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Promoting Intestinal Health and Growth Performance in Slow-Growing Pigs: Early Nutritional and Management Interventions

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Promoting Intestinal Health and Growth Performance in Slow-Growing Pigs: Early Nutritional and Management Interventions

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El Dr. José Francisco Pérez Hernández, catedràtic del departament de Ciència Animal i dels Aliments de la Facultat de Veterinària de la Universitat Autònoma de Barcelona,

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que el treball de recerca i la redacció de la memòria de la tesi doctoral titulada "Promoting Intestinal Health and Growth Performance in Slow-Growing Pigs: Early Nutritional and Management Interventions" han estat realitzats sota la seva direcció per en

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i perquè quedi constància, signa aquest document a Bellaterra,
a 18 de febrer del 2022.

Dr. José Francisco Pérez Hernández

Aquesta tesi reuneix el resultats dels estudis realitzats durant el meu doctorat que va començar el setembre del 2018 fins al juny del 2023 al Servei de Nutrició i Benestar Animal del Departament de Ciència Animal i dels Aliments de la Universitat Autònoma de Barcelona, i va comptar amb el suport financer d'una beca de Formació de Professorat Universitari (FPU18/00401) del Ministeri de Ciència, Innovació i Universitats del Govern d'Espanya.

Els estudis realitzats han format part de dos projectes RETOS que han rebut finançament del Ministeri de Economia, Industria i Competitivitat:

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Des d'abril fins a agost de 2021, vaig realitzar una estada doctoral al Teagasc Pig Development Department del Moorepark Animal and Grassland Research and Innovation Centre a Irlanda, sota la supervisió del Dr. Edgar García Manzanilla. Durant aquesta estada, vaig dur a terme un estudi que ha resultat un manuscrit descrit en el Capítol 5 d'aquesta tesi.

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Un cop tancat aquest capítol de la meva vida, arriba el moment de mirar enrere i recordar aquelles persones que m'han acompanyat en aquest camí i han contribuït a fer realitat aquesta tesi.

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Aquesta tesi és per a tots vatros.

Summary

The presence of slow-growing pigs in a batch results in an increase in body weight (BW) variability, leading to negative economic implications for the porcine industry. Ensuring optimal early-life development of piglets and facilitating their adaptation to the post-weaning environment and diet are crucial factors in reversing the trend of slow-growing pigs. Therefore, the present thesis aimed at improving our understanding of the pig's early-life factors related to slow growth and investigating various early intervention strategies to improve intestinal microbial colonization, intestinal health, pig welfare, and growth performance after weaning.

The development of piglets is significantly influenced by both the gut microbiota and their individual response to stress during early life. In **Chapter 3**, we explored the connections between fecal microbiota characteristics, stress biomarkers in hair, and piglet growth performance during the lactation and nursery periods. Fast-growing pigs exhibited higher levels of cortisone in their hair and a lower cortisol-to-cortisone ratio at the end of the nursery period, indicating an increased 11β-HSD type 2 enzymatic activity. Additionally, piglet growth in both periods was associated with differences in gut microbiota and short-chain fatty acid (SCFA) concentration in feces at the end of the nursery period. Notably, the growth during lactation was associated with more pronounced differences, highlighting the critical influence of microbial colonization during this period on shaping the piglets' adult microbiota. Moreover, butyrate concentration showed a positive correlation with growth in both stages, suggesting that it may play a role in promoting piglet growth.

These findings were the basis for the hypothesis of **Chapter 4**, which aimed to examine the effects of the supplementation of low-dose supplementation (30 to 60 mg/day) of xylo-oligosaccharides (XOS) on suckling piglets. This intervention stimulated the development of fiber-degrading and SCFA-producing microbial populations during the lactation period and also in the nursery period. Nevertheless, it had no effect on growth performance.

Post-weaning gastrointestinal dysfunctions are primarily attributed to the stress response observed in pigs. As a measure to attenuate stress, in **Chapter 5**, we aimed to analyze the impact of keeping litters together after weaning, instead of mixing them, along with providing a low nutrient and energy density dietary regime (-10% energy, protein, and lysine) during the nursery period on their growth performance and welfare during the whole production cycle. Keeping litters together showed few effects on growth performance but it benefited pig's welfare when they were moved to the nursery and growing-finishing accommodation and when they were fed the low-density dietary regime. In addition, this strategy resulted in a similar BW variability within the pen than the pigs segregated by BW at the end of the nursery period except in pigs fed the low-density dietary regime. The low-density dietary regime caused a growth retardation that could not be fully compensated for during the growing-finishing period. It also exacerbated the competition for feed and the incidence of damaging behaviour when it was provided in a single-space feeder.

This thesis also evaluated strategies to facilitate the proper adaptation of the pig to the nursery diet and reduce the BW variability within the batch. In **Chapter 6**, we investigated the effect of partially substituting a high inclusion of soybean ingredients in the pre-starter diet with either spray-dried plasma (SDP), porcine digestible peptides (PDP), or a combination of both. These animal-sourced ingredients improved piglets' growth at the end of the pre-starter period. Additionally, the combination of PDP and SDP upregulated the expression of genes associated with intestinal function.

In **Chapter 7**, we evaluated the effect of providing piglets with a higher allowance of a pre-starter diet, enriched with SDP and PDP, until they reached a targeted BW, instead until a fixed date. However, in our experimental conditions, the higher allowance of pre-starter feed until pigs achieved 7.9 kg did not provide any benefit in growth performance or BW variability at the end of the nursery period.

Finally, in the general discussion, we deliberated the applicability of the studied intervention strategies in commercial farms, and we proposed future research directions based on our findings.

Resum

La presència de porcs de creixement lent en un lot es tradueix en un augment de la variabilitat del pes corporal (PC), la qual cosa comporta implicacions econòmiques negatives per a la indústria porcina. Garantir un desenvolupament òptim dels garrins en les primeres fases de la seva vida i facilitar-ne l'adaptació a l'entorn i la dieta després del deslletament són factors crítics per invertir la tendència dels porcs de creixement lent. D'aquesta manera, l'objectiu d'aquesta tesi és millorar la nostra comprensió dels factors relacionats amb el creixement lent a les primeres etapes de vida del porc i investigar diverses estratègies d'intervenció primerenques per millorar la colonització microbiana de l'intestí, la salut intestinal, el benestar del porc i el seu creixement després del deslletament.

El desenvolupament dels garrins està significativament influït tant per la microbiota intestinal com per la resposta individual a l'estrès durant les primeres etapes de vida. Al **Capítol 3**, explorem les connexions entre les característiques de la microbiota fecal, els biomarcadors d'estrès al pèl i el rendiment del creixement dels garrins durant els períodes de lactació i de transició. Els porcs de creixement ràpid van mostrar majors nivells de cortisona al pèl i una menor relació cortisol-cortisona al final del període de transició, la qual cosa indica una major activitat de l'enzim 11β-HSD tipus 2. A més, el creixement dels garrins en ambdós períodes es va associar a diferències en la microbiota intestinal i en la concentració d'àcids grassos de cadena curta (AGCC) a la femta al final del període de transició. Particularment, el creixement durant la lactància es va associar amb diferències més pronunciades, cosa que posa en relleu la influència de la colonització microbiana durant aquest període en la formació de la microbiota adulta dels garrins. A més, la concentració de butirat va mostrar una correlació positiva amb el creixement en les dues etapes, la qual cosa suggereix que pot tenir un paper en la promoció del creixement dels garrins.

Aquestes troballes van constituir la base de la hipòtesi del **Capítol 4**, l'objectiu del qual era examinar els efectes de la suplementació de dosis baixes (30 a 60 mg/dia) de XOS en garrins lactants. Aquesta intervenció va estimular el desenvolupament de poblacions

microbianes degradadores de fibra i productores de AGCC durant el període de lactació i també durant el període de lactància. Tot i això, no va tenir efectes sobre el creixement.

Les disfuncions gastrointestinals post-deslletament s'atribueixen principalment a la resposta d'estrès observada als porcs. Com a mesura per atenuar l'estrès, al **Capítol 5** ens vam proposar analitzar l'impacte de mantenir les ventrades juntes després del deslletament, en lloc de barrejar-les, juntament amb el subministrament d'un règim dietètic de baixa densitat de nutrients i energia (-10% de energia, proteïna i lisina) durant el període de transició. Es van avaluar els efectes sobre el creixement i el benestar durant tot el cicle de producció. Mantenir les ventrades juntes va tenir pocs efectes sobre el creixement, però va millorar el benestar dels porcs quan se'ls va traslladar a les sales de transició i de creixement-acabat i també quan se'ls va subministrar el règim dietètic de baixa densitat. A més, aquesta estratègia va donar lloc a una variabilitat del pes corporal dins del corral semblant a la dels porcs segregats per pes corporal, excepte en el cas dels porcs alimentats amb el règim dietètic de baixa densitat. Aquest règim alimentari va provocar un retard del creixement que no es va poder compensar totalment durant el període de creixement-acabat. També va augmentar la competència pel pinso i la incidència de comportaments perjudicials quan se subministrava en una menjadora d'una sola boca.

En aquesta tesi també es van avaluar estratègies per facilitar la correcta adaptació del porc a la dieta de transició i reduir la variabilitat del pes corporal dins el lot. Al **Capítol 6**, vam investigar l'efecte de substituir parcialment una alta inclusió d'ingredients de soja a la dieta “pre-starter” per SDP, PDP o una combinació de tots dos. Aquests ingredients d'origen animal van millorar el creixement dels garrins al final del període “pre-starter”. A més, la combinació de PDP i SDP va augmentar l’expressió de gens associats a la funció intestinal.

Al **Capítol 7**, vam avaluar l'efecte de subministrar als garrins una major quantitat de dieta “pre-starter”, que incloïa SDP i PDP, fins que arribessin a un pes corporal desitjat, en lloc de fins a una data fixa. Tot i això, en les nostres condicions experimentals, la major ració de pinso “pre-starter” fins que els porcs van arribar als 7,9 kg no va aportar cap benefici en el creixement ni en la variabilitat de pesos al final del període de transició.

Finalment, a la discussió general, vam deliberar sobre l'aplicabilitat de les estratègies d'intervenció estudiades a granges comercials, i vam proposar futures línies de recerca basades en les nostres troballes.

Resumen

La presencia de cerdos de crecimiento lento en un lote se traduce en un aumento de la variabilidad del peso corporal, lo que conlleva implicaciones económicas negativas para la industria porcina. Garantizar un desarrollo óptimo de los lechones en las primeras fases de su vida y facilitar su adaptación al entorno y a la dieta tras el destete son factores críticos para invertir la tendencia de los cerdos de crecimiento lento. De este modo, el objetivo de la presente tesis es mejorar nuestra comprensión de los factores relacionados con el crecimiento lento en las primeras etapas de vida del cerdo e investigar diversas estrategias de intervención tempranas para mejorar la colonización microbiana del intestino, la salud intestinal, el bienestar del cerdo y su crecimiento tras el destete.

El desarrollo de los lechones está significativamente influido tanto por la microbiota intestinal como por su respuesta individual al estrés durante sus primeras etapas de vida. En el **Capítulo 3**, exploramos las conexiones entre las características de la microbiota fecal, los biomarcadores de estrés en el pelo y el rendimiento del crecimiento de los lechones durante los periodos de lactación y de transición. Los cerdos de crecimiento rápido mostraron mayores niveles de cortisona en el pelo y una menor relación cortisol-cortisona al final del periodo de transición, lo que indica una mayor actividad de la enzima 11β-HSD tipo 2. Además, el crecimiento de los lechones en ambos periodos se asoció a diferencias en la microbiota intestinal y en la concentración de ácidos grasos de cadena corta (AGCC) en las heces al final del periodo de transición. Particularmente, el crecimiento durante la lactancia se asoció con diferencias más pronunciadas, lo que pone de relieve la influencia de la colonización microbiana durante este periodo en la formación de la microbiota adulta de los lechones. Además, la concentración de butirato mostró una correlación positiva con el crecimiento en ambas etapas, lo que sugiere que puede desempeñar un papel en la promoción del crecimiento de los lechones.

Estos hallazgos constituyeron la base de la hipótesis del **Capítulo 4**, cuyo objetivo era examinar los efectos de la suplementación de dosis bajas (30 a 60 mg/día) de XOS en lechones lactantes. Esta intervención estimuló el desarrollo de poblaciones microbianas

degradadoras de fibra y productoras de AGCC durante el periodo de lactación y también en el periodo de transición. Sin embargo, no tuvo efectos sobre el crecimiento.

Las disfunciones gastrointestinales post-destete se atribuyen principalmente a la respuesta de estrés observada en los cerdos. Como medida para atenuar el estrés, en el **Capítulo 5** nos propusimos analizar el impacto de mantener las camadas juntas tras el destete, en lugar de mezclarlas, junto con el suministro de un régimen dietético de baja densidad de nutrientes y energía (-10% de energía, proteína y lisina) durante el periodo de transición. Se evaluaron los efectos sobre su crecimiento y bienestar durante todo el ciclo de producción. Mantener las camadas juntas tuvo pocos efectos sobre el crecimiento, pero mejoró el bienestar de los cerdos cuando se les trasladó a las salas de transición y de crecimiento-acabado y cuando se les suministró el régimen dietético de baja densidad. Además, esta estrategia dio lugar a una variabilidad del peso corporal dentro del corral similar a la de los cerdos segregados por peso corporal, excepto en el caso de los cerdos alimentados con el régimen dietético de baja densidad. El régimen alimentario de baja densidad provocó un retraso del crecimiento que no pudo compensarse totalmente durante el periodo de crecimiento-acabado. También exacerbó la competencia por el pienso y la incidencia de comportamientos dañinos cuando se suministraba en un comedero de una sola boca.

En esta tesis también se evaluaron estrategias para facilitar la correcta adaptación del cerdo a la dieta de la transición y reducir la variabilidad del peso corporal dentro del lote. En el **Capítulo 6**, investigamos el efecto de sustituir parcialmente una alta inclusión de ingredientes de soja en la dieta “pre-starter” por SDP, PDP o una combinación de ambos. Estos ingredientes de origen animal mejoraron el crecimiento de los lechones al final del periodo “pre-starter”. Además, la combinación de PDP y SDP aumentó la expresión de genes asociados a la función intestinal.

En el **Capítulo 7**, evaluamos el efecto de suministrar a los lechones una mayor cantidad de dieta “pre-starter”, que incluía SDP y PDP, hasta que alcanzaran un peso corporal deseado, en lugar de hasta una fecha fija. Sin embargo, en nuestras condiciones experimentales, la mayor ración de pienso “pre-starter” hasta que los cerdos alcanzaron los

7,9 kg no aportó ningún beneficio en el crecimiento ni en la variabilidad de pesos al final del periodo de transición.

Finalmente, en la discusión general, deliberamos sobre la aplicabilidad de las estrategias de intervención estudiadas en granjas comerciales, y propusimos futuras líneas de investigación basadas en nuestros hallazgos.

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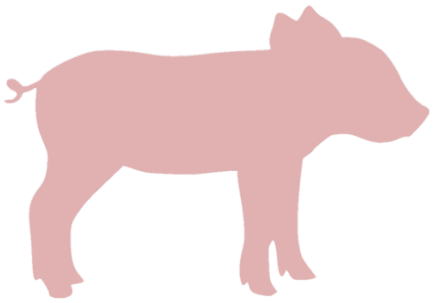
Abbreviations

ADFI	Average daily feed intake
ADG	Average daily gain
AGCC	"Àcids grassos de cadena curta"
ANOVA	Analysis of variance
ASF	African swine fever
ASV	Amplicon sequence variant
BCFA	Branched-chain fatty acids
BL	Body lesions
BW	Body weight
CF	Crude fiber
CP	Crude protein
CV	Co-efficient of variation
E. coli	*Escherichia coli*
EC	Enzyme commission
EU	European Union
FCR	Feed conversion ratio
FDR	False discovery rate
FOS	Fructo-oligosaccharides
G:F	Feed efficiency
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GOS	Galacto-oligosaccharides
IFN-γ	Interferon-gamma
IL	Interleukin
IUGR	Intrauterine growth restricted
KEGG	Kyoto Encyclopedia of Genes and Genomes
KO	Kyoto orthologs
LPS	Lipopolysaccharide

Lys	Lysine
MDA	Malonaldehyde
NE	Net energy
OTU	Operational taxonomic unit
PBS	Phosphate-buffered saline
PC	“Pes corporal”
PCA	Principal component analysis
PCoA	Principal coordinates analysis
PDP	Porcine digestible peptides
PERMANOVA	Permutational multivariate analysis of variance
PICRUSt2	Phylogenetic investigation of communities by reconstruction of unobserved states
ROS	Reactive oxygen species
SCFA	Short-chain fatty acids
SD	Standard deviation
SDP	Spray-dried plasma
SID	Standardized ileal digestible
T-AOC	Total anti-oxidation capacity
TNF-α	Tumor necrosis factor-alpha
VFA	Volatile fatty acids
XOS	Xylo-oligosaccharides



Chapter 1

Literature Review

1.1. Introduction: Current Challenges of the Pig Production Industry

The pig industry has significantly evolved during the last decades introducing improvements in nutrition, health, breeding, and genetics which have led to significant growth of its production capacity. This growth has been deaccelerated during the last few years, due to the impact of the African swine fever (ASF) outbreak across Asia in 2018 and the COVID-19 human pandemic in 2020, although the Asian producers are governing a fast recovery (OECD/Food and Agriculture Organization of the United Nations, 2022). In Spain, the swine industry has become one of the key sectors for the economy, positioning itself as the most relevant sector for Spanish livestock farming with more than 40% of the Final Livestock Production (Subdirección General de Producciones Ganaderas y Cinegéticas, 2021). In fact, Spain is the third largest producer country worldwide (after China and the United States) and the first in the European Union (EU) after having recently overtaken Germany (OECD/Food and Agriculture Organization of the United Nations, 2022) (Figure 1.1).

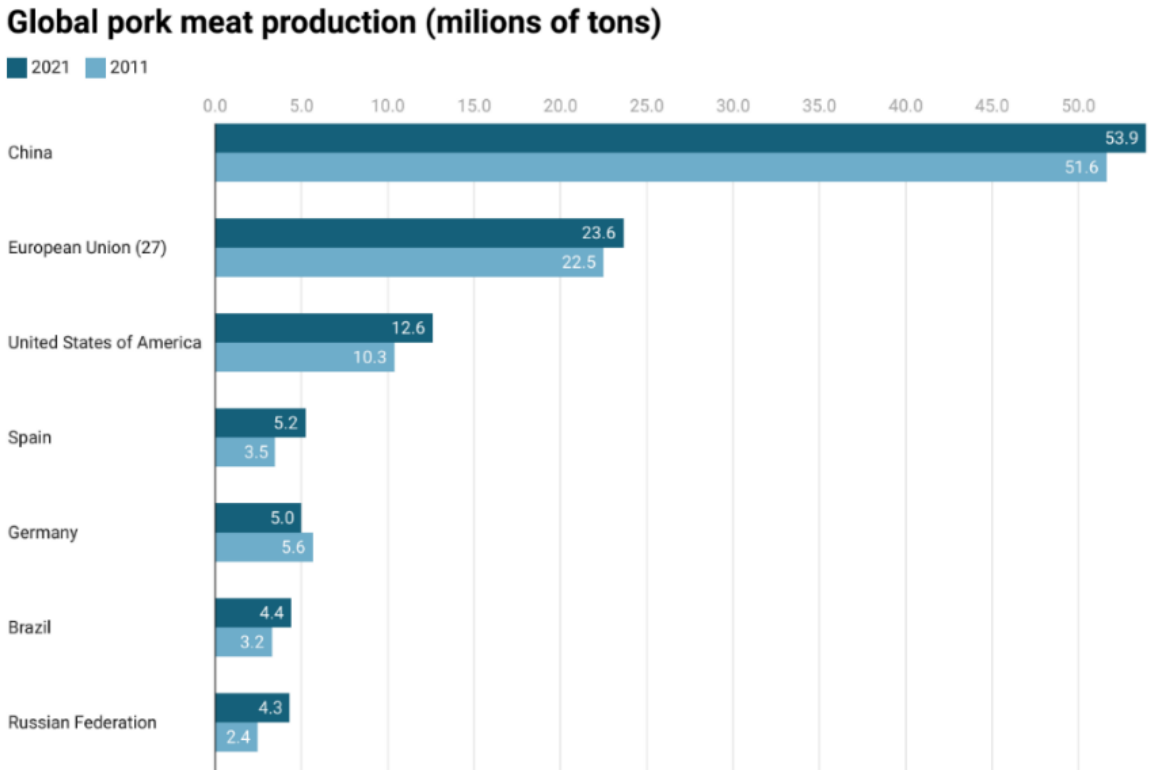


Figure 1. 1. Annual pork meat production in the 6 major global producers in 2011 and 2021 (OECD/FAO, 2022b).

Pork meat production in Spain has grown a 49% in the last decade, contrasting with the moderate 5% growth of the overall EU and the declining trend of important producers such as Germany (-13%). The increase in the Spanish pork production indices has been sustained by the high demand from the Asian markets affected by the ASF and the competitiveness of the sector in the world market.

In the coming years, pig production in Spain and the EU will have to attend to society's growing concern about antimicrobial resistance, farm sustainability, and animal welfare (Akash et al., 2022). The EU has already introduced regulations on the use of antimicrobials (European Parliament and the Council of the European Union, 2019a, 2019b), the ban on the use of zinc oxide (European Commission, 2016a), and other regulations designed to improve animal welfare, such as the group housing of sows during the gestation period (Council of the EU, 2009) and avoiding tail-docking (European Commission, 2016). Moreover, society's environmental awareness will push the pig sector to minimize the number of resources used, its carbon footprint, and the nitrogen residues emitted into the air and soil. This involves the reduction of the feed-food competition and the avoidance of imported ingredients, replacing them with food industry by-products. As well, as to improve the efficiency of nutrient utilization and the management of the increasing manure production (Lassaletta et al., 2019). Therefore, the principal challenge of the EU pig production sector will be to reduce its negative impacts on the environment and satisfy animal welfare needs while maintaining the sector's profitability (Renaudeau & Dourmad, 2022).

However, there are still certain obstacles that hinder the fulfillment of the EU agenda. First, during the last decades, the genetic selection of sows that farrow larger litters has decreased the average birth body weight (BW) and increased the number of lightweight piglets at birth (Beaulieu et al., 2010; Matheson et al., 2018; Peltoniemi et al., 2021), which often remain stunted and can't catch up their heavier counterparts, leading to greater BW variability in the batch. This situation is aggravated by the extended practice of early weaning the piglets, which increases the incidence of enteric diseases (Lallès et al., 2004) and increases growth variability. Slow-growing pigs represent a critical shadow cost, that

causes enormous economic losses to the pig industry. Traditional methods of controlling post-weaning diarrhea were the use of antimicrobials and therapeutic doses of zinc oxide, but EU restrictions on its use have opened a new scenario that requires the application of new strategies to minimize the impact of weaning on the efficiency of the production system.

Part of our research group trajectory has been dedicated to exploring approaches to improve the adaptation to weaning focused on the period after the separation from the sow, including feeding and management proposals aimed at improving piglet intake, reducing stress, improving diet digestibility and/or controlling growth and the adherence of opportunistic pathogens. As well, there are several post-weaning nutritional strategies available to improve the piglet's health and performance during the nursery period (J.-P. Lallès et al., 2007; Heo et al., 2013; Bonetti et al., 2021). However, given the evolving market demands, it is essential to continually develop cost-effective and nutritionally adequate alternatives that can sustain farm efficiency and economic sustainability. As well, the traditional management of commercial farm practices needs to be revised to adapt to the increasing volume of slow-growing pigs as well as to satisfy animal welfare needs, giving a response to the increasing concern of society for this issue. On the other hand, there is a growing body of evidence suggesting that early-life microbial colonization plays a key role in gut maturation, metabolic development, and immune system development and offers a "window of opportunity" to prepare the piglet for the challenge of weaning and enhance its future performance (Gensollen et al., 2016b; C. H. F. Hansen et al., 2012; Nowland et al., 2019; Thompson et al., 2008). Nevertheless, further investigation is needed to comprehend how the microbiota can be modified consistently in piglets, leading to long-term benefits.

To address the needs of the swine industry, as well as fill gaps in current knowledge, the primary objective of this thesis is to identify key piglet traits that are associated with early-life growth. By doing so, we can develop targeted intervention strategies to support the growth of slow-growing pigs. Additionally, this thesis aims to analyze the effectiveness of pre- and post-weaning nutritional and management interventions to improve the overall health, growth performance, and welfare of the piglets. In the following sections of this

literature review, we will delve into the existing research on the risk factors associated with slow growth and the significance of gut microbial colonization in pig health and development. As well, we will discuss the current literature on the intervention strategies that are evaluated in this thesis.

1.2. Economic Impact of Slow-Growing Pigs

Pig producers' decisions regarding when and how to market their pigs are influenced by the desired market weight set by the slaughterhouse. However, in conventional farms, there might exist a subgroup of pigs that may require additional time to achieve the slaughter weight. Animals belonging to this subset are called slow-growing pigs and are estimated to represent 10-15% of the batch (Calderón Díaz et al., 2017; S. L. Douglas et al., 2013; He et al., 2016). The presence of slow-growing pigs increases the BW variability within the herd, posing a significant challenge to production efficiency and a critical shadow cost in the current swine production systems (Patience et al., 2004).

Slow-growing pigs prolong the use of barn facilities and limit the efficiency of their utilization in the all-in-all-out system (S. L. Douglas, Edwards, et al., 2014; Patience et al., 2004). Farmers may opt to keep slow-growing pigs with a subsequent batch, but this practice increases the risk of disease transmission (Calderón Díaz et al., 2017). Additionally, packing plants impose penalties on pigs that fall below the weight standards, which results in a reduced market value for these animals (S. L. Douglas, Edwards, et al., 2014; Tolosa et al., 2021). Moreover, studies have shown that slow-growing pigs are at a greater risk of mortality during each production phase compared to their counterparts, which represents another hidden cost for swine producers (S. L. Douglas, Edwards, et al., 2014; Larriestra et al., 2006). In summary, slow-growing pigs pose a significant concern for the porcine industry, impacting the efficiency of the production cycle and causing significant economic losses (S. L. Douglas, Edwards, et al., 2014; López-Vergé, Gasa, Farré, et al., 2018; Patience et al., 2004). The swine industry requires practical solutions to reduce BW variability, facilitate management and minimize economic losses.

1.3. Risk Factors of Slow Growth

Pigs may experience slow growth at any stage between birth and slaughter, which can result in greater variation in body weight among individuals within a group (S. L. Douglas et al., 2013). This growth limitation is often attributed to various risk factors that can arise during the prenatal period and throughout the pig's life stages. These factors may include the initial birth BW, genetic potential, parity of the dam, management, and environmental conditions, among others (Camp Montoro et al., 2020; S. L. Douglas et al., 2013; López-Vergé, Gasa, Farré, et al., 2018; Quiniou et al., 2002a). Within this framework, certain factors are inherent to the piglet, accounting for the differences in growth rates observed among piglets under similar conditions (Figure 1.2). In many instances, reduced growth is the result of complex interactions between these factors.

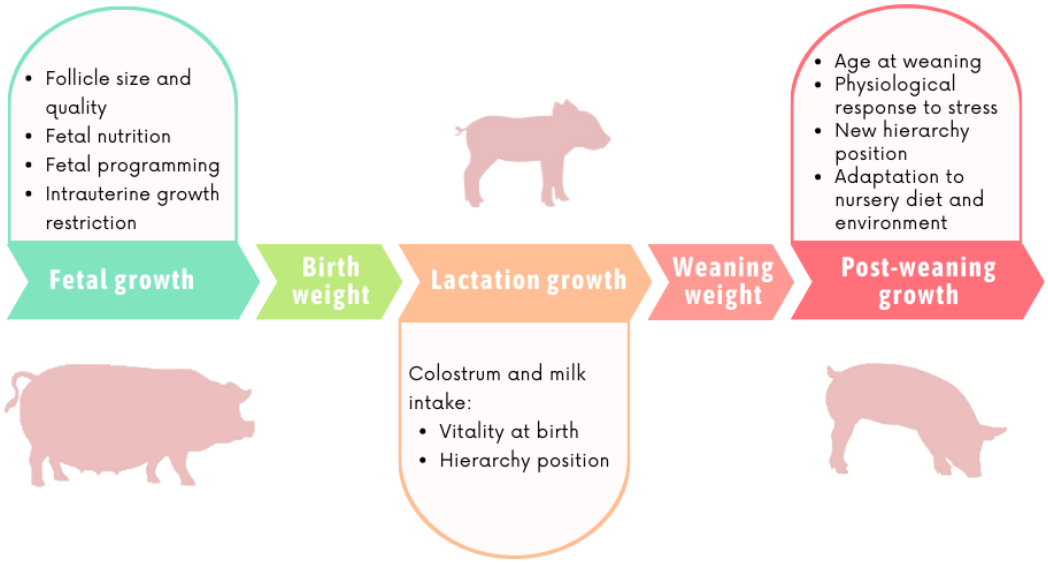


Figure 1. 2. Diagram illustrating the major inherent risk factors contributing to slow growth in piglets throughout gestation, lactation, and post-weaning periods.

1.3.1. Birth Weight and the Prenatal Period

Birth weight is a crucial factor that impacts piglet growth during postnatal life (S. L. Douglas et al., 2013; He et al., 2016). Low birth weight piglets require more time to reach market weight (Camp Montoro et al., 2020; S. L. Douglas et al., 2013; López-Vergé, Gasa,

Farré, et al., 2018), tend to have lower growth efficiency (Huting et al., 2018; Paredes et al., 2012; Quiniou et al., 2002a) and are more vulnerable to disease (Rutherford et al., 2013), which makes it challenging for them to catch up to heavier piglets throughout the production cycle (Quiniou et al., 2002a; Beaulieu et al., 2010). As well, lighter piglets face a higher risk of pre-weaning mortality (Ferrari, 2010).

The birth weight of piglets is a result of the fetal development during the gestation period, which is driven by the genetic potential of the progenitors but is also influenced by several factors fundamentally dependent on the sow (Fowden et al., 2006; López-Vergé, Gasa, Farré, et al., 2018). These factors include the size and quality of the follicle (López-Vergé, Gasa, Farré, et al., 2018), direct transfer of maternal hormones (Fowden et al., 2006), the capacity of the sow's uterus (Foxcroft et al., 2006), and alterations in placental function and nutrient supply (Fowden et al., 2009). As well, sow parity (S. L. Douglas et al., 2013) and its nutrition during the gestation period (Martineau & Badouard, 2009) may also influence birth BW and its variability. During gestation, adverse conditions in the intrauterine environment may impact not only fetal development but also have persistent effects beyond birth and across multiple generations (Ji et al., 2017). This phenomenon is known as fetal programming and refers to the long-term impact of environmental factors on the developing fetus (Barker & Clark, 1997; Kwon & Kim, 2017). It involves covalent modifications of nucleotide bases in DNAs without changes in the sequence that lead to modified activities of metabolic pathways and homeostatic control processes (Burdge et al., 2007). For instance, Villagómez-Estrada et al. (2022) observed that small BW piglets had a distinct gene expression profile indicative of impaired gut development and nutrient absorption, higher pro-inflammatory responses, and the activation of a stress signaling pathway that persisted until the end of lactation. Any limitation in uterine capacity or nutrient provision can lead to intrauterine growth restriction (IUGR), resulting in impaired fetal growth and organ development during gestation (Wu et al., 2006). Pigs exposed to IUGR, which currently represent 30–40% of all piglets (S. L. Douglas et al., 2013), show lower postnatal growth and survival compared to normal-weight littermates (Hansen et al., 2019), along with deficient immune function (Amdi et al., 2020) and impaired intestinal integrity

and health (Tang & Xiong, 2022). Fetal programming determines both the quantity and type of muscle fibers (Barker & Clark, 1997; G. Wu et al., 2006), which tends to be lower in low-birth-weight pigs (Nissen et al., 2004; Gondret et al., 2005; Gondret et al., 2006). A reduced number of muscle fibers can negatively impact their future growth performance by limiting lean growth and promoting fat deposits, ultimately leading to poorer pork quality (Gondret et al., 2006; Rehfeldt et al., 2008; Rehfeldt & Kuhn, 2006).

The endocrine state of the sow can also influence fetal programming (Fowden et al., 2006). Prenatal stress has been shown to impact critical traits related to offspring growth, physiology, and behavior through the effect of glucocorticoids and catecholamines (Otten et al., 2015). Maternal stress seems to affect fetal growth and differentiation of fetal tissues, leading to reduced birth weight (Bate et al., 1991; Kranendonk et al., 2006; Otten et al., 2015), impaired immune function, vitality at birth (Bate et al., 1991; Tuchscherer et al., 2002; Otten et al., 2007; Otten et al., 2015), and reduced growth at weaning (Jarvis et al., 2006; Muns, Manzanilla, et al., 2014). Nonetheless, there are disparities in research indicating that variables such as the timing of stress exposure or the nature of stimuli play a vital role in determining the result (Jarvis et al., 2006; D. Couret et al., 2009; David Couret et al., 2009; Otten et al., 2015).

In light of the evidence, birth weight and fetal programming are the primary factors that influence the probability of suffering slow growth during a pig's early life.

1.3.2. Lactation Period

In recent decades, the problem of low-birth-weight piglets has become increasingly relevant due to the genetic selection of sows with higher prolificity. These changes have brought a decline in the average birth weight (Peltoniemi et al., 2021), resulting in an increase in the proportion of light-born piglets and the frequency of intrauterine growth-restricted (IUGR) piglets (Beaulieu et al., 2010; Matheson et al., 2018). Additionally, genetic selection for leaner meat has led to piglets being born with insufficient body reserves (Palomo Yagüe, 2019), which do not meet their early life needs for maintenance, thermoregulation, and activity (le Dividich et al., 2005). This scenario makes colostrum intake during the first hours of life a critical need. In fact, in modern pig farms, insufficient

colostrum consumption is one of the primary reasons for neonatal mortality and reduced growth, particularly among low birthweight piglets, which have lower energy reserves and less capacity for thermoregulation (Herpin et al., 2002; Devillers et al., 2011; Decaluwé et al., 2014a).

The importance of colostrum intake during the first few days of a piglet's life goes beyond its nutritional function. Colostrum promotes rapid hypertrophy and hyperplasia of the gastrointestinal tract to quickly adapt from transplacental to enteral nutrition after birth (Zabielski et al., 2008). In addition, since the sow's epitheliochorial placenta does not transfer macromolecules during gestation, colostrum provides immunoglobulins to the piglet to keep him immunologically protected during its initial phase of life (Baintner 1986; le Dividich et al., 2005) and growth factors that are crucial for its development (Sanderson & Walker, 1993). The transfer of these components into the blood stops abruptly between 18 and 36 hours after birth during intestinal closure (Weström et al., 1984), which is one of the steps of the development of the epithelial barrier function (Moeser et al., 2017). However, the induction of intestinal closure is influenced by colostrum components and humoral factors released in response to feeding. If the pig is starving, intestinal closure can be delayed (Ekström & Weström, 1992). The posterior process of epithelial barrier development is presumably driven by genetic programming, microbial colonization, and colostrum and milk bioactive substances, but the underlying mechanisms are not yet clear (Moeser et al., 2017).

The increase in sow prolificity has resulted in a decrease in colostrum consumption per piglet, as the quantity of colostrum produced is unrelated to litter size (Decaluwé et al., 2014b). Moreover, although the sow can increase the volume of milk produced as the size of the litter grows, it is not directly proportional to the number of piglets she nurses (Auldist et al., 1998). This circumstance is exacerbated by the fact that the number of teats has not increased proportionally to the increment in the number of piglets born alive, resulting in enhanced competition for the already limited resources and increased mortality (Vasdal et al., 2011). Both birth weight and the vitality of piglets at birth, which might be impaired by a situation of fetal hypoxia during farrowing (Alonso-Spilsbury et al., 2005), are

determinants in the capacity of the piglets to compete for resources. Piglets with low birth weight and/or reduced vitality may take longer to perform their first suckle and may struggle to compete for access to the best teats and to stimulate them, which raises the risk of inadequate colostrum intake (Le Dividich et al., 2017; Muns et al., 2016). Additionally, as piglets have a clear hierarchy and always suckle the same teat during the whole lactation, those who have initially chosen the sow's front teats will grow faster than piglets suckling the inguinal teats because of differences on milk production (De Passille & Rushen, 1989; Del Águila et al., 2017). Insufficient colostrum and milk supply to piglets during this critical early period may compromise the piglet's viability and represent a significant risk factor for the emergence of slow-growing pigs and for pre-weaning mortality (Edwards & Baxter, 2014). However, the implementation of strategies like cross-fostering, creep feeding, and milk replacers might potentially mitigate this risk (Muns et al., 2016).

1.3.3. The Impact of Weaning

In the current pig production system, weaning is the most stressful event in a piglet's life due to the combination of changes in diet, social, and environmental life conditions it involves. The piglet is separated from the mother, handled, and transported to a different physical environment where they are mixed with piglets from other litters (Campbell et al., 2013). Mixing triggers fights for the re-establishment of the social hierarchy in the nursery pen, which aggravates the experienced stress and can result in physical injuries (Camerlink et al., 2021). Additionally, weaning represents an abrupt change in diet from sow's milk to a plant-based diet which contains many ingredients and vegetable structures that piglets have not eaten before (J.P. Lallès et al., 2007). In natural conditions, piglet's weaning is a gradual process that extends until 10 to 12 weeks of age, when the gastrointestinal epithelial, immune, and nervous systems are almost fully mature. However, in commercial pig farms, it is an abrupt process occurring at 3-4 weeks of age when both the gastrointestinal tract and the immune system of the pig are still immature (Moeser et al., 2017). The combination of stressors occurring at weaning causes a delay in the onset of solid food intake of mostly 24 to 48 hours and reduced consumption in the week following weaning, leading to an impairment of growth performance, which is known as post-weaning

growth check (Dong & Pluske, 2007; J.-P. Lallès et al., 2007). The stress associated with weaning is a welfare concern and the reduction in growth during the first days following weaning results in enormous economic losses to the swine industry with increased days to market (Heo et al., 2013).

Stress is thought to cause most of the post-weaning gastrointestinal dysfunctions, such as changes in small intestine structure and enzyme function, temporary increases in mucosal permeability, imbalanced absorption and secretion of electrolytes, and altered patterns of local inflammatory cytokines (Moeser et al., 2017). These functional disturbances caused by early weaning can persist into adulthood (Pohl et al., 2017). The mechanism by which weaning stress produces these harmful effects is an activation of the corticotropin-releasing factor stress signaling pathways, which lead to inflammation, mast cell activation, and release of proinflammatory cytokines, causing a breakdown of the intestinal mucosa and an increase of the epithelial permeability (Moeser, Klok, et al., 2007). It seems that early-weaned pigs show higher early and chronic activation of these pathways compared to pigs weaned at older ages, which might explain why weaning age is a major factor influencing the severity of post-weaning gastrointestinal damage (Moeser, Ryan, et al., 2007; Smith et al., 2010). Piglets weaned in a week of difference show differences in intestinal permeability immediately after weaning (Moeser, Ryan, et al., 2007) which can persist for up to 9 weeks post-weaning (Smith et al., 2010). As well, extending the lactation period results in an increased weaning weight, which is one of the main predictors of nursery performance (S. L. Douglas et al., 2013; He et al., 2016; Paredes et al., 2012). Heavy pigs at weaning have a more mature digestive system (Cranwell et al., 1997; Pluske et al., 2003) and a higher feed intake post-weaning (Magowan et al., 2011a) which results in a greater capacity to adapt to the post-weaning environment and avoid slow growth and mortality (Main et al., 2004; Main et al., 2005; López-Vergé et al., 2019). However, piglets within a batch are frequently sourced from sows with differing farrowing dates and subsequently weaned on a fixed day. This lag time between farrowing dates within a batch has the potential to increase the variability in post-weaning growth, presenting a risk factor for slow growth that needs to be considered.

Moreover, post-weaning anorexia may play a significant role in mediating the negative effects of weaning stress (Lallès et al., 2004). Pearce et al. (2013) demonstrated that pigs whose feed intake was restricted to mimic that of heat-stressed pigs experienced similar changes in intestinal integrity indicators. This suggests that limiting feed intake may trigger some of these physiological stress responses. Pigs that exhibit delayed feeding behavior after weaning may suffer from higher mortality rates and poor post-weaning performance due to a more important gastrointestinal disruption (Huting et al., 2021; Pluske, 2016). In this regard, **implementing management and nutritional strategies that facilitate the proper adaptation of the digestive system from the sow's milk to the solid-based diet offered after weaning is essential to help alleviate the "growth check" associated with weaning** (Blavi et al., 2021; Huting et al., 2021; Pluske, 2016).

Additionally, the adaptability of piglets to the new environment and their capacity to deal with weaning stress varies among individuals, which also contributes to growth variability. In this regard, research suggests that behavioral characteristics and biological traits interact to influence a piglet's response to stressful situations (Ursinus et al., 2013). Studies attempting to evaluate individual behavioral characteristics have identified two "coping styles": proactive and reactive pigs. Proactive pigs tend to be more fearless, dominant, aggressive, and less flexible to a changing environment than reactive animals (Hessing et al., 1993; Bolhuis et al., 2006). These "coping styles" also affect the piglet's neurobiological response to stressors, with proactive animals displaying high sympathetic reactivity and low or moderate hypothalamic-pituitary-adrenal axis responsiveness to stressors, while reactive individuals exhibit the opposite patterns (Koolhaas et al., 1999; Koolhaas et al., 2007; Kanitz et al., 2019). As well, differences in immune system humoral response have been identified between the different "coping styles" (Kanitz et al., 2019). Besides, specific genetic traits have been linked to variations in social behavior and reactions to stress-inducing stimuli, which have demonstrated an impact on growth performance after weaning (Camerlink, Ursinus, et al., 2018; O'Connell et al., 2005). The piglets' hierarchy during lactation and teat choice is also correlated with the piglets' stress response to weaning. Piglets that suckle on anterior teats, which usually are the biggest

piglets, tend to experience more nutritional deprivation, while those that suckle on posterior teats, which are the smallest, may experience more stress from maternal separation (Mason et al., 2003).

This correlation between pig behavior and specific biological mechanisms is likely to be crucial in explaining individual differences in piglet growth after weaning. Therefore, **understanding the characteristics of piglets associated with their individual capacity to deal with weaning stress can help in developing management strategies to support their adaptive responses and help reduce growth variability in the post-weaning period**.

1.4. The Role of Gut Microbiota in Pig Development and Performance

The intestinal tract of animals is not just a simple digestive system; it is a complex ecosystem populated by diverse bacterial communities that have a profound impact on the host's health and physiological functions (Sommer & Bäckhed, 2013). These microbial communities have co-evolved with the host, forming a symbiotic relationship that involves constant crosstalk between the bacteria and their host (Knight et al., 2017). This crosstalk has been found to play a crucial role in regulating the immune system, maintaining the gut barrier function, and modulating metabolic processes (Cebra, 1999; Jandhyala et al., 2015; Palmer et al., 2007; Sommer & Bäckhed, 2013).

1.4.1. Early-Life Gut Colonization

The microbial colonization of the pig gut was traditionally believed to begin at birth. However, a recent study has identified bacteria in the spiral colon of stillborn piglets, suggesting that initial colonization might occur during the prenatal period (Nowland et al., 2021). At birth, the bacteria from the mother’s vagina, feces, nipples, colostrum, and the environment will shred in the piglet’s body and digestive system establishing the initial gut microbiota (Konstantinov et al., 2006; Jost et al., 2014; Chen et al., 2018). During the microbial colonization of piglet’s gut, bacterial population abundances change rapidly as they grow, going through a period of microbial succession. In this process, microbiota

diversity and richness increase until reaching an adult-like microbiota (Pajarillo et al., 2014; Saladrigas-García et al., 2022). This colonization is influenced by multiple factors, such as genetics, the mother's microbiome, colostrum and milk, and the environment during the initial days of life (Konstantinov et al., 2006; Jost et al., 2014; Bian et al., 2016; Chen et al., 2018).

Although the microbiota is a dynamic system, the first colonizing bacteria largely drives its establishment and development (Nowland et al., 2019). In fact, studies in humans reveal that the most crucial time for microbiome development is during the first 1-3 years of life, and any dysbiosis during this period can lead to disease (Palmer et al., 2007; Robertson et al., 2019). During this early-life period, the gut microbiota presents special plasticity and can be easily altered but afterward, the microbiome becomes more stable and harder to change (Thriene & Michels, 2023). The shift from milk to solid food is believed to contribute to this stability (Palmer et al., 2007). In agreement, the initial influence of the sow's microbiota decreases with the introduction of solid feed or the weaning process (Bian et al., 2016). Weaning will produce major and abrupt changes to the microbiota which will also lead to a more stable state (Pajarillo et al., 2014; Saladrigas-García, D'Angelo, Ko, Nolis, et al., 2021). Thus, evidence suggests that the crucial time for microbiome formation in pigs is prior to weaning.

1.4.2. The Crosstalk Between Gut Microbiota and Immune System

During this early-life period, intestinal microbial colonization plays a pivotal role in the development and programming of the neonatal immune system (Hansen et al., 2012; Nowland et al., 2019). This microbiota-immune system relationship is complex and multifactorial and involves the interplay between the microbiota, pathogens, and the diet.

Since birth, bacterial colonization represents the first stimulus for the immune system, by primarily increasing the number of circulating antimicrobial-specific antibodies (Cebra, 1999). Subsequently, mucosa-associated microbiota will influence health and survival, affecting susceptibility to diseases and even modulating cognitive development (Duarte & Kim, 2022; Nowland et al., 2019), resulting in long-lasting consequences throughout an individual's lifetime (Hansen et al., 2012; Nowland et al., 2019). The gut

microbiome regulates the immune system's ability to distinguish between commensal and pathogenic bacteria (Burkey et al., 2009; Pluske et al., 2018). Both hematopoietic and non-hematopoietic cells of the innate immune system have the capacity to detect microorganisms and their byproducts, which allows the host to elaborate an appropriate physiological response (Thaiss et al., 2016). The host's response influences the composition and functional capabilities of the microbiota and promotes the proliferation of beneficial species (Levy et al., 2015). When the balance between the host immune system and the gut microbiota is disrupted, dysbiosis is created and increases susceptibility to diseases such as diarrhea (Patil et al., 2020).

