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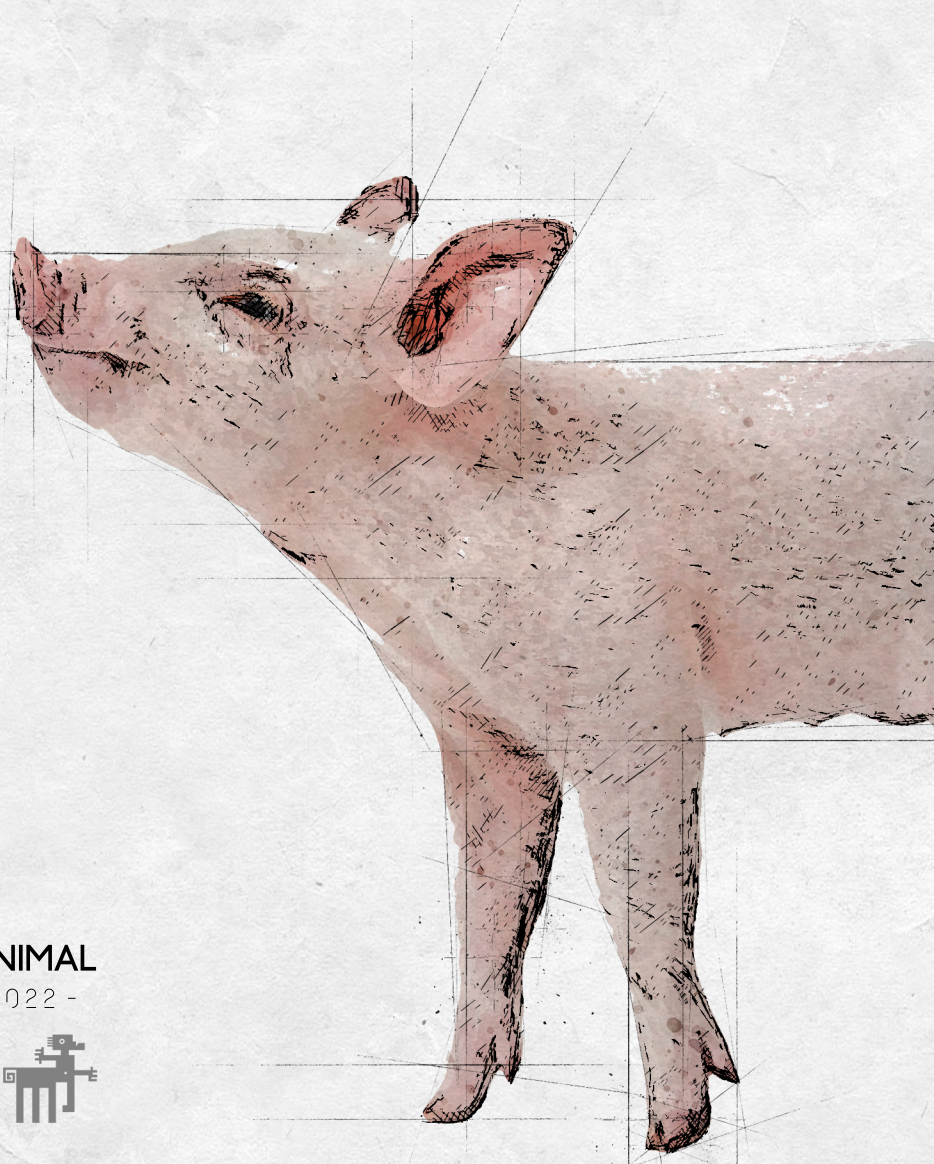

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Development of intestinal microbiota in the commercial pig and early intervention strategies to improve this process

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FACULTAT DE VETERINÀRIA

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Certifican:

Que la memoria titulada **"Development of intestinal microbiota in the comercial pig and early intervention strategies to improve this process",** presentada por Mireia Saladrigas García con la finalidad de optar al grado de Doctor en Veterinaria, ha sido realizada bajo su dirección y, considerándola finalizada, autorizan su presentación para que sea juzgada por la comisión correspondiente.

Y para que conste a efectos oportunos, firman la presente en Bellaterra, 1 de febrero de 2022.

Dra. Susana María Martín Orúe

Dra. Matilde D'Angelo

"A promise made must be a promise kept"
Aristotle

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"It has long been an axiom of mine that the little things are infinitely the most important"
Sir Arthur Conan Doyle, The Memoirs of Sherlock Holmes.

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Summary

Among the multitude of challenges the pig has to cope with after birth, weaning is the most critical period with frequent clinical episodes and therapeutic use of antibiotics. Improving the adaptation of the animals during this period would reduce mortality rates and variability of batches and would also contribute to reducing the risk of antimicrobial resistance. In short, manipulating the intestinal microbiota is undoubtedly a strategy to consider in pig production to improve the health, well-being, and productivity of the animals. The greater knowledge of the pig microbiota is allowing the design of different intervention strategies that seek to facilitate the establishment of a robust intestinal microbiota with a positive impact on the immune response, physiology, and animal metabolism. These strategies include dietary manipulation, the inclusion of functional ingredients and/or food additives, or the incorporation of new management guidelines on the farm, mainly at an early age.

It, therefore, seemed opportune to conduct a comprehensive study proposing strategies that allow us to modulate the development of the intestinal microbiota of pigs to improve their health and productive performance. The present doctoral thesis aims to focus on those early events that occur in the first days of life of the piglets that could determine significant changes in the performance of the animals in the following stages of life. Moreover, the project aims to explore specific applications in the commercial practice addressed to improve the health and productivity of pigs and to reduce the use of antibiotics.

In **Chapter 4** the development of piglet gut microbiota from birth to weaning in the commercial practice was explored. Moreover, how different farm environments could condition this process was also assessed. To achieve this, two trials were performed. In Trial 1, two farms were selected, and an intensive fecal sampling (5 time points/animal) was performed. In Trial 2, four farms were selected, and a simplified sampling pattern (2 time points/animal) was performed. The results showed that alpha diversity was strongly affected by age, with an increased richness of species through time. Beta diversity

decreased after weaning, suggesting a convergent evolvement among individuals. Regarding the structure of the microbiota, a clear clustering of the samples according to age was observed. The early intestinal colonizers were found to belong to *Bacteroides*, *Escherichia-Shigella*, *Clostridium sensu stricto 1*, and *Fusobacterium* genera. During lactation, the higher relative abundances of *Bacteroides* and *Lactobacillus* genera were correlated with a milk-oriented microbiome. As the piglets aged and after weaning, increasing abundances of genera such as *Prevotella*, *Butyricimonas*, *Christensenellaceae* R-7 group, *Dorea*, *Phascolarctobacterium*, *Rikenellaceae* RC9 gut group, *Subdoligranulum*, and *Ruminococcaceae UCG-002* were observed. These changes demonstrated the adaptation of the piglets to a cereal-based diet rich in oligosaccharides and starch. On the other hand, the farm environment was shown to have an impact on biodiversity and specific taxa, evidencing the influence of different environments and rearing systems on the gut microbiota development in the young piglet. In Trial 1 piglets receiving intramuscular amoxicillin (days 2-5 of life) and being offered an acidifying rehydrating solution (Alpha farm) showed a greater alpha diversity and increased *Lactobacillus* counts throughout the study. Differences related to farms were more noticeable after weaning than during lactation. In Trial 2, the only farm that did not offer an acidified rehydrating solution showed a lower alpha diversity (day 2 of life) and increased abundance of *Enterobacteriaceae* (both at 2 and 21 days). The use of in-feed antibiotics in the sows was also associated with changes in several taxonomic piglet microbial groups.

In **Chapter 5**, the effect of the commercial early weaning on gut microbiota, intestinal gene expression, and serum metabolomic response was assessed via an integrated -omic approach combining 16S rRNA gene sequencing, the OpenArray gene expression technology, and ^{1}H-NMR spectroscopy. For that, a total of 14 piglets from different litters were sampled for blood, jejunum tissue and cecal content two days before, and three days after weaning. The results showed a clearly differential ordination of the cecal microbiota. Moreover, higher abundances of *Roseburia*, *Ruminococcus*, *Coprococcus*, *Dorea* and *Lachnospira* genera were observed in weaned piglets compared to suckling piglets, evidencing the quick microbial changes of the piglets' gut microbiota. The downregulation of *OCLN*, *CLDN4*, *MUC2*, *MUC13*, *SLC15A1*, and *SLC13A1* genes, also evidenced the negative impact of weaning on the gut barrier and digestive functions. The metabolomic approach pinpointed sig-

nificant decreases in choline, LDL, triglycerides, fatty acids, alanine, and isoleucine and increases in 3-hydroxybutyrate after weaning. Moreover, the correlation between microbiota and metabolome datasets revealed the existence of metabolic clusters interrelated to different bacterial clusters.

Probiotics have been largely used in the swine industry due to their capacity to promote the healthy growth of pigs. Moreover, different probiotic strains when administered to sows during gestation and/or lactation have been shown to have positive effects on both sow and piglet performance. In **Chapter 6**, the effect of long-term administration of two Bacillus strains ($5x10^8$ cfu/kg feed of *Bacillus subtilis* or *Bacillus amyloliquefaciens*) was tested on 98 breeding sows and their litters during three complete reproductive cycles. The results showed a significant increase in the total born piglets in both supplemented groups, although no significant differences were observed on sow's feed intake, body weight, or fat mobilization. *Bacillus amyloliquefaciens* supplementation also increased the number of born-alive and weaned piglets. Moreover, Nuclear Magnetic Resonance analysis showed an impact of *Bacillus amyloliquefaciens* on milk composition. A different fecal microbiota structure in supplemented sows was found, with changes at phylum, family, and genus level that were more marked at day 8 after farrowing, suggesting a relevant role of probiotics reestablishing a new intestinal balance after labor. Microbial shifts were also seen in the piglets, with a clearer impact post-weaning than in lactation. In this regard, correlations between microbial groups of sows and piglets showed a higher link with weaned (d33) than with lactating pigs (d21) reinforcing the idea of an early mother imprinting whose effect lasts beyond weaning.

Finally, it has been suggested that animals reared in an environment that enables the expression of play behavior are better prepared to cope with unfavorable situations at a later stage of life, such as weaning stress. In **Chapter 7** the possible ability of early socialization and an enriched neonatal environment to improve the adaptation of piglets to weaning was assessed. A total of 24 sows and their litters were allotted into an enriched treatment in which piglets from two adjacent pens were combined from day 14 of life and farrowing crates were enriched with toys since birth. The results showed a differential ordination of the cecal microbiota after weaning. Moreover, the serum metabolome suggested a reduced energetic metabolism in enriched

animals, as evidenced by shifts in triglycerides and fatty acids, VLDL/LDL, and creatine regions. The *TLR2* gene showed to be downregulated in the jejunum of enriched pigs after weaning. The integration of gene expression, metabolome, and microbiota datasets confirmed that differences between barren and enriched neonatal environments were evident only after weaning, showing that improvements in adaptation to weaning could be mediated by a better response to the post-weaning stress.

Resumen

Entre la multitud de desafíos que el cerdo tiene que enfrentar después del nacimiento, el destete es el período más crítico, con episodios clínicos frecuentes y uso terapéutico de antibióticos. Mejorar la adaptación de los animales durante este período reduciría las tasas de mortalidad y la variabilidad de los lotes y también contribuiría a reducir el riesgo de resistencia a los antimicrobianos. En definitiva, la manipulación de la microbiota intestinal es sin duda una estrategia a tener en cuenta en la producción porcina para mejorar la salud, el bienestar y la productividad de los animales. Un mayor conocimiento de la microbiota porcina está permitiendo el diseño de diferentes estrategias de intervención que buscan facilitar el establecimiento de una microbiota intestinal robusta con un impacto positivo en la respuesta inmune, la fisiología y el metabolismo animal. Estas estrategias incluyen la manipulación dietética, la inclusión de ingredientes funcionales y/o aditivos alimentarios, o la incorporación de nuevas pautas de manejo en la granja, principalmente a una edad temprana.

En este contexto, era oportuno realizar un estudio exhaustivo proponiendo estrategias que permitieran modular el desarrollo de la microbiota intestinal de los cerdos para mejorar su salud y rendimiento productivo. La presente tesis doctoral tiene como objetivo centrarse en aquellos eventos tempranos que ocurren en los primeros días de vida de los lechones que podrían determinar cambios significativos en el rendimiento de los animales en las siguientes etapas de la vida. Además, el proyecto tiene como objetivo explorar aplicaciones específicas en la práctica comercial dirigidas a mejorar la salud y la productividad de los cerdos y reducir el uso de antibióticos.

En el **Capítulo 4** se exploró el desarrollo de la microbiota intestinal de los lechones desde el nacimiento hasta el destete en la práctica comercial. Además, también se evaluó cómo los diferentes entornos de diferentes granjas podrían condicionar este proceso. Para lograr esto, se realizaron dos ensayos. En el Ensayo 1, se seleccionaron dos granjas y se realizó un muestreo fecal intensivo (5 puntos de tiempo/animal). En el Ensayo 2, se seleccionaron cuatro granjas y se realizó un patrón de muestreo simplificado (2 puntos de

tiempo/animal). Los resultados mostraron que la diversidad alfa se vio fuertemente afectada por la edad, con una mayor riqueza de especies a través del tiempo. La diversidad beta disminuyó después del destete, lo que sugiere una evolución convergente entre los individuos. En cuanto a la estructura de la microbiota, se observó una clara agrupación de las muestras según la edad. Se encontró que los primeros colonizadores intestinales pertenecían a los géneros *Bacteroides*, *Escherichia-Shigella*, *Clostridium sensu stricto 1* y *Fusobacterium*. Durante la lactancia, las mayores abundancias relativas de los géneros *Bacteroides* y *Lactobacillus* se correlacionaron con un microbioma orientado a la leche. A medida que los lechones envejecieron y después del destete, se observaron abundancias crecientes de géneros como *Prevotella*, *Butyricimonas*, *Christensenellaceae R-7 group*, *Dorea*, *Phascolarctobacterium*, *Rikenellaceae RC9 gut group*, *Subdoligranulum* y *Ruminococcaceae UCG-002*. Estos cambios demostraron la adaptación de los lechones a una dieta basada en cereales rica en oligosacáridos y almidón. Por otro lado, se demostró que el entorno de la granja tiene un impacto en la biodiversidad y los taxones específicos, evidenciando la influencia de diferentes ambientes y sistemas de cría en el desarrollo de la microbiota intestinal en el lechón joven. En el Ensayo 1, los lechones que recibieron amoxicilina intramuscular (días 2-5 de vida) y se les ofreció una solución rehidratante acidificante (granja Alpha) mostraron una mayor diversidad alfa y un aumento de los recuentos de *Lactobacillus* a lo largo del estudio. Las diferencias relacionadas con las granjas fueron más notables después del destete que durante la lactancia. En el Ensayo 2, la única granja que no ofreció una solución rehidratante acidificada mostró una menor diversidad alfa (día 2 de vida) y una mayor abundancia de *Enterobacteriaceae* (tanto a los 2 como a los 21 días). El uso de antibióticos en el alimento en las cerdas también se asoció con cambios en varios grupos microbianos taxonómicos de los lechones.

En el **Capítulo 5** se evaluó el efecto del destete temprano comercial sobre la microbiota intestinal, la expresión génica intestinal y la respuesta metabolómica sérica a través de un enfoque ómico integrado que combina la secuenciación del gen 16S rRNA, la tecnología de expresión génica OpenArray y la espectroscopia ^{1}H-NMR. Para ello, se tomaron muestras de sangre, tejido de yeyuno y contenido cecal de un total de 14 lechones de diferentes camadas dos días antes y tres días después del destete. Los resultados mostraron una agrupación claramente diferencial de la microbiota cecal. Además, se

observaron mayores abundancias de los géneros *Roseburia*, *Ruminococcus*, *Coprococcus*, *Dorea* y *Lachnospira* en lechones destetados en comparación con lechones lactantes, lo que evidencia los rápidos cambios microbianos de la microbiota intestinal de los lechones. La regulación a la baja de los genes *OCLN*, *CLDN4*, *MUC2*, *MUC13*, *SLC15A1* y *SLC13A1*, también evidenció el impacto negativo del destete en la barrera intestinal y las funciones digestivas. El enfoque metabolómico identificó disminuciones significativas en colina, LDL, triglicéridos, ácidos grasos, alanina e isoleucina y aumentos en el 3-hidroxibutirato después del destete. Además, la correlación entre la microbiota y los conjuntos de datos de metaboloma reveló la existencia de grupos metabólicos interrelacionados con diferentes grupos bacterianos.

Los probióticos se han utilizado en gran medida en la industria porcina debido a su capacidad para promover el crecimiento saludable de los cerdos. Además, se ha demostrado que diferentes cepas probióticas cuando se administran a las cerdas durante la gestación y / o la lactancia tienen efectos positivos tanto en el rendimiento de la cerda como en el de los lechones. En el **Capítulo 6**, se probó el efecto de la administración a largo plazo de dos cepas de *Bacillus spp.* ($5x10^8$ ufc/kg de alimento de *Bacillus subtilis* o *Bacillus amylo-liquefaciens*) en 98 cerdas reproductoras y sus camadas durante tres ciclos reproductivos completos. Los resultados mostraron un aumento significativo en el total de lechones nacidos en ambos grupos suplementados, aunque no se observaron diferencias significativas en la ingesta de alimento de la cerda, el peso corporal o la movilización de grasa. La suplementación con *Bacillus amylo-liquefaciens* también aumentó el número de lechones nacidos vivos y destetados. Además, el análisis de Resonancia Magnética Nuclear mostró un impacto de *Bacillus amyloliquefaciens* en la composición de la leche. Se encontró una estructura diferente de la microbiota fecal en cerdas suplementadas, con cambios a nivel de filo, familia y género que fueron más marcados en el día 8 después del parto, lo que sugiere un papel relevante de los probióticos en el restablecimiento de un nuevo equilibrio intestinal después del parto. También se observaron cambios microbianos en los lechones, con un impacto más claro después del destete que en la lactancia. En este sentido, las correlaciones entre los grupos microbianos de cerdas y lechones mostraron un mayor vínculo con los destetados (d33) que con los cerdos lactantes (d21), reforzando la idea de una huella temprana de la madre cuyo efecto dura más allá del destete.

Finalmente, se ha sugerido que los animales criados en un entorno que permite la expresión del comportamiento de juego están mejor preparados para hacer frente a situaciones desfavorables en una etapa posterior de la vida, como el estrés por destete. En el **Capítulo 7** se evaluó la posible capacidad de socialización temprana y un ambiente neonatal enriquecido para mejorar la adaptación de los lechones al destete. Un total de 24 cerdas y sus camadas fueron asignadas a un tratamiento enriquecido en el que los lechones de dos corrales adyacentes se combinaron desde el día 14 de vida y los boxes de maternidad se enriquecieron con juguetes desde el nacimiento. Los resultados mostraron una ordenación diferencial de la microbiota cecal después del destete. Además, el metaboloma sérico sugirió un metabolismo energético reducido en animales enriquecidos, como lo demuestran los cambios en las regiones de triglicéridos y ácidos grasos, VLDL / LDL y creatina. El gen *TLR2* demostró estar regulado a la baja en el yeyuno de los cerdos enriquecidos después del destete. La integración de los conjuntos de datos de expresión génica, metaboloma y microbiota confirmó que las diferencias entre ambientes neonatales vacíos y enriquecidos eran evidentes solo después del destete, lo que demuestra que la mejora en la adaptación al destete podría estar mediada por una mejor respuesta al estrés posterior al destete.

Resum

Entre la multitud de desafiaments que el porc ha d'enfrontar després del naixement, el deslletament és el període més crític, amb episodis clínics freqüents i l'ús terapèutic d'antibiòtics. Millorar l'adaptació dels animals durant aquest període reduiria les taxes de mortalitat i la variabilitat dels lots i també contribuiria a reduir el risc de resistència als antimicrobians. En definitiva, la manipulació de la microbiota intestinal és sens dubte una estratègia que cal tenir en compte en la producció porcina per millorar la salut, el benestar i la productivitat dels animals. Un coneixement més gran de la microbiota porcina permet el disseny de diferents estratègies d'intervenció que busquen facilitar l'establiment d'una microbiota intestinal robusta amb un impacte positiu en la resposta immune, la fisiologia i el metabolisme animal. Aquestes estratègies inclouen la manipulació dietètica, la inclusió d'ingredients funcionals i/o additius alimentaris, o la incorporació de noves pautes de maneig a la granja, principalment a una edat primerenca.

En aquest context, era oportú dur a terme un estudi exhaustiu proposant estratègies que permetessin modular el desenvolupament de la microbiota intestinal dels porcs per millorar la seva salut i rendiment productiu. Aquesta tesi doctoral té com a objectiu centrar-se en aquells esdeveniments primerencs que ocorren en els primers dies de vida dels garrins que podrien determinar canvis significatius en el rendiment dels animals en les etapes de la vida futures. A més, el projecte té com a objectiu explorar aplicacions específiques a la pràctica comercial dirigides a millorar la salut i la productivitat dels porcs i reduir l'ús d'antibiòtics.

Al **Capítol 4** es va explorar el desenvolupament de la microbiota intestinal dels garrins des del naixement fins al deslletament a la pràctica comercial. A més, també es va avaluar com els diferents entorns de diferents granges podrien condicionar aquest procés. Per aconseguir això, es van fer dos assajos. A l'Assaig 1, es van seleccionar dues granges i es va realitzar un mostreig fecal intensiu (5 punts de temps/animal). A l'Assaig 2, es van seleccionar quatre granges i es va realitzar un patró de mostreig simplificat (2 punts de temps/animal). Els resultats van mostrar que la diversitat alfa es va veure

fortament afectada per l'edat, amb més riquesa d'espècies a través del temps. La diversitat beta va disminuir després del deslletament, cosa que suggereix una evolució convergent entre els individus. Quant a l'estructura de la microbiota, es va observar una agrupació clara de les mostres segons l'edat. Es va trobar que els primers colonitzadors intestinals pertanyien als gèneres *Bacteroides*, *Escherichia-Shigella*, *Clostridium sensu stricto 1* i *Fusobacterium*. Durant la lactància, les majors abundàncies relatives dels gèneres *Bacteroides* i *Lactobacillus* es van correlacionar amb un microbioma orientat a la llet. A mesura que els garrins van créixer i després del deslletament, es van observar abundàncies creixents de gèneres com *Prevotella*, *Butyricimonas*, *Christensenellaceae R-7 group*, *Dorea*, *Phascolarctobacterium*, *Rikenellaceae RC9 gut group*, *Subdoligranulum* i *Ruminococcaceae UCG-002*. Aquests canvis demostren l'adaptació dels garrins a una dieta basada en cereals rica en oligosacàrids i midó. D'altra banda, es va demostrar que l'entorn de la granja té un impacte en la biodiversitat i taxons específics, evidenciant la influència de diferents ambients i sistemes de cria en el desenvolupament de la microbiota intestinal del garrí jove. A l'Assaig 1, els garrins que van rebre amoxicil·lina intramuscular (dies 2-5 de vida) i se'ls va oferir una solució rehidratant acidificant (granja Alpha) van mostrar una major diversitat alfa i un augment dels recomptes de *Lactobacillus* al llarg de l'estudi. Les diferències relacionades amb les granges van ser més notables després del deslletament que durant la lactància. A l'Assaig 2, l'única granja que no va oferir una solució rehidratant acidificada va mostrar una menor diversitat alfa (dia 2 de vida) i més abundància d'*Enterobacteriaceae* (tant als 2 com als 21 dies). L'ús d'antibiòtics a l'aliment a les truges també es va associar amb canvis en diversos grups taxonòmics microbians en els garrins.

Al **Capítol 5** es va avaluar l'efecte del deslletament primerenc comercial sobre la microbiota intestinal, l'expressió gènica intestinal i la resposta metabolòmica sèrica mitjançant un enfocament òmic integrat que combina la seqüenciació del gen 16S rRNA, la tecnologia d'expressió gènica OpenArray i la espectroscòpia ^{1}H-NMR. Per fer-ho, es van prendre mostres de sang, teixit de jejú i contingut cecal d'un total de 14 garrins de diferents garrinades dos dies abans i tres dies després del deslletament. Els resultats van mostrar una agrupació clarament diferencial de la microbiota cecal. A més, es van observar majors abundàncies dels gèneres *Roseburia*, *Ruminococcus*, *Coprococcus*, *Dorea* i *Lachnospira* en garrins deslletats en comparació amb garrins lactants,

la qual cosa evidencia la rapidesa dels canvis de la microbiota intestinal dels garrins. La regulació a la baixa dels gens *OCLN*, *CLDN4*, *MUC2*, *MUC13*, *SLC15A1* i *SLC13A1*, també va evidenciar l'impacte negatiu del deslletament a la barrera intestinal i les funcions digestives. L'enfocament metabolòmic va identificar disminucions significatives en colina, LDL, triglicèrids, àcids grassos, alanina i isoleucina i augments en el 3-hidroxibutirat després del deslletament. A més, la correlació entre la microbiota i els conjunts de dades de metaboloma sèric va revelar l'existència de grups metabòlics inter-relacionats amb diferents grups bacterians.

Els probiòtics s'han utilitzat en gran mesura a la indústria porcina a causa de la seva capacitat per promoure el creixement saludable dels porcs. A més, s'ha demostrat que diferents soques probiòtiques quan s'administren a les truges durant la gestació i/o la lactància tenen efectes positius tant en el rendiment de la truja com en el dels garrins. Al **Capítol 6**, es va provar l'efecte de l'administració a llarg termini de dos soques de *Bacillus spp.* ($5x10^8$ ufc/kg d'aliment de *Bacillus subtilis* o *Bacillus amyloliquefaciens*) en 98 truges reproductores i les seves garrinades durant tres cicles reproductius complets. Els resultats van mostrar un augment significatiu en el total de garrins nascuts en ambdós grups suplementats, encara que no es van observar diferències significatives en la ingesta d'aliment de la truja, el pes corporal o la mobilització de greix. La suplementació amb *Bacillus amyloliquefaciens* també va augmentar el nombre de garrins nascuts vius i deslletats. A més, l'anàlisi de Ressonància Magnètica Nuclear va mostrar un impacte de *Bacillus amyloliquefaciens* a la composició de la llet. Es va trobar una estructura diferent de la microbiota fecal en truges suplementades, amb canvis a nivell de fílum, família i gènere que van ser més marcats al dia 8 després del part, cosa que suggereix un paper rellevant dels probiòtics en el restabliment d'un nou equilibri intestinal després del part. També es van observar canvis microbians als garrins, amb un impacte més clar després del deslletament que a la lactància. En aquest sentit, les correlacions entre els grups microbians de truges i garrins van mostrar un vincle més gran amb els deslletats (d33) que amb els porcs lactants (d21), reforçant la idea d'una empremta primerenca de la mare, l'efecte de la qual dura més enllà del deslletament.

Finalment, s'ha suggerit que els animals criats en un entorn que permet l'expressió del comportament de joc estan més ben preparats per fer front a

situacions desfavorables en una etapa posterior de la vida, com l'estrès per deslletament. Al **Capítol 7** es va avaluar la possible capacitat de socialització primerenca i un ambient neonatal enriquit per millorar l'adaptació dels garrins al deslletament. Un total de 24 truges i les seves garrinades van ser assignades a un tractament enriquit en què els garrins de dos corrals adjacents es van combinar des del dia 14 de vida i els boxes de maternitat es van enriquir amb joguines des del naixement. Els resultats van mostrar una ordenació diferencial de la microbiota cecal després del deslletament. A més, el metaboloma sèric va suggerir un metabolisme energètic reduït en animals enriquits, com ho demostren els canvis a les regions de triglicèrids i àcids grassos, VLDL/LDL i creatina. El gen *TLR2* va demostrar estar regulat a la baixa al jejú dels porcs enriquits després del deslletament. La integració dels conjunts de dades d'expressió gènica, metaboloma i microbiota va confirmar que les diferències entre ambients neonatals buits i enriquits eren evidents només després del deslletament, cosa que demostra que la millora en l'adaptació al deslletament podria estar mediada per una millor resposta a l'estrès posterior al deslletament.

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List of abbreviations and acronyms

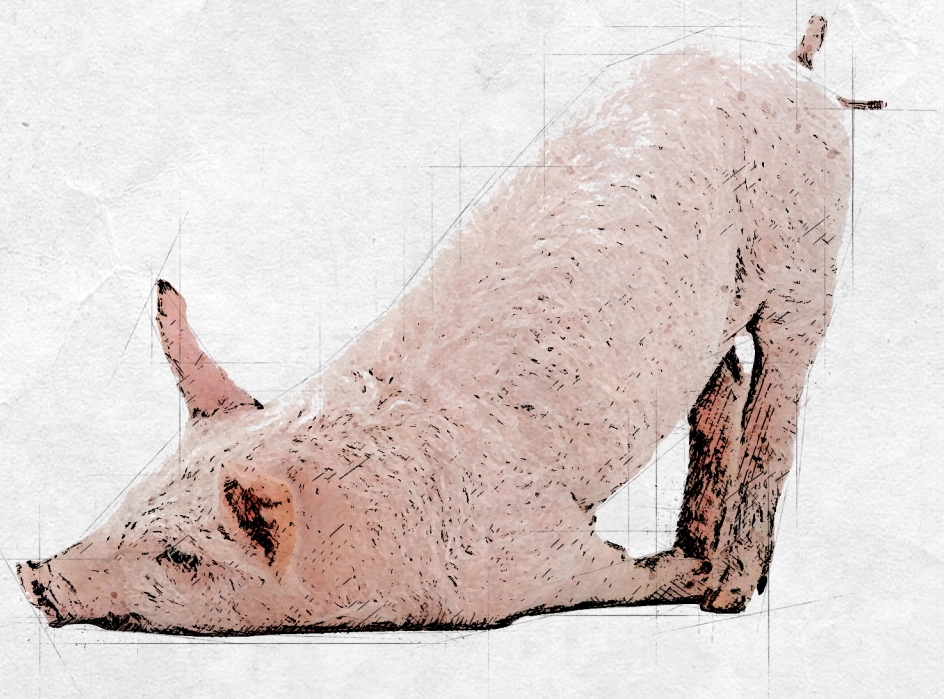
AA	Amino acids
ADFI	Average daily feed intake
ADG	Average daily gain
ADP	Adenosine diphosphate
ANOSIM	Analysis of similarities
ANOVA	Analysis of variance
APP	*Actinobacillus pleuropneumoniae*
ARRIVE	Animal Research: Reporting of In Vivo Experiments
ASV	Amplicon sequence variant
ATP	Adenosine triphosphate
AUC	Area under the curve
BCFA	Branched-chain fatty acids
BW	Body weight
CCK	Cholecystokinin
cDNA	Complementary deoxyribonucleic acid
CFU	Colony-forming unit
CLDN4	Claudin-4
CLDN15	Claudin-15
CP	Crude protein
CSS	Cumulative sum scaling
DADA2	Divisive amplicon denoising algorithm 2
DFM	Direct-fed microbials
DNA	Deoxyribonucleic acid
DOHaD	Developmental origins of health and disease
EFSA	European Food Safety Authority
ELISA	Enzyme-linked immunosorbent assay
EMA	European Medicines Agency
ETEC	Enterotoxigenic *Escherichia coli*

EU	European Union
FDR	False discovery rate
^{1}H-NMR	Proton nuclear magnetic resonance
HNMT	Histamine N-Methyltransferase
HSP27	Heat shock protein 27
HSPB1	Heat shock protein family B member 1
HTS	High-throughput sequencing
IFNγR1	Interferon γ receptor 1
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IGF1R	Insulin-like growth factor 1 receptor
IL	Interleukin
IL-17A	Interleukin-17A
IL-23	Interleukin-23
IL-33	Interleukin-33
IUGR	Intrauterine growth restricted
KEGG	Kyoto Encyclopedia of Genes and Genomes
LAB	Lactic acid bacteria
LBW	Low birth weight
LDL	Low-density lipoprotein
LPS	Lipopolysaccharide
MHA	Methionine hydroxy analogue
MUC2	Mucin-2
MUC13	Mucin-13
NCBI	National Center for Biotechnology Information
NMDS	Non-metric multidimensional scaling
NMR	Nuclear magnetic resonance
NRC	National Research Council
OCLN	Occludin
OPLS-DA	Orthogonal partial least squares discrimination analysis
OTU	Operational taxonomic units

PCA	Principal components analysis
PCR	Polymerase chain reaction
PERMANOVA	Permutational multivariate analysis of variance
PICRUSt	Phylogenetic Investigation of Communities by Reconstruction of Unobserved States
PLS-DA	Partial least-squares discriminant analysis
PPARGC1α	Peroxisome proliferator-activated receptor gamma coactivator 1-α
PRRS	Porcine Reproductive and Respiratory Syndrome
QIIME	Quantitative Insights Into Microbial Ecology
RA	Relative abundance
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
ROC	Receiver operating characteristic
RT-PCR	Quantitative reverse transcription polymerase chain reaction
SCFA	Short-chain fatty acids
SEM	Standard error of the mean
SI	*Sucrase-Isomaltase*
SID	Standardized ileal digestible
SLC15A1	Solute Carrier Family 15 Member 1
SLC13A1	Solute Carrier Family 13 Member 1
STTD	Standardized total tract digestible
TLR2	Toll like receptor 2
TMAO	trimethylamine-N-oxide
TSP	Sodium-3´-trimethylsilylpropionate-2,2,3,3-d4
UPGMA	Unweighted pair-wise grouping method with hierarchical arithmetic mean grouping
VIP	Variable importance in projection
VLDL	Very low-density lipoprotein
VMP	Veterinary medicinal products
ZnO	Zinc oxide

Chapter 1

General introduction



General introduction

The swine industry is of crucial importance in the Spanish economy. Within livestock productions, pig production ranks first in terms of its economic significance. Spain is the country of the European Union (EU) with the largest pig population (see **Figure 1.1**) and the second, behind Germany, in pork production (see **Figure 1.2**). In fact, Spain is the fourth largest producer worldwide (after China, the US, and Germany). In recent years, the swine industry has grown notably, both in production, in censuses and in the number of farms, thanks to the push from foreign markets supported, in turn, by the competitiveness of the sector in the world market. Likewise, both global pork production and global pork consumption are expected to increase by 2030 (OECD and Food and Agriculture Organization of the United Nations, 2021).

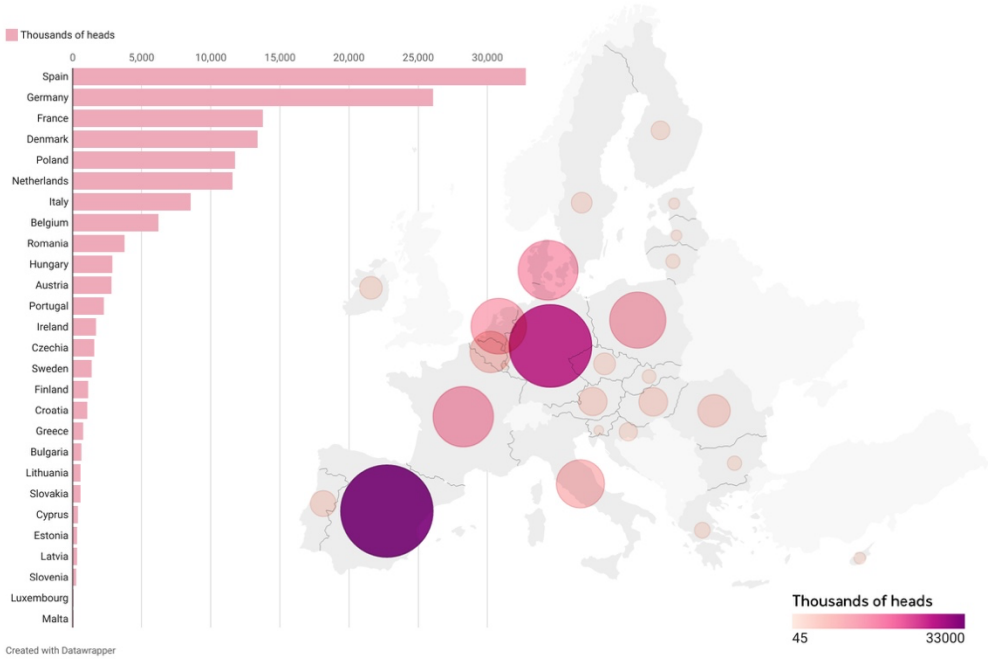


Figure 1.1. EU-27 pig population (annual data from 2020). Source: Eurostat Online Database at https://ec.europa.eu/eurostat/data/database.

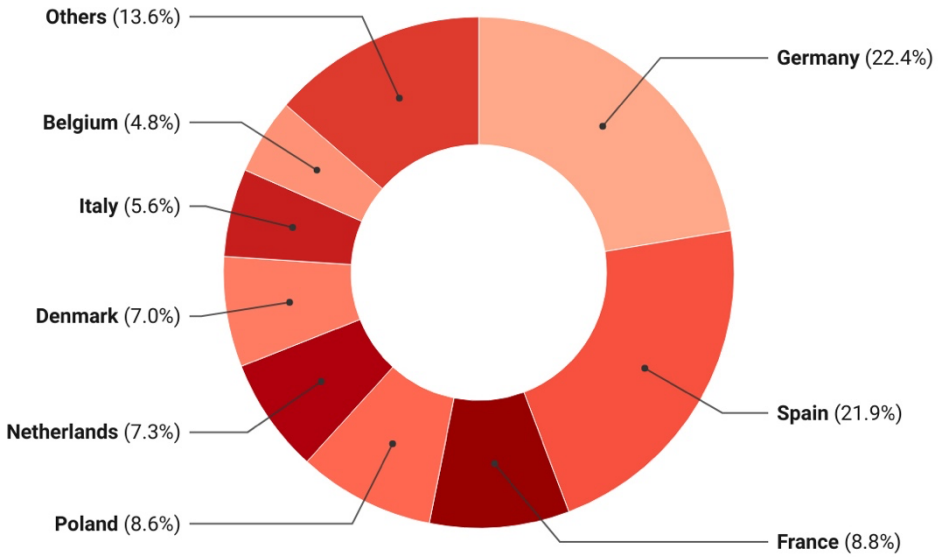


Figure 1.2. EU-27 meat production (annual data from 2020). Source: Eurostat Online Database at https://ec.europa.eu/eurostat/data/database.

Pig production is nowadays characterized by being one of the most technified and intensive livestock activities. It aims to achieve higher number of piglets per sow per year and a more efficient fattening of the pigs through the continuous efforts to improve genetics, handling and feeding practices. However, there are numerous clinical episodes or sanitary conditions that compromise productive yields and determine a high use of antibiotics in feed.

Spain is the seventh country with the highest sales of veterinary antimicrobial agents for food-producing animals among the European countries (ESVAC 11th Report, 2021), being the swine industry the livestock sector that consumes the highest amount of antibiotics. Antibiotic growth promoters such as tylosin, bacitracin, virginiamycin, and chlortetracycline have been used in piglets to promote growth performance through increased feed conversion and weight gain that leads to healthier animals (Kim *et al.*, 2012). However, there are increasing concerns related to the development of antibiotic-resistant bacterial strains and antibiotic residues in meat products and animal feces (Juntunen *et al.*, 2010; Holman and Chénier, 2015). Moreover, the potential adverse impact of these antibiotic resistance genes on human health has been

increasing. Despite the fact that since 2006, in Spain, as in the rest of the European Union, antibiotics cannot be used for growth promotion (Regulation (EC) N° 1831/2003), under EU law, antibiotics can still be used for routine disease prevention. This means, for example, that it remains legal for a prescription to be written for mass medication of animals via feed or drinking water. However, in recent years, driven by the urgency of a reduction in the use of antimicrobials in livestock, several European regulations have been drawn up in order to guarantee the highest level of protection for public health, animal health and the environment. Among them, Regulation (EU) 2019/6, on veterinary drugs, and Regulation (EU) 2019/4, on the manufacture, marketing and use of medicated feed, by which veterinary drugs and medicated feed may only be used with veterinary prescription. In this context, the Veterinary Medicines Regulation allows farm antibiotic use for metaphylaxis only when the risk of spread of an infection or of an infectious disease in the group of animals is high and where no other appropriate alternatives are available and, therefore, routinely administration and prophylaxis use (with few exceptions) are then forbidden. Moreover, in addition to the ban of antibiotic use as growth promoters, the European Medicines Agency (EMA) concluded in 2017 that the overall benefits of zinc oxide (ZnO) for the prevention of diarrhea in piglets do not outweigh the risks for the environment, withdrawing marketing authorizations for ZnO-based veterinary medicinal products (VMP) across the European Union by June 2022. Therefore, it is essential to develop alternative methods to diminish or eliminate the therapeutic use of antibiotics and ZnO from food production, both due to the European regulatory framework and due to social (consumer perception) and environmental (contamination and emergence of antibiotic resistances) aspects.

