21	5.03	0.46	47.50	1.51
	LNnT	21.98	2.17	24.26	1.74	5.25	0.71	51.48	4.34
	2'FL + 3'SL	24.25	1.52	25.36	1.78	4.91	0.29	54.53	2.53
	2'FL + 6'SL	22.94	0.54	19.74	5.92	5.60	0.22	48.28	6.30
	2'FL + LNnT	22.72	1.72	19.33	5.15	5.44	0.49	47.49	4.46
	3'SL + 6'SL	24.13	4.10	26.22	1.69	5.78	0.60	56.13	5.98
	3'SL + LNnT	20.54	3.87	20.97	6.48	5.46	0.31	46.97	9.71
	6'SL + LNnT	36.92	13.08	39.58	13.92	7.81	2.02	84.31	28.99
	2'FL + 3'SL + 6'SL	22.72	1.63	24.95	1.74	5.34	0.36	53.01	3.17
	2'FL + 3'SL + LNnT	20.68	1.71	25.49	1.78	5.51	0.27	51.69	3.51
	2'FL + 6'SL + LNnT	31.15	8.67	29.52	8.54	6.81	2.24	67.49	19.43
	3'SL + 6'SL + LNnT	30.82	9.48	20.46	11.73	6.49	1.42	57.77	22.63
	All four	23.61	2.91	16.31	8.24	5.42	0.91	45.34	12.07
P value (Treatment:SCFA)		0.469		0.533		0.473		0.498	

4.3.3 Microbiota

A total of 51 samples were processed to extract microbial DNA and a total of 3,244,036 reads were generated through 16S rRNA sequencing of the hypervariable V4 region. After screening and filtering 16,788 high-quality unique reads remained.

4.3.3.1 Alpha and Beta Diversity

Alpha diversity metrics Chao1, Shannon and Simpson did not significantly differ between treatments (all $p > 0.9$). The highest values across all indices were produced by treatment 2-3 (716 ± 113 , Chao1; 3.13 ± 0.040 , Shannon; 0.876 ± 0.010 , Simpson) and the lowest value across all indices was treatment 6-L (503 ± 4.73 , Chao1; 2.83 ± 0.114 , Shannon; 0.829 ± 0.028 , Simpson).

Beta diversity was also not significantly impacted by treatment (*Bray-Curtis*; $F = 0.914$; $R^2 = 0.301$; $p = 0.637$).

4.3.3.2 Relative Abundance: Phylum Level

Microbiome analysis revealed phyla present across all samples were Actinomycetota, Bacillota, Bacteroidota, Pseudomonadota, Spirochaetota, Synergistota, and Thermodesulfobacteriota. Colostrum was the only treatment dominated by Bacteroidota ($65.9 \pm 6.78\%$), with other treatments having more Bacillota (mean abundance $58.9 \pm 2.8\%$) than Bacteroidota (mean abundance $38.0 \pm 2.72\%$), and generally more equal Bacteroidota to Bacillota ratios than colostrum (Figure 4.2). Pseudomonadota was consistently the third most abundant phylum across all groups, including colostrum, ranging from $1.84 \pm 0.306\%$ to $3.95 \pm 1.75\%$ and averaging at a relative abundance of $2.67 \pm 0.602\%$. Other present phyla were recorded at negligible abundances across all groups ($< 1\%$).

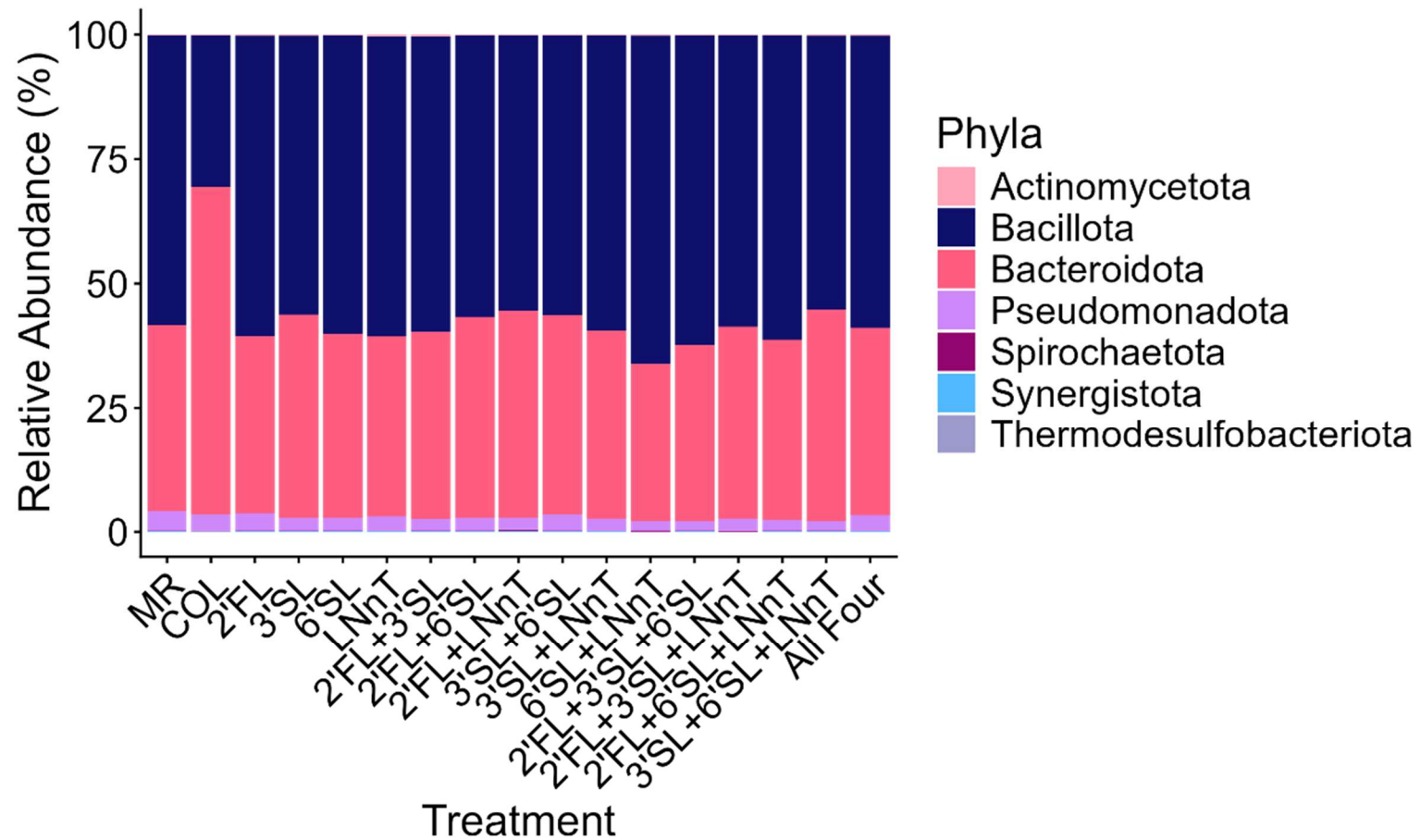


Figure 4.2 Percentage relative abundance of phyla present after fermentation of various treatments. Abbreviations from left to right: Milk replacer; colostrum; 2'-fucosialyllactose; 3'-sialyllactose; 6'-sialyllactose; lacto-N-neotetraose.

4.3.3.3 Relative Abundance: Genus Level

Consistently prevalent genera included *Bacteroides*, *Streptococcus*, *Lachnospiraceae_unclassified*, *Lachnospiraceae_UCG-010*, *Escherichia-Shigella*, and high proportions of *Other*; as calculated by the sum of any genera at < 1% relative abundance across all treatments.

The top three genera produced in nine of the oligosaccharide treatments were *Streptococcus* (mean $30.1 \pm 3.13\%$), *Bacteroides* (mean $24.5 \pm 2.01\%$), and *Lachnospiraceae_unclassified* (mean $13.8 \pm 1.58\%$). These appeared in that order of prevalence in groups 2-3-6, 2-3-L, 2-6-L, 2-6, 2'FL, 3-L, 6-L, 6'SL, and LNnT. Another seven treatments had the same most prevalent genera but in a different order with *Bacteroides* as most abundant (mean $28.0 \pm 1.63\%$), followed by *Streptococcus* (mean $25.0 \pm 0.97\%$) and then *Lachnospiraceae_unclassified* (mean $14.1 \pm 1.14\%$); this occurred in groups 2-3, 2-L, 3-6-L, 3-6, 3'SL, and All Four. Colostrum shared *Bacteroides* at most abundant but with an increased relative abundance compared to other treatments ($51.9 \pm 7.74\%$), with the proportion of *Other* genera being second most abundant (11.1%) and *Lachnospiraceae_unclassified* third ($8.28 \pm 1.32\%$). Distributions of genera appearing at > 1% in at least one treatment are displayed in Figure 4.3.