Moreover, the metabolites produced by the intestinal microbiota can have a direct influence on the immune system and the intestinal epithelium integrity. Short-chain fatty acids (SCFA) are the major microbial metabolites produced by the fermentation of carbohydrates and the carbon chain of amino acids. The SCFAs produced by the microbiota represent an extra energy supply but they also decrease the pH in the intestine, promote gastrointestinal motility and inhibit the proliferation of opportunistic pathogens (Den Besten et al., 2013). Among the SCFAs, butyrate is the preferred energy source used by colonocytes, which is involved in the maintenance of the gut barrier function and has been related to anti-inflammatory pathways (J. Tan et al., 2014). Butyrate is considered a health-promoting metabolite who plays an important role in cell proliferation and development and has regulatory functions on the metabolism (Den Besten et al., 2013). Additionally, acetate can induce the activation of regulatory T cells (Smith et al., 2013), and propionate has functions that inhibit the growth of pathogens (Levison, 1973). Thus, the production of SCFAs in the distal part of the gastrointestinal tract improves the gut's functioning by helping to form and protect the intestinal barrier and regulate host defense and inflammation.

Research in germ-free animals has revealed that the lack of gut microbiota during early life leads to impairments in the development of lymphoid organs and a decreased production of lymphocytes (Bauer et al., 2006; Macpherson & Harris, 2004). As well, it produces structural and functional deficiencies in the gastrointestinal tract such as thin villi,

weak peristalsis, decreased intestinal surface area, reduced blood flow in the villus, and prolonged cell cycle time (Jandhyala et al., 2015). However, when germ-free pigs are colonized by any kind of microbiota, most of the functional immune system components develop likewise the conventional pigs (Inman et al., 2012). Although most of these abnormalities are age-independent and can be corrected by adding bacteria at any age, there are some cellular defects that can only be restored during a limited time in early life, otherwise full intestinal immune development will not be achieved. This finding evidences that there is a critical period during early life in which microbial interactions participate in the development of the immune system (El-Aidy et al., 2013; Gensollen et al., 2016b). This "microbial programming" is crucial not only for proper immune system functioning in later stages but also for gut maturation, metabolic development, and piglet growth (Thompson et al., 2008).

1.4.3. The Impact of Gut Microbiota at Weaning

When weaning occurs, the shift in diet and environment as well as the experienced stress leads to alterations in the intestinal microbiota of piglets. After weaning, gut microbiota richness increases (Pajarillo et al., 2014; Chen et al., 2017; W. Wang et al., 2019; Choudhury et al., 2021; Saladrigas-García, D'Angelo, Ko, Nolis, et al., 2021) and beta diversity, described as the variability among individual piglets, decreases, suggesting that after overcoming the weaning stress, the microbiota advances to a more adult-like state (Chen et al., 2017; Choudhury et al., 2021; Saladrigas-García, D'Angelo, Ko, Nolis, et al., 2021). Additionally, the sudden diet change during the weaning transition induces a rapid shift from a milk-oriented microbiota to the proliferation of bacterial populations adapted to the solid plant-based nursery diet (Gresse et al., 2017). This abrupt alteration of the gut microbial ecosystem can critically increase the risk of gastrointestinal diseases (Gresse et al., 2017). For instance, the decline of lactic acid-producing *Lactobacillus* during weaning raises gut pH, which increases the risk of pathogen proliferation (J.P. Lallès et al., 2007). As previously mentioned, weaning stresses promote the impairment of the epithelial barrier function and the activation of proinflammatory pathways in the gastrointestinal system, which provide a favorable environment for the expansion of pathogenic enterobacteria

(Baümler & Sperandio, 2016). Additionally, this process coincides with the decrease in immunoglobulin supply from milk (Dunne-Castagna et al., 2020). Intestinal dysbiosis together with the weakened defenses against pathogens potentially contributes to the development of postweaning diarrhea and enteric infections (Konstantinov et al., 2006; J.P. Lallès et al., 2007; Gresse et al., 2017).

The literature describes that the gut microbiota profile during early life influences the probability to develop post-weaning diarrhea. Dou et al. (2017a) observed that piglets susceptible to post-weaning diarrhea could be discriminated from the healthy ones as early as seven days of age. Healthy animals had a higher abundance of Prevotellaceae, Lachnospiraceae, Ruminocacaceae, and Lactobacillaceae, compared to the susceptible pigs. Karasova et al. (2021) also confirmed the existence of bacterial markers of health or post-weaning diarrhea 3 days before weaning. Even in the absence of post-weaning diarrhea, weaning causes a growth check due to the transitory malabsorption of nutrients and subclinical decline in animal health. However, some pigs display good resilience and are little affected in terms of growth and health (Revilla et al., 2019). It seems that piglets that present a more mature microbiota, capable of metabolizing complex carbohydrates found in nursery diets, such as those with a greater abundance of *Prevotella*, appear to be less affected by weaning and experience a lower reduction in growth (Choudhury et al., 2021; Luise et al., 2021; Luo et al., 2022).

1.4.4. The Role of Gut Microbiota in Feed Efficiency, Feed Intake and Growth.

Gut microbiota has been shown to affect various physiological functions apart from immune system development, including nutrient digestion and absorption, and energy metabolism (Den Besten et al., 2013). As a result, it plays a relevant role in the feed efficiency of pigs and in the regulation of certain metabolic processes (McCormack et al., 2017). Several studies have revealed correlations between specific bacterial genera and feed efficiency traits, with those related to the metabolism of dietary polysaccharides being linked to higher feed efficiency during the weaning-to-finishing phase (McCormack et al., 2017; Tan et al., 2017; Yang et al., 2017; Bergamaschi et al., 2020). These findings suggest that the distinctive microbiota present in pigs with high feed efficiency may be more

competent at digesting dietary carbohydrates, which are transformed in SCFA and represent an extra energy source for the host (Vigors et al., 2016). However, the connection between gut microbiota and feed efficiency appears to be influenced by both the pigs' diet and gender (Verschuren et al., 2018). Specifically, the impact of microbiota on feed efficiency is more pronounced in male pigs and when they are offered fibrous diets (Verschuren et al., 2018). Additionally, Metzler-Zebeli et al. (2018) identified that certain mucosal bacteria and microbial metabolites in the luminal area were more closely related to differences in feed efficiency and cecal gene expression than others. However, the authors were unable to determine whether these changes caused variations in feed efficiency or were a result of changes in the pig's physiology or feeding behavior related to feed efficiency. Indeed, the feeding behavior and appetite of pigs also seem to be influenced by gut microbiota. For instance, research has found a link between enterotypes and feed intake in growing-finishing pigs (Yang et al., 2018). Specifically, pigs with a Prevotella-dominant enterotype show a higher feed consumption compared to those with a Treponema-dominant enterotype (Yang et al., 2018). It has been proposed in other species that gut microbiota can synthesize mimetic proteins of peptide hormones, which activate anorexigenic pathways, influencing the appetite of the host (Breton et al., 2016). However, further research is needed to confirm these findings.

Overall, the literature describes that gut microbiota plays a vital role in regulating various physiological functions that affect growth, including feed efficiency and immune system development. Consequently, several studies have attempted to establish links between gut microbial populations and growth in pigs (Gaukroger et al., 2020; Mach et al., 2015; Ramayo-Caldas et al., 2016). Their findings suggest that early-life factors play a significant role in determining the composition of microbiota. Birth weight, in particular, has been found to impact fecal microbiota richness and composition during the first two months of life (N. Li et al., 2018; Gaukroger et al., 2020). Additionally, early-life gut microbial composition and long-term performance traits have also shown a connection. For instance, higher levels of *Lactobacillus*, unclassified *Prevotellaceae*, and *Ruminococcaceae* UCG-005 in feces during the first two weeks of life have been associated with improved growth

performance until day 56 of life (Gaukroger et al., 2020). Other studies have shown that piglets with a *Ruminococcaceae*-dominated enterotype tend to have better growth during lactation, while those with a *Prevotella*-dominated enterotype exhibit lower growth rates during lactation (Mach et al., 2015) but higher average daily gain after weaning (Mach et al., 2015; Ramayo-Caldas et al., 2016). These findings demonstrate that differences in the gastrointestinal microbiota associated with performance are time-specific, highlighting the evolution of the intestinal microbiota as piglets grow (Gaukroger et al., 2020; Mach et al., 2015).

The interplay between gut microbiota and host phenotype is very complex, with microbiota acting as both a cause and an effect (Figure 1.3). Several studies describe associations between the composition of gut microbiota and performance metrics. However, these correlations do not necessarily indicate causality. Either as cause or consequence, or concomitantly to other changes, the **gut microbiota is an important factor involved in a pig's performance and, thus, a potential target for interventions to improve the pig production efficiency**. **The early postnatal phase offers a "window of opportunity" when interventions can take advantage of microbiota plasticity and promote the establishment of desired microbial populations** (Bian et al., 2016; C. Cheng et al., 2018). As well, during this period, gut microbiota participates in the programming of gut maturation, metabolic development, and immune system development (Thompson et al., 2008). Nevertheless, additional research is necessary to understand how the microbiota can reliably and consistently be altered to establish changes in piglets that result in life-long benefits. **By monitoring the microbiota profiles of pigs that have experienced rapid or slow growth during early life, we may be able to identify gut microbiota characteristics linked to growth performance that persist in later stages**. This knowledge might help in the design of effective interventions targeting their gut microbiota that mitigate the risk of slow growth in pigs.

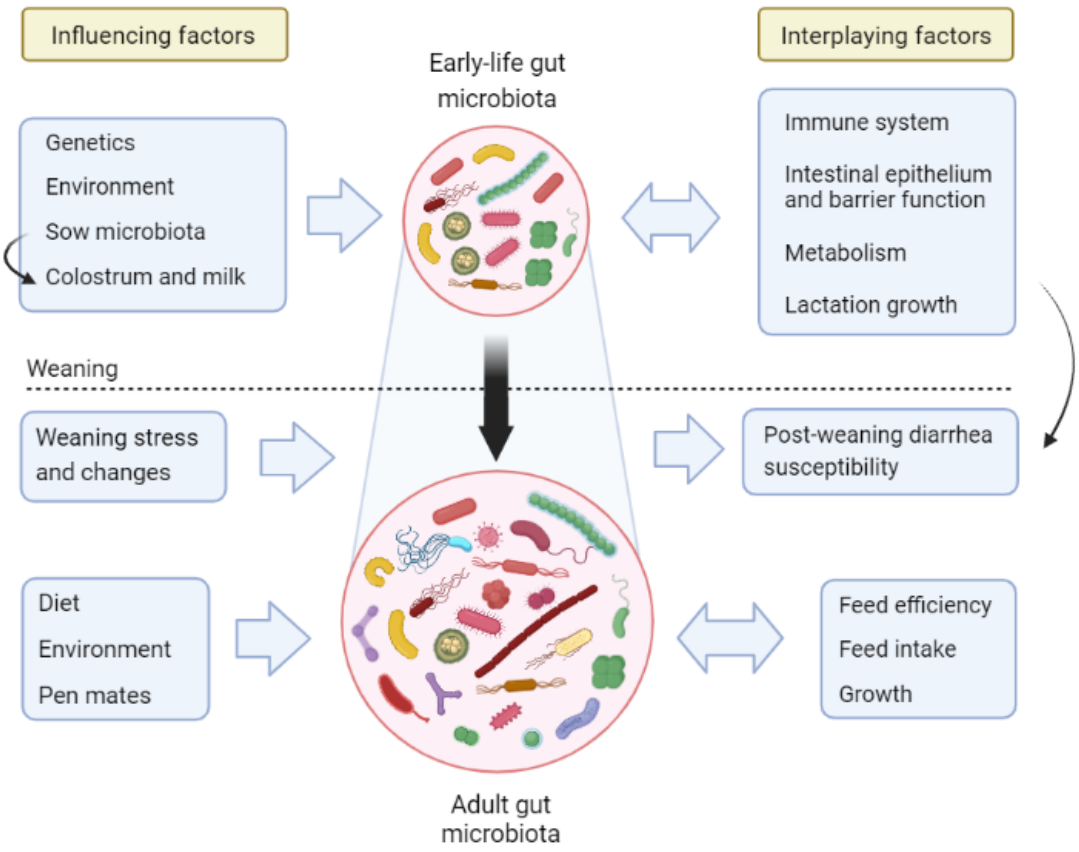


Figure 1. 3. Diagram of the interplay between the gut microbial colonization and maturation, the host phenotype, and the extrinsic factors. Created in *biorender.com*.

1.5. Pre-weaning and Post-weaning Management and Nutritional Strategies to Improve Pig's Growth and Health.

In previous sections of this revision, we have discussed the early-life risk factors that contribute to the development of slow-growing pigs. We also have highlighted the negative impact these animals have on production efficiency and the associated economic losses. To tackle this issue and facilitate the management of these pigs, pig producers may utilize a range of strategies. Nevertheless, research indicates that the effectiveness of these interventions varies depending on the timing of their implementation (S. L. Douglas et al., 2013; López-Vergé, Gasa, Farré, et al., 2018).

1.5.1. Opportunities for Intervention to Address Slow Growth.

As previously discussed, birth BW is a major risk factor for slow growth in the post-natal period. For this reason, there are strategies aiming at improving birth weight through improved prenatal management and sow nutritional strategies (Vos et al., 2014; Blavi et al., 2021). However, a study conducted by López-Vergé et al. (2018) found that changes in the weight category of pigs before weaning are frequent, as shown by the low correlation coefficients between their birth weight and the time it takes for them to reach market weight (Figure 1.4). Literature supports that light birth pigs can upgrade their BW category in posterior stages, suggesting that light pigs have the potential to compensate during postnatal growth (Douglas et al., 2013; López-Vergé et al., 2018; Montoro et al., 2020). Nevertheless, it is crucial to differentiate between those that are born small for their gestational age and those that suffer from IUGR (Foxcroft et al., 2006). Proportionally small piglets may not necessarily experience the same long-term consequences as those with IUGR, which is believed to lead to permanently stunted growth (Huting et al., 2018; G. Wu et al., 2006). Moreover, the severity of IUGR can vary among pigs and depends on the duration and stage of gestation (Wu et al., 2006). To reduce the incidence of IUGR in pigs, several studies have explored nutritional strategies for sows (Van Ginneken et al., 2022). For instance, maternal supplementation with a blend of phytogenic actives (Reyes-Camacho et al., 2020; Reyes-Camacho et al., 2021) or partial maternal diet supplementation with organic trace minerals (Villagómez-Estrada et al., 2021) had positive effects on neonatal piglet gene expression, indicating its potential as strategies to mitigate the negative effects of IUGR. Despite these efforts, when piglets are born with IUGR, farms still opt to cull them as there is a lack of nutritional and management strategies that reverse their long-term stunting during the suckling or post-weaning phase (Van Ginneken et al., 2022). However, culling pigs has a negative animal welfare perception in society and also leads to economic losses for farmers. **If optimal management and dietary conditions are provided to lighter piglets, there is still a chance to improve their condition.**

In contrast, weaning weight shows a higher correlation with the time to reach market weight compared to birth weight (Douglas, Edwards, et al., 2014; López-Vergé et al.,

2018), and this correlation becomes stronger using the weight at the end of the nursery period and subsequent stages. Pigs born light but who achieve a high weaning weight due to experiencing above-average growth during lactation have the potential to continue showing a nursery growth comparable to that of heavier piglets at birth (Camp Montoro et al., 2020; Paredes et al., 2012; Surek et al., 2019). However, this also means that pigs that start the nursery period within a low BW category are more likely to stay in that category by the end of the cycle. For instance, Camp Montoro et al. (2020) determined that the probability to reach the target slaughter weight at 22 weeks of age decreased in pigs < 6.7 kg BW at 28 days of weaning age. In fact, growth during the entire nursery period is more strongly associated with weaning weight than with average daily gain during the first week after weaning (Faccin et al., 2020). This suggests that improving weaning weight may have a greater impact on nursery performance than implementing post-weaning feeding and management strategies designed to boost growth rate (Faccin et al., 2020; Huting et al., 2017; Van Barneveld & Hewitt, 2016; Wolter & Ellis, 2011). Therefore, it can be stated that the lactation period provides a "window of opportunity" to implement strategies to improve the performance of slow-growing pigs, since the effectiveness of these interventions decrease as they grow up (López-Vergé, Gasa, Farré, et al., 2018) (Figure 1.4). There are multiple interventions that can be applied during the lactation period such as cross-fostering (Deen & Bilkei, 2004; Muns, Silva, et al., 2014), creep feeding (Blavi et al., 2015; Solà-Oriol and Gasa, 2017; Muns and Magowan, 2018), using milk replacer (Azain et al., 1996; Blavi et al., 2015; de Greeff et al., 2016; Muns et al., 2017; Baxter et al., 2020) or split suckling (Solà-Oriol & Gasa, 2017). In the present thesis, we will focus on the provision of prebiotics and stimbiotics during the lactation period to take advantage of the plasticity of the microbiota during early life and promote changes with long-term influence on pig performance.

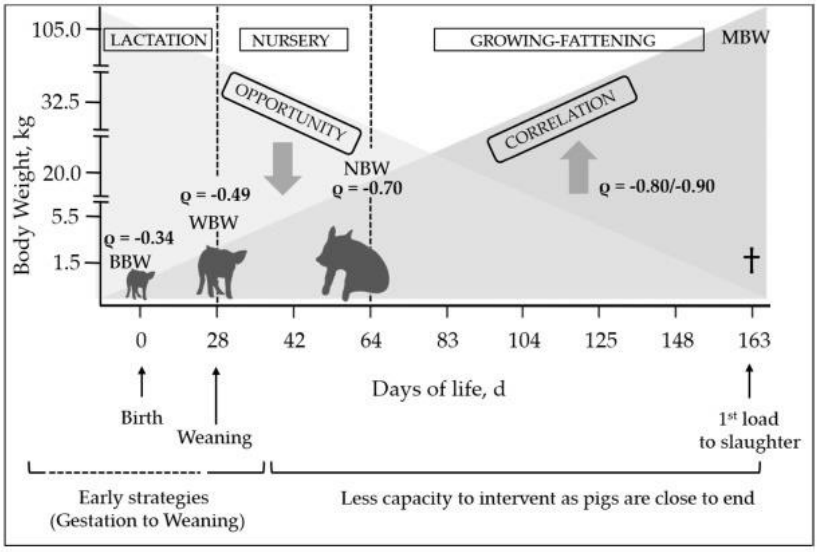


Figure 1. 4. Effect of birth and weaning body weight until slaughter, adapted from (Blavi et al., 2021). The spearman correlation coefficient (ρ) indicates the correlation between body weights at different production phases with the number of days to reach a market body weight of 105 kg. BBW: birth body weight; WBW: weaning body weight; NBW: nursery body weight; MBM: market body weight. † means slaughterhouse.

Although the importance of weaning weight in subsequent performance, slow-growing pigs still have an opportunity to reverse their situation by showing compensatory growth during the post-weaning period. However, this capacity is influenced by the previous history of the pig (Huting et al., 2018; Montoro et al., 2020). For instance, pigs that are born heavy but are weaned light due to poor growth during lactation have an advantage in compensatory growth post-weaning compared to those that are born and weaned light (Huting et al., 2018a; Montoro et al., 2020; Surek et al., 2019; Zeng et al., 2019). It is possible for these lighter piglets at weaning to achieve a similar BW to the heavier pigs at weaning by the end of the production cycle (Camp Montoro et al., 2020), but their success will reside in the management and feeding strategies performed during the post-weaning period. For instance, some farmers wean slow-growing pigs at a later stage by split weaning, which involves weaning the heavier piglets and leaving the lightweight piglets on the sow for a longer time (Pluske & Williams, 1996, Abraham et al, 2004). Other management strategies aim to reduce weaning stress by providing environmental enrichment (Oliveira et al., 2016) or performing early socialization with other litters while piglets still stay with their mothers (Ko et al., 2021; Saladrigas-García, D'Angelo, Ko, Traserra, et al., 2021). However, in this

thesis, we will focus on the strategy of keeping litter groups in nursery pens instead of mixing them with pigs from other litters.

Additionally, to facilitate the adaptation of weaned piglets to their post-weaning diet, it is essential to offer a high-quality starter diet containing easily digestible and palatable ingredients (J.-P. Lallès et al., 2007). Moreover, a variety of nutritional strategies and interventions have been developed to enhance the health of piglets during this stage. These include reducing dietary protein content, increasing fiber intake, and including feed functional additives. Among the wide variety of feed additives available in the market, there are enzymes to enhance feed digestibility, prebiotics, and probiotics to protect against gut dysbiosis, and other substances such as organic acids, essential oils, functional amino acids, spray-dried plasma, hydrolysate peptides, and egg yolk antibodies to promote gut function and health (J.-P. Lallès et al., 2007; Heo et al., 2013; Bonetti et al., 2021). Moreover, several studies have shown positive effects of increasing the quantity of the first post-weaning diets (A. L. Craig et al., 2020; S. L. Douglas, Wellock, et al., 2014; Huting et al., 2017; Lawlor et al., 2002; Magowan et al., 2011a, 2011b; Muns & Magowan, 2018). In this thesis, we will explore the supplementation of animal by-products in the starter diet to optimize gut functionality in the post-weaning period. As well, we will evaluate a strategy that involves allowing pigs to consume the pre-starter diet containing these animal by-products until they reach a target body weight, rather than offering it until a predetermined date.

1.5.2. Early-Life Prebiotics and Stimbiotics to Modulate the Microbiota and its Long-Term Effects.

Research in pigs has shown correlations between gut microbiota and performance (Dou et al., 2017a; Gaukroger et al., 2020; Mach et al., 2015a; McCormack et al., 2017) and the ability to modulate it to improve feed efficiency and average daily gain (Y. Li et al., 2018; McCormack et al., 2019). Other research lines are focused on manipulating microbiota for improving herd health and reducing the dependence of the pig industry on antimicrobials, especially during the postweaning period (Heo et al., 2013). Pursuing the two objectives, nutritional strategies showing the potential to favor the growth of a healthy microbial population during early life have gained considerable attention (Huting et al., 2021). Among

these strategies, probiotics, prebiotics, and stimbiotics have shown a major role, but this review will only focus on the use of prebiotics and stimbiotics.

1.5.2.1 Prebiotics and Stimbiotics in Pig Nutrition

Prebiotics have been largely used in pig nutrition because of their beneficial effects on the gut microbiota (Gresse et al., 2017; Nowland et al., 2019). Prebiotics are dietary fibers, mostly belonging to non-starch oligosaccharides, that cannot be hydrolyzed or absorbed in the first part of the digestive system. Therefore, they reach the colon where they are selectively fermented and cause specific alterations in the composition and/or activity of the gut microbiome, leading to a beneficial impact on the host's physiological status (Ducatelle et al., 2014). Prebiotics support the normal proliferation and differentiation of intestinal cells by promoting the growth of bacteria that produce short-chain fatty acids, such as butyrate-producing bacteria (Fouhse et al., 2016). Studies have shown that prebiotics can increase the proportion of Lactobacillus in the gut microbiota of weaning piglets and reduce the presence of potentially harmful bacteria, like Clostridium (Jiao et al., 2014) and Enterobacteriaceae (Castillo et al., 2008; O'Doherty et al., 2010). As well, prebiotics have demonstrated the potential to prevent post-weaning diarrhea or palliate its effects by combating *E. coli* pathogenic stains. For instance, galacto-oligosaccharides (GOS) and mannan- oligosaccharides reduce the in vitro adherence of *E. coli* F4 to intestinal mucins or porcine IPEC-J2 (Badia, Zanello, et al., 2012; Hermes et al., 2011; Sarabia-Sainz et al., 2013), and reduce the enterotoxigenic *E. coli* -induced expression of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF), and chemokines in these cells (Badia, Zanello, et al., 2012). Additionally, the supplementation of an alginate-derived oligosaccharide can alleviate the harm caused by *E. coli* F4 infection in weanling piglets by reducing apoptosis and promoting the growth of enterocytes in response to the challenge (Wan et al., 2018). All that evidence suggests that the use of prebiotics holds promise for enhancing pig health during early life. However, the emphasis of research has been on using prebiotics to decrease post-weaning diarrhea once are in the nursery phase, with a limited examination of their application during lactation.

Xylooligosaccharides (XOS) are sugar oligomers made up of 2–6 xylose units linked through β-(1→4)-linkages (Samanta et al., 2015) which have similar properties to the prebiotics. Their selective fermentation can change both the composition and activity of the gut microbiota, promoting the growth of Bifidobacterium and other beneficial fiber-degrading and SCFA-producing bacteria (Berger et al., 2021; M. Chen et al., 2020; J. B. Liu et al., 2018; Pan et al., 2019). Several studies have shown that XOS can improve gut health when supplemented in weaning pigs (J.B. Liu et al., 2018; Yin et al., 2019; Chen et al., 2021) and broiler diets (A. D. Craig et al., 2020; De Maesschalck et al., 2015; Ribeiro et al., 2018) and, in some cases, it enhances growth performance (Y. Chen et al., 2021; De Maesschalck et al., 2015). However, although the effects of XOS fulfill the definition of a prebiotic, they are described as a "stimbiotic", which is a new term defined as a "non-digestible but fermentable additive that can stimulate a fiber-degrading microbiome to increase fiber fermentability at doses which clearly are too low to contribute in a meaningful manner to short-chain fatty acid (SCFA) production" (González-Ortiz et al., 2019). Therefore, unlike prebiotics that are quantitatively fermented by the microbiome, stimbiotics simply improve the fermentation of fiber that is already present in the diet (González-Ortiz et al., 2019). For instance, supplementation of XOS at 0.1 – 0.2 g/kg has shown improvements in growth performance. Considering that the energy contribution of 0.1g of XOS added to 1 kg of feed represents a total of 0.3 kcal/kg of feed, it indicates that its mechanism of action cannot involve quantitative fermentation alone (J.B. Liu et al., 2018; Ribeiro et al., 2018; Yin et al., 2019). The increase in fiber fermentability caused by XOS may contribute to more oligosaccharide production, and therefore, SCFA production, which is considered an indirect mechanism of action to explain part of its health-related benefits (Bedford, 2019). However, the effects of XOS during the early life of piglets have been scarcely studied.

1.5.2.2 Supplementation of Prebiotics/Stimbiotics to Suckling Piglets Using Different Administration Methods

The common form to administer prebiotics or other functional additives to farm animals and ensure their consumption is their inclusion in the diet formula, as this is their main source of nutrients. However, the major source of nutrients for the suckling piglet is

maternal milk, where direct intervention is not possible. Given the critical influence of the sow in the early-life microbiota of their offspring through milk and feces (Bian et al., 2016), the supplementation of additives to the sow as an indirect method to modulate the gut microbial colonization of the piglets has been reviewed by previous thesis from our group (Mireia Saladrigas-García, 2022). Therefore, this thesis will focus on the methods for directly delivering prebiotics to piglets.

Since the feed intake is restricted in newborn piglets, researchers have investigated the benefits of providing oral supplements, such as prebiotics like inulin, β-glucans, and oligosaccharides (fructo-oligosaccharides (FOS) and GOS), to suckling piglets by oral gavage (Huting et al., 2021). Most studies have focused on administering these products daily for extended periods of time, ranging from 7 to 28 days, which may be longer than what is practical. The supplementation of prebiotics during early life generally demonstrated beneficial effects, although there are some discrepancies between studies (Table 1.1). Some studies observed modulations of the diversity indexes or improvements in the microbial composition, increasing the abundance of SCFA-producing bacteria (Schokker et al., 2018; Tian et al., 2019; Wu et al., 2020; Gao et al., 2021; Tian, Wang, Wang, et al., 2022), while others observed few changes in the microbiota associated to the treatment (Lépine et al., 2019; Ayuso et al., 2020; de Vries et al., 2020). Also, several studies demonstrated improvements in the epithelium morphology and/or barrier function (B. Li et al., 2018; Tian et al., 2018; Wu et al., 2020; Gao et al., 2021; Tian, Wang, Wang, et al., 2022) while others did not observe any effect (Schokker et al., 2018; Ayuso et al., 2020). Finally, some reports identified an anti-inflammatory effect of the prebiotics (Tian et al., 2018; Tian et al., 2019; Gao et al., 2021; Tian, Wang, Gao, et al., 2022), while others observed an upregulation of the expression of pro-inflammatory cytokines (Ayuso et al., 2020; Wu et al., 2020). Some of the discrepancies between studies seem to be associated with the type of prebiotic used, as the best results have been observed with the supplementation of GOS (Tian et al., 2018; Tian et al., 2019; Wu et al., 2020; Gao et al., 2021; Tian, Wang, Wang, et al., 2022; Tian, Wang, Gao, et al., 2022), while other prebiotics show more inconsistent results and have been less studied. Rather than proposing a strategy to be implemented in the farms, these

results establish a proof of concept of the potential of prebiotics supplementation for early-life programming. Therefore, research has explored alternative methods for administering additives to lactating piglets, such as its inclusion in supplemental milk or creep feed, with the goal of implementing a practical solution in farm practices.

Table 1. 1. Review of the main studies of oral gavage prebiotic supplementation in suckling piglets. The studies have been ordered by type of prebiotic supplemented and year of publication.

Prebiotic	Dose	Intervention period (Age)	Age at sampling	Effects versus a control oral gavage with water or physiological saline	Reference
Galacto-oligosaccharides (GOS)	1g/kg BW	d 0 - 7	d 8, d21	↑ growth during d14-21 Improves the jejunum barrier function: ↑ Small-intestine length at d8 ↓ Crypt depth at d21 ↑ mRNA expression of jejunal growth factors (d8 and d21) ↑ Protein expression of jejunal growth factors (d8) and tight junctions (d8 and d21) ↑ mRNA expression of jejunal nutrient transporters (d21) ↑ mRNA expression of cytokine TGF-β at d8, ↓ mRNA expression of cytokine IL-12 at d8 ↑ Jejunal lactase activity on d8, maltase activity and sucrase activity on d21	Tian et al., 2018
GOS	1g/kg BW	d 0 - 7	d 8, d 21	Promotes the growth of a healthy ileal microbiota, the production of SCFA and the expression of anti-inflammatory agents. = diversity indices (Shannon and Simpson) = richness estimators (Ace and Chao) ↑Firmicutes (d8), Lactobacillus and Unclassified Lactobacillaceae (d8, d21) ↓Proteobacteria and Steptococcus (d8), Clostridium sensu stricto (d8, d21), Escherichia, unclassified Bacteroidales and Alloprevotella (d21) ↓ pH ileal digesta (d8) ↑ Lactate, propionate, butyrate, and valerate (d8) and butyrate (d21) ↑ mRNA expression of defensins *PBD-1*, *PBD-3* (d8), *PBD-1* and cytokine *IL-10* (d21) ↑ endocrine peptide GLP-1 (d8)	Tian et al., 2019
GOS	1g/kg BW	d 1 to 13	d 14, 2h after a intraperitoneal injection with a LPS solution	Relieves the lipopolysaccharide (LPS)-induced colonic mucosa damage. ↓ malonaldehyde (MDA) level ↓ activity of reactive oxygen species (ROS) ↑ total anti-oxidation capacity (T-AOC) ↑ *norank_f__Muribaculaceae* and *Romboutsia* (colonic mucosa) ↓ *Alloprevotella*, *Campylobacter* and *Helicobacter* (colonic mucosa)	Gao et al., 2021

				↑ SCFA in the colonic digesta ↓ production of cytokines ↓ mRNA expression of MyD88-NF-κB ↑ mRNA expression of barrier function genes	
GOS	1g/kg BW	d 1 to 13	d 14, 2h after being intraperitoneally injected with a LPS solution	Relieves the LPS-induced inflammation response by: ↓ pro-inflammation cytokines (IL-1 β, IL-6, IL-8 and TNFα) level in the duodenum Relieve the LPS-induced oxidation by: ↑ T-AOC ↓ MDA level	Tian, Wang, Gao, et al., 2022
GOS	1g/kg suckling period and/or 2% in pre-starter diet.	Early-life GOS (ELG): d 1 to 7 by oral gavage. Post-weaning GOS (PWG): d 21 to 28	d 28	↑ post-weaning growth performance (PWG) ↓ post-weaning diarrhea frequency (ELG and PWG) ↑ACE and Chao-1 in colonic mucosa (PWG) ELG, PWG and its combination produce: ↑ butyrate producing microbiota in colonic mucosa (*Prevotellaceae* NK3B31 (ELG), *Prevotella 9* and *Prevotellaceae Unclassified* (PWG)) and colonic digesta (*Ruminococcaceae* UCG-014, *Faecalibacterium* (ELG), *Clostridium sensu stricto 1, Terrisporobacter* (PWG), *Dorea* and *Phascolarctobacterium* (Combined ELG + PWG)) ↑ SCFA production in colonic lumen ↓ inflammatory response by downregulation of MyD88-NF-κB signaling and ↓ production of cytokines ↑ expression of barrier function genes	Tian, Wang, Wang, et al., 2022
GOS + milk fat globule membrane + fructo-oligosaccharides (FOS)	1.2 g/kg BW	d 1 - 7	d 8, d 21	Modulated the gut microbiota in faeces and improved the intestinal barrier function: ↓α-diversity at d8 (Sobbs index, but not Shannon index) ↑α-diversity at d21 (Sobbs and Shannon index) ↑Lactobacillus (d8), Muribaculaceae, Christensenellaceae, Enterococcus and Romboutsia (d21) ↓Lachnospiraceae (d8), Eubacterium (coprostanoligenes group, d21) ↑Acetate and propionate (ileum), acetate (colon) ↑Gene expression of short-chain fatty acid receptors (ileum, colon) ↑Gene expressions of tight junctions, mucins and pro-inflammatory cytokines ↓Plasma diamine oxidase (d21)	Wu et al., 2020

FOS	10 g	d 2 - 14	d 14, d 25	Bifidogenic effect in the colon, changes in mucosal gene-expression profiles in the jejunum relating to intestinal barrier function and immunity (d14 and d25). = intestinal morphology	Schokker et al., 2018
Short-chain FOS	1 g	d 0 - 7; d 0 - 21	d 0, d7, d 21 (weaning) and d 35	↑ growth in piglets with average weight (between 25% and 75% quartiles) and reduced post-weaning mortality to 0% = composition and diversity of fecal microbiota = intestinal morphology ↑ mRNA expression of interferon-gamma (IFN-γ)	Ayuso et al., 2020
Inulin	0.5 to 2 g; 0.75 to 3 g	d 1 - 28	d 28	↑ morphology of the small intestine ↓ mRNA expression pro-inflammatory cytokines at mRNA in the large intestine The low inulin level was more beneficial, as it also increased the concentrations of propionate and iso-butyrate in the large intestine	Y. Li et al., 2018
Inulin	0.114 g/kg BW	d 2 - 23	d 23	↑Health status and postweaning feed efficiency = Composition and diversity of fecal microbiota	Lépine et al., 2019
Inulin	0.5 to 2 g; 0.75 to 3 g	d 1 - 28	d 28	Modest transcriptomic changes in the ileum: ↓ mRNA expression of ANGPTL4, APOA1 and AQP7, important genes in lipid metabolism.	Schroyen et al., 2021
β-glucans	50 to 200 mg every other day	d 2 - 27	d 27	Modest modulation of the gut microbiota in feces and immune system: ↓Shannon diversity in feces (d4, 8, 14, 26), no differences in jejunum, ileum and caecum digesta ↓Methanobrevibacter (d8, 14) ↑Fusobacterium and Ruminococcaceae (several time-points) ↓Proliferating NK immune cells and γδT cells in blood at d26 ↑IL-10 cytokine production by mesenteric lymph node cells (ex vivo)	de Vries et al., 2020

Supplemental milk is used in maternities to improve pre-weaning survival and performance in large litters and when the milking ability of the sow is impaired, although studies on its effectivity have yielded inconsistent results (Baxter et al., 2020). Apart from its nutritional function, it can be used to deliver probiotics to suckling piglets. The supplementation of milk formula with prebiotics, including polydextrose, FOS, GOS, and yeast β-glucans has been shown to affect gut morphology, digestive function, mucosal immune system, and microbiota in piglets (Hester et al., 2012; Alizadeh et al., 2016; Berding et al., 2016; Kruger et al., 2017; Fleming et al., 2019). However, these studies were using artificially reared piglets, mostly as a model for human infant formula, rather than for its use as a supplement for sow-reared piglets. Therefore, further research is needed to determine the effectiveness of this method of supplementation in sow-reared piglets.

The other method to deliver nutrients of interest to suckling piglets is through its inclusion in the creep feed. Creep feed is provided in the maternities to habituate the piglet to solid feed prior to weaning and may contribute to a reduction of pig BW variability from weaning onward (Solà-Oriol & Gasa, 2017). Pigs eating creep feed during lactation start to consume earlier in the nursery rooms and increase their feed intake and growth during the first days post-weaning (Bruininx et al., 2002; Kuller et al., 2007; Middelkoop et al., 2020; Sulabo, Tokach, Dritz, Goodband, DeRouchey, et al., 2010). Nevertheless, the literature shows inconsistent results regarding the effects of creep feed on gut development (Huting et al., 2021). Only a few studies reported subtle beneficial effects on the GIT morphology and function, mostly observed post-weaning (Cabrera et al., 2013; Kuller et al., 2007; Muns & Magowan, 2018; van der Meulen et al., 2010). However, creep feed contains a broad diversity of nutrients not found in milk, which explains why its consumption has been demonstrated to affect gut microbiota (Torrallardona et al., 2012; Bian et al., 2016). Its effects are dependent on the age at provision, its nutrient composition, and the intake level (R. C. Sulabo et al., 2010; van Hees et al., 2019).

Some authors have studied nutritional interventions in creep feed using prebiotics (Tran et al., 2014; Fouhse et al., 2019) or stimbiotics (Bai et al., 2021), although most interventions in creep feed aimed at modulating piglet microbiota have focused on

incorporating soluble and insoluble fibers (L. Zhang et al., 2016; Mu et al., 2017; van Hees et al., 2019; Bai et al., 2021; Choudhury et al., 2021; van Hees et al., 2021; van Hees et al., 2023). Most of the studies reveal significant effects on gut morphology, gut microbial colonization, and maturation of the mucosal immune system, but only two of them monitored if the piglets consumed the treatment (Choudhury et al., 2021; van Hees et al., 2023).

In fact, the main limitation of interventions through supplemental milk or creep feed is the variability in its consumption among littermates and different litters (Azain et al., 1996). In general, the onset of supplementary milk consumption starts much before creep feed. The literature describes that during the first two weeks after birth, 51% of piglets already started to eat formula milk (de Greeff et al., 2016) while only around 5% have eaten creep feed (Huting et al., 2018; van der Meulen et al., 2010). De Greff et al. (2016) described that supplemental milk can archive 87% of consumers at week 3 of lactation but other authors observed wide variations between litters, ranging from almost 0 to more than 20 liters consumed per piglet at the third week of lactation (Azain et al., 1996). On the other hand, the number of piglets consuming creep feed increases exponentially with age (Huting et al., 2018), reaching between 60% and 80% of consumers at the end of lactation depending on weaning age (R C Sulabo et al., 2010; Middelkoop et al., 2020). However, creep feed consumers also show an important variability in the intake, ranging from ~100 to 1500 g of creep feed per pig for a 28-day period (Barnett et al., 1989; Middelkoop et al., 2020). It seems that the variations in the intake of supplemental milk and creep feed can be explained by the intake of milk from the sow (Miller et al., 2012; Middelkoop et al., 2019). For example, piglets suckling the middle and posterior teats consume more creep feed than those suckling the anterior teats, which generally produce a higher quantity and quality of milk (Huting et al., 2017). It appears that those who cannot satisfy their needs with maternal milk are more likely to explore alternative sources of nutrients. Therefore, it is important to consider individual piglet behavior and preferences when designing feeding strategies to ensure an optimal supplementation and response to the treatment. Nevertheless, **given the difficulties to achieve high consumption of nutritional supplements during lactation,**

stimbiotics, such as XOS, represent an interesting candidate to modulate the microbiota as they have shown effectiveness at very low doses.

1.5.2.3 Long-Term Effects of Preweaning Interventions in the Microbiota

Earlier in this revision, we explored the concept of "microbial programming" during the suckling period, which refers to the fact that changes in the postnatal microbial colonization have a long-term impact on gut community structure, gut maturation, metabolic development, and immune system development (Thompson et al., 2008). However, following this period, weaning triggers major changes in the gut microbiota, leading to a more mature and stable state (Pajarillo et al., 2014; Saladrigas-García, D'Angelo, Ko, Nolis, et al., 2021). This microbial maturation results in a more homogenous structure and composition among piglets, which might blur the effects of interventions made during suckling (Chen et al., 2017; Saladrigas-García, D'Angelo, Ko, Nolis, et al., 2021). Although there is an important number of studies evaluating preweaning nutritional strategies in pigs, most of them only measured the effects until weaning. Only a small number of studies followed up on the progression of the effects during the nursery period, obtaining contrasting results. Supplementation of inulin by oral gavage and the inclusion of XOS in creep feed during the whole lactation improved post-weaning feed efficiency, in the case of XOS associated with an improved SCFA-production capacity of the microbiota (Lépine et al., 2019; Bai et al., 2021). However, van Hees et al. (2021) observed that neither the inclusion of a high fermentable nor a low-fermentable fiber source in creep feed affected post-weaning growth or the gastrointestinal health of piglets. Other studies evaluated the effects of extending the pre-weaning interventions with probiotics during the post-weaning period (Tran et al., 2014; Fouhse et al., 2019; Tian, Wang, Wang, et al., 2022). The 10% inclusion of yeast-dried milk in creep feed from d7 of lactation until d7 after weaning resulted in an improvement in performance during the first 28 days after weaning and an increase in microbial diversity at d7 (Tran et al., 2014). In addition, a 0.08% dietary supplementation of a yeast-derived mannan-rich fraction in creep feed during the lactation and post-weaning periods improved jejunal morphology and altered cecal microbial population at d7 postweaning, but the effects vanished at d21 (Fouhse et al., 2019). Finally,

Tian, Wang, Wang, et al. (2022) observed that 1g/kg of GOS supplementation by oral gavage during d1-7 of the suckling period improved piglet's gut microbial composition, increased SCFA concentration in the colon and reduced the inflammatory status of the gut at d7 post-weaning, but they reported that these effects were magnified when 2% GOS was also supplemented in nursery feed. Overall, **the long-term effects of pre-weaning interventions with prebiotics or stimbiotics may be inconsistent across studies. However, the potential benefits of this strategy are promising enough to encourage further research in this area**.

1.5.3. Avoiding Mixing Pigs at Weaning to Reduce Stress and Enhance Productivity

Commercial pig farms commonly mix pigs sorting them by body weight and sex at weaning and when they are transported to slaughter. However, farms may also mix pigs at the beginning of the grower-finisher period or at the late-finisher stage (Garcia & McGlone 2021). The purpose of this practice is to fit the group size to the pen dimensions and/or to minimize the variation of body weight (BW) in the pens, aiming to obtain a more uniform weight at slaughter (Peden et al., 2018). However, it has been observed that the initial decrease in BW variability within the pen obtained when mixing often increases to levels similar to those obtained without regrouping the pigs (O'Quinn et al., 2000). In addition, mixing unfamiliar pigs requires re-establishing social hierarchies through aggressive behavior, which lasts around 24 hours after mixing (Meese & Ewbank 1973; Stookey & Gonyou 1994) before a stable hierarchy is formed (Hayne & Gonyou 2006). Fights among pigs cause skin injuries and cause high levels of stress, which might have immunosuppressive effects (Morrow-Tesch et al., 1994; Peden et al., 2018). If mixing is performed during weaning, it can exacerbate the stress experienced by pigs during this period, which may delay the onset of feed intake and increase the risk of post-weaning diarrhea (J.P. Lallès et al., 2007). These effects frequently lead to a decrease in growth performance and deleterious effects on welfare (Stookey & Gonyou 1994; Hyun, Ellis, and Johnson 1998; Hyun, Ellis, Riskowski, et al., 1998; Coutellier et al., 2007; Camp Montoro et al., 2021; Camp Montoro et al., 2022).

The literature describes a significant effect of mixing on growth performance. Camerlink et al. (2021) reported poorer growth during the first week post-weaning after

mixing weaned pigs compared to maintaining litters of siblings as pen mates. As well, Jones et al., 2011 (Jones et al., 2011) studied how the number of littermates kept in the same group until slaughter affected the body weight gain and the backfat of the pigs. They determined that each additional sibling per group increased the average daily gain by 5.5 ± 1.6 g/day and the back fat by 0.12 ± 0.05 mm. The effect of regrouping at the beginning or in the late growing-finishing period on growth performance has been more extensively studied but showed contradictory results. Two recent studies from Camp Montoro et al. (2021; 2022) reported the detrimental effects of mixing pigs at 11 weeks of age on performance at 21 weeks of age compared to keeping them in intact litter groups. However, they did not identify any effect on the number of skin lesions. These results are in line with other authors who observed negative effects on performance when pigs were mixed at weaning and regrouped at the beginning (Hyun, Ellis, & Johnson 1998; Hyun, Ellis, Riskowski, et al., 1998) or in the late growing-finishing period (Stookey & Gonyou 1994). On the other hand, other reports described no effect of mixing on performance when pigs were mixed at weaning and the beginning of the growing-finishing period (O'Connell et al., 2005; Li & Johnston 2009). The explanation for the negative effects of mixing on performance provided by some authors relies on the higher energy expenditure in establishing a new social order within the group when they met the unfamiliar pigs rather than in a reduced feed intake (Coutellier et al., 2007). However, Stookey & Gonyou (1994) reported that the effects of mixing finishing pigs in growth performance were not immediate and the delay might be explained by the stress that persist after the dominance fights subsided.