In this sense, the trajectory of our research group (SNiBA) has been focused for years in the best approach to reinforce the natural defenses of the animals in front of pathogens, creating a healthier and more productive microbial ecosystem as mean to prevent the abusive use of antibiotics against the digestive disorders that might appear in commercial practice. Over the past few years, several strategies have been investigated to smooth the weaning period and to improve feed intake and growth of pigs, from new probiotic, prebiotic and symbiotic treatments to short- and medium-chain fatty acids as feed additives for the promotion of gut health in weanling pigs. However, until

now most of the research to improve the adaptation of the piglet to weaning has been focused on the post-weaning period. The window of late gestation and/or lactation could be seen as an opportunity to provide the animal with adequate epigenomic programming and particularly an appropriate modulation of the immune system.

Until recently, it was considered that mammals are primarily determined by DNA sequence and its linear translation into RNA and protein (known as the Central Dogma of molecular biology). However, this conception has been superseded by the "fluid genome" by which the animal organism is interconnected with its environment through different cycles of epigenomic programming and reprogramming (Shenderov and Midtvedt, 2014). These processes are especially decisive in the first stages of life and thus in the human species it is said that the first 1000 days of life are the most important stage of our existence. During the maturation process, which leads to the definitive establishment of the indigenous microbiota, there is a sequence of substitution of some microbial groups for others. This colonization pattern is not arbitrary, and each stage of this process plays a fundamental role in the interactions established with the host genome (Lewis *et al.*, 2012). Therefore, the process of microbial colonization of the intestine after birth plays a crucial role in the development of the neonatal immune system of mammals with implications throughout their lives (Hansen *et al.*, 2012; Houghteling and Walker, 2014). An adequate colonization maintains the homeostasis of the immune system and directly influences the probability of the development of pathologies in the future, such as, for example, diarrhea from post-weaning syndrome.

Early microbial colonization results from factors such as genetics, microbial exposure, both to the mother and the environment, and the use of antibiotics. This, in turn, sets off the crosstalk between the microbiome and the host mediated by changes in nutrition, immunity, barrier function, metabolism, and gene expression (**Figure 1.3**). The discovery of these complex communication mechanisms between the microbiota and the host, and the identification of positive scenarios that may contribute to reducing the use of antibiotics theoretically provide the basis for future therapeutic opportunities.

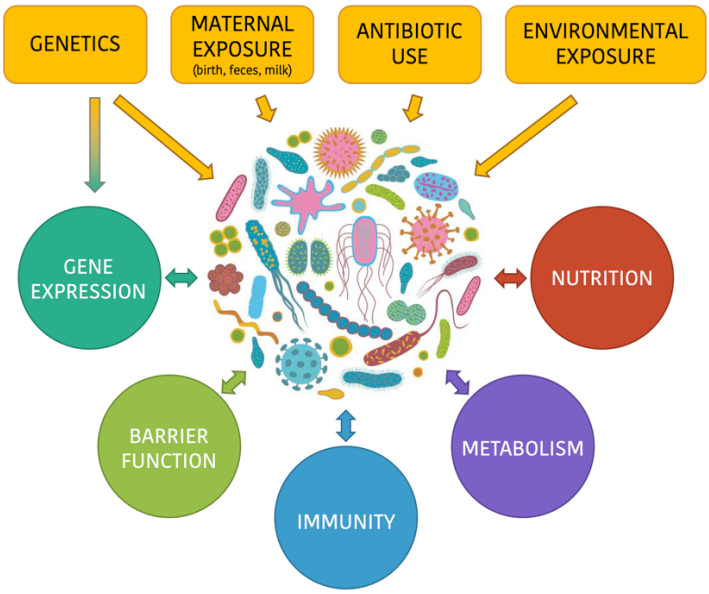


Figure 1.3. Diagram of the crosstalk between the microbiome and intestinal homeostasis. Adapted from Houghteling *et al.* (2014).

Among the multitude of challenges the pig has to cope with after birth, weaning is the most critical period with frequent clinical episodes and therapeutic use of antibiotics. Improving the adaptation of the animals during this period would reduce mortality rates and variability of batches. This would also contribute to reducing the risk of antimicrobial resistance. In short, manipulating the intestinal microbiota is undoubtedly a strategy to consider in pig production in order to improve the health, well-being, and productivity of the animals. The greater knowledge of the pig microbiota is allowing the design of different intervention strategies that seek to facilitate the establishment of a robust intestinal microbiota with a positive impact on the immune response, physiology, and animal metabolism. These strategies include dietary manipulation, the inclusion of functional ingredients and/or food additives, or the incorporation of new management guidelines on the farm, mainly at an early age.

It, therefore, seems opportune to conduct a comprehensive study proposing strategies that allow us to modulate the development of the intestinal microbiota of pigs in order to improve their health and productive

performance. The present project (AGL2016-75463-R), funded by the Ministry of Economy, Industry and Competitiveness (MINECO) of Spain within the framework of Proyectos I+D+I Convocatoria RETOS 2016 and the State Plan for Scientific and Technical Research and Innovation, aims to a more in-deep study of those early events that occur in the first days of live of the piglets that could determine significant changes in the performance of the animals in the following stages of life. Moreover, the project aims to explore specific applications in the commercial practice addressed to improve the health and productivity of pigs and to reduce the use of antibiotics.

Chapter 2

Literature review

Literature review

2.1. Early-life development of the newborn piglet

It is becoming more and more evident that the development and productive future of an animal is determined from its earliest stage in the mother's womb. Research in evolutionary biology, developmental biology, and animal physiology suggests that environmental processes influencing the susceptibility to disease in adulthood operate during the periconceptional, fetal, and early phases of life. This "developmental origins of health and disease" (DOHaD) hypothesis is based on the definition of developmental plasticity – the ability of an organism to change its structure and function during critical time windows in response to environmental stimuli, a process that then becomes irreversible (Gluckman and Hanson, 2004).

> "The plasticity of development decreases with age (Hanson and Gluckman, 2014). Thus, not only is future development often most susceptible to events that occur earlier in development, but the greatest benefits from interventions are likely to occur when these are applied as early as possible."

Gestation length in sows generally lasts for 114 to 116 days, with 10% of sows farrowing before 114 days and 25% of sows farrowing after 116 days of gestation (Vanderhaeghe *et al.*, 2011). During this time, fetal programming can promote lifelong changes in the function of various organ systems by inducing integrated adjustments in the mature phenotype, a process underpinned by epigenetic mechanisms and influenced by prediction of the mature environment (Gluckman, Hanson and Beedle, 2007). These changes, in turn, provide a basis for chronic diseases later in life. Therefore, the risk of developing some diseases in adulthood is influenced not only by genetic and adult lifestyle factors but also by environmental factors acting in early life. Experimental studies in animals have documented many examples of fetal programming, with recent studies showing that alterations in maternal nutrition during gestation can have long-term effects on the offspring

(Godfrey and Barker, 2001). For example, sow nutrition has been shown to affect the development of fetal skeletal muscle and adipose tissue, which exert long-term effects on the growth performance and meat quality of offspring (Du *et al.*, 2015). Similarly, dietary supplementation of arginine throughout gestation resulted in a 24% increase in live litter weight in pigs (Mateo *et al.*, 2007), and supplementation of arginine to lactating sows increased piglet body weight gain by 11% through d 21 postpartum (Mateo *et al.*, 2008). Therefore, in addition to genetic background, proper fetal development is also important to maximize the health and growth potential of animals. However, the maternal impact on offspring growth and development is not only limited to the fetal stage, and increasing evidence points to the importance of lactation on the offspring. Maternal high energy intake during lactation affects milk composition, which has a long-term effect on the offspring's metabolism (Vogt *et al.*, 2014). Moreover, maternal milk composition is altered by maternal diet, providing another opportunity to modulate neonatal nutrition and the transference of passive immunity and thus, offspring development and subsequent productive performance.

One way to intervene in the development of the newborn piglet is through changes in the composition of the intestinal microbiota, which exerts a direct influence on the host's health. (Fouhse, Zijlstra and Willing, 2016; Guevarra *et al.*, 2019; Patil, Gooneratne and Ju, 2020; Wang *et al.*, 2020; Duarte and Kim, 2021). The gut microbial ecosystem is fundamental due to its role in promoting intestinal maturation, immune system modulation, and consequently the enhancement of the health and growth performance of the pig. The intestinal microbiota protects against colonization by pathogens by bacterial competition and interaction (H. Y. Cheng *et al.*, 2019). The disruption of the healthy microbial community during the neonatal period may lead to the overgrowth of indigenous pathobionts and induction of pro-inflammatory status (Mulder *et al.*, 2009; Schmidt *et al.*, 2011). Therefore, given the importance of the critical period offered by the gestation and lactation of the sow as well as the first days of the piglet's life and given the impact of the intestinal microbiota on the development and health of the individual, it is essential to investigate the process of microbial colonization that occurs in commercial pig practice, as well as those intervention strategies that could allow improving the performance and welfare of both sows and their litters.

2.1.1. Maternal imprinting and acquisition of the gut microbiome after birth

The process of microbial colonization of the gut after birth plays an important role in the development of the neonatal immune system of mammals with implications during their whole life (Hansen *et al.*, 2012; Houghteling and Walker, 2014; Nowland *et al.*, 2019). The structure and composition of the gut microbiota in animals are determined by many factors, including genetics, age, breed, diet, and the surrounding environmental conditions during birth. In piglets, initial exposure is believed to occur during passage through the birth canal (Houghteling and Walker, 2014; Patil, Gooneratne and Ju, 2020), along which they also encounter maternal intestinal bacteria (Makino *et al.*, 2013). However, recent studies have suggested that the microbial contact of the offspring with their mothers may begin already before birth (Jiménez *et al.*, 2008; Mshvildadze *et al.*, 2010; Aagaard *et al.*, 2014) and imply the existence of bacterial communities in the placenta, amniotic fluid, and meconium of healthy pregnancies (Perez-Muñoz *et al.*, 2017). These findings have led many scientists to challenge the "sterile uterus paradigm" and propose that the acquisition of the microbiome begins in the uterus, an idea that would fundamentally change the understanding of the acquisition of the gut microbiota. Therefore, maternal influences on fetal gut colonization should be considered as the first stage of intestinal microbiota development. Up to now, little is known about the maternal influences on fetal gut colonization. Studies so far have only shown that the maternal status during pregnancy is related to the intestinal microbiota development after birth. In humans, mothers' body mass index, weight, and weight gain during pregnancy can influence the composition and development of infant gut microbiota after birth (Collado *et al.*, 2008). Fecal Bacteroides and Staphylococcus concentrations were significantly higher in infants of overweight mothers during the first six months, whereas the abundance of bacteria related to Akkermansia muciniphila, Staphylococcus, and Clostridium difficile was lower in infants of normal-weight mothers and of mothers with normal weight gain during pregnancy (Collado *et al.*, 2010).

Immediately after birth, environmental and maternal bacteria, including colonization via the vagina, nipple surface, and milk, quickly colonize offspring gut and establish the initial microbiota of the piglet (Konstantinov *et al.*, 2006; Jost *et al.*, 2014; Xue Chen *et al.*, 2018). The mother's fecal, vaginal

and nipple microbiota probably represent the most influential inoculum source for the development of the offspring microbiota, due to intimate contacts during and after birth (Xue Chen *et al.*, 2018). In this context, the entero-mammary pathway has been also suggested as one of the possible routes of microbial transfer from the maternal gut to the mammary gland and, consequently, to the newborn piglet (Selvamani *et al.*, 2021). As a matter of fact, breast milk in humans has been shown to be capable of delivering certain intestinal microorganisms from the mother to the offspring using the entero-mammary route (Rodríguez, 2014). As reviewed by Barba-Vidal, Martín-Orúe and Castillejos (2019), *Lactobacillus* species and even gut-associated obligate anaerobes such as *Bifidobacterium* breve have been reported to be efficiently delivered through this pathway. However, the entero-mammary route in pigs has hardly been studied in the literature and there are very few studies that propose it, and they focus particularly on the possible transfer of IgA and probiotics through milk (Salmon, 2002; Zanello *et al.*, 2013; Zhang *et al.*, 2018). Therefore, to our knowledge, no direct entero-mammary pathway has been described yet in the swine species.

"The initial intestinal microbial colonization of the newborn piglet occurs from the environment at birth (Xue Chen *et al.*, 2018), being the mother the main donor (birth canal, feces, milk, surface of the nipple)."

The first colonizers in the immature intestine, between birth and 2 days of life, are facultative anaerobes, *Enterobacteriaceae*, *Enterococci*, and *Staphylococci*, and then anaerobes such as *Bifidobacteria*, *Bacteroides*, and *Clostridia* enter the immature intestine and settle (Patil, Gooneratne and Ju, 2020). Some gut-associated microbiota, such as *Bifidobacterium*, *Bacteroides*, *Parabacteroides*, and members of the clostridial class (*Blautia*, *Clostridium*, *Collinsella*, and *Veillonella*) are shared among maternal feces, breast milk, and neonatal feces, meaning that milk maternal gut can be one of the important vehicles in the vertical transfer of the maternal gut microbiota to the newborn (Xue Chen *et al.*, 2018). Lactose, which is a major sugar present in milk, acts as a prebiotic and can elicit the development of a highly diverse microbiota in the prenatal gastrointestinal tract of growing animals (Call *et al.*, 2018). As previously explained, several studies have shown the gastrointestinal tract of the early

postnatal pig to be colonized predominantly by *Clostrideaceae* and *Enterobacteriaceae* species (Inoue *et al*., 2005; Konstantinov *et al*., 2006). Petri, Hill and Van Kessel (2010), further estimated that 34% of the total microbial population present at 6h of age is from the family *Clostridiaceae*, which is seen to reduce to 1% by 20 days. Moreover, a secondary colonization of predominantly *Lactobacillaceae* species has been described beginning at 3 days of age in consistency with previous reports (Inoue *et al*., 2005; Konstantinov *et al*., 2006; Petri, Hill and Van Kessel, 2010). Interestingly, *Streptococcaceae* were identified 6 h after birth and appeared to predominate all locations of the gastrointestinal tract briefly between 1 and 3 days of age before displacement by *Lactobacillaceae* and *Clostridiaceae*. As stated, in the first 5 days after birth, the microbial community is dominated by strict aerobes and facultative anaerobes, which are gradually replaced almost entirely by strict anaerobes (starting from day 7 up to day 22) (Inoue *et al*., 2005). However, a high level of individuality has been reported to occur in 1- and 2-week-old piglets, indicating that there is considerable randomness to the process of acquiring microbes (Thompson, Wang and Holmes, 2008). Although this gut community in very young piglets might be highly dynamic, the microbial community is known to stabilize by day 28. The development of major immune system induction elements occurs approximately 2 weeks after birth, and by 4 weeks significant concentrations of IgA are evident (Inoue *et al*., 2005). The first significant change in intestinal microbial diversity occurs in piglets on days 4–7 when the number of *Clostridium perfringens* organisms declines due to the activity of IgA inherited from the mother. Hence, one of the first significant changes in gut microbiota occurs in piglets during their first weeks of life, when the number of *Clostridium* and *Escherichia/Shigella* organisms declines due to the activity of IgA. Decreases in the abundances of *Clostridium*, *Fusobacterium,* and *Escherichia-Shigella* with the age of the piglets have also been observed by several other authors (Pajarillo *et al*., 2014; Frese *et al*., 2015; Mach *et al*., 2015; Chen *et al*., 2017; Luise, Le Sciellour, *et al*., 2021). A steady increase in *Enterobacteriaceae* has been described from weaning (approximately 28 days) to 5 days post-weaning; however, they are seen to significantly decline after day 11 post-weaning (Dou *et al*., 2017).

As the piglets grow, the intestinal microbiome rapidly undergoes a remarkable shift from the initial microbial groups to the establishment of an

adult-like microbial community, experiencing in between a period of microbial successions (Isaacson and Kim, 2012; Pajarillo *et al.,* 2014; Guevarra *et al.,* 2019). In this sense, aging has been associated with the increased abundance of *Firmicutes* and the decreased abundance of *Proteobacteria*, *Fusobacteria,* and *Actinobacteria (Slifierz, Friendship and Weese, 2015; Chen et al., 2017).* In commercial pig husbandry, the abrupt dietary shift from sow milk to solid-feed-based diets poses a challenge to piglets during early-life development. During the pre-weaning phase, microbiome composition is dominated by a milk-oriented microbiome composed of families like *Bacteroidaceae* and *Lactobacillaceae* (Frese *et al*., 2015), which rapidly change after weaning. For instance, the butyrate-producing genera such as *Prevotella*, having a very low abundance in suckling piglets, dramatically increase post-weaning due to the availability of complex oligosaccharides and polysaccharides in the feed (Frese *et al*., 2015; Mach *et al*., 2015; Zhao *et al*., 2018). The rapidly changing microbiome of the young piglets seems to increase in microbial diversity and richness in the suckling phase and gradually stabilizes postweaning (Petri, Hill and Van Kessel, 2010; Kim *et al*., 2011; Frese *et al*., 2015; Slifierz, Friendship and Weese, 2015; Chen *et al*., 2017). The changes in the gut microbiota of piglets produced around weaning are discussed in more detail in section 2.2., dedicated to the impact of weaning in the young piglet and the role of the intestinal microbiota.

"The initial pattern of microbial maturation is essential as the gut microbiota is fundamental for adequate development and programming of the mucosal immune response (Schokker *et al*., 2015)."

As stated above, the mother's microbiota plays an important role in the development of the gut microbiota of offspring. Thus, factors that affect the maternal gut microbiota during delivery and lactation should also be considered. In this regard, subtherapeutic doses of antibiotics in pigs can adversely affect the microbiota, whereas the inclusion of probiotics, prebiotics, and fiber appear to negate these effects and enhance diversity (Holman and Chénier, 2014; Patil, Gooneratne and Ju, 2020). On the one hand, the use of antibiotics is a two-edged weapon, as it destroys both pathological and

beneficial microbes indiscriminately, allowing loss of gut microbiota or the so-called dysbiosis (Neuman *et al.*, 2018; Kelly *et al.*, 2021). Maternal antibiotic treatment has been a subject of study especially in the field of human medicine. It has been recently found that antibiotic use during pregnancy alters the commensal vaginal microbiota of women (Stokholm *et al.*, 2014). Moreover, amoxicillin administration to sows during late gestation has been reported to impact both, sow vaginal and fecal microbial diversities (Arnal *et al.*, 2014; de Greeff *et al.*, 2020). Together, these results indicate that maternal antibiotic treatment may influence the gut microbial colonization of offspring through changing maternal microbiota composition. In addition, it is well known that maternal antibiotic residues can be transferred from mothers to their offspring via breastfeeding (Mathew, 2004). However, it is unclear to what extent the maternal antibiotic treatment affects the intestinal microbiota development in the offspring. To date, very few studies have analyzed the effect of the administration of antibiotics in the mothers in their offspring, and there are especially few studies focused on the effects produced in their intestinal microbiota. In pigs, Arnal *et al.*, (2014) reported a significant effect on the microbiota of the small intestine of the offspring but not of the colon. Moreover, some effects of maternal antibiotic treatment on the gut physiology and morphology of the offspring have been seen in early-life (Arnal *et al.*, 2014, 2015; Lin *et al.*, 2018; de Greeff *et al.*, 2020; Trevisi *et al.*, 2021). On the other hand, the use of probiotics emerged as a promising strategy to improve sow reproductive performance by increasing feed consumption along with lactation, reducing fat mobilization, promoting milk production, and increasing litter weight (Alexopoulos *et al.*, 2004; Böhmer, Kramer and Roth-Maier, 2006; Kritas *et al.*, 2015; Hayakawa *et al.*, 2016; Zhang *et al.*, 2020). Several studies have also evidenced that when probiotics are administered to sows, positive effects can be also seen in the performance of piglets, with increases in growth rates (Kritas *et al.*, 2015; Betancur *et al.*, 2021; Crespo-Piazuelo *et al.*, 2021) and reduction in the clinical signs of post-weaning diarrhea (Alexopoulos *et al.*, 2004; Taras *et al.*, 2005, 2006; Betancur *et al.*, 2021). Moreover, probiotics have been demonstrated to be transferred from the mother to the piglet through contact with maternal feces (Jadamus, Vahjen and Simon, 2001; Kenny *et al.*, 2011). The possible effects derived from probiotic supplementation are discussed in more detail in section 2.3.2., dedicated to the use of probiotics as an early intervention strategy. In addition to probiotics, many other strategies have been studied by

supplementing the sows with functional ingredients and/or zootechnical additives modulators of the intestinal microbiota such as phytogenic compounds, prebiotics (fibrous ingredients and non-digestible oligosaccharides) or symbiotics, among others (Roca *et al.*, 2014; Patil, Gooneratne and Ju, 2020). For example, among the most recent studies, phytogenic additive supplementation has been shown to increase litter size while modifying the fecal microbiota of sows and their offspring (Nowland, Stanley, *et al.*, 2021). Hall, Wilkinson and Le Bon (2021), reported that sows supplemented with oregano essential oil during lactation showed an increase in the relative abundance of *Lactobacillaceae* family, as well as an increase in *Fibrobacteriaceae* and *Akkermansiaceae* families, important for fiber digestion. On the other hand, and concerning fiber intake, dietary inulin (1.6%) has been reported to regulate sow gut microbiota by decreasing *Cyanobacteria* and *Streptococcus* counts, increasing at the same time the relative abundance of *Desulfovibrio*, CF231, and *Prevotella*, among others (H. Li *et al.*, 2021). In another study, other fibrous ingredients were studied such as alfalfa meal, beet pulp and soybean skin (Boshuai *et al.*, 2022). As a result, the increased dietary fiber affected gut microbiota by increasing the relative abundance of proinflammatory bacteria, while decreasing anti-inflammatory bacteria. Moreover, the total short-chain fatty acid and the butyric acid content was higher in sows during lactation. In short, the interaction of dietary additives and gut microbes can produce microbiota shifts and bioactive metabolites, which are of great significance to sows' metabolism and reproductive performance, as well as piglet health and performance.

Apart from the mother's microbiota, the early exposure to microbes in the surrounding environment of newborns could also be a strong influencing factor in the development of the intestinal microbiota. In addition to animal age and genetic background (Kubasova *et al.*, 2018), the structure and activity of gut microbiota can differ between animals depending on various other factors including dietary influence (Frese *et al.*, 2015; Everaert *et al.*, 2017; Le Sciellour *et al.*, 2018), rearing farm (Vigors, O' Doherty and Sweeney, 2020; Luise, Le Sciellour, *et al.,* 2021), sanitary status (Bearson *et al.,* 2013), antimicrobial use (Bosi *et al.*, 2011; Looft *et al.*, 2014; Mu *et al.,* 2017; Soler *et al.,* 2018) and husbandry practices (Wen *et al.*, 2021), among others. Some studies have shown that the surrounding environment during postnatal development can have a long-term impact on gut community structure

(Thompson, Wang and Holmes, 2008). Different early-life environments were shown to be associated with major differences in mucosa-adherent microbial diversity in the ileum of adult pigs (Mulder *et al.*, 2009). The raising environment highly contributed to the microbial distribution in piglets at an early age. Pigs housed in a natural outdoor environment showed a dominance of Firmicutes, in particular *Lactobacillus*, whereas pigs housed in a hygienic indoor environment had reduced Lactobacillus and higher numbers of potentially pathogenic phylotypes (Mulder *et al.*, 2009). In this regard, the hygienic characteristics of the housing environment might also play an important role in shaping the gut microbiota. This has been demonstrated by comparing the gut microbiota of piglets exposed to soil (Zhou *et al.*, 2016; Vo *et al.*, 2017) or raised in disparate environments (Mulder *et al.*, 2009). Moreover, excessive hygienic conditions and limited microbial exposure during early life have shown significant impairment in the microbiota of adult pigs (Schmidt *et al.*, 2011). Therefore, aside from the maternal factor, the surrounding environments also have a huge influence on the development of the gut microbiota in newborn piglets.

Likewise, it has been shown that stress during the early-life period may also induce a long-lasting impact on the establishment of gut microbiota, gut biology, disease susceptibility, and growth performances of offspring pigs (Moeser *et al.*, 2007; Schokker *et al.*, 2014, 2015; Chen *et al.*, 2017; Moeser, Pohl and Rajput, 2017; Mu *et al.*, 2017; Y. Li, Guo, *et al.*, 2018). This is especially relevant in swine production in which differences in husbandry along the first days of life could have a long-lasting impact on animal health and productive outcome. For instance, some authors have described how different exposure to stress, or the use of antibiotics can determine changes in the gut microbial colonization of piglets 8 days after birth with implications in the immune development (Schokker *et al.*, 2014). Evidence has also been published defining differences in the fecal microbiota of piglets of as early as 7 days of life, determining their susceptibility to suffering post-weaning diarrhea four weeks later (Dou *et al.*, 2017). Similarly, different stressors such as high temperatures, transportation, weaning, and overcrowding can also worsen the diversity of the microbiota (Patil, Gooneratne and Ju, 2020).

Finally, and as previously described, antibiotic treatment is a common disturbance that impacts the intestinal microbiota, both in the mother and in

the piglet. Correspondingly, antibiotic administration at an early age also influences the colonization process of the microbiota in the gut of young piglets. Moreover, in commercial practice piglets are frequently exposed to antibiotics early in life primarily to prevent outbreaks of intestinal and respiratory diseases. It is for this reason that several studies have been conducted to assess the effects of early-life administration of antibiotics on the intestinal microbiota of piglets. For example, in newborn piglets, a single parenteral dose of long-lasting amoxicillin at the first day of life was reported to cause a significant decrease of fecal microbiota diversity for at least five weeks after administration (Janczyk *et al.*, 2007). Likewise, a single dose of tulathromycin administered to 4-day old piglets was able to modulate the gut microbiota for a prolonged period, as reported by Schokker *et al.* (2014). In that case, the antibiotic treatment increased the relative abundance of anaerobic bacteria including *Bifidobacterium*, *Eubacterium*, *Faecalibacterium prausnitzii*, and *Solobacterium moorei*, and decreased the relative abundance of facultative bacteria such as *Staphylococcus aureus*. Moreover, in mice, neonatal amoxicillin treatment significantly altered the levels of *Lactobacilli* and led to a significant impact on the diversity of the intestinal *Lactobacillus* community (Yuan *et al.*, 2010). These studies demonstrate how antibiotics may shape the intestinal microbiota of young piglet, and strongly suggest a link between antibiotic supplementation and gut microbiota dysbiosis. Because the intestinal microbiota at an early age is more dynamic and less resilient than that of adults (Choudhury, Middelkoop, Boekhorst, *et al.*, 2021), antibiotic-induced patterns at early age, and their impact later in life would deserve more studies.

2.1.2. The role of nutrients on early development of the gastrointestinal system

Once the piglet is born, the gastrointestinal must adapt quickly to the transition from parenteral nutrition (via the placenta) to enteral nutrition (colostrum/milk) (Pluske, 2016). The intake of colostrum and milk regulates the development of the gastrointestinal tract and causes rapid body and organ growth. In fact, within the first four postnatal weeks, the weight of the piglet increases more than fivefold, with the gastrointestinal tract organs growing faster than many other organs of the body (Zabielski, Godlewski and

Guilloteau, 2008). However, there is, to our knowledge, no literature available assessing the composition of the sow's milk with the intestinal development of the piglet.

During the earliest stages of the piglets' life, their survival and growth are highly dependent on the nutrients, growth factors, and protective components provided by sow colostrum and milk. The gastrointestinal tract of the piglet is developed during an extended period that starts before birth and continues until the post-weaning phase. In fact, at the time of weaning, the gastrointestinal tract of a young pig is still developing (McCance, 1974; Everaert *et al.*, 2017) and undergoing rapid changes in gut microbiota colonization, digestive system, and immune development (Pajarillo *et al.*, 2014; Frese *et al.*, 2015; Pluske, Turpin and Kim, 2018; Xiong *et al.*, 2019). Although many studies evaluate the effect of a solid diet (including creep-feeding and dietary fiber) on the intestinal development of the newborn piglet, even in the early stages of life, very few describe their process of intestinal development. As represented in **Figure 2.1.**, after birth, the small intestinal tissue weight increases by up to 70% during the first postnatal day with weight gain primarily confined to the mucosal layer (Xu, Mellor, *et al.*, 1992; Zhang, Malo and Buddington, 1997). In parallel, there are rapid increases in small-intestinal length and diameter (Widdowson, Colombo and Artavanis, 1976; Xu, Mellor, *et al.*, 1992), and the mucosal cell population increases by approximately 50% during the first day and doubles by the third day after birth (Xu, Mellor, *et al.*, 1992). Marked elongation of the villi occurs with the estimated absorption surface area of the small intestine increasing by about 50% during the first postnatal day and about 100% during the first 10 postnatal days in naturally suckled piglets (Smith and Jarvis, 1978; Xu, Mellor, *et al.*, 1992). Villus-like structures are observed in the cecum and the proximal colon in piglets at birth and 1 d after birth, but not in piglets 3 d after birth. Xu, Tungthanathanich, *et al.*, (1992) speculated that such villus-like structures might have a functional significance during the transition to complete dependence on oral nutrition in newborns. In this context, a primary function of the colon is to absorb water, electrolytes, and short-chain fatty acids (SCFA) produced from microbial digestion; however, the retention of dietary carbohydrates through the action of colonic bacteria and production of SCFA relies on inoculation with the microbial population acquired from the sow and the environment.

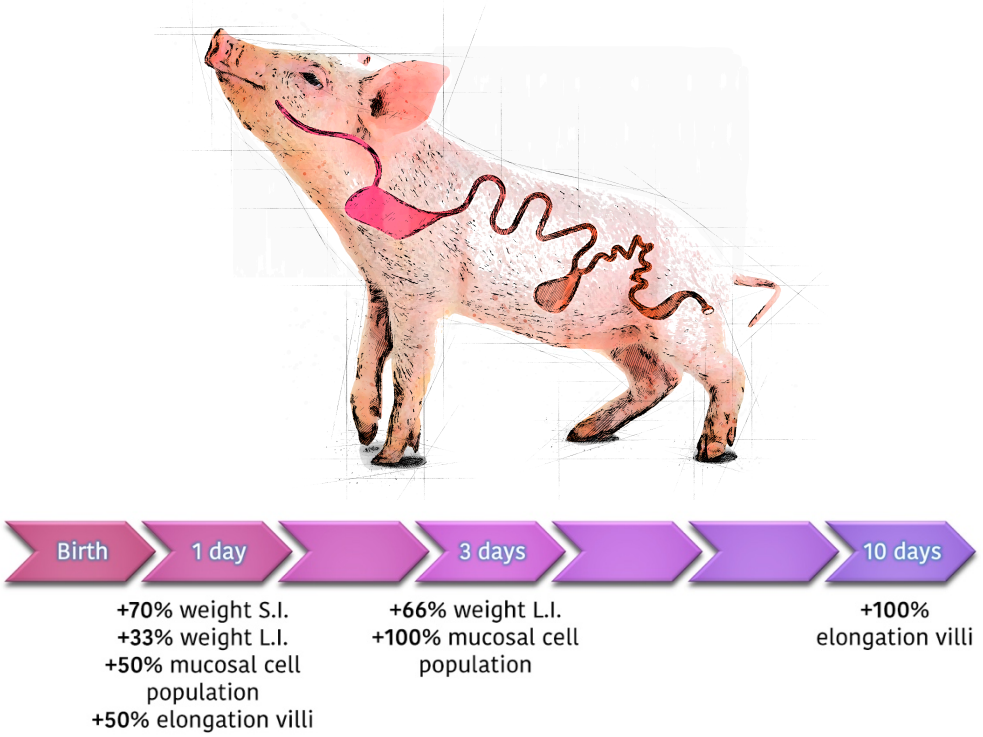


Figure 2.1. Schematic representation of the growth and morphological changes in the small and large intestines of piglets after birth. Percentages represent the percentual increase concerning the measurement at birth. S.I. = Small intestine; L.I. = Large intestine. Data extracted from Xu, Mellor, *et al.* (1992).

As previously stated, immediately after birth, piglets start intestinal nutrition by suckling the sow to obtain colostrum and milk. Maternal milk provides energy and nutrients including lactose, milk oligosaccharides, amino acids, and fat (Kim, 2013) that activate digestive functions and in turn alter the environment for intestinal microbiota colonization (Everaert *et al.*, 2017; Liu, Zeng, *et al.*, 2019). The quantity of milk ingested during lactation may potentially affect the health and performance of the host through modulation of the intestinal microbiota, as suggested by Wylensek *et al.* (2020). In their study, heavier piglets have been shown to have a greater abundance of Bacteroidetes, *Bacteroides*, and *Ruminococcaceae* and a lower abundance of *Actinobacillus porcinus* and *Lactobacillus amylovorus* compared with lighter piglets. The nutritional components of maternal milk include oligosaccharides that contribute greatly to the development of the intestinal microbiota

(Salcedo *et al.*, 2016). In addition, Schokker *et al.* (2018) reported that oral fructooligosaccharide administration to suckling pigs increased the relative abundance of *Lactobacillaceae* and *Bifidobacteriaceae* in colonic digesta and enhanced barrier function whereas reducing the expression of cytokine signaling in jejunal mucosa. Besides the nutrients in colostrum and milk, the bioactive compounds including immunoglobulins, antimicrobial, anti-inflammatory factors, and microbiota also contribute to the intestinal microbiota establishment and development especially in neonatal pigs with an immature immune system (Xiyue Chen *et al.*, 2018).

The composition and the abundance of the developing microbiota are largely affected by dietary factors. This is why diet is one of the most important factors affecting the intestinal microbiota of pigs from birth through the finishing phase, but especially in early-life (Mulder *et al.*, 2009; Schokker *et al.*, 2014; Merrifield *et al.*, 2016; Duarte and Kim, 2021). At the same time, the gut microbiota plays an important role in carbohydrate metabolism and helps in nutrient uptake after metabolizing various food components, particularly indigestible polysaccharides (H. Yang *et al.*, 2017). Therefore, for dietary nutrients to be efficiently metabolized, a population of healthy gastrointestinal tract microbes is highly important, as this can lead to improved digestion and efficient absorption and utilization of nutrients via the pig gut mucosal membrane (Patil, Gooneratne and Ju, 2020). Since the gut microbiota plays a pivotal role in nutrient digestibility, the differences in gut microbiome composition may impact feed efficiency (Bergamaschi *et al.*, 2020).

Since the first introduction of complementary feed for suckling piglets, such as creep-feed or milk replacer, the diet becomes a major force that shapes the composition and activity of the gut microbiota (Konstantinov *et al.*, 2006). This is evident from the fact that the introduction of solid foods drives the immature gut microbiome into an adult type (Frese *et al.*, 2015), and that an alteration in gut microbiota has been shown after weaning or dietary changes. There are three main different strategies in addition to solid feed to favor the acquisition of nutrients and the development of the piglet gastrointestinal tract and microbiota: creep-feed, liquid creep-feed (same solid feed but mixed with water or milk), and milk replacers (Blavi *et al.*, 2021). On the one hand, creep-feeds are highly palatable and easily digestible diets that are offered to lactating pigs after the first week or ten days of lactation. Creep-

feeding is one of the most common earliest feeding practices with solid feed to promote a suitable transition at weaning, as piglets may become familiarized early with a solid diet and they start to consume it early after weaning (Bruininx *et al.*, 2002; Solà-Oriol and Gasa, 2017). Moreover, piglets fed with liquid creep-feed have been reported to have higher villi after weaning (Deprez *et al.*, 1987). However, it is important to remark that not all pigs within a litter consume creep-feed and only a certain proportion of pigs (about 45% to 65%) within the litter are creep-feed eaters (Bruininx *et al.*, 2002; Pluske, Kim, *et al.*, 2007; Sulabo, Tokach, *et al.*, 2010). On the other hand, providing milk replacer supplementation has been reported to increase piglet survival (Kobek-Kjeldager *et al.*, 2020). In addition, milk replacer supplementation may help piglets to become familiar with solid feed after weaning according to gradually replacing milk formula with solid feed as a result of the feeding strategy used.

"The first aim of complementing milk with a creep feed is mainly to anticipate the ability of piglets to secrete the digestive enzymes needed to digest solid feeds."

Interestingly, recent studies show that the largest change in bacterial composition occurs in pigs that are between 21 and 33 days of age, which is the period of time that the animal transitions from a primarily milk-based diet to one containing solid feed (Frese *et al.*, 2015; Bian *et al.*, 2016; De Rodas *et al.*, 2018; Guevarra *et al.*, 2019; Li *et al.*, 2020). These results were consistent along all the gastrointestinal tract, including duodenum, ileum, cecum, and colon (De Rodas *et al.*, 2018). Moreover, even when on a solid diet during different stages of growth, the change was gradual indicating the role of diet in influencing the composition of gut microbiota (Li *et al.*, 2020).

After creep-feeding as a transition strategy at the end of lactation, the composition of the initial solid diet after weaning can also influence the intestinal microbial development of the piglet. Although most of the dietary proteins are digested and absorbed in the small intestine, the undigested protein reaches the large intestine and is fermented by the microbiota. It is important to note that microbiota in the small intestine also can ferment proteins however, to a lower extent (Davila *et al.*, 2013). The level of crude

protein in the diet can increase the nitrogen availability as well as the pH of the digesta, favoring the proliferation of proteolytic bacteria and potential pathogens (Kim, Chen and Parnsen, 2019). The major bacteria fermenting protein in the small intestine include *Klebsiella spp.*, *E. coli*, *Streptococcus spp.*, *Succinivibrio spp.*, *Mitsuokella spp.*, and *Anaerovibrio spp.* (Dai *et al.*, 2010). However, in the large intestine of monogastric animals, the proteolytic activity has been mainly attributed to the genera of *Bacteroides*, *Propionibacterium*, *Streptococcus*, *Fusobacterium*, *Clostridium*, and *Lactobacillus* (Davila *et al.*, 2013). Protein fermentation in the intestine produces amino acids (AA), short-chain fatty acids (SCFA), branched-chain fatty acids (BCFA), and polyamines that are known to affect intestinal health. In this context, the use of highly digestible protein supplements may reduce the availability of protein for microbial fermentation. (Iakhno *et al.*, 2020) reported that the use of yeast, replacing 40% of crude protein in the diet, reshaped the microbiota in the ileal and colonic digesta of nursery pigs.