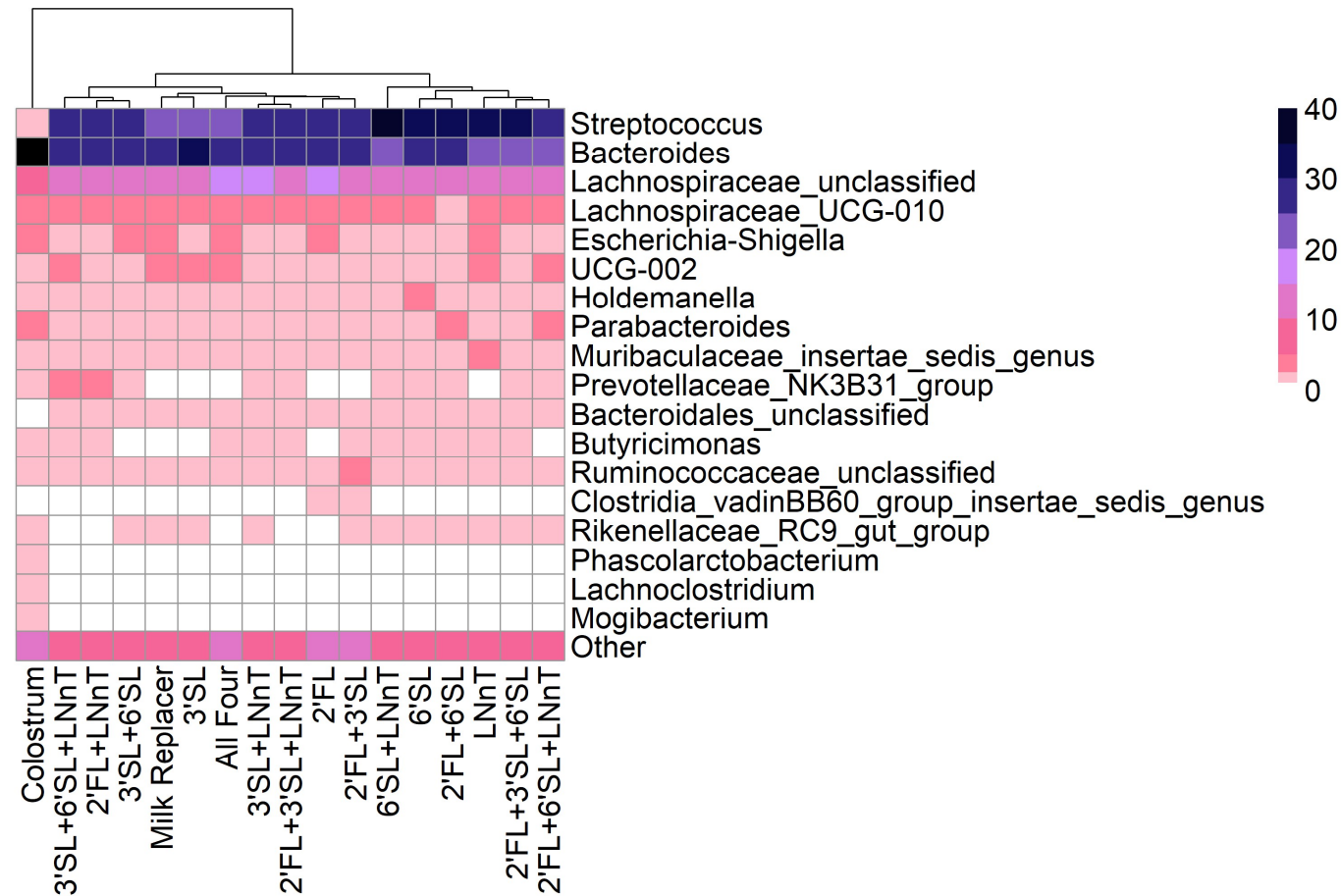


Figure 4.3 Heatmap indicating relative abundance of genera that were above 1% in at least one treatment. 'Other' indicates sum of genera at <1% relative abundance. Oligosaccharide abbreviations: 2'FL, 2'-fucosialyllactose; 3'SL, 3'-sialyllactose; 6'SL, 6'-sialyllactose; LNnT, lacto-N-neotetraose. Colour denotes magnitude of abundance where pale boxes are lesser and dark boxes are increased, as per right-hand key. Dendrogram clusters columns based on Bray-Curtis dissimilarity

4.4 Discussion

Milk oligosaccharides are structurally diverse, thus elicit distinct functional responses depending on microbial capacity and compound ratios. Colostrum and sow milk are naturally rich in PMO, resulting in many prebiotic benefits for the suckling piglet. However, due to production pressure to increase litter sizes, interventions such as provision of supplemental milk replacer have become necessary to support large litters and small piglets. The majority of piglet milk replacer on the market do not contain prebiotic compounds like sow milk (see Table 1.2 for summary of bioactive milk components) so to address the high pre-wean mortality rates, and due to the success in adding HMO to infant formula, here potential PMO combinations were explored *in vitro*. The aim of these experiments was to identify differences in fermentation parameters, microbiota composition and metabolite production between colostrum, milk replacer, and various milk oligosaccharide combinations (of 2'FL, 3'SL, 6'SL, and LNnT) in an *in vitro* model of the pre-wean piglet colon, to identify an optimal combination of HMO to research further in farm trials. It was hypothesised that a combination would elicit the most colostrum-like response, due to structure-dependent effects, and that a combination of 2'FL, 3'SL and LNnT would be most promising. This hypothesis was rejected likely? due to colostrum producing a unique microbiota.

4.4.1 Fermentation Kinetics

Colostrum produced the lowest mean maximum gas volume and lowest growth rate when fermenting with the faecal slurry, resulting in many significant pairwise differences to other treatments for these parameters. Fermentation kinetics were strongly driven by substrate type and accessibility as discussed previously in Section 3.7.3. A major component contributing to rapid fermentability is lactose which occurs at 6.08% (as fed, when prepared 135 g/L) in the milk replacer used in this study (see Table 3.2) whereas average colostrum lactose content is reported at 3-5% (Zhang et al., 2018; Inoue and Tsukahara, 2021). The colostrum used in this study was not analysed for macronutrient composition but it could be that the higher proportion of lactose in the milk replacer is having the greatest effect on fermentation kinetics, with addition of any milk oligosaccharide on top of this further enhancing fermentability. This would need to be tested...

Furthermore, livestock milk replacer is often based on bovine products (Pieper et al., 2016) where ingredients can be heat-treated and spray-dried resulting in highly soluble components (Bista et al., 2022; McCarthy et al., 2022), increasing substrate accessibility. Microbes will more readily ferment these accessible components in comparison to the structurally complex matrix of colostrum. The addition of PMO to the milk replacer is also providing accessible nourishment to the microbiota as oligosaccharides are highly fermentable (Houdijk et al., 1998; Difilippo, Pan, Logtenberg, Willems, et al., 2016; Difilippo, Pan, Logtenberg, Willems, et al., 2016). Providing the PMO treatments in simple formats, relative to the complexity seen in colostrum, could be why no kinetic differences were seen, compared to the milk replacer. Colostrum will contain a richer and more diverse composition of PMO, many of which could be long and/or complexly branched, whereas the chosen four structures added presently are shorter-chain structures, more likely to have a higher fermentation capacity (increased gas volume and growth rate) (Vester Boler et al., 2009b; Vester Boler et al., 2009a).

There was a significant difference in final pH between the colostrum condition and all other treatments tested, however, the mean pH for colostrum was 6.9 and the mean across all other groups was 6.5. Although statistically significant, this numeric difference of *circa* 0.4 units may have limited biological relevance as discussed previously (Section 3.6.3.1) due to the natural fluctuations seen *in vivo* (Li et al., 2012). Therefore a difference of this amount is to be expected when fermenting different matrices and is unlikely to cause significant ecological shifts.

4.4.2 Short Chain Fatty Acid Production

Concentration of acetate, butyrate, and propionate did not differ significantly across any groups, possibly due to some large SE values (see Table 4.3). Treatments with particularly large SE values were colostrum, 2-6-L, 3-6-L, and 6-L, possibly suggesting some inconsistent interactions between 6'SL and LNnT. As 6'SL is an acidic structure, and LNnT a neutral one, they offer different glycosidic linkages and monosaccharide constituents to the microbiota. Usually microbes will cleave sialic acid from acidic compounds, allowing other organisms to then consume the released lactose, but this is dependent on microbes possessing sialidase which can vary at the strain specific level (ten Bruggencate

et al., 2014). Such small variations in ability for the microbiota to ferment (or not) different substrates will no doubt impact the nonlinear cross-feeding networks, potentially amplifying into large numeric variation, as seen in the SCFA concentration data. Early onset differences across batches that could influence variation could be aspects like cell density distribution or micro-scale oxygen exposure, either of these then contributing to early domination of specific taxa, and thus different cross-feeding cascades and divergent SCFA profiles at the end.

Large variation has been seen in previous gas chromatography data in Chapter 3, and it should be acknowledged that the replacement of columns and cleaning of the autosampler could have impacted results from different batches. The machine and protocol used have not been validated across other more modern machines or methods, therefore absolute values must be considered relative within each experiment. That is to say, increases and decreases in values between groups are biologically meaningful, however, an exact numerical value cannot be compared like-for-like with values reported by other research groups.

Although not statistically significant, colostrum had the highest concentration of butyrate, which is beneficial for gut health as its used as an energy source for colonocytes (Donohoe et al., 2011), gut barrier function (Knudsen et al., 2018), and immune modulation (Furusawa et al., 2013). The enhanced butyrate production may reflect the broader diversity of fermentable substrates present in colostrum compared to milk replacer formulations. Butyrate is typically produced via acetyl-coA pathway or cross-feeding (lactate conversion) (Pryde et al., 2002; Duncan et al., 2004) and as certain taxa thrive on complex and resistant glycans, the greater butyrate production from colostrum may reflect enhanced cross-feeding interactions enabled by its complex matrix.

Enhancing butyrate production in this *in vitro* model would be a strong indicator for a beneficial treatment due to the numerous advantageous effects butyrate has. Butyrate is the preferred energy source for colonocytes over and above acetate or propionate (Clausen and Mortensen, 1995; Ritzhaupt et al., 1998) and can decrease intestinal permeability through promotion of cell differentiation (Pryde et al., 2002), modulating gene expression (Deng et al., 2024), and inducing apoptosis in tumour cells (Tailor et al., 2014; Salimi et al., 2017). These benefits have led to butyrate being classified as a protective agent

against colorectal cancer (Luo et al., 2023) and colitis (Recharla et al., 2023; Deng et al., 2024) in humans, with inadequate butyrate supply being associated with increase colitis risk (Pryde et al., 2002).

Beneficial supplementation of butyrate has been demonstrated *in vivo* in piglets (Lu et al., 2008; Xu et al., 2016). Lu et al. (2008) demonstrated that supplementation with 1 g/kg sodium butyrate enhanced growth performance, reduced pathogen counts, decreased inflammatory cytokine expression, and improved intestinal morphology in weanling piglets. At the pre-wean stage, Xu et al. (2026) demonstrated sodium butyrate supplementation modulated expression of many cytokines including IL-6, IL-8 and IL-10, as early as 8 days of age.