The effects of the fights during the re-establishment of hierarchy on pig welfare and performance during the first days after mixing are well documented. However, the effects of chronic aggression have received less attention, although its impact on welfare and performance seems notorious (Tan et al., 1991). The literature shows that aggressive behavior decreases within pens after a stable social order is established about two weeks after mixing the pigs (Stookey & Gonyou 1994). Nevertheless, stable groups still exhibit chronic aggression related to maintaining dominance relationships, which can be exacerbated by resource competition (Giersing & Studnitz 1997). The hierarchy defined

after regrouping determines which pigs have priority access to the feeder. In a situation where there is a feed restriction or a feeder space limitation, animals on the top of the dominance hierarchy will have the access to feeder granted, while the subordinate pigs might struggle to reach the feeder. This competition results in higher BW variability in the pen and a higher number of slow-growing pigs (López-Vergé, Gasa, Temple, et al., 2018). Additionally, limited feeder space has been linked to an increased incidence of body lesions (López-Vergé, Gasa, Temple, et al., 2018; Laskoski et al., 2019) and can force some pigs, especially those with slower growth rates, to alter their feeding patterns towards nocturnal hours, raising significant concerns about animal welfare (Young & Lawrence 1994). **Keeping intact litters in nursery pens can influence the social network and coping mechanisms of piglets when resources are limited, leading to potential benefits for welfare and reduced chronic aggression. Furthermore, this practice may positively impact the evolution of body weight variability**. Despite these potential benefits, there is a lack of research exploring the long-term effects of maintaining litter groups at weaning under resource limitations. As such, it is crucial to fulfill this knowledge gap and investigate the impacts of this practice.

1.5.4. Post-Weaning Supplementation of Animal By-Products to Optimize Gut Functionality

Weaning is associated with several stressors that cause a delay in the onset of solid food intake in piglets (J.-P. Lallès et al., 2007). Starvation can cause atrophy of the gastrointestinal epithelium, and reduction in the brush-border digestive enzyme activities and pancreatic secretions, which can impair the gut's digestive and absorptive capacity (Campbell et al., 2013). When the period of starvation ends, piglets may consume more feed than their gastrointestinal tract can handle, as their mucosa has not yet adapted to producing the necessary digestive enzymes for vegetable-based feeds (Miller et al., 1986). Furthermore, some ingredients of vegetal origin included in the pig feed contain anti-nutritional factors, such as antigens, trypsin inhibitors, and lectins, which can reduce nutrient availability (Li et al., 1991; Salgado et al., 2002). This may result in an increased flow of undigested feed in the distal parts of the gastrointestinal tract, made available for

microbial fermentation. The presence of fermentable substrates in pig diets can influence the microbial ecology in the gut and have health-promoting or detrimental effects on the host (Aumiller et al., 2015). The promotion of lactic acid bacteria and SCFA production, particularly butyrate, is believed to be advantageous for the host and can help maintain a healthy gut environment. Conversely, bacterial protein fermentation, which mostly occurs in the hindgut, poses a potential risk to intestinal barrier function, and may lead to an increase in enteric diseases caused by pathogens (Pieper et al., 2016). In the past, subtherapeutic doses of antibiotics were commonly used as growth promoters to tackle these issues. However, since their usage has been prohibited, alternative strategies are needed to minimize the risk of intestinal diseases. As a result, post-weaning diets now tend to contain lower amounts of protein but of higher quality (Pieper et al., 2016). One popular approach is to include animal protein in the formula, including whey protein, fish meal, spray-dried plasma (SDP), or protein hydrolysates from animal processing by-products. These protein sources have greater palatability than vegetal sources (Martínez-Puig et al., 2007; Solà-Oriol et al., 2010, 2011), which can accelerate the onset of feed intake after weaning.

From the industrial fractionation of blood from healthy animals it is obtained SDP, which is a protein-rich (70-80% crude protein (CP)) substance. The blood is collected using an anticoagulant and centrifuged to separate the blood cells. The resulting plasma is then concentrated and subjected to high-pressure spray drying, achieving a minimum temperature of 80°C. This process helps to preserve the biological activity of the proteins within SDP (Borg et al., 2002). Since its introduction as a protein source for pig diets in the late 1980s (Gatnau & Zimmerman 1990), numerous studies have highlighted the benefits of using SDP. A revision on the effects of SDP supplementation on weaning piglets (Pérez-Bosque et al., 2016) and a meta-analysis of its effects on performance (Balan et al., 2021) have been published, and both relate a robust beneficial effect of SDP on performance.

The effect of SDP was initially attributed to an increase in feed intake due to improved palatability of the diets (Ermer et al., 1994). However, the increased feed consumption may be also attributed to enhanced piglet health. Further studies evidenced

that SDP displayed growth benefits unrelated to food intake, indicating the existence of a distinct biological impact (Jiang et al., 2000). Apart from the benefits on performance, SDP has been shown to reduce the incidence of post-weaning diarrhea (Gatnau & Zimmerman 1991; van der Peet Schwering & Binnendijk GP 1995; Cain & Zimmerman 1997), to preserve the integrity of the gut mucosa, modulating the immune status and reducing mortality (Owusu-Asiedu et al., 2003; Pérez-Bosque et al., 2016; Pujols et al., 2016; Y. Zhang et al., 2016). These effects have also been observed in piglets experimentally challenged with E. Coli K 88 (Owusu-Asiedu et al., 2003; Torrallardona et al., 2003; Torrallardona et al., 2007) or fungal mycotoxins (Müller et al., 2018). In fact, SDP has been found to be particularly effective in younger pigs with immature immune systems (Torrallardona et al., 2002) or in lower sanitary conditions (Coffey & Cromwell 1995).

The presented evidence suggests that SDP supports the immune system, and it may act directly against pathogens (Coffey and Cromwell 1995; Bergstrom et al., 2014). The immunoglobulin-rich fraction in plasma is believed to be responsible for these beneficial effects (Pierce et al., 2005). Although weaning piglets cannot absorb immunoglobulins through the intestinal epithelium, these still contribute to the piglet's innate immune response by helping to neutralize toxins and opsonize pathogens in the lumen (Pérez-Bosque et al., 2016). Apart from the immunoglobulins, SDP is believed to contain a variety of bioactive components, such as hormones, growth factors, and bioactive peptides, which are known to be present in milk. These proteins can interact with immune cells present in the mucosa, thereby altering the cytokine environment (Pérez-Bosque et al., 2016). In fact, SDP can decrease the production of pro-inflammatory cytokines (such as TNF-α, IL-8, and interferon-gamma (IFN-γ)) (Bosi et al., 2004) and increase the production of the anti-inflammatory cytokine IL-10 in the intestinal mucosa (Maijó et al., 2012). This reduction in immune system activation by SDP has been associated with improved villous height, reduced cellularity (Nofrarías et al., 2006), and increased intestinal tightness (Y. Zhang et al., 2016), even in non-inflamed pigs (Peace et al., 2011). In addition to its effects on the intestine, SDP has also been found to modulate the immune response in other mucosal areas. For instance, when weaned pigs were fed SDP in the presence of the swine influenza

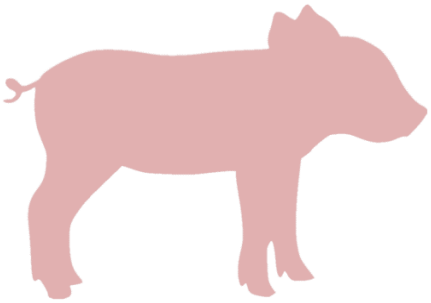
virus, they exhibited reduced lung lesions compared to those on a non-supplemented diet (Campbell et al., 2011). Moreover, SDP was able to prevent the increase in activated lymphocyte populations during lung inflammation induced by LPS (Maijó et al., 2012). Including SDP in post-weaning diets is widely recognized as an effective approach for improving piglet health and performance. However, its restricted availability and elevated cost are limiting factors for its incorporation into the formula. Therefore, in pig nutrition, there is a tendency to search for cheaper alternatives with similar properties.

Porcine digestible peptides (PDP) is a coproduct of the heparin industry and are obtained from the enzymatic hydrolysis of the porcine intestinal mucosa. Scientific evidence supports that PDP can be used as a cheaper alternative to other sources of high-quality protein. For instance, PDP has demonstrated high palatability and is equally preferred by weaned piglets compared to SDP, fish meal, and whey protein (Figueroa et al., 2016; Solà-Oriol et al., 2011). In addition, using PDP in the diet of weaning piglets has resulted in comparable growth performance to these other protein sources. First, Cho et al. (2010) observed that weanling pigs fed diets with PDP had similar or better growth performance than those fed diets with SDP. Meanwhile, J. H. Kim et al. (2000) observed that replacing 6% of SDP with PDP resulted in reduced growth performance, but a combination of 3% SDP and 3% PDP showed equivalent growth. Moreover, Myers et al. (2014) observed a similar growth performance in animals fed PDP and fish meal, while Solà-Oriol et al. (2010) found improved growth performance, feed intake, and efficiency in animals fed PDP compared to fish meal. Additionally, PDP has shown similar digestibility of the most essential amino acids compared to fish meal (Sulabo et al., 2013).

The inclusion of PDP in weanling diets has shown improvements in villus height of the small intestine compared to some sources of intact protein like SDP (Borda et al., 2005) or fish meal (Xin et al., 2001), which might be associated with a positive impact on nutrient uptake. In addition, the beneficial impact of PDP on growth performance may be due in part to its content of short-chain peptides that are more easily absorbed by pigs than intact proteins (Gilbert et al., 2008) or even than free amino acids (Rérat et al., 1988). Despite the evidence of a beneficial impact of PDP on growth performance and gut morphology, there

is no existing literature that investigates the potential impact of PDP on intestinal function. However, these findings suggest that **substituting (completely or partially) the inclusion of SDP with PDP in starter diets might be an effective strategy to reduce the cost of the diet while maintaining the benefits in growth performance and intestinal function**.

These two additives are hypothesized to provide benefits during the initial phase of the nursery period. However, lighter pigs at weaning may have a less developed digestive tract compared to their average counterparts (Michiels et al., 2013). Therefore, they might benefit from extending the provision of these ingredients for a longer period.



Chapter 2

Hypothesis and Objectives

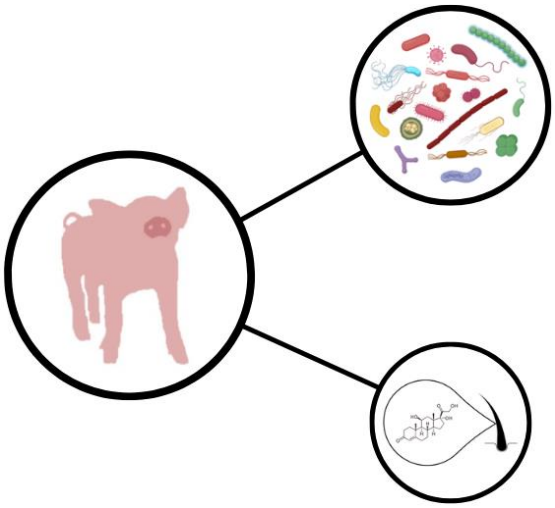
The general hypothesis of this thesis is that the piglet's early-life environment plays a crucial role in their subsequent development. Thus, providing appropriate nutrition and management practices during lactation and post-weaning periods is key to promoting adequate microbial colonization, improving intestinal health, and enhancing welfare, which will benefit their future growth performance. The characterization of the gut microbiota, the stress biomarkers, the intestinal gene expression, and the behavior of the pigs will provide relevant data about the mechanisms that influence the early-life determinism of pigs and their growth response. Therefore, the starting hypotheses of this Ph.D. dissertation were:

I. Piglet's early-life gut microbial colonization and the stress response they experience before and after weaning are causal drivers of their individual growth responses during lactation and nursery periods.
II. Supplementing XOS to suckling piglets can potentially modulate the initial colonization of their intestinal tract, promoting the growth of butyrate-producing bacteria and leading to a more mature microbiota that is more prepared to handle the weaning transition. Furthermore, this may influence the establishment of a distinct microbiota population post-weaning.
III. Providing a stable social environment for piglets, where they can remain with their littermates after weaning, might mitigate the detrimental effects of social stress related to weaning and other challenges on their growth performance and welfare, with major effects in slow-growing pigs.
IV. The replacement of plant-based protein sources with highly digestible protein sources in the pre-starter diet of piglets may enhance their performance post-weaning, as well as promote intestinal health and function.
V. Allowing piglets to consume the pre-starter diet until they reach a target body weight, rather than offering it until a fixed date will have a positive impact on their performance and contribute to reducing batch variability.

In this context, the general objective of the present doctoral thesis was to investigate the factors from birth to the post-weaning period that influences the piglet's response capacity and future productive performance, making emphasis on the experienced stress and the interaction between gut microbiota and the host. Furthermore, this thesis aimed to evaluate different nutritional and management interventions that promote an adequate environment for the development of the piglet and help him to deal with the weaning stress, especially to improve the performance of those piglets that initially present the lowest efficiency. Thus, the specific objectives were:

- **Chapter 3**: Find links between the fecal microbiota composition, fermentation capacity, and glucocorticoid biomarker levels in piglets' hair and their growth performance during lactation and nursery periods.
- **Chapter 4**: Examine whether the supplementation of XOS to suckling piglets on a daily basis affects their fecal microbiota composition and functionality at the end of the suckling period, and also during the nursery period while exposed to identical diet and environmental conditions.
- **Chapter 5**: Analyze the impact of keeping litters together after weaning, instead of mixing them, and providing them with a low nutrient and energy density dietary regime during the nursery period on their growth performance and welfare during the nursery and growing-finishing period.
- **Chapter 6**: Determine the effect of partially substituting a high inclusion of soybean ingredients in the pre-starter diet with either SDP, PDP, or the combination of both in the growth performance of piglets and the expression of genes related to intestinal function in their gut tissue.
- **Chapter 7**: Evaluate the effects of providing piglets a higher allowance of a pre-starter diet which includes SDP and PDP, until they reach a targeted BW, on their growth performance and the BW variability of the batch.

The following chapters will detail the methodology used to evaluate the previously mentioned hypothesis and goals, along with the main findings observed in this thesis. The findings and conclusions drawn from each section will be further discussed in Chapter 8.



Chapter 3

Fecal Microbiota and Hair Glucocorticoid Concentration Show Associations with Growth during Early Life in a Pig Model

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Abstract

Identifying characteristics associated with fast or slow growth during early life in a pig model will help in the design of nutritional strategies or recommendations during infancy. The aim of this study was to identify if a differential growth during lactation and/or the nursery period may be associated with fecal microbiota composition and fermentation capacity, as well as to leave a print of glucocorticoid biomarkers in the hair. Seventy-five commercial male and female pigs showing extreme growth in the lactation and nursery periods were selected, creating four groups (First, lactation growth, d0–d21; second, nursery growth, d21–d62): Slow_Slow, Slow_Fast, Fast_Slow, and Fast_Fast. At d63 of life, hair and fecal samples were collected. Fast-growing pigs during nursery had higher cortisone concentrations in the hair ($P < 0.05$) and a tendency to have a lower cortisol-to-cortisone ratio ($P = 0.061$). Both lactation and nursery growth conditioned the fecal microbiota structure ($P < 0.05$). Additionally, fast-growing pigs during nursery had higher evenness ($P < 0.05$). Lactation growth influenced the relative abundance of eight bacterial genera, while nursery growth affected only two bacterial genera ($P < 0.05$). The fecal butyrate concentration was higher with fast growth in lactation and/or nursery ($P < 0.05$), suggesting it has an important role in growth, while total SCFA and acetate were related to lactation growth ($P < 0.05$). In conclusion, piglets' growth during nursery and, especially, the lactation period was associated with changes in their microbiota composition and fermentation capacity, evidencing the critical role of early colonization on the establishment of the adult microbiota. Additionally, cortisol conversion to cortisone was increased in animals with fast growth, but further research is necessary to determine its implications.

2.1. Background

Early life experiences and environmental conditions have a critical role in the growth and development of mammals. On the one side, the early life microbial colonization of the gut, shaped by factors such as host genetics, environment, diet, immunological pressure, and antibiotics (Francino 2014; Gensollen et al., 2016b; Gresse et al., 2017), may influence the programming of the mucosal immune response and the development of the gut barrier function, conditioning their propensity to develop certain health disorders (Francino 2014; Gensollen et al., 2016b; Gresse et al., 2017). Evidence suggests that a disruption of microbial colonization might cause lifelong deficits in growth and development (Robertson et al., 2019). On the other hand, early life stress has also been linked to physical and psychological sequelae later in life, including alterations in the immune system (Fogelman & Canli 2019; Smith & Pollak 2020). Extended exposure to glucocorticoids leads to increases in the sympathetic nervous system, hypothalamic pituitary adrenal axis, and inflammatory markers (Fogelman & Canli 2019; Smith & Pollak 2020) and has been associated with an impairment of growth and development both in humans and animal models (Abrantes et al., 2019; Mukherjee et al., 2022). Therefore, studying the differences in the microbial colonization process and the experienced stress during early life among individuals with differential growth responses might help to understand which conditions are associated with optimal growth during this period.

Humans and pigs share a great physiological similarity in digestive and associated metabolic processes. In fact, the neonatal piglet has become a good model for the study of pediatric nutrition and metabolism (Heinritz et al., 2013). In addition, it is well-established as a model for assessing interactions between microbiota and health, as it exhibits similar scenarios to humans, such as weaning diarrhea (Heinritz et al., 2013). The individual growth response of piglets during their early life on a commercial farm is affected by multiple factors. During lactation, colostrum and milk intake are the primary drivers of piglet growth (López-Vergé, Gasa, Farré, et al., 2018). Initial differences in the birth BW and vitality among piglets will influence the sibling competition for the sow's nutrients and may have a major

effect on survival and growth (S. L. Douglas, Edwards, et al., 2014; Milligan et al., 2002). Piglet's weaning is a stressful event involving separation from the sow, an abrupt change in diet from sow's milk to a plant-based diet, and the establishment of new social hierarchies in the nursery pens (J.P. Lallès et al., 2007). Afterward, the postweaning growth will highly depend on the management and nutritional strategies conducted to accelerate piglet adaptation to the solid diet and the new housing conditions (J. M. Campbell et al., 2013; Huting et al., 2021). When the stressful stimuli of weaning overpower a piglet's adaptation, it can lead to chronic stress, poor performance, and increased mortality (Sutherland et al., 2006; Campbell et al., 2013; Martínez-Miró et al., 2016). Piglets belonging to the same farrowing and weaning batch are exposed to very similar housing and environmental conditions, and they share a large part of their genetic background. These similarities and the challenges they face during the lactation and nursery periods make them a good model to study the factors that determine their individual growth responses and development during early life. Thus, identifying the characteristics of pigs with different growth rates during these periods is the first step to understanding those mechanisms and to designing individualized strategies to stimulate the development of slow-growing individuals.

In this study, it was hypothesized that gut microbiota, as well as the experienced stress during the lactation and nursery periods, might have a relationship with the piglets' individual growth responses during these periods. Therefore, the aim of this study was to identify associations among the growth performance during the lactation and nursery periods with the fecal microbiota composition, its fermentation capacity, and the levels of glucocorticoid biomarkers in their hair.

2.2. Materials and Methods

2.2.1. Animals, Housing, and Diet

The study was conducted in the farrowing room of a commercial farm from farrowing day to the day of weaning (d21) and in the nursery unit of the same farm during the 41 days post-weaning (d62).

A total of 324 commercial male and female piglets ((Landrace × Yorkshire) × Pietrain) from a single weaning batch (weaned at d21 ± SD 0.8 days of life) were weighed at birth (d0) and at d21 of lactation (weaning). A subset of 70 pigs with slow growth during lactation (139 ± SD 20.4 g/d, ranging from 97 g/d to 173 g/d, belonging to the 10–42% percentile of the initial population) and 81 pigs with fast growth during lactation (248 ± SD 21.2 g/d, ranging from 208 g/d to 295 g/d, belonging to the 60–93% percentile of the initial population) were weighed again at the end of nursery period (d62 of life). Then, pigs with slow growth during the nursery period from each subset were selected (6–30% percentile from the slow lactation subset and 5–26% percentile from the fast lactation subset) and also pigs with fast growth during the nursery period (76–100% percentile from the slow lactation subset and 79–100% percentile from the fast lactation subset), obtaining four groups as a factorial design: slow growth during the lactation and nursery periods (Slow_Slow; *n* = 18; 9 males + 9 females), slow growth during lactation but fast during the nursery period (Slow_Fast; *n* = 19; 10 males + 9 females), fast growth during lactation but slow during the nursery period (Fast_Slow; *n* = 19; 9 males + 10 females), and fast growth during the lactation and nursery periods (Fast_Fast; *n* = 19; 10 males + 9 females). The growth characteristics of each group are described in Table 3.1.

Table 3. 1. Mean body weight (BW) and average daily gain (ADG) of the animals included in each group for the lactation and nursery periods. Each mean is followed by its corresponding SEM.

Group [1]	Group Size (*n*)	Lactation ADG, g/d	Nursery ADG, g/d	Birth BW, kg	Weaning BW, kg	Nursery BW, kg
Fast_Fast	19	246 ± 5.2 [a]	273 ± 7.1 [a]	1.45 ± 0.026 [a]	6.6 ± 0.09 [a]	17.9 ± 0.33 [a]
Fast_Slow	19	244 ± 4.6 [a]	128 ± 3.7 [c]	1.31 ± 0.069 [ab]	6.4 ± 0.09 [a]	11.7 ± 0.20 [c]
Slow_Fast	19	148 ± 3.3 [b]	238 ± 5.7 [b]	1.35 ± 0.074 [ab]	4.4 ± 0.11 [b]	14.2 ± 0.31 [b]
Slow_Slow	18	138 ± 5.9 [b]	112 ± 3.5 [d]	1.21 ± 0.056 [b]	4.1 ± 0.11 [b]	8.7 ± 0.17 [d]
p-value		<0.001	<0.001	0.041	<0.001	<0.001

[1] Fast_Fast: pigs showing fast growth during the lactation and nursery periods, Fast_Slow: pigs showing fast growth during the lactation period and slow growth during the nursery period, Slow_Fast: pigs showing slow growth during the lactation period and fast growth during the nursery period, and Slow_Slow: pigs showing slow growth during the lactation and nursery periods. [a,b,c,d] Values with different letters in the same column are significantly different, according to ANOVA and Tukey's adjust.

Sows and their litters were housed in individual farrowing pens (2.6 × 1.8 m^2) on a partially slatted floor with a heated floor pad for piglets, equipped with a farrowing crate, an individual feeder, and nipple drinkers for sows and piglets. The temperature in the farrowing room was automatically controlled (22–24 °C). Water and feed were offered ad libitum to the sows. Piglets had access to water and were offered creep feed (2558 kcal NE/kg, 19.7% CP, and 1.370 digestible Lys) since d7 of lactation.

At weaning (d21), piglets were moved to the nursery unit without transport. Piglets were allocated in 2 pens blocked by sex (280 pigs/pen) and mixed with other animals that were not monitored since birth. Each pen (60 m^2) was equipped with eight commercial feeders (Tolva EVO-800D, Porinox, Olot, Spain) and eight nipple bowl drinkers to provide ad libitum access to feed and water. The floor was completely slatted, and the temperature and ventilation rates were controlled using central and forced ventilation with an automatic cooling system. The first two days post-weaning, all animals were offered the creep feed diet and then were fed a pre-starter diet (2430 kcal NE/kg, 18.94% CP, and 1.29% standardized ileal digestible (SID) Lys) until d10 post-weaning. Afterward, a starter diet (2500 kcal NE/kg, 17.5% CP, and 1.18% SID Lys) was offered until the end of the nursery period. All diets were formulated to meet the requirements for the maintenance and growth of newly weaned piglets (National Research Council, 2012) (Table 3.2).

Table 3. 2. Diets offered to the animals included in the trial.

Ingredient, %.	Creep Feed	Pre-Starter	Starter
Wheat	17.8	23.0	17.5
Barley	7.0	10.0	30.7
Maize	1.2	1.4	21.7
Rapeseed	-	-	3.0
Sweet milk whey	15.0	10.0	-
Broken rice	15.0	15.0	-
Spray dried yogurt	13.2	-	-
Soy protein concentrate	8.4	3.7	-
Soybean meal 47% crude protein (CP)	-	13.0	-
Soybean meal 44% CP	-	-	12.3
Extruded soybeans	-	6.0	-
Porcine digestible peptides (PDP) 62% CP	5.8	2.1	-
PDP 50% CP	-	-	5.7
Animal plasma 80% CP	1.7	1.7	-
Skimmed milk	3.3	-	
Extruded cereals [1]	3.0	-	-
Lard	1.0	1.5	3.3
Lactose	-	2.8	-
Sucrose	2.2	1.7	-
Sugar beet pulp	2	1.8	0.5
Wheat bran	1.6	3.8	-
Lignocellulose 65% crude fiber (CF)	-	-	1
Vit-Min premix [2]	0.5	0.5	0.5
Liquid lysine 50%	0.61	0.62	0.99
DL-Methionine	0.26	0.24	0.22
L-Threonine	0.19	0.18	0.27
L-Valine	0.16	0.08	0.09
L-Tryptophane	0.10	0.07	0.07
Histidine	-	-	0.11
Isoleucine	-	-	0.03
Mono calcium phosphate	-	0.42	1.10
Calcium carbonate	-	0.19	0.71
Salt	-	0.26	0.25
Calculated composition			
NE, kcal/kg	2558	2430	2500
Ash, %	5.1	5.0	5.2
Crude protein, %	19.7	18.9	17.5
Ether extract, %	4.7	4.1	6.6
Crude fiber, %	1.9	3.0	5.4
Starch, %	29.8	30.0	39.1
Calcium, %	0.550	0.650	0.700
Total *p*, %	0.484	0.515	0.626
Digestible *p*, %	0.333	0.300	0.423

SID amino acids[3]			
Lys, %	1.372	1.294	1.187
Met, %	0.578	0.497	0.453
Met + Cys, %	0.822	0.774	0.712
Thr, %	0.891	0.839	0.794
Trp, %	0.301	0.284	0.251

[1] Composition: 50% maize, 30% barley, and 20% wheat.
[2] Provided per kilogram of diet: 12,000 IU of vitamin A (acetate), 2000 IU of vitamin D3 (cholecalciferol), 250 IU of vitamin D (25-hydroxicholecalciferol), 75 mg of vitamin E, 2 mg of vitamin K3, 3 mg of vitamin B1, 7 mg of vitamin B2, 7.33 mg of vitamin B6, 15 mg of vitamin B12, 17 mg of D-pantothenic acid, 45 mg of niacin, 0.2 mg of biotin, 1.5 mg of folacin, 80 mg of Fe (chelate of amino acids), 100 mg of Zn (chloride), 12.5 mg Zn (chelate of amino acids), 12.5 mg of Mn (chloride), 0.3 mg of Se (inorganic), 2.04 mg of BHT, 400 UI of endo-1,4 beta-xylanase, and 250 OTU of 6-phytase.
[3] Standardized ileal digestible amino acids.

2.2.2. Sample Collection

At the end of the nursery period (d63), fecal and hair samples were collected from the piglets included in the study. An aliquot of the feces was stored in 2 mL sterile cryotubes for analysis of the fecal microbiota, and another aliquot of 5g was stored in Ziplock bags for the analysis of SCFA. Both aliquots were snap-frozen in dry ice and afterward kept at −80 °C. Hair samples were obtained with scissors from the lumbar area, cutting it as close to the skin as possible.

2.2.3. Cortisol and Cortisone Hair Concentration Analysis

The protocol used for cortisol and cortisone extraction was performed as described by López-Arjona et al. (2020). Briefly, the hair samples were weighed (250 mg), placed in a polypropylene tube, and covered with isopropanol (5 mL). The tube was mixed at room temperature, centrifuged (1500× *g*, 1 min), and the isopropanol discarded. The samples were washed again with isopropanol and left at RT until completely dry. Next, the hair was pulverized to a fine powder in a homogenizer (Precellys Evolution homogenizer, Bertin Technologies, France) and incubated with 1 mL of methanol for 18 h at room temperature with continuous gentle agitation for steroid extraction. Samples were then centrifuged (2000× *g*, 5 min). The samples were evaporated to dryness in a Speed Vac Concentrator (Concentrator 5301, Eppendorf). The dry extracts were reconstituted with 0.1 mL of phosphate-buffered saline (PBS) and stored at –80 °C until analysis. Cortisol and cortisone

hair concentrations were measured by sensitive assays based on AlphaLISA technology that also enabled the estimation of 11β-hydroxysteroid dehydrogenase type 2 activity (López-Arjona et al., 2020). The assay validation was performed as described by López-Arjona et al. (2020). The assays were precise (imprecision <12%) and accurate (recovery range, 80–115%) for cortisol and cortisone determination.

2.2.4. Short-Chain Fatty Acid Analysis

Short-chain fatty acids and lactic acid determinations were performed on feces by gas liquid chromatography. Sequentially, the samples were submitted to an acid–base treatment followed by an ether extraction and derivatization with N-(tertbutyldimethylsilyl)-N-methyl-trifluoroacetamide plus 1% tertbutyldimethylchlorosilane agent, using the method of Richardson et al. (1989), modified by M.T. Jensen et al. (1995). The volatile fatty acids measured were acetate, propionate, butyrate, iso-butyrate, valerate, iso-valerate, succinate, and formate.

2.2.5. 16S rRNA Gene Sequencing

The composition and structure of the microbial communities present in the fecal samples preserved at −80 °C were determined through a 16S rRNA gene sequence-based analysis. Bacterial DNA was extracted from 250 mg of each fecal sample using the commercial MagMAX CORE Nucleic Acid Purification Kit 500RXN (Thermo Fisher, Barcelona, Spain) following the manufacturer's instructions. A negative control and a Mock Community control (Zymobiomics Microbial Community DNA) were included to ensure the quality of the analysis. The amplification of the samples was performed using specific primers for the V3–V4 regions of the 16S rRNA DNA:

(F5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3', R5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3') (Klindworth et al., 2013). The preparation of libraries was carried out in Microomics Systems SL (Barcelona, Spain). The amplification was performed after 25 PCR cycles using the Illumina Miseq sequencing 300 × 2 approach.

For sequencing data bioinformatics, the sequence reads generated were processed using QIIME version 2019.4 software (Bolyen et al., 2019). The taxonomic assignment of phylotypes was performed using a Bayesian Classifier trained with Silva V4 database version 138 (99% OTU full-length sequences) (Wang et al., 2007). A detailed description of all further steps in the bioinformatic analysis is available in our previous publication (González-Solé et al., 2022).

2.2.6. Statistical Analysis

All the statistical analyses were performed with open-source software R v4.0.3 (R Foundation for Statistical Computing, Vienna, Austria). Growth performance data were analyzed with ANOVA as a complete randomized design, together with Tukey's test for multiple comparisons. Data of the SCFA concentrations in feces and cortisol and cortisone in hair were analyzed using a linear mixed model as a factorial arrangement (lactation growth x nursery growth). The model included lactation and nursery growth as fixed effects, their interaction, sex as a covariable, and the lactation mother as a random effect. Normality and homoscedasticity were checked with the Shapiro–Wilk test and Levene's test, respectively. A Box–Cox transformation was performed on the cortisol concentration data, and a logarithmic transformation on cortisone and the cortisol-to-cortisone ratio for is statistical analysis after the Shapiro–Wilk test revealed it did not have a normal distribution. Alpha diversity was calculated using the vegan package (Oksanen and F. Guillaume Blanchet, Michael Friendly, Roeland Kindt, Pierre Legendre, Dan McGlinn, Peter R. Minchin, R. B. O'Hara, Gavin L. Simpson, Peter Solymos, M. Henry H. Stevens, Eduard Szoecs, Helene Wagner 2020) from raw counts (OTU level), including observed OTUs, Pielou's Evenness, and the Shannon Index. An ANOVA test was performed to test group differences for the alpha diversity. A principal coordinates analysis (PCoA) was calculated using beta diversity distance matrices (Bray–Curtis). A permutational multivariate analysis of variance (PERMANOVA) was used to test the effects of day and treatment and their interaction on the Bray−Curtis distance between samples. Microbial diversity was analyzed as a factorial arrangement with treatment and sampling day as the main factors. A differential abundance analysis was performed using the metagenomeseq package (Paulson, Olson, et

al., 2013) to examine differences in the genus level. Separate analyses were performed to analyze the effects of lactation and nursery growth. A cumulative sum scaling (CSS) (Paulson, Colin Stine, et al., 2013) normalization of the raw counts was performed, and a zero-inflated Gaussian mixture model was used for the analysis. Relative abundances were used to plot taxon abundances at the phylum and family levels for each group. Log2 fold changes were calculated using relative abundances for the taxonomical groups that showed different abundances between the growth categories at each stage. *p*-Values for the differential abundance analysis were corrected by the false discovery rate (FDR). The pig was considered the statistical unit in all analyses, and statistical significance and tendencies were considered at $P \leq 0.05$ and $0.05 < P \leq 0.10$, respectively. Significance and tendencies were considered using the FDR value instead of the *p*-value in the microbiota differential abundance analysis.

2.3. Results

The growth characteristics of the experimental groups and the results of the statistical comparisons are detailed in Table 3.1 (See Table S3.1 for additional information regarding the statistical analyses). Birth BW was significantly higher in the Fast_Fast group than in the Slow_Slow group ($P < 0.05$). In congruence with the experimental design, lactation growth and weaning BW were different between the fast lactation and slow lactation groups. Additionally, nursery growth and the final BW were different among all groups, starting with the highest values for the Fast_Fast group and followed by the Slow_Fast, Fast_Slow, and Slow_Slow groups.

2.3.1. Cortisol and Cortisone Concentrations in Hair

The cortisol and cortisone concentration levels in hair and the cortisol-to-cortisone ratio for each group are represented in Table 3.3. The cortisone levels were higher in the animals with a fast growth rate during nursery ($P < 0.05$) than their slowest counterparts. The cortisol-to-cortisone ratio also showed a tendency to be lower in the fast-growing pigs

during the nursery period (P = 0.065). No differences between the sexes were identified (Table S3.2).

Table 3. 3. Cortisol and cortisone concentration levels in hair and calculated cortisol-to-cortisone ratio for each growth group.

Item		Cortisol (pg/mg)	Cortisone (pg/mg)	Cortisol/Cortisone
Lactation Growth	**Nursery Growth**			
Fast	Fast	19.2	188	0.27
	Slow	17.4	86	0.54
Slow	Fast	20.3	105	0.56
	Slow	19.1	115	0.67
SEM [1]		1.06	33.1	0.163
Lactation growth				
Fast		18.3	140	0.40
Slow		19.7	110	0.62
SEM		0.74	22.3	0.115
Nursery growth				
Fast		19.7	153	0.39
Slow		18.2	99	0.60
SEM		0.74	23.4	0.110
***p*-value [2]**				
Lactation growth		0.130	0.329	0.227
Nursery growth		0.463	0.041	0.069
Lactation x Nursery growth		0.852	0.174	0.157

[1] Standard error of the mean. [2] *p*-value obtained from the factorial ANOVA test.

The sample size for the cortisol analysis: Fast_Fast (19), Fast_Slow (19), Slow_Fast (19), and Slow_Slow (18). The sample size for cortisone and the cortisol/cortisone ratio analysis: Fast_Fast (n = 18, outliers = 1), Fast_Slow (n = 16, outliers = 3), Slow_Fast (n = 13, outliers = 6), and Slow_Slow (n = 13, outliers = 5). Outliers correspond to cortisone values below the limit of detection (<2 pg/mg).

2.3.2. Microbiota Structure and Biodiversity

After quality control, an average of 65,358 ± 14,527 high-quality reads for each fecal sample was generated. The microbial community alpha diversity measured as observed in the OTUs, Pielou's Evenness Index, and Shannon Index are summarized in Table 3.4. The microbiota of the animals with fast growth during the nursery period showed a higher evenness (P < 0.05) than their slowest counterparts. No differences between the sexes were identified (Table S3.2).

Table 3. 4. Alpha diversity measured as observed in the OTUs, Pielou's Evenness Index, and Shannon Index for pigs showing fast and slow growth during the lactation and nursery periods.

Item	Observed OTUs	Evenness	Shannon
Lactation growth			
Fast	354	0.786	6.63
Slow	343	0.781	6.55
SEM [1]	14.9	0.0078	0.109
Nursery growth			
Fast	353	0.797	6.72
Slow	345	0.770	6.46
SEM	15.1	0.0074	0.105
***p*-value [2]**			
Lactation growth	0.330	0.568	0.384
Nursery growth	0.969	0.016	0.127
Lactation × Nursery growth	0.526	0.985	0.904

[1] Standard error of the mean. [2] *p*-values obtained from the factorial ANOVA test.
The sample size for the alpha diversity analysis: Fast_Fast (*n* = 19), Fast_Slow (*n* = 18, missing samples = 1), Slow_Fast (*n* = 17, missing samples = 2), and Slow_Slow (*n* = 17, missing samples = 1).

The beta diversity analysis at the OTU level using the Bray–Curtis distance revealed that the microbial structure of the animals was different according to their growth in both the lactation and nursery periods ($P < 0.05$). The principal coordinate analysis (PCoA) based on the Bray–Curtis distance matrix exposed a different clustering of the individuals according to their growth during each period, although the 2D representation only preserved 10.8% of the total variance (Figure 3.1). The sum of the first five principal coordinates preserved 21.72% of the variance.

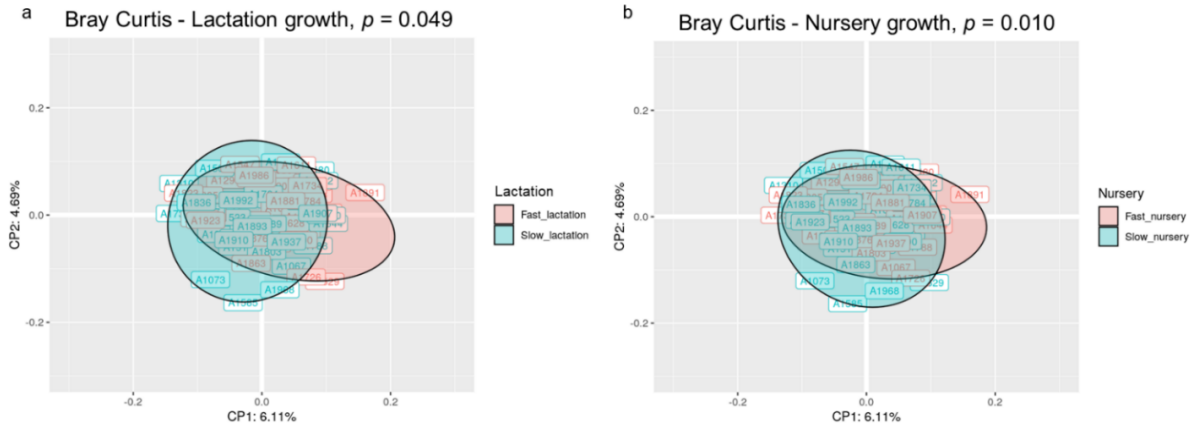


Figure 3. 1. Principal coordinate analysis (PCoA) ordination performed by Bray–Curtis dissimilarities representing the microbiome compositions of all animals clustered by lactation growth (**a**) and nursery growth (**b**). The *p*-value corresponds to the permutational analysis of variance (PERMANOVA) test for each factor. Sample size for the PCoA: Fast_Fast (*n* = 19), Fast_Slow (*n* = 18, missing samples = 1), Slow_Fast (*n* = 17, missing samples = 2), and Slow_Slow (*n* = 17, missing samples = 1).

2.3.3. Fecal Microbiota Composition

The microbiota composition of each group, represented as the relative abundance of the main phyla and families, is depicted in Figure 3.2. Firmicutes was the predominant phyla (80%), followed by Bacteroidota (11%), Patescibacteria (3%), and Actinobacteriota (3%), representing together 96% of the fecal microbiome. At the family level, Ruminococcaceae (14%), Erysipelotrichaceae (14%), the (Eubacterium) coprostanoligenes group (13%), and Lachnospiraceae (12%) were the predominant groups.

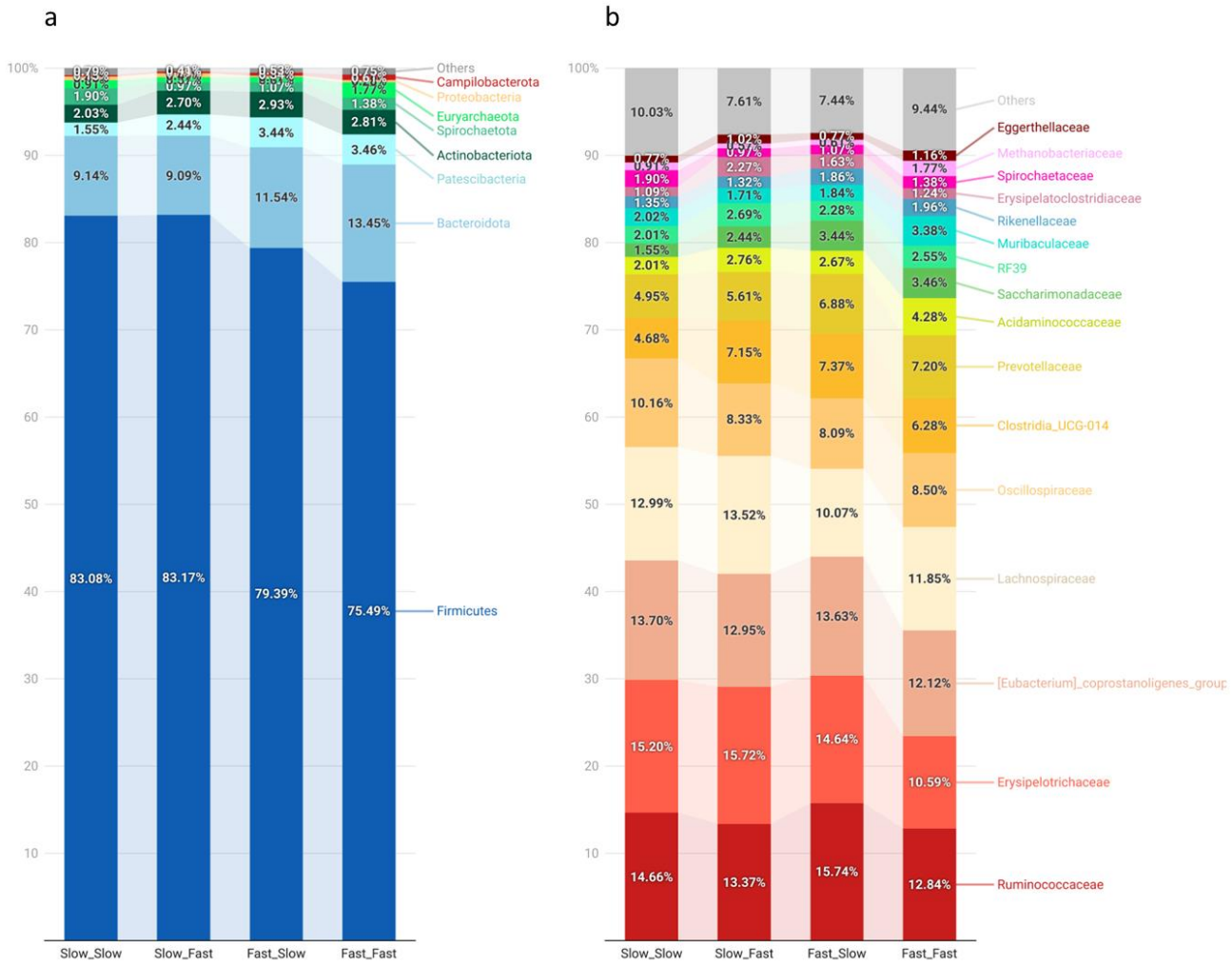


Figure 3. 2. Relative abundances (RA) of the main phyla (a) and families (b) observed in the analysis of the microbiota of piglets by massive sequencing of the 16S rRNA gene. The figure was created with the online open-source tool Datawrapper (http://datawrapper.de; accessed on 2/11/2022). Fast_Fast: pigs showing fast growth during the lactation and nursery periods (*n* = 19), Fast_Slow: pigs showing fast growth during the lactation period and slow growth during the nursery period (*n* = 18, missing samples = 1), Slow_Fast: pigs showing slow growth during the lactation period and fast growth during the nursery period (*n* = 17, missing samples = 2), and Slow_Slow: pigs showing slow growth during the lactation and nursery periods (*n* = 17, missing samples = 1).

Pigs' growth during lactation was associated with a few statistical differences in the main groups at the phylum and family levels. Microbial populations belonging to the Patescibacteria phyla showed higher abundance in the fast lactation growth group (3%) than the slow counterparts (2%; $P < 0.05$). Firmicutes was numerically lower in the pigs showing fast growth during the lactation period (Fast_Slow, 79%; Fast_Fast, 75%) compared to the ones showing slow growth (83%). This difference was mainly counterbalanced by an increased abundance of Bacteroidetes, as well as the abovementioned Patescibaceria phylum.

In Figure 3.3, the Log2 fold changes were calculated for the taxa that showed significant differences between the fast and slow growth groups in each period at the genus level. Fast growth during the lactation period was associated with a higher abundance of the genera *dgA-11* gut group, *Candidatus Saccharimonas*, *Colidextribacter*, *Bacteroidales RF16* group, *Campylobacter* ($P < 0.05$), and *Lachnospiraceae XPB1014* group ($P = 0.054$), while the fast growth during the nursery period was associated with a higher abundance of the genera *Streptococcus* ($P < 0.05$), *Dialister* ($P = 0.056$), *(Eubacetrium) ventriosum* group ($P = 0.056$), and *Lactobacillus* ($P = 0.092$). On the other hand, slow growth during the lactation period was associated with a higher abundance of the genus *(Eubacterium) ruminantium* group, *Lachnospiraceae UCG-002*, *Lachnospiraceae NK4A136* group ($P < 0.05$), *Candidatus Soleaferrea* ($P = 0.054$), and *Lachnospiraceae AC2044* group ($P = 0.093$), while the slow growth during nursery period was associated with the *Anaerofustis* ($P < 0.05$) and *Terrisporobacter* ($P = 0.056$) genera. All genera that showed differences between groups presented relative abundances < 1%, except for *Candidatus* Saccharimonas, which was 2.7%.