In addition to protein content, dietary fiber is another dietary compound highly related to intestinal microbiota, as it can influence gut health positively (Jha *et al.*, 2019). Dietary fiber is also implicated in gastrointestinal tract development and mucosal changes in pigs (Knudsen, Hedemann and Lærke, 2012; Van Hees *et al.*, 2019). The fermentable fibers pass through the small intestine undigested and act as a substrate for the distal gut microbiota, stimulating microbial fermentation and SCFA production in the colon. The predominant SCFA formed (approximately 95%) are acetic, propionic and butyric acid, although some other organic acids can be detected as well, such as lactic, succinic, isovaleric, and isobutyric acid (Rios-Covian *et al.*, 2016). However, special attention is commonly given to butyric acid because it serves as a major source of energy for colonic epithelial cells and has been proposed to exert several beneficial effects in the establishment and maintenance of homeostasis in the colon mucosa, including colonocyte growth and proliferation (Hamer *et al.*, 2008; Berni Canani, Di Costanzo and Leone, 2012; Leonel and Alvarez-Leite, 2012; Den Besten *et al.*, 2013; van der Beek *et al.*, 2017; Xiong *et al.*, 2019). Acetate, lactate, propionate, and butyrate help in digestive tract development by potentiating the gut epithelia (Montagne, Pluske and Hampson, 2003). These acids also inhibit the growth of *Salmonella*, *Escherichia coli*, and *Clostridium* (Pickard *et al.*, 2017). In this regard dietary fibers have been reported to decrease the diarrhea incidence

in pigs around the time of weaning (Wellock *et al.*, 2008; Molist *et al.*, 2014; Superchi *et al.*, 2017). The inclusion of insoluble non-starch polysaccharides such as pectin, cellulose, gums, and hemicelluloses stimulate the growth of commensals within the gut, increase the short-chain organic acid production, lower the colon pH and increase the length of the intestinal villi and delay transit time, thereby allowing a longer period for degradation of fibrous material by microbiota in the colon (Knudsen, Hedemann and Lærke, 2012; Ivarsson *et al.*, 2014; Balasubramanian, Lee and Kim, 2018).

Likewise, new studies suggest that fiber can be utilized by the intestinal microbiota in cross-feeding or a cell-dependent action. This may indicate that supplementation of a single enzyme may affect those bacteria utilizing the target subtract and the oligosaccharides released (Feng *et al.*, 2018). Recently, it was also reported that by including xylanase in pig diet, the fecal and ileal counts of beneficial lactobacilli could be increased while simultaneously reducing the *Escherichia coli* counts (Balasubramanian, Lee and Kim, 2018). Moreover, the arabinoxylo-oligosaccharides (AXOS) release by enzymes would affect both luminal and mucosa-associated microbiota differently from those directly supplemented (Petry *et al.*, 2021). Therefore, feed formulation is an important tool in manipulating the intestinal microbiota to promote the health and performance of pigs.

2.13. Early postnatal development of the immune system

The gastrointestinal epithelium and underlying lamina propria are continually exposed to a harsh luminal environment, which includes massive amounts of toxins, antigens, pathogens, etc. In this environment, the gut must provide a barrier to pathogenic and antigenic components in the lumen to prevent an overwhelming immune activation and potentially sepsis, which is critical for host survival (Moeser, Pohl and Rajput, 2017; Pluske, Turpin and Kim, 2018). As described by Moeser, Pohl and Rajput (2017), the intestinal epithelial cells act as immune sentinel cells by recognizing pathogenic signal molecules and secreting interleukins (IL) and growth factors (e.g., IL-17A, IL-33, IL-23, and transforming growth factor-β), which have important immuno-modulatory properties. However, simultaneously, the gastrointestinal system must efficiently transport luminal nutrients, water, and electrolytes, which are

vital for maintenance and growth, and selectively uptake dietary and microbial antigens to facilitate proper development and education of the mucosal immune system (Pluske, Turpin and Kim, 2018). To perform these divergent functions, the gastrointestinal system is equipped with multiple layers of sophisticated barrier mechanisms. On the other hand, the enteric nervous system, through the constant release of an array of neurochemicals, plays a central role in gut motility, secretion and absorption, and modulation of epithelial permeability. The nervous system is also a major regulator of systemic and local gastrointestinal immune responses via neuro-immune synapses and can modulate bacterial toxin sensing and adherence (Downing and Miyan, 2000; Fernandez-Cabezudo *et al.*, 2010; Dhawan *et al.*, 2012; Moeser, Pohl and Rajput, 2017). Therefore, the gastrointestinal barrier is comprised of a multi-layered system of host defense mechanisms provided by the intestinal epithelial cells, and components of the immune and enteric nervous system (Moeser, Pohl and Rajput, 2017). Moreover, the resident immune cells and related lymphoid structures in the gut constitute the largest immune organ in the body (Moeser, Pohl and Rajput, 2017; Pluske, Turpin and Kim, 2018).

The piglet is born with low body energy stores and devoid of serum immunoglobulins (Le Dividich, Rooke and Herpin, 2005). Moreover, because of the epitheliochorial nature of the porcine placenta, the newborn piglet must acquire maternal immunoglobulin G (IgG) from ingested colostrum for passive immune protection until the immune system of the piglet becomes fully developed (Le Dividich, Rooke and Herpin, 2005). Therefore, the intake of colostrum and milk during the early days of life plays a crucial role in the survival of newborn piglets and for proper growth and development (Curtasu, Theil and Hedemann, 2016). The post-natal period is marked by the maturation of the epithelial barrier and transport functions, and immune and enteric nervous systems that are almost complete by 12 to 14 weeks of age (Moeser, Pohl and Rajput, 2017). The developmental processes occurring this time exhibit a high degree of plasticity and shape the adult phenotype and function of the gastrointestinal barrier. Therefore, stressful or inflammatory disturbances during this time can have long-lasting consequences.

During the fetal stage, the placenta supplies nutrients, growth factors, and protective factors through the umbilical cord (Hurley, 2015; Curtasu, Theil and

Hedemann, 2016). After farrowing, the newborn pig relies only on colostrum as its sole source for serum antibody and milk for its intestinal antibody during most of the post-natal period (Bourne, 1976). Colostrum and sow's milk initially provide the piglet with energy and protective passive immunity as well as important growth and immune factors, including lactose, oligosaccharides, amino acids, fat, enzymes, growth factors, and immunoglobulins (Noblet *et al.*, 1997; Rooke and Bland, 2002; Le Dividich, Rooke and Herpin, 2005; Kim, 2013; Hurley, 2015; Moeser, Pohl and Rajput, 2017). However, although colostrum is very digestible and both colostral energy and nitrogen are retained with very high efficiency in the offspring, the IgG concentrations vary widely between individual sows both in initial concentration and in the rate at which concentrations decline during the first 24 h of life. The piglets can only absorb intact IgG before gut closure, which occurs in the first 24 h of life and is induced by intakes of colostrum which are insufficient to maintain piglet body weight (Le Dividich, Rooke and Herpin, 2005). As a result, the amounts of intact IgG absorbed by the piglet vary widely. Therefore, the consumption of colostrum in sufficient amounts to meet the energy requirements of the piglet is a major determinant for survival (Vallet, Miles and Rempel, 2013; Vallet *et al.*, 2015; Ogawa *et al.*, 2016). As for milk consumption during lactation, it is very important to highlight that its composition is very different from that of colostrum (Kim, 2013). The immunoglobulin A (IgA) concentration reduces from 21.2 to 6.7 mg/mL 18 h following farrowing (Klobasa, Werhahn and Butler, 1987). The reduction in the IgA concentration can be related to the variation on the intestinal microbiota during lactation. IgA binds to pathogens impairing their replication (Moor *et al.*, 2017) and helps to prevent bacterial adhesion to intestinal epithelial cells (Dunne-Castagna, Mills and Lönnerdal, 2020). These reasons explain why current research in sow feeding strategies is directed toward improving the quantity of colostrum and milk to maximize the survival and growth of piglets (Curtasu, Theil and Hedemann, 2016).

The first three months of postnatal life represent a major maturational period of gastrointestinal development in the pig (**Figure 2.2.**). During this time, intestinal epithelial, immune and enteric nervous system (ENS) phenotype and function change dramatically as the neonate adapts to life in the extra-uterine environment (Moeser, Pohl and Rajput, 2017). In the piglet, immunoglobulin-producing cells first appear in the gut at the end of the first

week of life and reach a mature profile after one month. During this period, the piglet is likely to be capable of responding to orally presented antigens (Bourne, 1976). At approximately 2 weeks after birth, the development of major immune system induction elements occurs, and by 4 weeks significant concentrations of serum IgA are evident (Inoue *et al.*, 2005). At the time of weaning, the gastrointestinal tract of the young piglet is still developing (McCance, 1974; Everaert *et al.*, 2017) and undergoing rapid changes in gut microbiota colonization, digestive system, and immune development (Pajarillo *et al.*, 2014; Frese *et al.*, 2015; Pluske, Turpin and Kim, 2018; Xiong *et al.*, 2019; Choudhury, Middelkoop, de Souza, *et al.*, 2021). Throughout this entire stage, birth and weaning represent the major challenges to the developing immune system as it must adapt to gut microbial colonization and milk and feed antigens. In addition to a rapid epithelial barrier establishment, additional exogenous and endogenous factors act to suppress immune activation. For example, milk-derived immunoglobulins (e.g., immunoglobulin A, IgA), maternal leukocytes, and milk glycans can act modulating and neutralizing intestinal microbes. Additionally, mothers' milk provides anti-inflammatory cytokines and peptides, which suppress neonatal toll-like receptor (TLR) and inflammatory cytokine expression (Newburg and Walker, 2007; Moeser, Pohl and Rajput, 2017).

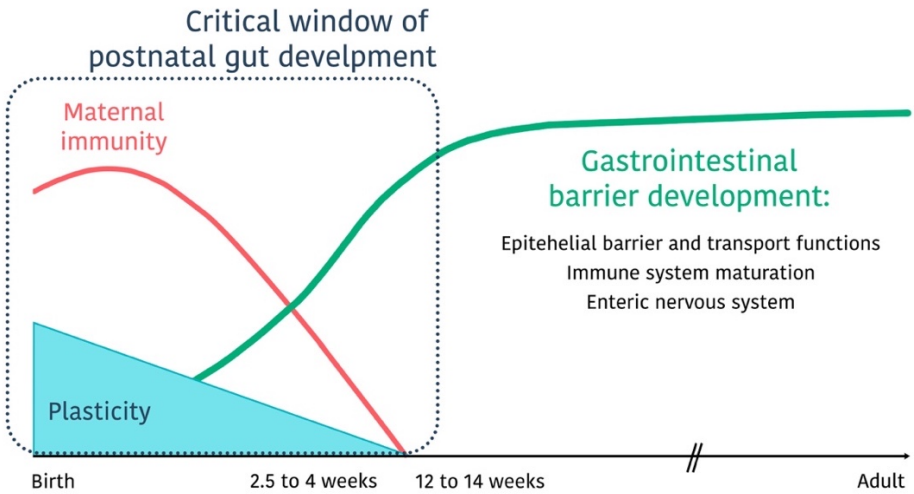


Figure 2.2. Temporal development of the gastrointestinal barrier function development in the newborn piglet. Adapted from Moeser, Pohl and Rajput (2017).

Many approaches have been examined to demonstrate the critical association between gut microbiota and the host's innate and acquired immune system. As a result, the microbiota is known to influence not only the local intestinal immune system but also systemic immunity (Smith, McCoy and Macpherson, 2007; Wu and Wu, 2012; Patil, Gooneratne and Ju, 2020). Microbiota colonization after birth is the most important trigger for immune system development and early modulation of microbiota is bound to modify the future immune phenotype of the host (Hansen *et al.*, 2012; Matamoros *et al.*, 2013; Xu *et al.*, 2020). In addition, the establishment of a beneficial microbiota is important during the suckling stage as piglets still have an immature immune system and they are dependent on sow's milk to prevent colonization and overgrowth of opportunistic pathogens (Castillo *et al.*, 2007).

"Early-life microbial exposure is of particular importance to growth, development of the immune system, and health (Dou *et al.*, 2017; Guevarra *et al.*, 2019)."

The complex interactions occurring in the gastrointestinal tract between nutrition, the gut mucosa, and the microbiota are key mounting an appropriate immune response (Pluske, Turpin and Kim, 2018). The mucosa-associated microbiota acts as the frontline defender against pathogens by competitive exclusion and immune status modulation (Brandtzaeg, 2007; Belkaid and Hand, 2014; Ma *et al.*, 2018; H. Y. Cheng *et al.*, 2019; Duarte and Kim, 2021). The production of IgA induced by microbial fermentation modulates bacterial colonization and limits pathogen entrance through the intestinal epithelial cells (Che *et al.*, 2014; Gutzeit, Magri and Cerutti, 2014). Moreover, these secretory IgA concentrations are positively correlated with *Prevotella* abundance and increased animal growth (Mach *et al.*, 2015). Furthermore, the intestinal microbiota is shown to support the maturation of the intestinal epithelial cells and their barrier functions, promoting homeostasis of the intestinal immune system (Kabat, Srinivasan and Maloy, 2014; N. Li *et al.*, 2018; De Vries and Smidt, 2020). Similar to the impacts on long-term microbial colonization, continued microbial exposure during piglet development is important for balancing the immune cell population (Inman *et al.*, 2010).

In summary, a healthy gut results from positive interactions between the microbiome and host (Patil, Gooneratne and Ju, 2020). In this context, the epithelial barrier function and the mucosal immune system are vital components (Burkey, Skjolaas and Minton, 2009; Pluske, Turpin and Kim, 2018). When the balance between host immunity and microbiota is disrupted, the risk for dysbiosis and progression of diseases such as diarrhea and swine dysentery is increased (Tamboli *et al.*, 2004).

2.2. The impact of weaning in the young piglet and the role of the intestinal microbiota

In commercial swine production, the suckling-weaning transition is the most critical period for piglet health. As summarized in **Figure 2.3.**, pigs face nutritional, environmental, physiological, and social challenges causing weaning stress (Montagne *et al.*, 2007; Campbell, Crenshaw and Polo, 2013; Gresse *et al.*, 2017). Weaning is usually performed at an early age between 3 and 4 weeks of age, a period in which piglets are usually vulnerable to infectious diseases because of weaning stress and immaturity of intestinal tract (J. C. Kim *et al.*, 2012), while in nature systems it occurs gradually around 2-3 months of age (Michiels *et al.*, 2013; Moeser, Pohl and Rajput, 2017; Salazar *et al.*, 2018). Therefore, even if strategies are adopted during the suckling period to improve the host maturation, pigs have not completely developed enough to digest vegetable substrates or to achieve immune competence at weaning (Trevisi *et al.*, 2021), leading to perturbations in gut microbiota, host physiology, and immune function (Konstantinov *et al.*, 2006).

Weaning stress leads to a disruption in the intestinal microbiota, being one of the major factors contributing to post-weaning infections (Konstantinov *et al.*, 2006; Gresse *et al.*, 2017). The stress of being removed from the sow, mixed into a new environment, and abruptly withdrawn from the sow's milk is associated with a volatile gut microbial ecosystem and lowered defenses against pathogen entry, leading to increased risk of disease, in particular, post-weaning diarrhea (Konstantinov *et al.*, 2006; Lallès *et al.*, 2007a; Fouhse, Zijlstra and Willing, 2016). The diet being fed and ingested is another major factor in the modulation of the intestinal microbiota (Frese *et al.*, 2015; Niu *et al.*, 2015; Bian *et al.*, 2016; Tilocca *et al.*, 2017; X. Wang *et al.*, 2019). The shift

in the intestinal microbiota profile around weaning is attributed mainly to the abrupt transition from liquid milk to a solid plant-based diet that affects the physicochemical conditions and the substrate availability in the intestine (Bian *et al.*, 2016), in addition to the reduction in the immunoglobulin supply from milk (Dunne-Castagna, Mills and Lönnerdal, 2020). The stress caused as a result of weaning not only changes microbial composition and function but also alters the microbial metabolic profiles in the intestine (Y. Li, Guo, *et al.*, 2018; Das *et al.*, 2020).

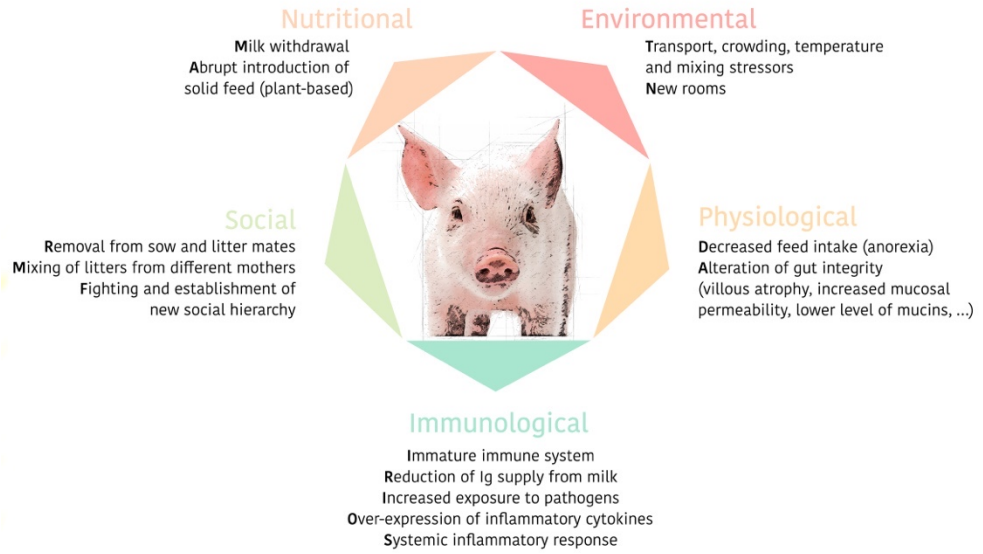


Figure 2.3. Schematic representation and brief summary of the challenges faced by the piglet at weaning.

Another crucial factor leading to dysbiosis and post-weaning diarrhea is low feed and water intake post-weaning (Lallès *et al.*, 2007a). During the first week of adaptation to solid feed, piglets experience a critical period of low voluntary feed intake, which results in the alteration of gut integrity (Lallès *et al.*, 2004, 2007b), characterized by shortened villous length (Pluske, Hampson and Williams, 1997), disturbed absorptive-secretory electrolyte and fluid balances, increased mucosal permeability (Miller and Skadhauge, 1997; Vente-Spreeuwenberg *et al.*, 2001; Boudry *et al.*, 2004), decreased enzymatic activities (Pluske, Hampson and Williams, 1997), activation of heat shock proteins in the mucosa (David, Grongnet and Lallès, 2002), as well as a

lowered level of mucins (Lopez-Pedrosa *et al.*, 1998) and goblet cell density (Brown *et al.*, 1988; Núñez *et al.*, 1996). Furthermore, the intestine atrophy is accompanied by an immediate over-expression of inflammatory cytokines along the intestine of the piglets' (Pié *et al.*, 2004) and also a systemic inflammatory response (Piñeiro *et al.*, 2009). The alteration of the intestinal microbiota due to weaning stress also changes the bioactive compounds (Y. Li, Guo, *et al.*, 2018) and the expression of genes related to nutrient metabolism (Meng *et al.*, 2020). After 5-15 days from weaning, the recovery of the impairment of the villi starts, with changes in microbiota and distal parts of the gut (Hampson, 1986; Pluske *et al.*, 2003; Montagne *et al.*, 2007). Therefore, a 1–2-week adaptative phase to more complex solid cereal-based diets is then observed.

The impact of weaning on piglet gut microbiota has been widely reported in the literature (Kim *et al.*, 2011; Pajarillo *et al.*, 2014; Slifierz, Friendship and Weese, 2015; Zhao *et al.*, 2015; Frese *et al.*, 2015; Mach *et al.*, 2015; Niu *et al.*, 2015; Chen *et al.*, 2017; Holman *et al.*, 2017; Y. Li, Guo, *et al.*, 2018; Guevarra *et al.*, 2018, 2019; X. Wang *et al.*, 2019; Ke *et al.*, 2019; Choudhury *et al.*, 2020). Several authors have described an increased diversity after weaning (Pajarillo *et al.*, 2014; Frese *et al.*, 2015; Niu *et al.*, 2015; Slifierz, Friendship and Weese, 2015; Chen *et al.*, 2017; Ke *et al.*, 2019; X. Wang *et al.*, 2019; Choudhury *et al.*, 2020). Diversity results are, however, contradictory with some other studies reporting decreased alpha diversity during the early period after weaning (Hu *et al.*, 2016; Gresse *et al.*, 2017; Han *et al.*, 2018; Y. Li, Guo, *et al.*, 2018), with a later increase from weaning to adulthood. Higher diversity in the gut microbiota has been related to a more mature gut ecosystem and agrees with the concept of functional redundancy, which supports that additional taxa add redundancy to specific functions, helping the ecosystem to preserve its resilience and stability after environmental stress (Naeem, Kawabata and Loreau, 1998; Konopka, 2009; Holman and Chénier, 2014; Chen *et al.*, 2017). Discrepancy between studies might be due to differences in weaning age or the post-weaning sampling time-point. As previously mentioned, two phases have been described after weaning, being the first one an acute phase (0-5 days after weaning) and the second a recovery phase (5-15 days after weaning), therefore, sampling time election is decisive for homogeneous results. The variability of the microbiota among individual piglets (beta diversity) is known to decrease after weaning (Chen *et al.*, 2017; Choudhury

et al., 2020; Luise, Le Sciellour, *et al.*, 2021), suggesting that after the weaning stress the microbiota shifts toward maturation.

> "The early-life microbial colonization with potentially beneficial and diverse gut microbes can influence the maintenance of intestinal homeostasis and prevent gut dysbiosis (Pluske, Turpin and Kim, 2018; Guevarra *et al.*, 2019; Nowland *et al.*, 2019)."

Gresse *et al.* (2017) stated that weaning transition is characterized by a decrease in the abundance of bacteria belonging to the Lactobacillus group and an increase in the abundance of facultative anaerobes, including bacteria belonging to the *Enterobacteriaceae*, *Proteobacteriaceae*, *Clostridiaceae*, and *Prevotellaceae* families (Chen *et al.*, 2017; Gresse *et al.*, 2017). It is well known that the reduction of lactic acid-producing bacteria (Lactobacillus) during weaning raises intestinal pH, increasing disease susceptibility because low gut pH is bacteriocidal (Lallès *et al.*, 2007a; J. C. Kim *et al.*, 2012). Post-weaning diarrhea is characterized by reductions in healthy bacteria, including bacteria from the *Lactobacillaceae* family, and increases in pathogenic *Escherichia coli* (Konstantinov *et al.*, 2006; Lallès *et al.*, 2007a).

Li *et al.* (2018) reported that weaning increased mainly *Lachnospiraceae*, *Negativicutes*, *Selenomonadales*, *Campylobacterales*, whereas decreased *Campylobacter*, *Porphyromonadaceae*, *Alloprevotella*, *Barnesiella*, and *Oscillibacter*. Moreover, after weaning the relative abundance of *Prevotella* increased in weaned piglets with the introduction of a plant-based diet (Guevarra *et al.*, 2018).

Pajarillo *et al.* (2014), assessed the fecal bacterial diversity during the weaning transition. As a result, the preweaning microbial community consisted primarily of the phyla Firmicutes (54%) > Bacteroidetes (38.7%) > Proteobacteria (4.2%) > Spirochetes (0.7%) > Tenericutes (0.2%). Although the same major phyla prevailed after weaning, the relative proportions varied, with Bacteroidetes (59.6%) > Firmicutes (35.8%) > Spirochetes (2.0%) > Proteobacteria (1%) and Tenericutes (1%). Thus, Firmicutes and Bacteroidetes accounted for more than 90% of the fecal bacterial community during both

the pre-weaning and postweaning stages. However, although Firmicutes accounted for the initial prominent phyla, a shift toward Bacteroidetes was observed after weaning. Among the genera, *Bacteroides*, *Blautia*, *Dorea*, *Escherichia*, and *Fusobacterium* were determined to be the most abundant pre-weaning, whereas *Prevotella* and *Clostridia* increased after weaning.

All the previous observations are largely in agreement with several studies which exemplify *Prevotella* as a prominent microbe in the typical post-weaning microbiota together with species belonging to *Roseburia*, *Faecalibacterium*, *Ruminococcus*, *Lachnospira*, *Dorea*, *Blautia*, *Subdoligranulum* (Kim *et al.*, 2011; Pajarillo *et al.*, 2014; Frese *et al.*, 2015; Mach *et al.*, 2015; Slifierz, Friendship and Weese, 2015; Ramayo-Caldas *et al.*, 2016; Y. Li, Guo, *et al.*, 2018; Guevarra *et al.*, 2018, 2019; Choudhury *et al.*, 2020; Luise, Le Sciellour, *et al.*, 2021). The drastic increase in the relative abundance of *Prevotella* is likely due to the established capacity of the members of the genus to metabolize plant-derived non-starch polysaccharides to short-chain fatty acids (Flint and Bayer, 2008; Ivarsson *et al.*, 2014), which are prominent constituents of the cereal-based weaner diet. *Prevotella spp*. has also been known to degrade polysaccharides in the plant cell wall by producing enzymes, such as β-glucanase, mannase, and xylanase (Flint and Bayer, 2008). Therefore, the abrupt change to a solid cereal-based diet and the withdrawal of milk explains the decrease of the previously mentioned genera and the increase of propionate- and butyrate-producing genera including *Phascolarctobacterium*, *Rikenellaceae RC9 gut group*, and *Dorea*, *Butyricimonas*, *Lachnospira*, and *Subdoligranulum*, respectively, among others (Zhao *et al.*, 2018). Microorganisms belonging to the *Lachospiraceae* genera, such as *Lachnospira*, *Coprococcus*, and *Dorea*, have been reported to begin to emerge after weaning (Y. Li, Guo, *et al.*, 2018). The genera belonging to *Lachnospiraceae* and *Ruminococcaceae* families are adapted to metabolize a wide range of complex oligosaccharides and polysaccharides while producing short-chain fatty acids. Altogether, the higher abundance of propionate- and butyrate-producing genera in older piglets, adapted to digest resistant starches and dietary fibers to convert them to short-chain fatty acids, show the quick microbial transformation of the piglets' gut microbiota to cope with diets rich in complex carbohydrates, as these abundance shifts occur in a short period of time. Therefore, the porcine microbiota rapidly evolves through time, towards a homogeneous and stable microbiome structure.

According to Ke *et al.* (2019), maturation of the intestinal microbiota normally occurs around 80 d of age in pigs, whereas Zhao *et al.* (2015) indicated that the intestinal microbiota is relatively stable at 6 months of age. However, the intestinal microbiota is dynamically affected by several factors including dietary components and the host immune system maturation (Duarte and Kim, 2021). Therefore, it can be suggested that the maturation of intestinal microbiota occurs during early life from weaning when pigs receive plant-basal diets to the finishing phase, which is also related to the maturation of the immune system (Honda and Littman, 2016).

2.3. Intervention strategies

The modulation of intestinal microbiota towards a more beneficial microbial community in the earliest stages of life can be a key factor in enhancing intestinal health and therefore increasing the growth performance of nursery pigs (Duarte and Kim, 2021). Actually, during early life stages, the intestinal microbiota is dynamic and can be easily influenced by environmental conditions (Koenig *et al.*, 2011; Collado *et al.*, 2012; Schokker *et al.*, 2014); therefore, modulating the intestinal microbiota development in early life is an exceptional strategy to maintain host health in later life.

During the last decades, several nutritional approaches have been examined to lower the incidence of health problems around weaning (Lallès *et al.*, 2007a). Altogether, the effect of the numerous feed additives promoting health and growth response in pigs can be associated with changes in the intestinal microbiota. The most studied strategies to promote animal health and reduce the need for antibiotic use include dietary fiber and copper, prebiotics, probiotics, postbiotics, enzymes, phytobiotics (essential oils, phenolic compounds, and resins), and microbial transplants. However, in the following sections, we will focus on two intervention strategies that play a role in modulating the gut microbiota of suckling pigs: non-dietary strategies based on management practices and the use of probiotics. In both cases, the intervention period reviewed focuses on the postnatal period as an effective window for modulating the developmental dynamics of pigs.

2.3.1 Non-dietary strategies

Pigs reared under conventional intensive production are often housed in stimulus-poor, barren environments, which offer little potential to facilitate their natural behaviors like socialization, exploration, and rooting (Studnitz, Jensen and Pedersen, 2007; Giuliotti *et al.*, 2019; Luo *et al.*, 2020). These barren conditions can also cause chronic stress in pigs, causing gastrointestinal pathophysiology (de Jong *et al.*, 1998; Chaloupková, Illmann, Neuhauserová, *et al.*, 2007; Carreras *et al.*, 2016) and immunity alteration (Reimert *et al.*, 2014; Li *et al.*, 2017; Luo, Geers, *et al.*, 2017; Luo, van Dixhoorn, *et al.*, 2017). Moreover, the recent growing social concern for animal welfare (Giuliotti *et al.*, 2019) and the urgency for a reduction in the use of antibiotics in livestock, are added factors for the search of new ways to improve pig health and welfare.

According to a European Commission Directive (2001/93/EC), to improve the welfare of pigs, these "must have permanent access to a sufficient quantity of materials to enable proper investigation and manipulation activities". Numerous studies have proven that, as opposed to barren housing, enrichment of the environment with materials such as straw bedding or peat, can increase play behavior in pigs (Bolhuis *et al.*, 2005, 2006; Chaloupková, Illmann, Bartoš, *et al.*, 2007). It has been suggested that animals reared in an environment that enables the expression of play behavior are better prepared to cope with unfavorable situations at a later stage of life (Spinka, Newberry and Bekoff, 2001), such as weaning stress and its consequences. However, in addition to the materials and objects used for the enhancement of the surroundings, environmental enrichment can also be a larger space, a fragrance, or even music (Nowicki and Klocek, 2012; Martin, Ison and Baxter, 2015; Silva *et al.*, 2017; X. Li *et al.*, 2019; J. Li *et al.*, 2021). Furthermore, the socialization of piglets in the farrowing environment is another kind of environmental enrichment, namely social enrichment, involving direct contact with conspecifics. Finally, although dietary strategies will be discussed later, the role of nutrition should also be noted. In this sense, nutrition can be a special enrichment, especially the way it is provided to animals (for example, hidden in the substrate) might have some benefits for improving pig activity and natural rooting behavior (Reimert *et al.*, 2013).

Although many studies investigate the effect of environmental enrichment and early socialization, few focus on the adaptation of the piglet to weaning since the vast majority are focused on the reduction of tail-biting and skin injuries due to the fighting for the establishment of social hierarchy. A review of those studies carried out during the early stage, with a positive impact on adaptation to weaning can be found in **Table 2.1.**

Table 2.1. Compilation of studies evaluating the effect of early-life enrichment (environmental, social, or both) on parameters related to stress and welfare of the piglet after weaning.

Enrichment type	Detailed circumstances	Period	Weaning age	Main observations	Reference
Environmental	Increased space allowance and straw	From birth until weaning	28 days	Increased play behavior during lactation and decreased food competition after weaning.	(Chaloupková, Illmann, Bartoš, *et al.*, 2007)
Environmental	Increased space allowance, straw, wood shavings, peat and branches.	From birth until weaning	29 days	Increased chewing, food exploration and play behavior after weaning	(Oostindjer *et al.*, 2010)
Environmental	Substrate (wood bark) or hanging objects	From birth until 10 days after weaning	25 days	Providing substrate decreased piglets' stress at weaning (salivary cortisol)	(C. H. Yang, Ko, *et al.*, 2018)
Environmental	Additional space in the pen (8.6 m^2) with straw, sawdust, and peat as substrates and an alternated daily toy.	From birth until weaning	29 days	Better ability to cope with weaning stress by showing an increased BW and feed intake after weaning	(Luo *et al.*, 2020)
Social	Socialization between litters from two adjacent pens	From day 10 until weaning.	30 days	Socialized pigs formed a stable hierarchy more rapidly after weaning, benefitting them in the longer term.	(D'Eath, 2005)
Social	Socialization between litters from three adjacent pens	From day 12 until weaning.	28 days	Less agonistic behavior after weaning and improved performance.	(Hessel, Reiners and Van den Weghe, 2006)

Social	Socialization in a group housing system	From day 10 until weaning.	28 days	Reduced social stress at weaning and increased weight gain after weaning	(Kutzer *et al.*, 2009)
Social	Socialization between litters from three adjacent pens	From day 14 until weaning.	21 days	Increased agonistic behavior the first day of socialization but reduced after weaning. Greater ADG after weaning.	(Ledergerber *et al.*, 2015)
Social	Socialization between litters from two adjacent pens	From day 14 until weaning.	26 days	Increased agonistic behavior after socialization, but fewer injuries at later regrouping than control (weaning)	(Camerlink *et al.*, 2018)
Social and environmental	Social (between two adjacent pens) and environmental (straw, moist peat, wood shavings, jute bags and broom branches)	Social enrichment: From day 13 until weaning. Environmental enrichment: From birth onwards	31 days	Reduced disease susceptibility to co-infection of PRRSV and A. *pleuropneumoniae* in weaned pigs	(van Dixhoorn *et al.*, 2016)
Social and environmental	Social (between two adjacent pens) and environmental (six enrichment objects or toys per pen)	Social enrichment: From day 14 until weaning. Environmental enrichment: From birth onwards	25 days	Increased exploration pre-weaning, mitigation of weaning stress, and reduced aggression post-weaning until slaughter.	(Ko et al., 2020)
Social and environmental	Social (between two adjacent pens) and environmental (six enrichment objects or toys per pen)	Social enrichment: From day 14 until weaning. Environmental enrichment: From birth onwards	25 days	Improved short-term performance after regrouping and reduced time to reach targeted market weight (105 kg).	(Ko et al., 2021)

2.3.1.1. Environmental enrichment

One of the goals of environmental enrichment programs is to increase the animal's ability to cope with behavioral and physiological challenges such as environmental variation. Environmental enrichment should stimulate animals' visual, somatosensory, and olfactory systems, and the key idea is that these objects should provide an aspect of novelty (Nithianantharajah and Hannan, 2006). Taking into consideration the fact that pigs may lose attention towards the object within a few days, it is of crucial importance to sustain the animals' interest by the frequent replacement or renewal of enrichments (Godyń, Nowicki and Herbut, 2019). Moreover, the pigs should have enough materials to play with at the same time. The effects of environmental enrichment have been studied in piglets before and after weaning. Providing the enrichment in early, neonatal housing conditions may lead to better social behavior, welfare, and later life functioning, putatively via its effect on brain development and functioning (Mora, Segovia and del Arco, 2007; Kuzumaki *et al.*, 2011; Martin, Ison and Baxter, 2015; do Prado *et al.*, 2016). Furthermore, a growing number of studies have demonstrated that enriched housing can also positively influence the level of natural antibodies (Reimert *et al.*, 2014; Luo, Geers, *et al.*, 2017; Luo, van Dixhoorn, *et al.*, 2017). A key aspect seems to be to provide the enrichment at the neonatal stage (Godyń, Nowicki and Herbut, 2019)

Luo *et al.* (2020) found that enriched housed pigs were better able to cope with weaning transition, as they gained more weight and had a higher feed intake during the first days after weaning. This could be related to the finding that enriched pigs also tend to consume more creep-feed before weaning, which is known to stimulate post-weaning feed intake and thereby reduces the weaning-related complications (Bruininx *et al.*, 2002, 2004; Sulabo, Jacela, *et al.*, 2010). The better post-weaning performance of enriched pigs might reflect an increased adaptability of piglets reared in enriched conditions to stressful processes such as weaning (Oostindjer *et al.*, 2010; C. H. Yang, Ko, *et al.*, 2018). However, it has been shown that post-weaning environmental enrichment alone, irrespective of pre-weaning housing, also improves performance and health of newly weaned piglets (Oostindjer *et al.*, 2010), which may be mediated by preserving gut functioning (Pluske, Durmic, *et al.*, 2007; Staals *et al.*, 2007), either through intake of substrates or through stress reduction (Fraser, 1985; Fraser *et al.*, 1991). Taking this aspect into

consideration, Yang *et al.* (2018) found that piglets that had access to wood bark during the neonatal stage had a lower level of cortisol after weaning.

An enriched environment during the early life of piglets is known to positively influence behavioral development and stress adaptation later in life (Oostindjer *et al.*, 2011) by providing piglets with the appropriate social skills and stress coping capabilities (Brunson *et al.*, 2003). For instance, rearing conditions consisting of an outdoor pasture with loose housed sows in the first 6 weeks of life suppressed the development of social stress in adult life (De Jonge *et al.*, 1996), and pre-weaning substrate enrichment, in the form of wood shavings and chopped straw, decreased the number of agonistic encounters at a later age (Munsterhjelm *et al.*, 2009). Access to straw, wood shavings, wood bark, or even pieces of newspaper have also shown a positive effect on piglet behavior during lactation (Telkänranta *et al.*, 2014; van Dixhoorn *et al.*, 2016; C. H. Yang, Ko, *et al.*, 2018).

Among the many potential environmental enrichment strategies, animal welfare programs try to implement external stimuli to relieve stress, which may promote the health and growth of the animal. The effective design of enrichment programs relies upon an improved understanding of animal cognition (Nawroth *et al.*, 2019). While the mechanism underlying cognitive and behavioral responses to stimuli remain largely unresolved, the positive impact of enrichment on animal physiology is apparent. For example, music is a simple enrichment tool that lessens stress in dogs (Lindig, McGreevy and Crean, 2020), horses (Lansade *et al.*, 2014; Wiśniewska *et al.*, 2019), and piglets (X. Li *et al.*, 2019). For instance, Li *et al.* (2021) reported that short-term music stimulus (8 d) reduced the stress response in growing pigs, whereas long-term music stimulus (60 d) enhanced the immune responses.

Aromas in pigs' surroundings may be another factor causing positive stimulation, which has been proven in some studies (Bench and Gonyou, 2006; Nowicki and Klocek, 2012; Nowicki *et al.*, 2015; Sartor *et al.*, 2018). For example, Bench and Gonyou (2006) showed that a soil-filled tray was very effective in the improvement of the weaned pigs' manipulating behavior. Nowicki *et al.* (2015) found that among the synthetic and natural aromas, weaned pigs preferred the most natural fragrances, such as moist soil, fresh grass, and dried mushrooms. Last but not least, Sartor *et al.* (2018), in addition

to different fragrances, used classical music and blue light LED lighting as enrichment in suckling piglets. As a result, among the fragrances of chamomile, lavender, lemon, and thyme used in the heated creeps, the piglets preferred thyme the most. Moreover, piglets showed a greater preference for creeps with blue artificial lighting. Although research about these strategies is still very immature, they show that there are non-dietary strategies capable of modulating the stress of piglets, which is why they should be taken into consideration and more studies on the effect of these techniques should be carried out, especially about the development during the early stage and their impact on weaning.

2.3.1.2. Early socialization

As recently reviewed, the process of weaning is a multifactorial stressor, in which nutritional, social, physical, and psychologic stressors are combined. Piglets weaned under commercial conditions are greatly stressed by maternal separation, abrupt changes in the diet, and the mixing of litters. When piglets are exposed to unfamiliar piglets, it often results in a period of vigorous fighting (Oostindjer *et al.*, 2010). Post-weaning aggression represents a significant cost to animal welfare and economic efficiency due to stress and injury. Although in commercial practice individual litters are separated in farrowing crates, previous studies have suggested that housing systems that allow pre-weaning socialization of piglets can reduce aggression after weaning (Morgan *et al.*, 2014; Salazar *et al.*, 2018). In addition, there is direct experimental evidence that the farm environment during the early life of the piglet influences the regulation of immune responses (Lewis *et al.*, 2012).

In this sense, early socialization not only facilitates positive behaviors and cognition abilities of post-weaning piglets but also has effects on social behavior and physiology of suckling piglets during the lactation (Ji *et al.*, 2021). Therefore, favoring social interaction between litters during lactation can improve the social adaptation of the piglet at the time of weaning (Morgan *et al.*, 2014; de Ruyter *et al.*, 2017; Salazar *et al.*, 2018) with a clear decrease in agonistic behavior between piglets (Hessel, Reiners and Van den Weghe, 2006; Ledergerber *et al.*, 2015; Martin, Ison and Baxter, 2015).

"The social skills obtained by co-mingling piglets in early life can enable them to establish a stable dominance hierarchy more quickly and efficiently at weaning, improving their adaptation and reducing social stress (D'Eath, 2005; Camerlink *et al.*, 2018; Salazar *et al.*, 2018; Wen *et al.*, 2021)."