4.4.3 Microbial Community Composition

Neither alpha- nor beta- diversity differed significantly between treatments, suggesting that overall microbial richness and evenness were not enhanced by the PMO added, and, additionally, the addition of PMO to the milk replacer base did not shift the microbial population sufficiently to result in any obvious clustering. However, the analysis may have lacked power to detect similarities due to low repetition number and high treatment number.

A previous study found that bacterial community diversity was higher in milk replacer groups compared to colostrum, and community compositional shifts were reported but without specific OTU differences (Poulsen et al., 2017). The work by Poulsen et al. (2017) was conducted across a period of days so it could be argued that the 24 hour fermentation period in the present study was not sufficient to see these differences emerge, however, differences are reported across other experiments from as little as 8 hours fermentation (see Section 4.3.1.3). As PMO treatments have not been added in previous experiments, it is worth considering that the PMO require longer to break down, due to structural complexity, and thus more than 24 hours could be needed to see the subsequent effects. The PMO were not included in the foregut simulation phase prior to fermentation because these structures are naturally resistant to foregut digestion (Gibson and Roberfroid, 1995; Bode, 2012), thus there was no justification to do so. This could explain why quite different communities present in colostrum, compared to PMO treatments, as the colostrum was pre-digested and perhaps

the components were more available to the microbes than the non-digested PMO.

The microbial communities, despite not showing overall dissimilarity, did differ (numerically) in specific OTU abundance. Colostrum fermentation was characterised by a relative enrichment of Bacteroidota and reduction in Bacillota compared with other treatments. The enrichment of Bacteroidota and, at the genus level, of *Bacteroides*, may reflect the greater structural complexity of colostrum-derived glycans compared with isolated oligosaccharide supplementation as *Bacteroides* are strong degraders of complex glycans and thrive on structurally-diverse substrates (Gong et al., 2025). *Bacteroides* contribute to butyrate production (Li et al., 2023) which explains the increased butyrate seen in the colostrum treatment. The benefits of butyrate, and by association, of *Bacteroides* extend to activation of T cell-mediated immune responses (Mazmanian et al., 2008) and reduction of pathogenic colitis inducing bacteria (Mazmanian, 2008). Ingestion of colostrum in *in vivo* studies has been characterised by increased *Bacteroides* abundance in the gut (Kubasova et al., 2017; Chen et al., 2018), therefore this is an expected result to see and confirms the model is able to somewhat mimic large trends seen when fermenting different substrates. Particularly low abundance of *Bacteroides* has been shown to be associated with post-weaning diarrhoea (Ren et al., 2022), suggesting the pooled faecal samples used are from healthy donors.

A notable result from the genera present in each treatment, is the reduction of *Streptococcus* and increased *Bacteroides* in the 3'SL treatment. This is noticeably more similar to what is seen in colostrum, compared to the very strong presence of *Streptococcus* in other treatments. Milk replacer and the combination of all four PMO also show comparably lower *Streptococcus* but not with the increased *Bacteroides*, while 3'SL has the next highest abundance of *Bacteroides* after colostrum. Although very tentative, these results may imply that 3'SL is one of the stronger PMO and, as it is the most abundant PMO in sow colostrum (Smith et al., 2026), may be utilised most effectively by the piglet microbiota.

4.4.4 Limitations

Unlike previous fermentation experiments (detailed in Chapter 3), this study did not perform a minimum of three replications per batch over three batches due to financial restrictions. The high treatment number relative to the low replication number is likely to have skewed statistical results and increased relative variation in measured parameters. As a preliminary and novel experiment, many treatments were used to identify any obvious interactions between PMO, as they have been previously reported to elicit varying and synergistic responses *in vitro* and *in vivo*.

The concentration of 2 g/L PMO added was justified from average concentrations seen in the colostrum of sows at the National Pig Centre (Chapter 2; Smith, et al. (2026)), however, this is considered low, relative to HMO presence in human breast milk, averaging 10-20 g/L (Albrecht et al., 2014; Durham et al., 2023). Comparison to concentrations used in literature reveals variance depending on type of oligosaccharide being studied. For example, *in vivo* piglet studies supplement 1% when investigating effects of GOS (Lee et al., 2023; Stanley et al., 2025b; Stanley et al., 2025a), and as little as 0.005% for XOS in piglets and poultry (Šimić et al., 2023; Stanley et al., 2025a), all studies reporting statistically significant treatment effects. Studies using HMO in piglets lie between with 0.2-0.4% (Hester et al., 2013; Li et al., 2014), both reporting significant treatment effects. Due to differences in oligosaccharide structures (discussed in section 1.4), it may be that different formulations require varying concentrations to be effective.

An important consideration regarding the translation of the present findings to commercial pig production is the economic feasibility of milk oligosaccharide supplementation. This is particularly relevant because HMO production is complex and mass-manufacturing is limited (Urashima et al., 2025; Liu et al., 2025). This can be helped by investigating particularly low doses of target prebiotics to limit the cost of an additive for use in commercial pig production. Doses of prebiotics such as PMO, GOS and FOS that are too low to contribute in a meaningful manner to microbiome community or SCFA production, yet strong enough to help stimulate fibre fermentation are deemed 'stimbiotics' (Bedford, 2019; Gonzalez-Ortiz et al., 2019). These may offer a compromise to the costly inclusion of PMO at prebiotic dose levels, and beneficial stimbiotic effects have been established in weaned pigs (Cho et al., 2020; Stanley et al.,

2025b; Stanley et al., 2025a) and poultry (Šimić et al., 2023; Davies et al., 2024; Toghyani et al., 2025), suggesting further investigation into efficacy in pre-weaned piglets is warranted.

Colostrum was used for comparison rather than mature sow milk due to the practical and ethical simplicity of colostrum sampling, as mentioned in Section 2.4. Mature milk requires oxytocin injection to the sow to stimulate milk let-down (Tao et al., 2010; Mudd et al., 2016), which, as a regulated procedure, requires home office approval (Animals (Scientific Procedures) Act, 1986), whereas colostrum can be freely collected by manual manipulation of the teat immediately prior to and during parturition.

Overall, the available evidence supports the view that colostrum is important in the establishment of the early-life microbial ecosystem (Le Dividich et al., 2005; Bailey et al., 2005; Devillers et al., 2011). The biological significance of colostrum lies partly in its timing as it provides PMO and other bioactive components at the point when the gut microbiome is first becoming established. Subsequent milk feeding then continues to shape this developing ecosystem through a changing supply of milk glycans and other substrates (Everaert et al., 2017; Liu et al., 2019; Trevisi et al., 2021; Song et al., 2026). The microbiome therefore represents a continuously developing system in which early colostrum exposure, continued milk feeding and later dietary transition are interconnected, rather than discrete events. Thus, the potential microbiome effects of early colostrum provision should be interpreted within the context of promoting early microbial colonisers, which play a key role in later life performance (Moeser et al., 2017; Li et al., 2018a; Li et al., 2019). Collection and comparison of mature sow milk to piglet milk replacer would be a logical progression for development of an optimal piglet milk replacer formula.

4.4.5 Conclusion

In conclusion, this study highlights the complexity of colostrum and the difficulty in synthetically simulating the matrix. The absence of differences between

treatments suggests that supplementation with selected PMO alone was insufficient to recapitulate the effects of whole colostrum, possibly due to the overwhelming effect of the highly fermentable piglet milk replacer substrate. The enrichment of Bacteroidota and *Bacteroides* alongside reductions in Bacillota and *Streptococcus* when fermenting colostrum suggest enhanced complex glycan degradation and potential cross-feeding interactions that promote butyrate production. In contrast, supplementation of piglet milk replacer with selected PMO did not replicate these compositional or functional effects, underscoring the importance of the colostrum matrix in shaping early-life microbial ecology.

Chapter 5 General Discussion and Conclusions

Milk oligosaccharides are structurally diverse glycans found in colostrum and milk of mammals at varying concentrations (Albrecht et al., 2014). In the immature gastrointestinal tract, these are resistant to foregut hydrolysis and pass into the hindgut to be fermented by resistant microbes; this characterises them as prebiotics (Gibson and Roberfroid, 1995; Gibson et al., 2017). Benefits of human milk oligosaccharides (HMO) are well-documented (reviewed by Bode (2012)), with functional effects including immunomodulation (Perdijk et al., 2019; Cheng, Kong, et al., 2020), proliferation of beneficial bacteria (Ward et al., 2006; Shen et al., 2011; Yu et al., 2013), and reduced adhesion of pathogens (Wang et al., 2020; Piotrowski et al., 2022). Given the physiological similarities between humans and pigs (Guilloteau et al., 2010; Odle et al., 2014), it is logical to explore the use of HMO/porcine milk oligosaccharides (PMO) as a prebiotic supplement in a similar fashion to the success seen in human infant studies.

It is of particular importance to explore nutritional interventions in the current pig production industry due to modern, hyper-prolific sow lines. These sows can produce a supernumerary litter, with piglets born alive outnumbering the functional teats on the sow (Rohrer and Nonneman, 2017; Icely et al., 2026), resulting in inadequate provision of nutrients to all piglets. This is exacerbated by increased number of low birth weight (LBW) piglets in large litters (Rutherford et al., 2013; Ward et al., 2020), who suffer reduced fitness and are most at risk of fatal casualty in early life. The industry has therefore seen an unfortunate rise in pre-wean mortality rate, associated with the increased number of piglets born (Canario, Cantoni, et al., 2006; Canario, Roy, et al., 2006; Rosendo et al., 2007), and this needs to be addressed for sustainability, profit-margins, and pig welfare. Feeding a supplementary milk replacer has some benefits (Kobek-Kjeldager et al., 2020; Icely et al., 2023), but generally livestock formula is lacking in prebiotic activity (Pieper et al., 2016; Icely, 2024) and piglet milk replacer may benefit from supplementary PMO, specifically for supporting gut health in the neonate and reduce mortality.

This thesis investigated the potential of PMO as a prebiotic by characterising which PMO occur naturally and at what concentration, whether there are associations between PMO and litter performance, and if PMO added

to piglet milk replacer would better support a healthy gut microbiota and associated metabolites in a novel *in vitro* fermentation model (Figure 3.25).