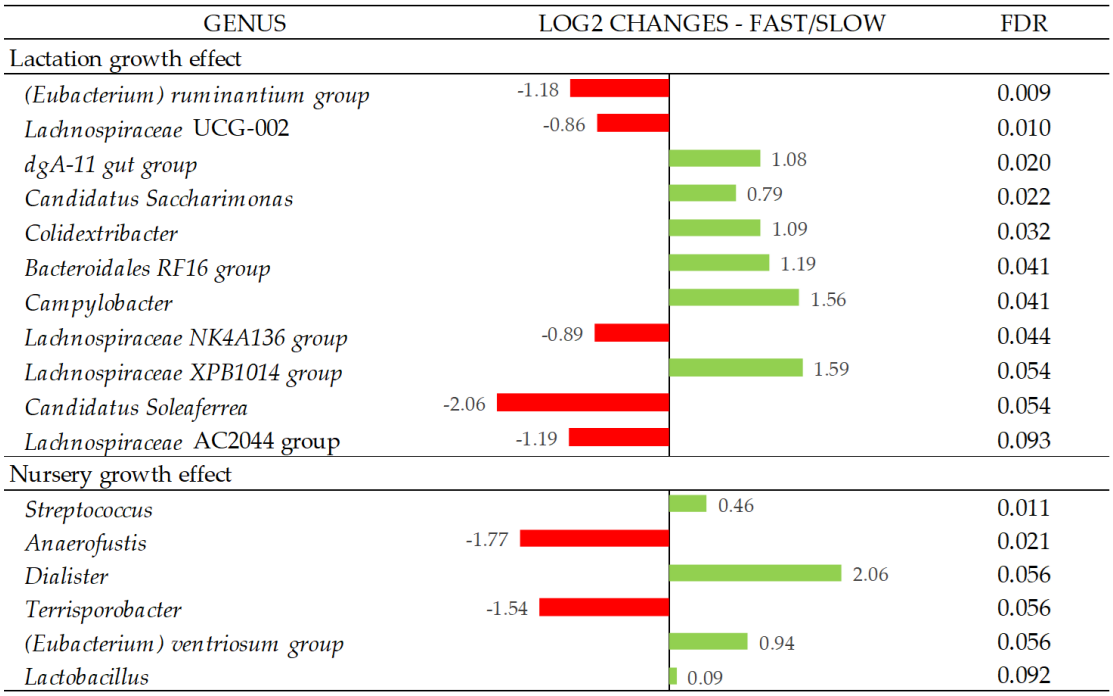


Figure 3. 3. Log2 changes between the fast- and slow-growth pigs during the lactation and nursery periods (fold discovery rate p-adjusted < 0.1) in microbial genera. Taxa are sorted by the level of significance (from higher to lower). Differences presented are based on all taxa detected in the samples per group. Sample size for the differential abundance analysis: Fast_Fast (*n* = 19), Fast_Slow (*n* = 18, missing samples = 1), Slow_Fast (*n* = 17, missing samples = 2), and Slow_Slow (*n* = 17, missing samples = 1).

2.3.4. Short-Chain Fatty Acid Concentrations in Feces

The SCFA concentration in the fecal samples showed differences between groups, which are depicted in Figure 3.4. The analysis of SCFA in feces revealed a higher concentration of total SCFA ($P < 0.05$) and a tendency to have higher concentrations of acetate ($P = 0.056$) in animals experiencing fast growth during the lactation period. Furthermore, fast growth was associated with the highest levels of butyrate in both periods ($P < 0.05$). No differences were identified between groups in the analysis of propionate (15.8 ± SD 3.83 µmol/g), iso-butyrate (1.1 ± SD 0.54 µmol/g), iso-valerate (0.8 ± SD 0.38 µmol/g), valerate (1.4 ± SD 0.54 µmol/g), succinate (1.6 ± SD 0.39 µmol/g), and formate (5.1 ± SD 0.21 µmol/g) ($P > 0.05$), and no interactions were observed for any of the SCFA measured ($P > 0.05$). No differences between sexes were identified (Table S3.2).

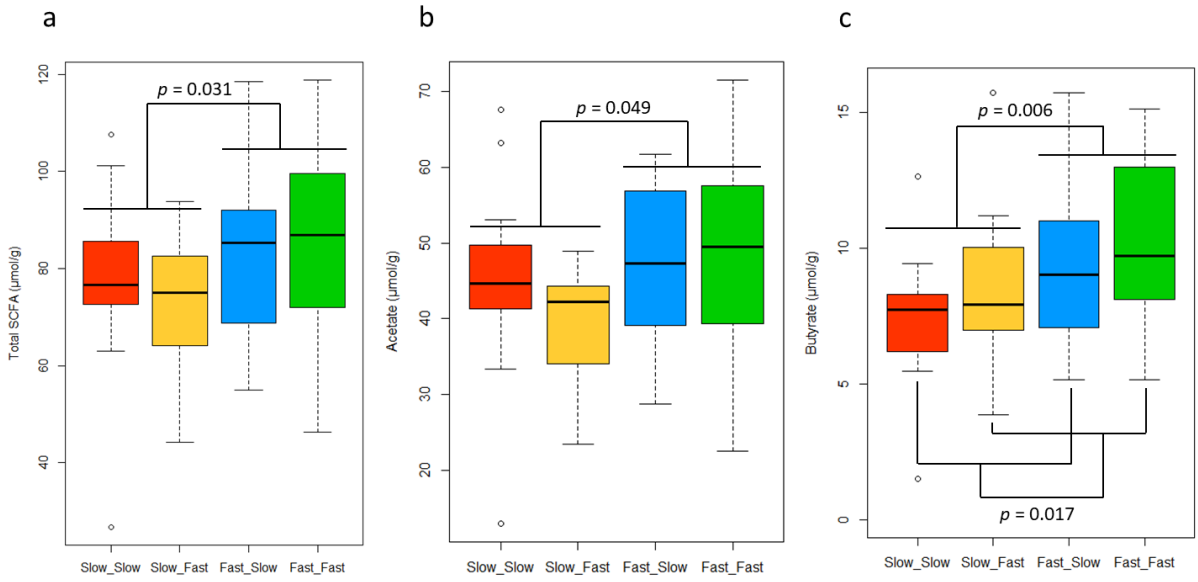


Figure 3. 4. Boxplot representing the total short-chain fatty acid (SCFA) (a), acetate (b), and butyrate (c) concentrations in feces for each group. The p-values correspond to the factorial ANOVA. ast_Fast: pigs showing fast growth during the lactation and nursery periods (*n* = 18, missing samples = 1), Fast_Slow: pigs showing fast growth during the lactation period and slow growth during the nursery period (*n* = 18, missing samples = 1), Slow_Fast: pigs showing slow growth during the lactation period and fast growth during the nursery period (*n* = 16, missing samples = 3), and Slow_Slow: pigs showing slow growth during the lactation and nursery periods (*n* = 17, missing samples = 1).

2.4. Discussion

In the present study, 75 pigs with different growth characteristics during early life were selected from a single weaning batch to determine their glucocorticoid levels in their hair, fecal microbiota composition, and fecal SCFA concentrations. The distribution of animals among the groups was performed according to the differences in growth during the lactation and nursery periods, while their BW was not considered. Due to that, there were differences in the birth BW among the experimental groups. Piglets within the Slow_Slow group were born smaller than the animals in the Fast_Fast group, which might be explained by the fact that piglets born small often remain stunted, presenting lower growth rates than their big counterparts (Camp Montoro et al., 2020; Quiniou et al., 2002b). Some light birth weight pigs have fewer muscle fibers (Gondret et al., 2005); they may show an insufficient colostrum intake (Muns et al., 2016), or they may have a retarded intestinal maturation (Michiels et al., 2013), which might compromise their growth for the rest of their lives.

Additionally, pigs in the Slow_Fast group were born numerically bigger than pigs in the Slow_Slow group. This is consistent with other studies that observed that piglets born with higher BW have a greater ability to show compensatory growth after having shown poor growth during the suckling phase (Camp Montoro et al., 2020; Huting et al., 2018). In addition, the weaning BW influenced nursery growth. Animals with a high weaning BW had faster growth than their smallest counterparts during nursery, despite being selected for the same nursery growth group. It is necessary to consider these differences, because they might have an influence on the results of the present investigation.

2.4.1. Cortisol and Cortisone in Hair

As part of this study, the hair concentrations of cortisol and cortisone at the end of the nursery period were analyzed, and the ratio of cortisol to cortisone was calculated. The use of cortisol as a stress biomarker is an extended practice in research with pigs and humans (Martínez-Miró et al., 2016). The use of hair samples has some advantages over other biological samples, such as blood or saliva, in that it can be collected noninvasively, it can indicate long-term concentrations, and the manipulation of the subject at the time of collection does not interfere with the results (Bacci et al., 2014; Stubsjøen et al., 2015). The analysis revealed higher concentrations of cortisone than cortisol in the hair of the pigs, a fact that has also been reported in previous studies (Stubsjøen et al., 2015; López-Arjona et al., 2020). Cortisone and the cortisol-to-cortisone ratio in hair, which are considered estimators of the 11β-HSD type 2 activity, have attracted much attention as biomarkers of stress (La Marca-Ghaemmaghami et al., 2013; Vanaelst et al., 2013; Zhang et al., 2013; Bacci et al., 2014; Stubsjøen et al., 2015; López-Arjona et al., 2020). Although chronic stress is recognized to have negative effects on growth in early life (Mousikou et al., 2021), there are no previous exploratory studies aiming to establish a relationship between growth and the metabolism of glucocorticoids. Pigs showing a fast growth rate during the nursery period, especially animals in the Fast_Fast group, showed higher levels of cortisone and a tendency to have a lower cortisol-to-cortisone ratio, suggesting that they have a higher 11β-HSD type 2 activity than slow-growing pigs. The activity of 11β-HSD type 2 in hair, which converts active cortisol to inactive cortisone, has been considered a biomarker of chronic

stress in different species. For instance, a previous study associated an increase in cortisone levels in the hair of sows with the stress experienced during the farrowing and lactation periods (Bacci et al., 2014). Additionally, research performed on humans associated psychosocial stress in young children with higher cortisone concentrations (Vanaelst et al., 2013). In addition, a study on sheep observed that a localized experimental infection could increase the deposition of cortisone in the hair at the site of infection (Stubsjøen et al., 2015). According to these previous studies, pigs with the fastest growth may have experienced higher levels of stress, although their growth was not affected. A possible explanation for that result is that pigs with a higher growth rate also present a higher feed intake and are more exposed to the competition for feed. However, there are discrepancies in the literature regarding the association between the activity of 11β-HSD type 2 and chronic stress, especially in other sample types different than hair. Studies in humans have observed that elderly people with high cortisol-to-cortisone ratios in spot urine had higher levels of perceived stress (Shimanoe et al., 2021) or that pregnant women with higher emotional support showed a higher metabolization of cortisol to cortisone in saliva (La Marca-Ghaemmaghami et al., 2013). Additionally, a decreased cortisol-to-cortisone ratio in 24-h urine was associated with an increase in performance in elite swimmers, which suggests that the increased inactivation of cortisol might protect the anabolic processes in the muscles against the deleterious effect of prolonged hypercortisolism (Atlaoui et al., 2004). Due to the discrepancies in the literature and the lack of complementary feeding and/or social behavioral information about the pigs in the study, we cannot reach any conclusion about the stress levels of the animals based on the glucocorticoid levels in their hair.

On the other hand, there is another isozyme participating in the metabolism of glucocorticoids, although it is discretely expressed in the hair follicle: 11β-HSD type 1 (Krozowski et al., 1999; Hennebert et al., 2007). It catalyzes the reverse reaction to that performed by 11β-HSD type 2. Cortisol can be metabolized into cortisone by 11β-HSD type 2, and cortisone can be transformed back into cortisol by 11β-HSD type 1. In a previous study, we identified an increased expression of 11β-HSD type 1 in the intestines of piglets

with lower BW both at birth and at weaning, which might indicate that fetal distress and the neonatal inflammatory condition can cause its long-term activation (Villagómez-Estrada et al., 2022). Therefore, although its activity is residual in hair follicles, we could speculate that 11β-HSD type 1 activity might be enhanced in slow-growing pigs, increasing the cortisol levels, the active form that exerts the inhibitory effects over muscle growth (Braun and Marks 2015). Additionally, 11β-HSD type 2 activity might be higher in fast-growing pigs, inactivating the cortisol to cortisone reaction. This hypothesis should be taken into closer consideration for future studies, as well as a possible gestational or neonatal imprinting of these two isoenzymes.

Differences in the birth BW between groups might have affected the glucocorticoid levels by possible gestational imprinting, but we cannot rule out a direct contribution of glucocorticoids deposited during the prenatal period, since the hair presented by the pigs at birth was not shaved due to the experimental logistics. Previous studies speculate that the time window for glucocorticoid deposition includes about one to two months prior to the date of collection, excluding the last 15 days (Bacci et al., 2014), but the exact timing is uncertain, so it is not known whether the gestational period could influence the levels obtained. However, the contribution of the hair grown before birth to the total hair length at the time of collection is small, suggesting that glucocorticoids deposited during the gestational period might have a reduced impact on the total levels obtained at the end of the nursery period.

2.4.2. Fecal Microbiota Composition

The analysis of the fecal microbiota of the piglets in the study revealed that Firmicutes and Bacteroidetes were the dominant phyla, in agreement with other studies (Holman et al., 2017; Mach et al., 2015; Niu et al., 2015; Pajarillo et al., 2014; Saladrigas-García, D'Angelo, Ko, Nolis, et al., 2021; X. Wang et al., 2019). However, few studies have researched the possible link between intestinal microbiota at a particular age and the previous individual growth traits. The 16S rRNA gene sequence-based analysis of the feces of the piglets at the end of the nursery period revealed differences in the microbial diversity and composition between animals showing a previous fast and slow growth in each period.

The beta diversity analysis showed differences in the microbial structure, in line with a previous study that observed differences in the microbiota of heavy and light pigs at d100 of life (Oh et al., 2020). Additionally, pigs with a fast growth during the nursery period had a significantly higher evenness than their slowest counterparts, in agreement with Han et al. (2017), who reported higher microbial diversity in the microbiota of heavy 9-week-old pigs compared to lighter pigs (Han et al., 2017). Research in human children aged 0–2 years also revealed that a reduced microbial diversity was predictive of a future growth deficit (Gough et al., 2015). However, this result conflicts with other studies that observed higher values in alpha diversity indexes in lighter pigs (Ramayo-Caldas et al., 2016; Panasevich et al., 2018; Oh et al., 2020). The relationship between intestinal microbial diversity and body weight is still a controversial issue.

2.4.3. Lactation Growth Effect on Fecal Microbiota and SCFA Concentrations

The study of the relationship between lactation growth and intestinal microbiota composition at the end of the nursery period can help us to improve our understanding of the impact of early microbial colonization in the posterior phases. Both in humans and pigs, the gut microbiota structure undergoes drastic changes during the weaning phase, shifting from bacterial populations oriented to degrade metabolites present in the mother's milk to bacterial populations adapted to degrade complex carbohydrates existent in the novel food offered at weaning (Homann et al., 2021; Saladrigas-García, D'Angelo, Ko, Nolis, et al., 2021). Nevertheless, several studies suggest that events and interventions during the lactation period have the potential to induce long-lasting effects on the immune system programming, barrier function, and bacterial composition, which are preserved beyond weaning (Francino 2014; Schokker et al., 2014; Schokker et al., 2015; González-Solé et al., 2022). Studies in humans showed contrasting results regarding the long-term repercussions of the early modulation of the microbiome. However, interventional studies on animal models are more convincing in this respect (Robertson et al., 2019). For instance, in a previous report, we observed that the supplementation of xylo-oligosacharides to piglets exclusively during the lactation period stimulated the growth of fiber-degrading bacterial populations in the post-weaning period (González-Solé et al., 2022).

The current study revealed that piglets' growth during the lactation period was associated with differences at the end of the nursery period in the fecal concentrations of SCFA and the relative abundance of certain microbial populations. First, although it was not a significant difference, piglets showing a fast growth during the lactation period had a numerically lower abundance of Firmicutes and higher abundance of Bacteroidetes than the slow-growth group. The ratio between Firmicutes and Bacteroidetes has been assumed to play a role in weight gain potential and the development of obesity (Ley et al., 2006). Generally, the literature describes a higher Firmicutes/Bacteroidetes ratio in obese or overweight animals and humans (Han et al., 2017; Kallus & Brandt, 2012; Koliada et al., 2017; Ley et al., 2006; Mach et al., 2015; Oh et al., 2020). Researchers explain these results by arguing that Firmicutes are more efficient in extracting energy from food than Bacteroidetes, thus promoting the more efficient absorption of calories and subsequent weight gain (Kallus and Brandt 2012; Koliada et al., 2017). However, some reports did not observe any relationship or even a decreased Firmicutes/Bacteroidetes ratio in obese individuals (Schwiertz et al., 2010; Magne et al., 2020). On the other hand, McCormack et al., observed lower Firmicutes counts in weaned piglets, which showed better feed efficiency in the posterior phases (McCormack et al., 2017), which could explain why this numerical difference was determined by lactation growth. Nevertheless, additional investigation is required to fully understand the relationship between the Firmicutes/Bacteroidetes ratio and the growth capacity of the host.

At the same time, piglets with a faster growth during lactation showed significantly higher levels of total SCFA in feces than the slow lactation growth group, independently of the nursery growth. The difference was mainly caused by the higher concentration of butyrate and the trend in the acetate levels in the fast-growth piglets during lactation. This result suggests that the microbiota of fast-growing pigs during lactation might have an increased capacity to produce SCFA from carbohydrate components present in the diet, which can be used as an extra energy source by the host. This is consistent with a study that reported a higher feed efficiency in pigs, with a higher total SCFA production in the caecum and a tendency to increase the butyrate ratio (Vigors et al., 2016). Furthermore, SCFAs have

functional properties that might contribute to a superior growth capacity other than representing an extra energy supply. Butyrate plays an important role in cell proliferation and development, has regulatory functions on the metabolism, and is considered a health-promoting molecule. Additionally, it represents the preferred energy source for the colonocytes and helps to guarantee a correct gut function (J. Tan et al., 2014). Additionally, acetate has an important regulatory role in body weight control and insulin sensitivity through its effects on lipid metabolism and glucose homeostasis (Hernández et al., 2019). However, during lactation, the main substrates for fermentation and SCFA production were the oligosaccharides in the sow's milk, whereas, at the time of sampling, they were the complex carbohydrates present in the plant-based ingredients of the nursery diet. This might indicate that pigs showing a good growth during lactation developed a microbiota with a better capacity for fiber degradation and SCFA production in the posterior stages. Further research is necessary to confirm this long-term influence and its long-term impact on the digestive and immune function.

The relative abundance analysis of the bacterial genus associated with lactation growth revealed subtle changes in bacterial populations with abundances <1%, with the only exception of *Candidatus Saccharimonas*, with a mean abundance of 2.7%, which had higher representativeness in fast-growing animals during lactation. This genus has a fermentative metabolism with acetate and lactate as the main products, which could have contributed to the higher concentration of acetate in the feces of these piglets.

2.4.4. Nursery Growth Effect on Fecal Microbiota and SCFA Concentrations

Growth during the nursery period would be expected to have a greater impact on the microbiota at the end of this stage than lactation growth. Nevertheless, the analysis revealed fewer changes associated with the nursery growth. The abundance of Firmicutes and Bacteroidetes was not significantly different between the fast and the slow nursery growth groups, and the numerical difference was contemptible.

The total SCFA concentration was not affected by nursery growth. However, fast-growing pigs during nursery had significantly higher levels of butyrate in feces than their slower counterparts. An increased butyrate concentration was associated with fast growth

during both the lactation and nursery periods, suggesting that it plays an important role in growth. Although it is difficult to identify the relevance of the causes and/or consequences associated with these changes on the gut microbiota and fermentation activity, the present findings may indicate that promoting the growth of butyrogenic bacterial populations might be a promising strategy for improving growth and redirect the trend of individuals with an impaired growth. This strategy has been tested in previous studies in swine. For instance, supplementing gestating sows with alfalfa meal, a highly fibrous ingredient, stimulated the growth of anti-inflammatory and butyrogenic bacteria in their gut, which led to an increase of butyrate concentration in their feces during lactation, as well as multiple benefits in the sows' health and performance (B. Liu et al., 2021). Alternatively, there is the possibility of supplementing exogenous butyrate for the organism. In fact, butyrate supplementation has already been explored in humans as a potential therapy for metabolic and intestinal diseases. In particular, due to its regulatory role of body weight gain and metabolism, it has been tested for treating obesity (H. Liu et al., 2018). In pigs, the supplementation of sodium butyrate in the pigs' diet proved to be a successful strategy to improve their growth performance, intestinal health, and morphology (Lu et al., 2008; Fang et al., 2014; Sun et al., 2020).

A relative abundance analysis of the bacterial genus based on nursery growth also showed fewer differences than for lactation growth. Nursery growth was associated with two significant differences versus the eight identified for lactation growth. These differences were identified in the microbial genera with low counts (<1% of relative abundance).

The aim of this study was to explore the characteristics of piglets with different adaptation capacities during the initial stages of life to identify opportunities to improve the growth of slow-growing individuals. This study only analyzed the differences among piglets showing extreme growth, which facilitated the detection of differences among groups with a limited number of animals sampled. However, this design did not provide information on the entire spectrum of pigs, which would have allowed the study of correlations among the different variables. In addition, sampling at various time periods throughout early life

instead of only one time at the end of the nursery period would have provided a better understanding of the causal effects among the growth, gut microbiota, and stress during this period. Another limitation of the present study were the initial differences in birth BW among the growth groups. Birth BW is an important factor associated with the pre- and postnatal development of the pig, and these differences might have introduced a confounding factor that should be taken into consideration in the interpretation of the results. Moreover, the analysis of stress biomarkers was not accompanied by behavioral observations or other indicators of stress, which prevented us from drawing a conclusion about the stress levels of the studied pigs. Furthermore, although the impact of prenatal glucocorticoid deposition in the analyzed hair during the nursery period was expected to be low, the fact that the pigs were not shaved at the beginning of the experimental period implied some uncertainty in the time window represented in the results.

Despite the limitations already described, the obtained results indicated that the events that occurred during both the lactation and nursery periods influenced the glucocorticoid deposition in hair, gut microbiota, and its fermentation capacity. A glucocorticoid analysis revealed that fast-growing pigs might have an increased 11β-HSD type 2 activity, converting cortisol to cortisone, although its implications on the stress status need further confirmation. Regarding the microbiota and SCFA profiles, a larger number of differences were identified associated with lactation growth than nursery growth. This observation adds further evidence to the hypothesis that microbial colonization and modulation during the lactation period have a critical influence on the development and configuration of adult microbiota. In addition, the preservation of differences between fast and slow growers during lactation until the end of the nursery period might indicate that early interventions in the microbiota might have a long-term effect from weaning onwards. Most of the differences associated with growth were not consistent between the lactation and nursery periods, which calls into question the effectiveness of modulating the microbiota during early ages to obtain growth improvement during the posterior stages. However, the fecal butyrate levels were positively associated with growth during both periods, suggesting that stimulating butyrogenic microbiota during early life could provide

an opportunity to redirect the growth of less efficient individuals. This study encourages the investigation of the implications of 11β-HSD type 2 activity on growth during early life, as well as the research of a possible gestational imprint on its activity. Additionally, future studies should examine the causal relationships among stress, microbiota, and growth, as well as effective strategies to modulate them to improve health and growth during early life.

2.5. Supplementary material

Table S3. 1. Mean body weight (BW) and average daily gain (ADG) of the animals included in each group for lactation and nursery periods. Each mean is followed by its corresponding SEM. DF: Degrees of freedom.

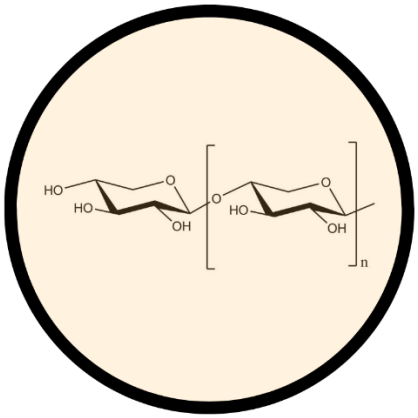
Group	Fast_Fast	Fast_Slow	Slow_Fast	Slow_Slow	DF	*F* value	*P* value
Lactation ADG, g/d	246 ± 5.2 a	244 ± 4.6 a	148 ± 3.3 b	138 ± 5.9 b	71	151.4	< 0.001
Nursery ADG, g/d	273 ± 7.1 a	128 ± 3.7 c	238 ± 5.7 b	112 ± 3.5 d	71	203.5	< 0.001
Birth BW, kg	1.45 ± 0.026 a	1.31 ± 0.069 ab	1.35 ± 0.074 ab	1.21 ± 0.056 b	71	2.904	0.040
Lactation BW, kg	6.7 ± 0.083 a	6.4 ± 0.094 a	4.5 ± 0.105 b	4.1 ± 0.112 b	71	123.6	< 0.001
Nursery BW, kg	17.9 ± 0.330 a	11.7 ± 0.196 c	14.2 ± 0.306 b	8.7 ± 0.170 d	71	215.1	< 0.001

Fast_Fast: Pigs showing fast growth during lactation and nursery periods; Fast_Slow: Pigs showing fast growth during lactation period and slow growth during nursery period; Slow_Fast: Pigs showing slow growth during lactation period and fast growth during nursery period; Slow_Slow: Pigs showing slow growth during lactation and nursery periods. DF: Degrees of freedom; a,b,c,d; Values with different letter in the same column are significantly different according to ANOVA and Tukey adjust.

Table S3. 2. Hair glucocorticoid concentration, alpha diversity and SCFA concentration for each sex, lactation growth group and nursery growth group.

	Lactation growth			Nursery growth			Sex			DF	*F* value				*P* value			
Item	Fast	Slow	SEM	Fast	Slow	SEM	Females	Males	SEM		Lactation growth	Nursery growth	Lactation x nursery growth	Sex	Lactation growth	Nursery growth	Lactation x nursery growth	Sex
Cortisol, pg/mg	18.3	19.7	0.74	19.7	18.2	0.74	19.1	18.8	0.77	68	2.29	0.54	0.03	0.52	0.130	0.463	0.852	0.472
Cortisone, pg/mg	140	109.6	22.3	18.20	98.90	0.602	135	118	22.8	53	0.95	4.18	1.85	0.47	0.329	0.041	0.174	0.492
Cortisol/cortisone	0.40	0.62	0.115	0.39	0.62	0.110	0.48	0.51	0.112	53	1.46	3.30	2.00	0.40	0.227	0.069	0.157	0.527
Observed OTUs	354	343	14.9	353	345	15.1	346	352	15.2	64	0.95	0.00	0.40	1.26	0.330	0.969	0.526	0.260
Eveness	0.786	0.781	0.0078	0.797	0.770	0.0074	0.778	0.790	0.0075	64	0.33	5.79	0.00	0.97	0.604	0.018	0.959	0.325
Shannon	6.63	6.55	0.109	6.72	6.46	0.105	6.55	6.64	0.107	64	0.76	2.32	0.01	0.69	0.411	0.137	0.929	0.407
Total SCFA, µmol/g	84.02	75.54	2.970	79.68	80.35	2.930	80.2	79.8	2.98	63	4.63	0.03	1.03	0.00	0.031	0.864	0.310	0.986
Acetate, µmol/g	47.33	42.39	1.848	43.56	46.17	1.823	45.5	44.5	1.86	63	3.85	0.95	1.43	0.07	0.049	0.331	0.231	0.797
Butyrate, µmol/g	10.20	8.05	0.589	9.67	8.58	0.581	9.0	9.3	0.60	63	7.53	5.86	0.00	0.14	0.006	0.017	0.964	0.711

DF: Degrees of freedom.



Chapter 4

Supplementation of Xylo-Oligosaccharides to Suckling Piglets Promotes the Growth of Fiber-Degrading Gut Bacterial Populations During the Lactation and Nursery Periods

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Abstract

Modulating early-life microbial colonization through xylo-oligosacharides (XOS) supplementation represents an opportunity to accelerate the establishment of fiber-degrading microbial populations and improve intestinal health. Ninety piglets from 15 litters were orally administered once a day from d7 to d27 of lactation with either 5 mL of water (CON) or 5 mL of a solution containing 30 to 60 mg of XOS (XOS). Supplementation ceased at weaning (d28) when all piglets were fed the same commercial pre-starter diet. Growth performance did not differ between treatments during the experimental period (d7 to d40). Piglet's fecal microbiota (*n* = 30) shifted significantly from the end of lactation (d27) to nursery period (d40) exhibiting an increase in microbial alpha diversity. Animals supplemented with XOS showed higher richness and abundance of fiber-degrading bacteria and short-chain fatty acid (SCFA) production at d27 and d40. Additionally, the predicted abundance of the pyruvate to butanoate fermentation pathway was increased in the XOS group at d40. These results show that supplementation of XOS to lactating piglets promotes fiber-degrading bacterial populations in their hindgut and differences observed in the nursery period suggest it can influence the microbiota in the long-term.

3.1. Background

Weaning is considered a critical period for piglets, when they suddenly need to deal with different stressful events. The challenge includes an abrupt diet change from sow's milk to plant-based dry solid diets, which contains many ingredients and vegetable structures that piglets have not eaten before (J. P. Lallès et al., 2007). Weaning also occurs when their gut barrier is still developing, which can induce long-lasting deleterious consequences on gut health (Moeser et al., 2017). As a result, growth performance of the pigs is impaired and there is a high risk of appearance of post-weaning diarrhea, which is associated with enormous economic losses for the pork industry (Gresse et al., 2017).

The gut microbiota during early-life is considered to have a decisive role in the development and programming of both the mucosal immune response and the establishment of the adult microbiota (Francino, 2014; Mulder et al., 2011; Schokker et al., 2014, 2015). There is increasing evidence that the existence of a "window of opportunity" occurs in the early life of animals when an intervention may determine an improvement of microbial colonization and, hence, benefit the immunity status of the pig (C. S. Cheng et al., 2019; Gensollen et al., 2016b).

Multiple management and feeding strategies in the early stages of weaning have been explored in order to take advantage of the plasticity of the microbiota during that period, the goal being to mitigate the negative impact of weaning and improve post-weaning performance (Blavi et al., 2021; Huting et al., 2021). One such strategy involves providing dietary fiber to suckling piglets to accelerate gut microbiome maturation, thus increasing populations capable of breaking down complex polysaccharides before they have to deal with the post weaning feed (Choudhury et al., 2021; Mu et al., 2017; L. Zhang et al., 2016). Other studies examined the oral supplementation of prebiotics, such as FOS of GOS, to suckling piglets to enable identifying a modulation of the gut microbiota, intestinal morphology and function (Huting et al., 2021).

Among dietary fiber compounds, xylo-oligosaccharides (XOS) are oligomers composed of 2–6 xylose units linked through β-(1→4)-linkages (Samanta et al., 2015). Their

selective fermentation has been shown to induce changes in both composition and activity of the gastrointestinal microbiota, stimulating the growth of butyrate-producing bacteria (Berger et al., 2021; M. Chen et al., 2020). Butyrate has been associated with benefits in intestinal health, such as the modulation of the mucosal inflammatory response and barrier function (J. Tan et al., 2014; Tremaroli & Bäckhed, 2012). In fact, several studies supplementing XOS into weaning pig (Y. Chen et al., 2021; J. B. Liu et al., 2018; Yin et al., 2019) and broiler (A. D. Craig et al., 2020; de Maesschalck et al., 2015; Ribeiro et al., 2018) diets have demonstrated such improvements in gut health, and in some cases these benefits have been associated with enhanced growth performance (Y. Chen et al., 2021; de Maesschalck et al., 2015). Overall, the beneficial effects of XOS fulfill the definition of prebiotic, although their mechanism of action seems to be specific. Their effects have been observed at supplementation levels of 0.1 – 0.2 g/kg, which are considered too low to be effective through its quantitative fermentation alone (J. B. Liu et al., 2018; Ribeiro et al., 2018; Yin et al., 2019). That is why it has been proposed to describe XOS as a "stimbiotic", which is a new term defined as a "non-digestible but fermentable additive that can stimulate a fiber-degrading microbiome to increase fiber fermentability at doses which clearly are too low to contribute in a meaningful manner to short-chain fatty acid (SCFA) production" (González-Ortiz et al., 2019). The increase in fiber fermentability may contribute to more oligosaccharide production, and therefore, SCFA production, which is considered an indirect mechanism of action to explain part of its health-related benefits (Bedford, 2019). Nevertheless, little research has been conducted on the effects of XOS supplementation in the early stages of a piglet's life.

This trial hypothesized that providing low doses of XOS to suckling piglets modulates the early-colonization of the piglet intestinal tract by stimulating the growth of fiber-degrading and butyrate-producing bacteria, and conditioning the establishment of a differential microbiota population in the hindgut after weaning. Therefore, the objective was to explore if daily supplementation of XOS to suckling piglets influences the fecal microbiota composition and its functionality at the end of the suckling period, but also

during the nursery period when piglets were exposed to the same diet and same environmental conditions.

3.2. Materials and Methods

The experimental procedures used were approved by the Ethical Committee on Animal Experimentation of the Universitat Autònoma de Barcelona (CEAAH 3817), and are in full compliance with national legislation following the EU-Directive 2010/63/EU for the protection of animals used for scientific purposes and designed in compliance with the ARRIVE guidelines.

3.2.1. Animals, Housing, and Diet

The study was conducted in the farrowing room of a commercial farm from day 7 of lactation to the day of weaning (d28) and in the nursery unit of the same farm from weaning to 12 days post-weaning (d40). Ninety commercial male and female piglets ((Landrace x Large White) x Duroc) from 15 litters were included in the trial. On d7 of lactation, six piglets from each litter with an average body weight (BW; 2.91 ± 0.42 kg) were selected for the experiment, ear-tagged and divided into two experimental treatments (3 piglets/treatment/litter) according to its BW on d7 and sex. From d7 to d27 of lactation, piglets in the control treatment (CON, *n* = 45) were individually administered 5 mL of water once a day while piglets in the supplemented group (XOS, *n* = 45) individually received 5 mL of a solution containing 30 mg XOS (AB Vista, Marlborough, UK) obtained from corn cob with a purity of 35% of XOS from d7 to d14 and 60 mg XOS from d15 to d27 by oral gavage. Sows and their litters were housed in individual farrowing pens (2.6 x 1.8 m^2), in a partially slatted floor with a heated floor pad for piglets, equipped with a farrowing crate, an individual feeder and nipple drinkers for sows and piglets. The temperature in the farrowing room was automatically controlled. Water and feed were offered *ad libitum* to the sows while piglets were offered only water. Piglets did not have access to creep feed during the lactation period.

At weaning (d28), supplementation ceased and piglets were moved to the nursery unit without transport. Piglets were randomly allocated in 6 pens blocked by sex (3 female pens and 3 male pens; 15 animals/pen), mixing animals from CON and XOS in the same pens. Each pen (3.20 m^2) was equipped with two commercial pan feeders (Maxi hopper, Rotecna, Spain) and a nipple bowl drinker to provide *ad libitum* access to feed and water. The floor was completely slatted, and the temperature and ventilation rates were controlled using central and forced ventilation with an automatic cooling system. All animals were fed a common conventional pre-starter diet formulated to contain 2470 kcal NE/kg, 18.8 CP/kg and 1.425% digestible lysine (Table 4.1) and to meet the requirements for maintenance and growth of newly weaned piglets (National Research Council 2012). No XOS were supplemented in the pre-starter diet.

Table 4. 1. Nursery diet offered to the animals included in the trial.

Item	Nursery diet
Ingredient, %	
Wheat	23.4
Extruded barley	20.0
Acid whey	10.0
Corn	10.0
Soybean protein concentrate	8.3
Soybean meal heat processed	7.0
Dextrose	4.0
Fish meal	3.0
Spray-dried plasma	3.0
Milk whey 50% Fat	2.5
Nucleus[1]	2.0
Beet pulp	2.0
Lard	1.85
Mono calcium phosphate	0.72
L-Lysine sulphate	0.72
Vitamin-Mineral premix[2]	0.4
L-Threonine	0.31
Calcium carbonate	0.31
DL-Methionine	0.29
Salt	0.20
L-Valine	0.08
Calculated composition	
NE, kcal/kg	2470
Ash, %	3.1
Crude Protein, %	18.8
Ether Extract, %	6.8
Crude Fiber	3.1

Starch	30.1
Calcium, %	0.542
Total P, %	0.620
Digestible P, %	0.496
Digestible amino acids[3]	
Lys, %	1.425
Met, %	0.562
Met+Cys, %	0.894
Thr, %	1.027
Trp, %	0.257

[1]Basic composition of the nucleus: yogurt, extruded soybean, micronized carob meal, nucleotides, hyperimmune egg and endo-1,4 beta-xylanase (420 UI/kg).

[2]Provided per kilogram of diet: 12000 IU of vitamin A (acetate); 2000 IU of vitamin D3 (cholecalciferol); 250 IU of vitamin D (25-hydroxicholecalciferol); 75 mg of vitamin E; 2 mg of vitamin K3; 3 mg of vitamin B1; 7 mg of vitamin B2; 7.33 mg of vitamin B6; 15 mg of vitamin B12; 17 mg of D-pantothenic acid; 45 mg of niacin; 0.2 mg of biotin; 1.5 mg of folacin; 80 mg of Fe (chelate of amino acids); 100 mg of Zn (chloride); 12.5 mg Zn (chelate of amino acids); 12.5 mg of Mn (chloride); 0.3 mg of Se (inorganic); and 2.04 mg of BHT.

[3] Standardized ileal digestible amino acids.

3.2.2. Performance Measurements and Sample Collection

Piglets were individually weighed on d7, d28 (weaning) and d40 of age. The ADG was calculated for the experimental period. At weaning, fecal samples were collected from a piglet with a medium body weight from each litter and treatment (*n* = 15). Same piglets were used to obtain fecal samples on d40. In both periods, an aliquot of the feces was stored in 2 mL sterile cryotubes, snap frozen in dry ice, and afterward kept at -80ºC for analyses of fecal microbiota. Another aliquot was stored in the Biofreezer tubes (Alimetric Diagnostics, Espoo, Finland) following the recommended protocol by the manufacturer for the analysis of SCFA. Considering that the present study had an exploratory will, both microbiota and SCFA characterization were performed in fecal samples and not in intestinal digesta to avoid euthanizing animals, following the principle of the three R's (Replacement, Reduction and Refinement) for more ethical use of animals in scientific research.

3.2.3. Short-Chain Fatty Acid Analysis

The fecal SCFA were analysed as free acids by gas chromatography. Briefly, 1 mL of H_2O was added to 1 g of ceca content, and 1 mL of a solution containing 20 mmol/L pivalic acid was incorporated as an internal standard. Afterwards, 1 mL of perchloric acid was

added, and SCFA were extracted by shaking the mixture for 5 min. After centrifugation, 50 µL of 4 mol KOH in 500 µL of supernatant were added to precipitate the perchloric acid in the supernatant. Saturated oxalic acid was added after 5 min, the mixture was incubated at 4°C for 60 min and then centrifuged at 18,000 × g for 10 min. The chromatography procedure used a glass column packed with 80/120 Carbopack B-DA/4% Carbowax 20 mol stationary phase (Supelco, Bellefonte, PA), using a flame ionization detector and helium as the carrier gas (Apajalahti et al., 2019). The acids measured were lactic acid and volatile fatty acids (VFA) which in turn comprised of acetic, propionic, butyric, iso-butyric, 2-methyl-butyric and iso-valeric acids. The sum of isobutyrate, 2-methyl butyrate and isovalerate results in branched-chain fatty acids (BCFA).

3.2.4. 16S rRNA Gene Sequencing

The fecal samples stored at -80ºC were used for the determination of the composition and structure of microbial communities present through a 16S ribosomal RNA gene sequence-based analysis. Bacterial DNA was extracted from 250 mg of each fecal sample using the commercial MagMAX CORE Nucleic Acid Purification Kit 500RXN (Thermo Fisher, Barcelona, Spain) following the manufacturer's instructions. In order to ensure the quality of the analysis, a negative control and a Mock Community control (Zymobiomics Microbial Community DNA) were included. Samples were amplified using specific primers for the V3-V4 regions of the 16S rRNA DNA (F5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3', R5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3')(Klindworth et al., 2013). The library preparation was performed in Microomics Systems SL (Barcelona, Spain). The Illumina Miseq sequencing 300 × 2 approach was used and amplification was performed after 25 PCR cycles.

For sequencing data bioinformatics, the sequence reads generated were processed using QIIME version 2019.4 software (Bolyen et al., 2019). The software package DADA2 was used for primer trimming, quality filtering, denoising, pair-end merging, and amplicon sequence variant calling (ASV, i.e., phylotypes) using qiime dada2 denoise-paired method

(Callahan et al., 2016). Also, Q20 was used as a quality threshold to define read sizes for trimming before merging (parameters: --p-trunc-len-f and --p-trunc- len-r). Reads were truncated at the place when the 25th percentile Phred score felt below Q20 for both forward and reverse reads. After quality filtering steps, the average sample size of reads was resolved and phylotypes were detected. To even sample sizes for the diversity analysis using qiime diversity core metrics-phylogenetic pipeline, ASV tables were subsampled without replacement. Bray-Curtis distances were calculated to compare community structure. Taxonomic assignment of phylotypes was performed using a Bayesian Classifier trained with Silva V4 database version 132 (99% OTUs full-length sequences) (Q. Wang et al., 2007).

3.2.5. Functional Predictions

The functional potential of the gut microbiota was explored by inferring metagenomics functionality from the 16S rRNA gene sequencing data using the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2) software (G. M. Douglas et al., 2020). Individual gene-family copy numbers for each ASVs was estimated after placing sequences into a reference phylogeny tree containing more than 20,000 full 16SrRNA genes from bacterial and archaeal genomes from the Integrated Microbial Genomes (IMG) database (Markowitz et al., 2012). Afterwards, ASVs are corrected by their 16S rRNA gene copy number, and pathway abundances are inferred on the basis of structured pathway against the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa et al., 2012) orthologs (KOs) and Enzyme Commission numbers (EC numbers) databases.

3.2.6. Statistical Analysis

Growth performance data and SCFAs concentration data were analyzed with ANOVA using the general linear model (GLM) procedure of statistical package SAS (version 9.4, SAS Institute Inc., Cary, NC). Growth performance was analyzed as a complete randomized design and SCFAs concentration as a factorial arrangement (treatment x day). Normality

and homoscedasticity were checked with Shapiro–Wilk test using the univariate procedure and Levene's test using the generalized linear model procedure, respectively. The LSMeans statement was used to calculate mean values for each parameter.

The statistical analysis of the fecal microbiota was performed in open-source software R v4.0.3 (R Foundation for Statistical Computing, Vienna, Austria). Alpha diversity was calculated using vegan package (Oksanen et al., 2020) from raw counts (OTU level) including observed OTUs and Shannon index. An ANOVA test was performed to test group differences for alpha diversity. Principal coordinates analysis (PCoA) were calculated using beta diversity distance matrices (Bray-Curtis). Permutational multivariate analysis of variance (PERMANOVA) was used to test the effects of day and treatment, and their interaction on the Bray−Curtis distance between samples. Microbial diversity was analyzed as a factorial arrangement taking treatment and sampling day as main factors and main effects are discussed for responses in which interaction was not significant. Differential abundance analysis was performed using the metagenomeseq package (Paulson, Colin Stine, et al., 2013) to examine differences in phylum level, family level, genus level and predicted pathway data. Separate analysis were performed for each sampling day to compare CON and XOS treatments and another one to compare between the two sampling days, independently of the treatment. A cumulative sum scaling (CSS) (Paulson, Olson, et al., 2013) normalization of the raw counts was performed and a zero-inflated Gaussian mixture model was used for the analysis. Relative abundances were used to plot taxon abundances at phylum and family level for each sampling day. Log_2-fold changes were calculated using relative abundances for the taxonomical groups that showed different abundance between treatments at each sampling day. *P*-values were corrected by the false-discovery rate. The pig was considered the statistical unit in all analyses and statistical significance and tendencies were considered at $P \leq 0.05$ and $0.05 < P \leq 0.10$, respectively.

3.3. Results

3.3.1. Growth Performance

The effects of XOS supplementation on growth performance of piglets are summarized in Table 4.2. No differences in body weight (BW) or average daily gain (ADG) were observed in any of the studied periods or the overall ($P > 0.05$).

Table 4. 2. Effects of the XOS supplementation on growth performance.

	Experimental groups[2]			
Items[1]	**CON**	**XOS**	**SEM**	***P* value[3]**
BW d7, kg	2.91	2.91	0.064	0.934
BW d28, kg	7.55	7.48	0.182	0.772
BW d40, kg	10.02	9.83	0.264	0.598
ADG d7-d28, kg	0.221	0.218	0.0077	0.782
ADG d28-d40, kg	0.195	0.196	0.0173	0.968
ADG d7-d40, kg	0.216	0.210	0.0074	0.604

[1]BW: Body weight; ADG: Average daily gain.

[2]CON: Piglets supplemented with water; XOS: Piglets supplemented with XOS.

[3]*P* value obtained from ANOVA test.

3.3.2. Microbiota Structure and Biodiversity

An average of 19,210 ± 9,042 high-quality reads were generated per sample after quality control, from 60 fecal samples. Microbiota community alpha diversity measured as observed OTUs and Shannon index are summarized in Table 4.3. No interactions were observed between treatments and sampling days. Higher richness values were obtained in both observed OTUs and Shannon index on the last sampling day ($P < 0.001$). In addition, piglets supplemented with XOS showed more OTUs when the treatment was analyzed as a main factor ($P = 0.019$).

Table 4. 3. Effect of XOS supplementation on observed OTUs and Shannon indexes from the analysis of the fecal microbiota.

Item	Observed OTUs	Shannon
Treatment effect[1]		
CON	128.8	3.92
XOS	155.1	4.06
SEM	9.27	0.081
Day effect		
d27	115.6	3.73
d40	168.3	4.24
SEM	9.27	0.081
***P* value**[2]		
Treatment	0.019	0.150
Day	< 0.001	< 0.001
Treatment x day	0.5691	0.459

[1]CON: Piglets supplemented with water; XOS: Piglets supplemented with XOS.