Ledergerber *et al.* (2015) reported that piglets socialized before weaning had a higher average daily gain (ADG) and lower occurrence of agonistic behavior during the first 6 hours after weaning compared to those piglets that were not socialized. Moreover, socially enriched pens contained fewer piglets displaying agonistic behavior than those without environmental enrichment during the first 12 hours after weaning. Therefore, socializing piglets in the nursery period was an effective means to reduce agonistic behavior between piglets after weaning, improving their performance. Furthermore, according to Šilerová *et al.* (2010) piglets from sow group housing systems, where several litters and sows lived together, were better prepared for weaning than those from individual housing of sows with litters because of increased freedom of movement and social contact as well as co-mingling litters before weaning affected piglet social behavior positively. Hessel, Reiners and Van den Weghe (2006) also reported less agonistic behavior in the initial 48 h after weaning in socialized piglets and a higher weight gain in the long run. By socializing unfamiliar piglets before weaning, stress due to mixing could be distanced in time from the other burdens of weaning, thereby improving performance.

The combination of both physical and social enrichment has been reported to have a substantial impact on piglets' socio-cognitive development (Martin, Ison and Baxter, 2015), improving their ability to cope with routine stressors. In this context, Ko *et al.* (2020) reported a lasting positive effect of enriching the neonatal environment both physically and socially, on piglet object exploration pre-weaning, mitigation of weaning stress, and reduced aggression post-weaning until slaughter. Moreover, in another study, enriching the neonatal environment improved the short-term performance after regrouping, benefitting the life-long performance by reducing time to reach market weight (Ko *et al.*, 2021).

In addition to early socialization among piglets, other social strategies have been studied. For instance, de Ruyter *et al.* (2017) reported that gradually reducing sow contact in lactation produced a reduction in cortisol concentration in response to weaning, evidencing that piglets suffered less maladaptive behaviors probably due to a reduction in weaning stress. Therefore, pigs with better social and cognitive skills can improve their ability to cope with routine stressors by improving their well-being and intestinal health.

2.3.1.3. The bond between environmental enrichment and microbiota

The gut microbiota has been shown to play a pivotal role not only in digestive health but also in the development of the immune response, metabolism, conversion efficiency, body composition, and quality of meat or even in reproductive performance or resistance to stress (Schokker *et al.*, 2014; Zhang, 2014; Mu *et al.*, 2017). Furthermore, the gut microbiota has emerged as a key player in the regulation of the bidirectional communication network through the so-called microbiota-gut-brain axis (Mayer, Tillisch and Gupta, 2015; Foster, Rinaman and Cryan, 2017; Patil, Gooneratne and Ju, 2020), contributing to neurophysiological regulation through the release of bacterial metabolites with the ability to modulate animal behavior and response. Van Dixhoorn *et al.* (2016) reported a decrease of stress-related behaviors around weaning in pigs reared under enriched housing conditions compared to conventionally housed pigs. In addition, the fecal microbiota was slightly but significantly affected by housing conditions during lactation. This finding is in line with previous studies, where environmental conditions were shown to be an important factor driving gut microbiota (Thompson, Wang and Holmes, 2008; Mulder *et al.*, 2009).

Early exposure to the microbiota of different animals through early socialization of suckling piglets could also accelerate the maturation process in the piglet and contribute to the construction of a more diverse and robust adult ecosystem. For instance, Wen *et al.* (2021) assessed environmentally enriched housing combined with early socialization and concluded that it positively drives important aspects of the development of the immune system and the establishment of gut microbiota in early life. In their study, enrich-

ment consisted of straw, moist peat, wood shavings, jute bags, and branches of a broom. As a result, the structure of the fecal microbiota of enriched pigs significantly differed as early as on day 12 of life with a decrease of Enterococcus and an increase in the relative abundance of a *Prevotella*, *Christensenellaceae* (R-7 group), *Ruminococcus*, *Ruminiclostridium*, and *Phascolarctobacterium* in enriched pigs. The increased genera are known to be involved in the degradation of a wide range of plant-derived polysaccharides and production of short-chain fatty acids (Wu *et al.*, 2011; La Reau and Suen, 2018), suggesting that exposure to socially and environmentally enriched housing accelerated the maturation of early-life microbiota composition towards plant-based diet consumption in enriched pigs. These results might be explained by the fact that the bedding materials used for the enriched group (straw, moist peat, and wood shavings) contained plant-derived compounds (carbohydrates, fibers) that were ingested by the animals through rooting behavior during the suckling period (Wen *et al.*, 2021). The faster maturation of pig gut microbiota observed by Wen *et al.* (2021) was also reported by Vo *et al.* (2017), where only exposure to soil (from day 4 to day 13 postpartum) accelerated the maturation in pig gut microbial composition compared to conventionally reared piglets.

Interestingly, Wen *et al.* (2021) also found that the inter-individual variation of colonic microbiota was significantly larger in control pigs than in enriched pigs on day 61 of life. In this regard, Chen *et al.* (2017) showed that the inter-individual variation between different piglets is significantly higher during suckling and decreases markedly upon weaning, suggesting that gut microbiota successively stabilizes and converges with age. Hence, a lower inter-individual variation in enriched piglets after weaning shows that continuous exposure to social and environmental enrichments resulted in a more homologous gut microbiota.

Therefore, early social and environmental enrichment may be profitable for piglets not only by reducing agonistic behaviors and changing immune competence but also by accelerating the maturation of gut microbiota. Moreover, taking into account the pivotal role that the gut microbiota plays in the maturing development of the young piglet, non-dietary intervention strategies might contribute to improving the health and welfare of pigs as well as reducing antibiotic and ZnO use in pig feed.

2.3.2. Probiotics as an early intervention strategy

Between the dietary strategies designed to promote the establishment of a robust microbiota early life in the piglets, the use of probiotics is probably the one that has received the most attention. Probiotics, also known as direct-fed microbials (DFM), have been largely used in the swine industry to promote the healthy growth of pigs. Probiotics are defined as "live microorganisms that, when fed in adequate amounts, confer a health benefit to the host" (Hill *et al.*, 2014). Probiotics can be used to prevent and treat microbial imbalance by altering intestinal populations, epithelial lining, and the gut-associated lymphoid tissues (Metzler, Bauer and Mosenthin, 2005). Moreover, commensal microbes that show some benefit to the host can be potentially considered probiotics (Duarte and Kim, 2021).

> "The practical applications of probiotics with effects on performance parameters such as average daily gain, feed conversion, morbidity, and mortality are of great importance to the pig industry."

The potential mechanisms by which probiotics can modulate the intestinal microbial ecology are presented in **Figure 2.4.** The roles of probiotics are intrinsic and related to the host microbiota including competition for nutrients, adhesion sites on the intestinal mucosa, production of lactic acid, short-chain fatty acids, and antimicrobial compounds (Abriouel *et al.*, 2011; Valeriano, Balolong and Kang, 2017; Barba-Vidal, Martín-Orúe and Castillejos, 2019). As consequence, these factors, enhance the intestinal barrier function and modulate the immune system (Markowiak and Śliżewska, 2018; Duarte, Tyus and Kim, 2020). Lactic acid and short-chain fatty acids produced by the probiotics change the microenvironment in the intestinal lumen, favoring the proliferation of beneficial bacteria (such as Lactobacilli) normally related to lower pH (Dowarah *et al.*, 2018). Conversely, the lower pH and the growth of beneficial bacteria lead to an unfavorable environment for the growth of pathogens (Luise *et al.*, 2019). Antimicrobials produced by some probiotics also help to reduce the proliferation of pathogens (Barba-Vidal *et al.*, 2017). Moreover, bacteria that utilize the metabolites produced by probiotics can also be affected (W. Wang *et al.*, 2019). Probiotics also improve the efficacy

of nutrient digestion, absorption, and utilization with subsequent improvement in production performance (Kenny *et al.*, 2011). Additionally, probiotics can affect the immune system which then, in turn, alters the intestinal microbiota composition (Roselli *et al.*, 2017). All in all, probiotic supplementation leads to much higher growth rates and body weight gain, meeting the production target in the pig industries.

> "Probiotic supplementation increases beneficial bacteria within the pig gut (such as *Lactobacilli*) and decreases the population of harmful bacteria such as *Escherichia coli* and *Clostridia spp.* (Siggers *et al.*, 2008; Veljović *et al.*, 2017)."

The European ban on antibiotic use as a growth promoter in feed left the pig industry seeking viable alternatives to prevent losses due to illness and suboptimal growth. Probiotics were already in widespread use in the livestock, poultry, and companion animal sectors as an aid in treatment and as a promoter of intestinal health (Chaucheyras-Durand and Durand, 2010). In piglets, the prevention of post-weaning diarrhea using probiotics has therefore been the focus of much research.

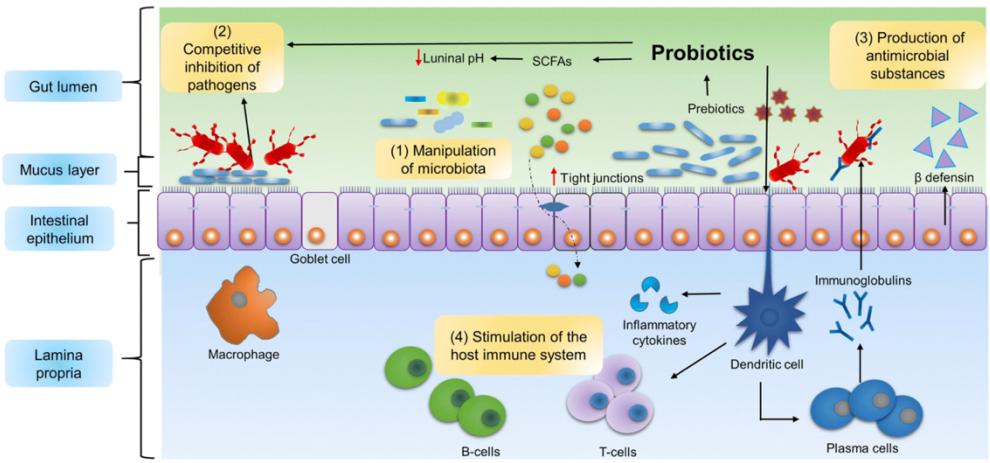


Figure 2.4. The potential mechanisms by which probiotics affect intestinal microbial ecology. Probiotics may act through the following mechanisms: (1) manipulation of the microbiota by changing luminal pH, (2) competitive inhibition of pathogen, (3) production of antimicrobial substances, and (4) stimulation of the pig's immune system. Extracted from Guevarra *et al.* (2019).

In pigs, the gastrointestinal tract is already colonized by a wide range of bacteria just a few days after birth. The microorganisms used as probiotics must therefore possess the property to colonize the gut and compete with the potentially harmful microbes (Kenny *et al.*, 2011). Moreover, after probiotic ingestion by the host, the probiotic encounters several stress factors such as low pH in the stomach and bile in the small intestine (Hou *et al.*, 2015). These factors must be taken into consideration when selecting the most suitable strain.

2.3.2.1 Intervention through an improved performance of the sow

Dietary probiotic supplementation in sows not only aims to improve piglet microbial gut colonization indirectly by changes in the mother's microbiota, but also to improve piglet health and welfare by improving sow reproductive and maternal performance. In this regard, several studies in the literature have pointed out that supplementation of sows with probiotics during gestation and lactation may promote milk production and reduce the mobilization of reserves, improving body condition at the end of lactation (Jeong *et al.*, 2015; Kritas *et al.*, 2015; Hayakawa *et al.*, 2016; Menegat *et al.*, 2019). Moreover, a reduction in the weaning-estrus interval has also been reported (Alexopoulos *et al.*, 2004; Böhmer, Kramer and Roth-Maier, 2006; Kritas *et al.*, 2015; Hayakawa *et al.*, 2016).

Probiotic supplementation to gestating sows has also been shown to increase feed consumption during late gestation and lactation (Alexopoulos *et al.*, 2004; Böhmer, Kramer and Roth-Maier, 2006; Jeong *et al.*, 2015; Kritas *et al.*, 2015; Hayakawa *et al.*, 2016) as well as nutrient digestibility (Upadhaya *et al.*, 2015; Hu, Kim and Kim, 2021). Moreover, *Bacillus* strains commonly possess amylase and protease activities (Lee *et al.*, 2012; Ahmed *et al.*, 2014), simultaneously improving the activities of host lipases and proteases. Increased feed consumption and better nutrient utilization during lactation can be translated into higher colostrum quality, milk quality, and quantity, which may partially explain the better growth and higher piglet weaning weights described by other researchers (Alexopoulos *et al.*, 2004; Böhmer, Kramer and Roth-Maier, 2006; Taras *et al.*, 2006; Scharek-Tedin *et al.*, 2015). In this regard, the supplementation with probiotics during gestation and

lactation has been reported to induce beneficial effects on the milk composition of rats (Azagra-Boronat *et al.*, 2020). Moreover, in that study authors demonstrated that although the milk microbiota was not modified, the probiotic was able to reach the milk. To date, some probiotics have been reported to modulate the immune response of the sow herd (Medina *et al.*, 2007) or even litter immunity (Scharek-Tedin *et al.*, 2015; Hayakawa *et al.*, 2016). The inclusion *of Bacillus subtilis* in lactating sows has been reported to be beneficial for milk production and increase the concentration of IgG (Ayala *et al.*, 2016). Moreover, in fecal samples, probiotic administration has been reported to slightly increase the total IgA concentration (Hayakawa *et al.*, 2016).

In this context, the addition of a novel mixed probiotic culture in pregnant sows has been reported to influence the piglets' gut colonization with beneficial bacteria and reduce the number of *Enterobacteriaceae* (Veljović *et al.*, 2017). Additionally, supplementing sows with *Enterococcus faecium* during the previous month to labor has been reported to modify the fecal microbiota of the mother with some translated impact on their litters (Starke *et al.*, 2013). Moreover, *Bacillus subtilis* probiotic-fed sow progenies have been reported to show a similar fecal microbial population than their mothers (Menegat *et al.*, 2019). On the other hand, Buddington *et al.* (2010) showed the persistence of *Lactobacillus acidophilus* and *Bifidobacterium* lactis 14 days after birth in piglets from probiotic-supplemented mothers and immediately separated after birth, demonstrating the possibility of maternal-to-offspring transmission of probiotics. In this context, Demecková *et al.* (2002), demonstrated a change in the lactic acid bacteria:coliform ratio in sow feces after feeding a *Lactobacillus plantarum* fermented feed during gestation. In turn, a reduction of the fecal contamination of the environment by pathogens was expected. However, some inconsistency in gut microbial responses from probiotic-fed sows and their suckling piglets has led some researchers to hypothesize that response to probiotics differs in each individual (Starke *et al.*, 2013). Additionally, as previously reviewed in the section on the acquisition of the microbiota after birth, the entero-mammary route in pigs has hardly been studied in the literature, particularly on the possible transfer of probiotics through milk. Therefore, further research is needed to confirm the existence of a direct entero-mammary route in the swine species.

The natural exposition of the piglet to sow's feces together with the possibility of an entero-mammary route for microbial transfer (Jost *et al.*, 2014; Xue Chen *et al.*, 2018; Jiang *et al.*, 2019; Liu, Zeng, *et al.*, 2019), open the possibility of gut microbiota modulation in the piglet through probiotic supplementation to the sow. Furthermore, the mother's imprinting on the piglet could occur even before its birth. In a recent study, microbial colonization of the spiral colon occurred in stillborn pigs, suggesting microbial exposure before birth (Nowland, Kirkwood, *et al.*, 2021). After birth, colostrum and milk intake is essential for the formation of the piglet's gut microbiota. As demonstrated by Liu, Zeng, *et al.* (2019), maternal milk microbes were primarily responsible for the colonization of the small intestine, contributing approximately 90% bacteria throughout the first 35 days of neonatal life. Moreover, this study also shows how this initial impact of breast milk on the piglet is gradually replaced by maternal fecal microbes.

2.3.2.2. The importance of early-life supplementation

The process of early microbial colonization of the gastrointestinal tract after birth plays a crucial role in the development of the neonatal immune system of piglets with implications throughout their lives (Hansen *et al.,* 2012). In this context, dietary strategies and specifically probiotics, have gained considerable attention. Probiotics can be directly delivered to the piglets but also can be used as an indirect strategy throughout their administration to their mothers. Different probiotic strains when administered to sows during gestation and/or lactation have been shown to have positive effects on the performance of piglets.

The gastrointestinal tract of newborn piglets is believed to be colonized at birth through environmental bacteria, primarily from the dam (Houghteling and Walker, 2014; Xue Chen *et al.*, 2018; Patil, Gooneratne and Ju, 2020). At 5 weeks of age, the piglet's microbiota is considered to have reached a stable phase (Thompson, Wang and Holmes, 2008), therefore, the developmental window in which the gut community is in a transition phase is relatively short. The colonization of the neonatal gut is random and even within the same environment piglets develop unique bacterial compositions (Thompson, Wang and Holmes, 2008). Moreover, the early development of the microbiota

has been shown to have a long-term influence on intestinal parameters (Schultz *et al*., 2004; Jansman *et al*., 2012). For instance, Harvey *et al.* (2005) conducted a study with a single dose of probiotic in the newborn piglet and obtained lasting effects after weaning. The inclusion of specific probiotics, such as *Lactobacillus casei*, *Lactobacillus reuteri*, and *Lactobacillus acidophilus* markedly improved growth rate and body weight gain in piglets, showing at the same time higher counts of *Lactobacillus spp.* and lower *Escherichia coli* counts in feces (Wells, Yen and Miller, 2005). In another study, germ-free piglets fed a *Lactobacillus* probiotic had lower colonization densities with *Clostridium perfringens* (Siggers *et al*., 2008). Therefore, probiotic supplementation during the early life of the piglets could decrease the colonization of pathogenic bacteria and promote health and growth on a long-term basis by altering the bacterial composition.

2.3.2.3. Most common probiotics in pig feed and their main effects

Lactobacillus spp, *Bacillus spp*, *Enterococcus faecium*, and *Saccharomyces cerevisiae* have dominated the research in pig probiotic use (Kenny *et al*., 2011). Probiotics used for pigs are therefore often lactic acid bacteria (LAB) since they can survive the gastric acid and are already a part of the pigs' intestinal microbiota. Lactic acid bacteria are characterized by being gram-positive, non-sporulating, and by producing lactic acid as a product of the fermentation of carbohydrates (Meng *et al*., 2010), which elicits an inhibitory effect against *Escherichia coli* and enterobacteria (Li *et al*., 2015). Probiotic supplementation with LAB is believed to benefit the host through competition with pathogenic bacteria for nutrients and absorption sites, competition for intestinal epithelium binding sites, and production of compounds that are toxic to pathogens and by stimulating the immune system (Quigley, 2011; Heo *et al*., 2013). For instance, it has been shown that the supplement of LAB to neonatal piglets can benefit their health by regulating the formation of the microbiota in the gut (Siggers *et al*., 2008), and thereby, decreasing problems that can occur around weaning. Moreover, probiotics have been shown to have the most effects for the host animal when the microbiota is still unstable, for example, after birth, the time around weaning, or after movement to a new environment (Jensen, 1998)

"*Bacillus spp.* enables mutual growth with LAB and *Lactobacillus*, *Bifidobacterium*, and *Enterococcus* are lactic acid producing bacteria commonly used in probiotic mixtures due to their characteristics (Pringsulaka *et al.*, 2015; Yang *et al.*, 2015; Kimelman and Shemesh, 2019; He *et al.*, 2020)."

In **Table 2.2.** the main studies that analyzed the effect of the administration of probiotics during the gestation and lactation phases and their impact on the piglet are shown. In summary, very few studies have analyzed the effect of supplementing only the creep-feed of suckling piglets. Despite this, many have studied the combined effect of maternal supplementation during lactation (in feed) together with the possible added effect of supplementing the piglet's creep-feed during lactation. Dietary supplementation was found to be the most studied method, especially due to its high practicality and simplicity, necessary in a commercial farm environment. This is why there are also fewer studies carried out with oral supplementation since they require more time and effort to individually administer the oral doses to all individuals.

The easiest microbes to manipulate and, therefore, include in animal feed, are those that produce spores (Kenny *et al.*, 2011). Spores are extremely robust and stable yet non-replicating under normal storage conditions. Although several spore-forming species of the genus *Bacillus* (*B. subtilis*, B*. licheniformis,* and B*. cereus var toyoi*) have been used in the pig industry, these organisms are not usually part of the indigenous porcine gut microbiota; they are however common soil bacteria, which are likely to be transient passengers through the guts of most outdoor reared pigs.

Bacillus-based probiotics such as *Bacillus subtilis* and *Bacillus amyloliquefaciens* are gram-positive, spore-forming bacteria that germinate but do not proliferate in the gastrointestinal tract (Zani *et al.*, 1998; Alexopoulos *et al.*, 2001, 2004; Taras *et al.*, 2005; Stamati *et al.*, 2006; Baker *et al.*, 2013; Kritas *et al.*, 2015; Hayakawa *et al.*, 2016; Kimelman and Shemesh, 2019; Zhang *et al.*, 2020; Hu, Kim and Kim, 2021). *Bacillus* species have been shown to be effective in maintaining a favorable balance of microflora in the gastrointestinal tract and in improving growth, feed utilization, and gut health

(Zani *et al*., 1998; Alexopoulos *et al*., 2001, 2004; Taras *et al*., 2005; Stamati *et al*., 2006; Baker *et al*., 2013; Kritas *et al*., 2015; Hayakawa *et al*., 2016; Zhang *et al*., 2020; Hu, Kim and Kim, 2021). The germination of *Bacillus* spores avoids pathogenic bacteria from binding on the intestinal epithelium, showing inhibitory activity against the *E. coli*, *S. aureus*, *S. typhimurium*, and *C. perfringens* (Guo *et al*., 2006; Larsen *et al*., 2014). However, the main mode of action of *Bacillus*-based probiotics is through the production of several extracellular enzymes including α-amylases, cellulase, metalloproteases, and proteases that enhance digestibility and absorption of nutrients (Ahmed *et al*., 2014). These enzymes create a favorable environment for the growth and colonization of beneficial bacteria in the gastrointestinal tract, particularly *Lactobacillus* species (Kimelman and Shemesh, 2019; Menegat *et al*., 2020). Additionally, the bacteriocins (subtilin and barnase) produced by *B. subtilis* and *B. amyloliquefaciens* (Lisboa *et al*., 2006; Ulyanova, Vershinina and Ilinskaya, 2011) have bactericidal effects against pathogenic bacteria such as Clostridium perfringens and *Escherichia coli*. Moreover, Kimelman and Shemesh (2019) have recently demonstrated the antagonistic potential of the *Bacillus subtilis* against *Staphylococcus aureus* through activating the antimicrobial lipopeptide production pathway.

The main results obtained after dietary supplementation with probiotic *Bacillus* strains in sows during pregnancy and/or lactation and in piglets during the lactation phase are presented in **Table 2.2.** Generally, the supplementation with *Bacillus* strains (*B. altitudinis*, *B. cereus*, *B. licheniformis*, *B. mesentericus*, and *B. subtilis*) has shown positive results both in the maternal reproductive performance and in the performance of the piglet, both concerning its growth, as well as its intestinal health and immune development. For instance, the administration of probiotic *Bacillus* strains in the mother's diet and the piglet creep-feed has shown beneficial effects on body weight, average daily gain, and feed conversion ratio in numerous studies (Zani *et al*., 1998; Alexopoulos *et al*., 2001; Taras *et al*., 2005; Kritas *et al*., 2015; Hayakawa *et al*., 2016; Crespo-Piazuelo *et al*., 2021). Likewise, the same result has also been observed in studies that have evaluated the supplementation of probiotics only in sows (Alexopoulos *et al*., 2004; Stamati *et al*., 2006; Baker *et al*., 2013; Jeong *et al*., 2015; Davis *et al*., 2020; Zhang *et al*., 2020; Hu, Kim and Kim, 2021), demonstrating the importance of the effect of the sow in the neonatal environment. In this sense, Alexopoulos *et al.*

(2004) and Stamati *et al.* (2006), also reported lower mortality rates and less incidence of diarrhea when *Bacillus* strains were supplemented only to mothers. This could be due to the modulating effect of probiotic strains on maternal feces and intestinal colonization of piglets. In fact, in studies carried out on sows and their litters and, in those carried out only on sows, changes have been observed in the microbiota of the piglet (Baker *et al.*, 2013; Kritas *et al.*, 2015; Hayakawa *et al.*, 2016). For instance, lower counts of pathogenic bacteria such as *Escherichia coli* or *Clostridium perfringens* (Kritas *et al.*, 2015; Hayakawa *et al.*, 2016) and higher counts of beneficial strains such as *Bifidobacterium* or *Lactobacillus* (Baker *et al.*, 2013; Hayakawa *et al.*, 2016) have been reported in the gut microbiota of the piglets. On the other hand, Zhang *et al.*, (2017) reported that pigs receiving *Bacillus* probiotics increased the abundance of mucosa-associated *Clostridium*, *Lactobacillus*, and *Turicibacter* increasing the expression of atonal BHLH transcription factor 1 (Atoh1) upregulating the goblet cells proliferation in the ileum. The greater number of goblet cells increased Mucin 2 (MUC2) production, preserving the intestinal barrier function.

Enterococcus faecium is another probiotic strain that has shown its potential to improve the growth and intestinal health of piglets. For example, Taras *et al.* (2006) studied the effect of supplementing *Enterococcus faecium* NCIMB 10415 to sows and piglets, detecting a decrease in the incidence of diarrhea in piglets. On the other hand, Starke *et al.* (2013) demonstrated the existence of a modulating effect on the microbiota, with higher *Lactobacillus* counts in the small intestine of piglets. Furthermore, *Enterococcus faecium* DSM 7134 supplementation only in sows has also shown positive effects on body weight and the incidence of diarrhea of their litters (Böhmer, Kramer and Roth-Maier, 2006; Lan and Kim, 2020). In the same way, as the probiotic *Bacillus* strains, *Enterococcus faecium* was also able to modify the bacterial microbiota of the piglets through supplementation only in sows (Lan and Kim, 2020), increasing the *Lactobacillus* and *Enterococci* in the piglet feces and decreasing the *Escherichia coli* counts.

Other probiotics, such as several *Lactobacillus* strains or the yeast *Saccharomyces cerevisiae*, have also shown beneficial effects when administered to both, mothers and piglets, including greater average daily weight gain (Jurgens, Rikabi and Zimmerman, 1997) or the ability to modulate

the fecal microbiota of the progeny (Shin *et al*., 2019), with greater diversity and richness of species in the intestinal ecosystem of piglets, associated with greater robustness and resilience and, therefore, with a more mature microbiota (Naeem, Kawabata and Loreau, 1998; Konopka, 2009; Holman and Chénier, 2014; Chen *et al*., 2017). In the same way, as with the probiotic strains of *Bacillus* or *Enterococcus faecium*, the supplementation only in sows of strains of the genera *Lactobacillus*, *Pediococcus*, and the yeast *Saccharomyces cerevisiae* have shown in numerous studies improvements in the body weight of piglets (Shen *et al*., 2011; Wang *et al*., 2014; Apić *et al*., 2017; Liu *et al*., 2020; Betancur *et al*., 2021), a decrease in litter mortality (Apić *et al*., 2017; Liu *et al*., 2020; Betancur *et al*., 2021), and a decreased incidence of diarrhea (Apić *et al*., 2017; Liu *et al*., 2020; Betancur *et al*., 2021). Moreover, Veljović *et al.* (2017) also showed the ability of a set of probiotics to modulate the microbial diversity of the piglet, reducing, in turn, the enterobacteria in feces. More specifically, *Lactobacillus* probiotics can decrease inflammation, measured as lower expression or serum inflammatory cytokine, which can help divert nutrients toward growth (Qiao *et al*., 2015). Shin *et al.* (2019) reported that *Lactobacillus plantarum* probiotic supplemented to pigs from lactation to 4 weeks after weaning increased the microbiota diversity and richness, the growth of lactic acid bacteria, and relative abundance of *Erysipelotrichaceae*, *Sphaerochaetaceae*, *Spirochaetaceae*, and *Christensenellaceae*, whereas it reduced the abundance of *Prevotellaceae* in fecal samples. In addition, feeding *Lactobacillus* derived from the pig intestine as probiotics (as some kind of fecal transplant) reduced the abundance of *Enterobacteriaceae* including pathogenic *Escherichia coli*, reduced the incidence of diarrhea (Huang *et al*., 2004; De Angelis *et al*., 2007), enhanced immune response during infection (Naqid *et al*., 2015), and increased weight gain (Konstantinov *et al*., 2008). Furthermore, the treatment of suckling pigs with *Lactobacillus reuteri* can protect by reducing intestinal pH through lactic acid production via *Bifidobacterium spp*., subsequently reducing the abundance of *E. coli* (Hou *et al*., 2015). With regards to yeasts and, in particular, *Saccharomyces cerevisiae*, they have also been successfully used as probiotics modulating the intestinal microbiota and enhancing the small intestinal health of nursery pigs (Xu *et al*., 2018; Elghandour *et al*., 2020; Zhaxi *et al*., 2020).

Finally, supplementation only in suckling piglets has been the subject of research, especially in recent years (see **Table 2.2.**). In these types of studies,

the use of oral doses instead of feed supplementation is more common. Oral probiotic supplementation during lactation has repeatedly shown improvements in body weight and piglet performance (Xu *et al.*, 2018; Y. Wang *et al.*, 2019; Haupenthal *et al.*, 2020; Luise, Spinelli, *et al.*, 2021), as well as the ability to reduce the incidence of diarrhea (Xu *et al.*, 2018; Y. Wang *et al.*, 2019) and even litter mortality (Xu *et al.*, 2018). Luise et al. (2021) studied the effect of a single oral dose at the beginning of the piglet's life and its effect throughout lactation with *Enterococcus faecium* lactiferm WS200 and *Saccharomyces cerevisiae var. boulardii* CNCM-1079. In their study, a simple dose of probiotic was able to improve the performance and modulate the intestinal microbiota, with changes in specific taxonomic groups. In fact, several studies have reported changes in the intestinal microbiota of suckling piglets after oral doses of probiotics (Siggers *et al.*, 2008; Zhang *et al.*, 2011; Luise, Spinelli, *et al.*, 2021; Moturi *et al.*, 2021). The supplementation of creep-feed only in suckling piglets with a mixture of probiotics has also been associated with lower incidences of diarrhea (Tissopi *et al.*, 2019) and the modulation of the intestinal microbiota, with decreases in coliform counts in the colon and increases in bifidobacteria in the ileum and colon (Shim *et al.*, 2005).

Therefore, it is common in the literature to observe beneficial effects after probiotic supplementation in both sows and their litters, with improvements in piglet growth and performance and gut health. The gestation and lactation period offer a great opportunity to condition the microbial colonization of the piglet from its earliest stage.

Table 2.2. Summary of the main probiotic supplementation studies carried out in sows and piglets before weaning, that is, during the gestation and lactation stages (in sows) or lactation (piglets). The studies and results associated with post-weaning are not presented. The studies have been ordered by the supplemented animal (sow and/or piglet), type of administration of the probiotic (in feed, oral administration, or creep-feed), and by probiotic strain.

Supplemented animals	Form of administration	Probiotic	Dose[a]	Main observations[b]	Reference
Sows and piglets	In feed	*Bacillus altitudinis* WIT588	G $4x10^9$, L $1.2x10^{10}$, P $1x10^9$ spores/day	↑BW, ↑FCR , ↑ SI absorptive capacity	(Crespo-Piazuelo *et al.*, 2021)
Sows and piglets	In feed	*Bacillus cereus* (CenBiot)	$0.5\text{-}1x10^6$ spores/g feed	↑BW, ↑ADG, ↑FCR, ↓ Diarrhea index	(Zani *et al.*, 1998)
Sows and piglets	In feed	*Bacillus cereus* CIP5832 (Paciflor)	$8.5x10^5$ cfu/g feed	↑ BW, ↑ ADG, ↑ FCR	(Alexopoulos *et al.*, 2001)
Sows and piglets	In feed	*Bacillus cereus var toyoi* NCIMB 40112	G $2.6x10^5$, L $4x10^5$, P $1.3x10^6$ cfu/g feed	↑ADG ↑FCR ↓ Diarrhea index	(Taras *et al.*, 2005)
Sows and piglets	In feed	*Bacillus cereus var. toyoi* NCIMB 40112	G $2.6x10^5$, L $4.0x10^5$, P $1.3x10^6$ cfu/g feed	↑ fecal IgA before weaning, ↓ fecal IgG after weaning	(Scharek *et al.*, 2007)
Sows and piglets	In feed	*Bacillus subtilis C-3102*	3×10^5 cfu/g feed	↑BW, ↑ADG, ↓ *E. coli* and *Clostridium spp.* in feces	(Kritas *et al.*, 2015)
Sows and piglets	In feed	*Bacillus subtilis* C-3102	3×10^5 cfu/g feed	↑ *SCFA* in distal SI and colon, ↓ villus atrophy and crypt deepening in SI	(Michiels *et al.*, 2016)
Sows and piglets	In feed	*Bacillus subtilis C-3102*	G $5x10^5$, L $1x10^6$, P $5x10^5$ cfu/g feed	↓ADG, ↓ADFI, ↑ *Bacillus spp.* counts in feces	(Menegat *et al.*, 2019)

Sows and piglets	In feed	*Bacillus mesentericus* TO-A + *Clostridium butyricum* TO-A + *Enterococcus faecalis* T-110	$1\times10^8 + 1\times10^8 + 1\times10^9$ cfu/g feed	↑BW, ↑FCR, ↑ *Bifidobacterium* counts (ileum), ↓ Diarrhea index, ↑ Villus height and ↑ Villus:crypt ratio	(Hayakawa *et al.*, 2016)
Sows and piglets	In feed	*Enterococcus faecium NCIMB 10415*	G $1.6x10^6$, L $1.2x10^6$, P $0.17x10^6$ cfu/g feed	↓ Diarrhea index	(Taras *et al.*, 2006)
Sows and piglets	In feed	*Enterococcus faecium* NCIMB 10415 (SF68)	G $1.6x10^6$, L $1.2x10^6$, P $0.17x10^6$ cfu/g feed	↓ fecal IgA and IgG after weaning	(Scharek *et al.*, 2007)
Sows and piglets	In feed	*Enterococcus faecium* NCIMB 10415	G+L $4.2\text{-}4.3x10^6$, P $5.1x10^6$ cfu/g feed	None	(Martin *et al.*, 2012)
Sows and piglets	In feed	*Enterococcus faecium* NCIMB 10415 (SF68)	G+L $4.2\text{-}4.3x10^6$, P $5.1x10^6$ cfu/g feed	↑ *Lactobacillus* (SI), fecal microbiota modulation	(Starke *et al.*, 2013)
Sows and piglets	In feed (sows), in water (piglets)	*Lactobacillus plantarum* JDFM LP11	$2.5x10^7$ cfu/mL	↑ diversity and richness in feces, ↑ Villus height and crypt depth SI	(Shin *et al.*, 2019)
Sows and piglets	In feed	*Saccharomyces cerevisiae*	1.5×10^{10} live cells/g feed	↑ ADG, ↑ FCR	(Jurgens, Rikabi and Zimmerman, 1997)
Sows only	In feed	*Bacillus cereus var toyoi* (Toyocerin)	$5x10^8$ spores/g feed	↑ BW, ↓Mortality, ↓ Diarrhea index	(Stamati *et al.*, 2006)

Sows only	In feed	*Bacillus licheniformis* DSM5749 + *Bacillus subtilis* DSM5750 (Bioplus 2B)	$1.3x10^6$ spores/g feed	↑BW ↓Mortality ↓ Diarrhea index	(Alexopoulos *et al.*, 2004)
Sows only	In feed	*Bacillus subtilis*	$3.75x10^5$ cfu/g feed	↑BW, ↑ADG, ↑ *Lactobacillus* (ileum and colon), ↓ *E. coli* (colon) and *Clostridium perfringens* (ileum)	(Baker *et al.*, 2013)
Sows only	In feed	*Bacillus subtilis* + *Lactobacillus acidophilus*	$1.2\times10^7 + 1.15\times10^6$ cfu/g feed	↑ BW	(Jeong *et al.*, 2015)
Sows only	In feed	*Bacillus subtilis* PB6	$4.0x10^8$ cfu/kg feed	↑ BW and ADG at weaning, ↑ lactation survival rate	(Zhang *et al.*, 2020)
Sows only	In feed	*Bacillus subtilis* (combination of two strains)	$3.7x10^5$ cfu/g feed	↑BW, ↑ADG, ↑FCR, ↑ADFI, ↑ LAB counts on d3 after weaning	(Davis *et al.*, 2020)
Sows only	In feed	*Bacillus subtilis* + *Bacillus licheniformis*	$1x10^9$ cfu/g each	↑BW ↑ADG	(Hu, Kim and Kim, 2021)
Sows only	In feed	*Bifidobacterium lactis* BI-07	$1x10^{10}$ cfu/day	Fecal presence of the probiotic in 80% of piglets 24h after birth	(Buddington *et al.*, 2010)
Sows only	In feed	*Enterococcus faecium* DSM 7134 (Bonvital)	$5x10^8$ cfu/g feed	↑BW, ↓Pig loss	(Böhmer, Kramer and Roth-Maier, 2006)

Sows only	In feed	*Enterococcus faecium* DSM 7134	2.7-5.4×10^8 cfu/kg feed	↑ BW, ↑ ADG, ↑ FCR, ↑ *Lactobacillus* and *Enterococci* in feces, and ↓ *E. coli,* ↓ Diarrhea index	(Lan and Kim, 2020)
Sows only	In feed	*Lactobacillus acidophilus* (WN0074 and NCFM)	1x10^{10} cfu/day	Fecal presence of the probiotic in 53% of piglets 24h after birth	(Buddington *et al.*, 2010)
Sows only	In feed	*Lactobacillus johnsonii XS4*	6.0×10^9 cfu/kg feed	↑ BW	(Wang *et al.*, 2014)
Sows only	In feed	*Lactobacillus helveticus BGRA43 + Lactobacillus fermentum BGHI14 + Streptococcus thermophilus BGVLJ1-44*	200 ml of mixed probiotic culture (10^8 cfu/ml) in feed	↑ Microbiota diversity, ↓ *Enterobacteriaceae* in feces	(Veljović *et al.*, 2017)
Sows only	Oral dose	*Lactobacillus plantarum CAM6*	10 ml with 1x10^9 cfu/ml	↑ BW, ↓ Mortality, ↓ Diarrhea index, ↑ physiological status	(Betancur *et al.*, 2021)
Sows only	In feed	*Pediococcus acidilactici* ZPA017	2.4×10^9 cfu/kg feed	↑ BW, ↓ Mortality, ↓ Diarrhea index	(Liu *et al.*, 2020)
Sows only	In feed	*Saccharomyces cerevisiae* fermentation product	G 12 g/d, L 15 g/d	↑BW	(Shen *et al.*, 2011)
Sows only	In feed	*Saccharomyces cerevisiae CNCM I-4407 (Actisaf Sc47®)*	600g of Actisaf Sc47® per ton of feed	↑BW, ↓Mortality, ↓ Diarrhea index	(Apić *et al.*, 2017)
Suckling piglet	Oral dose	*Bacillus subtilis C-3102*	20x10^6 cfu/kg BW	↑ *Bacillus spp.* counts in feces	(Menegat *et al.*, 2020)

Suckling piglet	Oral dose	*Enterococcus faecium lactiferm* WS200	4 ml with $1x10^{10}$ cfu	↑ BW, ↑ ADG, microbial alteration with ↑ *Lachnospiraceae*	(Luise, Spinelli, *et al.*, 2021)
Suckling piglet	Oral dose	*Lactobacillus delbrueckii*	Increasing doses with $5x10^{9}$ cfu/mL	↑ antioxidant capacity and intestinal immune response (↑ secretory IgA, cytokines and chemokines in intestinal mucosa)	(Y. Li *et al.*, 2019)
Suckling piglet	Oral dose	*Lactobacillus salivarius* B1	$5x10^{9}$ cfu/ml	↑ intestinal immunocompetent cells, ↑ IL-6 and TLR2 intestinal gene expression, ↑ maturation fecal microflora	(Zhang *et al.*, 2011)
Suckling piglet	Oral dose	*Lactobacillus salivarius*	10 ml with $1x10^{8}$ cfu/mL	↑ *Lactobacillus* and villus height in the duodenum, jejunum, and ileum	(Moturi *et al.*, 2021)
Suckling piglet	Oral dose	*Lactobacillus rhamnosus* GG (ATCC 53103)	2 ml with $5x10^{8}$ cfu/ml	↑ BW, ↑ ADG, ↓ Diarrhea index, ↑ immunologic intestinal barrier	(Y. Wang *et al.*, 2019)
Suckling piglet	Oral dose (bolus)	*Bifidobacterium animalis* (DSM15954) + *Lactobacillus acidophilus* (DSM13241) + *Lactobacillus casei* (ATCC55544) + *Lactobacillus pentosus* (DSM14025) + *Lactobacillus plantarum* (DSM13367)	$1x10^{9}$ cfu/g each ($5x10^{9}$ cfu/3ml peptone-water)	↓ *Clostridium perfringens* and coliforms in SI and colon, commensal *Lactobacillus* more closely associated with enterocytes	(Siggers *et al.*, 2008)

Suckling piglet	Oral dose	*Lactobacillus plantarum* + *Lactobacillus casei* + *Lactobacillus gasseri* + *Enterococcus faecium*	2 mL with $2.0x10^6$ + $1.0x10^6$ + $1.0x10^6$ + $1.0x10^6$ cfu/mL	↑ ADG, ↑ duodenal villus	(Haupenthal *et al.*, 2020)
Suckling piglet	Oral dose	*Bifidobacterium bifidum* + *Lactobacillus acidophilus* + *Lactobacillus plantarum* + *Enterococcus faecium* + *Saccharomyces cerevisiae*	1 mL with $3.3x10^6$ + $3.3x10^6$ + $1.7x10^6$ + $1.7x10^6$ + $3.3x10^5$ cfu/mL	↑ ADG, ↑ duodenal villus	(Haupenthal *et al.*, 2020)
Suckling piglets	Oral gavage (10 ml/day)	*Saccharomyces cerevisiae* S288c	$2.0x10^8$ CFU/ml	↑BW, ↑ADFI, ↓Mortality, ↓ Diarrhea index	(Xu *et al.*, 2018)
Suckling piglet	Oral dose	*Saccharomyces cerevisiae var. boulardii* CNCM-1079	4 ml with $1x10^{10}$ cfu	↑ BW, ↑ ADG, microbial alteration with ↑ *Erysipelotrichaceae*	(Luise, Spinelli, *et al.*, 2021)
Suckling piglet	Creep-feed	*Lactobacillus acidophilus* + *Lactobacillus bulgaricus* + *Bacillus subtilis* + *Saccharomyces cerevisiae*	$9x10^8$ + $2x10^6$ + $9x10^8$ + $8x10^6$ cfu/g	↓ coliforms in colon, ↑ bifidobacteria in ileum and colon	(Shim *et al.*, 2005)
Suckling piglet	Creep-feed	*Enterococcus faecium* + *Lactobacillus acidophilus* + *Lactobacillus casei* + *Lactobacillus plantarum*	$1x10^6$ cfu/g each	↓ Diarrhea index and severity	(Tissopi *et al.*, 2019)

[a] CFU = Colony forming units; G = Gestation; L = Lactation; P = Creep-feed suckling piglets.
[b] BW = Body weight; FCR = Feed conversion ratio; ADG = Average daily gain; ADFI = Average Daily Feed Intake; SCFA = Short-chain fatty acid; SI = Small intestine; LAB = Lactic acid bacteria.