5.1 Main Findings

5.1.1 *In Vitro* Fermentation Model Development

Through review of existing literature, PMO were identified as potential prebiotic additives to support the reductive milk replacer currently available for pre-wean piglets. Determining how to assess PMO efficacy in the pre-wean piglet led to investigation of *in vitro* models to avoid low-throughput and ethically challenging *in vivo* trials. It was concluded that batch fermentation facilitates a cost-effective and high-throughput design, but experimental parameters varied greatly between studies. This variation, in conjunction with limited publications on the pre-wean piglet, prompted the systematic development of a novel protocol which is accessible and repeatable. It was concluded that alterations made to the inoculum and/or the substrate elicited the most significant changes, once using a suitable buffer. In addition to biological relevance, methodological robustness was considered. Particularly the need for precise and consistent handling at fermentation termination and sample processing. An approach of 12 or 14 hour incubation periods, while seeming optimal for avoiding substrate depletion, will result in extended hours of labour and may not be experimentally practical nor support rigor and reproducibility of the method. Lack of significant difference in microbiota between 12 and 24 hours concluded that a 24 hour incubation be the most practical (Figure 3.9 and Figure 3.10). The significant substrate-driven shifts should be strongly considered if negating a foregut simulation prior to fermentation (as done by many (Smiricky-Tjardes et al., 2003; Vester Boler et al., 2009a; Li et al., 2012; Nissen et al., 2022)). While the development of an *in vitro* model of the pre-wean piglet colon using batch fermentation does not represent direct biological findings, it was essential for enabling the functional evaluation of PMO as described in Chapter 4.

5.1.2 Variation in Porcine Milk Oligosaccharides

Concentrations of PMO identified in colostrum of modern sows were highly varied between individuals, particularly of compounds that were abundant across the sample population. Parity did not explain the variation seen, which is verified

in previous studies quantifying PMO (Wei et al., 2018; Trevisi et al., 2020), despite other compositional factors being dependant on parity (Nuntapaitoon et al., 2020; Amatucci et al., 2022). Although the sows used in this study were of similar genetic background and were maintained under standardised management conditions, individual genetic variation may still have contributed to differences in milk oligosaccharide profiles. The synthesis of milk oligosaccharides is dependent on the coordinated activity of multiple glycosyltransferases and other enzymes involved in glycan biosynthesis. Consequently, differences in the expression or activity of these enzymes between individuals could influence the types and relative abundance of oligosaccharides produced by the mammary gland. Blood group and secretor phenotype represent established examples of genetic variation affecting glycan synthesis in humans. Explanation of structural variation stems from blood type, with those expressing secretor genes able to produce fucosylated HMO, where non-secretors cannot (Newburg et al., 2004). Furthermore, numerous factors were not characterised, such as differences in metabolic status or presence of subclinical disease, which could affect colostrum composition. Therefore, the observed variation in oligosaccharide profiles may have reflected biological differences between sows that were not eliminated by the use of similar genetics and standardised management.

The high variation seen in PMO in Chapter 2, and complex interactions reported in HMO/PMO studies previously (Kavanaugh et al., 2013; Zehra et al., 2018; Kong et al., 2019) coincide with the large standard error (SE) values reported, particularly across SCFA production when fermenting PMO combinations. It was speculated that because LNnT was a constant in groups showing large SE values in Chapter 4, that this could be manifestation of synergy between various PMO. In general, the inability to significantly shift the microbiota (or other parameters) closer to that produced when fermenting colostrum is explained better when we understand how varied colostrum oligosaccharides are, both across and between sows. The novel *in vitro* model developed in this thesis could be used in future studies to investigate the synergistic interactions in more depth, potentially characterising effects that variation in PMO profile have on the gut microbiota, without the need for invasive *in vivo* work.

Due to the pig population used for sampling in this thesis, it is likely the conclusions are primarily translatable across similar UK indoor herds. The variation seen in PMO across the population of sows used here suggests great diversity in colostrum composition even in sows of close genetic make-up and identical environmental conditions. As none of our recorded parameters were associated with the variation seen in colostrum composition, it is difficult to speculate how an outdoor herd may differ, other than the likelihood of similar variation occurring. It is likely that sialylated PMO still dominate the oligosaccharide profiles seen in outdoor herds due to previous reports in a range of sow populations (Tao et al., 2010; Mudd et al., 2016; Salcedo et al., 2016; Wei et al., 2018; Trevisi et al., 2020). Work quantifying PMO in colostrum or milk of outdoor sows would need to be repeated to confirm any hypothesised similarities to indoor herds.

Many ‘unconfirmed’ structures were identified in Chapter 2, all of which appeared at relatively high concentrations. As discussed in that chapter, inconsistent and diverse methods make cross-comparison between studies and research groups very difficult. Based on similar abundance and reported mass, it is likely that at least two ‘unconfirmed’ structures have been found elsewhere (Tao et al., 2010; Salcedo et al., 2016; Wei et al., 2018) but knowledge and understanding of these is still minimal. Further development and application of complementary analytical techniques will be required to confirm the structures of these ‘unconfirmed’ oligosaccharides. In particular, purification of individual compounds followed by NMR spectroscopy, in combination with HPLC-MS/MS and comparison with authentic standards where available, could provide greater structural resolution and allow the linkage and configuration of these structures to be determined. Such approaches may help establish whether the structures tentatively identified in this study correspond to those previously reported in porcine milk. Regardless, the structures shared with humans, such as 2’FL, 3’S_L, 6’S_L and LNnT, will remain the most researched and therefore the safest to focus on for pre-wean piglet supplementation, particularly given their approval by food standards authorities already.

5.1.3 Porcine Milk Oligosaccharides and Piglet Performance

Previous studies have reported benefits of supplementing LNnT to piglets (Hester et al., 2013; Li et al., 2014) and the structure is strongly associated with

increasing *Bifidobacteria* proliferation (Cordeiro et al., 2023; Li et al., 2023), which has been shown to be positive for pre-wean piglet health (Hansen et al., 2022; Hansen et al., 2025). We identified that LNnT concentration decreased when litter size increased and speculated that this may be occurring due to energy trade-off in the sow. The metabolic requirement of producing a larger litter may require a higher energy input and this reduction of energy available may be characterised in lower synthesis of LNnT. Alternatively, the sow may prioritise synthesis and production of energy dense components such as lactose or critical immunoglobulins over PMO in colostrum. This supports hypotheses suggesting that litter proliferation has outpaced the sow's physiological capacity to provide adequate nutrients (Andersen et al., 2011; Andersson et al., 2016), which is one possible explanation for the increased mortality seen in large litters (Nowak et al., 2024). This further supports the notion of adding selected PMO to milk replacer formula, particularly in larger litters when natural concentrations of PMO may be compromised. When LNnT was fermented in the *in vitro* model, it exhibited the highest mean maximum gas volume, suggesting it ferments well with piglet faecal inoculum and therefore could be efficiently utilised by the colonic microbiota. These findings strengthen previous work identifying LNnT as a good candidate for supplementation to the pre-wean piglet for enhancing gut health.

5.1.4 Microbial Utilisation of Porcine Milk Oligosaccharides

The most abundant PMO identified in Chapter 2 was 3'SL which was expected based on previous studies reporting that sow milk is highly sialylated (Tao et al., 2010; Mudd et al., 2016; Salcedo et al., 2016; Wei et al., 2018; Trevisi et al., 2020). Interestingly, when fermenting 3'SL in my *in vitro* model, this was the compound able to achieve a more 'colostrum-like' microbiota than other PMO tested, specifically interpreting relative abundance of *Streptococcus* and *Bacteroides*. *Bacteroides* are a desirable species in the piglet gut due to their production of butyrate (Li, et al., 2023), and the beneficial immunomodulatory effects of butyrate such as upregulation of mucins (Gaudier et al., 2004; Hatayama et al., 2007), and proliferation of colonocytes (Donohoe et al., 2011), both reducing intestinal permeability (Knudsen et al., 2018). *Streptococcus*, as a lactose-utilising species, is found commensally in suckling piglets (Li et al., 2018b; Mu et al., 2019) due to the high quantity of lactose provided in sow milk. This more 'colostrum-like' microbiota produced when adding 3'SL to milk replacer

may be due to the starting microbiota being well-adapted to fermentation of 3'SL, as this was the most concentrated PMO found in sow colostrum in Chapter 2. If we assume concentration of 3'SL does not significantly decrease between colostrum and mature milk, as previously evidenced (Tao et al., 2009; Chen et al., 2018), then it is likely the faecal inoculum used *in vitro* was better adapted towards efficient utilisation of 3'SL. This is further supported in the *in vitro* model data in Chapter 4 by fermentation kinetics as 3'SL was one of few treatments that did not significantly differ to colostrum regarding maximum gas volume and growth rate asymptotes.

5.1.5 Nutritional Intervention Applications

Although no optimal combination of PMO has been identified yet, findings in this thesis suggest that PMO play a role in pre-wean health and performance, driven by the prebiotic effects. The evidence in existing literature supports biological efficacy, many structures have been approved for safe use (Zhu, Luo, et al., 2022), and PMO qualify to be added as a complementary feed material (EFSA, Regulation 767/2009). However, this does not necessarily translate into practical application within commercial pig production. The economic feasibility of a nutritional intervention is an important consideration, as producers must balance the cost of supplementation against measurable returns, for example, reduced mortality, improved growth, reduced disease incidence/ medication costs, or improved feed efficiency. This distinction is particularly relevant for milk oligosaccharides, for which production at commercially relevant scale remains technically demanding and comparatively costly.

The PMO structures investigated in this thesis are analogous to HMO, and due to their structural complexity, manufacturing HMO can involve sophisticated chemical, enzymatic or microbial production systems together with substantial downstream purification requirements (Bode et al., 2016; Palur et al., 2023). Although microbial fermentation has improved the scalability and economic feasibility of HMO production (Liu et al., 2025), purification and process scale-up remain important manufacturing challenges (Bode et al., 2016; Urashima et al., 2025). Thus, the cost of producing purified oligosaccharides at a scale and price suitable for animal-feed applications may represent a greater barrier to adoption than demonstrating biological efficacy alone. Importantly, a robust commercial cost cannot be inferred from the prices of research-grade HMOs, as used in

Chapter 4. Consequently, the present study cannot determine whether the supplementation strategy would be economically viable under commercial production conditions because a verified price for feed-grade HMO at the required scale was not available. Rather than representing a limitation of biological efficacy, this highlights the need for future studies to evaluate the cost-effectiveness of HMO supplementation using commercially available material and realistic production-scale pricing.