[2]*P* value obtained from ANOVA test.

Beta diversity analysis at the OTU level using the Bray-Curtis distance revealed that the microbiota structure changed from d27 to d40 ($P < 0.001$). No differences were attributed to treatment as the main effect. However, pairwise PERMANOVA revealed that XOS and CON tended to have a different diversity composition on d40 ($P = 0.076$). The Principal Component Analysis (PCoA) based on the Bray-Curtis distance matrix shows a distinctive clustering corresponding to the two sampling days (Figure 4.1).

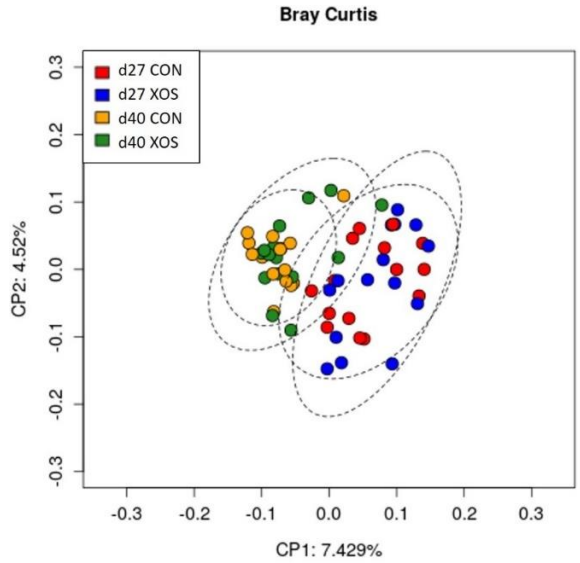


Figure 4. 1. Principal coordinate analysis (PCoA) ordination performed by Bray-Curtis dissimilarities representing the microbiome composition for all animals. d27 CON (red): fecal samples of piglets supplemented with water on day 27; d27 XOS (blue): fecal samples of piglets supplemented with XOS on day 27; d40 CON (yellow): fecal samples of piglets supplemented with water on d40; and d40 XOS (green): fecal samples of piglets supplemented with XOS on d40.

3.3.3. Composition of Gut Microbiota

The relative abundance of the main phyla and families observed are presented in Figure 4.2. The predominant phyla were Firmicutes (58%) and Bacteroidetes (22%) followed by Euryarchaeota (6%), Proteobacteria (5%) and Actinobacteria (5%), representing together 96% of the fecal microbiome. At the family level, *Rumminococcaceae* (24%), *Erysipelotrichaceae* (11%) and *Lachnospiraceae* (11%) were the predominant groups.

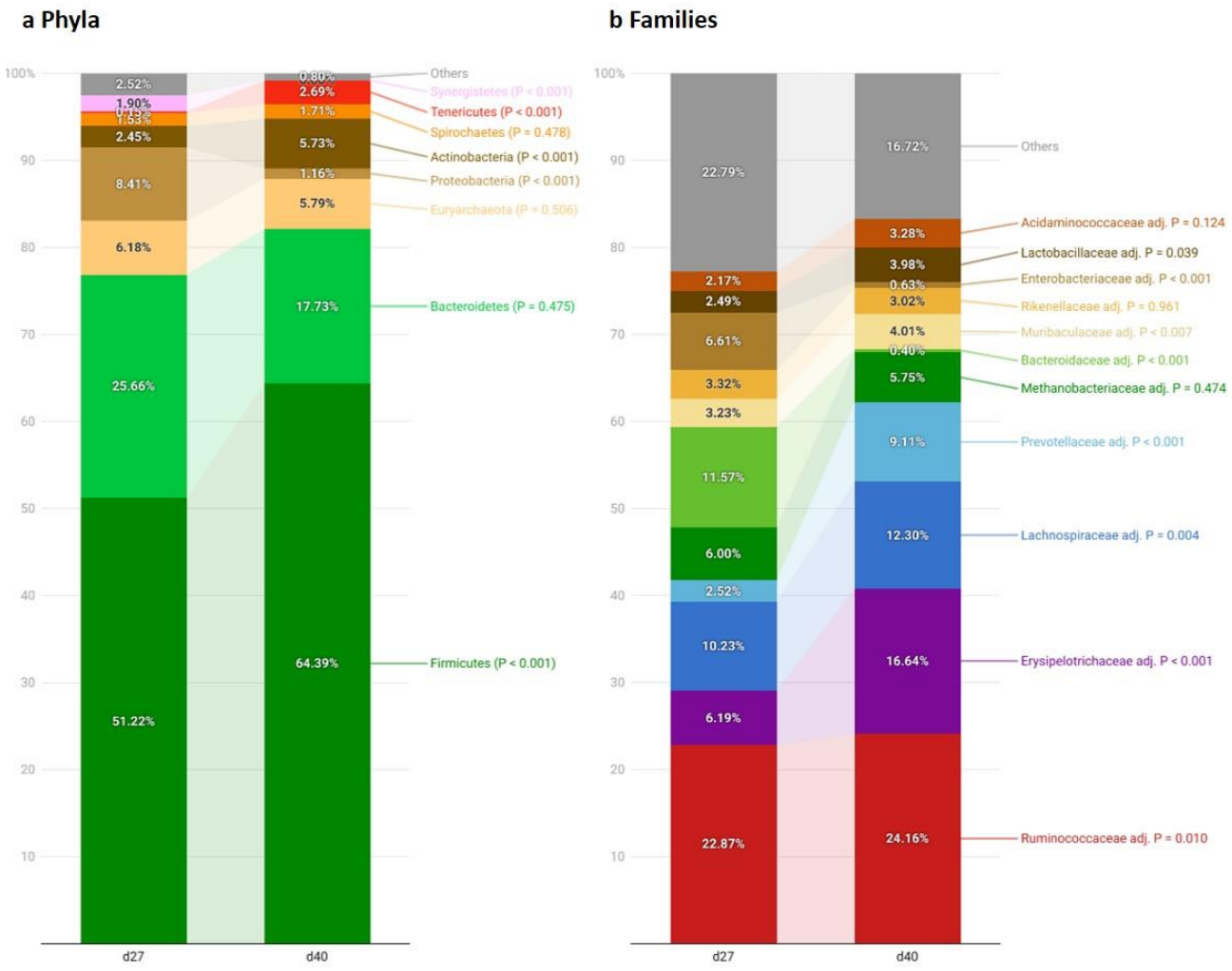


Figure 4. 2. Relative abundances (RA) of the main phyla (**a**) and families (**b**) observed in the analysis of the microbiota of piglets by massive sequencing of the 16S rRNA gene. Figure created with the online open-source tool Datawrapper (http://datawrapper.de).

From d27 to d40, the relative abundance of Firmicutes increased from 51% to 64% ($P < 0.001$) while Bacteroidetes numerically decreased (26% to 18%; $P = 0.475$). The relative abundance of Proteobacteria decreased (8% to 1%; $P < 0.001$) and Actinobacteria increased (2% to 6%; $P < 0.001$) with age. At the family level, an increase of four predominant groups was identified, including *Ruminococcaceae* ($P = 0.01$), *Erysipelotrychaceae* ($P < 0.001$), *Lachnospiraceae* ($P = 0.004$) and *Prevotellaceae* ($P < 0.001$). Meanwhile, the relative abundance of the *Bacteroidaceae* family significantly dropped ($P < 0.001$).

Log_2 fold changes were calculated for the taxa that showed significant differences among groups at the family and genus levels (Figure 4.3). On d27, XOS supplementation promoted the proliferation of families *Helicobacteraceae*, *Methanomethylophilaceae*, *Saccharimonadaceae*, *Eubacteriaceae* and *Spirochaetaceae* compared to CON piglets. At the genus level, *Helicobacter*, *Candidatus Methanomethylophilus*, *Ruminococcaceae* UCG-013, *Prevotella* 7, *Erysipelotrichaceae* UCG-009, *Romboutsia*, *Erysipelotrichaceae* UCG-004, *Olsenella*, *Ruminiclostridium* 5, *Ruminococcus* 1 and *Ruminiclostridium* 9 were increased compared to CON piglets ($P < 0.05$). In contrast, *Blautia* genus was significantly lower in the XOS group compared to CON ($P = 0.030$). On d40, XOS supplementation increased the family taxon *Bacteroidaceae* and also the genus *Romboutsia*, *Bacteroides*, *Senegalimassilia*, *Agathobacter*, *Moryella*, *Ruminiclostridium* 6 and *Lachnospiraceae* NK4A136 group ($P < 0.05$). The relative abundance of *Agathobacter* genus was lower in XOS-supplemented piglets ($P = 0.034$).

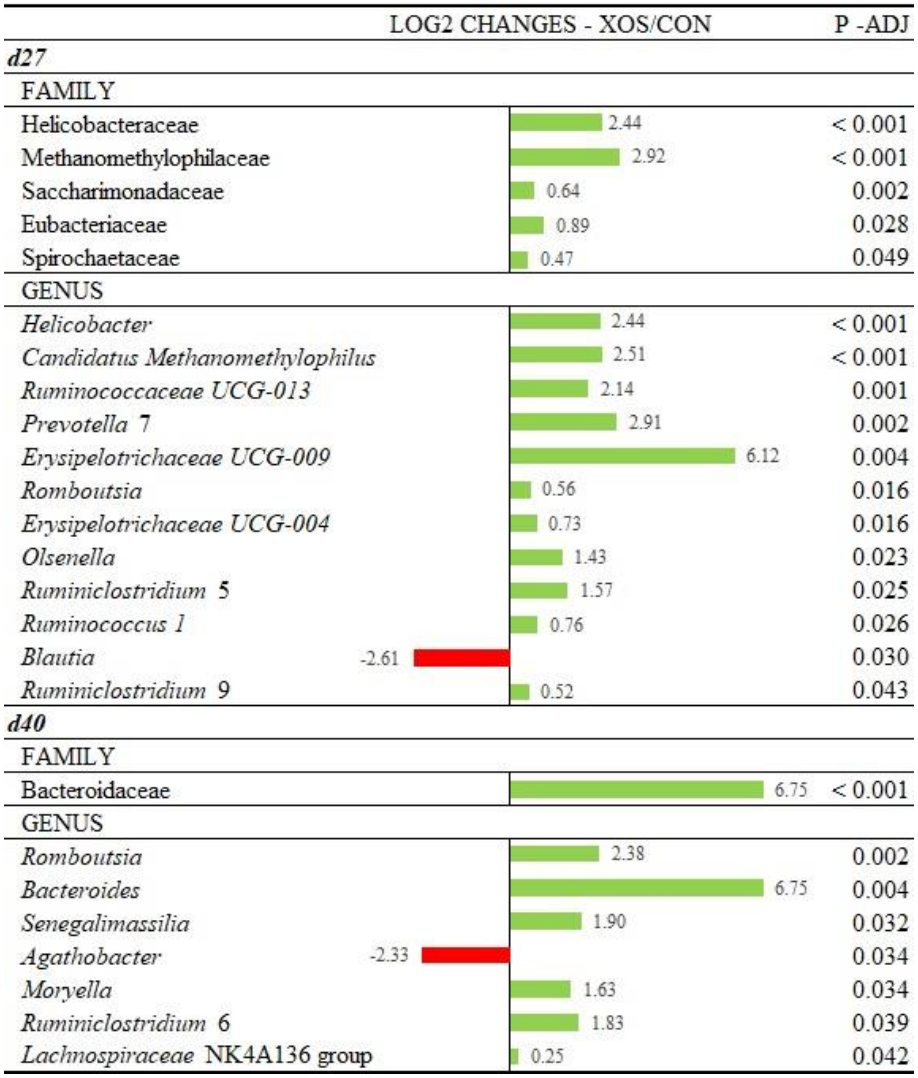


Figure 4. 3. Log_2 changes promoted by xylo-oligosacharides supplementation (fold discovery rate p-adjusted < 0.05) in microbial families and genera on d27 and d40. Taxa are sorted by level of significance (from higher to lower). Differences presented are based on all taxa detected in samples per treatment.

3.3.4. Predicted Functionality of the Gut Microbiota

The functional capacity of the gut microbiota was predicted by using PICRUSt2 based on 16S rRNA gene amplicon sequences. A heatmap representation of all predicted pathways revealed major differences of functional potential of the microbiota between d27 and d40, while the differences between CON and XOS animals were subtle (Figure 4.4). A more in-depth analysis of the abundance differences in the pathway metabolic data between both treatment groups showed that 186 out of 349 pathways significantly changed from d27 to d40, while supplementation of XOS significantly changed the relative abundance of 3 and

24 pathways out of 349, at 27 and 40 days of age, respectively. Interestingly, the results showed an increase of pathway CENTFERM-PWY, also known as “pyruvate fermentation to butanoate”, in XOS-supplemented animals on d40 (*P* = 0.045).

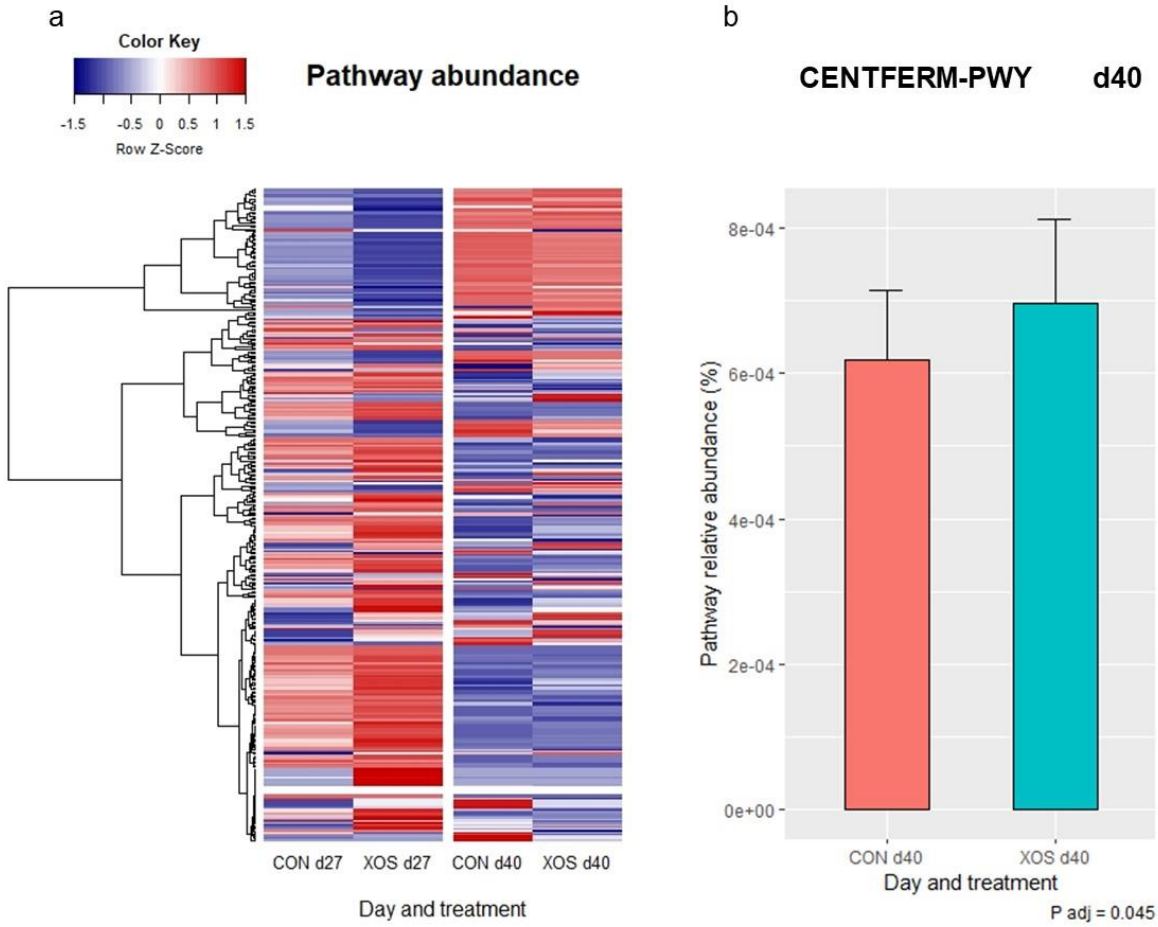


Figure 4. 4. Predicted functionality of the fecal microbiota. The potential functionality of the gut microbiota was inferred from 16S rRNA gene amplicon sequences in feces collected from control piglets (CON) and piglets supplemented with xylooligosacharides (XOS) at the end of lactation period (d27) and during the nursery period (d40). (**a**) Heatmap representing the mean relative abundance of each predicted pathways in each treatment. The color represent the Z-scores (row-scaled relative abundance) from low (blue) to high values (red). Predicted pathways (rows) were clustered by the average method. (**b**) Bar plot representing the relative abundance of the CENTFERM-PWY predicted pathway at d40. Error bar represents standard error of the mean. d27 CON: fecal samples of piglets supplemented with water on day 27; d27 XOS: fecal samples of piglets supplemented with XOS on day 27; d40 CON: fecal samples of piglets supplemented with water on d40; and d40 XOS: fecal samples of piglets supplemented with XOS on d40. Figure created by using open-source software R v4.2.0. (https://www.r-project.org/foundation/) and the gplots package (https://cran.r-project.org/web/packages/gplots/).

3.3.5. Short-Chain Fatty Acids Analysis

The SCFA in the fecal samples on d27 and d40 are summarized in Table 4.4. No interactions were observed for any of the SCFA measured ($P > 0.05$). Supplementation with XOS did not influence SCFA ($P > 0.05$). Feces of piglets of 40 days of age had higher concentrations of total SCFAs, acetic acid, propionic acid, butyric acid, lactic acid and volatile fatty acid ($P < 0.001$).

Table 4. 4. Effect of XOS supplementation in total SCFAs concentration in feces, expressed in mM.

Item[1]	Total SCFAs	Acetic A.	Propionic A.	Butyric A.	Valeric A.	Lactic A.	BCFAs	VFAs
Treatment effect[2]								
CON	81.62	45.05	14.94	11.00	2.43	1.43	6.81	80.17
XOS	84.71	48.43	15.81	10.11	2.64	1.06	6.67	83.66
SEM	4.912	2.070	1.243	1.315	0.362	0.234	0.683	4.955
Day effect								
d27	60.91	34.62	8.67	7.18	2.31	0.51	7.65	60.38
d40	105.42	58.86	22.08	13.93	2.76	1.98	5.83	103.45
SEM	4.683	2.094	1.132	1.277	0.318	0.238	0.737	4.709
***P* value[3]**								
Treatment	0.657	0.253	0.621	0.634	0.684	0.266	0.889	0.620
Day	<0.001	<0.001	<0.001	<0.001	0.242	<0.001	0.105	<0.001
Treatment x day	0.605	0.917	0.703	0.266	0.944	0.808	0.280	0.597

[1]SCFAs: Short-chain fatty acids. BCFAs: Branched-chain fatty acids; VFAs: Volatile fatty acids.
[2]CON: Piglets supplemented with water; XOS: Piglets supplemented with XOS.
[3]*P* value obtained from ANOVA test.

3.4. Discussion

Supplementing monogastric animals with XOS has proven to be positive in the past, increasing performance, promoting the growth of beneficial microbial populations and enhancing intestinal health (Bai et al., 2021; Y. Chen et al., 2021; A. D. Craig et al., 2020; De Maesschalck et al., 2015; J. B. Liu et al., 2018; Pan et al., 2019; Ribeiro et al., 2018; Yin et al., 2019). In this study, XOS was provided to suckling piglets at very low doses (30 to 60 mg/day) to explore their likely effects as stimbiotics, with the aim of modulating the early-colonization of the gut microbiota and the establishment of a higher fibrolytic environment with age. Inclusion of soluble fiber, such as oligosaccharides, in the diet of newly weaned piglets has been considered a risk factor for pig health and growth in some reports,

especially under poor sanitary conditions, due to a limited digestive capacity of piglets (Montagne et al., 2012). In this trial, XOS did not influence piglet's performance during suckling (d28) nor the post-weaning period (d40), indicating that their digestive system tolerated the supplementation of low doses of XOS during this period without conditioning their performance. No differences were also observed on the SCFAs concentration in feces.

3.4.1. Evolution of Gut Microbiota from Suckling to Nursery Phase

The process of weaning is associated with a combination of environmental, social and dietary stressors that are known to generate changes in the piglet's gut microbial ecosystem (J. P. Lallès et al., 2007; Moeser et al., 2017). The high throughput sequencing of the 16S RNA gene from the feces of the piglets revealed changes in the microbial diversity and composition between the end of lactation and the nursery period. The number of observed OTUs, that is an indicator of richness, and the Shannon index, that reflects richness and evenness, increased in the post-weaning period. This result agrees with other studies (L. Chen et al., 2017; Saladrigas-García, D'Angelo, Ko, Nolis, et al., 2021) that reported an increase in alpha diversity indexes after weaning transition, suggesting the maturation of the gut microbiota during that period. Alpha diversity is considered an indicator of gut ecosystem maturation, because a higher diversity of bacterial species suggests a higher "functional redundancy" that helps the microbial ecosystem maintain its resilience, resistance and stability after environmental stresses (Konopka, 2009; Naeem & Li, 1997). Beta diversity analysis corroborated that pre-weaning and post-weaning gut populations had different microbiota structures.

The microbial composition of piglets' feces from this study are in the line with pig microbiota described in the literature (Holman et al., 2017). Firmicutes and Bacteroidetes were the dominant phyla in piglets feces regardless of age, in agreement with other studies (Holman et al., 2017; Mach et al., 2015; Niu et al., 2015; Pajarillo et al., 2014; Saladrigas-García, D'Angelo, Ko, Nolis, et al., 2021; X. Wang et al., 2019). Firmicutes was the main phylum in the whole period of the study and increased its relative abundance in the nursery period. The abundance of Proteobacteria drastically decreased in the nursery period, as has been reported previously (L. Chen et al., 2017; Niu et al., 2015; Pajarillo et al., 2014).

Proteobacteria include multiple opportunistic pathogens, thus, its reduction might reflect the maturation of gut microbiota during the weaning transition. From lactation to the nursery period, at the family level, the relative abundance of *Bacteriodaceae* abruptly decreased at the same time that *Ruminococcaceae*, *Lachnospiraceae* and *Prevotellaceae* increased. These shifts might be explained by the diet change from sow's milk during lactation to a solid plant-based diet as reported in other studies (L. Chen et al., 2017; Frese et al., 2015; Pajarillo et al., 2014; Saladrigas-García, D'Angelo, Ko, Nolis, et al., 2021; X. Wang et al., 2019). *Bacteroideaceae* members have the ability to metabolize complex oligosaccharides present in the sow milk (Marcobal et al., 2011), while *Ruminococcaceae*, *Lachnospiraceae* and *Prevotellaceae* families are adapted to break down oligosaccharides and polysaccharides present in the nursery feed (Zhao et al., 2018).

Differences observed in the fecal microbial communities between pre- and post-weaning were concurrent with relevant changes in the predicted functionality of the microbiota obtained from PICRUSt2 analysis. This indicates that the different gut populations found in each period also had a distinct metabolic profile. Indeed, the expansion of fibrolytic bacteria in the nursery period was correlated with an increase in total SCFAs, VFAs, acetic acid, propionic acid, butyric acid and lactic acid concentrations in feces observed in the same period, as has been described in other studies (Van Beers-Schreurs et al., 1998).

3.4.2. Effects of XOS Supplementation on Gut Microbiota

The main objective of the present study was to explore the effect of providing low doses (30 to 60 mg/d) of XOS to suckling piglets in their fecal microbiota at the end of lactation and in the nursery period. Provision of XOS to pigs has been extensively shown to modulate their gut microbiota, promoting the growth of *Bifidobacterium* and other beneficial fiber-degrading and SCFA-producing bacteria (Y. Chen et al., 2021; J. B. Liu et al., 2018; Pan et al., 2019b), even at very low doses (González-Ortiz et al., 2019; Yin et al., 2019). In this study animals supplemented with XOS showed a higher OTU richness in the fecal microbiota, suggesting that XOS promoted an earlier microbial maturation. In addition, beta

diversity analysis revealed that microbial populations of the XOS group tended to differ from the CON group in the nursery period.

The effect of XOS in the hindgut microbiota of the piglets at the end of lactation was characterized by an increase in the relative abundance of some taxa associated to fiber fermentation such as *Eubacteriaceae* at the family level and *Ruminococcaceae* UCG-013, *Prevotella* 7, *Ruminiclostridium* 5, *Ruminoccocus* 1 and *Ruminiclostrium* 9 at the genus level. These groups are considered beneficial for the host health due to their capacity to produce SCFAs through the fermentation of complex polysaccharides, and in addition, some of them have been associated to lower diarrhea incidence in suckling pigs (Dou et al., 2017). Furthermore, members of *Eubacteriaceae* family and *Prevotella* genus had demonstrated positive correlations with elevated pig growth performance (Amat et al., 2020; Oh et al., 2020; Ramayo-Caldas et al., 2016). Supplementation of XOS also increased the abundance of *Erysipelotrichaceae*. Members of the *Ruminococcaceae*, *Prevotellaceae* and *Erysipelotrichaceae* families are considered typical post-weaning bacteria (L. Chen et al., 2017; Saladrigas-García, D'Angelo, Ko, Nolis, et al., 2021), and their enrichment in XOS-supplemented piglets at the end of the lactation phase suggests that XOS supplementation accelerates gut microbiota maturation. In contrast, *Blautia* relative abundance was lower in XOS-supplemented pigs even though it is considered as a genus with probiotic characteristics, capable of fermenting carbohydrates for SCFAs production (X. Liu et al., 2021).

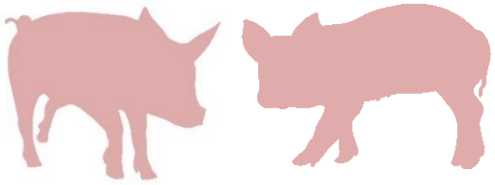
At 12 days post-weaning, differences in the relative abundances of specific taxa were also observed. Like at the end of lactation, XOS supplementation increased the abundances of genera related to fiber fermentation and SCFAs production in the nursery period, such as *Bacteroides*, *Moryella*, *Ruminiclostridium* 6 and *Lachnospiraceae* NK4A136. Interestingly *Bacteroides* was found to be 6.75 log-2 fold higher in XOS-supplemented piglets compared to CON. *Bacteroides* are major primary degraders of oligo- and polysaccharides with a close relationship with XOS, containing the most expanded glycolytic gene repertoires that target xylan degradation (Mendis et al., 2018). In fact, different *in vitro* tests have demonstrated that XOS stimulates *Bacteroides* growth in the microbial

ecosystem (M. Chen et al., 2020; Míguez et al., 2018). The abundance of *Lachnospiraceae* in mice fed a high-fat diet was increased by XOS, increasing the formation of butyric acid in the cecum (Berger et al., 2021).

Functional profile predictions demonstrated differences in the pathway abundance between CON and XOS in both periods. However, more differences were found during the nursery period, which is consistent with the tendency for the microbial structure of both treatments to be different in the nursery period. The higher relative abundance of the predicted pathway "pyruvate fermentation to butanoate" found in XOS-supplemented pigs in the nursery period is in agreement with the higher presence of fermenters producing SCFAs, including butyrate, such as *Bacteroides*, *Moryella*, *Ruminiclostridium* 6 and *Lachnospiraceae* NK4A136. Actually, SCFAs decrease the pH in the intestine, promote gastrointestinal motility and inhibit the proliferation of opportunistic pathogens (Den Besten et al., 2013). Among them, butyrate is the preferred energy source used by colonocytes, which is involved with the maintenance of the gut barrier function and has been related in anti-inflammatory pathways (J. Tan et al., 2014). Nevertheless, neither the increase of certain SCFA-producing bacterial populations observed in the XOS-supplemented piglets nor the increase in the predicted pathway of butyrate production in the nursery period resulted in any change in fecal SCFAs concentrations. It is more likely to observe changes in the production of SCFA using *in vitro* tests including XOS (M. Chen et al., 2020; Smiricky-Tjardes et al., 2003), while there is more uncertainty to see such effects from *in vivo* studies (Bai et al., 2021; Y. Chen et al., 2021; Pan et al., 2019). Most SCFAs (95%) produced by the microbiota are quickly absorbed by the mucosa, while only 5% are excreted in the feces (Den Besten et al., 2013). In fact, several studies have reported that increases in SCFAs concentration in portal blood could not be predicted from the concentrations in the intestinal lumen (Kuller et al., 2007; Nakatani et al., 2018). Therefore, SCFAs concentration found in the feces might have a limited value in identifying their production in the intestine and future studies should use cecum or intestinal digesta for its determination instead.

The nursery diet offered to both treatments did not include XOS but it contained xylanases, which are expected to hydrolyze dietary arabinoxylan molecules and release XOS in the gut of post-weaned piglets. In spite of this, the animals supplemented with XOS during the suckling period still maintained differences in microbial populations, structure and functions in the nursery period compared to CON animals, which confirms the hypothesis that an early-supplementation of suckling piglets with XOS can accelerate the establishment of a different microbial population with age. The results of this study agree with Bai et al. (2021), who studied the effect of adding XOS in the creep feed of suckling piglets, and showed an increase in the predicted microbial ability for carbohydrate digestion and absorption capacity in the feces at weaning and a higher xylanase activity at 27 days after weaning, even though they were fed the same diet after weaning. Several studies evaluating dietary interventions, such as the supplementation of prebiotics, fiber or functional ingredients to modulate the functioning of the gastrointestinal tract in suckling piglets have been published (Huting et al., 2021; Pluske, 2016), but only a few of them have investigated the long-term effects post-weaning. These studies described a disappearance of the differences in the gut microbial populations generated during the suckling period after weaning occurs (Choudhury et al., 2021; Fouhse et al., 2019). The process of gut microbiota maturation supposes a homogenization and stabilization of its structure and populations that might blur the effects of certain interventions made during suckling (L. Chen et al., 2017; Saladrigas-García, D'Angelo, Ko, Nolis, et al., 2021). The persistence of the effects of XOS in the gut microbiota after weaning emphasizes its potential in modulating the early microbial colonization of piglets' gut and suggests it might have effects on the long-term performance and health.

In summary, the supplementation of low doses (30 to 60 mg/d) of XOS to piglets during the suckling period promoted the growth of fiber-degrading and SCFA-producing microbial populations in the hindgut. The effects of supplementing XOS only during lactation are still observed in the nursery period, when all piglets were on the same diet and environment, suggesting that XOS conditions the establishment of a differential microbiota population in the long term.



Chapter 5

Effect of Mixing at Weaning and Nutrient Density of the Weaner Diet on Growth Performance and Welfare of Pigs to Slaughter

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Abstract

Mixing pigs at weaning can compromise pig welfare and growth. Therefore, grouping littermates together may allow a diet nutrient and energy density reduction during the nursery period to reduce feed cost without affecting slaughter weight. This study investigated the combined effect of mixing and reducing dietary energy and nutrient density on growth performance, body lesions (BL), and behaviour in pigs from weaning to slaughter. Forty-eight litters [554 pigs, 11-12 pigs/litter; Danish Duroc × (Large White × Landrace)] were included in the trial. At 28 days of age, pigs were weaned and housed in nursery rooms in litter groups (INTACT, n = 24) or mixed with other litters and grouped by weight to reduce within-pen pig weight variation (MIXED, n = 24). A dietary regimen meeting pigs' nutritional requirements (CON) and a low-density dietary regimen (LOW; -10% energy and protein) completed a 2x2 factorial arrangement (Mixing x Diet, n=12). On day 74 of age, pigs moved to the grower-finisher accommodation without further mixing and all pigs received the CON dietary regimen. Mixing increased feed conversion ratio (FCR) by 4.0 % during the nursery period ($P = 0.003$). Nursery pigs fed LOW experienced a growth retardation which was maintained until slaughter (-2.6 kg slaughter weight; $P = 0.025$). Initial differences in the co-efficient of variation (CV) between MIXED (10.4%) and INTACT (17.6%; $P < 0.001$) pigs were reduced in CON pens but not in LOW pens (interaction $P = 0.025$) at the end of the nursery period. MIXED pigs had more fights and BL ($P < 0.001$) at weaning and showed more aggression ($P = 0.003$) after being moved to the grower-finisher rooms. At the end of the nursery period, MIXED pigs fed LOW showed the highest number of aggressive behaviours around the feeder (interaction; $P = 0.003$) and pigs fed LOW showed more damaging behaviour ($P < 0.001$). In conclusion, mixing animals at weaning had limited impact on growth performance but impaired welfare which was aggravated by energy and nutrient reduction in the nursery diet. Decreasing dietary nutrient density in the nursery stage retarded growth, which could not be compensated for during the growing-finishing period.

4.1. Background

Mixing or regrouping of pigs is a common management practice used in intensive production systems whereby pigs are sorted into groups by body weight (BW) and/or sex (Peden et al., 2018). Unfamiliar pigs are mixed together during all stages of the production cycle (and during transport and lairage)(van Staaveren et al., 2015) for a variety of reasons including to reduce variation in litter size (i.e. cross fostering), to separate gilts destined as replacements, to adjust group size to the dimensions of the pens, to reduce within-pen bodyweight variation and to achieve more homogenous slaughter weights (Conte et al., 2012). However, the initial reduction in BW variability within pens achieved by mixing often increases to values similar to those obtained without mixing the animals (O'Quinn et al., 2000). Additionally, social hierarchies need to be established every time unfamiliar pigs are re-grouped. Under intensive commercial practice, pigs establish the dominance hierarchy by fighting and other aggressive strategies (Stookey & Gonyou, 1994) that persist for approximately the first 24h after mixing (Meese & Ewbank, 1973), until a relatively stable hierarchical structure is established (Hayne & Gonyou, 2006). Fights result in skin lesions, have physiological effects, and increase susceptibility to infection due to the immunosuppressive effects of stress (Morrow-Tesch et al., 1994; Peden et al., 2018). These problems often result in an impairment of growth performance in addition to negative effects on welfare (Camp Montoro et al., 2021, 2022; Coutellier et al., 2007; Hyun, Ellis, & Johnson, 1998; Hyun, Ellis, Riskowski, et al., 1998; Stookey & Gonyou, 1994). Furthermore, the acute social stress resulting from the mixing of pigs during weaning, combined with the stress of being separated from their mother and the abrupt dietary and environmental change, can have negative consequences for pig health (J. P. Lallès et al., 2007). Once the dominance hierarchy is established after mixing, aggression can continue in the long term due to space restrictions and competition for access to resources [15]. Less attention is paid to the effects of this chronic aggression, although its impact on growth is likely to be significant (S. S. L. Tan et al., 1991).

Reducing the energy and nutrient density of the nursery diets is a strategy to reduce the feed cost and it does not necessarily involve a reduction in growth performance along the production cycle when pigs can compensate during later stages for the slower growth rate after weaning. Additionally, pigs show the capacity to increase their feed intake to maintain energy consumption when dietary energy density is reduced (Aymerich et al., 2020; Q. Li & Patience, 2017; Pichler et al., 2020). While the feed intake capacity of pigs increases as they get older, pigs weighing less than 20 kg may not have the capacity to compensate for severe reductions in energy density (J. W. Kim et al., 2021). In addition, when feeder space is limited, such as is the case with single-space feeders, the number of skin lesions associated with competition among pigs for access to the feeder increases (López-Vergé, Gasa, Temple, et al., 2018). Therefore, diluting the energy density of the feed, which will increase the average daily feed intake (ADFI), in combination with a limitation in the number of feeder spaces, increases competition for access to food thereby increases aggression in the longer term i.e. chronic aggression.

Our hypothesis was that allowing piglets to remain with their littermates in a stable social group following weaning would attenuate the impact of the social stress associated with weaning and the subsequent performance of chronic aggressions within the group. Additionally, we expected that providing a low-density diet during the nursery period would have less impact on the growth of pigs kept as intact litters and that any reduction in growth would be compensated for by providing a diet with a higher energy and nutrient density during the growing-finishing period. Thus, the objective of the present study was to determine the impact of mixing and providing low-density nursery diets to pigs on lifetime growth and welfare.

4.2. Materials and Methods

The experimental procedures used in this study were approved by the Teagasc Animal Ethics Committee (TAEC 204/2018) and were in full compliance with national

legislation following the EU-Directive 2010/63/EU for the protection of animals used for scientific purposes and designed in compliance with the ARRIVE guidelines.

4.2.1. Animals, Housing, and Diet

The present trial was conducted at the Teagasc Pig Research Facility in Fermoy, Co. Cork, Ireland. Two batches of 24 litters with a total of 264 and 288 Danish Duroc × (Large White × Landrace) piglets (docked and un-castrated males), were weaned at 28 days of age and housed in pens of 11 pigs/pen in batch 1 and 12 pigs/pen in batch 2. Pens were allocated to 4 treatments in a 2 x 2 factorial arrangement (Mixing x Diet, n = 12). For the mixing treatment, pigs were in groups of intact litters (LITTER) or mixed with unfamiliar pigs from other litters to reduce the within pen BW variation (MIXED). MIXED pens were composed of big or small mixed-sex pigs. LITTER pens were adjusted to 11 or 12 pigs (depending on the batch) by removing pigs where necessary, preserving a normal distribution of individual BW. For diet, pens were allocated to a dietary regimen meeting the nutritional requirements of the pigs (CON) or a low-density regimen (LOW) with -10% net energy (NE) and -10% standard ileal digestible Lysine (SID Lys)) of CON. Pigs received a starter diet at weaning for 11 days followed by a link diet from d12 to d22 post weaning and a weaner diet from d23 to d46 post-weaning when the nursery phase ended (Table 5.1). Nursery pens were equipped with fully slatted plastic floors (2.5 × 2 m) with automatic environmental control. Each pen had a single-space (33 cm) wet-dry feeder (BA19100, Verba, Netherlands) with inset nipple drinker and a supplementary bowl drinker (SS Drinker, Rotecna, Spain). Nursery pens were enriched with a rubber spiked ball (Easyfix Luna 142, Easyfix, Galway, Ireland).

Pigs were moved to the finisher accommodation at 74 days of age (31.6 ± 3.4 kg BW), keeping the same pen composition as in the nursery rooms. Finisher pens had fully slatted concrete floors (2.4 × 4.2 m) with automatic environmental control, containing one single-space (33 cm) wet-dry feeder (MA19100, Verba, Netherlands) with inset nipple drinker and a supplementary bowl drinker (SS Drinker, Rotecna, Spain). Finisher pens were enriched with a larch wood post. All pigs were fed a single soybean meal-maize-wheat based finisher diet (Table 5.1) up to target slaughter weight and remained in the facility until the

first group of pigs reached 110 kg of BW when all pigs were sent for slaughter. Water and pelleted feed were provided ad libitum during the trial.

Table 5. 1. Experimental diets offered to the animals included in the trial.

	Experimental diets						
	Starter		Link		Weaner		Finisher
Item	CON	LOW	CON	LOW	CON	LOW	
Ingredients, %							
Barley	5.0	5.0	6.8	59.3	49.6	73.7	41.1
Maize	23.1	37.7	30.0	-	-	-	-
Wheat	-	-	10.0	6.2	21.7	6.0	39.0
Soybean meal 48%	14.3	10.1	18.7	11.3	16.3	14.1	16.5
Full fat soya	13.1	10.0	7.0	10.0	5.0	3.0	1.1
Whey permeate (Lactoflo)	20.0	20.0	15.0	7.5	-	-	-
Skim dried milk	12.5	12.5	5.0	-	-	-	-
Soya oil	8.50	1.38	3.82	1.60	4.00	-	-
Lysine HCl 78.8%	0.622	0.606	0.672	0.736	0.593	0.523	0.427
DL-Methionine	0.362	0.300	0.318	0.296	0.217	0.168	0.100
L-Threonine	0.364	0.329	0.342	0.354	0.271	0.230	0.190
L-Tryptophan	0.140	0.134	0.127	0.113	0.057	0.045	0.022
L-Valine	0.129	0.107	0.126	0.152	0.062	0.017	-
Limestone flour	0.700	0.700	0.750	0.900	1.050	1.050	1.100
Salt	0.300	0.300	0.300	0.300	0.300	0.300	0.300
Mono Dicalcium Phosphate	0.550	0.550	0.700	0.900	0.550	0.550	0.100
Vitamin-Mineral Premix nursery [1]	0.300	0.300	0.300	0.300	0.300	0.300	-
Vitamin-Mineral Premix finisher [2]	-	-	-	-	-	-	0.100
Phytase	0.010	0.010	0.010	0.010	0.010	0.010	0.010
Calculated composition							
Dry matter, %	91.1	90.0	89.4	88.2	87.8	87.2	87.3
Net Energy (NE), kcal/kg	2882	2594	2616	2354	2462	2216	2342
Ash, %	6.23	5.98	5.65	5.42	4.70	4.76	4.05
Protein, %	20.0	18.0	19.0	17.1	17.7	16.6	16.7
Ether Extract, %	12.18	5.03	6.82	4.73	6.34	2.18	2.66
Neutral Detergent Fiber, %	6.05	6.85	8.08	13.99	13.99	16.15	14.02
Calcium, %	0.819	0.801	0.754	0.767	0.737	0.730	0.652
Total P, %	0.586	0.581	0.567	0.584	0.489	0.497	0.389
Digestible P, %	0.462	0.459	0.423	0.423	0.332	0.336	0.246
Na, %	0.340	0.341	0.263	0.182	0.131	0.131	0.132
Cl, %	0.914	0.916	0.736	0.556	0.352	0.355	0.317
SID amino acids[3]							
Lys, %	1.528	1.375	1.414	1.272	1.200	1.080	1.000

Met, %	0.680	0.603	0.591	0.513	0.447	0.389	0.324
Cys, %	0.238	0.226	0.258	0.254	0.276	0.264	0.279
Met + Cys, %	0.917	0.825	0.848	0.763	0.720	0.648	0.600
Thr, %	0.993	0.894	0.919	0.827	0.780	0.702	0.671
Trp, %	0.336	0.303	0.311	0.280	0.240	0.216	0.200
Val, %	0.963	0.866	0.891	0.802	0.756	0.680	0.666

[1] Premix provided per kilogram of complete diet: Cu from copper sulphate, 100 mg; Fe from ferrous sulphate monohydrate, 90 mg; Mn from manganese oxide, 47 mg; Zn from zinc oxide, 120 mg; I from potassium iodate, 0.6 mg; Se from sodium selenite, 0.3 mg; vitamin A as retinyl acetate, 2.1 mg; vitamin D3 as cholecalciferol, 25 µg; vitamin E as DL-alpha-tocopheryl acetate, 100 mg; vitamin K, 4 mg; vitamin B12, 15 µg; riboflavin, 2 mg; nicotinic acid, 12 mg; pantothenic acid, 10 mg; choline chloride, 250 mg; vitamin B1, 2 mg; and vitamin B6, 3 mg.

[2] Premix provided per kilogram of complete diet (Diet 4): Cu from copper sulphate, 15 mg; Fe from ferrous sulphate monohydrate, 24 mg; Mn from manganese oxide, 31 mg; Zn from zinc oxide, 80 mg; I from potassium iodate, 0.3 mg; Se from sodium selenite, 0.2 mg; vitamin A as retinyl acetate, 0.7 mg; vitamin D3 as cholecalciferol, 12.5 µg; vitamin E as DL-alpha-tocopheryl acetate, 40 mg; vitamin K, 4 mg; vitamin B12, 15 µg; riboflavin, 2 mg; nicotinic acid, 12 mg; pantothenic acid, 10 mg; vitamin B1, 2 mg; vitamin B6, 3 mg.

[3] Standardized ileal digestible amino acids.

4.2.2. Productive Performance

Pens of pigs were weighed every two weeks until d151 of age, before they were sent to slaughter. Average daily gain (ADG) was calculated for every 2-week interval. Feed intake was recorded daily at pen level, added for every 2-week period and ADFI was calculated. FCR was calculated as $\frac{\text{kg of feed consumed}}{\text{BW gain}}$ for each 2-week period. Pigs were also weighed individually at weaning (d28), transfer from nursery to finisher (d74) and before slaughter (d151) to determine the CV within the pen.

4.2.3. Animal Behaviour Measurements

Animal behaviour was recorded by direct observation 1 day post-weaning (d29), three days before transfer to the grower-finisher rooms (d71), one day after the transfer (d75) and at d150, before pigs started to go to slaughter. All pens were observed for three 5 min observations (15 min in total per pen) between 08:00 and 16:00 hours. All occurrences of aggression, aggression around the feeder, damaging and sexual behaviour were recorded (Table 5.2). Observations were equally distributed across pens and time for each recording period.

Table 5. 2. Ethogram for the recording of negative behaviours in mixed and intact groups of pigs on two diets (adapted from O'Driscoll et al., 2013).

	Behaviour	Description
Aggression	Fight	Sustained aggressive biting/pushing by ≥2 pigs
	Bite	Biting aggressively at head/body of other pig
	Head knock	Hitting vigorously with head against body/head of other pig
	Parallel pressing	Pressing shoulders against body of other pig, pushing
Aggression around feeder	Displacement from feeder	Pig displaced from feeder by another pig
	Bite at feeder	Biting at body of other pig, at feeder
	Head Knock at feeder	Hitting vigorously with head against body/head of other pig, at feeder
	Climb at feeder	Placing two front hoofs on the body/head of another pig, at feeder
Damaging	Belly nose	Repeated thrusting of snout into belly of another pig
	Ear bite	Ear of other pig in mouth
	Tail bite	Tail of other pig in mouth
Sexual	Sexual mount	Placing two front hoofs on the body/head of another pig

4.2.4. Body Lesion Counts

Following the Welfare Quality® criteria (Welfare Quality ® Assessment Protocol for Pigs, 2009), the body of the pigs was divided into anterior, mid and posterior part. A body lesion (BL) was defined as either surface penetration of the epidermis or penetration of the muscle tissue (Welfare Quality ® Assessment Protocol for Pigs, 2009). All skin lesions in each location were counted individually as BL, recorded on a check sheet, and summed up to obtain the total number of BL per pig (Welfare Quality ® Assessment Protocol for Pigs, 2009). Lesions arising from damaging behaviour were scored according to severity (ears: 0 – 4 and tails: 0 – 3), both scales are described in (Chou et al., 2018). Lesions were counted and ears and tails were scored 2 days post-weaning (d30), two weeks later (d42), two days before the transfer to grower-finisher rooms (d72), two days after the transfer (d76), at d144 and at d151, before pigs started to go to slaughter.