Chapter 3

Hypothesis and objectives

Hypothesis and objectives

The present doctoral thesis is part of the project AGL2016-75463-R, funded by the Ministry of Economy, Industry, and Competitiveness (MINECO) of Spain within the framework of Proyectos I+D+I Convocatoria RETOS 2016 and the State Plan for Scientific and Technical Research and Innovation, which aimed to delve into the study of early factors in the life of the piglet that can determine its response capacity and subsequent productive performance. The project pays special attention to those mechanisms that are established through communication between the microbiota and the host during the first weeks of life. For that, different positive scenarios based on providing the animal with adequate interaction with its environment during the perinatal period (nutrients, microorganisms, handling) were proposed. Some of them, such as the use of probiotics in sows or the environmental enrichment of newborn piglets, are included in this work.

Therefore, the starting hypotheses of this Ph.D. dissertation were:

a) There is a pattern of gut microbial colonization during the early life of the piglets that can vary due to differences in the environment and husbandry practices of the farms.

b) This pattern of perinatal gut microbial colonization of piglets is relevant for the development of the digestive and immune function and can have an impact on the animal response after weaning.

c) The inclusion of probiotic microorganisms in the sow's diet during pregnancy and lactation period can modulate the gut colonization pattern of the piglet in the post-natal period through its mother.

d) Combining early socialization and environmental enrichment in the nursery can improve the intestinal colonization of suckling piglets and their adaptation to the stress of weaning.

Consistent with the initial hypotheses, the main objective of the project was, therefore, to delve into the study of factors early in the life of the piglet that can determine its response capacity after weaning, paying special attention to

those that are established through communication between the microbiota and the host during the first weeks of life. Moreover, this project aimed to provide evidence on the importance of the different events conditioned by the handling and feeding situations of the sows and their litters during pregnancy and/or lactation.

Thus, the specific objectives were:

1) Determine, using massive sequencing techniques of the 16S rRNA gene (Illumina MiSeq), the succession of microbial colonization that occurs in commercial practice and identify differentiated patterns based on different farm scenarios (**Chapter 4**).

2) To provide further clarity in the complex interactions between the piglets and their intestinal microbiota during the suckling and weaning period, by using newly developed methodologies for the study of the intestinal microbiota (High-Throughput sequencing of the 16S RNA gene with Illumina-Mi Seq), host gene-expression with the OpenArray® technology and a metabolomic approach by Nuclear Magnetic Resonance (NMR) (**Chapter 5**).

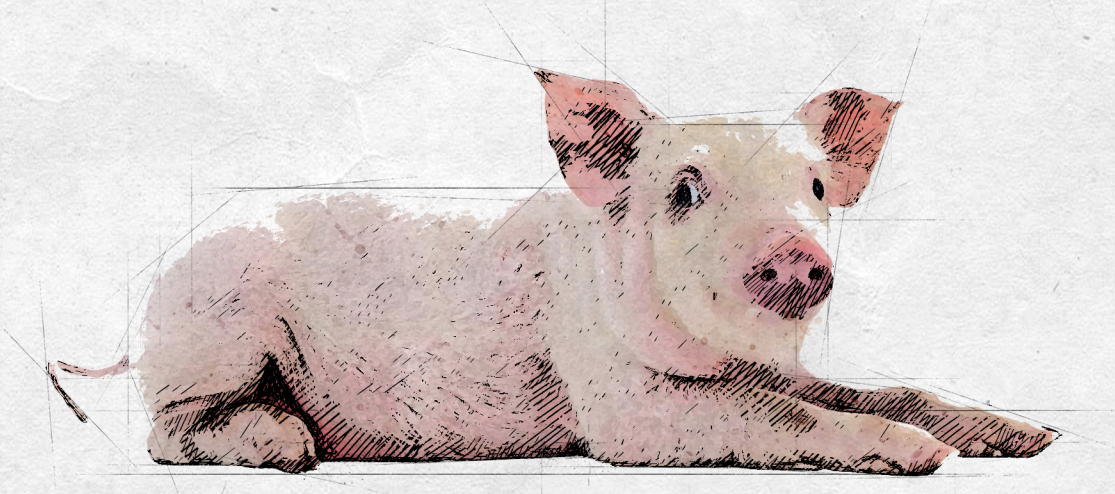
3) To determine whether the inclusion of one of two *Bacillus* probiotic strains (5x108 cfu/kg feed): *Bacillus subtilis* strain EB15 (DSM 25841) or *Bacillus amyloliquefaciens* strain ZM16 (DSM 25840) in the mother's diet during gestation and the lactation period is capable of modifying the piglet's intestinal microbiota in the post-natal period, and to determine medium-term changes in the productive response after weaning (**Chapter 6**).

4) Demonstrate the relevance of the combined effects of early socialization and neonatal enriched environment during lactation on the pattern of cecal microbial colonization, the jejunal gene expression, the serum metabolome and the intestinal physiology of the piglets before and after weaning, and the ability of piglets to adapt to weaning stress (**Chapter 7**).

To assess each of these objectives, four different trials were performed. Results are shown and discussed in Chapters 4 to 7.

Chapter 4

An insight into the commercial piglet's microbial gut colonization: from birth towards weaning



An insight into the commercial piglet's microbial gut colonization: from birth towards weaning

4.1. Abstract

Background: The establishment of the gut microbiota can be influenced by several perinatal factors, including, most importantly, the maternal microbiota. Moreover, early-life environmental variation affects gut microbial colonization and the intestinal health of offspring throughout life. The present study aimed to explore the development of piglet gut microbiota from birth to weaning in the commercial practice and also to assess how different farm environments could condition this process. To achieve this objective, we performed two trials. In a first Trial, we selected 2 farms in which we performed an intensive sampling (5 samples /animal) to characterize the gut colonization pattern during the first days of life and to identify the time window with the greatest impact. Both farms differed in their health status and the use of antimicrobials. In a second Trial, we selected 4 additional farms with variable rearing conditions and a simplified sampling pattern (2 samples/ animal). Faecal samples were obtained with swabs and DNA was extracted by using the PSP® Spin Stool DNA Kit and sequencing of the 16S rRNA gene (V3-V4 region) performed by Illumina MiSeq Platform.

Results: Alpha diversity was strongly affected by age, with an increased richness of species through time. Beta diversity decreased after weaning, suggesting a convergent evolvement among individuals. Regarding the structure of the microbiota, a clear clustering of the samples according to age was observed. The present study contributes to better understanding the microbiome development during the transition from birth to weaning in commercial conditions. We pinpointed the early intestinal colonizers belonging to *Bacteroides*, *Escherichia-Shigella*, *Clostridium sensu stricto 1*, and *Fusobacterium* genera. During lactation, the higher relative abundances of *Bacteroides* and *Lactobacillus* genera were correlated with a milk-oriented

microbiome. As the piglets aged and after weaning, increasing abundances of genera such as *Prevotella*, *Butyricimonas*, *Christensenellaceae R-7 group*, *Dorea*, *Phascolarctobacterium*, *Rikenellaceae RC9 gut group*, *Subdoligranulum*, and *Ruminococcaceae UCG-002* were observed. These changes indicate the adaptation of the piglets to a cereal-based diet rich in oligosaccharides and starch. The farm showed to have an impact on biodiversity and specific taxa, evidencing the influence of different environments and rearing systems on the gut microbiota development in the young piglet. In Trial 1 piglets receiving intramuscular amoxicillin (days 2-5 of life) and being offered an acidifying rehydrating solution (Alpha farm) showed a greater alpha diversity and increased Lactobacillus counts throughout the study. Differences related to farms were more noticeable after weaning than during lactation. In Trial 2, the only farm that did not offer an acidified rehydrating solution showed a lower alpha diversity (day 2 of life) and increased abundance of Enterobacteriaceae (both at 2 and 21 days). The use of in-feed antibiotics in the sows was also associated with changes in several taxonomic piglet microbial groups.

Conclusions: In conclusion, during the first weeks of life, the pig microbiota showed a relevant succession of microbial groups towards a more homogeneous and stable ecosystem better adapted to the solid dry feed. In this relevant early-age process, the rearing conditions, the farm environment, and the antimicrobial use in piglets and mothers determine changes that could have a relevant impact on gut microbiota maturation. More research is needed to elucidate the relative impact of these farm-induced early changes life-long in the growing pig.

Keywords: pig, suckling piglet, weaning, microbiota, colonization, lactation, gut health, commercial farm.

4.2. Background

The process of microbial colonization of the gut after birth plays an important role in the development of the neonatal immune system of mammals with implications during their whole life (Houghteling and Walker, 2014). Immediately after birth, environmental and maternal bacteria, including colonization via the vagina, nipple surface, and milk, quickly colonize offspring gut and establish the initial microbiota of the piglet (Konstantinov *et al.*, 2006; Jost *et al.*, 2014; Xue Chen *et al.*, 2018). The intestinal microbiota protects against colonization by pathogens by bacterial competition and interaction (H. Y. Cheng *et al.*, 2019). The disruption of the healthy microbial community during the neonatal period may lead to the overgrowth of indigenous pathobionts and induction of pro-inflammatory status (Mulder *et al.*, 2009; Schmidt *et al.*, 2011). It has been shown that stress, diet, management practices, and antimicrobial compounds during the early-life period may induce a long-lasting impact on the establishment of gut microbiota, gut biology, disease susceptibility, and growth performances of offspring pigs (Jurgens, Rikabi and Zimmerman, 1997; Thompson, Wang and Holmes, 2008; Schmidt *et al.*, 2011; Isaacson and Kim, 2012; Schokker *et al.*, 2014, 2015; Zhang, 2014; Mu *et al.*, 2017). This is especially relevant in swine production in which differences in husbandry and farm environment along the first days of life, could have a long-lasting impact on animal health and productive outcome. For instance, some authors have described how different exposure to stress, or the use of antibiotics can determine changes in the gut microbial colonization of piglets 8 days after birth with implications in the immune development (Schokker *et al.*, 2014). Evidence has also been published defining differences in the faecal microbiota of piglets of as early as 7 days of life, determining their susceptibility to suffering post-weaning diarrhoea four weeks later (Dou *et al.*, 2017), emphasizing the potential of the early microbiota establishment on the development of the immune response. It is, therefore, crucial to accurately determine the early-life development of the gut microbiome to eventually unravel microbiome effects on host phenotype.

In commercial pig husbandry, weaning is an abrupt event comprising a dietary shift from sow milk to solid-feed-based diets, which poses a challenge to piglets during early-life development. During the pre-weaning phase, microbiome composition is dominated by a milk-oriented microbiome

composed of families like *Bacteroidaceae* and *Lactobacillaceae* (Frese *et al*., 2015; Saladrigas-García, D'Angelo, Ko, Nolis, *et al*., 2021), which rapidly changes after weaning when a solid cereal-based diet is introduced. For instance, butyrate-producing genera such as *Prevotella*, having a very low abundance in suckling piglets, dramatically increase post-weaning due to the availability of complex oligosaccharides and polysaccharides in the feed (Frese *et al*., 2015; Mach *et al*., 2015; Zhao *et al*., 2018). The rapidly changing microbiome of the young piglets seems to increase in diversity and richness along with the suckling phase and gradually stabilizes postweaning (Kim *et al*., 2011; Frese *et al*., 2015; Slifierz, Friendship and Weese, 2015; Chen *et al*., 2017; Saladrigas-García, D'Angelo, Ko, Nolis, *et al*., 2021). However, despite the recent advances in the knowledge of the development of intestinal microbiota in the young pig, there is still a lack of knowledge regarding how this patter can be influenced by the farms' management practices and the microbiological environment in which piglets are raised.

Considering the great importance of the early gut microbiota development for pig future health and productive life, the objective of this work was, therefore, to determine, using massive sequencing techniques of the 16S rRNA gene (Illumina MiSeq), the succession of microbial colonization that occurs in those piglets reared in commercial conditions to identify pattern changes characteristic of the management guidelines. For that purpose, a longitudinal analysis of the faecal microbiota along the first days of life was performed in six different farms differing in their sanitary status, management guidelines, and particularly in the use of antimicrobials in piglets and sows.

4.3. Methods

4.3.1. Animal ethics and experimental design

The experimental work was approved by the Animal Ethics Committee at the Autonomous University of Barcelona. Faecal swab collections were performed as per standard operative procedures approved by the Animal Ethics Committee.

A longitudinal analysis of the development of the faecal microbiota of piglets reared under commercial conditions was carried out in two different stages of the research. A total of 6 farms (S1 and S2) were selected, two were included in a first Trial and four in a second Trial. The first Trial included an intensive sampling during the first days of life (5 time-points each animal on days 2, 7, 14, 21, and 36 of life), and the second Trial a simplified sampling pattern (2 time-points each animal on days 2 and 21). Stool samples were collected from the piglets by rectal swab. General information about the six farms involved in the study can be found in **Table 4.1.**

The two farms included in the first Trial (Alpha and Bravo farms) were selected based on their different health status and use of antimicrobials. Whereas Alpha could be considered a high standard farm with a low incidence of pathologies, Bravo frequently coursed episodes of pleuropneumonia (*Actinobacillus pleuropneumonia*, APP) and swine dysentery (*Brachyspira hyodysenteriae*). Alpha was involved in an antibiotic reduction program and piglets received an intramuscular dose of amoxicillin (15 mg amoxicillin/kg BW) between their second and fifth day of life; moreover, they were given an oral rehydrating and acidifying solution the first week (Hidracid®, MEVET S.A.U, Lleida, Spain) including dextrose, sodium chloride, glycerine, mono-basic potassium phosphate, mono, di and triglycerides of medium-chain fatty acids (C4, C8, and C10), potassium chloride and organic acids (formic acid, sodium formate, propionic acid, sodium citrate). In Bravo, piglets did not receive any antibiotic treatment, although the mothers received a tulathromycin injection on day 16 post-partum (2.5 mg tulathromycin/kg BW). The piglets were also given an oral rehydrating solution the 1st week of life (Hidravall®, MEVET S.A.U, Lleida, Spain) including dextrose, sodium chloride, monopotassium phosphate, and potassium chloride. In both farms, piglets were offered creep feed without zinc oxide. Mothers received a lactating non-medicated feed (NMF). After weaning the piglets were mixed according to the usual management procedures in each farm. The piglets were fed a feed without ZnO in all cases. In each farm, 10 litters were randomly selected, and one 1 piglet per litter was sampled. The same piglet was sampled on d2, d7, d14, and d21 of lactation and d14 after weaning (d36 of life), obtaining a total of 100 samples for Trial 1. However, in Alpha farm, the faecal sampling after weaning had to be anticipated due to an imminent treatment of the piglets (d7 after weaning and d29 of life) because of a diarrhoea outbreak.

Table 4.1. Additional general information about each one of the six farms involved in the study.

	Trial 1		Trial 2			
Farm ID	**Alpha**	**Bravo**	**Charlie**	**Delta**	**Echo**	**Foxtrot**
Number of sows	1330	5144	3240	1800	518	2790
Production schedule	Weekly	Weekly	Weekly	Weekly	4-week period	Weekly
Biosafety level	High	High	High	High	High	High
Closed-cycle farm	No	No	No	No	No	No
Farm involved in an AB reduction program	Yes	No	No	No	No	No
Sow vaccination program	PRRS, porcine parvovirus, swine erysipelas, Aujeszky, Actinobacillus pleuropneumonia (APP), colibacillosis and rotavirus in gilts	PRRS, porcine parvovirus, swine erysipelas, Aujeszky, APP (toxoid), Mycoplasma hyopneumoniae, swine influenza, colibacillosis + clostridiosis, rotavirus in gilts and swine dysentery autovaccine	PRRS, porcine parvovirus, swine erysipelas, Aujeszky and colibacillosis	PRRS, porcine parvovirus, swine erysipelas, Aujeszky and colibacillosis	PRRS, porcine parvovirus, swine erysipelas, Aujeszky, colibacillosis and rotavirus in gilts	PRRS, porcine parvovirus, swine erysipelas, Aujeszky, swine influenza, colibacillosis and porcine circovirus
Recurring sanitary problems	No	APP and B. *hyodysenteriae*	No	No	*B. hyodysenteriae*	*B. hyodysenteriae*

Piglet processing at birth	Tail docking, iron administration by injection, oral administration of coccidiostat and antibiotic treatment (injected)	Tail docking, iron administration by injection and oral administration of coccidiostat	Tail docking, iron administration by injection, oral administration of coccidiostat and antibiotic treatment (injected)	Tail docking, iron administration by injection, oral administration of coccidiostat and antibiotic treatment (injected)	Tail docking, iron administration by injection, oral administration of coccidiostat and antibiotic treatment (injected)	Tail docking, iron administration by injection, oral administration of coccidiostat and antibiotic treatment (injected)
Regular use of creep-feeding	Yes	Yes	Yes	Yes	Yes	Yes
Use of antimicrobials in creep-feeding (including ZnO)	No	No	No	No	No	No
Regular use of re-hydrating solution during lactation	Yes	Yes	Yes	Yes	Yes	No
Piglet age at weaning	21 days	21 days	28 days	21 days	21 days	21 days
Cross fostering and litter management at birth	Nursing mothers at 24 hours. Minimal movements	Equalization of litters trying to minimize movements. Piglets are collected only during the 1st week.	Minimal movements	Minimal movements	Minimal movements	Minimal movements
Medicated maternal feed during lactation	No, **NMF** diet	No, **NMF** diet[1]	No, **NMF** diet	No, **NMF** diet	Yes, **ABF** diet[2]	Yes, **ABF** diet[2]

[1] Bravo farm sows routinely received a tulathromycin injection on day 16 postpartum (2.5 mg tulathromycin/kg BW).
[2] ABF diet consisted of the same NMF diet supplemented with 600 ppm of lincomycin

For the second Trial of the study, four farms were selected (Charlie, Delta, Echo and Foxtrot). In two of them (Charlie and Delta) sows were fed NMF and in the other two (Echo and Foxtrot) sows received medicated feed with lincomycin (600 ppm) (ABF). Piglets from all farms were offered creep feed without ZnO and piglets from Charlie, Delta and Echo farms a rehydrating oral solution during the first week of life (Hidravall®, MEVET S.A.U, Lleida, Spain). In each farm 15 litters were randomly selected, sampling one piglet per litter. The same piglet was sampled on d2 and d21. Due to some casualties on d21, ultimately 102 paired samples (d2 + d21 from the same animal) were analysed. Information regarding the composition of lactation diets (NMF and ABF) is provided in **Annex 2: Table S4.1.**

4.3.2. Faecal DNA extraction and 16S rRNA sequencing

Stool samples were taken with the Stool Collection Tubes with DNA Stabilizer (STRATEC Molecular GmbH, Berlin, Germany). DNA was extracted from 1.4 mL of each stool sample using the PSP® Spin Stool DNA Kit (STRATEC Molecular GmbH, Berlin, Germany) according to the manufacturer's instructions following the optimization steps for bacterial DNA enrichment. DNA concentration and purity were verified with the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA.). For high-throughput sequencing of the faecal microbiota, the MiSeq® Reagent Kit V2 (500 cycles) (Illumina, San Diego, CA, USA) was used and the V3-V4 region of 16S rRNA was targeted. All subsequent steps were performed on the MiSeq Illumina instrument.

4.3.3. Bioinformatics and statistical analysis

Raw sequencing reads (Fastq files) were independently processed, aligned and categorized using Divisive Amplicon Denoising Algorithm 2 (DADA2) (Callahan *et al.*, 2016), which was run as an R script (in R v.4.0.2) using its R package (dada2 v.1.16.0). Sequence reads were filtered using the recommended DADA2 parameters (that is, an expected error threshold of 2) and chimeras were removed. Afterwards, sequences were processed into Amplicon Sequences Variants (ASV) at 99% of identity. ASV were classified to the

lowest possible taxonomic level based using the SILVA reference database (v138) provided by the SILVA web service (Quast *et al.*, 2013). The diversity patterns within the ASV table were analysed using a custom bioinformatics pipeline implemented in R 4.0.2 (http://www.r-project.org). Support for DADA2 in R was achieved through the phyloseq package (v.1.32.0; available at https://joey711.github.io/phyloseq/) (McMurdie and Holmes, 2013). The alpha diversity metrics were calculated using the phyloseq "estimate_richness" function from the rarefied ASV tables and using the microbiome package (v.1.10.0) (Lahti *et al.*, 2017). The observed species, the Chao1 index, the Simpson and inverse Simpson metrics and the Shannon diversity measures were estimated. For beta diversity, measurements were calculated using the Whittaker index (Whittaker, 1960) and the betadisper () function of the vegan package (v.2.5.6) (Oksanen *et al.*, 2013) using relative abundances. To compare any differential effects, an ANOVA analysis was performed for alpha richness and diversity. The Non-metric multidimensional scaling (NMDS), analysis of similarities (ANOSIM), permutational analysis of variance (PERMANOVA) and the method of grouping of unweighted pairs with hierarchical grouping of arithmetic mean (UPGMA), all of them based on the distance of Bray-Curtis, were carried out to test the significance of differences in overall microbial composition. The normalization of the raw counts was performed using cumulative sum scaling (CSS) (Paulson, Stine, *et al.*, 2013) and the differential abundance analysis was performed following the metagenomeSeq package (v.1.30.0) (Paulson, Talukder, *et al.*, 2013). The taxa were aggregated at the phylum, family and genus level and expressed as compositional data. Environmental (farm or diet group) and host-covariates (age) were all considered factors that might modulate the diversity, structure and profile of the microbial communities. All p-values from multiple testing were corrected with a false discovery rate according to the procedure by Benjamini–Hochberg (Benjamini and Hochberg, 1995). Differences were expressed as significant if adjusted $p \leq 0.05$ or tendencies if $0.05 < p < 0.1$. Results are presented as the mean ± the standard error.

4.4. Results

4.4.1. Trial 1: Intensive sampling in two farms with different health statuses

4.4.1.1. Changes in microbiota structure and biodiversity

An average of 55,190 ± 6,265 amplicon sequence variants (ASV) were obtained per sample, for a total of 100 samples of faecal content, with an increase in species richness as the piglets aged (P=0.002). Higher richness values and reads were also detected in piglets from Bravo compared to Alpha farm (P<0.001 and P=0.007 for species richness and reads, respectively).

Regarding changes in the ecosystem structure with age and environmental factors, PERMANOVA multivariate analysis showed that both, farm and animal age, showed significant effect shaping the gut communities (P<0.001). The non-metric multidimensional scaling (NMDS) based on the Bray–Curtis distance, showed five clusters that matched with piglet age (**Figure 4.1.**). The diversity indices also revealed important differences related to farms and ages. Following the richness results, greater alpha diversity was observed in Bravo farm and with the increasing age of piglets (P<0.001), as presented in **Annex 2: Table S4.2.** In addition, measured by the ANOSIM Bray-Curtis dissimilarities matrix, beta diversity also showed increased values with age (P=0.001) suggesting that as the piglets grow and the gut community becomes more diverse, the divergence among animals increases.

To better analyse the impact of the farm, a complementary analysis was performed by sampling day. PERMANOVA within each sampled age (**Annex 2. Figure S4.1.**) showed a clear effect of the farm on community composition on all sampling days (P < 0.01). Along lactation differences between farms in alfa and beta diversity were significant at days 2, 7 and 14 of life, showing Bravo farm higher values (P < 0.05 for all indices). However, no differences were found at day 21, probably due to the establishment of a more mature microbiota. Finally, after weaning a large impact of the farm of origin was observed (PERMANOVA, P < 0.001), in part due to the advanced sampling in the Alpha farm (7 days earlier than in the Bravo farm). Differences in the alpha and beta diversities between farms were also seen after weaning (P<0.001).

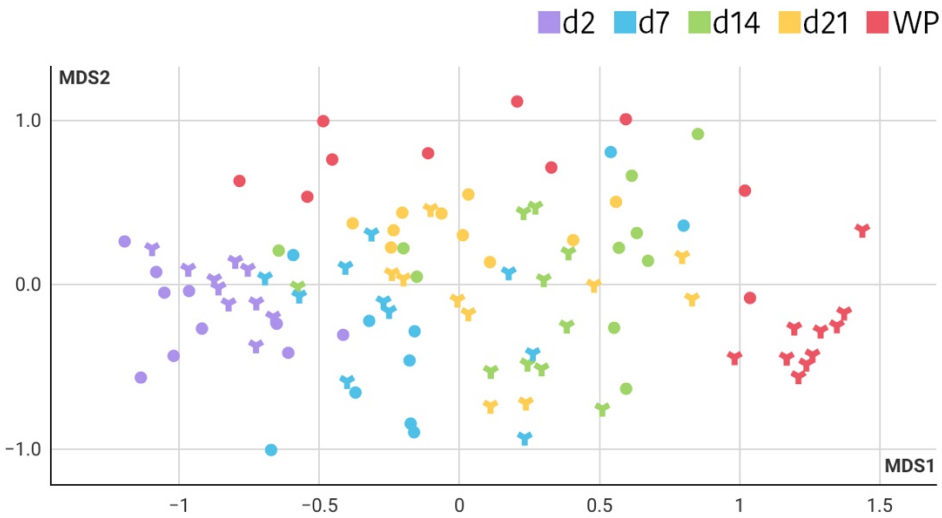


Figure 4.1. NMDS of the relative abundances of ASV in Trial 1. Different colours have been used to highlight each sampling day. The farm of origin is indicated with a different shape, round for the Alpha farm and with a 3-pointed star for the Bravo farm. The age factor separates individuals into distinct clusters. The significant effect of the farm is observed more clearly when performing the analysis by sampling day (**Annex 2. Figure S4.1.**).

4.4.12. Changes in taxonomic groups

The temporal progression in the relative abundances of the main families is represented in **Table 4.2** and **Figure 4.2**. In the sampling closest to birth (day 2 of life), the microbiota of the piglets was composed of bacteria belonging to the phyla Firmicutes (36.98%), Proteobacteria (29.68%), Fusobacteria (18.34%) and Bacteroidetes (14.30%). Within the Proteobacteria, the main families observed were *Enterobacteriaceae* (24.62%), of which 10.30% were identified as *Escherichia/Shigella*, and *Pasteurellaceae* (4.50%). The phylum Bacteroidetes was represented by its main genus *Bacteroides* (11.89%), while the phylum Firmicutes was represented by a greater variety of families and genera, such as *Clostridiaceae* (18.56%), represented mainly by *Clostridium sensu stricto 1* (4.05 %), *Lachnospiraceae* (7.87%), *Streptococcaceae* (3.41%) and *Lactobacillaceae* (1.97%), with a great relative abundance of its genus *Lactobacillus* (4.21%). Finally, the high presence of bacteria from the *Fusobacteriaceae* family (18.31%) and its genus *Fusobacterium* (10.04%) was found very characteristic of young piglets (d2 and d7).

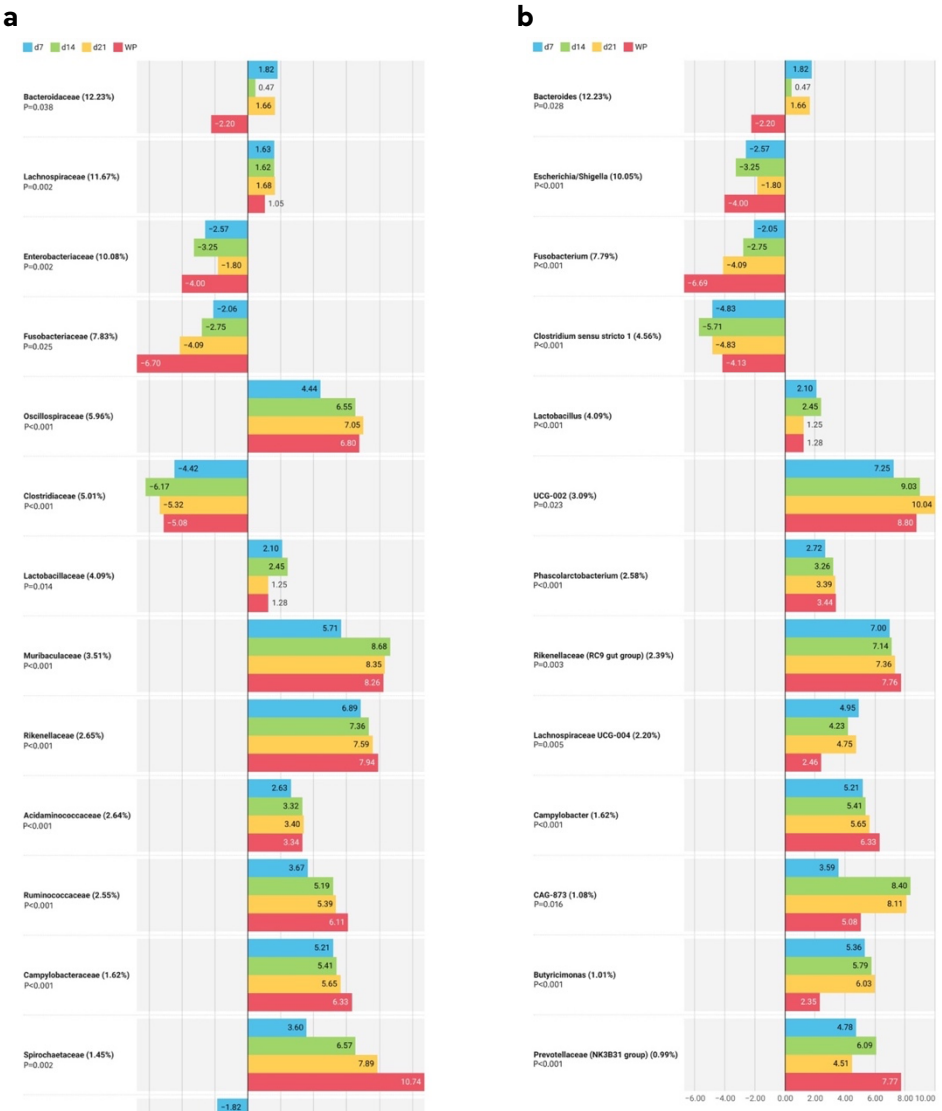


Figure 4.2. Differentially abundant taxa from faecal content (ln change and P-value<0.05) among repeated samplings at family (**a**) and genus (**b**) level. Only predominant (>1%) significant families and genera are presented; positive values and negative values indicate greater and lower abundance, respectively, in repeated samplings (d7, d14, d21 and weaned piglet) compared to new-born piglets (d2); taxa are sorted according to the general mean of relative abundance (the average of the entire trial, indicated between brackets in %) and in decreasing order. Figure created with the online open-source tool Datawrapper.

Table 4.2. Relative abundances (%) of the families present in the faecal microbiota of the piglets in a percentage higher than 0.5%. Results came from 20 piglets sampled from two different farms along the first days of life. Faecal samples were collected on days 2, 7, 14 and 21 of lactation and 14 days after weaning (36 days of life). Results are presented in decreasing order of abundance concerning the general average. The colours represent the phyla to which the families belong. Significant changes are highlighted in **bold**.

	d2	d7	d14	d21	WP	SEM	P-value
Bacteroidaceae	10.64	17.28	10.07	14.97	8.18	1.185	**0.038**
Lachnospiraceae	7.87	12.26	14.84	14.56	8.81	0.648	**0.002**
Enterobacteriaceae	24.62	4.67	6.44	6.66	8.03	1.272	**0.002**
Prevotellaceae	3.12	9.71	7.42	7.29	11.87	0.891	0.116
Fusobacteriaceae	18.31	11.04	4.11	2.19	3.51	1.057	**0.025**
Oscillospiraceae	0.32	5.40	7.66	7.85	8.58	0.625	**0.000**
Clostridiaceae	18.56	1.03	1.66	1.23	2.59	0.892	**0.000**
Lactobacillaceae	1.97	6.19	5.26	3.99	3.03	0.513	**0.014**
no_match[a]	0.23	3.25	5.91	4.46	6.53	0.368	**0.012**
Muribaculaceae	0.04	3.23	5.54	5.27	3.47	0.455	**0.000**
Rikenellaceae	0.01	2.72	3.46	3.93	3.13	0.325	**0.000**
Acidaminococcaceae	0.47	3.48	3.46	2.95	2.86	0.221	**0.000**
Ruminococcaceae	0.20	2.64	3.81	2.75	3.36	0.301	**0.000**
Streptococcaceae	3.41	1.06	2.05	0.46	1.55	0.303	0.116
Campylobacteraceae	0.14	1.64	2.37	1.50	2.43	0.259	**0.000**
Spirochaetaceae	0.00	0.82	0.81	0.87	4.73	0.292	**0.002**
Pasteurellaceae	4.50	0.90	0.70	0.65	0.38	0.244	**0.006**
Erysipelotrichaceae	0.17	0.95	1.49	1.59	1.82	0.145	**0.019**
Marinifilaceae	0.16	1.00	1.37	2.23	1.12	0.151	**0.000**
Christensenellaceae	0.00	0.61	1.37	2.00	1.59	0.176	**0.000**
Veillonellaceae	1.96	1.34	0.68	0.75	0.39	0.143	0.168

Tannerellaceae	0.22	1.03	0.82	1.17	1.17	0.109	**0.004**
Selenomonadaceae	0.01	1.13	0.78	1.77	0.36	0.252	0.239
Desulfovibrionaceae	0.17	0.80	0.94	1.27	0.73	0.072	**0.000**
Enterococcaceae	0.85	0.56	0.97	0.83	0.32	0.167	0.999
Sutterellaceae	0.25	0.65	0.52	0.48	0.27	0.055	0.082
Erysipelatoclostridiaceae	0.02	0.38	0.50	0.58	0.61	0.057	**0.007**
p-2534-18B5_gut_group	0.00	0.15	0.26	0.50	1.10	0.107	0.239
Anaerovoracaceae	0.00	0.43	0.47	0.29	0.72	0.057	**0.000**
Butyricicoccaceae	0.53	0.45	0.34	0.21	0.26	0.058	0.190
Succinivibrionaceae	0.00	0.36	0.25	0.37	0.72	0.095	**0.003**
UCG-010	0.00	0.15	0.20	0.28	0.84	0.052	0.065
Akkermansiaceae	0.00	0.29	0.01	0.65	0.40	0.096	**0.006**
Helicobacteraceae	0.01	0.42	0.06	0.13	0.57	0.083	**0.003**
Synergistaceae	0.00	0.02	0.16	0.70	0.20	0.053	0.130
Bacteroidales_RF16_group	0.00	0.08	0.08	0.13	0.48	0.048	0.999

[a]no_match = Not assigned taxa.

■: *Firmicutes* ■: *Bacteroidetes* ■: *Fusobacteria* ■: *Proteobacteria*
■: *Spirochaetes* ■: *Verrucomicrobiota* ■: *Synergistetes* □: None

At one week of life (d7), a large decrease in the relative abundance of the phylum Proteobacteria (6.89%) was observed, mainly due to the large decrease also observed in the *Enterobacteriaceae* family (4.67%), despite the fact that the relative abundance of *Escherichia/Shigella* genus level remained high (12.38%). There was a significant increase in the relative abundance of Bacteroidetes (35.64%), due to the increase in abundance of genera such as *Prevotella* (6.34%), *Rikenellaceae RC9 gut group* (1.94%) and *CAG-873* (1.82%). Firmicutes remained the predominant phylum (41.25%), although important changes were observed in its distribution, with increases in families such as *Lachnospiraceae* (12.26%), *Lactobacillaceae* (6.19%), *Oscillospiraceae* (5.40%) and *Acidaminococcaceae* (3.48%) and a large decrease in the *Clostridiaceae* family (1.03%), despite the fact that the relative abundance of

the genus *Clostridium sensu stricto 1* remained high (5.21%). Fusobacteria decreased slightly (11.95%), although at genus level *Fusobacterium* continued to predominate (10.00%).

On day 14 and day 21, the microbial composition showed a more similar outcome to each other. Firmicutes and Bacteroidetes established themselves as the predominant phyla. The abundance of Fusobacteria dropped to a great extent (4.11% and 2.19% at d14 and d21, respectively) and Proteobacteria stabilized with a relative abundance at the phylum level of 10%. At this stage, there were no major changes in the families or genera present in the microbiota. Moreover, a higher evenness was observed, as the relative abundances of taxonomic groups seemed to equal each other in abundance, with less prevalence of specific genera as in the first days of life (evenness: 0.88 ± 0.043; 0.91 ± 0.027; 0.92 ± 0.024; 0.92 ± 0.036 and 0.95 ± 0.013 for day 2, 7, 14, 21 and WP respectively). Thus, higher abundances of bacteria belonging to Firmicutes were observed, such as *Lachnospiraceae* (14.84% and 14.56% at d14 and d21, respectively), *Oscillospiraceae* (7.66% and 7.85%), *Ruminococcaceae* (3.81% and 2.75%), *Erysipelotrichaceae* (1.49) % and 1.59%) and *Christensenellaceae* (1.37% and 2.00%). A slight decrease was also observed in the relative abundance of *Lactobacillaceae* (5.26% and 3.99% at d14 and d21, respectively), and in the Bacteroidetes families, such as *Bacteroidaceae* (10.07% and 14.97% at d14 and d21, respectively) and *Prevotellaceae* (7.42% and 7.29% at d14 and d21, respectively).