One of the obstacles to translation of this work to outdoor herds is the practicality of providing supplemental milk replacer to piglets outdoors. An alternative strategy to positively affect piglet gut health, may be in perinatal nutritional management. Le Bourgot *et al.* (2026) demonstrated modulation of the gut microbiota in pre-wean piglets when the sow had been given a short-chain FOS treatment from 28 days pre-partum, to 2 days post-partum. The beneficial effects of this perinatal treatment extended to reporting increased feed efficiency in piglets post-weaning, characterised by reduced feed conversion ratio (Le Bourgot *et al.*, 2026). Supplementing sow diets may offer a more cost- and labour- effective route to enhancing piglet health and performance, however it should be considered that concentrations will likely be increased for sow administration due to increased bodyweight and to accommodate absorption by the sow. In addition to assessment of piglet performance parameters, such as recorded in Chapter 2, the colostrum or milk produced by supplemented sows could be collected and fermented in the developed *in vitro* model in Chapter 3. This would provide timely data on the impact supplementing the sow with PMO has on the piglet gut microbiota without inducing piglet loss.

5.2 Strengths and Limitations

A major strength of this thesis is the integration of analytical characterisation with the subsequent investigation of PMO functionality. PMO present in the colostrum of hyper-prolific sows were characterised, including the identification of potentially novel structures, under strongly controlled sow management conditions that minimised environmental variation. Multiple analytical techniques were used to increase confidence in PMO identification and quantification, while statistical approaches accounted for hierarchical data and applied *post-hoc* corrections to reduce the risk of overestimating significance. Importantly, the mean PMO concentration established in this chapter was carried forward to inform

concentrations used in subsequent experiments, strengthening the biological relevance of the *in vitro* investigations. This provides a clear progression from characterising PMO abundance *in vivo* to investigating their potential biological effects.

To facilitate this investigation, a novel *in vitro* batch fermentation model was developed to mimic the colonic environment of the pre-wean piglet. The model generated complementary measures of fermentation kinetics, SCFA production and microbial community structure, with validation against *post-mortem* samples demonstrating preservation of key differences. The model therefore provided a controlled and accessible approach for investigating microbial community shifts without the ethical, logistical and financial constraints associated with *in vivo* trials. Its relative simplicity and lack of specialist equipment also provides opportunities to refine experimental conditions, including concentration screening, before progressing to animal studies, potentially reducing the number of animals required for these.

The final data chapter demonstrates the application of this model to address a biological question arising from the initial PMO characterisation. Fermentation of colostrum and commercial milk replacer revealed differences in microbial community structure, providing preliminary evidence that milk substrate can influence the developing pre-wean piglet microbiome. Applying the model to individual PMO structures further enabled their potential as stimbiotics to be explored without the ethical and financial burden of *in vivo* experimentation. Although further replication is required to establish the reliability and biological significance of these findings, the combined approach demonstrates a progression from PMO characterisation, through methodological development, to preliminary investigation of their functional potential. As this work was a singular batch *in vitro* model with a 24 hour incubation, it is limited to highlighting short-term effects. Running consecutive batches would provide a way of testing short-term delivery of PMO on long-term microbiota structure as the fermentation fluid can be collected and used to inoculate consecutive batches. For this, only the initial substrate would contain PMO treatment and subsequent substrate used in the consecutive batches would contain no PMO. Short-term supplementation of probiotics has been shown to elicit positive immunomodulation responses 22 days after cessation of treatment (Hansen et al., 2022). Short-term delivery by

Hansen et al. (2022) was characterised as every two days over a 4 week period, which, by comparison to my studies, is a prolonged administration. There was significant microbiome restructuring compared to the control group from day three, however, we cannot determine how immediate this effect was, as day three was the earliest sampling point.

As a preliminary study, the sample size used in Chapter 2 was modest, therefore, a larger repeat of the study may offer more statistical power and subsequent insight into PMO and litter performance associations previously uncaptured. It would be of particular interest to determine the significance of 2'FL concentration on pre-weaning mortality as this became insignificant *post-hoc*. Further to this, sampling mature sow milk may provide additional strength to the applicability of results as it is hypothesised that milk, being the main source of nutrition during the suckling period, will have greater effect on performance parameters than colostrum.

As suggested in Chapter 4, it is likely PMO need to be added in greater concentrations than have been here. This work has already begun using a continuous *in vitro* model based on work by Davis Birch et al. (2023), adapted for modelling the piglet weaning transition (Moura et al. *in prep*). The aim of this preliminary study is to determine if PMO supplemented media is more able to recover from a pathogen challenge than the control. If successful, the batch fermentation developed in Chapter 3 (Figure 3.25) could be of use to screen various concentrations prior to inclusion in the more labour- and time-intensive continuous model. This concentration screen is also recommended prior to designing any feeding trials on farm to maximise likelihood of effects.

5.3 Conclusions and Implications

Quantification of PMO in colostrum confirmed the reduction in nutrient provision associated with increase litter size, as evidenced in LNnT concentration. The potential for 2'FL to be associated with pre-wean mortality requires further exploration with a larger population, but given its integral role in infant gut health, is concluded to be a candidate for inclusion in milk replacer, along with LNnT. Finally, as the most abundant structure, and most promising PMO after fermentation, 3'SL is suggested as a prebiotic also. High pre-wean piglet mortality and increased numbers of LBW piglets requires intervention, either at the individual level by targeting vulnerable piglets, or at the litter level. It is concluded that PMO be considered for addition as a prebiotic, or stimbiotic, to supplemental milk to increase piglet robustness and vitality in the suckling period and increase likelihood of successful weaning.

This thesis identified PMO in sow colostrum and highlighted the variation in concentrations from a modern sow population. A novel batch fermentation protocol was developed to mimic the pre-wean piglet hindgut and enable testing of PMO effects on microbiota and metabolites *in vitro*. Using this model, I showed how different PMO impact fermentation and microbial parameters and highlighted how challenging replication of the colostrum matrix may be. In conclusion, there are some PMO which are good targets for inclusion as an additive to supplemental milk to support gut health and piglet performance.

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Appendix A

A random data set was used to confirm the hypothesis that gilts produce smaller litters. This was historic data from the National Pig Centre as provided for use to practice data analysis methods. The sows were farrowing from December 2022 to March 2023 and included parities 1-6, the distribution of the population is displayed in Table 5.1. A total of 203 sows were in the sample and this was based randomly on which sows had their parity available on the computerised system.

Table 5.1 Distribution of sow parity in sample population

Parity	Number of Sows
1	35
2	36
3	24
4	49
5	57
6	2

Parities 2-6 were grouped as 'multiparous' and parity 1 sows as 'gilts'. An independent samples t-test was conducted following confirmation that litter size and number of piglets born alive were normally distributed. This resulted in confirmation that litter size is significantly smaller in the first parity ($t = -4.43$; $df = 55.2$; $p < 0.001$), with gilts producing a mean number of 15.5 piglets, and multiparous sows producing 18.1 piglets. Number born alive was also tested and found significant ($t = -3.79$; $df = 47.7$; $p < 0.001$) with an average of 14.5 piglets born alive by gilts, and 16.9 by sows.

Appendix B

Table 5.2 Porcine milk oligosaccharide concentrations (g/L) per sow

SowID	Concentration, g/L											Total
	2'FL	3'GL	GSL 1	GSL 2	3'SL	6'SL	S-LN-Tri	LNnT	LSTc	LNnH	SLNnH	
220	0.014	0.075	0.472	0.172	1.332	0.066	0.062	0.042	0.013	0.013	0.018	2.279
310	0.000	0.203	0.673	0.310	2.176	0.067	0.776	0.033	0.016	0.031	0.032	4.318
311	0.008	0.098	1.063	0.213	1.663	0.089	0.286	0.023	0.008	0.021	0.034	3.505
551	0.003	0.026	0.239	0.056	0.413	0.017	0.047	0.014	0.000	0.007	0.005	0.827
552	0.009	0.084	0.196	0.169	1.108	0.044	0.232	0.027	0.006	0.013	0.010	1.898
560	0.003	0.151	0.191	0.279	1.265	0.067	0.979	0.038	0.016	0.041	0.045	3.075
574	0.003	0.109	0.350	0.258	1.184	0.063	0.082	0.040	0.004	0.041	0.038	2.172
576	0.006	0.097	0.086	0.212	0.622	0.065	1.279	0.038	0.022	0.034	0.047	2.509
593	0.009	0.220	0.140	0.345	2.268	0.067	0.326	0.035	0.007	0.048	0.053	3.518
636	0.003	0.069	0.208	0.141	1.165	0.036	0.111	0.037	0.008	0.021	0.018	1.815
642	0.003	0.172	0.122	0.287	1.248	0.061	1.059	0.064	0.027	0.061	0.081	3.185
644	0.005	0.208	0.084	0.343	2.333	0.059	0.076	0.034	0.006	0.025	0.026	3.198
719	0.010	0.073	0.432	0.223	2.029	0.061	0.132	0.025	0.012	0.024	0.023	3.045
743	0.003	0.048	0.239	0.182	0.919	0.079	0.099	0.060	0.015	0.020	0.026	1.690
759	0.019	0.143	0.637	0.304	1.212	0.057	0.917	0.039	0.020	0.023	0.029	3.401
761	0.093	0.065	0.052	0.320	0.976	0.069	0.009	0.012	0.010	0.036	0.022	1.663
765	0.013	0.087	0.141	0.173	1.069	0.048	0.036	0.028	0.006	0.011	0.010	1.622
855	0.000	0.078	0.265	0.146	0.974	0.035	0.580	0.012	0.000	0.016	0.015	2.120
858	0.022	0.119	0.215	0.255	1.163	0.044	0.196	0.072	0.018	0.028	0.031	2.162
860	0.000	0.051	0.111	0.136	0.608	0.036	0.289	0.037	0.014	0.018	0.018	1.317
862	0.005	0.076	0.265	0.203	0.769	0.048	0.043	0.036	0.006	0.017	0.016	1.484
873	0.000	0.147	0.158	0.312	0.924	0.060	0.077	0.037	0.015	0.028	0.022	1.781
876	0.000	0.152	0.090	0.262	2.144	0.072	0.050	0.048	0.008	0.042	0.040	2.909