4.2.5. Statistical Analyses

All data were analysed in open-source software R v4.0.3 (R Foundation for Statistical Computing, Vienna, Austria). Each pen was considered as the experimental unit for all data

analyses. Growth performance data, BL counts and ear and tail scores were analyzed using general linear mixed models and behaviour data were analyzed using a generalized linear mixed model for a Poisson distribution. The model included mixing, diet and their interaction as fixed effects, and batch as a random effect. Growth performance data analysis used initial BW as a covariable while BL and behaviour observations analysis used the BW at the time of the measurement as a covariable. Differences between treatment groups were calculated as $\frac{\text{Grup A}-\text{Group B}}{\text{Group B}}$. Multiple means comparisons were done using Tukey-Kramer's correction when the analysis revealed an interaction between mixing and diet. Alpha for determination of significance and tendencies were 0.05 and 0.10, respectively.

4.3. Results

4.3.1. Body Weight, Feed Intake, and Feed Efficiency Traits

The growth performance results are summarized in Table 5.3. As there was no Mixing x Diet interaction for the growth performance results, only the main effects of each factor will be described. Mixing did not affect BW, ADG or ADFI during the experimental period ($P > 0.05$) but caused a 4.0% increase in the FCR during the d28 to d74 period ($P = 0.003$). Mixing showed no effect on growth performance during the period from d28 to d151.

Table 5. 3. Effect of mixing at weaning or keeping pigs in litter groups and two dietary regimens fed in the nursery period on the productive performance of pigs from weaning to slaughter.

	Dietary regimen [2]							
	CON		LOW			*P* value		
Trait [1]	Litter	Mixed	Litter	Mixed	SEM[3]	Mixing	Diet	Mixing × Diet
Productive performance								
BW d28, kg	8.38	8.43	8.43	8.43	0.294	0.932	0.936	0.926
BW d74, kg	33.1	32.8	30.1	30.4	0.98	0.832	< 0.001	0.485
BW d151, kg	118.8	117.3	114.6	116.2	2.17	0.933	0.025	0.176
ADFI d28 - d74, kg	0.772	0.791	0.757	0.785	0.0193	0.215	0.534	0.760
ADFI d74 - d151, kg	2.503	2.452	2.410	2.428	0.0512	0.595	0.093	0.300
ADFI d28 – d151, kg	1.855	1.831	1.792	1.814	0.0392	0.970	0.291	0.543
ADG d28 - d74, kg	0.538	0.527	0.475	0.477	0.0128	0.637	< 0.001	0.496
ADG d74 - d151, kg	1.113	1.097	1.097	1.114	0.0188	0.999	0.998	0.204
ADG d28 – d151, kg	0.898	0.884	0.864	0.876	0.016	0.950	0.182	0.416
FCR d28 - d74	1.43	1.51	1.60	1.65	0.033	0.003	< 0.001	0.580
FCR d74 - d151	2.25	2.23	2.20	2.18	0.023	0.367	0.008	0.950
FCR d28 – d151	1.94	1.96	1.97	1.98	0.016	0.442	0.127	0.725

[1] BW: Body weight; ADG: average daily gain; ADFI: average daily feed intake; FCR: feed conversion ratio.

[2] CON: dietary regimen meeting pigs' nutritional requirements; LOW: low-density dietary regime with -10% of energy and protein of CON during the nursery period, but with the same dietary regimen as CON during the growing-finishing period.

[3] Standard error of the mean.

[a,b,c] Within rows, significant differences between groups ($P < 0.05$).

Pigs fed the LOW diet gained 57 g less per day and had a 2.72 kg lower BW at the end of the nursery period (d74) compared to pigs fed the CON diet ($P < 0.001$), but both treatments had a similar ADFI ($P > 0.05$). As a result, the FCR for LOW pigs deteriorated by 10.3 % ($P < 0.001$). During the growing-finishing period, when all animals were offered the same diet, no differences in ADG were observed ($P > 0.05$), but pigs that were fed the LOW diet during the nursery period had a 2.4 % better FCR ($P = 0.008$) and tended to have lower ADFI ($P = 0.093$). Pigs from the LOW regime had a 2.65 kg lower slaughter weight than CON pigs ($P = 0.025$).

Mixing reduced the coefficient of variation (CV) for pig weight at weaning (d28): 10.4% in MIXED groups compared to 17.6% in LITTER groups ($P < 0.001$; Figure 5.1). There was a Mixing x Diet interaction for the CV for pig weight at d74 ($P = 0.025$). The initial difference in CV between MIXED and LITTER groups was reduced in LITTER groups fed the

CON diet (13.1% and 13.6%, respectively) but not in those fed the LOW diet (13.6% and 18.1%, respectively). At the end of the growing-finishing period, the differences in CV for BW between MIXED (9.7%) and LITTER pens (11.1%) tended towards significance (P = 0.084).

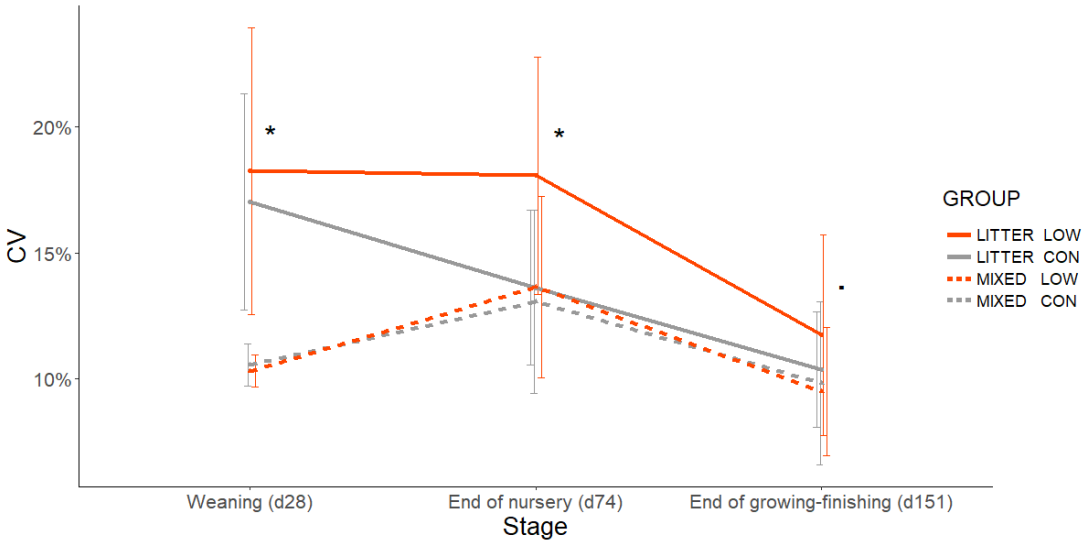


Figure 5. 1. Evolution of the coefficient of variation for within pen pig weight during the experimental period for each group (mean ± SD). Weaning: mixing effect (P < 0.001); End of nursery (d74): interaction mixing × diet (P = 0.025); End of growing-finishing: mixing effect (P = 0.084).

4.3.2. Body Lesion Counts and Ear and Tail Lesion Scores

The total BL counts and the ear and tail lesion scores are summarized in Table 5.4. At weaning, MIXED pigs showed 358% more BL and higher ear lesion scores compared to LITTER pigs (P < 0.001). However, LITTER pigs tended to show higher ear lesion scores both at the end of the nursery period (P = 0.057) and at the beginning of the growing-finishing period (P = 0.063). At the beginning of the growing-finishing period, pigs fed the LOW diet during the nursery period showed 14.5% more BL than pigs fed the CON diet (P < 0.009).

Table 5. 4. Effect of mixing at weaning or keeping pigs in litter groups and dietary regimen on the total body lesion (BL) counts, ear and tail lesion scores in pigs from weaning to finish.

	Dietary regimen [2]							
	CON		LOW			*P* value		
Trait [1]	Litter	Mixed	Litter	Mixed	SEM [3]	Mixing	Diet	Mixing × Diet
Weaning, d30								
Total BL	11.6	36.8	9.9	42.4	2.92	< 0.001	0.523	0.161
Ear score	0.237	0.676	0.135	0.857	0.081	< 0.001	0.621	0.058
Tail score	0	0	0	0	-	-	-	-
Nursery, d42								
Total BL	4.6	4.2	4.5	3.4	0.84	0.296	0.891	0.790
Ear score	0.015	0.015	0.029	0.032	0.0087	0.577	0.966	0.800
Tail score	0.007	0.000	0.000	0.008	0.1350	0.655	0.889	0.865
End of nursery, d71								
Total BL	24.3	22.8	23.0	24.7	1.38	0.908	0.091	0.259
Ear score	0.146	0.000	0.399	0.063	0.1350	0.057	0.496	0.268
Tail score	0.014	0.008	0.007	0.000	0.0087	0.419	0.295	0.991
Beginning of growing-finishing, d76								
Total BL	23.5	26.1	27.2	29.6	1.96	0.155	0.009	0.517
Ear score	0.118	0.000	0.363	0.022	0.1330	0.063	0.627	0.399
Tail score	0.007	0.000	0.000	0.014	0.0078	0.642	0.845	0.151
End of growing-finishing, d151								
Total BL	19.8	18.8	19.5	18.1	1.31	0.311	0.760	0.832
Ear score	0.000	0.000	0.000	0.008	0.0038	0.294	0.231	0.336
Tail score	0.021	0.023	0.000	0.000	0.0151	0.950	0.182	0.885

[1] Mean number of body lesions counted on each pig. Total body lesions (BL): Sum of body lesions (BL) from the anterior, middle, and posterior body sections in each pig; Ear score: ear lesions on a scale from 0 – 4. Tail score: tail lesions on a scale from 0 – 3.

[2] CON: dietary regimen meeting pigs' nutritional requirements; LOW: low-density dietary regime with -10% of energy and protein than CON during the nursery period, but with the same dietary regimen as CON during the growing-finishing period.

[3] Standard error of the mean.

4.3.3. Pig Behaviour

Behaviour data are detailed in Table 5.5. After weaning, the number of aggressive behaviours and aggressive behaviours around the feeder showed an interaction between mixing and diet ($P < 0.001$; $P = 0.005$). MIXED pigs fed the LOW diet performed the highest number of aggressive behaviours, followed by LITTER pigs fed the LOW diet, with MIXED

pigs fed the CON diet showing fewer aggressive behaviours than pigs in pens fed the LOW diet. LITTER pigs fed the CON diet showed fewer aggressive behaviours around the feeder at weaning than the rest of groups ($P < 0.005$ interaction). MIXED pigs showed more damaging ($P = 0.042$) and sexual behaviour ($P = 0.039$) towards other pen mates than LITTER pigs.

Table 5. 5. Effect of mixing or being in litter groups at weaning and two dietary regimens on the occurrence of different negative behaviours in pigs from weaning to finish.

	Dietary regimen [1]							
	CON		LOW			*P* value		
Trait [1]	**Litter**	**Mixed**	**Litter**	**Mixed**	**SEM[3]**	**Mixing**	**Diet**	**Mixing × Diet**
Post-weaning, d29								
Aggression	3.79 [bc]	2.96 [c]	4.25 [b]	7.66 [a]	1.159	0.005	<0.001	<0.001
Feeder	0.67 [b]	2.00 [a]	1.88 [a]	2.04 [a]	0.597	0.024	0.037	0.005
Damaging	2.33	2.58	2.29	3.46	0.455	0.042	0.191	0.254
Sexual	0.75	1.08	0.46	1.21	0.449	0.039	0.674	0.184
End of nursery, d70								
Aggression	3.25 [a]	1.58 [b]	2.83 [ab]	3.17 [a]	0.685	0.195	0.257	0.023
Feeder	2.75 [bc]	1.08 [c]	3.92 [ab]	4.75 [a]	1.048	0.515	<0.001	0.003
Damaging	3.66	3.5	5.42	6.5	0.978	0.471	<0.001	0.398
Sexual	2.08	1.92	1.17	1.67	0.528	0.667	0.131	0.331
Beginning of growing-finishing, d75								
Aggression	6.17	8.58	4.25	7.67	1.720	<0.001	0.066	0.292
Feeder	3.58	3.75	3.33	2.92	1.146	0.814	0.313	0.564
Damaging	4.08	4.58	5.42	3.75	0.847	0.304	0.666	0.075
Sexual	2.33	1.25	1.25	1.17	0.467	0.110	0.110	0.257
End of growing-finishing, d150								
Aggression	2.33 [b]	3.92 [ab]	4.92 [a]	2.58 [b]	0.911	0.445	0.286	<0.001
Feeder	2.58	2.33	2.17	1.58	0.696	0.263	0.181	0.574
Damaging	3.00	1.75	3.83	2.08	0.597	<0.001	0.198	0.814
Sexual	0.00	0.33	0.75	0.58	0.227	0.600	0.372	0.986

[1] Occurrence of different behaviours for a period of 15 minutes by pen.

[2] CON: dietary regimen meeting pigs' nutritional requirements; LOW: low-density dietary regime with -10% of energy and protein than CON during the nursery period, but with the same dietary regimen as CON during the growing-finishing period.

[3] Standard error of the mean.

[a,b,c] Within rows, significant differences between groups ($P < 0.05$).

At the end of the nursery period (d70), MIXED pigs fed the CON diet showed fewer aggressive behaviours than MIXED pigs fed the LOW diet and LITTER pigs fed the CON diet (interaction; P = 0.023). The analysis also revealed an interaction in the number of aggressive behaviours around the feeder (P = 0.003): MIXED pigs fed the LOW diet performed more aggression at the feeder than pigs fed the CON diet and pigs in pens fed the LOW diet performed more of these behaviours compared to MIXED pigs fed the CON diet. Pigs fed the LOW diet showed more damaging behaviours than pigs fed the CON diet (P < 0.001).

At the beginning of the growing-finishing period, MIXED pigs showed more aggressive behaviours (P < 0.001). Pigs in pens fed the LOW diet showed a tendency to perform fewer aggressive behaviours (P = 0.066).

At the last observation before slaughter (d150), the number of aggressive behaviours showed an interaction between mixing and diet (P < 0.001). LITTER pigs fed the LOW diet showing more aggressive behaviours than LITTER pigs fed the CON diet and MIXED pigs fed the LOW diet. In addition, LITTER pigs performed more damaging behaviours than MIXED pigs.

4.4. Discussion

4.4.1. Growth Performance

Re-grouping of pigs at weaning requires the establishment of a new social order (Meese & Ewbank, 1973) through aggressive behaviour, which results in skin lesions, psychological stress (Peden et al., 2018) and compromised productive performance (Camerlink et al., 2021). We hypothesized that apart from the initial fights for hierarchy establishment at re-grouping, MIXED pigs would show a poorer social stability along the production cycle, displaying more conflicts and chronic aggression when dealing with a nutritionally low-density diet. Pigs in MIXED groups showed higher levels of aggression and had more body lesions post-weaning compared to pigs kept in LITTER groups. Additionally, the results support that a low-density diet interacts with mixing at weaning, as aggression

was more prevalent in MIXED groups fed the LOW diet. In line with previous reports (Aymerich et al., 2020; J. W. Kim et al., 2021; Q. Li & Patience, 2017; Pichler et al., 2020), nursery pigs fed a low energy and protein diet were unable to increase their voluntary feed intake to compensate for the reduced dietary nutrient and energy density and so their growth was reduced. Consequently, the FCR of pigs fed the LOW diet was 10.3% poorer during the nursery period.

Although pigs expend a high amount of energy fighting to establish the social order (Coutellier et al., 2007) and the associated stress response is energy costly, mixing did not affect weight gain of pigs. Mixing caused a slight numerical increase in feed intake, which resulted in a worsened FCR during the nursery period. These findings are in contrast to previous studies that identified a negative effect of regrouping involving an impairment of growth. Camerlink et al. (2021) reported poorer growth during the first week post-weaning after mixing weaned pigs compared to keeping them in littermate pens. Additionally, other authors associated the regrouping of pigs at the beginning of or during the growing-finishing period with a reduction in growth (Camp Montoro et al., 2021, 2022; Coutellier et al., 2007; Hyun, Ellis, & Johnson, 1998; Hyun, Ellis, Riskowski, et al., 1998; Stookey & Gonyou, 1994). It may be that under the conditions of the current study that the aggression exhibited due to mixing was not sufficiently intense to compromise growth, but this is difficult to confirm since most previous studies did not include behavioural data.

One of the goals of mixing pigs at weaning is to reduce the variability in pig BW within the pen. Previous studies observed that while the co-efficient of variation (CV) for within pen pig weight was initially reduced due to mixing, it then gradually increased until the end of the growing and finishing period when mixed groups had similar CV for pig weight as pen-groups of littermates (i.e. not remixed) (O'Quinn et al., 2000; Tindsley & Lean, 1984). Indeed, Tindsley & Lean (1984) proposed that a certain degree of variation in BW between individuals within a group is a necessary component of group social dynamics and that groups of animals will therefore tend towards such variation. Accordingly, the CV for pig weight increased among pigs in MIXED groups and converged with the CV in BW of LITTER groups fed the CON dietary regime. However, the CV in BW of LITTER groups fed the LOW

diet did not decrease to the levels observed in the other three treatment groups. This result is probably explained by the fact that the lighter pigs in LITTER pens had reduced feed intake capacity. Therefore, they could not ingest sufficient nutrients and energy from the low-density diet to catch up the heaviest littermates. In the same line, S. L. Douglas, Wellock et al. (2014) found that light birth weight pigs benefitted more from a high specification post-weaning diets than their normal birthweight counterparts. In growing pigs, Hastad et al. (2020) demonstrated that increasing the dietary energy density for pigs from 30 kg BW mainly favored the growth of the lighter half of the pigs and reduced the within-pen CV of bodyweight at slaughter. Aymerich et al. (2022) also showed that severely limiting dietary SID Lys:NE below nutritional requirements can negatively affect the within-pen CV of pig weight of growing pigs (28–63 kg BW), mainly because the dietary challenge restricted the growth of the lightest pigs. This interaction between the initial BW homogeneity of the pen and dietary regime density was also observed by Magowan et al. (2011a). These authors observed the highest within-pen CV for ADG when they provided a low energy density dietary regime to pigs heterogeneously grouped from weaning to 20 weeks of age, while the lowest CV for ADG was observed when uniformly grouped pigs were fed an energy and nutrient-rich dietary regime.

At the beginning of the growing-finishing period, all treatment groups were moved to finisher accommodation without further mixing and all groups were fed an amino-acid and energy rich finisher diet. For the rest of the trial, MIXED and LITTER pens did not show any differences in growth parameters. This is in contrast with the results of Jones et al. (2011), who reported improved growth during the growing-finishing period in mixed groups of pigs that included full siblings compared to those that did not, although contrary to our study, these pigs underwent another regrouping at the beginning of this growing-finishing period. After providing a growing-finisher diet to meet the dietary requirements of all pigs during the realimentation period, pigs that were fed the LOW diet during the nursery period exhibited the same BW gain and tended to show a lower ADFI than those fed the CON regimen during the nursery period, which translated into a lower FCR for the former. However, animals fed the LOW diet continued to be 2.6 kg lighter until slaughter. The better

efficiency of pigs fed the LOW diet during the growing-finishing period was probably explained by their lower BW at the start of this period, as pigs become less efficient as they grow (Noblet & van Milgen, 2004). The dietary change at transfer to growing finishing accommodation reduced the within-pen variation in BW of the LITTER pigs that had been fed the LOW diet during the nursery period. By the end of the study, within pen variation in pig weight was no different to that of the other groups, most probably because the lighter pen-mates had the opportunity to show some degree of compensatory growth.

4.4.2. Behaviour Observations and Lesion Scores

Establishment of the dominance hierarchy in mixed groups was reflected in an increased number of aggressive behaviours, aggressive behaviours around the feeder, body lesions (358% increase) and ear injuries relative to pigs kept in litter groups in the 24 to 48 hours post weaning. The number of aggressive behaviours, including aggression at the feeder, showed an interaction with the diet, which is difficult to explain given the short time it was offered to the pigs before the behavioural observations were performed and the low feed intake in the first hours post-weaning. However, it has been suggested that after abrupt weaning, piglet sill have dependence on high oleic acid lipids as found in milk and prefer feeds with a higher lipid inclusion than typically offered in weaner diets (Weng, 2017). Therefore, the superior fat content of the CON diet (12.18%) compared to the LOW diet (5.03%) might have helped piglets to cope better with the transition from sow's milk to the starter diet, which might have reduced the stress level and the social tension among pigs. However, the dietary regimen had no effect on the number of body lesions which are a good proxy for aggressive behaviour on commercial farms (Stukenborg et al., 2011; Turner et al., 2009). In the current study, the body lesions probably showed a more realistic picture of the aggressive interactions that occurred after mixing the pigs, as aggressive behaviour caused by mixing generally subsides within 24 hours (Meese & Ewbank, 1973), while in the current study behaviour observations were only performed after this time.

During the same post-weaning observation, LITTER pigs showed a lower frequency of damaging oral behaviour towards other pigs, including belly nosing, tail biting and ear

biting than MIXED pigs. The expression of damaging behaviours likely reflected the more stressed state of the mixed animals after weaning (Dybkjær, 1992). Mixed pigs showed more ear lesions, although, in this stage, they were more likely caused by aggression than by oral ear manipulation. Tail scores were non-existent at the post weaning inspection, indeed they were low throughout the study and did not differ between treatments probably reflecting the fact that the pigs were docked (Lahrmann et al., 2017). Additionally, mixed groups showed more mounting behaviour than litter groups after weaning. Mounting may occur when the dominant pig settles its rank (Camerlink, Farish, et al., 2018) or to demonstrate the dominance status (Fredriksen et al., 2008) and probably was part of the set of behaviours associated with establishment of the dominance hierarchy. These results are in line with Camerlink et al. (2021), who also identified an increase in sexual mounting in regrouped pigs at weaning. However, they did not observe increases in the performance of damaging behaviour in response to mixing, probably because of the large between-pen variability found in their observations.

There is normally a reduction in aggressive behaviour between mixed pigs once a new stable social order is established, approximately two weeks after mixing (Stookey & Gonyou, 1994). In agreement, the number of body lesions did not differ between treatment groups two weeks after weaning in the current study. However, in socially stable groups chronic aggression may persist, refining previously established social relationships and are often triggered during competition for limited resources (Giersing & Studnitz, 1997). In our study, the number of aggressive behaviours and aggression around the feeder at the end of the nursery period increased where the LOW dietary regimen was fed to MIXED groups of pigs. Pigs fed the LOW diet, especially the ones that were mixed performed more aggression associated with the feeder than pigs fed the CON diet at the end of the nursery period. At the time of the observations (d70), pigs were close to reaching 30kg BW and probably already had some capacity to increase their physical feed intake to increase their energy and amino acid intake on the LOW regimen. The low-density diet, especially when provided via a single-spaced feeder, might have stimulated increased competition for access to feed and, consequently, increased the number of aggressive behaviours around the feeder. This

could have increased the risk of conflicts given the limited feeder space (Bakare et al., 2014), resulting in high levels of stress and aggression (Boumans et al., 2018). The level of aggression and aggression around the feeder was lowest in MIXED pigs fed the control diet. However, the nutritional treatment only had a significant effect in pigs in the MIXED pens. This result suggests that when pigs are fed the control diet, mixed animals might perform less chronic aggression. However, when they are fed a reduced nutrient and energy density diet and potentially face an increased competition for access to feed, they may display more chronic aggressions compared to groups of littermates. Nevertheless, the differences in the aggressive behaviour observed among groups were not reflected in the number of skin lesions.

Pigs fed the LOW dietary regimen showed more damaging behaviour towards pen mates at the end of nursery period, which might reflect that these pigs were nutritionally limited. When growth or immune functioning are limited by nutrient availability, pigs can increase their foraging behaviour to satisfy their nutritional needs (M. B. Jensen et al., 1993). If rooting substrates are not sufficiently available, pigs can redirect their foraging and exploratory behaviour to nosing, chewing, or sucking certain body parts of their pen mates, which could end up in vigorous biting leading to wounds (van der Meer et al., 2017). Others have described an increase in the occurrence of damaging behaviours such as ear and tail biting when protein requirements were not fulfilled (M. B. Jensen et al., 1993; van der Meer et al., 2017). However, the increased damaging behavior in our study was not reflected in increased ear and/or tail lesion scores. In contrast, pigs in litter pens showed a tendency to have higher ear lesion scores. This was a consequence of two pens that showed an outbreak of ear attacks performed by a single pig in each case. Therefore, it is likely that he role of the experimental treatments in the higher ear lesion scores was probably minimal in the current study.

When pigs were moved to the grower-finisher rooms, there was a general increase in aggressive behaviour. This effect was already described by Moore et al. (1994) who found that pigs from static groups fought more after rehousing than pigs of similar size housed in dynamic groups. Nevertheless, this increase in aggressions was minor in the pigs kept in

litter groups, suggesting they preserved a better social group stability than those in MIXED groups. Despite this, the total skin lesion counts at the beginning of growing-finishing period did not show a difference between pigs in MIXED and LITTER pens.

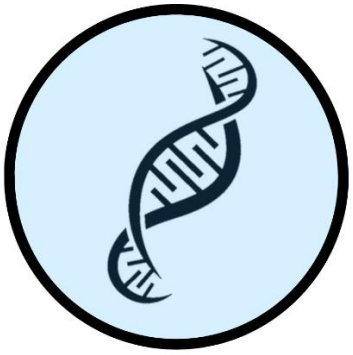
After substituting the low-density nursery diet by the growing-finishing diet with a higher nutritional density, the differences between groups in aggression around the feeder disappeared. Even pigs that received the LOW diet during nursery period showed a tendency to perform less aggression after the dietary change. This emphasizes the significant role that competition for feed played in the development of chronic aggression. However, pigs previously fed the LOW dietary regime during the nursery period showed more skin lesions. The explanation for this contrasting finding may be that these lesions possibly resulted from the aggression around the feeder performed during the last days of nursery phase, which was higher in the pigs fed the LOW diet.

At the end of growing-finishing period, there was an interaction between mixing and diet for the counts of aggressive behaviour. LITTER pigs fed the LOW diet showed more aggressive behaviours than LITTER pigs fed the CON diet and MIXED pigs fed the LOW diet. LITTER pigs also performed more damaging behaviours than MIXED pigs. These differences are difficult to interpret but given the passage of time since weaning/mixing and the dietary challenge it is likely that these findings are not biologically relevant. In addition, the lesion scoring did not corroborate these behavioural differences between treatments.

4.5. Conclusions

Overall, the practice of mixing pigs at weaning triggered fights for hierarchy re-establishment and increased stress in pigs immediately after weaning as reflected by the increased amount of damaging behaviour. Chronic aggression was reduced by mixing at the end of the nursery period but not when a low nutrient and energy density diet was fed. Furthermore, chronic aggression was increased in mixed pigs after being moved to the grower-finisher accommodation. Thus, although mixing animals at weaning had a limited impact on pig growth, it had a detrimental effect on welfare and should be avoided, especially when pigs are fed low nutrient and energy density diets.

Provision of a low-density diet during the nursery period caused a growth retardation that could not be compensated for during the growing-finishing period. In addition, it increased the variation in BW in litter-mate pens, possibly because lightweight pigs within the pen were especially affected by the low-density dietary regimen. Furthermore, diets in this experiment were fed from single-space feeders which likely aggravated competition for feed and contributed to the increase seen in the performance of damaging behaviour at the end of the nursery period when the low-density diet regimen was fed.



Chapter 6

Porcine Digestible Peptides (PDP) in Weanling Diets Regulates the Expression of Genes Involved in Gut Barrier Function, Immune Response and Nutrient Transport in Nursery Pigs

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Abstract

This study was conducted to investigate the effects of dietary supplementation of porcine digestible peptides (PDP), spray-dried plasma (SDP), or a combination of both, on growth performance and the expression of genes related to intestinal function of weaned pigs. A total of 180 piglets (Trial 1) and 198 piglets (Trial 2) were used to evaluate the partial substitution of soybean ingredients with 2% SDP or 2% PDP (Trial 1), and with 3% SDP or the combination of 1% SDP and 2% PDP (SDP-PDP; Trial 2) during the pre-starter period (d 0-14). The gene expression of 56 genes was quantified in a qPCR platform in jejunum and ileum samples obtained from piglets on d 14 after weaning (Trial 2). Piglets fed SDP, PDP and SDP-PDP had a higher body weight (BW), average daily gain (ADG) and feed efficiency (G:F) than the soybean control on day 14 ($P < 0.05$). In addition, the combination of SDP and PDP upregulated ten genes in jejunum samples ($P < 0.05$) related to intestinal function. More research is needed to confirm that gene expression upregulation by PDP in combination with SDP has an impact on intestinal function and to elucidate its underlying mechanisms.

5.1. Background

Weaning is a stressful period for piglets as they have to deal with a change from sow milk to a less digestible, plant-based dry solid diet that contains many ingredients that the pig has not eaten before (J. P. Lallès et al., 2007). One major consequence of weaning is a reduction in feed intake, which in turn causes a reduction in the villi height in the small intestine (J. P. Lallès et al., 2007) and a disruption of the gut microbiota ecosystem with a loss of diversity (Gresse et al., 2017). After weaning, piglets are more susceptible to gut inflammatory problems and their intestinal function can be affected. This often leads to post-weaning diarrhea and increased mucosal permeability (Pluske, 2013). A strategy for helping piglets to reduce intestinal disturbances during this period is to reduce the inclusion of less-digestible vegetal protein sources in their feed, like soybean ingredients, and substitute them with high-quality digestible animal protein sources. Certain protein sources, like spray-dried plasma (SDP) contain biologically active components that give them physiological or regulatory functions beyond their nutritional value (Bhat et al., 2015). In particular, SDP has shown potential for modulating the immune response, reducing intestinal mucosa inflammation and maintaining its integrity (Bosi et al., 2004; Pérez-Bosque, Amat, et al., 2018; Pérez-Bosque, Miró, et al., 2018).

Porcine digestible peptides (PDP) are a coproduct of the heparin industry and are obtained from the enzymatic hydrolysis of porcine intestinal mucosa. Currently, PDP can be used in postweaning diets as a cheapest alternative for SDP, fish meal and other sources of high-quality protein in terms of preference and digestibility (Figueroa et al., 2016; Solà-Oriol et al., 2011). The ability of PDP to increase villus height suggests that they may improve nutrient uptake (Xin et al., 2001). However, there is no available literature exploring possible effects of PDP on intestinal function when replacing major soy protein ingredients in the diet. In the present study, in a first trial we hypothesize that including PDP and SDP in a high crude protein (CP) diet partially substituting a high content of soybean ingredients might improve piglet performance after weaning (Trial 1). In a second trial we also hypothesize that partially substituting soybean meal (SBM) in weanling diets with SDP or a

combination of SDP and PDP could improve piglet performance and the expression of genes related to intestinal function (Trial 2).

5.2. Materials and Methods

The experimental procedures used in the two trials were approved by the Ethical Committee on Animal Experimentation of the Autonomous University of Barcelona (CEAAH 3817), and are in full compliance with national legislation following the EU-Directive 2010/63/EU for the protection of animals used for scientific purposes.

5.2.1. Animals, Housing, and Diet

5.2.1.1. Trial 1

The first trial was conducted in the weanling unit of a commercial farm throughout the pre-starter period (d 0 to 14 post-weaning). A total of 180 male and female weaned commercial piglets ([Landrace x Large White] x Pietrain, weaned at d 28) with a body weight (BW) of 7.5 ± SD 1.15 kg were moved to the nursery unit. These animals were not given previous access to creep feed during lactation. Piglets were distributed into two blocks according to initial BW (heavy piglets: 8.6 ± SD 0.03 kg; light piglets: 6.4 ± SD 0.02 kg). Each block contained 9 pens of 10 animals to which the three experimental treatments were randomly assigned (6 pens or replicates/treatment). Each pen (3.2 m^2 in floor area) had a commercial non-lidded hopper (TR5, Rotecna, Agramunt, Spain) and a nipple waterer to ensure ad libitum feeding and free water access.

Table 6. 1. Composition of the experimental diets used on trial 1, % as fed basis.

	Experimental diets[1]		
Item	CON	SDP	PDP
Ingredient, %			
Maize	36.9	38.7	38.8
Wheat	16.0	16.0	16.0
Extruded Soybeans	15.0	11.4	11.2
Barley	13.0	13.0	13.0
Soybean meal 44% CP	6.8	6.8	6.8
Soybean protein concentrate 56% CP	5.6	5.6	5.6
Sweet milk whey	2.5	2.5	2.5
Spray-dried plasma 80% CP	-	2.0	-
Porcine digestible peptides 62% CP	-	-	2.0
Mono calcium phosphate	1.34	1.37	1.30
Calcium carbonate	0.62	0.64	0.67
L-Lysine HCL	0.55	0.49	0.52
Salt	0.53	0.40	0.16
Vitamin-Mineral premix[2]	0.40	0.40	0.40
DL-Methionine	0.27	0.23	0.57
L-Threonine	0.25	0.21	0.23
L-Valine	0.15	0.11	0.18
L-Tryptophan	0.09	0.08	0.10
Calculated composition			
NE, kcal/kg	2470	2470	2470
Dry Matter, %	89.1	89.0	89.1
Ash, %	5.4	5.3	5.4
Crude Protein, %	19.5	19.7	19.5
Calcium, %	0.650	0.655	0.650
Total P, %	0.678	0.671	0.671
SID amino acids[3]			
Lys, %	1.280	1.280	1.280
Met, %	0.509	0.470	0.829
Cys, %	0.222	0.262	0.213
Met+Cys, %	0.768	0.768	1.078
Thr, %	0.832	0.832	0.832
Trp, %	0.282	0.282	0.282
Val, %	0.896	0.896	0.934
Analyzed composition			
Dry Matter, %	89.1	88.8	88.8
Ether Extract, %	5.1	4.2	4.3
Neutral Detergent Fiber, %	10.5	10.2	10.0
Crude Protein, %	17.9	18.2	18.4

[1]CON: Control diet; SDP: Diet with 2% spray-dried plasma inclusion; PDP: Diet with 2% porcine digestible peptides inclusion. [2]Supplied the following per kg of diet: 7,000 IU of vitamin A (acetate); 500 IU of vitamin D3 (cholecalciferol); 250 IU of vitamin D (25-hydroxicholecalciferol); 45 mg of vitamin E; 1 mg of vitamin K3; 1.5 mg of vitamin B1; 3.5 mg of vitamin B2; 1.75 mg of vitamin B6; 0.03 mg of vitamin B12; 8.5 mg of D-pantothenic acid; 22.5 mg of niacin; 0.1 mg of biotin; 0.75 mg of folacin; 20 mg of Fe (chelate of amino acids); 2.5 mg of Cu (sulphate); 7.5 mg of Cu (chelate of glycine); 0.05 mg of Co (sulphate); 40 mg of Zn (oxide); 12.5 mg Zn (chelate of amino acids); 12.5 mg of Mn (oxide); 7.5 of Mn (chelate of glycine); 0.35 mg of I, 0.5 of Se (organic); 0.1 mg of Se (inorganic).
[3] Standardized ileal digestible amino acids.

Treatments consisted of three different iso-protein pre-starter diets: a control diet (CON) with a high content of soybean ingredients and two extra diets with partial replacement of the extruded soybeans by 2% SDP (AP 820 P, APC Europe S.L., Granollers, Spain) or 2% PDP (Palbio 62SP, Bioiberica S.A.U., Palafolls, Spain). The basal pre-starter diet was formulated to contain 2,470 kcal of net energy (NE)/kg, 19.5% CP/kg and 1.28% digestible lysine (Table 6.1) to meet the requirements for maintenance and growth of newly weaned piglets (FEDNA, 2013). Diets were presented in mash form and were fed ad libitum for fourteen consecutive days. No antimicrobials or ZnO were used in the experimental diets.

5.2.1.2. Trial 2.

The second trial was conducted in a different commercial farm throughout the pre-starter (d 0 to 14 post-weaning) and starter period (d 14 to 35 post-weaning). A total of 198 male and female weaned commercial crossing piglets ([Landrace x Large White] x Pietrain, weaned at d 21) with a BW of 5.7 ± SD 0.60 kg were moved to the nursery unit without transport to be used in the trial. Piglets had access to creep feed during lactation. Animals were distributed into two blocks according to initial BW (heavy piglets: 6.3 ± SD 0.02 kg; light piglets: 5.1 ± SD 0.01 kg) and each block contained 9 pens of 11 animals to which three experimental treatments were randomly assigned (6 replicates for each treatment). Each pen (3 m^2 in floor area) had a commercial non-lidded hopper (TR5, Rotecna) and a nipple waterer to ensure ad libitum feeding and free water access. The different characteristics of this farm compared to the farm of the first trial forced to make some changes to the experimental design of the experiment.

Treatments consisted of three different iso-protein pre-starter diets: a CON diet with a high content of SBM and two extra diets with partial replacement of the SBM by 3% Spray-dried Plasma (SDP; AP 820 P, APC Europe S.L.) or a combination of 1% Spray-dried Plasma and 2% Porcine Digestible Peptides (SDP-PDP; Palbio 62SP, Bioiberica S.A.U.). The basal pre-starter diet was formulated to contain 2,470 kcal NE/kg, 20.5% CP/kg and 1.35% digestible lysine (Table 6.2) and to meet the requirements for maintenance and growth of newly weaned piglets (National Research Council, 2012). The pre-starter diets were fed ad libitum for

fourteen consecutive days and a common starter diet was also fed ad libitum from d 15 until d 35 post-weaning (Table 6.2). No antibiotics, alternative antimicrobials or ZnO were included in the diets.

Table 6. 2. Composition of the experimental diets used on trial 2, % as fed basis.

	Experimental diets[1]			
Item	CON	SDP	SDP-PDP	Starter
Ingredients, %				
Maize	29.68	32.53	32.11	29.79
Soybean Meal 47% CP	25.82	20.6	21.34	21.34
Wheat	16	16	16	15
Barley	6.5	6.5	6.5	20
Dextrose	6.5	6.5	6.5	-
Sweet Milk Whey	6.5	6.5	6.5	-
Potato Protein	2.5	2.5	2.5	-
Lard	2.67	2.38	2.31	6.53
Di-calcium phosphate	1.72	1.77	1.67	1.56
Spray-dried plasma (80% CP)	-	3	1	-
Porcine digestible peptides 62% PB	-	-	2	-
Salt	0.47	0.27	0.03	0.48
Vitamin-Mineral Premix[2]	0.40	0.40	0.40	0.40
L-Lysine HCL (78)	0.46	0.39	0.39	0.51
DL-Methionine	0.27	0.24	0.25	0.25
L-Threonine	0.21	0.16	0.17	0.24
Calcium Carbonate	0.11	0.11	0.17	0.55
L-Valine	0.11	0.09	0.08	0.14
L-Tryptophan	0.08	0.07	0.09	0.07
Calculated composition				
NE, kcal/kg	2470	2470	2470	2653
Ether Extract, %	4.69	4.43	4.39	8.61
Neutral Detergent Fiber, %	7.87	7.57	7.61	9.92
Crude Protein, %	20.51	20.50	20.50	18.50
Calcium, %	0.65	0.65	0.65	0.73
Total P, %	0.70	0.68	0.68	0.65
SID amino acids[3]				
Lys, %	1.350	1.350	1.350	1.230
Met, %	0.545	0.504	0.539	0.478
Met+Cys, %	0.810	0.810	0.810	0.720
Thr, %	0.878	0.878	0.878	0.780
Trp, %	0.297	0.297	0.297	0.264
Analyzed composition				
Dry Matter,%	90.3	92.3	91.8	89.3
Ether Extract, %	4.6	4.3	4.1	8.4
Neutral Detergent Fiber, %	9.7	7.7	8.2	-
Crude Protein, %	19.7	18.7	19.6	17.7

[1]CON: Control diet; SDP: Diet with 3% spray-dried plasma inclusion; SDP-PDP: Diet supplemented with a combination of 1% spray-dried plasma and 2% porcine digestible peptides.
[2]Supplied the following per kg of diet: 7,000 IU of vitamin A (acetate); 500 IU of vitamin D3 (cholecalciferol); 250 IU of vitamin D (25-hydroxicholecalciferol); 45 mg of vitamin E; 1 mg of vitamin K3; 1.5 mg of vitamin B1; 3.5 mg of vitamin B2; 1.75 mg of vitamin B6; 0.03 mg of vitamin B12; 8.5 mg of D-pantothenic acid; 22.5 mg of niacin; 0.1 mg of biotin; 0.75 mg of folacin; 20 mg of Fe (chelate of amino acids); 2.5 mg of Cu (sulphate); 7.5 mg of Cu (chelate of glycine); 0.05 mg of Co (sulphate); 40 mg of Zn (oxide); 12.5 mg Zn (chelate of amino acids); 12.5 mg of Mn (oxide); 7.5 of Mn (chelate of glycine); 0.35 mg of I, 0.5 of Se (organic); 0.1 mg of Se (inorganic).
[3] Standardized ileal digestible amino acids.

5.2.2. Data and Sample Collection

Piglets were ear-tagged and individually weighed at weaning (d 0) and d 14 post weaning in trial 1 and at weaning (d 0), d 7, d 14 and d 35 post-weaning in trial 2. Feed disappearance from each hopper was measured throughout the experimental period. The average daily feed intake (ADFI), average daily gain (ADG) and feed efficiency (G:F) were calculated for the experimental period.

One piglet per pen was sedated with a combination of zolazepam/tiletamine and xylazine, and was euthanized with a pentobarbital injection on d 14 in trial 2. A portion of 0.5 cm of jejunum and ileum tissues were collected at 30 cm and 1.3 cm from the ileo-cecal valve respectively. Intestinal sections were rinsed in phosphate-buffered saline (PBS) and immediately snap frozen in 1 mL of RNAlater (Deltalab, Rubí, Spain). Samples were stored at -80 ºC until analysis.

5.2.3. Proximate Analysis of Diets

Diet proximate analyses from both trials were performed following the Association of Official Agricultural Chemists methodology: dry matter (AOAC 934.01 (AOAC, 1990)), ash (AOAC 942.05 (AOAC International, 2007)), ether extract (AOAC 2003.05 (AOAC International, 2007)) and crude protein (AOAC 968.06 (AOAC International, 2005)). Neutral-detergent fiber was determined according to the method of Van Soest et al. (1991).

5.2.4. Gene Expression Study by qPCR

Gene expression was quantified by RT-qPCR to study the expression of 56 genes in two intestinal tissues in an Open Array Real-Time PCR Platform (Applied Biosystems, Waltham,

MA, USA) by the Servei Veterinari de Genètica Molecular at the Veterinary Faculty of the Universitat Autònoma de Barcelona (Spain).

The pre-amplified product was diluted 1:10 with 0.1X Tris-EDTA pH 8.0 and 6 µL was transferred to 384-well plates. These were analyzed in duplicate in Taqman Open Array gene expression plates custom-designed in a QuantStudio 12K Flex Real-Time PCR system (ThermoFisher Scientific, Waltham, MA, USA). One sample was used as an inter-plate control to check the replication of results from different plates.

5.2.5. Open Array Design

A list of 56 genes related to intestinal health were selected according to the bibliography (Badia, Lizardo, et al., 2012; Chmielewska et al., 2013; Garrick et al., 2003; S. L. Hansen et al., 2009; Khan & Asif, 2015; Kröger et al., 2013; P. Liu et al., 2014; Schnoor et al., 2009; S. H. Smith et al., 2014; Takeda et al., 2003; Tamura et al., 2008; Taupin & Podolsky, 2003; Xu et al., 2015; Zhou et al., 2012) and included: (1) genes participating in the barrier function (*OCLN*, *ZO1*, *CLDN1*, *CLDN4*, *CLDN15*, *MUC2*, *MUC13* and *TFF3*); (2) genes that play an important role in the immune response, such as pattern recognition receptors, cytokines, chemokines and stress proteins (*TLR2*, *TLR4*, *IL1B*, *IL6*, *IL8*, *IL10*, *IL17A*, *IL22*, *IFNG*, *TNF*, *TGFB1*, *CCL20*, *CXCL2*, *IFNGR1*, *HSPB1*, *HSPA4*, *REG3G*, *PPARGC1A*, *FAXDC2* and *GBP1*); (3) genes coding for enzymes and hormones implicated in the digestion process (*GPX2*, *SOD2*, *ALPI*, *SI*, *DAO1*, *HNMT*, *APN*, *IDO1*, *GCG*, *CCK*, *IGF1R* and *PYY*); (4) genes involved in nutrient transport (*SLC5A1*, *SLC16A1*, *SLC7A8*, *SLC15A1*, *SLC13A1*, *SLC11A2*, *MT1A*, *SLC30A1* and *SLC39A4*); (5) genes involved in the stress response (*CRHR1*, *NR3C1* and *HSD11B1*); and (6) four reference genes (*ACTB*, *B2M*, *GAPDH* and *TBP*).

Primers were designed spanning exon-exon boundaries or at different exons using the PrimerExpress 2.0 software (Applied Biosystems) for 55 genes. The *IL8* gene primer was pre-designed by the company due to its complexity (see Table S6.1). Possible residual genomic DNA amplification and primer dimer formation were controlled. Finally, a customized Open Array panel containing 56 genes was obtained.