Finally, after weaning, a greater variety of species and evenness among them was observed. Firmicutes (43.69%) and Bacteroidetes (31.27%) were kept as predominant phyla, followed by Proteobacteria (15.50%). The Spirochaetes phylum increased significantly in this period, with a relative percentage of 4.74%. Fusobacteria maintained levels similar to those observed before weaning (3.52%). At the family level, an increase was observed with respect to the previous sampling in *Prevotellaceae* (11.87%) and *Spirochaetaceae* (4.73%), and a decrease in *Lachnospiraceae* (8.81%). At the genus level, a decrease of *Escherichia/Shigella* was observed with respect to the lactating piglets (5.31%).

The effects of farm of origin (Alpha or Bravo) in the relative abundances of main families and genera is represented in **Figure 4.3.**

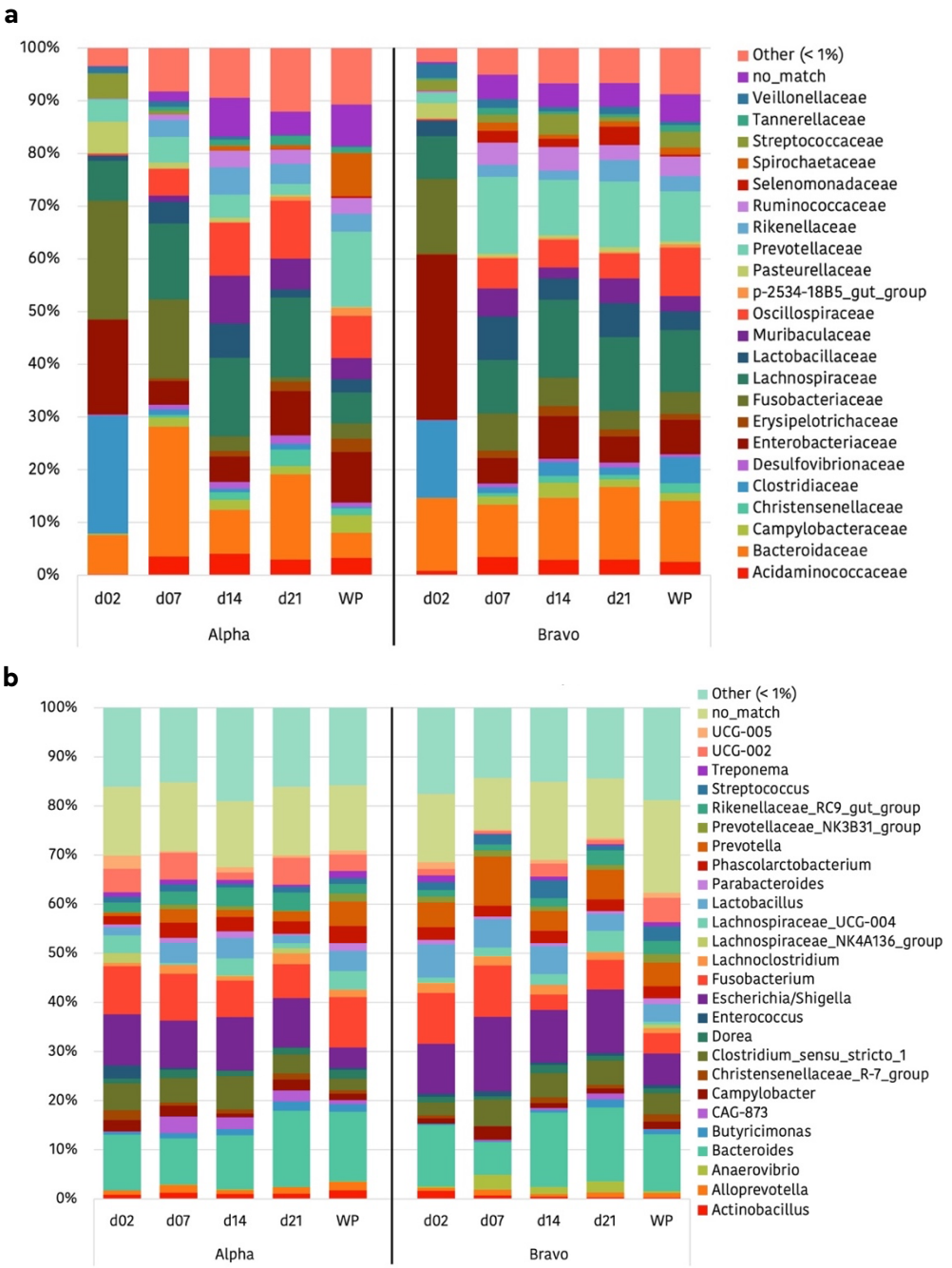


Figure 3. Bar diagram of the relative abundances (%) of the main (>1%) families (**a**) and genera (**b**), organized by farm (Alpha or Bravo) and by age. WP = Weaned piglets.

The taxonomic groups that showed significant differences between farms along the different sampling days can be found in **Annex 2: Figure S4.2.** considering only the taxa detected in at least half of the samples. In general terms, it was observed that at day 2 of life and after weaning, the impact of the farm was greater than at days 7, 14 and 21 of life, since a higher number of differential taxa were detected. After weaning, differences between farms were manifested with significant changes in more than 19 genera. It is interesting to remark the lower numbers of *Enterobacteriaceae* and *Escherichia/Shigella* genera observed in Alpha farm on days 2 and 7 of life (2.9 and 3.4 negative ln change respectively) and the opposite pattern registered after weaning (3.2 positive ln change compared to Bravo farm).

4.4.2. Trial 2: Impact of the farm management practices on piglet microbiota

4.4.2.1 Changes in microbiota structure and biodiversity

An average of 74,429 ± 3,611 amplicon sequence variants (ASV) were obtained per sample for a total of 102 samples of faecal content. As found in the previous trial, a greater richness was observed in 21-day-old piglets when compared to 2-day-old piglets ($P < 0.001$). Among the different farms, no difference was detected in terms of richness, although a higher number of reads was observed in the Foxtrot farm ($P = 0.057$).

Regarding changes in the microbial ecosystem with age, farm environmental factors and the use of medicated feed in the sows, PERMANOVA multivariate analysis showed that all factors considered (age, farm and medicated feed) had a significant impact shaping the gut communities ($P<0.001$ for age factor and $P=0.001$ for farm and feed). However, the non-metric multidimensional scaling (NMDS) based on the Bray–Curtis distance, only found significant the effect of age ($P<0.001$) with two clear clusters between 2-day-old piglets and 21-day-old piglets (**Figure 4.4.**).

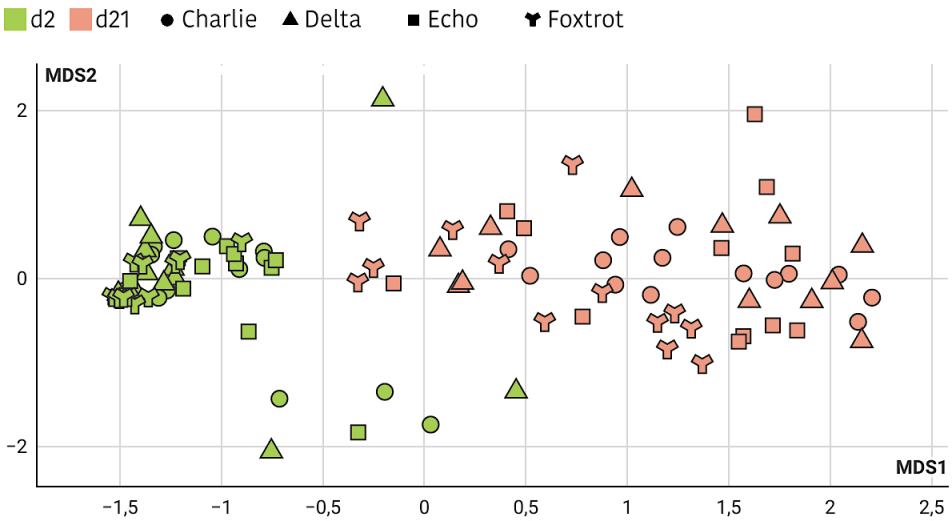


Figure 4.4. NMDS of the relative abundances of ASV in Trial 2. Different colours have been used to highlight each sampling day and different shapes to distinguish each farm. Sampling day clearly separated individuals into distinct clusters (P<0.001).

The diversity indices also revealed important differences between farms, use of medicated feed in sows and piglet age. In accordance with the richness results, a greater alpha diversity was observed in 21-day-old piglets compared to day 2, as presented in **Annex 2: Table S4.3.** Moreover, Delta and Foxtrot farms were found to have significantly lower alpha-diversities for Observed species, Chao1 and Shannon indices than Charlie and Echo (P=0.033, P=0.034 and P=0.027, respectively for farm effect) despite at day 2 of life decreases in alpha-diversity were only found in Foxtrot farm (**Figure 4.5.**). No significant impact of the use of medicated diet was found.

Regarding beta diversity calculated by means of the Whittaker index, no differences were found, neither between the sampling days, nor between farms or the use of medicated feed in sows. However, the multivariate analysis of ecological communities by the ANOSIM Bray-Curtis dissimilarities matrix, showed a significant effect of farm (P=0.011 and P=0.001, for d2 and d21, respectively) and of use of medicated feed in sows (P=0.011 and P=0.001, for d2 and d21, respectively). In 2-day-old piglets, a greater similarity was observed between the samples from the Foxtrot farm, while the rest of the farms presented similar results between them. However, on the 21st day of

life, it was the Charlie farm that stood out for its greater similarity between samples. Regarding the use of medicated feed in mothers, in 2-day-old piglets, a greater similarity was observed between the microbiota of the piglets from medicated mothers (ABF), while on day 21 the piglets from the non-medicated groups (NMF) showed a greater similarity among each other.

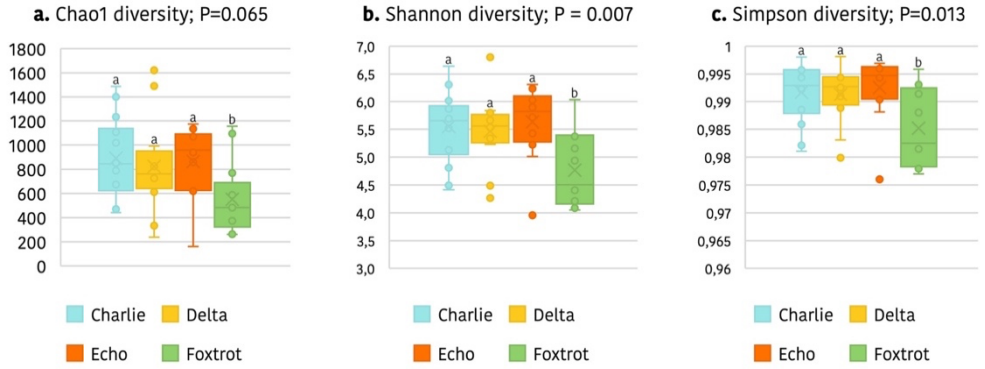


Figure 4.5. Box plot of the alpha diversity calculated using the Chao1 (**a**), Shannon (**b**) and Simpson indices (**c**) of the four farms included in Trial 2 in 2-day-old piglets. Foxtrot farm showed lower alpha diversity values than the rest of the farms.

4.4.2.2. Changes in taxonomic groups

Proteobacteria and Firmicutes constituted the two predominant phyla in the faecal microbiota of 2-day-old piglets, contributing with a 58.04 and 30.57% of the relative abundance, respectively. Bacteroidetes (7.09%) and Fusobacteria (2.80%), with a representation greater than 1% in relative abundance, were also considered predominant phyla. At day 21 of life, a very different scenario was observed, with Firmicutes and Bacteroidetes being the two predominant phyla in the faecal microbiota of the piglet, contributing 49.19 and 35.73% of the relative abundance, respectively. Proteobacteria (9.73%) and Fusobacteria (1.14%), were also considered predominant phyla. Changes at genus level with the age of the pig were also remarkable and are shown in **Figure 4.6.** From a total of 476 genera detected only 27 taxa represented a relative abundance greater than 1%.

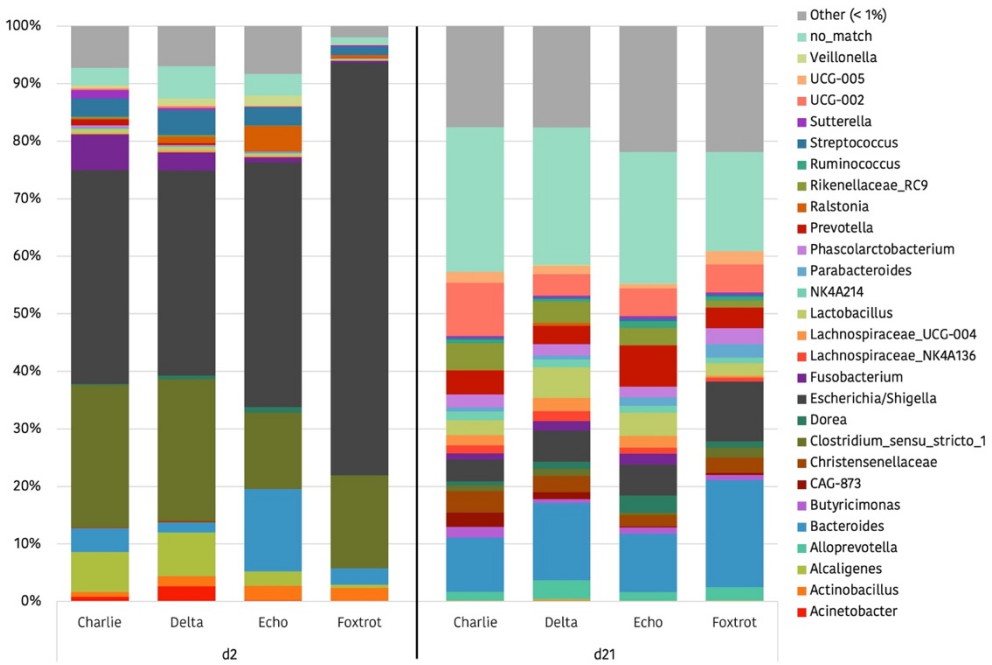


Figure 4.6. Bar diagram of the relative abundances (%) of the main genera (>1%) in Trial 2, organized by farm (Charlie, Delta, Echo or Foxtrot) and by sampling day. In Echo and Foxtrot farms the sows received medicated feed.

Regarding the possible effects of the farm on microbial taxonomy, **Tables 4.3. and 4.4.** show the relative abundances of the predominant families (>0.5%), in the different farms, on days 2 and 21 respectively, and **Figure 4.7.** shows differences at genus level. Two days after birth, significant higher levels for the *Bacteroidaceae* family were detected at the Echo farm. *Moraxellaceae* family was the lowest in the Foxtrot and the highest in the Delta farm. Trends were detected for other families, with remarkable higher levels (P= 0.059) of *Enterobacteriaceae* in the Foxtrot farm (**Table 4.3.**). At genus level, *Sutterella*, *Desulfovibrio* and *Blautia* also showed different abundance between farms (P = 0.025; P = 0.035 and P = 0.006 respectively) (**Figure 4.7.**). At day 21 of life, significant lower levels of *Lachnospiraceae* and *Fusobacteriaceae* and higher levels of *Enterococcaceae* were observed in the Foxtrot farm (**Table 4.4.**). At genus level UCG-002, *Desulfovibrio* and *Butyricimonas* also showed different abundance between farms (P <0.001; P = 0.016 and P = 0.001, respectively) (**Figure 4.7.**).

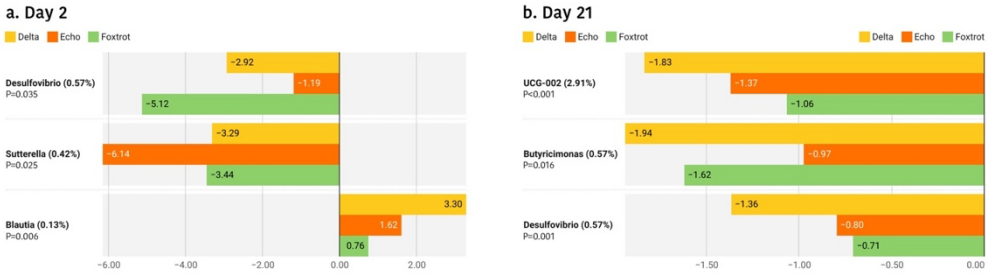


Figure 4.7. Differentially abundant taxa from faecal content (ln change and P-value<0.05) among farms at genus level in 2-day-old (**a**) and 21-day-old (**b**) piglets. Only predominant (>0.5%) significant genera are presented; positive values and negative values indicate greater and lower abundance, respectively, among farm environments (Delta, Echo and Foxtrot) compared to Charlie farm; taxa are sorted according to the general mean of relative abundance (the average of the entire trial, indicated between brackets in %) and in decreasing order. Figure created with the online open-source tool Datawrapper (http://datawrapper.de).

Table 4.3. Relative abundances (%) of the families present in the faecal microbiota of 2-day-old piglets from Trial 2 in a percentage higher than 0.5%, classified by farm and in decreasing order of abundance in relation to the general average. The colours represent the phyla to which the families belong. In Echo and Foxtrot farms, sows received medicated feed; in Charlie, Delta and Echo farms piglets were offered a rehydrating solution during lactation. Significant changes are highlighted in **bold**.

		Charlie	Delta	Echo	Foxtrot	SEM	P-value
	Enterobacteriaceae	37.38	35.76	44.72	71.82	4.393	0.059
	Clostridiaceae	26.33	25.55	14.93	17.03	2.699	0.999
	Alcaligenaceae	7.31	7.84	2.78	0.59	1.728	0.999
	Bacteroidaceae	3.13^{a}	1.50^{a}	11.19^{b}	2.33^{a}	0.996	**0.031**
	Streptococcaceae	3.33	4.44	3.45	1.54	0.605	0.079
	Lachnospiraceae	1.73	4.58	4.16	1.42	0.620	0.183
	Fusobacteriaceae	5.88	2.94	0.64	0.25	0.996	0.653
	Pasteurellaceae	0.99	2.22	3.18	2.37	0.480	0.359
	Burkholderiaceae	0.23	1.00	4.18	0.48	0.859	0.647
	Moraxellaceae	1.20^{ab}	3.03^{a}	1.04^{ab}	0.12^{b}	0.655	**0.030**
	no_match^{a}	0.98	1.91	0.62	0.20	0.304	0.506

	Veillonellaceae	0.54	1.20	1.75	0.17	0.236	0.094
	Peptostreptococcaceae	0.63	0.39	1.48	0.17	0.175	0.094
	Prevotellaceae	1.36	0.74	0.18	0.04	0.221	0.889
	Sutterellaceae	1.48	0.32	0.11	0.20	0.184	0.295
	Lactobacillaceae	0.69	0.54	0.42	0.21	0.079	0.629
	Oscillospiraceae	0.48	0.63	0.37	0.04	0.151	0.135
	Enterococcaceae	0.34	0.77	0.13	0.09	0.063	0.219
	Muribaculaceae	0.26	0.25	0.02	0.01	0.068	0.999

[a]no_match = Not assigned taxa.

■: *Firmicutes* ■: *Bacteroidetes* ■: *Fusobacteria* ■: *Proteobacteria* □: None

Table 4.4. Relative abundances (%) of the families present in the faecal microbiota of 21-day-old piglets from Trial 2 in a percentage higher than 0.5%, classified by farm and in decreasing order of abundance in relation to the general average. The colours represent the phyla to which the families belong. In Echo and Foxtrot farms, sows received medicated feed; in Charlie, Delta and Echo farms piglets were offered a rehydrating solution during lactation. Significant changes are highlighted in **bold**.

	Charlie	**Delta**	**Echo**	**Foxtrot**	**SEM**	**P-value**
Bacteroidaceae	9.37	13.95	10.18	19.05	1.459	0.252
Lachnospiraceae	10.99[a]	14.12[a]	12.75[a]	8.31[b]	0.677	**0.043**
Oscillospiraceae	14.50	8.62	9.34	10.92	0.600	0.901
Prevotellaceae	8.31	8.37	11.95	7.48	0.979	0.407
Enterobacteriaceae	3.76	5.48	5.33	10.26	1.155	0.107
no_match[a]	6.40	6.50	6.07	4.57	0.484	0.219
Muribaculaceae	8.06	5.52	5.60	3.43	0.697	0.270
Ruminococcaceae	4.47	4.28	6.93	5.26	0.421	0.747
Rikenellaceae	5.36	4.31	3.79	1.97	0.367	0.051
Lactobacillaceae	2.56	5.34	4.00	2.03	0.397	0.107
Christensenellaceae	4.53	4.01	2.09	2.98	0.316	0.382
Acidaminococcaceae	2.44	2.14	1.92	2.77	0.131	0.329
Erysipelotrichaceae	1.38	2.06	1.89	2.42	0.245	0.562
Tannerellaceae	0.75	0.72	1.68	2.34	0.176	0.782

	Marinifilaceae	2.01	0.73	1.44	1.04	0.173	0.500
	Desulfovibrionaceae	1.63	0.90	1.40	1.11	0.085	0.365
	p-2534-18B5_gut_group	1.96	1.24	0.79	0.69	0.225	0.747
	Clostridiaceae	0.94	1.18	0.40	1.69	0.264	0.181
	Fusobacteriaceae	1.19[a]	1.30[a]	1.54[a]	0.05[b]	0.321	**0.005**
	Spirochaetaceae	0.94	0.84	0.89	1.26	0.132	0.999
	Enterococcaceae	0.32[a]	0.64[a]	0.34[a]	1.83[b]	0.286	**0.005**
	UCG-010	0.83	0.82	0.67	0.40	0.064	0.155
	Comamonadaceae	0.26	0.05	0.66	1.64	0.320	0.433
	Campylobacteraceae	0.61	0.28	1.24	0.42	0.156	0.400
	Anaerovoracaceae	0.45	0.86	0.67	0.49	0.073	0.999
	Methanobacteriaceae	0.60	0.54	0.49	0.36	0.050	0.175
	Veillonellaceae	0.34	0.47	0.68	0.38	0.075	0.490
	Erysipelatoclostridiaceae	0.36	0.31	0.58	0.37	0.068	0.719
	F082	0.12	0.06	0.06	0.13	0.031	0.182

[a]no_match = Not assigned taxa.

■: *Firmicutes* ■: *Bacteroidetes* ■: *Fusobacteria* ■: *Proteobacteria*
■: *Spirochaetes* ■: *Euryarchaeota* □: None

Regarding the possible impact of the use of medicated feed in the microbiota of the progeny, **Figure 4.8.** shows those taxonomic groups (families and genera) that were significantly modified at day 2 and 21 of life. At day 2 of life, piglets from dams fed non-medicated feed (NMF) showed statistically significant lower abundances of Firmicutes and Fusobacteria phyla explained by the lower detected levels of *Fusobacteriaceae*, *Prevotellaceae*, *Sutterellaceae*, *Enterococcaceae* and *Acidaminococcaceae* families. Regarding genera, *Fusobacterium*, *Prevotella*, *Phascolarctobacterium*, *Sutterella* and *Enterococcus* also showed significant lower abundances. Moreover, NMF farms were associated with higher counts of the *Enterobacteriaceae* family and the *Escherichia/Shigella* genera. On day 21 of life, changes were only observed in minor taxonomic groups (**Figure 4.8.**).

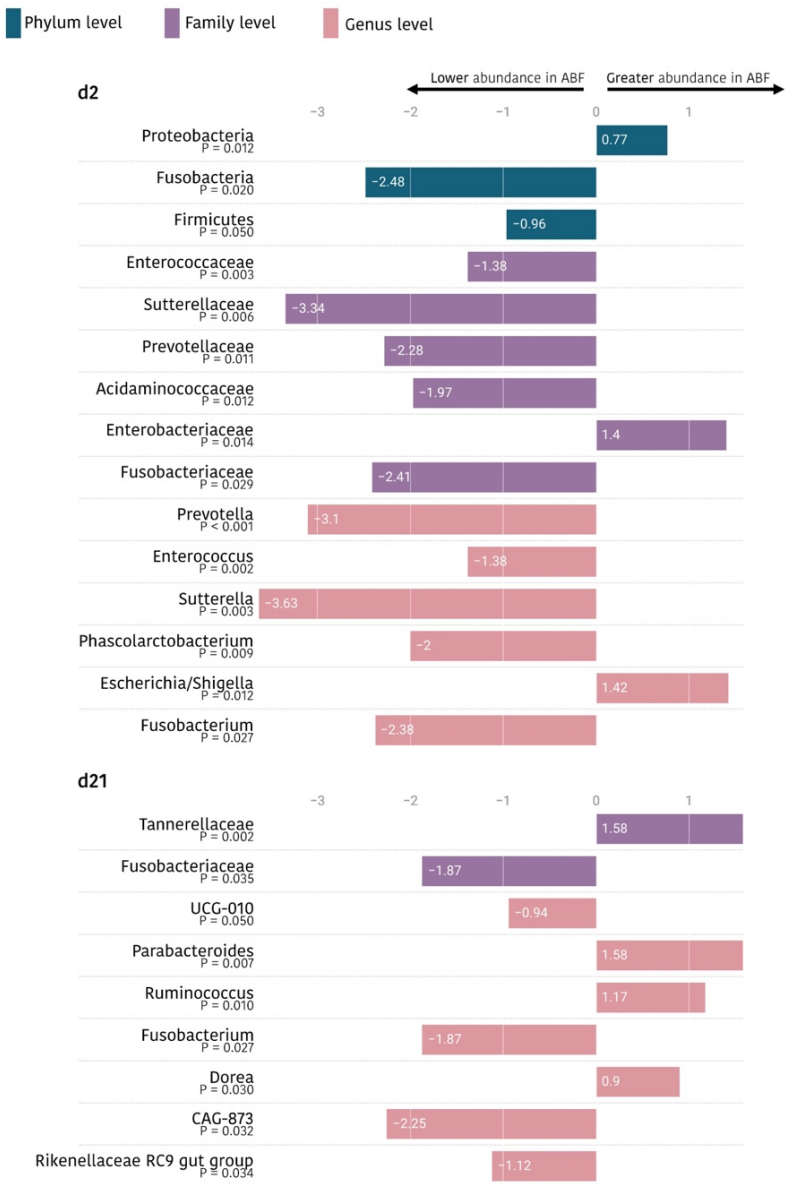


Figure 4.8. Ln changes in taxa promoted by sow feed (sows receiving (ABF) vs not (NMF) medicated feed; ln change and P-value<0.05) at phylum, family and genus level in the microbiota of piglets. Positive values and negative values indicate higher and lower abundance, respectively, in piglets from ABF-fed mothers. Taxa are sorted by level of significance (from higher to lower). Only taxa with relative abundances higher to 0.5% are included in the figure. The presented differences are based only on taxa detected in at least half of the animals per sampling. Figure created with the online open-source tool Datawrapper (http://datawrapper.de).

4.5. Discussion

4.5.1 The pattern of microbial colonization during the first weeks of life

After birth, the intestinal microbiome of the piglet rapidly undergoes a remarkable shift from the initial microbial groups which are present during the first days of life to the establishment of an adult-like microbial community, experiencing in between a period of changing microbial successions (Isaacson and Kim, 2012; Pajarillo *et al.*, 2014; Guevarra *et al.*, 2019). This initial pattern of microbial maturation is essential as the gut microbiota is fundamental for an adequate development and programming of the mucosal immune response (Schokker *et al.*, 2015). Moreover, a "window of opportunity" has been described during this early-life development stage, where any disruption may have long-lasting impacts on health and welfare (Nowland *et al.*, 2019). Therefore, understanding the dynamics of the young piglet gut microbiota during the first weeks of life is certainly concerning as its major impact on the future health and growth performance of pigs.

Several authors have affirmed that age and weaning are the driving factors of microbiota development, pointing out specific changes in specific taxonomic groups at certain ages literature (Kim *et al.*, 2011; Pajarillo *et al.*, 2014; Frese *et al.*, 2015; Slifierz, Friendship and Weese, 2015; Chen *et al.*, 2017; Guevarra *et al.*, 2018; X. Wang *et al.*, 2019). For instance, the gradual increase observed in the microbial diversity of piglets as they aged, in the present study, is in accordance with several previous works (Pajarillo *et al.*, 2014; Frese *et al.*, 2015; Niu *et al.*, 2015; Slifierz, Friendship and Weese, 2015; Chen *et al.*, 2017; Ke *et al.*, 2019; X. Wang *et al.*, 2019; Choudhury *et al.*, 2020; Saladrigas-García, D'Angelo, Ko, Nolis, *et al.*, 2021). A higher diversity in the gut microbiota has been related to a more mature gut ecosystem and is in agreement with the concept of functional redundancy, which supports that additional taxa add redundancy to specific functions, helping the ecosystem to preserve its resilience and stability after environmental stress (Naeem, Kawabata and Loreau, 1998; Konopka, 2009; Holman and Chénier, 2014; Chen *et al.*, 2017). Diversity results are, however, contradictory with some other studies that have reported a decreased alpha diversity during the early period after weaning (Hu *et al.*, 2016; Han *et al.*, 2018; Y. Li, Guo, *et al.*, 2018), with a later increase from weaning to adulthood. This discrepancy might be due to

differences among studies in weaning age or the post-weaning sampling time-point but also to differences in management and feeding practices or in the microbiological environment of the farm. Regarding variability of the microbiota among individual piglets (interindividual Bray–Curtis distances), several authors have described that it tends to diminish as the piglets age (Chen *et al.*, 2017; Choudhury *et al.*, 2020; Luise, Le Sciellour, *et al.*, 2021; Saladrigas-García, D'Angelo, Ko, Nolis, *et al.*, 2021), suggesting that gut microbiota structure of piglets tends to converge among animals as they age to establish of a homogenous, rich and stable microbiota composition after weaning. However, results from the present work showed that this evolution is not always present, as in the Trial 1 beta diversity was increased as the piglets grew. It is possible that under the complex and challenging environmental conditions the animals have to face in the commercial practice, the evolution of their gut ecosystems will diverge more widely than when they are reared under highly standardized experimental conditions.

Firmicutes followed by Bacteroidetes were found to be the dominant phyla across all experimental samplings except for 2-day-old piglets. Although this is consistent with the majority of earlier studies (Kim *et al.*, 2011; Pajarillo *et al.*, 2014; Frese *et al.*, 2015; Mach *et al.*, 2015; Chen *et al.*, 2017; Ke *et al.*, 2019; X. Wang *et al.*, 2019), there are a few reports with Bacteroidetes or Proteobacteria (Slifierz, Friendship and Weese, 2015; Zhao *et al.*, 2015) as the pre-dominant phyla. However, the age of the piglet plays an important role in this result. In the present study, we observed that in 2-day-old piglets from Trial 1 Proteobacteria was found to be almost as abundant as Firmicutes and in Trial 2, Proteobacteria doubled Firmicutes in relative abundance. Therefore, the Proteobacteria phylum stands out in this study for its important weight at the time closest to birth. The decreasing abundance of Fusobacteria observed in Trial 1 during the first weeks of life has also been reported by several other authors (Pajarillo *et al.*, 2014; Niu *et al.*, 2015; Slifierz, Friendship and Weese, 2015; Ke *et al.*, 2019; Choudhury *et al.*, 2020; Saladrigas-García, D'Angelo, Ko, Nolis, *et al.*, 2021). However, some studies have not reported the presence of this bacterial group at all (Frese *et al.*, 2015; Guevarra *et al.*, 2018). This is in consonance with the results observed in Trial 2, where much lower abundances than those previously reported in Trial 1 were detected. Such differences in taxonomic abundance could, to some extent, be due to variability factors such as the study design and conditions, pig genetics,

environmental factors including the time of the year the sampling was performed, the sampling procedures or the analytical procedures. A trend towards higher abundance was observed for the phyla Spirochaetes as the piglets aged in both Trials, as observed by Pajarillo *et al.* (2014). At the family level, relative abundances of *Enterobacteriaceae*, *Clostridiaceae*, *Fusobacteriaceae*, and *Veillonellaceae* declined over time while those of *Prevotellaceae*, *Ruminococcaceae*, *Spirochaetaceae*, *Rikenellaceae*, *Erysipelotrichaceae* and *Succinovibrionaceae* increased. Similar results have also been reported in previous studies (Kim *et al*., 2011; Pajarillo *et al*., 2014; Guevarra *et al*., 2019; Luise, Le Sciellour, *et al*., 2021). However, contrary to what was reported by these authors, we did not observe a decrease in *Bacteroidaceae* family in Trial 1 or an increase in *Veillonellaceae* family. *Bacteroidaceae*, *Lactobacillaceae*, *Lachnospiraceae* and *Streptococcaceae* families showed oscillations in their relative abundances throughout the repeated samplings, generally increasing during lactation and slightly decreasing after weaning.

The present study pinpointed the early intestinal colonizers belonging to *Bacteroides*, *Escherichia-Shigella*, *Clostridium sensu stricto 1* and *Fusobacterium* genera. This is in accordance with Petri, Hill and Van Kessel (2010), who reported the genera *Escherichia*, *Clostridium*, *Fusobacterium*, *Streptococcus*, and *Enterococcus* to be the earliest colonizers of the pig gut, between birth and 2 days. However, a high level of individuality has been reported to occur in 1- and 2-week-old piglets, indicating that there is considerable randomness to the process of acquiring microbes (Thompson, Wang and Holmes, 2008). Although this gut community in very young piglets might be highly dynamic, the microbial community is known to stabilize by day 28. Decreases in the abundances of *Clostridium*, *Fusobacterium* and *Escherichia-Shigella* with the age of the piglets have also been observed by several other authors (Pajarillo *et al*., 2014; Frese *et al*., 2015; Mach *et al*., 2015; Chen *et al*., 2017; Luise, Le Sciellour, *et al*., 2021; Saladrigas-García, D'Angelo, Ko, Nolis, *et al*., 2021). During lactation, the genera *Bacteroides* and *Lactobacillus* acquire a greater relative importance with respect to the rest of the genera and this fact have been correlated with a milk-oriented microbiome (Frese *et al*., 2015). *Bacteroides* has been reported to use wide range of both milk oligosaccharides and host-derived glycans (Marcobal *et al*., 2010) and *Lactobacillus* is a well-known lactate producer from lactose (Schwab and Gänzle, 2011). Moreover, whereas *Fusobacterium* has been positively correlated to

intestinal diseases (Allen-Vercoe and Jobin, 2014; Hermann-Bank *et al.*, 2015; Liu *et al.*, 2015), *Lactobacillus* has been labelled as a major player in the establishment and the maintenance of the bacterial homeostasis after birth (Konstantinov *et al.*, 2006).

The second significant change in the microbial community of the piglet occurs in the period around weaning. In the present study, the increase in genera such as *Prevotella*, *Butyricimonas*, *Christensenellaceae R-7 group*, *Dorea*, *Phascolarctobacterium*, *Rikenellaceae RC9 gut group*, *Subdoligranulum* and *Ruminococcaceae UCG-002* stands out. This is largely in agreement with previous observations which exemplify *Prevotella* as a prominent actor in the typical post-weaning microbiota together with species belonging to *Roseburia*, *Faecalibacterium*, *Ruminococcus*, *Lachnospira*, *Dorea*, *Blautia*, *Subdoligranulum* (Kim *et al.*, 2011; Pajarillo *et al.*, 2014; Frese *et al.*, 2015; Slifierz, Friendship and Weese, 2015; Mach *et al.*, 2015; Ramayo-Caldas *et al.*, 2016; Y. Li, Guo, *et al.*, 2018; Guevarra *et al.*, 2018, 2019; Choudhury *et al.*, 2020; Luise, Le Sciellour, *et al.*, 2021; Saladrigas-García, D'Angelo, Ko, Nolis, *et al.*, 2021). The drastic increase in relative abundance of *Prevotella* is likely due to the established capacity of the members of this genus to metabolize plant-derived non-starch polysaccharides to short-chain fatty acids (Flint and Bayer, 2008; Ivarsson *et al.*, 2014), which are prominent constituents of the cereal-based weaner diet. Therefore, the abrupt change to a solid cereal-based diet and the withdrawal of milk would explain the decrease of the previously mentioned genera and the increase of propionate- and butyrate-producing genera. Altogether, the higher abundance of propionate- and butyrate-producing genera in older piglets, adapted to digest resistant starches and dietary fibres, show the quick microbial transformation of the piglets' gut microbiota to cope with diets rich in complex carbohydrates.

4.5.2. The impact of farm management and environment on gut microbial colonization of piglets

In the present study, 6 commercial farms were included in order to determine the extent to which variations in farm environment and rearing conditions influence the microbiota development after birth. Farm variability was relatively controlled since all the selected farms were indoors, close-cycle,

geographically located in the same region of Spain, and vertically integrated using the same breed and feed formula. Moreover, farms included in Trial 1 or Trial 2 were sampled in the same temporal period. Differences among farms were mostly due to the metaphylactic use of antimicrobials (injected after birth to piglets and/or given to the sows as medicate feed) and rehydrating solutions during lactation. Farms were also selected based on differences in historical records of digestive (*Brachyspira hyodysenteriae*) and respiratory (*Actinobacillus pleuropneumonia*, APP) diseases (see **Table 4.1.** for additional information). After Trial 1, days 2 and 21 were chosen as the most relevant sampling days in Trial 2 to characterize the evolution pattern of the gut microbial ecosystem after birth, and to assess possible factors influencing this process. Day 2 would reflect one of the moments of greater variability in the establishment process of the first colonizers and day 21 would give a relatively accurate picture of the first ecological equilibrium reached by the gut microbiota after birth.

As expected, significant differences among farms were found in the microbial ecosystem of piglets in both trials. In Trial 1, greater alpha diversity was found in Bravo farm, whereas in Trial 2, lower alpha diversity was observed in Foxtrot farm in 2-day-old piglets. This difference among farms was also demonstrated by the fact that several taxa were influenced by the rearing farm. Although the impact of the effect of the rearing farm on specific taxa has not been analysed in detail in the literature, changes in the microbiota of the piglets have been reported (Vigors, O' Doherty and Sweeney, 2020; Luise, Le Sciellour, *et al.*, 2021). Indeed, in addition to animal age and genetic background (Kubasova *et al.*, 2018), the structure and activity of gut microbiota can differ among animals depending on various factors including dietary influence (Frese *et al.*, 2015; Everaert *et al.*, 2017; Le Sciellour *et al.*, 2018), sanitary status (Bearson *et al.*, 2013), antimicrobial use (Bosi *et al.*, 2011; Looft *et al.*, 2014; Mu *et al.*, 2017; Soler *et al.*, 2018) and husbandry practices (Saladrigas-García, D'Angelo, Ko, Traserra, *et al.*, 2021; Wen *et al.*, 2021), among others. For instance, in Trial 1, piglets from Alpha farm received an intramuscular dose of amoxicillin (15 mg amoxicillin/kg BW) between their second and fifth day of life, which could explain the lower alpha diversity found in Alpha piglets. Although most of the existing literature has focused on the study of oral administration of antibiotics, a recent study has evidenced significant dysbiosis regardless of administration route in mice (Kelly *et al.*,

2021). Moreover, amoxicillin has been related to profound dysbiotic effects, with population richness and diversity significantly reduced, in orally treated pigs (Bosi *et al*., 2011). In Trial 1 we also observed that 2 and 7-days-old piglets from the Alpha farm presented lower abundances of pathogenic bacteria such as *Clostridium sensu stricto 1* and *Escherichia-Shigella*, whereas *Lactobacillus* counts were increased in Bravo piglets in most of the time-points (d2, d7, d21 and after weaning). Similar results have been reported in orally treated pigs with amoxicillin, where the antibiotic intervention decreased the abundance of *Lactobacillus* and other lactic acid bacteria (Bosi *et al*., 2011; Mu *et al*., 2017). However, all these differences could had been due not only to the use of amoxicillin but also to differences in the composition of the oral solutions used in both farms that in Alpha not only included electrolytes but also a blend of mono, di and triglycerides of medium chain fatty acids, together a blend of acidifiers. With time and increasing age, the piglets from the Bravo farm ended up showing a more mature microbiota after weaning, with greater abundances of butyrogenic genera such as *Prevotella*, *Coprococcus*, *Faecalibacterium* and *Dorea*, among many others, as well as lower abundances of *Escherichia-Shigella* and *Fusobacterium* that could had been due to a lower selective pressure from antimicrobial prophylaxis. However, differences between farms after weaning in Trial 1 should be regarded with caution as the Alpha farm was sampled earlier than Bravo (7 vs. 14 days post-weaning) due to an imminent metaphylactic treatment of the piglets after first signs of diarrhoea outbreak. This could explain the higher abundances of *Escherichia-Shigella* and *Fusobacterium* in Alpha piglets and the slower development of the fermentative bacteria.