SowID	Concentration, g/L											Total
	2'FL	3'GL	GSL 1	GSL 2	3'SL	6'SL	S-LN-Tri	LNnT	LSTc	LNnH	SLNnH	
908	0.007	0.042	0.134	0.124	0.908	0.052	0.008	0.033	0.008	0.012	0.012	1.339
916	0.000	0.186	0.192	0.293	1.271	0.062	0.106	0.041	0.011	0.029	0.027	2.218
918	0.008	0.071	0.432	0.173	0.630	0.055	0.419	0.022	0.000	0.010	0.010	1.829
919	0.002	0.052	0.130	0.175	1.142	0.053	0.162	0.034	0.010	0.023	0.021	1.803
926	0.000	0.180	0.135	0.326	1.438	0.075	0.353	0.057	0.010	0.047	0.049	2.670
958	0.000	0.062	0.443	0.135	0.689	0.046	0.368	0.017	0.000	0.010	0.016	1.787
965	0.007	0.050	0.180	0.144	0.551	0.052	0.416	0.030	0.011	0.014	0.017	1.472
973	0.006	0.122	0.187	0.242	1.053	0.036	0.345	0.047	0.009	0.030	0.024	2.099
997	0.003	0.110	0.146	0.176	0.783	0.028	0.336	0.030	0.021	0.018	0.013	1.664
1017	0.010	0.164	0.164	0.301	0.718	0.042	0.600	0.045	0.019	0.040	0.034	2.136
1029	0.000	0.050	0.614	0.170	0.731	0.076	0.141	0.042	0.011	0.019	0.030	1.884
1070	0.003	0.035	0.133	0.081	0.333	0.015	0.217	0.018	0.000	0.010	0.008	0.852
1071	0.018	0.049	0.218	0.167	0.719	0.036	0.068	0.052	0.009	0.012	0.011	1.358
1073	0.011	0.041	0.506	0.134	0.941	0.040	0.065	0.045	0.012	0.015	0.019	1.828
1076	0.020	0.144	0.082	0.240	1.713	0.041	0.562	0.045	0.018	0.031	0.034	2.931
1092	0.088	0.091	0.090	0.306	0.800	0.038	0.055	0.025	0.006	0.053	0.020	1.572
1096	0.007	0.183	0.309	0.289	2.015	0.091	0.044	0.058	0.034	0.028	0.045	3.100
1101	0.002	0.189	0.309	0.353	1.033	0.064	0.476	0.089	0.027	0.050	0.046	2.639
1164	0.020	0.039	0.419	0.121	1.156	0.029	0.090	0.045	0.010	0.012	0.016	1.956
1184	0.010	0.064	0.258	0.189	1.142	0.055	0.026	0.063	0.019	0.012	0.018	1.856
1191	0.013	0.057	0.266	0.169	1.118	0.035	0.036	0.043	0.008	0.014	0.017	1.775
1192	0.009	0.047	0.295	0.172	0.824	0.046	0.039	0.035	0.016	0.013	0.014	1.510

Table 5.3 Summary of univariate mixed-effects models testing associations between individual PMOs and piglet level performance variables

Performance variable	PMO ¹	Estimate	Test statistic	Raw P value	FDR ² adjusted P value
Piglet bodyweight at day one	2'FL	0.00771	0.262	0.795	0.795
	3'GL	-0.0405	-1.52	0.137	0.302
	3'SL	-0.0496	-1.83	0.0754	0.259
	6'SL	-0.0335	-1.22	0.230	0.421
	LNnH	-0.0457	-1.71	0.0943	0.259
	LNnT	-0.0087	-0.281	0.780	0.795
	LSTc	-0.0207	-0.697	0.490	0.673
	GL1	0.0146	0.511	0.612	0.748
	GL2	-0.046	-1.74	0.0891	0.259
	S-LN-Tri	-0.0281	-1.03	0.310	0.488
	SLNnH	-0.0459	-1.72	0.0932	0.259
Piglet bodyweight at day seven	2'FL	0.0511	1.17	0.250	0.714
	3'GL	0.0347	0.839	0.406	0.714
	3'SL	-0.0177	-0.413	0.681	0.833
	6'SL	-0.00252	-0.0600	0.953	0.953
	LNnH	0.0313	0.755	0.455	0.714
	LNnT	-0.0441	-0.951	0.347	0.714
	LSTc	0.028	0.623	0.537	0.738
	GL1	-0.0839	-2.03	0.0493	0.543
	GL2	0.0523	1.29	0.206	0.714
	S-LN-Tri	-0.0454	-1.10	0.279	0.714
	SLNnH	-0.0128	-0.306	0.761	0.838

Performance variable	PMO ¹	Estimate	Test statistic	Raw P value	FDR adjusted P value
Piglet bodyweight at day 28	2'FL	0.134	1.00	0.323	0.977
	3'GL	0.0261	0.204	0.839	0.977
	3'SL	-0.00381	-0.029	0.977	0.977
	6'SL	0.00709	0.055	0.956	0.977
	LNnH	0.0648	0.507	0.615	0.977
	LNnT	-0.224	-1.62	0.113	0.977
	LSTc	-0.00525	-0.038	0.970	0.977
	GL1	-0.159	-1.23	0.228	0.977
	GL2	0.0657	0.518	0.607	0.977
	S-LN-Tri	-0.061	-0.473	0.638	0.977
	SLNnH	0.0117	0.091	0.928	0.977

¹Porcine milk oligosaccharide: 2'-fucosyllactose (2'FL); 3'-galactosyllactose (3'GL); 3'-sialyllactose (3'SL); 6'-sialyllactose (6'SL); lacto-N-neohexaose (LNnH); lacto-N-neotetraose (LNnT); sialyllacto-N-neotetraose c (LSTc); galactosyllactose 1 (GL1); galactosyllactose 2 (GL2); sialyl-lacto-N-triose (S-LN-Tri); sialyl-lacto-N-neohexaose (SLNnH)

²False discovery rate using the Benjamini-Hochberg step-up procedure

Table 5.4 Summary of univariate mixed-effects models testing associations between individual PMOs and sow level performance variables

Performance variable	PMO ¹	Estimate	Test statistic	Adjusted R-squared	Raw P value	FDR ³ adjusted P value
Pre-weaning mortality ²	2'FL	-0.510	-1.99	0.332	0.0466	0.435
	3'GL	0.647	1.18	0.250	0.237	0.435
	3'SL	0.0666	0.102	0.238	0.919	0.919
	6'SL	2.18	0.650	0.235	0.516	0.634
	LNnH	0.615	1.06	0.244	0.288	0.452
	LNnT	-15.4	-1.28	0.299	0.202	0.435
	LSTc	-6.27	-0.359	0.244	0.719	0.791
	GL1	-0.661	-1.48	0.299	0.139	0.435
	GL2	0.0850	0.646	0.235	0.518	0.634
	S-LN-Tri	0.208	1.70	0.270	0.0899	0.435
	SLNnH	0.741	1.31	0.249	0.191	0.435
Birth litter size	2'FL	0.00281	0.127	-0.0229	0.899	0.932
	3'GL	-0.000927	-0.086	-0.0231	0.932	0.932
	3'SL	0.00473	0.573	-0.0155	0.570	0.776
	6'SL	-0.000874	-0.506	-0.0172	0.616	0.776
	LNnH	-0.00812	-0.797	-0.00835	0.430	0.776
	LNnT	-0.00163	-3.44	0.198	0.00130	0.0143
	LSTc	-0.000657	-2.08	0.0700	0.0438	0.241
	GL1	0.0154	1.26	0.0129	0.216	0.776
	GL2	-0.0362	-0.836	-0.00688	0.408	0.776
	S-LN-Tri	0.0216	0.478	-0.0179	0.635	0.776
	SLNnH	-0.00915	-0.853	-0.00622	0.398	0.776

Performance variable	PMO	Estimate	Test statistic	Adjusted R-squared	Raw P value	FDR adjusted P value
Parity	2'FL	-0.037	-0.861	-0.0059	0.394	0.619
	3'GL	0.0141	0.671	-0.0127	0.506	0.696
	3'SL	0.0187	1.17	0.00812	0.250	0.507
	6'SL	0.00533	1.61	0.035	0.115	0.507
	LNnH	-0.00204	-0.100	-0.023	0.921	0.921
	LNnT	-0.00147	-1.43	0.023	0.161	0.507
	LSTc	-0.000717	-1.12	0.00556	0.271	0.507
	GL1	0.0266	1.10	0.00485	0.277	0.507
	GL2	-0.0106	-0.120	-0.0229	0.902	0.921
	S-LN-Tri	0.105	1.20	0.00979	0.237	0.507
	SLNnH	0.00539	0.254	-0.0217	0.801	0.921

¹Porcine milk oligosaccharide: 2'-fucosyllactose (2'FL); 3'-galactosyllactose (3'GL); 3'-sialyllactose (3'SL); 6'-sialyllactose (6'SL); lacto-N-neohexaose (LNnH); lacto-N-neotetraose (LNnT); sialyllacto-N-neotetraose c (LSTc); galactosyllactose 1 (GL1); galactosyllactose 2 (GL2); sialyl-lacto-N-triose (S-LN-Tri); sialyl-lacto-N-neohexaose (SLNnH)

²Adjusted R-squared for pre-weaning mortality are conditional pseudo-R-squared values following Nakagawa and Schielzeth (2012)

³False discovery rate using the Benjamini-Hochberg step-up procedure

Appendix C

Ethical approval forms for relevant procedures.