5.2.6. RNA Extraction and cDNA Preparation

Total RNA was obtained from 100 mg of frozen intestinal tissues with the RiboPure kit (Ambion, Austin, TX, USA) following the manufacturer's protocol. RNA concentration and purity was calculated with a NanoDrop ND-1000 spectrophotometer (NanoDrop products, Wilmington, DE, USA). RNA integrity was checked with Agilent Bioanalyzer-2100 equipment (Agilent Technologies, Santa Clara, CA, USA) following the producer's protocol. One microgram of total RNA was reverse-transcribed into cDNA in a final volume of 20 µl. The High-Capacity cDNA Reverse Transcription kit (Applied Biosystems) and random hexamer primers were used, and the following thermal profile was applied: 25 ºC, 10 min; 37 ºC, 120 min; 85 ºC, 5 sec; 4 ºC hold. A total of 25 ng of cDNA sample was pre-amplified using a 2X TaqMan® PreAmp Master Mix and a 0.2X Pooled Taqman Gene Expression Custom Assays in a final volume of 10 µl. The thermal cycling conditions for the pre-amplification reactions were 10 min at 95 ºC; 14 cycles of 15 sec at 95 ºC and 4 min at 60 ºC; and a final step of 10 min at 99 ºC. The pre-amplified cDNA product was stored at -20 ºC until use.

5.2.7. Gene Expression Data Analysis

Gene expression data were collected and analyzed using the ThermoFisher Cloud software 1.0 (Applied Biosystems) applying the $2^{-\Delta\Delta Ct}$ method for relative quantification and using the sample with the lowest expression as a calibrator. Some parameters were adjusted: the maximum cycle relative threshold allowed was 26, amplification score < 1.240, quantification cycle confidence > 0.8, and the maximum standard deviation allowed between duplicates was set at < 0.38. Samples that did not fit these requirements or had an inconclusive amplification status were deleted. Relative quantification values were checked for normalization by a $\log_{10}$ transformation, and all the statistical analyses were performed with the R 3.4.3 software (R Development Core Team, 2013) and Bioconductor (Gentleman et al., 2004). We carried out a one-way ANOVA and calculated the Benjamini-Hochberg false discovery rate (FDR *Q*-value) to control multiple *P*-values (Benjamini & Hochberg, 1995), setting an upper bound for the expected proportion of false significant tests, that is, false significant treatment differences in mean expression levels between

treatments. Pairwise post hoc treatment comparisons were carried out using Tukey's honest significant difference test (Tukey, 1949). Statistical differences between results for the treatments were identified at *P*-values and *Q*-values under 0.05 for the ANOVA and Tukey's analysis and for the FDR, respectively.

A principal component analysis (PCA) was performed with samples as cases and gene $\log_{10}$-expressions as variables. The function PCA of the FactoMiner R-library (Lê et al., 2008) was used for dimension reduction and visualization in the first two principal dimensions. The variables factor map was restricted to genes showing cos2-qualities over 0.45 and significant differences. Finally, the *heatmap* visualization method was used to obtain double clustering both for genes (with a correlation-based distance:

$$d = (1 - r)/2, \quad (1)$$

in which d is distance and r is the correlation coefficient, and the complete linkage hierarchical clustering) and for samples (with Euclidean distance and Ward's D2 method). These methods were chosen based on the following: the Euclidian distance between two samples adds all the squared differences in the log-expression level of them in each gene, and then the Ward's D2 linkage method uses the Euclidean squared-distance to cluster in a way that minimizes the increment of the variance into the resulting clusters. Correlation based distance is preferred for obtaining the gene clusters because the expression levels in different genes may not be comparable (see Murtagh & Legendre (2014), for Ward's method and Everitt (1974), for clustering methods). The function heatmap.2 in the R-library gplots was used (Warnes et al., 2015).

5.2.8. Performance Data Statistical Analysis

Production performance data were analyzed with ANOVA using the generalized linear model procedure of the statistical package SAS® (version 9.4, SAS Inst.Inc., Cary, NC). Normality and homoscedasticity were checked with Shapiro-Wilk test using the univariate procedure and Levene's test using the generalized linear model procedure respectively. Data were analyzed taking the experimental treatment and the block of weight as the main factors. Their corresponding interaction was also included in the model. The statistical unit

was the pen of 10 pigs in trial 1 and the pen of 11 pigs in trial 2. The results are presented as least square means taking into account the Tukey adjustment. The level of significance considered was $\alpha = 0.05$.

5.3. Results

5.3.1. Growth Performance

The productive performance of piglets during the first two weeks after weaning (Trial 1) is summarized in Table 6.3. Animals fed SDP and PDP showed higher BW, ADG and G:F ($P < 0.05$) than piglets in the control group.

Table 6. 3. Effect of the experimental treatments on growth performance of piglets in trial 1.

	Experimental diets[3]				
Item[2]	CON	SDP	PDP	SEM	*P*-value[1]
BW, day 0, g	7505	7517	7534	12.0	0.241
day 14, g	9438[b]	10381[a]	9990[a]	138.9	0.001
ADFI 0-14d, g/d	236[b]	295[a]	250[b]	11.3	0.002
ADG 0-14d, g/d	138[b]	205[a]	175[a]	11.8	0.002
G:F 0-14d	0.554[b]	0.695[a]	0.700[a]	0.0413	0.034

[1]*P*-values come from the ANOVA test.

[2]BW: body weight; ADFI: average daily feed intake; ADG: average daily gain; G:F: feed efficiency.

[3]CON: Control diet; SDP: Diet with 3% spray-dried plasma inclusion; SDP-PDP: Diet supplemented with a combination of 1% spray-dried plasma and 2% porcine digestible peptides.

[a,b]Different letters in the same row indicate significant statistical mean differences in the two diets (Tukey's test *P*-value <0.05).

For trial 2, the productive performance results are shown in Table 6.4. Piglets of the SDP and SDP-PDP group showed greater BW and ADG and G:F than CON piglets on d 14 ($P < 0.05$). Although a numeric difference in BW was observed at d 35 between the supplemented animals and the CON group, no significant differences in BW, ADG or G:F were observed.

Table 6. 4. Effect of the experimental treatments on growth performance of piglets in trial 2.

	Experimental diets[3]				
Items[2]	CON	SDP	SDP-PDP	SEM	*P*-value[1]
BW, day 0, g	5724	5720	5721	46.9	0.859
day 7, g	5956[b]	6344[a]	6254[ab]	88.3	0.023
day 14, g	7165[b]	7894[a]	7871[a]	152.7	0.008
day 35,g	15270	16252	15960	460.3	0.335
ADFI, day 0-14, g	267	282	283	5.96	0.139
day 14-35, g	483	508	525	25.9	0.532
ADG, day 0-14, g	103[b]	155[a]	153[a]	10.7	0.007
day 14-35, g	364	398	382	22.0	0.571
G:F, day 0-14	0.380[b]	0.548[a]	0.540[a]	0.0383	0.046
day 14-35	0.761	0.782	0.732	0.0501	0.840

[1]*P*-values come from the ANOVA test.

[2]BW: body weight; ADFI: average daily feed intake; ADG: average daily gain; G:F: feed efficiency.

[3]CON: Control diet; SDP: Diet with 3% spray-dried plasma inclusion; SDP-PDP: Diet supplemented with a combination of 1% spray-dried plasma and 2% porcine digestible peptides.

[a,b]Different letters in the same row indicate significant statistical mean differences in the two diets (Tukey's test *P*-value <0.05).

5.3.2. Intestinal Mucosa Gene Expression Values

After performing qPCR of the intestinal tissue, 37 genes were detected as expressed in jejunum tissue and 27 genes in ileum tissue from the 54 target genes initially included in the Open Array panel. The conservation of one tissue sample from the jejunum and seven from the ileum was affected and amplification was not possible. Gene expression results of the ileum tissue were not considered due to the high amount of lost samples of this intestinal section.

The results of the analysis of gene expression in the jejunum intestinal tissue (Trial 2) are shown in Table 6.5. Although only two genes showed statistical differences between groups ($P < 0.05$, $Q < 0.05$), other 8 genes showed a trend to be modified by the diets ($P < 0.05$, $Q < 0.2$). The *CLDN15* and *TFF3* genes from the barrier function group and the *SLC11A2/DMT1* gene from the nutrient transport group showed a tendency to higher expression for the SDP-PDP treatment compared to the CON treatment ($P < 0.05$). From the immune response functional group, the SDP-PDP group showed a trend to have higher expression level in three genes: *GBP1* compared to the SDP group, *IFNGR1* compared to the CON group, and *TLR4* compared to the other two groups ($P < 0.05$). Finally, the SDP-PDP

group had a higher expression level than the CON and SDP groups in two enzyme-coding genes (*HNMT* and *APN*) that only was significant for *HNMT (Q* < 0.05*).* The gene coding for a protein that metabolizes oxidation products, *SOD2*, also showed a significant increase when compared SDP-PDP group with the CON and SDP groups.

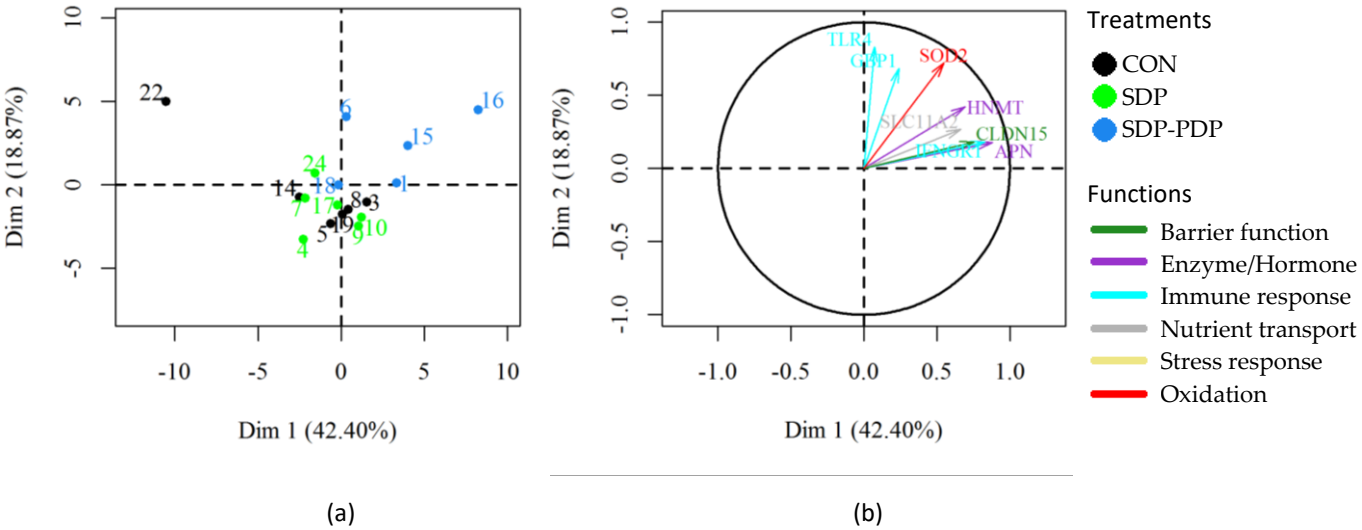
Table 6. 5. Effect of the experimental treatments on relative gene expression on jejunum mucosa after pre-starter period. Gene expression values are presented as ratios of cycle relative threshold value for each gene normalized to that of the reference sample. P-values come from the ANOVA test and FDR is the false discovery rate.

		Experimental diets[2]			Contrast		*Q*-value
Function	Genes[1]	CON	SDP	SDP-PDP	Statistic	*P*-value	(FDR)
Barrier function	CLDN15	0.51^{b}	0.81^{ab}	1.05^{a}	4.984	0.027	0.139
	TFF3	1.15^{b}	1.07^{ab}	1.83^{a}	4.290	0.035	0.145
Immune response	TLR4	1.54^{b}	1.31^{b}	2.86^{a}	8.052	0.005	0.059
	GBP1	2.40^{ab}	1.80^{b}	4.20^{a}	5.711	0.015	0.127
	IFNGR1	0.69^{b}	0.74^{ab}	1.10^{a}	4.661	0.028	0.139
Nutrient transport	SLC11A2/ DMT1	0.37^{b}	0.71^{ab}	1.24^{a}	3.862	0.046	0.169
Enzyme/ Hormone	HNMT	0.86^{b}	0.91^{b}	1.56^{a}	14.111	$4x10^{-4}$	0.015
	APN	1.59^{b}	1.40^{b}	3.40^{a}	4.577	0.030	0.139
Oxidation	SOD2	1.04^{b}	0.95^{b}	1.71^{a}	11.030	0.001	0.022

[1]CLDN15: Claudin 15; TFF3: Trefoil Factor 3; SLC11A2/DMT1: Solute carrier family 11 member 2/ divalent metal transporter 1 (DMT1); TLR4: Toll like receptor 4; GBP1: Guanylate binding protein; IFNGR1: Interferon-gamma receptor 1; HNMT: Histamine N-Methyltransferase; APN: Alanyl Aminopeptidase; SOD2: Superoxide dismutase 2.
[2]CON: Control diet; SDP: Diet with 3% spray-dried plasma inclusion; SDP-PDP: Diet supplemented with a combination of 1% spray-dried plasma and 2% porcine digestible peptides (2%).
[a,b]Different letters in the same row indicate significant statistical mean differences in the two diets (Tukey's test *P*-value <0.05).

A PCA was carried out to determine the correlation in the gene expression values among samples distributed in the three diet groups (Figure 6.1). The sample identifier numbers are represented in the individual factor map (a) and are colored according to the diet assigned to each sample. The variables factor map (b) shows which genes are correlated along the samples. The 2D representation preserves 60% of the total variance (61.27). The results show a correlation in the gene expression pattern. In this tissue, eight of the nine significant genes (*APN*, *HNMT*, *SLC11A2*, *CLCN15*, *IFNGR1*, *TLR4*, *GBP1* and *SOD2*) are well represented in 2D (long arrows) and all of them fall in the first quadrant. Furthermore, the

blue colored samples in the SDP-PDP group fall in the same quadrant or near to it, which indicates medium to high expression levels. Therefore, these plots clearly show a relationship between the highest expression levels in these significant genes and the SDP-PDP diet group. The CON and SDP groups are mixed and do not show high expression values.



(a) (b)

Figure 6. 1. Principal component analysis (PCA): (**a**) Samples' picture from jejunum (individuals factor map); (**b**) Gene expressions arrow diagram from jejunum (variables factor map); CON = control diet; SDP = diet with 3% spray-dried plasma inclusion; SDP-PDP = diet supplemented with a combination of 1% spray-dried plasma and 2% porcine digestible peptides. Samples are labeled with different colors depending on the treatment, and the gene expressions arrows diagram on the right. Only those genes with $P < 0.05$ and having a long arrow have been depicted. Each arrow color indicates a different functional group. Trial 2.

A heatmap was also made in order to observe overall similarities among the gene expression profiles of the animals (Figure 6.2). On the heatmaps, the samples from the SDP-PDP treatment tend to group in pairs with others in the same group in the first step (the shortest branches). In addition, most SDP-PDP samples fall on one side of the dendrogram, the side with the highest number of red pixels in many genes, indicating a higher level of expression of these genes within the SDP-PDP group. Clustering of genes does not show any evidence of an association by functional group.

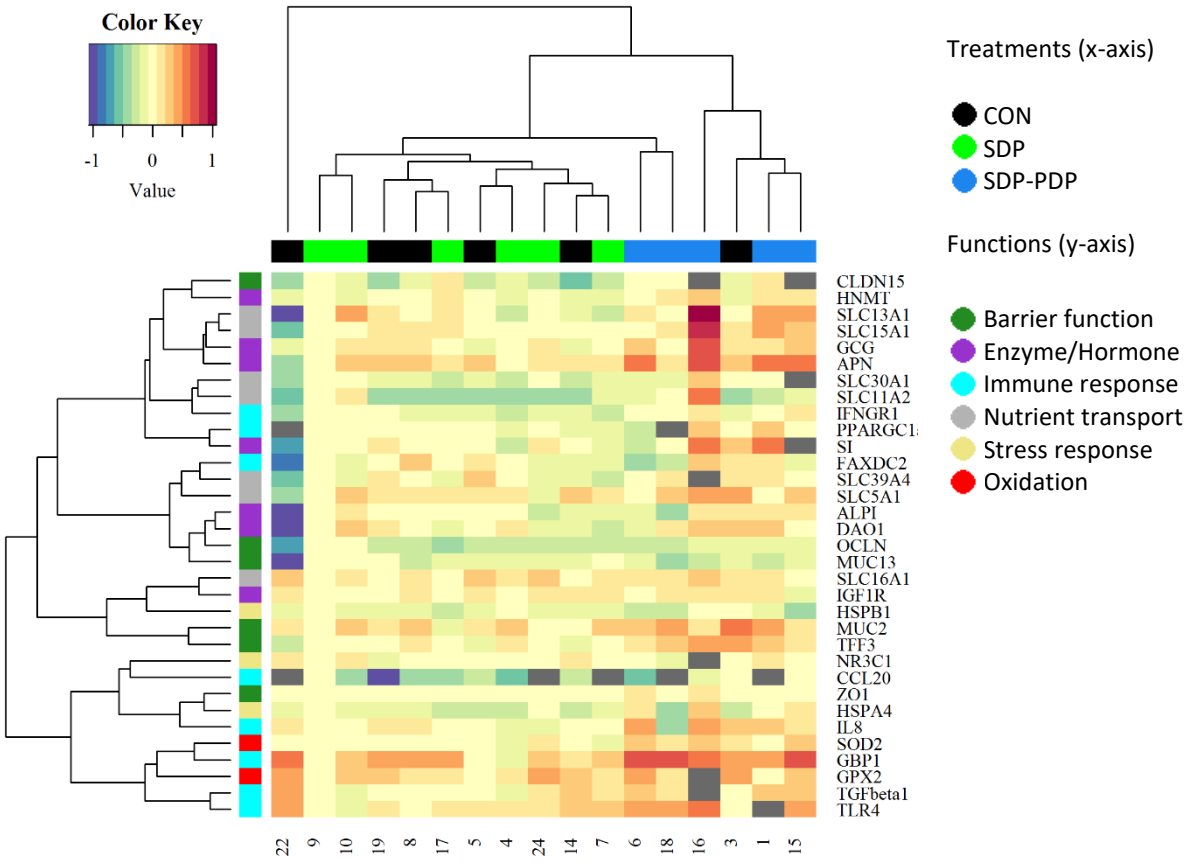


Figure 6. 2. Heatmap representing the gene expression of each sample from jejunum in each gene on trial 2. CON = control diet; SDP = diet with 3% spray-dried plasma inclusion; SDP-PDP = diet supplemented with a combination of 1% spray-dried plasma and 2% porcine digestible peptides. Genes are organized in rows and samples in columns. Samples are labeled with different colors representing every treatment and genes depending on its functional group.

5.4. Discussion

This study found that substituting soybean ingredients with 2-3% PDP and/or SDP to the diets of weaned piglets increased growth rate early after weaning. There are two main reasons that could be suggested and contribute to the observed results, such as the reduction of the negative impact of the soybean ingredients and the beneficial effects of the PDP and SDP. Unfortunately, our experimental design does not allow to discriminate between them. The diets in the present study were formulated to contain a high inclusion of soybean ingredients, such as soybean meal 44% or 47% CP, extruded soybeans and

soybean protein concentrate 56% CP. The β-sheet structures present in the secondary structure of raw legume proteins and the intermolecular β-sheet aggregates derived from heating are negatively correlated with feed digestibility values (Carbonaro et al., 2012). The β-sheet structures represent more than 30% of the secondary structure of soybean seeds, while they represent less than 10% in the ingredients of animal origin (Pieper et al., 2016). Consequently, the flow of protein into the distal parts of the GIT tends to be faster when more soybean ingredients are included, which promotes protein fermentation and selective growth of proteolytic bacteria (Pieper et al., 2016). Furthermore, soybeans contain anti-nutritional factors, such as antigens, trypsin inhibitors and lectins, which can produce digestive disorders and reduce nutrient availability (D. F. Li et al., 1991; Salgado et al., 2002). The consequences of high inclusion of soybean ingredients, aggravated by the situation of post-weaning anorexia, can lead to intestinal disorders, such as post-weaning diarrhea (Domeneghini et al., 2006; Prohászka & Baron, 1980). This can negatively affect the animal growth performance. In the present study, the reduction of soy ingredients accounts for 3-5% of the diet, being replaced by SDP and/or PDP. In contrast, proteins of animal origin as well as animal protein hydrolysates have higher palatability than vegetal proteins (Martínez-Puig et al., 2007; Solà-Oriol et al., 2010, 2011), which can translate into an increased feed intake after weaning.

The substitution of soybean ingredients with SDP improved the productive performance at the end of pre-starter period of both trials. The results reported herein are similar to other studies that showed that SDP improved performance, especially when piglets were challenged with experimental infection and did not receive in feed medication (Torrallardona, 2010). Spray-dried plasma is commonly used because it can stimulate feed intake due to its palatability (Ermer et al., 1994), as observed in Trial 1. Its beneficial effects are explained by the preservation of blood immunoglobulins, growth factors, and bioactive peptides or compounds during the spray-drying process. These components can interact with the gut-associated lymphoid tissue (Pérez-Bosque et al., 2016), therefore preserving the small intestinal barrier function and reducing intestinal inflammation and damage (Bosi et al., 2004).

Consumption of feed containing the hydrolysates, PDP or SDP-PDP, enhanced the productive performance of piglets compared to their corresponding control group at the end of the pre-starter period, and these groups were equivalent to the SDP group. This result is in accordance with other authors who also obtained similar growth performance by feeding PDP and SDP in early-weaned piglets (Myers et al., 2014). Some research data have shown improved growth performance, feed intake and efficiency of animals fed PDP compared to other high-quality protein sources like fish meal (Solà-Oriol et al., 2010). Other studies found out that the inclusion of PDP in weanling diets improved villus height of the small intestine compared to some sources of intact protein like SDP (Borda et al., 2005) or fish meal (Xin et al., 2001), which can be considered a good indicator of nutrient uptake. Part of the beneficial effects of PDP on growth performance could be due to its content of short-chain peptides that are more easily absorbed by pigs than intact proteins (Gilbert et al., 2008) or even free amino acids (Rérat et al., 1988).

This is the first study performed using gene expression to provide some inputs about the effects of PDP on intestinal function. The outcome of the PCA individual factor map of the gene expression shows that the factorial scores of the SDP-PDP group tended to be closer to each other and more separate from the CON and SDP group scores. In addition, the correspondence of most of the arrows representing the significant genes in the PCA variables map and most of the samples in the SDP-PDP group in a similar position indicates that there is a relationship between the higher expression levels in these genes and the differences that the SDP-PDP group showed from the CON and SDP groups. Heatmap representation also helped to visualize a partial clustering of the samples from the SDP-PDP treatment in the jejunum and ileum. In line with this, the statistical ANOVA and Tukey's test showed a stronger effect of the SDP-PDP diet than SDP with respect to the CON diet. Although all results agree, ANOVA and Tukey's test deal with mean treatment values while heatmaps and PCA-plots reflect individual performance. The combination of inferential treatment comparison techniques (ANOVA and Tukey, essentially) and powerful exploratory methods (PCA and heatmap) provides a clearer and dual (individuals and genes) idea of the differences in gene expression.

Proteins coded by the genes analyzed in the current study participate in the barrier function of gut cells, in nutrient transport in the mucosa, in digestion, in the immune response and in the metabolization of oxidation products.

Some trends in the jejunum gene expression due to diets may indicate a potentiation of the epithelial structure by SDP-PDP, because, for example, the TFF3 gene participates in epithelial restitution and maintenance of intestinal mucosa integrity (Taupin & Podolsky, 2003). However, the CLDN15 gene, which expression was also modified, codes for a pore-forming protein (Khan & Asif, 2015), which is important for the normal-sized morphogenesis of the small intestine (Tamura et al., 2008).

Diets also had an effect on the expression in the jejunum of genes related to the immune response and metabolization of oxidation products. Again, the SDP-PDP diet showed more changes than the SDP diet alone. Expression of immune response genes TLR4, IFNGR1 and GBP1 showed a trend to increase in the SDP-PDP group; TLR4 compared to the CON and SDP groups; IFNGR1 compared to the CON group; and GBP1 compared to the SDP group.

TLR4 is a receptor involved in the recognition of lipopolysaccharide, a major cell wall component of Gram-negative bacteria (Takeda et al., 2003), and IFNGR1 is part of the receptor that mediates the biological effects of IFN-γ (Xing et al., 2010). The TLR4 and IFNGR1 genes have been reported to be upregulated in animals under stress and infection conditions in order to activate the innate immune response and fight against pathogens properly (Badia, Lizardo, et al., 2012; P. Liu et al., 2014; Vaure & Liu, 2014). Thus, the tendency for upregulation of TLR4 and IFNGR1 might suggest that piglets fed with SDP-PDP seem to be more prepared for controlling infective processes and other intestinal challenges that can occur during the weaning period. In addition, GBP1 is a GTPase that regulates the inhibition of proliferation and invasion of endothelial cells. It protects against epithelial apoptosis induced by inflammatory cytokines and subsequent loss of the barrier function (Schnoor et al., 2009). Upregulation of GBP1 by the SDP-PDP diet, although without the statistically significance, is probably related to IFNGR1 upregulation because GBP1

expression is strongly induced by IFN-γ (Schnoor et al., 2009), although an upregulation of IFN-γ was not observed in this study.

Focusing on nutrient transport, only an up-regulatory trend in the expression of the SLC11A2/DMT1 gene was found in animals fed with the SDP-PDP combination compared to the CON group. The divalent metal transporter (SCL11A2/DMT1) is located on the apical surface of the enterocyte and is involved in the intestinal Fe uptake (Garrick et al., 2003). SLC11A2/DMT1 gene expression is upregulated in circumstances of low Fe intake (S. L. Hansen et al., 2009) and hyperglycemia conditions (L. Zhao et al., 2019). We have no evidence of differences in the Fe content or glycemic levels among experimental diets; therefore, the reason why the SDP-PDP treatment influenced the expression of SLC11A2/DMT1 should be researched further.

The enzyme-coding gene HNMT was upregulated and the gene APN showed a trend to increase due to the SDP-PDP diet. Kröger et al. (2013) reported that high dietary inclusion of fermentable CP increased the HNMT expression in the colon, which is a histamine-degrading enzyme. They determined that the histamine catabolism activity of HNMT counter-regulated the increased production of this biogenic amine, reducing the fecal score of the piglets fed with a high fermentable CP diet. Considering that all diets had an elevated CP level, an increase in this enzyme could show that SDP-PDP was attenuating the inflammatory effects of histamine more efficiently than other groups. On the other hand, APN is a Zn-dependent enzyme that takes part in the final digestion of peptides (Bank et al., 2008). Its upregulation in the small intestine has been documented with products considered beneficial for intestinal health, such as the probiotic *Lactococcus lactis* (Xu et al., 2015). Therefore, its increase due to the SDP-PDP diet may also be considered a positive physiological change.

Regarding the antioxidant defense mechanisms, we observed here that the expression of the SOD2 gene was also increased in the SDP-PDP group compared to both the SDP and CON groups. This mitochondrial enzyme is considered the first defense against reactive oxygen species (ROS) formed during normal cell metabolism (Chmielewska et al., 2013). Elimination of ROS by SOD2 can be considered as an anti-inflammatory effect due to the

important role that ROS plays in triggering and promoting inflammation (C. Li & Zhou, 2011). As well as the TLR4 gene, expression of SOD2 is stimulated by lipopolysaccharides but cytokines or ROS can also upregulate it (Visner et al., 1990). Thus, changes in SOD2 gene expression can be derived from the TLR4 upregulation also induced by the SDP-PDP intervention.

The gene expression results of this trial might indicate that the combination of PDP and SDP had some effects on intestinal function, although most of the differences found among treatments only could be considered as a trend ($P < 0.05$, $Q < 0.2$). First of all, the tendency for upregulation of TFF3 and CLDN15 could be considered a potentiation of the epithelial structure of the gut, which would be related with the increase of villi height observed in previous studies with PDP (Borda et al., 2005). Secondly, we could speculate that animals fed PDP-SDP might be more prepared for controlling infective processes and defending against other hazardous processes due to the up-regulatory trend in the expression of the immune response genes (TLR4, IFNGR1 and GBP1) and the upregulation of genes related to the degradation of toxic products of metabolism (SOD2 and HNMT). In addition, the trend to increase the expression of the APN gene could suggest an improvement in the digestion of protein. The underlying mechanisms that produced these effects are still unknown and the literature exploring possible functional effects of PDP is still scarce. More research is needed to confirm that gene expression upregulation by PDP in combination with SDP has an impact on intestinal function and to elucidate the underlying mechanisms that are responsible of these effects. Furthermore, it might be interesting to investigate if the effects on gene expression observed in this study are only attributable to the addition of PDP or if it is a synergy of PDP with SDP what is producing the effects, as SDP alone did not show them.

5.5. Conclusions

The present study suggests that substituting soybean products with animal protein sources like PDP or SDP increases growth performance of weanling piglets at the end of pre-starter period. In addition, it indicates that PDP can substitute or complement SDP because it showed the same effect on the growth performance of the piglets during this period. Furthermore, changes on the gene expression of the jejunum produced by the SDP-PDP diet suggest that this treatment might be able to produce beneficial effects in the epithelial structure of the gut, in the defensive capacity of the intestine against threats and the digestion of proteins. More research is needed to confirm that gene expression upregulation by PDP in combination with SDP has an impact on intestinal function and to elucidate the underlying mechanisms that are responsible of these effects.

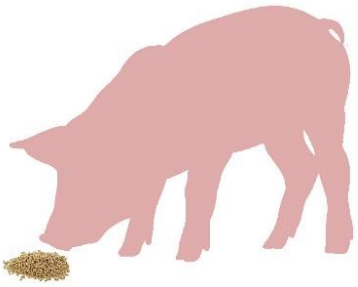
5.6. Supplementary Tables

Table S6. 1. List of primers used for the analyses of gene expression of the 56 genes by Open Array Real-Time PCR custom designed.

Gene	Name	Primer Forward (5´-3´)	Primer Reverse (5´-3´)	Probe (5´-3´)
OCLN	Occludin	CAGGTGCACCCTCCAGATTG	CAGGCCTATAAGGAGGTGGACTT	TGACATCAGCCATGTCAT
ZO1	Zonula occludens 1	GCTATGTCCAGAATCTCGGAAAA	TGCTTCTTTCAATGCTCCATACC	TCACCATCTTTTTACAACTAC
CLDN1	Claudin-1	CTTCGACTCCTTGCTGAATCTGA	CTTCCATGCACTTCATACACTTCAT	ACAGCACTTTGCAAGC
CLDN4	Claudin-4	CCTCCGTGCTGTTCCTCAA	GAGGCACAAGCCCAGCAA	CCTTGTGGCACTTTG
CLDN15	Claudin-15	GCTATCTCCTGGTATGCCTTCAA	GGGACTTCCACACTCCTTGGT	ACTTCTTCGACCCCTTGTA
MUC2	Mucin 2	AAGGACGACACCATCTACCTCACT	GGCCAGCTCGGGAATAGAC	CATGGTCAGCACCCCG
MUC13	Mucin 13	CAGTGGAGTTGGCTGTGAAAAC	ATCAAGTTCTGTTCTTCCACATTCTTG	TCCTCTCATTAAGATCAAAC
TFF3	Trefoil factor 3	AGAACCTGCCCGTGACCAT	CACACTGGTTCGCCGACAG	AGGCCAGGATGTTCT
TLR2	Toll-like receptor 2	CTCTCGTTGCGGCTTCCA	AAGACCCATGCTGTCCACAAA	CAAGGTCAACTCTCTG
TLR4	Toll-like receptor 4	CATCCCCACATCAGTCAAGATACT	TCAATTGTCTGAATTTCACATCTGG	ACAGCAATAGCTTCTCCA
IL1B	Interleukin 1 beta	GGTGACAACAATAATGACCTGTTATTTG	GCTCCCATTTCTCAGAGAACCA	ATGAAGTGCTGCACCC
IL6	Interleukin 6	CCAATCTGGGTTCAATCAGGAG	ACAGCCTCGACATTTCCCTTATT	AGATATACCTGGACTACCTC
IL8	Interleukin 8	GGAAAAGTGGGTGCAGAAGGT	GAGAATGGGTTTTTGCTTGTTGT	TACAGATATTTTTGAAGAGAACT
IL10	Interleukin 10	TGAGGCTGCGGCGCT	GAGCTTGCTAAAGGCACTCTTCA	AACAAGAGCAAGGCCGT
IL17A	Interleukin 17	CCAGACGGCCCTCAGATTAC	ATCTTCCTTCCCTTCAGCATTG	CCATGGACTCTCCAACG
IL22	Interleukin 22	TGTTCCCCAACTCTGATAGATTCC	GTTGTTCACATTTCTCTGGATATGCT	AGCTAAGCCAATGCCGTAT
IFNG	Interferon-gamma	TGACTTTGTGTTTTTCTGGCTCTT	CACTCTCCTCTTTCCAATTCTTCAA	ATCCTAAAGGACTATTTTAAT
TNF	Tumor necrosis factor alpha	CACCACGCTCTTCTGCCTACT	GACGGGCTTATCTGAGGTTTGA	CAAGGACTCAGATCATCGT
TGFB1	Transforming growth factor beta 1	GCGGCAGCTCTACATTGACTT	GACCTTGCTGTACTGAGTGTCTAGG	CCATGCCAATTTCTGCCT
CCL20	Chemokine (C-C motif) ligand 20	GACCATATTCTTCACCCCAGATTT	CACACACGGCTAACTTTTTCTTTG	ATCAATGCAATCATCTTT

CXCL2	Chemokine (C-X-C motif) ligand 2	CATGGTGAAGAAAATCATCGAGAA	GCCAGTAAGTTTCCTCCATCTCTCT	AACAAGAGCAGTGCCAAC
IFNGR1	Interferon gamma receptor 1	CATGTTACCCAAATCTTTGCTGTCT	CAGTATGCACGCTTGAAATTGTC	ATATATATCACCCATCACCTACC
HSPB1	Heat shock protein 27	CGAGGAGCTGACGGTCAAG	GCAGCGTGTATTTTCGAGTGAA	ACGGCTTCATTTCCCGGT
HSPA4	Heat shock protein 70	TCAATTGCCTGCGATTAATGAA	GAATGCCCCATGTCTACAAAAAC	CAGTTGCTCTTGCATATG
REG3G	Regenerating-islet derived protein 3 gamma	TGCCTGATGCTCCTGTCTCA	GGCATAGCAGTAGGAAGCATAGG	CCAAGGTGAAGATTC
PPARGC1A	Peroxisome proliferative activated receptor gamma, coactivator 1 alpha	CTCTGGAACTGCAGGCCTAA	TGGAGAAGCCCTAAAAGGGTTAT	ACCCACAACTCCTCCT
FAXDC2	Fatty acid hydrolase domain containing 2	CCATGACTACCACCATCTCAAGTT	GCAGGATCGTGTGTCTCTCGTA	TGTTCAAGCAGACCAAG
GBP1	Guanylate binding protein 1	AGAATCCATCACAGCAGACGAGTA	CGGATACAGAGTCGAGGCAGGTTAA	TCAAGCTTAAGAAGGGTACCAG
GPX2	Glutathione peroxidase 2	CAACCAATTTGGACATCAGGAG	GGGTAAAGGTGGGCTGGAAT	AGATCCTGAACAGCCTCA
SOD2	Superoxide dismutase	GGGTTGGCTCGGTTTCAA	CATGCTCCCACACGTCGAT	CTGCAAGGAACAACAGGTCT
ALPI	Intestinal alkaline phosphatase	ATGTCTTCTCTTTTGGTGGCTACA	GGAGGTATATGGCTTGAGATCCA	AAGCTCCGTTTTTGGCCT
SI	Sucrase-isomaltase	CGACCCCTTTTGCATGAGTT	AAGGCTGGACCCCATAGGAA	TTTAATGAAAAGCCAACCTG
DAO1	Diamine oxidase	GGAACCAACAGACCTTCAACTATCTC	TTCGGAATCCCAGGACCAT	CCGGACCCTTACTGGAAA
HNMT	Histamine N-methyltransferase	TGTTGAACCAAGTGCTGAACAAAT	ACTTTATGTTCTCGAGGTTTGATGTCTT	ACCAAGTACAAAGAGCTT
APN	Aminopeptidase-N	AGGGCAACGTCAAAAAGGTG	GTCAAAGCATGGGAAGGATTTC	ACACAGATGCAGTCTACAG
IDO1	Indoleamine 2,3 dioxygenase	TTGGCAAATTGGAAGAAAAAGG	CCGGAAATGAGAAGAGAATATCCAT	CCAGTGGGCCCATGACTTAC
GCG	Glucagon	AGGCGTGCCCAGGATTTT	CATCGTGACGTTTGGCAATG	CACCAAGAGGAACAAGAA
CCK	Cholecystokinin	CAGCAGGCTCGAAAAGCAC	AATCCATCCAGCCCATGTAGTC	CAGCCACAGAATAAGTGA
IGF1R	Insulin-like growth factor 1 receptor	CCGACGCGGCAACAAC	TCAGGAAGGACAAGGAGACCAA	CTACGTGAAGATCCGCCA
PYY	Peptide tyrosine tyrosine	CAGAGGTATGGGAAACGTGACA	CCTTCTGGCCACGACTTGAC	CAAACTGCTCTTCCCTGAA
SLC5A1	Solute carrier family 5 (sodium/glucose cotransporter) member 1	GGCCATCTTTCTCTTACTGGCA	TCCCACTTCATGAAAAGCAAAC	TTTATACGGATACCTTGCAGAC
SLC16A1	Monocarboxylate transporter 1	CCTTGTTGGACCTCAGAGATTCTC	CCAGTATGTGTATTTATAGTCTCCGTATATGTC	CCACCACTTTTAGGTCGTC

SLC7A8	Solute carrier family 7 (amino acid transporter light chain, L System) member 8	TGTCGCTTATGTCAC TGCAATGT	GACAGGGCGACGG AAATG	CTGTGACTTTTGGA GAGAA
SLC15A1	Solute carrier family 15 (oligopeptide transporter) member 1	GGTTATCCCTTGAGC ATCTTCTTC	AGTGCTCTCATTCC ATAGTAGGAAAA	TCAACGAGTTCTGT GAAAG
SLC13A1	Solute carrier family 13 (sodium/sulfate symporters) member 1	GGTACCTCCACCAAC TTGATCTTC	ATCCAAAGTTGATG CAGTGACAAT	ATTTCAATATGCGC TACCC
SLC11A2	Solute carrier family 11 (proton-coupled divalent metal ion transporter) member 2	GTCTTTGCCGAAGCG TTTTTT	ACCACGCCCCCTTT GTAGA	CCAACCAGCAGGTG GT
MT1A	Metallothionein 1A	TGAATCCGCGTTGCT CTCT	CAGGAGCAGCAGC TCTTCTT	ACGTGCAAAACCTG CAGA
SLC30A1	Solute carrier family 30 (zinc transporter) member 1	AATTGGACCGGACA GATCCA	TCTCTGATAAGATT CCCATTCACTTG	AAAAGTCCAGAAGT GATGC
SLC39A4	Solute carrier family 39 (zinc transporter) member 4	ATCTTTGGGCTCTTG CTCCTT	GCAGCCCCAGCAC CTTAG	CTGCTACCCACTAC GTCA
CRHR1	Corticotropin releasing hormone receptor 1	CAGGGCCCCATGATA TTGG	CCGGAGTTTGGTC ATGAGGAT	CTGATCAACTTTAT CTTCC
NR3C1	Glucocorticoid receptor	GGCAATACCAGGATT CAGGAACT	CCATGAGAAACAT CCATGAATACTG	TGACCAAATGACCC TCCT
HSD11B1	Hydroxysteroid (11-beta) dehydrogenase 1	GGTCAGAAGAAACT CTCAAGAAGGTG	GCGAAGGTCATGT CCTCCAT	TCTTCAGCACACTA CGTTG
GAPDH	Glyceraldehyde-phosphate-dehydrogenase	TTCGTCAAGCTCATT TCCTGGTA	TCCTCGCGTGCTCT TGCT	AATTTGGCTACAGC AACAG
ACTB	Actin, beta	CAAGGACCTCTACGC CAACAC	TGGAGGCGCGATG ATCTT	CACCACCATGTACC CAGG
TBP	TATA-box binding protein	CAGAATGATCAAACC GAGAATTGT	CTGCTCTGACTTTA GCACCTGTTAA	TTTGTCTCTGGAAA AGTTGT
B2M	Beta-2-microglobulin	TCACTCCTAACGCTG TGGATCA	CGGTTAGTGGTCTC GATCCC	AGCACGTGACTCTC GATA



Chapter 7

The Allowance of Pre-Starter Diet to Nursery Pigs Until Reaching a Targeted BW Does Not Affect the Body Weight Variability of the Batch at the End of the Nursery Period

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Manuscript under submission preparation

Abstract

First-phase feeds are formulated to help pigs reach a specific body weight before transitioning to a more affordable compound diet. However, these transitions typically take place on a fixed schedule, which may disadvantage the smallest pigs due to their less developed digestive systems, and their higher maintenance and growth requirements in relation to their feed intake capacity. This study aimed to investigate the effects of providing piglets with a higher allowance of a pre-starter diet until they reached a targeted body weight (BW) on their growth performance and the variability of body weight within the batch. A total of 528 male and female weaned commercial piglets ([Landrace x Yorkshire] x Pietrain, weaned at d 21) were divided into two blocks according to their weaning BW (MEDIUM pigs: 5.35 ± 0.31 kg; SMALL pigs: 3.97 ± 0.21 kg). Ten days after weaning, pigs were offered a starter feed (FIXED) or allowed to consume pre-starter feed until they reached a targeted BW (BWAPP), which was set as the average BW of MEDIUM pigs in the BWAPP strategy at d14 post-weaning. The final target BW was established at 7.9 kg of BW, which was achieved at d21 by SMALL pigs in the BWAPP strategy. The MEDIUM and SMALL pigs in the BWAPP strategy consumed 1.18 kg and 3.23 kg more pre-starter feed compared to the pigs in the FIXED strategy, respectively. From d10 to 14, MEDIUM pigs in the BWAPP strategy exhibited significantly higher ADG *(P* = 0.001), ADFI (*P* = 0.029), and a tendency towards decreased FCR (*P* = 0.069) compared to MEDIUM pigs in the FIXED strategy. From d10 to 21, SMALL pigs in the BWAPP strategy showed a tendency towards higher ADFI (*P* = 0.0.97) than SMALL pigs in the FIXED strategy. However, when the pigs in the BWAPP strategy were offered the starter diet, they showed numerically lower ADG and ADFI (*P* > 0.05) than pigs in the FIXED strategy, resulting in comparable growth performance for the entire experimental period (d0-36). In conclusion, providing a higher allowance of pre-starter feed to weaning pigs until they reached a target body weight did not result in any significant effect on growth performance or the variability of the batch. Future studies should investigate the potential impact of incorporating a target body weight for the allowance of starter feed and examinine the long-term effects of this strategy.

6.1. Introduction

The variation in growth rates among pigs in commercial herds can have detrimental economic consequences for producers (Patience et al., 2004). Thus, the implementation of strategies to mitigate its impact is essential. Although certain approaches, such as sorting pigs by weight after weaning, have gained popularity among farmers, it has been demonstrated that this practice only reduces variation within a pen at the time of sorting and does not have an effect without individualized treatment across different groups (O'Quinn et al., 2000).

First-phase feed plays a crucial role in minimizing any growth check at weaning and maximizing feed intake and growth performance during the nursery period but also in subsequent stages (Lawlor et al., 2002). The feeding programs of commercial farms include successive feeds formulated for the average pigs, that decrease in cost and specification. These regimens are designed to help pigs reach a specific body weight (BW) before transitioning to a more affordable compound diet (S. L. Douglas, Wellock, et al., 2014). However, these transitions occur on a predetermined day, without considering the BW variability that exists within the batch.

Under these circumstances, lighter piglets at weaning, who may have less developed digestive tracts compared to their average counterparts (Michiels et al., 2013), are expected to consume lower amounts of the initial diets, including specific nutrients or dairy ingredients. Furthermore, these lighter piglets will be provided with less digestible and cheaper diets, potentially before their digestive system tract is fully prepared to deal with them. This situation might lead to an escalation in the influx of undigested nutrients, including protein, into the distal segments of their gastrointestinal tract, whose microbial fermentation can have a detrimental effect on gut health (Pieper et al., 2016). This may impede their adaptation to post-weaning diets and can negatively impact their performance (Pluske et al., 2003).

Previous studies have already determined that providing increased amounts of a standard first-phase dietary regimen at weaning has positive effects on pigs' growth

performance (A. L. Craig et al., 2020; Huting et al., 2017; Lawlor et al., 2002; Magowan et al., 2011a, 2011b; Muns & Magowan, 2018). However, these studies provided the same additional amount of first-phase feed to all pigs, without considering that smaller pigs may have higher requirements for this particular feed. Hence, this study proposes a strategy that involves allowing pigs to consume the pre-starter diet until they reach a target body weight, rather than offering a limited amount of pre-starter, or offering it until a fixed date. It is hypothesized that this approach will have a positive impact on their performance and contribute to reducing batch variability.

In a prior study, we observed that including a combination of spray-dried plasma (SDP) and porcine digestible peptides (PDP) in a pre-starter feed was an effective strategy for improving the growth performance and intestinal functionality of weaned piglets. Therefore, the objective of this study was to investigate the effects of providing piglets a higher allowance of a pre-starter diet which included SDP and PDP, until they reached a targeted BW, on their growth performance and the BW variability of the batch.

6.2. Materials and Methods

The experimental procedures used in the trial were approved by the Ethical Committee on Animal Experimentation of the Autonomous University of Barcelona (CEAAH 3817), and are in full compliance with national legislation following the EU-Directive 2010/63/EU for the protection of animals used for scientific purposes.

6.2.1. Animals, Housing, and Diet

The trial was conducted in the weanling unit of a commercial farm throughout the nursery period (d 1 to 36 post-weaning). A total of 528 male and female weaned commercial piglets ([Landrace x Yorkshire] x Pietrain, weaned at d 21) with a BW of 4.7 ± 0.74 kg were moved to the nursery unit by truck to be used in the trial. Piglets had access to creep feed during lactation. Animals were individually ear-tagged and distributed into two blocks according to initial BW (MEDIUM pigs: 5.4 ± 0.31 kg; SMALL pigs: 4.0 ± 0.21 kg). Each block

contained 24 pens of 11 mixed-sex pigs. Each pen (3 m^2 in floor area) had a commercial non-lidded hopper (TR5, Rotecna, Spain) and a nipple waterer to ensure ad libitum feeding and free water access. All pens were offered the pre-starter diet immediately after weaning.