In Trial 2, differences were also observed among rearing farms. In addition to the recurring *B. hyodysenteriae* problems in Echo and Foxtrot farms, which implied the consumption of medicated feed by the mothers, the main management difference consisted of the absence of the rehydrating solution in the piglets from Foxtrot farm. Interesting in this farm it was observed a decreased alpha diversity at day 2 and marked increased abundances of Enterobacteriaceae both at 2 and 21 days of life. Moreover, at 21 days of life, significant changes in other microbial groups were also observed in this farm, such as a greater abundance of *Enterococcaceae* and a lower relative abundance of *Lachnospiraceae* and *Fusobacteriaceae*. Although it is not possible to assure that the observed changes are due to neither the initial antibiotic

treatment nor the re-hydrating solutions supplied during the first week of life of the piglets, it is evidenced that differential farm management guidelines, and particularly antimicrobial prophylaxis, can affect the development of the intestinal microbiota of the pig early in life.

Maternal antibiotic treatment has been also a subject of study especially in the field of human medicine. It has been recently found that antibiotic use during pregnancy alters the commensal vaginal microbiota of women (Stokholm *et al.*, 2014). Moreover, amoxicillin administration during late gestation has been reported to impact both sow vaginal and faecal microbial diversities (Arnal *et al.*, 2014; de Greeff *et al.*, 2020). Together, these results would suggest that maternal antibiotic treatment might influence the gut microbial colonization of the offspring through changing maternal microbiota composition. In addition, it is well known that maternal antibiotic residues can be transferred from mothers to their offspring via breastfeeding (Mathew, 2004). To date, very few studies have analysed the effect of the administration of antibiotics to the mothers on their offspring, and there are especially few studies focused on the effects produced in their intestinal microbiota. Arnal *et al.* (2014) reported a significant effect on the microbiota of the small intestine of the offspring but not of the colon. Moreover, some effects of maternal antibiotic treatment on the gut physiology and morphology of the offspring have been seen in early-life (Arnal *et al.*, 2014, 2015; de Greeff *et al.*, 2020; Trevisi *et al.*, 2021). In Trial 2 only farms Echo and Foxtrot gave the sows medicated feed during lactation. Two-day-old piglets from dams treated with medicated feed showed lower abundances of propionate- and butyrate-producing genera, such as *Phascolarctobacterium* and *Prevotella*, respectively. Lower abundances of *Enterococcus* were also observed. *Enterococcus* genera have recently gained attention due to their ability to produce bacteriocins recognized for their wide-range effectiveness on pathogenic and spoilage bacteria (Hanchi *et al.*, 2018). This is in consonance with the higher abundances of *Escherichia-Shigella* observed in piglets from dams treated with medicated feed. Piglets from treated dams also showed lower abundances of *Fusobacterium* at day 2 and 21 of life. Some authors have suggested that the abundance of *Fusobacteria*, is positively correlated with diarrheal swine diseases, such as the porcine epidemic diarrhoea and the new neonatal porcine diarrhoea (Hermann-Bank *et al.*, 2015; Liu *et al.*, 2015). On day 21 of life, lower abundances of the acetate- and butyrate-producing

genera *Rikenellaceae RC9 gut group* and *CAG-873*, respectively, were also observed in the piglets of mothers fed with medicated feed. Higher abundances of *Rikenellaceae RC9 gut group* are suggestive of a high level of functional redundancy for acetate in the swine gut microbiome (Holman *et al.*, 2017). A greater abundance of *Parabacteroides* was also observed in piglets from medicated dams. Higher abundances of *Parabacteroides* have been linked with lower BW and ADG (K. Wang *et al.*, 2019; Oh *et al.*, 2020). These findings suggest that maternal antibiotic treatment affected gut microbiota of offspring through the transfer of maternal gut microbiota to newborn piglets, although it is difficult to specify whether the maternal antibiotic treatment affected the microbiota development of offspring in a direct or indirect process. Little research has investigated the consequences of maternal antibiotic treatment with regards to the gut microbiome of their offspring and further research is needed.

4.6. Conclusions

Taken together, the present study confirms and refines the knowledge about the microbiome development during early-life stages in piglets. The initial colonization characterized by bacteria belonging to the *Clostridiaceace*, *Enterobacteriaceae* and *Fusobacteriaceae* families is progressively replaced by fermenting bacteria, essentially the acetate, propionate and butyrate-producing microorganisms. In this microbial development, the role of the dietary abrupt shift from sow milk to cereal-feed based diets stands out. The piglet rearing farm and the maternal antibiotic treatment in the feed showed a clear impact on the gut microbiota of their piglets, although the data presented in the present study are mainly descriptive and more studies are necessary to accurately determine the changes promoted by these factors.

4.7. Declarations

4.7.1. Data availability

The raw sequencing data employed in this article has been submitted to the NCBI's sequence read archive (https://www.ncbi.nlm.nih.gov/sra); BioProject: PRJNA800765.

4.7.2. Ethics declarations

Established principles of laboratory animal use and EU and Spanish laws related to animal experiments were adhered to in this study. The experimental study was approved by the Animal Ethics Committee at the Autonomous University of Barcelona. Faecal swab collections were performed as per standard operative procedures approved by the Animal Ethics Committee.

Chapter 5

Understanding host-microbiota interactions in the commercial piglet around weaning

Understanding host-microbiota interactions in the commercial piglet around weaning

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5.1. Abstract

Weaning is a critical period in the life of pigs with repercussions on their health and welfare and on the economy of the swine industry. This study aimed to assess the effect of the commercial early weaning on gut microbiota, intestinal gene expression and serum metabolomic response via an integrated-omic approach combining 16S rRNA gene sequencing, the OpenArray gene expression technology and ^{1}H-NMR spectroscopy. Fourteen piglets from different litters were sampled for blood, jejunum tissue and caecal content two days before (- 2d), and three days after (+ 3d) weaning. A clearly differential ordination of caecal microbiota was observed. Higher abundances of *Roseburia*, *Ruminococcus*, *Coprococcus*, *Dorea* and *Lachnospira* genera in weaned piglets compared to prior to weaning showed the quick microbial changes of the piglets' gut microbiota. Downregulation of *OCLN*, *CLDN4*, *MUC2*, *MUC13*, *SLC15A1* and *SLC13A1* genes, also evidenced the negative impact of weaning on gut barrier and digestive functions. Metabolomic approach pinpointed significant decreases in choline, LDL, triglycerides, fatty acids, alanine and isoleucine and increases in 3-hydroxybutyrate after weaning. Moreover, the correlation between microbiota and metabolome datasets revealed the existence of metabolic clusters interrelated to different bacterial clusters. Our results demonstrate the impact of weaning stress on

the piglet and give insights regarding the associations between gut microbiota and the animal gene activity and metabolic response.

5.2. Introduction

The process of weaning is one of the most stressful events for pigs in swine production. Pigs are subjected to biological stress marked by significant physiological, social, environmental, and nutritional challenges. Weaning is usually performed at around 3–4 weeks, where piglets are still vulnerable to infectious diseases because of stress and immaturity of the intestinal tract and immune system (J. C. Kim *et al.*, 2012). Therefore, during the first week of adaptation to solid feed, piglets experience low voluntary feed intake, which results in alteration of gut integrity (Lallès *et al.*, 2004, 2007b), characterized by shortened villous length (Pluske, Hampson and Williams, 1997) and increased mucosal permeability (Miller and Skadhauge, 1997; Boudry *et al.*, 2004). As a result, reduced pig health and performance are observed in commercial practice. In order to improve pig health and welfare during this time, a better understanding of the complex phenomena underlying is needed.

Recently, the association among gut microbiota, metabolites, and host physiology has gained increasing attention. The animal organism is believed to be interconnected with its environment through different cycles of epigenomic programming and reprogramming (Shenderov and Midtvedt, 2014). This epigenomic programming is the result of the interaction of metabolism and the microbiota, as well as their interaction with external factors such as diet, environmental exposure, or drugs. On the one hand, the process of intestinal microbial colonization has been shown to play a crucial role in the development of the neonatal immune system of mammals with implications throughout their lives (Hansen *et al.*, 2012). Early gut microbial colonization is affected by factors such as age, host genetics, diet, environment, disease, and maternal seeding (Costello *et al.*, 2012). This, in turn, sets off the crosstalk between the microbiome and the host driving changes in nutrition, immunity, barrier function, metabolism, and gene expression. Actually, the gut microbiota has been described to be involved in the intestinal epithelium differentiation (Sommer and Bäckhed, 2013), the

immune system development (Ivanov *et al*., 2009), and intestinal mucosal barrier maintenance (Garrett, Gordon and Glimcher, 2010). Adequate gut colonization in the piglet is therefore considered as a key element to maintain the homeostasis and promote an optimal training of the immune response with lifelong implications on the probability of developing pathologies (Kamada *et al*., 2013), such as post-weaning diarrhoea or other multifactorial diseases. On the other hand, it is well known that the intestinal microbiota plays a key role in gut health, participating in many metabolic pathways, such as nutrient digestion, absorption or lipid metabolism, and amino acid synthesis (Li *et al*., 2008). However, few studies focused on the complex interactions that occur in the pig organism during the suckling and weaning period.

The objective of this work was therefore to put a little bit of light in the complex crosstalk between the piglets and their intestinal microbiota early in life. For that, we studied changes in the gut ecosystem and the animal response under different commercial husbandry practices. We assessed changes in gut microbiota, intestinal gene expression, and serum metabolic profiles in piglets via an integrated omics approach combining 16S rRNA gene V3-V4 regions sequencing, the TaqMan OpenArray gene expression technology, and Proton Nuclear Magnetic Resonance (^{1}H-NMR) spectroscopy.

5.3. Materials and Methods

5.3.1. Animals and experimental design

The housing, management, husbandry, and slaughtering conditions of this experiment conformed to the European Union Guidelines (Directive 2010/63/EU). All experimental procedures used in this study were approved by the Animal and Human Experimental Ethical Committee of Universitat Autònoma de Barcelona (UAB) (permit code CEEAH 3817) and designed in compliance with the ARRIVE guidelines.

The trial was carried out in a commercial farm with a breeding stock of 1130 sows with 50-60 farrowing sows per batch in a closed cycle system. Sows in lactation and piglets until fattening were fed with commercial diets. Lactating and pre-starter diets were corn-soy based, but also incorporated other

cereals like wheat and barley (multi-cereal diets). Sows' and piglets' diets included *Saccharomyces cerevisiae* NCYC Sc 47 (E1702) (10^9 CFU/kg) and piglets' pre-starter diets also included 6-phytase and endo-1-4-beta-xylanase. Ingredients and additives included in each formula and their chemical composition can be found in the supplementary material (**Annex 2: Table S5.1.**). Pre-starter diets also included prescribed anti-microbials as metaphylaxis after first signs of disease in some individua to prevent the rest of the piglets from meningitis (Amoxicillin trihydrate, 250 mg/kg) and post-weaning diarrhoea (Oxytetracycline, (150 mg/kg) and ZnO (3,100 mg/kg)). Farm vaccination guidelines included: *Mycoplasma*, circo-virus, and Aujeszky for piglets, and swine influenza, *Escherichia coli*, PRRS, porcine parvovirus (PPV), and erysipelas for breeding sows.

A total of forty-eight sows and their litters (average litter size 14.1 ± 0.1) were included in this study. These animals formed part of a previously published study (Saladrigas-García, D'Angelo, Ko, Traserra, *et al.*, 2021) in which sows were randomly allocated into two groups (24 sows per group) balanced by sow parity. One group was subjected to conventional management in individual farrowing pens, whereas in the other group, piglet socialization was allowed by removing the separation fences between two sows from 14 days after delivery (for details see Saladrigas-García, D'Angelo, Ko, Traserra, *et al.* (2021)). The possible impact of these treatments on microbiota and animal response has been previously shown and discussed (Saladrigas-García, D'Angelo, Ko, Traserra, *et al.*, 2021).

Sows were fed twice a day and *ad libitum* water. Piglets were provided with *ad libitum* water and creep feed from two weeks of age. The piglets were weaned at 25 days of age and randomly mixed with other piglets into a total of 16 pens (40 piglets/pen, ca. 0.20 m^2/animal). Piglets housed in each pen had received the same management during the suckling period. Weaners were offered *ad libitum* commercial feed and water.

5.3.2. Sample extraction

Fourteen litters were sampled on two days before weaning (-2 d, n=14), and three days after weaning (+3 d, n=14). Seven litters had received a conven-

tional management and the other seven litters an enriched management. In each litter, one male piglet of medium birth weight was selected per sampling day. The piglets were sedated with an intramuscular injection containing 20 mg/kg of ketamine (Ketamidor) and 2 mg/kg of xylazine (Xilagesic) and humanely euthanized with an overdose of pentobarbital. Blood samples were collected after opening the abdominal cavity directly from the caudal vena cava and serum was obtained by centrifugation for 15 minutes at 3500 rpm and stored at -80°C. Jejunum tissue samples (1 cm^2) were collected from mid-jejunum (1 m after duodenum), washed thoroughly with PBS, and preserved in 1 mL of RNAlater (Deltalab, Rubí, Spain). Caecal content was collected directly from the cecum and immediately frozen in dry ice. Tissue and caecal samples were kept at -20°C until further analysis.

5.3.3. 16S rRNA gene sequencing

Deoxyribonucleic acid (DNA) was extracted from 250 mg of each caecal sample using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions following the optimization steps. DNA concentration and purity were checked with NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). For 16S rRNA gene high-throughput sequencing, amplicon libraries were prepared using Nextera XT Index Kits 16S V3–V4 Amplicon-Seq Kit (Illumina, San Diego, CA, USA). For sequencing on the MiSeq instrument, the generated libraries were placed in the reagent cartridge and loaded on the instrument along with the flow cell. The MiSeq Reagent Kit V2 (500-cycle) (Illumina, San Diego, CA, USA) was used. All subsequent steps were performed on the MiSeq Illumina instrument.

For sequencing data bioinformatics, the sequence reads generated were processed using Quantitative Insights Into Microbial Ecology (QIIME) version 1.9.1 software (Caporaso *et al.*, 2010). Open-reference OTU picking (Rideout *et al.*, 2014) at 97% identity was performed with bacterial 16S GreenGenes (v. 13_8) reference database (DeSantis *et al.*, 2006). A detailed description of all further steps in the bioinformatic analysis is available in our previous publication (Saladrigas-García, D'Angelo, Ko, Traserra, *et al.*, 2021).

5.3.4. Prediction of the functions of the microbial population

The predicted metagenome of caecal samples based on 16S rRNA gene sequencing data was analysed using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt v1.1.3) (Langille *et al.*, 2013) (http://picrust.github.io/picrust/) and the software STAMP v2.1.3. (Parks *et al.*, 2014) (https://github.com/dparks1134/STAMP). For this, a closed reference OTU table was created in QIIME using filter_otus_from _otu_table.py script and the Greengenes reference database v13.5 (DeSantis *et al.*, 2006). The generated OTU table was used as input for the 16S rRNA gene copy number normalization with the normalize_by_copy_number.py script. Functional predictions of Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthologs (KOs) were generated using the predict_metagenomes.py script, which was then summarized into KEGG pathways at KO level 3 using the categorize_by_function.py script. The software STAMP was finally used to identify functional pathways differentially expressed.

5.3.5. RNA extraction and gene expression study by qPCR

The expression of a total of 52 genes related to intestinal health was studied through a custom OpenArray plate (Applied Biosystems, Foster City, CA, USA). Full details of the method have been previously described by González-Solé *et al.* (2020). The method had been optimized to quantify the expression of relevant genes in the jejunum. Genes were selected based on previous knowledge and other reported works in the literature. GAPDH, ACTB, TBP, and B2M were used as housekeeping genes. RNA was extracted from 100 mg of frozen jejunum tissue with the RiboPure kit (Ambion, Foster City, CA, USA) following the manufacturer's protocol. A final cDNA volume of 6 µl from each sample was transferred per duplicate to a TaqMan OpenArray custom plate for gene expression and run in a QuantStudio 12K Flex Real-Time PCR System (ThermoFisher Scientific, Waltham, MA, USA). Gene expression data analysis was performed as specified by González-Solé *et al.* (2020). A list of the 52 genes included in the custom plate can be found in Supplementary information (**Annex 2: Table S5.2.**).

5.3.6. Nuclear Magnetic Resonance (NMR) spectroscopy

Metabolic profiles by ^{1}H-NMR spectroscopy were performed according to previous procedures (Saladrigas-García, D'Angelo, Ko, Traserra, *et al.*, 2021). Different endogenous metabolites were assigned from the ^{1}H-NMR spectra by comparing chemical shifts and multiplicities of peaks to free databases like the Human Metabolome Data Base (HMDB) (Wishart *et al.*, 2007), the Biological Magnetic Resonance Data Bank (BMRB) (Ulrich *et al.*, 2007) and published studies (Lindon, Nicholson and Everett, 1999; He *et al.*, 2009, 2012; Clausen *et al.*, 2011). Briefly, 400 µL of blood serum were mixed with 200 µL of a saline buffer 0.9% NaCl (wt/vol) in D_2O in the 5 mm NMR tube (Beckonert *et al.*, 2007), to obtain a mixture containing about 30% D_2O. The sample was subjected to spectral analysis at 14.1 T (600.13 MHz frequency for 1H) on a Bruker AVANCE II 600 spectrometer, equipped with a z-axis pulsed-field gradient 5 mm triple channel probe (TBI), BACS 60 automatic sample changer, and a BCU-Xtreme unit for temperature control. Proton spectra were acquired at 300.0 K. Further details about the acquisition of proton spectra and ^{1}HNMR data pre-processing details have already been published (Saladrigas-García, D'Angelo, Ko, Traserra, *et al.*, 2021).

Each spectrum was pre-processed before statistical analysis using TOPSPIN 3.6 (Bruker BioSpin, Germany). An exponential Fourier Transform (FT) using a line broadening factor of 0.3 was used. Each spectrum was aligned using lactate signal for calibration (1.33 ppm), automatic phase and baseline correction were applied with manual refinement when necessary. After that, spectral data set between 0.00 and 10.00 ppm was transferred to AMIX 3.9 package to automatically reduce it into integrated spectral regions of equal width (0.04 ppm), to exclude the spectral region between 4.78 and 4.66 ppm containing water signal and to normalize to total area. The final bucket table, containing 250 area regions of 0.04 ppm wide, was extracted and used for statistical analysis.

5.3.7. Statistical analysis

The details of the biostatistical analysis of caecal microbiota, the statistical analysis of gene expression and nuclear magnetic resonance, and the inte-

gration of gene expression, metagenomics, and metabolites can be found in Saladrigas-García, D'Angelo, Ko, Traserra, *et al.* (2021).

In brief, metagenomics biostatistics was performed in open-source software R v3.5.3. (Team, 2013) (https://www.r-project.org/foundation/) using the *phyloseq* package (McMurdie and Holmes, 2013) as support for QIIME in R. Cumulative sum scaling (CSS) (Paulson, Stine, *et al.*, 2013) normalization of raw counts and differential abundance analysis were performed following the metagenomeSeq package pipeline (Paulson, Talukder, *et al.*, 2013). Relative abundances were used to plot taxon abundances and statistical significance was assumed at $P<0.05$. For gene expression statistical analysis, open-source software R v3.5.3. was used. Two-way ANOVA was performed, and Benjamini-Hochberg false discovery rate (FDR) was used to adjust P-values. Statistical significance was assumed at $FDR<0.05$. Regarding ^{1}H-NMR statistical analysis, integral data from the bucket table was imported to SIMCA 14.1 software for multivariate data analysis. The validity of the prediction model was assessed in function of the values of model fitness (R^2) and predictive capacity (Q^2) parameters. The more contributing ^{1}H-NMR spectral bucket regions (0.04 ppm) for the discrimination between both groups were identified from a combination of VIP plot and S-plot analysis. Variables with VIP values ≥ 1 and located high up or low to the left corner of the S-plot were selected. The integration of gene expression, metagenomics, and metabolites was performed by using the open-source software R v3.6.1. and following the LinkHD package (Zingaretti *et al.*, 2019) pipeline (https://github.com/lauzingaretti/LinkHD).

Ultimately, for metabolome and microbiota correlation, a multivariate analysis of the data was performed to find statistically significant correlations. The Pearson correlation coefficients were calculated using InfoStat statistical software (Di Rienzo *et al.*, 2008). Hierarchical clustering of correlations coefficients between bacterial family counts and ^{1}H-NMR bucket regions and a heatmap were prepared using Multiple Array Viewer (MeV) software (CCCB, Boston, USA) (Saeed *et al.*, 2003), where red and green spots indicated, respectively, positive or negative Pearson correlations between variables.

In all of the previously mentioned analyses, significant differences were declared at $P \leq 0.05$, while $0.05 < P \leq 0.10$ were considered near significant trends.

5.4. Results

This work was part of a larger study that has been previously published (Ko *et al.*, 2020; Ko *et al.*, 2021; Saladrigas-García, D'Angelo, Ko, Traserra, *et al.*, 2021) that is recommended for complementary information. In those publications, we evaluated the impact of different management systems (including or not early socialization and environmental enrichment) on behavioural response (Ko *et al.*, 2020), performance (Ko *et al.*, 2021), and intestinal physiology and caecal microbiota (Saladrigas-García, D'Angelo, Ko, Traserra, *et al.*, 2021) of piglets. In the present work, our objective was to elucidate the changes induced by the commercial early weaning itself attending to changes in caecal microbiota, intestinal gene expression, and metabolomic response. We also aimed to integrate all these data in a holistic approach to better understand the relationships between microbiota and animal response.

5.4.1 Weaning-induced changes in the gut bacterial microbiome

An average of 78,562 ± 24,539 16S rRNA gene V3-V4 regions sequences per sample (ranging from 4,0061 to 132,201) with an average length of 460 bp were obtained from the 28 caecal content samples, with no differences in the number of reads between pre- and post-weaning piglets ($P = 0.424$) and rarefaction curves reaching the plateau phase. The sequences were assigned to 976 Operational Taxonomic Units (OTU) based on a 97% sequence similarity. The indices of *Chao1*, *observed species*, *Shannon,* and *Simpson* were calculated to estimate alpha diversity. As presented in **Figure 5.1.**, higher values were found after weaning ($P=0.015$, $P=0.017$, $P=0.013$, $P=0.080$; for *Chao1*, *observed species*, *Shannon* and *Simpson* indices, respectively), indicating an increase in the complexity of the gut ecosystem when animals are moved to the dry feed. Regarding beta diversity, a tendency was detected with the Whittaker index for a decrease after weaning ($P=0.062$) indicating

that ecosystems were more similar, and microbiota tends to converge between animals after weaning. Moreover, analyses of possible changes in the ecosystem structure related to weaning were performed using Anosim, Adonis, and Envfit tests, all of them based on Bray-Curtis distance. Highly significant differences between pre- and post-weaning piglets were found (P=0.0001, P=0.001, and P=0.0001, for Envfit, Anosim, and Adonis tests, respectively).

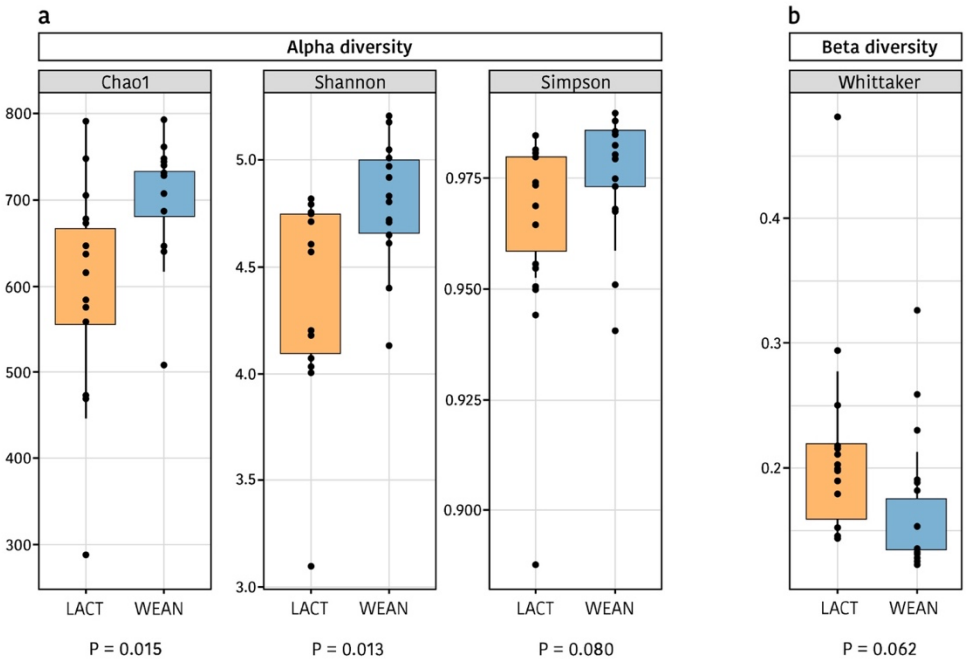

Figure 5.1. Box plot of the alpha (**a**) and beta (**b**) diversity during lactation (LACT) and after weaning (WEAN) based on the calculation of different indices: Chao1, Shannon, and Simpson for alpha diversity, and Whittaker for beta-diversity.

5.4.2. Changes promoted in particular taxonomic groups

Figure 5.2. shows the relative abundances obtained for suckling and weaned piglets at phylum and genus levels. *Firmicutes* and *Bacteroidetes* constituted the two predominant phyla in the caecal microbiota of the piglets, followed by *Proteobacteria* (7.76%), *Spirochaetes* (3.49%), and *Fusobacteria* (2.85%). At the genus level, *Prevotella* was found as the most predominant genus

(13.61%), followed by unclassified *Prevotella* (7.70%) and *Bacteroides* (4.79%) from *Bacteroidetes* phyla.

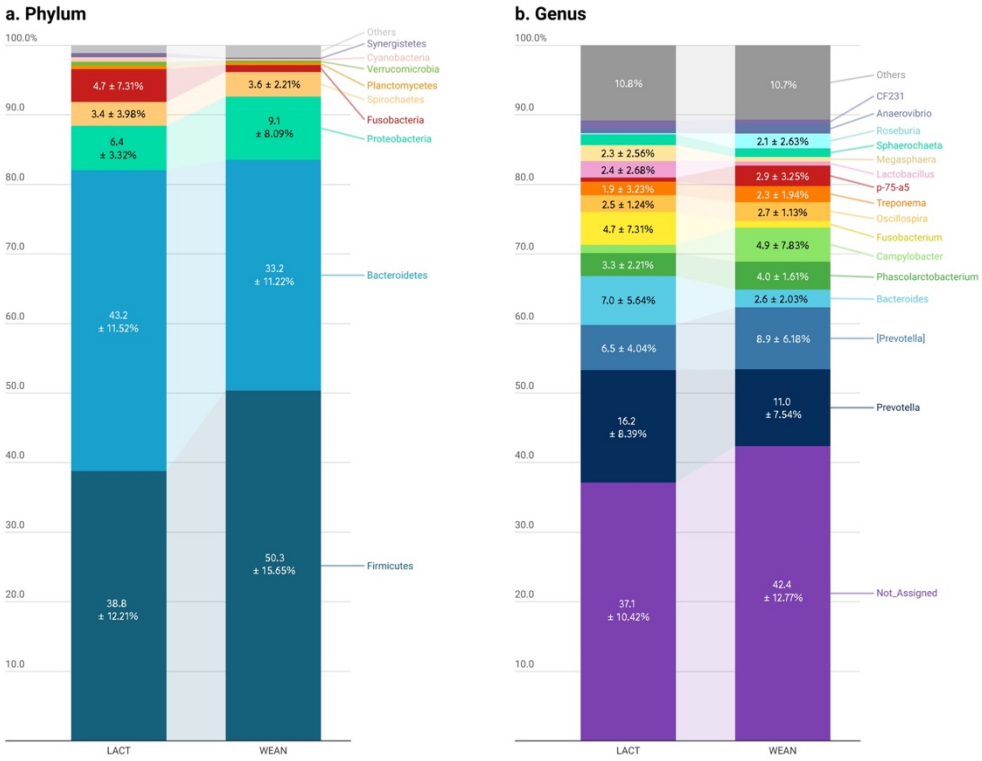

Figure 5.2. Bar plot of the relative abundances (RA) expressed in percentage of the phyla (**a**) and main genera (**b**) observed in the analysis of the microbiota of piglets by massive sequencing of the 16S rRNA gene. Bar plot LACT represents the relative abundances observed during lactation, while bar plot WEAN represents the values observed in weaned piglets. Only taxa with RA greater than 2.0% were annotated with RA percentage ± SD. Figure created with the online open-source tool Datawrapper (http://datawrapper.de).

Regarding the effect of weaning on microbial groups, although the most abundant phyla, *Firmicutes*, *Bacteroidetes,* and *Proteobacteria* revealed no significant shifts in their relative abundances as a whole, significant effects were seen in predominant families and genera within. Only the *Fusobacteria* phylum showed a remarkable decrease after weaning (P=0.005). At the family level, four predominant families showed significant reductions as the

piglets were weaned (**Table 5.1.**), including *Bacteroidaceae* (P=0.031), *Enterobacteriaceae* (P=0.031), *Fusobacteriaceae* (P=0.005), and *Lactobacillaceae* (P=0.003). At the same time, *Lachnospiraceae* and *Erysipelotrichaceae* increased significantly after weaning (P=0.031 and P=0.022, respectively). At the genus level, two predominant genera showed significant increases after weaning (**Figure 5.3.**), including *p-75-a5* (P=0.001) and *Roseburia* (P=0.012). The relative abundances of *Bacteroides*, *Fusobacterium*, *Lactobacillus*, and *Megasphaera* decreased in weaned piglets. Notably, *Bacteroides* and *Fusobacterium*, which were abundant in the gut of suckling piglets, declined from 7.01 and 4.72% to 2.58 and 0.99%, respectively for *Bacteroides* (P=0.019) and *Fusobacterium* (P=0.006), in a 5-day period. Among other non-predominant genera, several significant shifts were also detected, for example, an increased abundance of *Ruminococcus*, *Coprococcus*, *Dorea,* and *Lachnospira* in weaned piglets (P=0.020; P=0.018; P=0.038; and P=0.002, respectively).

Table 5.1. Composition of the caecal microbiota of piglets at the family level. Only main families (relative abundance >1%) and statistically significant families are included. Relative abundance results are expressed as a percentage (%) in decreasing order according to the general mean (the average of LACT and WEAN), and with the standard error of the mean (SEM), followed by the adjusted p-values (P-value) resulting from the com-parison between suckling (LACT) and weaned (WEAN) piglets.

	LACT	WEAN	SEM	P-value
Ruminococcaceae	12.92	18.51	1.404	0.6061
Prevotellaceae	16.21	10.94	1.555	0.2545
[Paraprevotellaceae]	8.64	10.16	0.993	0.9756
Veillonellaceae	7.98	6.67	0.854	0.6361
Lachnospiraceae	5.06	9.22	1.276	**0.0311**
Bacteroidaceae	7.00	2.58	0.894	**0.0311**
S24-7	3.84	3.61	0.342	0.6779
Campylobacteraceae	1.18	4.86	1.104	0.2384
Fusobacteriaceae	4.72	0.99	1.071	**0.0050**
Erysipelotrichaceae	1.45	3.81	0.496	**0.0216**
Clostridiaceae	1.80	3.22	0.296	0.1376
Spirochaetaceae	1.89	2.30	0.495	0.2317
Lactobacillaceae	2.40	0.63	0.422	**0.0033**
Sphaerochaetaceae	1.51	1.26	0.306	0.9756
Desulfovibrionaceae	0.98	1.15	0.149	0.9756
Enterobacteriaceae	1.66	0.44	0.256	**0.0311**
Pasteurellaceae	1.28	0.49	0.271	0.0576
Christensenellaceae	0.49	1.20	0.184	0.0759
[Odoribacteraceae]	1.15	0.48	0.193	0.0747
Dethiosulfovibrionaceae	0.48	0.10	0.078	**0.0311**
Coriobacteriaceae	0.19	0.39	0.075	**0.0476**
Victivallaceae	0.23	0.04	0.046	**0.0042**
Streptococcaceae	0.17	0.03	0.021	**0.0025**
Enterococcaceae	0.11	0.01	0.022	**0.0216**
Anaeroplasmataceae	0.00	0.07	0.033	**0.0025**

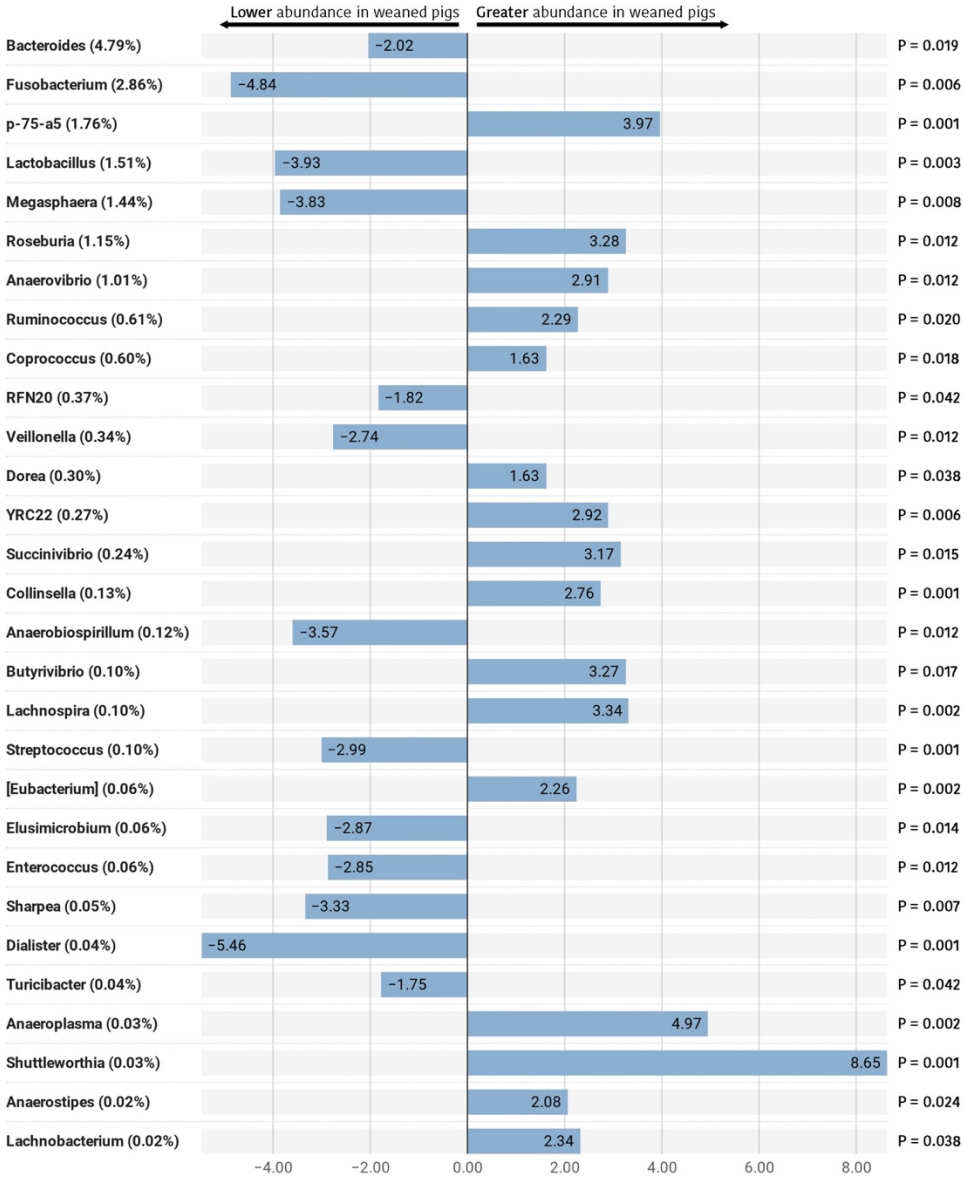

Figure 5.3. Differentially abundant taxa from caecal content (ln change and adjusted P-value<0.05) between suckling and weaned piglets at the genus level. All significant genera are presented; positive values and negative values indicate greater and lower abundance, respectively, in weaned animals (WEAN group) compared to suckling piglets (LACT); taxa are sorted according to the general mean of relative abundance (the average of LACT and WEAN, indicated between brackets in %) and in decreasing order. Figure created with the online open-source tool Datawrapper.

5.4.3. Predicted functions of the caecal microbiota

The inference of the functional profile of the caecal microbial community was predicted by using PICRUSt (Langille *et al.*, 2013) v1.1.3. A clear differentiation was observed between lactating and weaned piglets' microbiota related to several KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways (Kanehisa and Goto, 2000). During lactation, pathways related to metabolism, like glycan biosynthesis or lipids metabolism, cofactors and vitamins, and nucleotide metabolism, were highly represented. After weaning, functions related to cellular processes, such as cell motility and sporulation, or related to environment information processing, like membrane transport and signal transduction were higher (**Figure 5.4.**).

Going to a deeper level (KEGG level 3, presented in **Annex 2: Figure S5.1.**), lipid metabolism pathways, such as lipid biosynthesis proteins (P=0.005), energy metabolism pathways, such as carbon fixation pathways (P=0.019), and carbohydrate metabolism pathways, such as the citrate cycle (TCA cycle, P=0.028), among others, were more represented in suckling piglets. The pathways involved in the metabolism of purine (P=0.041) and alanine, aspartate, and glutamate metabolism (P=0.049), related to nucleotide and amino acid metabolism pathways, respectively, were also higher during lactation compared to suckling. Nicotinate and nicotinamide metabolism (P=0.008), related to the metabolism of cofactors and vitamins, increased also in weaned piglets compared to lactation.

Weaned piglets showed however a higher proportion of pathways involved in bacterial chemotaxis (P=0.004), bacterial motility proteins (P=0.007), and flagellar assembly (P=0.014), all related to cell motility. Pathways related to membrane transport, such as transporters (P=0.018) and ABC transporters (P=0.015), and signal transduction, such as the two-component system (P=0.015) were higher after weaning. Finally, lipopolysaccharide biosynthesis proteins (P=0.040), related to the glycan biosynthesis pathway, and sporulation pathway (P=0.014), showed higher values in weaned piglets.