Proposal

Staff and/or students of the University of Leeds planning to use animals for non-regulated scientific procedures in the UK

Section 1 Personal Details

Title and name	Mx Georgina Smith
Department	School of Biology
Ext	
E-mail	bsgos@leeds.ac.uk
Title of Project	Importance of milk oligosaccharides for performance in the pre-weaning piglet
Site where study to be undertaken	National Pig Centre, Spen Farm, LS24 9GF
Person/s who will be carrying out the work	Georgina Smith
Do you, or the person who will perform the study hold a personal licence under the Animals (Scientific Procedures) Act 1986?	NO Delete as appropriate
If so please give details	Applicant PIL number Researcher PIL Number

Section 2 Project Details

Description of work (include all intended manipulative procedures). Aim of project: To identify importance of milk oligosaccharides in the diet of pre-weaning piglets. Sows are selected and bred to produce large numbers of piglets in each litter as this maximises the profit made per sow and helps to meet increasing consumer demands for pork products. However, with litter size increasing, we see decreases in piglet birth weight and increased mortality rates, particularly during the first week of life^{1,2}. Colostrum is critical for neonates to consume within 24 hours of birth to provide initial energy supplies and passive immunity until their own immune system has developed. Colostrum contains high concentrations of many bioactive components, some of which are porcine milk oligosaccharides (PMOs)³. Milk oligosaccharides are complex bioactive sugar structures that have been shown to have immunomodulatory effects and benefit neonates with regards to health and performance by acting as a gut prebiotic⁴. Investigations into milk oligosaccharides in humans has led to them being a staple aspect of infant formula in order to minimize differences in health and mortality rates that were initially seen between formula-fed and breast-fed babies⁴. This project aims to explore the role of porcine milk oligosaccharides in the performance of the pre-wean piglet and will achieve this using the following objectives: analyse colostrum using mass spectrophotometry to ascertain oligosaccharide profile; use piglet faecal microbiome data and litter performance parameters to
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find any associations between oligosaccharide concentrations and litter health and performance; use an in vitro model of the piglet hindgut to ferment colostrum samples and identify 'gold standard' volatile fatty acid and microbial profiles and compare these findings to fermentation profiles of four most abundant oligosaccharides. This research hopes to identify the optimal ratios of milk oligosaccharides for improving the health and survival of pre-weaning piglets.

To achieve this, 2-3 mL of colostrum will be sampled from a range of functioning teats from a batch of sows (45-60 sows) within 24 hours of parturition. Power calculations using an effect size of 0.5 suggest a sample number of 27 (fermentation flasks) per treatment. Working to a 0.4-0.5 g input of dry matter (DM) per flask⁵ and a 21.9% DM content in colostrum⁶, this gives an approximate 60 mL colostrum needed including a 10% excess. Sows will be restrained in JYDEN freedom farrowing crates during the sampling (standard for sows at the farrowing stage) and 2-3 teats selected at random (colostrum quality has been shown to vary across teats⁷) to be gently manipulated to encourage secretion of colostrum. Samples will be pooled across all sows and collected directly into labelled 15ml falcon tubes to be frozen at -80°C until use. Per sow sampled from, 3-4 piglets from each litter will have faecal samples collected at day 7 when it is standard farm practice to weight piglets.

If relevant give details of animal housing and husbandry procedures which apply to your work. E.g. does the cage/pen space allocation (and other husbandry procedures) comply with the appropriate Home Office Code of Practice for the species and number of animals to be used?

No housing or husbandry practice will differ from usual management of farrowing sows at Spen Farm according to the Council Directive 2008/120/EC. Sows are moved into individual freedom farrowing pens (2.4 m²) one week prior to due date and locked into their crates ~24 hours prior to farrowing and released 2-3 days post-farrowing (varying on an individual basis at stockman's discretion). Sample collection will be taking place during the time the sows would be usually crated and does not require extension to the period they would otherwise be locked in for. All practices abide by the DEFRA Code of Practice for the Welfare of Pigs (2020) and Welfare of Farmed Animals (England) Regulations (2007). At day 7 piglets will be weighed as per standard farm practice and will coincide with collecting of faecal samples to limit unnecessary handling.

If work is to be done in the wild have you considered other legislation/requirements that will apply. Please give details.

N/A

What are the possible adverse effects on the animals and how will these be controlled?

Sows exhibiting discomfort (beyond what is expected as normal prior to parturition) or indicating hypersensitivity to the teat manipulation will be excluded from sampling. Approximate number of sows to be used to gather the volume required allows for some sows to be excluded without needing to recruit more. No adverse effects on the sow are expected as sampling will be opportunistic while sows are already crated and lactating. Taking colostrum from the sow will

result in less availability of this for piglets which could adversely affect their health so considerations have been made regarding parity selection and volume taken per sow based on previous literature. Primiparous sows have been suggested to produce less colostrum than multiparous sows^{8, 9, 10} and therefore will not be used. Parities 2+ produce sufficient colostrum to sample¹¹ and additionally, other studies have sampled ~15 ml of colostrum per sow^{11, 12} successfully therefore 2-3 ml at a significantly lower volume should not cause issue.

Addressing the 3 R's:

- Replacing the sows/colostrum is not a viable option due to this research relying on porcine colostrum analysis.
- Reducing the volume of colostrum taken has been considered by increasing number of sows to be used as the action of sampling will have no/minimal impact on the sow herself while reduction in volume taken will omit/minimise any consequences to the litter.
- Method of sampling has been refined to opportunistic sampling whereby no changes are made to the sow/piglets or environment in order to get the colostrum/faecal samples

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12. Davis HE, Jagger S, Toplis P, Miller HM. Feeding β -hydroxy β -methyl butyrate to sows in late gestation improves litter and piglet performance to weaning and colostrum immunoglobulin concentrations. *Animal Feed Science and Technology*. 2021 May 1;275:114889.

Have you read the Guidance on the operation of the Animals (Scientific Procedures) Act?	YES Delete as appropriate
Has confirmation been obtained from the Home Office that the above protocol, as described, would not be considered to include any regulated procedure that would require it to be licensed under the Animals (Scientific Procedures) Act 1986.	NO Delete as appropriate
Date confirmation received from the Home Office	
Name of individual from whom has confirmation been received	
Signed	
Date	

Section 3

The Named Veterinary Surgeon (NVS) has considered the proposal

entitled: Importance of milk oligosaccharides for performance in the pre-weaning piglet
submitted by: Georgina
Smith.....

and accepts that, as written, it describes work which is not regulated under the Animals (Scientific Procedures) Act 1986 (as amended).

This work may commence subject to the understanding that:

1. Before any further changes are made to the protocol as written such changes must, in the first instance, be discussed with the Named Animal Care and Welfare Officer (NACWO) and NVS.
2. The NVS will require the work to be stopped should it become known that changes have been made to the protocol without prior discussion.
3. Any adverse signs shown by any animal on the study, irrespective of whether the study is considered to be or not to be the cause, must be reported to the NACWO and NVS.
4. No procedures other than husbandry procedures normal for the species may be applied.

Signed  Date 03/10/23.....
Named Veterinary Surgeon

Signed  Date ..19/05/23.....
Applicant

Proposal

Staff and/or students of the University of Leeds planning to use animals for non-regulated scientific procedures in the UK

Section 1 Personal Details

Title and name	Mx Georgina Smith
Department	School of Biology
Ext	
E-mail	bsgos@leeds.ac.uk
Title of Project	Importance of milk oligosaccharides for performance in the pre-weaning piglet
Site where study to be undertaken	National Pig Centre, Spen Farm, LS24 9GF
Person/s who will be carrying out the work	Georgina Smith
Do you, or the person who will perform the study hold a personal licence under the Animals (Scientific Procedures) Act 1986?	NO Delete as appropriate
If so please give details	Applicant PIL number Researcher PIL Number

Section 2 Project Details

Description of work (include all intended manipulative procedures). Aim of project: To identify importance of milk oligosaccharides in the diet of pre-weaning piglets. Sows are selected and bred to produce large numbers of piglets in each litter as this maximises the profit made per sow and helps to meet increasing consumer demands for pork products. However, with litter size increasing, we see decreases in piglet birth weight and increased mortality rates, particularly during the first week of life^{1,2}. Colostrum is critical for neonates to consume within 24 hours of birth to provide initial energy supplies and passive immunity until their own immune system has developed. Colostrum contains high concentrations of many bioactive components, some of which are porcine milk oligosaccharides (PMOs)³. Milk oligosaccharides are complex bioactive sugar structures that have been shown to have immunomodulatory effects and benefit neonates with regards to health and performance by acting as a gut prebiotic⁴. Investigations into milk oligosaccharides in humans has led to them being a staple aspect of infant formula in order to minimize differences in health and mortality rates that were initially seen between formula-fed and breast-fed babies⁴. This project aims to explore the role of porcine milk oligosaccharides in the performance of the pre-wean piglet and will achieve this using the following objectives: analyse colostrum using mass spectrophotometry to ascertain oligosaccharide profile; use piglet faecal microbiome data and litter performance parameters to
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find any associations between oligosaccharide concentrations and litter health and performance; use an in vitro model of the piglet foregut and hindgut to ferment colostrum samples and identify 'gold standard' volatile fatty acid and microbial profiles and compare these findings to fermentation profiles of four most abundant oligosaccharides. This research hopes to identify the optimal ratios of milk oligosaccharides for improving the health and survival of pre-weaning piglets.

To achieve this, 10 mL of colostrum per sow will be sampled from a range of functioning teats from a batch of sows (45-60 sows) within 24 hours of parturition. Working to a 5 mL input for foregut digestion¹³ to recover 0.4-0.5 g input of dry matter (DM) per hindgut fermentation flask⁵, this gives an approximate 100 mL colostrum needed per batch of experiments including a 10% excess. This volume will allow for preliminary experiments to be undertaken before reviewing further requirements. Sows will be restrained in JYDEN freedom farrowing crates during the sampling (standard for sows at the farrowing stage) and 2-3 teats selected at random (colostrum quality has been shown to vary across teats⁷) to be gently manipulated to encourage secretion of colostrum. Samples will be split with some pooled across all sows and some retained as individual samples. All will be transported on ice to be frozen at -80°C until use. Per sow sampled from, 3-4 piglets from each litter will have faecal samples collected at day 7 when it is standard farm practice to weight piglets.