Ten days after weaning, pens were assigned to two experimental feed management strategies (12 replicates for each strategy). Half of the pigs within MEDIUM and SMALL BW categories were changed to the starter feed that same day (d10) following a fixed-day approach (FIXED). The remaining pigs were allowed to consume pre-starter feed until they reached a specific BW target, following a BW approach (BWAPP). The target BW was established as the average BW of MEDIUM pigs in the BWAPP strategy at d14 (Figure 7.1).

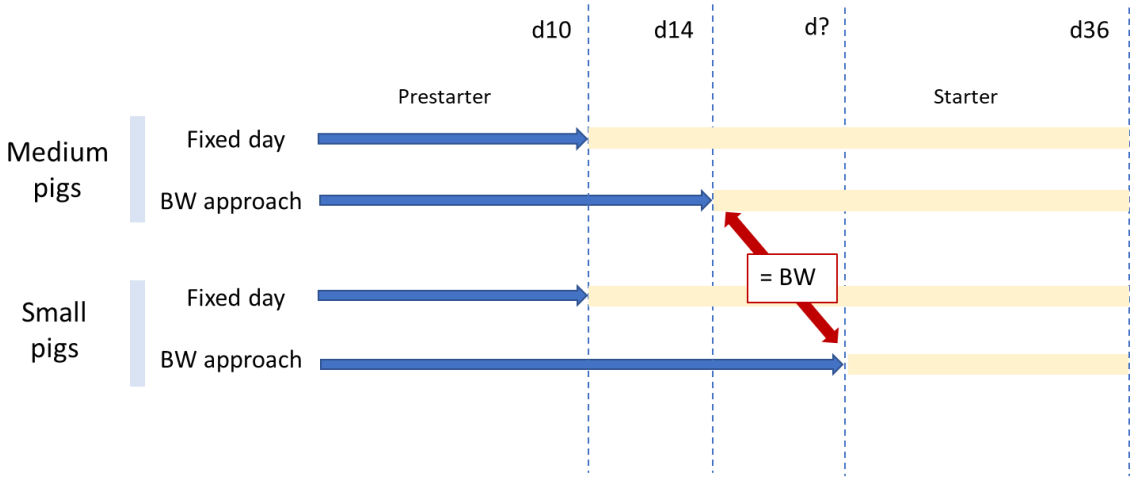


Figure 7. 1. Schematic representation of the experimental design of the study.

The pre-starter diet was formulated to contain 2,544 kcal of net energy (NE)/kg, 18.5% crude protein (CP), and 1.39% SID lysine (Table 7.1) to meet the nutritional requirements of newly weaned piglets (5-7 kg of BW) (FEDNA, 2013). The starter diet was formulated to contain 2,458 kcal of NE/kg, 17.89% CP, and 1.25% SID lysine (Table 7.1) to meet the nutritional requirements of nursery pigs (7-12 kg). No antimicrobials or ZnO were used in the experimental diets.

Table 7. 1. Composition of the experimental diets, % as fed basis.

	Diets	
Item	Pre-starter	Starter
Ingredients, %		
Corn	34.28	50.75
Broken rice	11	5
Wheat	6	10
Barley	3	7
Wheat bran	2.5	1
Soybean meal 47	10	12
Extruded soybeans	6.27	2.64
Soybean protein concentrate	3.32	6.64
Sweet milk whey	9	0
Lactose	3.5	0
Acid whey	2.18	0
Porcine digestible peptides 62% PB	2	0
SDP 80% PB	1	0
Sacarose	1.66	0
Lard	1	0.5
Mono calcium phosphate	0.56	1.06
Calcium carbonate	0	0.56
Salt	0.16	0.53
Vitamin-Mineral premix[1]	0.4	0.4
L-Lyisine 50	1.04	1.01
DL-Methionine	0.34	0.28
L-Threonine	0.31	0.29
L-Valine	0.25	0.20
L-Isoleucine	0.10	0.05
L-Triptophan	0.13	0.1
Calculated composition		
Dry Matter, %	89.5	87.8
Net Energy (NE), kcal/kg	2544	2458
Ash, %	4.6	4.6
Crude protein, %	18.5	17.9
Ether Extract, %	4.2	3.7
Starch, %	35.3	45.2
Neutral Detergent Fiber, %	7.0	8.8
Calcium, %	0.33	0.50
Total P, %	0.53	0.58
Digestible P, %	0.30	0.30
Na	0.35	0.22
Cl	0.36	0.36
SID amino acids[3]		

Lys	1.390	1.250
Met	0.593	0.514
Cys	0.239	0.240
Met+Cys	0.834	0.750
Thr	0.904	0.812
Trp	0.306	0.275
Val	0.973	0.875

[1]Supplied the following per kg of diet: 7,000 IU of vitamin A (acetate); 500 IU of vitamin D3 (cholecalciferol); 250 IU of vitamin D (25-hydroxicholecalciferol); 45 mg of vitamin E; 1 mg of vitamin K3; 1.5 mg of vitamin B1; 3.5 mg of vitamin B2; 1.75 mg of vitamin B6; 0.03 mg of vitamin B12; 8.5 mg of D-pantothenic acid; 22.5 mg of niacin; 0.1 mg of biotin; 0.75 mg of folacin; 20 mg of Fe (chelate of amino acids); 2.5 mg of Cu (sulphate); 7.5 mg of Cu (chelate of glycine); 0.05 mg of Co (sulphate); 40 mg of Zn (oxide); 12.5 mg Zn (chelate of amino acids); 12.5 mg of Mn (oxide); 7.5 of Mn (chelate of glycine); 0.35 mg of I, 0.5 of Se (organic); 0.1 mg of Se (inorganic).
[3] Standardized ileal digestible amino acids.

Pigs were individually weighed at weaning (d 0), d 10, and at d 36. Furthermore, pigs within each BW category were weighted at the moment of the feed change for the BWAPP group. A subset of SMALL pigs in the BWAPP strategy was weighted at d17 to estimate when they would reach the target BW.

Feed disappearance from each hopper was measured throughout the experimental period. The average daily feed intake (ADFI), average daily gain (ADG), and feed conversion ratio (FCR) were calculated for the experimental period.

6.2.2. Statistical Analysis

The data analysis was conducted using the open-source software R v4.0.3 (R Foundation for Statistical Computing, Vienna, Austria). To compare the growth performance between the two feeding strategies, separate T-tests were conducted for each BW category. Furthermore, the ADG, ADFI, and FCR of all groups during the period from day 0 to day 36 and the mortality were analyzed using a two-way ANOVA as a factorial arrangement. The model included the fixed effects of feeding strategy, BW category, and their interaction. The significance level for determining statistical significance was set at $\alpha = 0.05$, while tendencies were assessed at $\alpha = 0.10$.

6.3. Results

At d21, SMALL pigs on the BWAPP strategy reached a similar BW as the MEDIUM pigs on the BWAPP strategy at d14 (7.84 ± 0.36 kg and 7.90 ± 0.36 kg, respectively). As a result, the diet of SMALL pigs on the BWPP strategy was changed at d 21.

Pigs on the FIXED strategy consumed a total of 1.78 ± 0.254 kg (MEDIUM) and 1.66 ± 0.261 kg (SMALL) of pre-starter feed. Meanwhile, pigs on the BWAPP consumed 2.96 ± 0.254 kg (MEDIUM) and 4.89 ± 0.754 kg (SMALL) of pre-starter feed. Therefore, the MEDIUM and SMALL pigs in the BWAPP strategy consumed 1.18 kg and 3.23 kg more pre-starter feed than the pigs in the FIXED strategy, respectively.

The BW and CV within the pen throughout the experimental period are represented in Figure 7.2. No significant differences in BW or CV within the pen were observed between FIXED and BWAPP during the experimental period ($P < 0.05$).

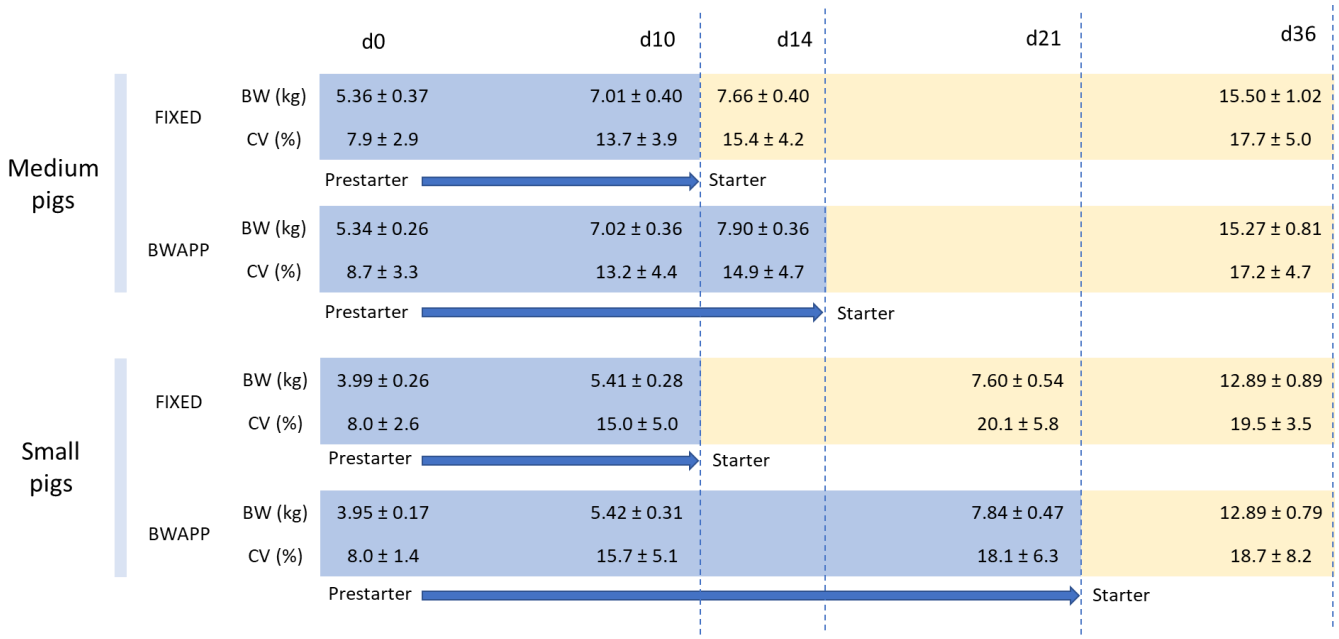


Figure 7. 2. Effect of the feeding strategy on the evolution of the body weight (BW) and coefficient of variance (CV) of small and medium pigs on two different feeding management strategies. Values are expressed as mean ± standard deviation. Differences in BW and CV between both strategies were non-significant ($P > 0.05$). FIXED: feeding strategy consisting in allowing pigs to consume pre-starter feed until d10 post-weaning; BWAPP: feeding strategy consisting in allowing pigs to consume pre-starter feed until achieving 7.9kg of BW.

The ADG, ADFI, and FCR calculations for each specific period are detailed in Table 7.2 and the calculations for the entire experimental period (d 0 - 36) are detailed in Table 7.3. From day 10 to day 14, MEDIUM pigs in the BWAPP strategy exhibited significantly higher ADG (*P* = 0.001) and ADFI (*P* = 0.029), and showed a tendency towards decreased FCR (*P* = 0.069) compared to MEDIUM pigs in the FIXED strategy. Similarly, from day 10 to day 21, SMALL pigs in the BWAPP strategy showed a tendency towards higher ADFI (*P* = 0.097) than SMALL pigs in the FIXED strategy. However, when the pigs in the BWA strategy were offered the starter diet (MEDIUM: days 14-36; SMALL: days 21-26) they showed numerically lower ADG and ADFI than pigs in the FIXED strategy, resulting in comparable growth performance for the entire experimental period (days 0-36). There were no differences in mortality between the different groups (Table 7.3).

Table 7. 2. Effect of the feeding strategy on the average daily gain (ADG), average daily feed intake (ADFI) and feed conversion ratio (FCR) of small and medium pigs on two different feeding management strategies for each period.

BW Category	Strategy[1]	d 0 - 10			d 10 - 14			d 14 - 36		
		ADG, g/d	ADFI, g/d	FCR	ADG, g/d	ADFI, g/d	FCR	ADG, g/d	ADFI, g/d	FCR
Medium pigs	FIXED	168	178	1.10	163	241	1.50	349	0.452	1.30
	BWAPP	163	185	1.11	220	277	1.27	335	0.446	1.34
SEM[2]		9.0	8.4	0.025	13.0	11.2	0.085	0.0084	0.0113	0.021
P value[3]		0.708	0.581	0.907	0.001	0.029	0.069	0.264	0.729	0.215

BW Category	Strategy[1]	d 0 - 10			d 10 - 21			d 21 - 36		
		ADG, g/d	ADFI, g/d	FCR	ADG, g/d	ADFI, g/d	FCR	ADG, g/d	ADFI, g/d	FCR
Small pigs	FIXED	141	166	1.17	199	265	1.34	339	488	1.45
	BWAPP	144	164	1.19	216	296	1.37	332	467	1.41
SEM[2]		9.2	7.4	0.045	7.4	12.5	0.052	8.4	11.1	0.032
P value[3]		0.798	0.843	0.773	0.118	0.097	0.728	0.590	0.193	0.433

[1] FIXED: feeding strategy consisting in allowing pigs to consume pre-starter feed until d10 post-weaning; BWAPP: feeding strategy consisting in allowing pigs to consume pre-starter feed until achieving 7.9kg of BW.

[2] Standard error of the mean.

[3] *P*-values come from the T test.

Table 7. 3. Effect of the feeding strategy on the average daily gain (ADG), average daily feed intake (ADFI), feed conversion ratio (FCR) and mortality of small and medium pigs on two different feeding management strategies for the whole experimental period.

BW Category	Strategy[1]	d 0 - 36			Mortality		
		ADG, g/d	ADFI, g/d	FCR	d 0 -10, %	d 10 -36, %	d 0 -36, %
Medium pigs	FIXED	277	353	1.27	0.8	3.5	4.1
	BWAPP	276	355	1.29	0.0	4.1	4.1
Small pigs	FIXED	242	330	1.33	0.8	6.6	7.4
	BWAPP	244	330	1.34	1.7	4.1	5.8
SEM[2]		8.1	9.1	0.020	0.76	1.68	1.82
P value[3]	Strategy	0.979	0.916	0.984	1.000	0.612	0.669
	BW Category	< 0.001	0.014	< 0.001	0.311	0.383	0.204
	Interaction	0.853	0.903	0.453	0.311	0.383	0.669

[1] FIXED: feeding strategy consisting in allowing pigs to consume pre-starter feed until d10 post-weaning; BWAPP: feeding strategy consisting in allowing pigs to consume pre-starter feed until achieving 7.9kg of BW.

[2] Standard error of the mean.

[3] *P*-values come from the T test.

6.4. Discussion

This study aimed to explore the potential advantages of increasing the pre-starter feed allowance until pigs reached a specific BW, as opposed to implementing a fixed-day feed change. However, contrary to the initial hypothesis, the study findings did not demonstrate any enhancement in growth performance when pigs received a higher pre-starter diet allowance until they reached 7.9 kg BW, compared to changing the feed at day 10 post-weaning. The feed change at d10 did lead to a temporary decrease in feed intake and growth for pigs following the fixed strategy. However, pigs on the BWAPP strategy also experienced slight growth retardation after the feed change, although it happened at a higher BW and maturational stage. Consequently, both groups of pigs ended the nursery period with similar BW.

These findings contradict previous studies that reported positive effects on growth or feed efficiency when pigs were offered a high allowance of first-phase diets (A. L. Craig et al., 2020; S. L. Douglas, Wellock, et al., 2014; Huting et al., 2019; Lawlor et al., 2002; Magowan et al., 2011a, 2011b; Muns & Magowan, 2018). However, it should be noted that the weaning weight and age in the present study (4.66 ± 0.74 kg and 21 days of age) were considerably lower than those in the previous studies that examined a similar feeding

strategy (> 7 kg average BW and > 28 days of age at weaning). Younger and smaller pigs are more vulnerable to the challenges of weaning and typically exhibit lower feed consumption, which likely influenced the results (Moeser et al., 2017). Therefore, comparisons must be approached with caution due to this crucial difference. Nevertheless, it is worth mentioning that this study differed from previous research in additional aspects that could have contributed to the divergent outcomes.

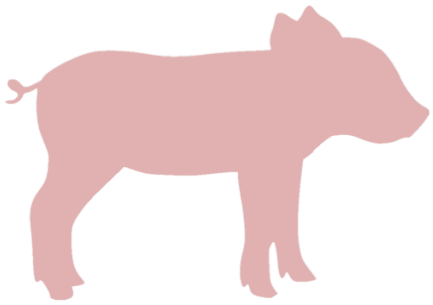
Firstly, most of those previous studies offered an extra 6kg (Lawlor et al., 2002; Magowan et al., 2011a, 2011b; Muns & Magowan, 2018) or 4 kg (A. L. Craig et al., 2020) of the first-phase diets. Meanwhile, in this study, the average difference in pre-starter consumed between the two strategies was 1.18 kg for MEDIUM pigs and 3.23kg for SMALL pigs. Therefore, it is possible the extra amount of pre-starter feed consumed by the pigs in the BWAPP strategy, particularly the MEDIUM pigs, may not be sufficient to determine a discernible effect on growth performance. However, (S. L. Douglas, Wellock, et al., 2014) reported that providing 3 extra kg of a first-phase diet benefited the growth of pigs with a light birth BW. Nevertheless, this effect was only observed when combined with a high-specification diet that exceeded the standard requirements of weaned pigs. Additionally, (Huting et al., 2019) observed a weight × feeding regime interaction effect when they provided an additional 2.7 kg of first-phase feed. Specifically, they observed that light pigs who consumed a larger quantity of first-phase diets were 1.2 kg heavier than the light pigs in the control group. However, this effect was not observed in heavy pigs, indicating that smaller pigs may particularly benefit from this feeding strategy. It is important to note, though, that this was a long-term effect, observed at 7 weeks post-weaning, when the pigs had already reached a weight of approximately 35 kg BW. This timeframe falls beyond the scope of this study's monitoring.

Furthermore, some of the studies that observed significant differences in growth performance employed a strategy that involves providing a higher quantity of the first two post-weaning diets, which are formulated with a high nutritional value, before transitioning to a third diet (grower or weaner diet) with lower energy, protein, and lysine content (A. L. Craig et al., 2020; Magowan et al., 2011a, 2011b; Muns & Magowan, 2018). In these trials,

the differences in growth became noticeable towards the end of the nursery period or even in the long term, after the pigs had already commenced consuming the grower diet. In contrast, the BWAPP strategy implemented in the current trial only extended the provision of the pre-starter diet, without introducing a distinct starter diet with potentially significant differences in nutritional composition. It is possible that if the starter diet had a poorer nutritional value compared to the pre-starter diet, the effects of the BWAPP strategy would have been more pronounced. Additionally, this suggests that including a target BW for the allowance of starter diet has potential as a strategy to reduce the variability in the batch. Future studies should also explore the growth performance of pigs under the BWAPP strategy until the slaughter stage in order to assess any potential long-term effects, as previously observed by other researchers.

6.5. Conclusions

In conclusion, the strategy of providing a higher allowance of pre-starter feed to weaning pigs until they reached a target body weight did not result in any significant effects on growth performance or the variability of the batch under the present conditions (5.4 ± 0.31 kg of initial BW for medium pigs; 4.0 ± 0.21 kg of initial BW for small pigs) and diet specifications (2,544 kcal/kg NE, 18.5% CP and 1.39% SID lysine for pre-starter; 2,458 kcal NE/kg, 17.89% CP and 1.25% SID lysine for starter diet). However, future studies should investigate the potential impact of incorporating a target body weight for the allowance of starter feed and examine the long-term effects of this strategy.



Chapter 8

General Discussion

Slow-growing pigs are a major concern for the porcine industry, as they reduce the efficiency of the production cycle and cause significant economic losses. Over the last few decades, the problem of variability in body weight within the batch has been exacerbated by the genetic selection of sows with higher prolificity. As a result, there is an increasing need to develop practical solutions that help reduce the prevalence of slow-growing pigs in farms and minimize economic losses. The present thesis aimed to address this need by focusing on two main areas of research: investigating early-life factors associated with slow growth and evaluating intervention strategies to enhance microbial colonization, intestinal health, welfare, and growth performance after weaning. The results of the studies performed in the thesis (Chapter 3 to Chapter 7) are discussed in detail in the following pages. The discussion includes the relationships between the various strategies, any limitations of the studies, and potential areas for future research in each of the topics covered.

7.1. Importance of Early-Life Microbial Colonization and Interventions to Modulate It

Early-life gut colonization plays a pivotal role in programming the maturation of the gut, metabolic development, and immune system development (Thompson et al., 2008). In light of the significant impact of microbiota on the strategic developmental processes of pigs, this thesis hypothesized that the early-life gut microbial colonization of piglets would be associated with their individual growth performance, as previously demonstrated in existing literature (Gaukroger et al., 2020; Mach et al., 2015a; Ramayo-Caldas et al., 2016). However, we further proposed that the pig's individual microbiota characteristics and fermentation activity at the end of the nursery period would reflect their growth performance during the lactation and nursery periods.

The study conducted in Chapter 3 showed that pig's growth during both the lactation and the nursery periods influences the structure and composition of its fecal microbiota and the SCFA concentration in their feces at the end of the nursery period.

However, we observed a larger number of differences in the relative abundance of bacteria and in the SCFA concentrations associated with lactation growth than nursery growth. This result is consistent with previous research, which has demonstrated that the microbiota is more malleable during the lactation period than the post-weaning period (Pajarillo et al., 2014; Saladrigas-García, D'Angelo, Ko, Nolis, et al., 2021). Additionally, it also demonstrates that microbial colonization during the lactation period has a crucial influence in shaping the pig adult microbiota, as proposed by previous authors (Nowland et al., 2019). To test this hypothesis, we supplemented low doses (30 to 60 mg/day) of XOS to piglets during the suckling period, as described in Chapter 4. Our intervention resulted in changes in the microbiota composition during the lactation period but also in the nursery period, when all piglets were on the same diet and environment. These results demonstrate that XOS supplementation during lactation can determine the establishment of a differential microbiota population in the post-weaning period. Consequently, these findings support the idea that the lactation phase presents a critical "window of opportunity" when interventions can take advantage of microbiota plasticity and facilitate the establishment of desired microbial populations (Bian et al., 2016; C. Cheng et al., 2018).

The correlation between microbial characteristics and growth rate was found to be generally inconsistent between the lactation and nursery periods. This suggests that certain microbiota features that promote growth during lactation may not have an impact on growth performance post-weaning. Nonetheless, elevated fecal butyrate levels were positively associated with growth during both periods, indicating that promoting the growth of butyrogenic microbiota during early life could redirect the growth trajectory of slow-growing pigs. This finding is in line with previous studies, which suggested that piglets with a more mature microbiota, capable of metabolizing complex carbohydrates found in nursery diets, are less affected by weaning and experience less reduction in growth (Choudhury et al., 2021; Luise et al., 2021; Luo et al., 2022). In Chapter 4, we choose the XOS due to its known effects in promoting the growth of SCFA-producing bacteria (Holman et al., 2017; Mach et al., 2015; Niu et al., 2015; Pajarillo et al., 2014; Saladrigas-García, D'Angelo, Ko, Nolis, et al., 2021; X. Wang et al., 2019). The intervention resulted in an

increase in the growth of fiber-degrading and SCFA-producing microbial populations in the hindgut during both the end of lactation and nursery periods. However, it had no significant effect on the concentration of SCFA in feces or growth performance. One possible explanation for this result is that the observed changes in the microbiota profile were not substantial enough to promote a significant increase in SCFA production and in growth performance. In this scenario, it might be interesting to assess the efficacy of varying dosages of XOS to identify the optimal dose. However, other potential explanations need to be considered.

Although the in vitro fermentation of XOS shows quite consistent improvements in SCFA production (Smiricky-Tjardes et al., 2003; Chen et al., 2020), previous in vivo studies have also struggled to observe these improvements (Bai et al., 2021; Y. Chen et al., 2021; Pan et al., 2019), possibly due to the quick absorption of this metabolites by the intestinal mucosa. Future research should use cecum or intestinal digesta for the determination of SCFA, as they might be more representative of the SCFA production by the microbiota (Den Besten et al., 2013; Nakatani et al., 2018).

On the other hand, the causal relationship between growth performance and increased butyrate production might be wrongly assumed. The microbiota can both cause and be affected by host phenotype, making it difficult to establish causality in observational studies like the one discussed in Chapter 3. The observed increase in butyrate concentration could potentially be attributed to a favorable growth environment, which may have also contributed to good growth performance. However, it remains uncertain whether elevated butyrate levels directly affect growth. To understand their relationship, future studies should control for covariates that could confound the effects, such as colostrum intake, feed intake pre- and post-weaning, environmental microbiota, and pig physiology among others. By collecting this information, we can gain a better understanding of the cause-and-effect relationship between growth and gut microbiota and identify interventions that can improve farm productivity and reduce the prevalence of slow-growing pigs.

The findings of this thesis indicate that an intervention with XOS in the gut microbiota of suckling pigs can modulate the microbiota even beyond weaning, despite not

showing an impact on growth. However, further research is necessary to comprehensively understand the impact of this intervention. This includes assessing its impact on intestinal health and studying the effects over a longer period, beyond the 12 days post-weaning evaluated in the present thesis. Additionally, alternative methods of administering XOS need to be explored, as the oral gavage of XOS is not practical for implementation in commercial farms.

In the literature review of this thesis, we examined several studies that used creep feed and supplementary milk as carriers for prebiotic and stimbiotic administration during the lactation period. While the results of these studies are promising, their effectiveness relies on piglets' consumption of the supplements, which can be highly variable among litters and within littermates. It seems that piglets unable to meet their nutritional needs with maternal milk are more likely to explore alternative sources of nutrients (Middelkoop et al., 2019; Y. J. Miller et al., 2012), which might include slow-growing pigs as they have a competitive disadvantage versus other littermates for the best teats (Huting et al., 2017). Therefore, these strategies would benefit the subset of pigs who need it the most. However, to maximize the number of animals that can benefit from XOS in early life, it is necessary to research ways to incentivize supplement consumption during suckling or develop alternative methods of administering additives during this period.

7.2. Impact of Stress in Growth Performance

Post-weaning gastrointestinal dysfunctions are predominantly attributed to the stress response exhibited by pigs (Moeser et al., 2017). Therefore, the differences in individual piglet adaptation to their new environment and their ability to cope with weaning stress may contribute to growth variability. In Chapter 3, we aimed to establish an association between the pig’s growth and its experienced stress, measured by glucocorticoid biomarkers in hair samples. The experiment revealed that pigs displaying fast growth during both the lactation and nursery periods exhibited higher levels of cortisone in their hair and tended to have a lower cortisol-to-cortisone ratio. This suggests an elevated

activity of the enzyme 11β-HSD type 2 in fast-growing pigs, which converts active cortisol into inactive cortisone. While this enzyme activity is generally considered a biomarker of chronic stress across various species (Vanaelst et al., 2013; Bacci et al., 2014; Stubsjøen et al., 2015), its interpretation lacks consensus, especially in sample types other than hair (Atlaoui et al., 2004; La Marca-Ghaemmaghami et al., 2013; Shimanoe et al., 2021). To enhance interpretation, future research should incorporate behavioral observations and supplementary stress indicators. Nevertheless, if we consider the interpretation that fast-growing pigs experience more chronic stress, one plausible explanation could be that pigs with a higher growth rate tend to consume more feed and, consequently, face greater competition for resources. Simultaneously, this implies that stress experienced during early life does not significantly impair growth performance. This contradicts studies that have shown stress to have detrimental effects on growth and be implicated as a cause of post-weaning intestinal disturbances (Moeser et al., 2017). However, an alternative explanation arises regarding the higher activity of the 11-HSD type 2 in fast-growing pigs. The inactivation of cortisol to cortisone by this enzyme potentially enables these animals to mitigate the adverse effects of cortisol on muscle growth, thereby facilitating their high growth performance even in stressful situations. Nonetheless, additional research is required to validate this hypothesis.

To mitigate stress at weaning and reduce social conflicts, the study in Chapter 5 focused on exploring the advantages of keeping littermates together during the nursery period instead of mixing them. The results indicated only slight variations in growth performance, with the mixed group exhibiting a mere 4% increase in FCR during the nursery phase. Moreover, the study design involved a reduction in nutrient and energy density, but it resulted in comparable growth retardation for both groups. Consequently, the social stress resulting from mixing did not have a significant impact on growth.

However, our findings contradict previous studies that have suggested that the acute and chronic stress experienced by pigs after mixing can compromise their growth performance (Stookey & Gonyou 1994; Hyun, Ellis & Johnson, 1998; Hyun, Ellis, Riskowski, et al., 1998; Coutellier et al., 2007; Camerlink et al., 2021; Camp Montoro et al., 2021; Camp

Montoro et al., 2022). It is possible the fights' intensity and the stress induced by mixing in our study was insufficient to compromise growth performance. Our research design involved regrouping pigs only once after weaning, with these groups maintained until slaughter. However, in commercial farms, pigs are frequently regrouped at different stages, such as the beginning or end of the growing-finishing period, to minimize body weight variability within pens and facilitate truck loading for slaughter. In such scenarios, keeping littermates together would likely offer a significant advantage in terms of growth compared to repeated remixing, considering the intense fighting that occurs each time pigs need to establish a new hierarchy.

Despite the initial reduction achieved in pen variability after segregating pigs based on BW, this effect diminished by the end of the nursery period, in agreement with other authors (O'Quinn et al., 2000). Notably, this effect was not observed in pigs fed a low-nutrient and density diet. However, the implications of this observation will be discussed in the following section. Consequently, the justification for mixing pigs and segregating them by weight to reduce pen variability is lost as long as they are offered a diet that does not restrict the growth potential of the pigs. Segregating pigs by weight may only be meaningful for implementing targeted interventions for slow-growing pigs.

Although the effects of mixing on growth performance were relatively small, the welfare benefits were evident. We observed reduced fighting and stress after weaning keeping littermates together due to the absence of hierarchy reestablishment, along with decreased chronic aggression when we provided a low nutrient and energy density diet. Furthermore, there was less aggression when the pigs were moved to finisher rooms. However, keeping littermates together during the nursery and growing-finishing period is a management practice that is only feasible in farrow-to-finish pig farms with adequate pen sizes. Maintaining the integrity of the litter becomes challenging when pigs need to be transported by truck to other facilities, particularly in farms with separated production stages. In such cases, previous studies have highlighted the importance of minimizing the number of times that the pigs are mixed to enhance their welfare. Additionally, when the pen size is too large to accommodate just one litter, mixing becomes unavoidable. However,

research has shown that keeping the maximum number of littermates together has a positive impact on their growth performance (Jones et al., 2011). Another strategy to reduce the social stress associated with mixing is to introduce early socialization of the piglets combined with enriching the pen while they are still suckling. This approach has been found to provide benefits in terms of both welfare and performance (Ko et al., 2021; Saladrigas-García, D'Angelo, Ko, Traserra, et al., 2021)

7.3. Impact of the Diet

Post-weaning anorexia may play a significant role in mediating the negative effects of weaning stress (Lallès et al., 2004). The implementation of management and nutritional strategies that facilitate the proper adaptation of the digestive system from the sow's milk to the solid-based diet offered after weaning is essential to help alleviate the "growth check" associated with weaning (Blavi et al., 2021; Huting et al., 2021; Pluske, 2016). In this regard, it is necessary to offer a high-quality starter diet containing easily digestible and palatable ingredients (J.-P. Lallès et al., 2007). Furthermore, the incorporation of additives designed to enhance piglet health during this stage may aid in the adaptation process.

Chapter 6 presents two trials that investigated the replacement of soybean ingredients in piglets' pre-starter diet (d0-14 post-weaning) with high-quality, digestible animal protein sources: PDP and SDP. In both trials, substituting soybean ingredients with PDP (2%), SDP (2% or 3%), or a combination of both (1% SDP + 2% PDP) improved piglets' growth performance at the end of the pre-starter period. In trial 2, the animals were followed until the end of the nursery period (d35 post-weaning), and the difference in BW between the animals fed SDP and SDP + PDP was numerically maintained, although it was not statistically significant. Previous reports have also highlighted the growth performance benefits of these two protein sources (Cho et al., 2010; Solà-Oriol et al., 2010; Myers et al., 2014; Pérez-Bosque et al., 2016; Balan et al., 2021). SDP, in particular, is known for its positive impact on intestinal function and immune support due to its various bioactive components, such as immunoglobulins, hormones, growth factors, and bioactive peptides

(Pérez-Bosque et al., 2016). Notably, our study provides novel evidence that PDP may also possess functional properties, as its combination with SDP upregulated several genes associated with gut epithelial structure, intestinal defense, and protein digestion, surpassing the effects of providing SDP alone.

To take advantage of the benefits of supplementing SDP + PDP in the postweaning diet to reduce the variability of the batch, we proposed a feeding strategy to benefit the growth of slow-growing pigs and support their adaptation. Chapter 7 investigated the impact of allowing pigs to consume pre-starter feed containing PDP + SDP until they reach a targeted body weight, as opposed to a fixed date. Accordingly, light pigs at weaning would consume a greater amount of pre-starter feed with a nutritional composition more suitable for their weight and maturity level, thus providing them with the intestinal function benefits observed in the previous study. However, this strategy did not significantly affect growth performance during the nursery period. Although pigs following the body weight approach exhibited a slight growth advantage during the pre-starter phase compared to those switched to a different feed earlier, this disparity vanished after their feed was changed as well. Allowing piglets to consume a feed appropriate for their maturation stage did not confer any growth benefits in our experimental conditions, even for the subset of slow-growing pigs, who consumed an additional 11 days of pre-starter feed and 3.23 kg more pre-starter feed than those in the fixed day strategy.

The findings from Chapters 6 and 7 suggest that the supplementation of PDP and SDPs may hold significance during the initial days post-weaning, supporting the pig's adaptation to solid feed and helping to overcome the transient period of intestinal damage that occurs during weaning. However, once the pig successfully overcomes the adaptation phase, the distinctive effects of these ingredients observed at weaning may diminish.

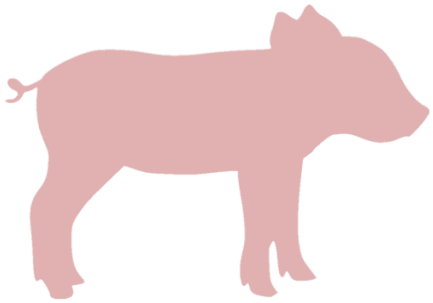
Meanwhile, in the experiment detailed in Chapter 3, reducing 10% of the net energy content, protein, and SID lysine of the diet during the whole nursery period determined significant differences in BW (2.7 kg) at the end of the nursery period that could not be compensated in the growing-finishing period. Additionally, the use of a low-density diet hindered the decrease in the CV of BW observed in groups of littermates compared to the

control diet. This could be attributed to the lighter pigs in the litter pens having a reduced feed intake capacity, thus being unable to consume sufficient nutrients and energy to show comparable growth to their larger counterparts. These findings support the hypothesis that slow-growing pigs may particularly benefit from a high-quality diet with improved digestibility. The divergent outcomes observed in Chapters 3 and 7 can be primarily explained by the magnitude of the differences between the two feeding regimes implemented in each experiment. The dietary regimes in Chapter 3 exhibited greater disparities in energy content (10% difference compared to the 3.4% in the Chapter 7 study) and were administered for a longer duration compared to the additional 4 or 11 days of pre-starter allowance in the body weight approach.

Previous studies that also provided higher allowances of first-phase diets observed a positive effect on pig growth (A. L. Craig et al., 2020; S. L. Douglas, Wellock, et al., 2014; Huting et al., 2019; Lawlor et al., 2002; Magowan et al., 2011a, 2011b; Muns & Magowan, 2018). However, in general, they offered higher quantities of first-phase diets compared to the control feeding regimens than we did and in some cases, the diet specifications were improved (S. L. Douglas, Wellock, et al., 2014). This suggests that the BW approach might help to reduce the BW variability of the batch if it also involves an improvement of the pre-starter energy requirements for the slow-growing pigs or includes a target BW for the starter diet allowance. However, it is worth considering that extending the supplementation of PDP or SDP to pigs may not be cost-effective, and instead, prioritizing the energy content and protein digestibility of the diet should be emphasized to promote the growth of slow-growing pigs.

The implementation of these nutritional strategies has the potential to improve pigs' growth performance and reduce batch variability. However, it is crucial to consider whether the benefits achieved in terms of performance outweigh the increased cost of the feed. The decrease of the nutrient and energy density in the diet performed in the study described in Chapter 3 is a strategy used to reduce the cost of the diet. The magnitude of reduction applied in our study might be too substantial for a commercial setting, as it was intended for research purposes. However, depending on the prevailing market prices of ingredients,

a decrease in dietary costs can potentially offset any productivity losses. In a scenario with low pork meat prices and high ingredient costs, offering a more affordable diet with lower levels of energy and protein can prove to be cost-effective. Nonetheless, our findings indicate that this approach can have adverse effects on the welfare of the animals when the feed is provided in single-space feeders. Therefore, if this strategy is considered appropriate, additional measures such as ensuring sufficient feeder space or providing enrichment materials for the pigs should be implemented to prevent the occurrence of harmful behaviors and ensure the overall welfare of the animals.



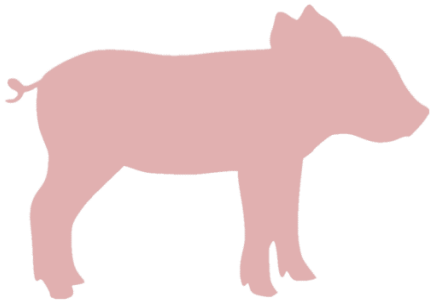
Chapter 8

Conclusions

The results of the studies described in Chapters 3 to 7 led to the following conclusions:

1. Pig's growth during the nursery period and especially the lactation period is associated with differences in fecal microbiota structure, composition, and SCFA concentration in their feces at the end of the nursery period. Specifically, butyrate concentration is positively correlated with growth in both stages.
2. The supplementation of low doses (30 to 60 mg/day) of XOS to suckling piglets stimulates fiber-degrading and SCFA-producing microbial population development in the hindgut. This supplementation can also influence the composition of the microbiota during the post-weaning period, but it has no effect on growth performance.
3. Fast-growing pigs show higher levels of cortisone in their hair and a lower cortisol-to-cortisone ratio at the end of the nursery period, which indicates an elevated 11β-HSD type 2 activity.
4. Keeping littermates together after weaning, instead of mixing them with other litters, has minimal effects on growth performance, resulting in a 4% decrease in FCR. However, this practice reduces fighting and the performance of damaging behavior immediately after weaning. In addition, it decreases the prevalence of chronic aggressions when pigs are provided a low-nutrient and energy-density diet or when animals are moved to the growing-finishing accommodation. Furthermore, mixing pigs and sorting them by BW fails to reduce BW variability within the pen in the long term.
5. A reduction of 10% in the net energy, the protein, and the lysine content of the nursery diets causes a growth retardation that cannot be fully compensated for during the growing-finishing period and increases the BW variability within littermate pens. It also exacerbates the competition for feed and the performance of damaging behaviour when it is provided in a single-space feeder.
6. Including PDP, SDP, or the combination of both in pre-starter feed improves piglets' growth at the end of the pre-starter period. Additionally, the combination of PDP and SDP upregulates the expression of genes associated with intestinal function.

7. Allowing piglets to consume pre-starter feed containing PDP and SDP until reaching a targeted BW (7.9 kg) instead of until a fixed date (d10 post-weaning) does not provide any benefit in growth performance or BW variability at the end of the nursery period.



Chapter 10

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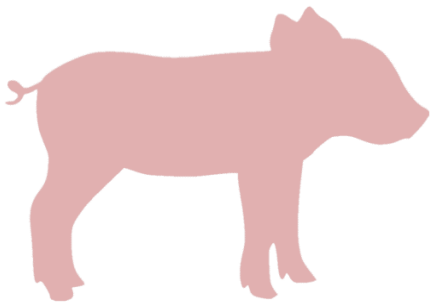

Chapter 11

Scientific Outcomes of this Ph.D. Thesis

10.1. Publications

1. **González-Solé, F.**, Solà-Oriol, D., Pérez, J.F. The allowance of pre-starter diet to nursery pigs until reaching a targeted BW does not affect the body weight variability of the batch at the end of the nursery period. (Under submission preparation)

2. **González-Solé, F.**, Camp Montoro, J., Solà-Oriol, D., Pérez, J. F., Lawlor, P. G., Boyle, L. A., Garcia Manzanilla, E. Effect of mixing at weaning and nutrient density of the weaner diet on growth performance and welfare of pigs to slaughter. Under revision at Porcine Health Management (2023).

3. **González-Solé, F.**, Solà-Oriol, D., Villagómez-Estrada, S., Melo-Durán, D., López, L.V., Villarroel Román, N., López-Arjona, M., Pérez, J.F. Fecal Microbiota and Hair Glucocorticoid Concentration Show Associations with Growth during Early Life in a Pig Model. *Nutrients* 2022; 14(21):4639. https://doi.org/10.3390/nu14214639

4. **González-Solé, F.**, Solà-Oriol, D., Ramayo-Caldas, Y., Rodriguez-Prado, M., González Ortiz, G., Bedford, M.R. & Pérez, J.F. Supplementation of xylo-oligosaccharides to suckling piglets promotes the growth of fiber-degrading gut bacterial populations during the lactation and nursery periods. *Sci Rep* 2022, 12, 11594. https://doi.org/10.1038/s41598-022-15963-4

5. Villagómez-Estrada, S., Pérez, J. F., Melo-Durán, D., **Gonzalez-Solè, F.**, D'Angelo, M., Pérez-Cano, F. J., and Solà-Oriol, D. Body weight of newborn and suckling piglets affects their intestinal gene expression. *J Anim Sci*, 2022; 100(6): skac161. https://doi.org/10.1093/jas/skac161

6. Forouzandeh, A., Blavi, L., Pérez, J. F., D'Angelo, M., González-Solé, F., Monteiro, A., Stein, H. H., and Solà-Oriol, D. (2022). How copper can impact pig growth: comparing the effect of copper sulfate and monovalent copper oxide on oxidative status, inflammation, gene abundance, and microbial modulation as potential mechanisms of action. *J Anim Sci,* 2022, 100(9), skac224. https://doi.org/10.1093/jas/skac224

7. Villagómez-Estrada, S., Pérez, J. F., van Kuijk, S., Melo-Durán, D., Forouzandeh, A., **Gonzalez-Solè, F**., D'Angelo, M., Pérez-Cano, F. J. and Solà-Oriol, D. Strategies of inorganic and organic trace mineral supplementation in gestating hyperprolific sow diets: effects on the offspring performance and fetal programming. *J Anim Sci,* 2021 July; 99(7): skab178. https://doi.org/10.1093/jas/skab178

8. Criado-Mesas, L., Abdelli, N., Noce, A. Farré, M., Pérez, J. F., Solà-Oriol, D., Martin-Venegas, R., Forouzandeh, A., **González-Solé, F**. and Folch, J. M. Transversal gene expression panel to evaluate intestinal health in broiler chickens in different challenging conditions. *Sci Rep*, 2021 Apr; 11:6315. https://doi.org/10.1038/s41598-021-85872-5

9. Crenshaw, J., del Río, L.L., Sanjoaquin, L., Simon, T., **González-Solé, F.**, Solà-Oriol, D., Rodríguez, C., Campbell, J. and Polo, J. Effect of spray-dried porcine plasma in peripartum sow feed on subsequent litter size. *Porc Health Manag, 2021;* 7:11. https://doi.org/10.1186/s40813-020-00180-0://doi.org/10.1038/s41598-021-85872-5

10. **González-Solé, F.**, Criado-Mesas, L., Villodre, C., García, W. C., Farré, M., Borda, E., Pérez-Cano F.J., Folch, J. M., Solà-Oriol, D. and Pérez, J. F. Porcine digestible peptides (PDP) in weanling diets regulates the expression of genes involved in gut barrier function, immune response and nutrient transport in nursery pigs. *Animals*, 2020; 10(12): 2368. https://doi.org/10.3390/ani10122368

10.2. Conference Oral Presentations

1. **González-Solé, F.**, Camp Montoro, J., Solà-Oriol, D., Pérez, J. F., Lawlor, P. G., Boyle, L. A., Garcia Manzanilla, E. Effect of mixing at weaning and dietary nutrient density on growth performance and welfare of pigs. ASAS Midwest Section Meeting Madison, USA, 12-15 th March of 2023.

2. **González-Solé F.,** Solà-Oriol, D., Villagómez-Estrada, D. Melo-Durán, López, L.V., Villarroel Román, N., Gasa, J., Pérez, J.F. Piglets with a differential growth response during lactation and/or the nursery period also show differences on hair glucocorticoid concentration and faecal microbiota. 7th EAAP International Symposium on Energy and Protein Metabolism and Nutrition (ISEP 2022). Granada, Spain, 12-15th September of 2022.

3. **González-Solé, F.**, Solà-Oriol, D., Ramayo-Caldas, Y., González Ortiz, G., Bedford, M. R and Pérez J. F . La suplementación de xilooligosacáridos a lechones lactantes promueve el crecimiento de bacterias degradadoras de fibra y productoras de AGCC en el intestino durante los períodos de lactación y transición. 15th Meeting of the Red Española de Bacterias Lácticas (REDBAL 2022). Valencia, Spain, 26-27 th May of 2022

10.3. Conference Poster Presentations

1. **González-Solé, F.**, Solà-Oriol, D., Ramayo-Caldas, Y., González Ortiz, G., Bedford, M. R and Pérez J. F. Supplementation of xylo-oligosaccharides to suckling piglets modulates their hindgut microbial populations during the lactation and nursery periods. 15th International Symposium of Digestive Physiology of Pigs. Rotterdam, Netherlands, 17-20 th May of 2022.