If relevant give details of animal housing and husbandry procedures which apply to your work. E.g. does the cage/pen space allocation (and other husbandry procedures) comply with the appropriate Home Office Code of Practice for the species and number of animals to be used?

No housing or husbandry practice will differ from usual management of farrowing sows at Spen Farm according to the Council Directive 2008/120/EC. Sows are moved into individual freedom farrowing pens (2.4 m²) one week prior to due date and locked into their crates ~24 hours prior to farrowing and released 2-3 days post-farrowing (varying on an individual basis at stockman's discretion). Sample collection will be taking place during the time the sows would be usually crated and does not require extension to the period they would otherwise be locked in for. All practices abide by the DEFRA Code of Practice for the Welfare of Pigs (2020) and Welfare of Farmed Animals (England) Regulations (2007). At day 7 piglets will be weighed as per standard farm practice and will coincide with collecting of faecal samples to limit unnecessary handling.

If work is to be done in the wild have you considered other legislation/requirements that will apply. Please give details.

N/A

What are the possible adverse effects on the animals and how will these be controlled?

Sows exhibiting discomfort (beyond what is expected as normal prior to parturition) or indicating hypersensitivity to the teat manipulation will be excluded from sampling. Approximate number of sows to be used to gather the volume required allows for some sows to be excluded without

needing to recruit more. No adverse effects on the sow are expected as sampling will be opportunistic while sows are already crated and lactating. Taking colostrum from the sow will result in less availability of this for piglets which could adversely affect their health so considerations have been made regarding parity selection and volume taken per sow based on previous literature. Primiparous sows have been suggested to produce less colostrum than multiparous sows⁸⁻¹⁰ and therefore will not be used. Parities 2+ produce sufficient colostrum to sample¹¹ and additionally, other studies have sampled 15-40 mL of colostrum per sow^{6, 11, 12} successfully therefore 10 mL should not cause issue.

Addressing the 3 R's:

- Replacing the sows/colostrum is not a viable option due to this research relying on porcine colostrum analysis.
- Reducing the volume of colostrum taken has been considered by increasing number of sows to be used as the action of sampling will have no/minimal impact on the sow herself while reduction in volume taken per sow will omit/minimise any consequences to the litter.
- Method of sampling has been refined to opportunistic sampling whereby no changes are made to the sow/piglets or environment in order to get the colostrum/faecal samples

References:

1. Canario L, Roy N, Gruand J, Bidanel JP. Genetic variation of farrowing kinetics traits and their relationships with litter size and perinatal mortality in French Large White sows. *Journal of Animal Science*. 2006 May 1;84(5):1053–8.
2. Koketsu Y, Iida R, Piñeiro C. A 10-year trend in piglet pre-weaning mortality in breeding herds associated with sow herd size and number of piglets born alive. *Porcine Health Management*. 2021 Dec 1;7(1):1–8.
3. Salcedo J, Frese SA, Mills DA, Barile D. Characterization of porcine milk oligosaccharides during early lactation and their relation to the fecal microbiome. *Journal of dairy science*. 2016 Oct 1;99(10):7733–43.
4. Walsh C, Lane JA, van Sinderen D, Hickey RM. Human milk oligosaccharides: Shaping the infant gut microbiota and supporting health. *Journal of Functional Foods*. 2020 Sep 1;72:104074.
5. Palowski A, Yang Z, Jang J, Dado T, Urriola PE, Shurson GC. Determination of in vitro dry matter, protein, and fiber digestibility and fermentability of novel corn coproducts for swine and ruminants. *Translational Animal Science*. 2021 Apr;5(2):txab055.
6. Lin C, Mahan DC, Wu G, Kim SW. Protein digestibility of porcine colostrum by neonatal pigs. *Livestock Science*. 2009 Apr 1;121(2-3):182–6.
7. Ogawa S, Tsukahara T, Tsuruta T, Nishibayashi R, Okutani M, Nakatani M, et al. The evaluation of secretion volume and immunoglobulin A and G concentrations in sow colostrum from anterior to posterior teats. *Animal Science Journal*. 2014;85(6):678–82.
8. Theil PK, Nielsen MO, Sørensen MT, Lauridsen C. Lactation, milk and suckling. *Nutritional physiology of pigs*. Danish Pig Research Centre, Copenhagen, Denmark. 2012:1–47.

9. King RH. Factors that influence milk production in well-fed sows. *Journal of Animal Science*. 2000 Jan 1;78(suppl_3):19-25.
10. Beyer M, Jentsch W, Kuhla S, Wittenburg H, Kreienbring F, Scholze H, Rudolph PE, Metges CC. Effects of dietary energy intake during gestation and lactation on milk yield and composition of first, second and fourth parity sows. *Archives of animal nutrition*. 2007 Dec 1;61(6):452-68.
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Date confirmation received from the Home Office	
Name of individual from whom has confirmation been received	
Signed	
Date	

Section 3

The Named Veterinary Surgeon (NVS) has considered the proposal

entitled:

Importance of milk oligosaccharides for performance in the pre-weaning piglet

submitted by: G Smith

and accepts that, as written, it describes work which is not regulated under the Animals (Scientific Procedures) Act 1986 (as amended).

This work may commence subject to the understanding that:

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2. The NVS will require the work to be stopped should it become known that changes have been made to the protocol without prior discussion.
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4. No procedures other than husbandry procedures normal for the species may be applied.

Signed  Date 19/4/2024
Named Veterinary Surgeon

Signed  Date ..18/04/24.....
Applicant

Proposal

Staff and/or students of the University of Leeds planning to use animals for non-regulated scientific procedures in the UK

Section 1 Personal Details

Title and name	Mx Georgina Smith
Department	School of Biology
Ext	
E-mail	bsgos@leeds.ac.uk
Title of Project	Importance of milk oligosaccharides for performance in the pre-weaning piglet
Site where study to be undertaken	National Pig Centre, Spen Farm, LS24 9GF
Person/s who will be carrying out the work	Georgina Smith
Do you, or the person who will perform the study hold a personal licence under the Animals (Scientific Procedures) Act 1986?	NO Delete as appropriate
If so please give details	Applicant PIL number Researcher PIL Number

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Using an in vitro model is a more accessible and higher throughput method than in vivo to assess the effects of oligosaccharides, however for the model to be valid and reliable it must accurately reflect the hindgut environment of a pre-weaning piglet.

Three healthy piglets aged between 1 and 3 weeks of age are required to be sacrificed to validate the in vitro model data. Due to the immaturity and diversity of the gut in the pre-weaning period, three piglets will provide more reliable conclusions than using only one while still limiting the number as much as possible.

Piglets will be selected at random when meeting the following criteria: not on any existing research trials, no non-standard medication administered since birth, within normal weight boundaries for age, no current signs or history of disease.

Piglets will be removed from the pen and transported to the on-site lab facilities according to the standard operating procedures (7.03: Dissections, National Pig Centre, 2023). Piglets will be stunned using a captive-bolt gun and exsanguinated according to Schedule 1 Animals (Scientific Procedure) Act 1986. The gastrointestinal tract will be dissected out and samples taken (digesta and tissue) from the proximal, middle, and distal colon.

Consideration was given to sampling from piglets found already dead, however, there is concern in identifying if the piglets were healthy before death. If unhealthy piglets are sampled from to validate the in vitro work, it will compromise the integrity of the model. The cause of death least likely to be associated with an existing disease would be fatal crushing injury from the sow, however, this could compromise the intestinal integrity and microbiome through the injury and subsequent contamination. Additionally, the more vulnerable (possibly diseased or low birth weight) piglets have a higher chance of being laid on. Another concern is microbiota change after death. There is not a large breadth of literature published on how microbial communities are impacted post-mortem so it is possible that samples from found-dead piglets may not be a true reflection of the live piglet colon. The studies read⁷ take samples over days rather than hours and do indeed find significant differences in microbial diversity but it could be argued there might not be significance if the pig is fresher than 24 hours post-mortem. Regardless, for this study to be reliable, any chance for inaccuracy must be eliminated as this in vitro colon model will reduce the need for piglet sacrifice in the future.

Consideration was also given to sharing of resource however no piglets are being culled for other studies at the NPC in the near future nor are there any standard farm practices that would allow advantage to be taken of ready culled piglets. Tissue will be banked or shared with other projects where appropriate to maximise use of each piglet.

If relevant give details of animal housing and husbandry procedures which apply to your work. E.g. does the cage/pen space allocation (and other husbandry procedures) comply with the appropriate Home Office Code of Practice for the species and number of animals to be used?

The piglets will be taken from the farrowing houses at the National Pig Centre, University of Leeds. Piglets are kept in 2.4m x 2.4m farrowing pens with the sow until 4 weeks of age.

If work is to be done in the wild have you considered other legislation/requirements that will apply. Please give details.

N/A

What are the possible adverse effects on the animals and how will these be controlled?

N/A

References:

1. Canario L, Roy N, Gruand J, Bidanel JP. Genetic variation of farrowing kinetics traits and their relationships with litter size and perinatal mortality in French Large White sows. *Journal of Animal Science*. 2006 May 1;84(5):1053–8.
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Have you read the [Guidance on the operation of the Animals \(Scientific Procedures\) Act?](#)

YES

Delete as appropriate

Has confirmation been obtained from the Home Office that the above protocol, as described, would

NO

Delete as appropriate

not be considered to include any regulated procedure that would require it to be licensed under the Animals (Scientific Procedures) Act 1986.

Date confirmation received from the Home Office

Name of individual from whom has confirmation been received

Signed

Date

Section 3

The Named Veterinary Surgeon (NVS) has considered the proposal

entitled:

.....

..... Importance of milk oligosaccharides for performance in the pre-weaning piglet

G. Smith submitted by:

.....

.....

and accepts that, as written, it describes work which is not regulated under the Animals (Scientific Procedures) Act 1986 (as amended).

This work may commence subject to the understanding that:

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Signed  Date 22/04/2024

Named Veterinary Surgeon

Signed  Date 22/04/23

Applicant