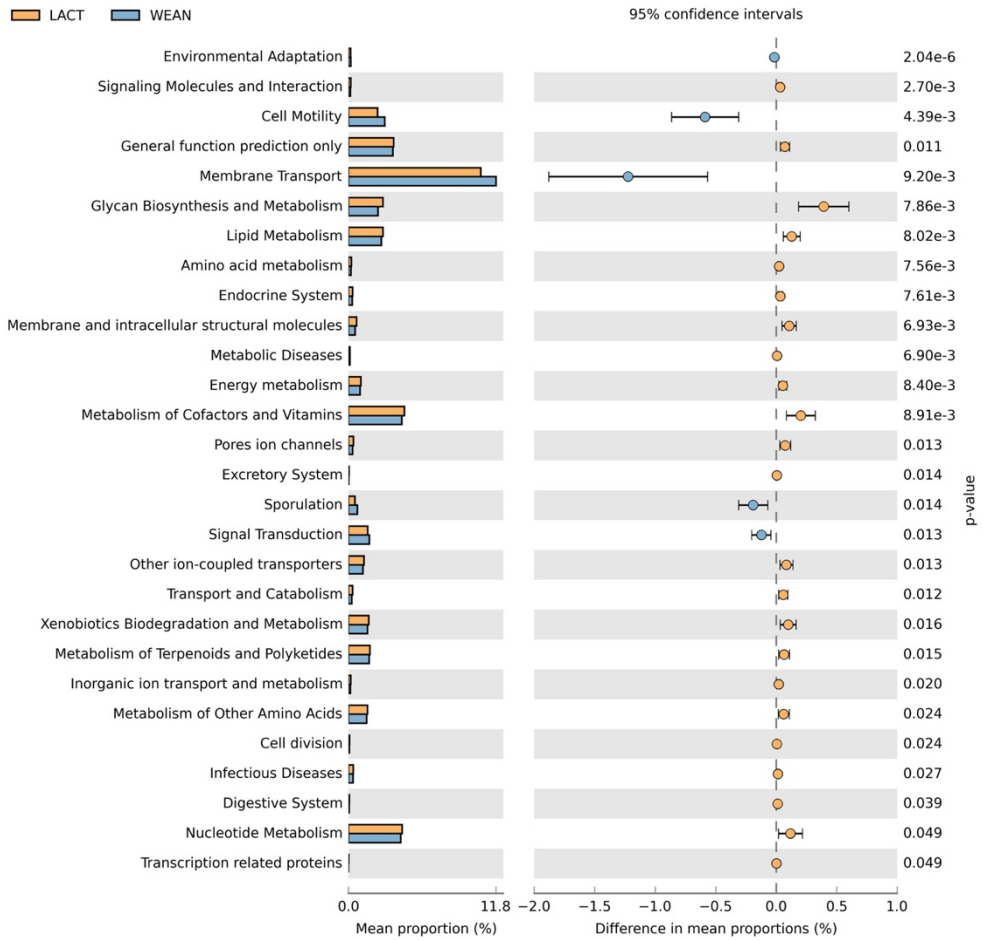

Figure 5.4. Significant differing caecal microbiota pathways between suckling and weaned piglets (KEGG level 2). All sequence reads were used to predict functions against the KEGG database (Kanehisa and Goto, 2000) (http://www.genome.jp/kegg/) by means of PICRUSt (Langille *et al*., 2013) v.1.1.3. (http://picrust.github.io/picrust/) bioinformatics software package. Difference values are expressed as the difference from pre- to post-weaning. Figure created with the software package STAMP (Parks *et al*., 2014) v2.1.3. (https://github.com/dparks1134/STAMP).

5.4.4. Changes induced in the jejunal gene expression

Jejunum samples from the fourteen piglets were collected shortly before and after weaning to analyse the expression of several genes related to intestinal functionality by using the OpenArray technology. The 51 genes analysed

were grouped into six categories for easier understanding according to whether they were related to: barrier function (BF), immune response (IR), nutrient transport (NT), enzyme/hormone encoders (EH), stress indicators (ST) or housekeeping (HK).

Several genes from all functional groups showed significant changes after weaning as shown in **Table 5.2.** Moreover, some additional genes related to barrier function, tended to be downregulated (*CLDN4* and *MUC2*, P=0.064 and P=0.074 respectively) or increased (*CLDN15*, P=0.079) in weaned piglets.

Table 5.2. Statistically significant differences observed in jejunal gene expression between suckling and weaned piglets. Values are expressed as Crt values ± Standard Deviation. Each analysed gene was classified based on its functionality: barrier function (BF), immune response (IR), nutrient transport (NT), enzyme/hormone encoders (EH), or stress indicators (ST). The p-values have been adjusted by the FDR method. A brief description of the genes analysed is given in **Annex 2: Table S5.2.**

Function	Gene	Suckling (Crt value ± SD)	Weaned (Crt value ± SD)	P-value
BF	*OCLN*	7.8 ± 0.47	7.1 ± 0.28	0.0004
BF	*MUC13*	2.4 ± 1.1	1.1 ± 0.35	0.0007
IR	*IFNGR1*	4.7 ± 0.8	3.3 ± 0.35	0.0001
IR	*PPARGC1α*	7.3 ± 0.55	8.0 ± 0.59	0.0154
NT	*SLC16A1*	6.6 ± 0.74	7.8 ± 0.62	0.0014
NT	*SLC15A1*	4.6 ± 1.73	3.5 ± 0.99	0.0176
NT	*SLC13A1*	6.3 ± 2.16	4.4 ± 0.68	0.0098
NT	*SLC30A1*	4.8 ± 0.64	3.7 ± 0.51	0.0003
NT	*SLC39A4*	4.6 ± 0.75	6.1 ± 0.57	0.0002
EH	*SI*	2.9 ± 1.21	1.5 ± 0.74	0.0029
EH	*HNMT*	5.3 ± 0.59	4.6 ± 0.37	0.0031
EH	*CCK*	7.4 ± 0.62	9.5 ± 1.13	0.0002
EH	*IGF1R*	6.3 ± 0.64	7.5 ± 0.74	0.0003
ST	*HSD11B1*	8.8 ± 0.69	9.8 ± 1.01	0.0117

5.4.5. Weaning-induced changes in serum metabolome (^{1}H-NMR spectra)

Twenty-eight serum samples were prepared and analyzed from 14 suckling piglets and 14 weaned piglets. Among the different endogenous metabolites assigned there were LDL, VLDL, lipids, unsaturated lipids, leucine, valine, isoleucine, lactate, alanine, adipate, acetate, N-acetyl glycoproteins, O-acetyl glycoproteins, glutamine, glutamate, pyruvate, creatine, choline, trimethylamine-N-oxide (TMAO), glucose, creatinine, tyrosine, and phenylalanine.

To identify potential differences between serum metabolites profiles of pre- and post-weaning piglets, an untargeted metabolomics approach using ^{1}H-NMR was also applied. In order to reduce the number of variables, filtering of ^{1}H-NMR bucket table was done by significant differences on Student's t-test (P-value ≤ 0.2) between the integrated buck regions of suckling and weaned piglets. To evaluate the global metabolic profile of serum samples collected from piglets in both periods, a blinded to age groups study by principal component analysis (PCA) of ^{1}H-NMR datasets was performed from the filtered ^{1}H-NMR bucket table. **Figure 5.5a** shows a biplot representation of PCA [$R^2x_{(cum)}$=0.82, $Q^2_{(cum)}$=0.30] from the reduced data, in which a clear pattern of separation between suckling and weaned piglets along PC1 could be observed, indicating that both piglets' groups were metabolically differentiated. To identify the key metabolites that influence this grouping, a study taking into account the age groups was made by an orthogonal partial least squares discriminant analysis (OPLS-DA) approach (**Figure 5.5b**). The supervised OPLS-DA model [$R^2x_{(cum)}$=0.38, $R^2y_{(cum)}$=0.84, $Q^2_{(cum)}$=0.60] developed a perfect separation into the two clusters with high fitness R^2 and accepted predictive ability Q^2 parameters ($R^2y_{(cum)}$ and $Q^2_{(cum)}$ >0.5). Moreover, the cross-model validation (**Annex 2: Figure S5.2a**) and the permutation test (100 times) (**Annex 2: Figure S5.2c**), both indicate that the developed OPLS-DA approach was positive and valid, confirming the distinction among both piglets' groups. Also, a value of 1.0 for the area under the curve (AUC) corresponding to the receiver operating characteristic (ROC) plot (**Annex 2: Figure S5.3.**) indicated a strong discrimination power for the OPLS-DA classifier model. To find the most relevant ^{1}H-NMR regions that contribute to the differentiation between suckling piglets from weaned piglets, an S-plot was performed (**Annex 2: Figure S5.4.**). From this plot, the key ^{1}H-NMR buckets that affect the discrimination were identified. These

regions were also screened according to their corresponding variable importance in the projection (VIP) values of the OPLS-DA model. Fifteen from the total 250 spectral regions were found as the more contributing (**Table 5.3.**), of which 12 regions had integral values that differed significantly between both groups (Student's t-test P-value ≤0.05). The discriminant metabolites that showed higher levels in suckling piglets were choline, lipids (including triglycerides and fatty acids), LDL, alanine, isoleucine, and probably also TMAO, whereas in weaned piglets those with higher levels were 3-hydroxybutyrate, ethanol, valine, and adipate (**Table 5.3.**).

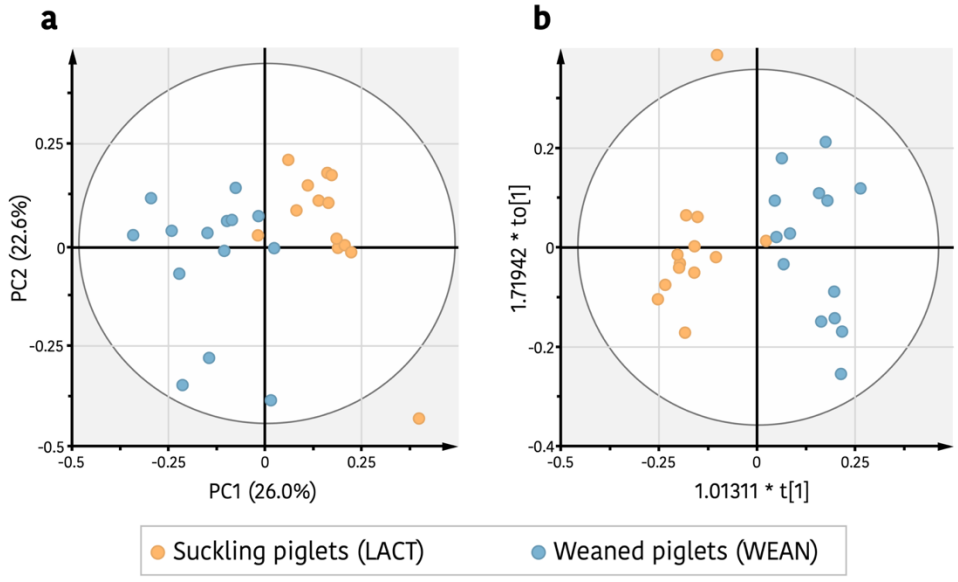

Figure 5.5. Weaning effect on serum metabolic profile of the piglets. (**a**) Principal components analysis (PCA) score plot of serum metabolites set from suckling piglets (orange) and weaned piglets (blue). (**b**) Orthogonal partial least squares discrimination analysis (OPLS-DA) score between suckling piglets (orange) and weaned piglets (blue).

Table 5.3. Statistically significant key metabolites that differentiate serum of weaned piglets from suckling piglets.

^{1}H Chemical shift ppm (Central bucket point)	Metabolite	Moieties	KEEG IDs	Weaned vs suckling		
				Fold change[a] weaning/ lactation	P-value[b]	VIP[c]
3.22	Choline	$\mathrm{N-(CH_3)_3}$	C00114	0.7	0.0002	4.36
1.14	3-hydroxy-butyrate	$\mathrm{\gamma CH_3}$	-	6.6	0.0001	4.11
3.66	Ethanol isoleucine	$\mathrm{CH_2}$ $\mathrm{\alpha CH}$	C00469 C00407	1.4	0.0350	2.88
3.54	AA + Glucose			1.3	0.0385	2.82
1.22	Lipids[e]	$CH_3(CH_2)_n$	-	0.7	0.0127	2.04
0.82	LDL[f]	$\mathrm{CH_3^*(CH_2)_n-}$	-	0.6	0.0030	1.99
2.26	Valine	βCH	C00183	1.5	0.0044	1.63
1.50	Alanine	βCH_3	C00041	0.6	0.0209	1.57
0.98	Valine isoleucine	γCH_3	C00183 C00407	1.3	0.0300	1.56
1.02	Valine isoleucine	γCH_3	C00183 C00407	1.5	0.0018	1.42
2.22	Valine	βCH	C00183	1.7	0.0764	1.28
1.98	Isoleucine	βCH	C00407	0.7	0.0007	1.25

[a] Fold change was calculated by dividing the mean of the normalized integral of each plasma metabolite in the former by the mean of the normalized integral of each plasma in the latter. Fold change >1 indicates that the metabolite was incremented, whereas fold change <1 indicates the metabolite was reduced.

[b] P-values were derived from Student's t-test.

[c] VIP value was derived from OPLS-DA with a threshold of 1.0.

[d] TMAO: Trimethylamine-N-oxide.

[e] Lipids: Triglycerides and fatty acids.

[f] LDL: Low-density lipoprotein.

5.4.6. Integration of the omics technologies

Gene expression, caecal microbiota, and metabolomics datasets were integrated by using the open-source software R (Team, 2013) v3.6.1. and the LinkHD (Zingaretti *et al*., 2019) package. The objective of this approach was to analyse these heterogeneous datasets to verify from a holistic point of view that weaning was determinant defining different clusters of samples. Therefore, confirming our hypothesis, with the added value to explore the connections (i.e: correlations) between the gene expression, metabolomics, and caecal microbial communities. Furthermore, the use of LinkHD allowed us to highlight the most informative variables within each dataset, as well as to identify which dataset was most relevant for the sample stratification. Although no model was applied in the statistical design, the samples were stratified into two clusters in a blind analysis, that aligned quite well with the groups of suckling and weaned piglets. The different ordination was mainly explained by the changes observed in piglet microbiota but also by the differential distribution of metabolites. Gene expression did not appear to contribute significantly to the cluster ordination probably due to the low number of input variables (52 genes) compared with the microbial and metabolites datasets. In total, 93 OTUs and 12 metabolites were found as discriminating between groups although the metabolites were not able to be identified. The discriminant relevant families included among others, *Fusobacteriaceae* (P=0.0010), *Bacteroidaceae* (P=0.0011), *Enterobacteriaceae* (P=0.0012), *Lactobacillaceae* (P=0.0013), *Erysipelotrichaceae* (P=0.0138), and *Prevotellaceae* (P=0.0424). Meanwhile, at the genera level, the significant discriminant genera were among others, *Fusobacterium* (P=0.0010), *Bacteroides* (P=0.0011), *Lactobacillus* (P=0.0013), *Megasphaera* (P=0.0014), and *Ruminococcus* (P=0.0157).

In addition to the stratification in clusters and the identification of the most relevant variables, LinkHD also related the existing multivariate correlation (RV) between the different datasets (**Figure 5.6.**). Thus, it was observed that the strongest association was that between the metabolites and 16S (RV values: Genes & 16S = 0.37; Genes & Metabolites = 0.22; 16S & Metabolites = 0.53).

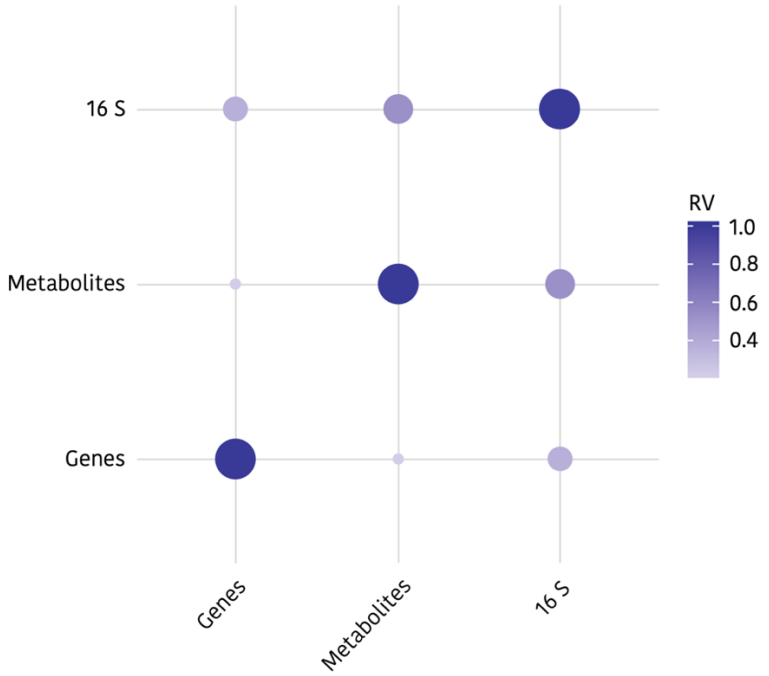

Figure 5.6. Correlation plot between gene expression (Genes), caecal microbiota (16 S) and metabolomic (Metabolites) datasets. The correlation between caecal microbiota and serum metabolome is much higher than with jejunal gene expression (Multivariate correlation values (RV): Genes & 16S = 0.37; Genes & Metabolites = 0.22; 16S & Metabolites = 0.53). Figure created by using open-source software R (Team, 2013) v3.5.3. (https://www.r-project.org/foundation/) and the LinkHD (Zingaretti *et al.*, 2019) package (https://github.com/lauzingaretti/LinkHD)

5.4.7. Integration of gut microbiome and serum metabolome data

Considering the relevance of microbiota and metabolomic data in the LinkHD analysis, an additional integration approach was performed by correlating the caecal microbiota with the blood serum metabolome. The objective was to elucidate possible relationships between the intestinal microbiota and animal metabolism, exploiting the wide range of responses found around weaning. A multivariate analysis of the data was performed looking for significant correlations among the traits (**Annex 2: Table S5.3**). The variables integrated were ^{1}H-NMR bucket area regions of 0.04 ppm width (58) and bacteria family counts (22), obtained from nursing piglets (n=14) and weaned piglets (n=14).

The 58 bucket regions, from the total of 250 regions of the spectra, were selected based on previous assigned soluble metabolites by Clausen *et al.* (2011) and He *et al.* (2009, 2012) The metabolites that showed significant Pearson correlation coefficients $|r| \geq 0.37$ (P-value ≤ 0.05) with the relative abundance of bacterial at family taxonomic level can be found in **Annex 2: Table S5.4.**

Furthermore, to better understand the putative relationships between gut microbiota and serum metabolites, a global analysis was performed by hierarchical clustering of Pearson correlations coefficients (**Figure 5.7.**). This analysis evidenced four clusters for metabolite buckets (illustrated as cluster I-IV in **Figure 5.7.**) and 3 major clusters for bacteria families (illustrated as A, B, and C). Interestingly, the clusters evidenced for metabolite buckets corresponded to certain chemical or metabolic categories. In this way, Cluster I included mainly glucose; Cluster II appears to be related to protein metabolism including aromatic (Tyr, Phe) and other amino acids (Ala, Gln, Glu) and glucose metabolism (glucose, pyruvate, and lactate); Cluster III mostly included branched-chain amino acids (Ile, Val) and also compounds related to energy metabolism (creatin/creatinine, 3-hydroxybutyrate, and acetate) and Cluster IV included buckets mostly related to lipid metabolism (unsaturated lipids, lipids [triglycerides and fatty acids], LDL, VLDL, choline). Regarding microbial clusters, Cluster A conformed by *Mogibacteriaceae*, *Coriobacteriaceae*, *Lachnospiraceae*, *Clostridiaceae*, *Ruminococcaceae*, *Spirochaetaceae*, and *Streptococcaceae* families, showed to be in general terms positively correlated with Cluster I and cluster III and negatively correlated with Cluster IV. Cluster B, including *Alcaligenaceae*, *Campylobacteraceae*, *Lactobacillaceae*, *Paraprevotellaceae*, *Porphyromonadaceae*, *Erysipelotrichaceae*, *Desulfovibrionaceae*, S24-7, *Prevotellaceae* and *Veillonellaceae*, differed from Cluster A in that Cluster B was negatively correlated with Cluster I and positively correlated with Cluster II. Finally, Cluster C, including *Bacteroidaceae*, *Dethiosulfovibrionaceae*, *Odoribacteraceae*, *Enterobacteriaceae*, and *Victivallaceae* families, differed from Clusters A and B in that it was strongly negatively correlated with cluster I, II, and III, and weakly positively correlated with Cluster IV.

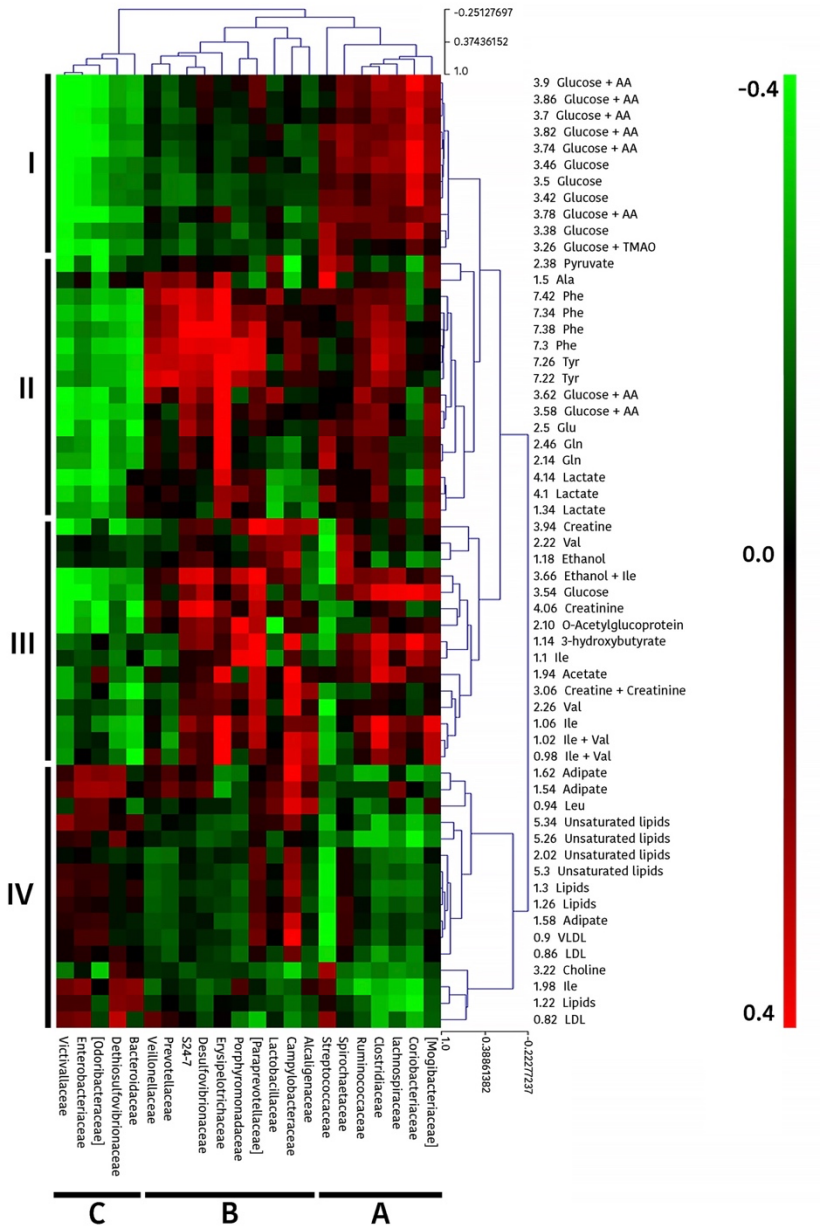

Figure 5.7. Heatmap showing the correlation analysis between gut microbiota and serum metabolome (^{1}H-NMR bucket area regions) in piglets. Red or green spots indicate positive or negative Pearson correlations between variables, respectively, and the colour intensity is directly proportional to the correlation coefficient. Four clusters (designated as I, II, III, or IV) are identified for metabolite buckets, and 3 major clusters (named A, B, and C) for bacterial families. Figure created by using open-source software R (Team, 2013) v3.5.3. (https://www.r-project.org/foundation/).

5.5. Discussion

The set of environmental, dietary, and social changes to which piglets are subjected at weaning, together with their immaturity, both intestinal and immune, are the source of relevant changes in the animal response and in the microbial ecosystem that symbiotically habit in its gastrointestinal tract (Lallès *et al.*, 2004; Moeser *et al.*, 2007; Gresse *et al.*, 2017). It is nowadays recognized that these changes can be decisive for the appropriate development and growth of the animal (Hansen *et al.*, 2012). A better understanding of these phenomena, therefore, appears as an essential tool to improve the productivity and welfare of piglets. The aim of the present study was to evaluate the impact of weaning on caecal microbial colonization, the jejunal gene expression, and the serum metabolome of the piglets to better understand the changes produced around weaning.

The high throughput sequencing of the 16S RNA gene showed that, in general terms, the diversity and community structure of caecal microbiota were in consonance with the predominant taxa described previously for healthy piglets (Holman *et al.*, 2017). The species richness and diversity of caecal microbiota were increased in piglets after weaning as reported by other studies (Pajarillo *et al.*, 2014; Mach *et al.*, 2015; Niu *et al.*, 2015; Zhao *et al.*, 2015; Chen *et al.*, 2017), where a continuous increase in alpha diversity of gut microbiota during weaning transition was observed. A higher diversity in the gut microbiota has been related to a more mature gut ecosystem and is in agreement with the concept of functional redundancy, which supports that additional taxa add redundancy to specific functions, helping the ecosystem to preserve its resilience and stability after environmental stresses (Naeem, Kawabata and Loreau, 1998; Konopka, 2009). Diversity results are, however, contradictory with other studies that have reported a decreased alpha diversity during the early period after weaning (Hu *et al.*, 2016; Han *et al.*, 2018; Y. Li, Guo, *et al.*, 2018), with a later increase from weaning to adulthood. This controversy could be due to differences between studies in the day samples were collected but also to differences in management and feeding practices or in the microbiological environment of the farm. In this sense, there may be also great differences between results obtained in controlled studies in experimental facilities and those carried out in conventional farms, where the stress to which the animals are subjected can be very different. Even

between commercial farms, the husbandry conditions around weaning can vary according to the size of the farm, the environmental and sanitary conditions, and different country-associated regulations. In this regard results obtained in this study must be contextualized in a medium-size farm, with a closed cycle system, weaning pigs at 25 days, and using medicated feed in the pre-starter period as metaphylaxis. Obviously, all these conditioning factors, and particularly the use of antimicrobials, can have a differential impact on other farms. When we use here the term weaning, its meaning goes further than the weaning itself, including also others conditioning factors associated. It is therefore important to be aware of the limitations of this study when translating their conclusions to other production systems. Regarding beta diversity, the interindividual Bray–Curtis distances between individuals decreased after weaning according to results reported by Chen *et al.* (2017), suggesting that the gut microbiota structure of piglets tends to converge between animals after weaning.

Bacteroidetes, Firmicutes, and Proteobacteria constituted the three predominant phyla in the caecal microbiota of piglets, both pre- and post-weaning, as reported in several studies (H. B. Kim *et al.*, 2012; Hu *et al.*, 2016; Chen *et al.*, 2017; Holman *et al.*, 2017; Y. Li, Guo, *et al.*, 2018). The most predominant phylum was Bacteroidetes in suckling piglets and Firmicutes in weaned piglets. However, there is no consensus on the predominant phylum for piglets after weaning. Whereas some authors describe Firmicutes (Chen *et al.*, 2017) as the main phylum, others have reported Bacteroidetes as the most abundant in weaned piglets (Pajarillo *et al.*, 2014; Hu *et al.*, 2016). Again, the different environments, diets, and sampling dates among experiments could explain this disparity. Fusobacteria, which was a predominant phylum during lactation, was significantly reduced in our study after weaning, as reported previously (Pajarillo *et al.*, 2014; Niu *et al.*, 2015; Hu *et al.*, 2016; Chen *et al.*, 2017). Some authors have suggested that the abundance of Fusobacteria, and therefore, Fusobacterium, is positively correlated with diarrheal swine diseases, such as the porcine epidemic diarrhoea and the new neonatal porcine diarrhoea (Hermann-Bank *et al.*, 2015; Liu *et al.*, 2015). Although a high individual variability is observed at the genus level, *Bacteroides* and *Lactobacillus* showed remarkable decreases after weaning, in consonance with several previous studies (Frese *et al.*, 2015; Mach *et al.*, 2015; Chen *et al.*, 2017; Gresse *et al.*, 2017; Han *et al.*, 2018). Other genera, such as

Fusobacterium and *Megasphaera* were also higher in suckling piglets, as stated by Chen *et al.* (2017) Nonetheless, some authors have reported increases in *Megasphaera* abundance after weaning (Slifierz, Friendship and Weese, 2015; Han *et al.*, 2018; Y. Li, Guo, *et al.*, 2018). The higher relative abundances of *Bacteroides* and *Lactobacillus* in suckling piglets have been correlated with a milk-oriented microbiome (Frese *et al.*, 2015). On the one hand, *Bacteroides* has been reported to use a wide range of both milk oligosaccharides and host-derived glycans (Marcobal *et al.*, 2010). On the other hand, *Lactobacillus* is a well-known lactate producer by consuming simple milk sugars such as lactose (Schwab and Gänzle, 2011). Moreover, whereas *Fusobacterium* has been positively correlated to intestinal diseases (Allen-Vercoe and Jobin, 2014; Hermann-Bank *et al.*, 2015; Liu *et al.*, 2015), *Lactobacillus* has been labelled as a major player in the establishment and the maintenance of bacterial homeostasis after birth (Konstantinov *et al.*, 2006). Therefore, the abrupt change to a solid cereal-based diet and the withdrawal of milk explain the decrease of the previously mentioned genera and the increase of butyrate-producing genera including *Roseburia*, *Ruminococcus*, and *Lachnospira*, among others (Zhao *et al.*, 2018). Actually, and in consonance with other authors (Mach *et al.*, 2015; Slifierz, Friendship and Weese, 2015; Chen *et al.*, 2017), an increased abundance of *Roseburia* was observed in piglets after weaning. Moreover, other *Lachospiraceae* genera, such as *Lachnospira*, *Coprococcus*, and *Dorea*, began to emerge after weaning. Although similar results were observed by Y. Li, Guo, *et al.* (2018), a decreased abundance of *Lachnospira* after weaning was reported by Frese *et al.* (2015). In agreement with other studies, *Ruminococcus* showed an increased abundance in weaned piglets (Frese *et al.*, 2015). The genera belonging to *Lachnospiraceae* and *Ruminococcaceae* families are adapted to metabolize a wide range of complex oligosaccharides and polysaccharides while producing short-chain fatty acids. Indeed, *Roseburia* is a major contributor to the metabolic network of carbohydrate utilization and production of butyrate (Duncan, Louis and Flint, 2004). Altogether, the higher abundance of *Roseburia*, *Ruminococcus*, *Coprococcus*, *Dorea* and *Lachnospira* genera in weaned piglets, adapted to digest resistant starches and dietary fibres to convert them to SCFA, show the quick microbial change of the piglets' gut microbiota to cope with diets rich in complex carbohydrates, as these abundance shifts occur in a short period of time.

These taxonomic changes observed in the intestinal microbiota were consistent with the results obtained for the functional metabolic pathways of the caecal microbiota with PICRUST analysis. It was observed that the pathways related to different metabolic routes, mostly energy and protein metabolism, were more represented in the suckling piglets, whereas the pathways related to bacterial processes and environment and information processing, such as cell motility, membrane transport, sporulation, or signal transduction were higher in weaned piglets. As it is well known, piglets experience anorexia and intestinal disorders shortly after weaning (Lallès *et al.*, 2004). As a result, the gastrointestinal environment and functionality are severely affected. In this context, it seems consistent that metabolic pathways related to nutrient metabolism do not result in a competitive advantage within the gut microbiota ecosystem. Other studies have reported comparable results with a decrease in many metabolic pathways such as carbon fixation pathways, lipid biosynthesis proteins, citrate cycle (TCA cycle), and cofactor metabolism after weaning (Y. Li, Guo, *et al.*, 2018), suggesting that the reduction in the nutrient availability related to post-weaning anorexia might be part of the microbiota adaptation process. On the other hand, the observed increase in the representativeness of pathways related to cell motility, membrane transport, or signal transduction suggests that during the post-weaning phase those functions would confer competitive advantages to certain microbial groups. An adapted microbiota, as that found at the end of the lactating period, would prioritize the metabolic activity specialized in using a particular set of nutrients provided in this case by milk. However, shortly after weaning the microbiota would give priority to pathways related to adaptation to the new environment, among them, chemotaxis, motility, flagellar function, construction of the cytoskeleton, and the activation of transporters. This transition can turn into a chance for opportunistic pathogens such as E. coli to proliferate and cause post-weaning diarrhoea. Actually, flagellar assembly and bacterial motility proteins, pathways exhibited between others by E. coli, showed higher representativeness after weaning and could be regarded as an index of potential virulence factors (Haiko and Westerlund-Wikström, 2013).

Regarding the possible impact of weaning on animal response and particularly on intestinal functionality, results from the OpenArray technology showed a significant decrease in the jejunal gene expression of *OCLN*, *CLDN4*,

MUC2, and *MUC13*. *OCLN* and *CLDN4* are transmembrane proteins of the tight junction (TJ) (McCarthy *et al.*, 1996; Markov, Aschenbach and Amasheh, 2015). A downregulation in the expression of *OCLN* has also been reported associated with the weaning process (Hu *et al.*, 2013). Moreover, *MUC2* and *MUC13*, which encode a gel-forming-mucin and a membrane-bound mucin respectively, were also downregulated in weaned piglets, reducing its protective effect on the intestinal epithelium. Actually, the downregulation of MUC genes has also been associated with the presence of pathogenic bacteria, such as ETEC (Zhou *et al.*, 2012).

Related to the immune function, a downregulation of *IFNγR1* and an upregulation of *PPARGC1α* were observed after weaning. *IFNγR1* is a cytokine receptor that encodes the ligand-binding chain of the gamma interferon receptor that has been reported to be upregulated by ETEC (Liu *et al.*, 2014). On the other hand, *PPARGC1α*, is an endogenous regulator of intestinal inflammation (D'Errico *et al.*, 2011), with an inhibitory effect on pro-inflammatory cytokines (Moraes, Piqueras and Bishop-Bailey, 2006). It has been reported to be upregulated during the transient suckling-weaning period in the jejunum of rats (Mochizuki, Yorita and Goda, 2009). Therefore, the upregulation we observed in *PPARGC1α* in weaned piglets could be explained by weaning, constituting another factor to be considered in the downregulation of the inflammatory cytokines encoding genes mentioned above including *IFNγR1*.

Within the hormone/enzyme encoders category, *SI* and *HNMT*, two genes encoding digestive enzymes, were downregulated after weaning, whereas the *CCK* and *IGF1R*, two genes encoding digestive hormones, were upregulated. The enzyme encoded by the *SI* gene, is responsible for the digestion of dietary carbohydrates, whereas *HNMT* encodes a histamine-degrading enzyme. A downregulation in the expression of SI has been reported related to weaning (Zhu *et al.*, 2012). As for the hormone encoders, *CCK* is involved in several activities such as satiety regulation, enzyme secretion, gut motility, and anxiety, whereas *IGF1R* is an important regulator of intestinal cell growth and differentiation (Jones and Clemmons, 1995). Although in this study both genes showed increased expressions in weaned piglets, other authors have reported a downregulation after weaning (Tang, Van Kessel and Laarveld, 2002; Zhu *et al.*, 2014). A higher expression of *CCK* and *IGFR1* could be

suggestive of a gut compensatory effect to increase the piglet digestion processes and stimulate the gut development after weaning. However, further research is required concerning the roles of *CCK* and *IGFR1* in these adaptive processes.

In the nutrient transporters category, a greater expression of *SLC16A1* – encoder of the monocarboxylate transporter 1 (MCT1) protein – was observed after weaning. The MCT1 mediates the absorption of lactate and microbial-derived SCFAs across cell membranes (Ganapathy *et al.*, 2013) and has been reported to be upregulated by butyrate (Villodre Tudela *et al.*, 2015). Therefore, an increased expression of *SLC16A1* could be explained by an increased microbial fermentative activity after weaning with the production of lactate and other SCFAs. The *SLC15A1* and *SLC13A1* genes – encoders of the peptide transporter 1 (PEPT1) protein and sodium/sulfate symporters (NaS1), respectively – were downregulated in weaned piglets. *SLC15A1* has been related to absorption of protein digestion products (Adibi, 1997), whereas *SLC13A1* has been shown to play a role in the intestinal barrier function through sulfomucins (Markovich, 2014). Thus, lower expressions of these genes carry negative repercussions for gut health and nutrient digestion. In addition, some authors have reported decreases in the expression of *SLC15A1* and *SLC13A1* due to the presence of pathogenic bacteria such as ETEC or Lawsonia intracellularis (Trevisi *et al.*, 2012, 2018; Smith *et al.*, 2014). To end with nutrient transporters, two genes related to zinc transport also showed significant changes. While *SLC30A1* – encoder of the zinc transporter 1 (ZnT1) protein, related to the transport of zinc to the extracellular matrix – increased, the *SLC39A4* gene – encoder of the zinc transporter ZIO4, and related to zinc uptake from the gut lumen – decreased after weaning. *SLC39A4* has been shown to be downregulated by high dietary ZnO in piglets (Martin *et al.*, 2013) and, particularly, in piglets challenged with ETEC (Sargeant *et al.*, 2010), whereas *SLC30A1* has been shown to be upregulated by high dietary ZnO (Martin *et al.*, 2013). Therefore, these results are according to what would be expected by the introduction of the weaning diet with pharmacological doses of ZnO. Finally, the *HSD11B1* gene, encoder of the 11β-Hydroxysteroid dehydrogenase type 1 enzyme, also known as cortisone reductase, and responsible for reducing cortisone to cortisol was upregulated in weaned piglets. A greater expression of *HSD11B1* after weaning could be related to a

higher cortisol production, which is used as a marker of the levels of stress in piglets after weaning (Martínez-Miró *et al.*, 2016).

Consistent with the changes observed in the jejunal gene expression around weaning, ^{1}H-NMR results, also evidenced the relevant impact of weaning on the animal metabolomic response. Within 5 days between samplings, animals showed a quite different metabolomic pattern. The reduced serum levels of LDL, lipids (triglycerides and fatty acids), choline, and the increased levels of the ketone body β-hydroxybutyrate after weaning, support the concept that weaning affects the metabolism of energy. Tucker *et al.* (2010) described β-hydroxybutyrate as a potential biomarker of metabolic stress as a result of food and/or water restriction or deprivation. In fact, the stressful condition of weaning generally results in reduced feed intake during the first week after weaning since the piglets must adapt from a digestible and palatable maternal liquid milk to a solid dry diet. We can hypothesize that the significant increment of β-hydroxybutyrate observed 72h after weaning could be the signal of an incipient (or previous) ketosis state considering fatty acids levels were not increased. Furthermore, it is known that serum levels of most amino acids undergo marked shifts in the neonatal period and in catabolic conditions. In this sense, reduced levels of the amino acid Ile after weaning could be due to its consumption as a precursor for ketonic bodies production that contribute to the increment of β-hydroxybutyrate. Likewise, lower levels of serum Ala in weaned piglets could be a consequence of its consumption during gluconeogenesis in the liver to provide glucose to extrahepatic cells and tissues (Wu, 2009). However, it is worth noting that the metabolic profile of an organism is just a snapshot at a given time whereas metabolism is something dynamic and complex that can hardly be evaluated from a single picture.

The blinded integration of gene expression, metabolome and metagenome datasets with LinkHD R package confirmed the disparity between suckling and weaned piglets clustering them in two groups and also the higher correlation between caecal microbiota and serum metabolome compared to jejunal gene expression. The hierarchical clustering of Pearson correlation coefficients between the metabolomic changes and the shifts observed in microbiota showed a clustering pattern (**Figure 5.7.**) that could suggest rational associations between gut microbiota structure and animal metabolic

response. Four clusters of metabolite buckets (clusters I-IV) and 3 clusters of bacterial families (clusters A-C) could be identified. Interestingly, most of the metabolites that were increased (like 3-hydroxybutyrate, valine, and ethanol) or decreased (among them choline, Ile, LDL, and lipids) after weaning were within Cluster III or Cluster IV, respectively. Moreover, all bacteria families conforming Cluster A (except *Streptococcaceae*) increased after weaning while those conforming Cluster C decreased after weaning. Considering that Cluster A is positively correlated with Cluster III and negatively with Cluster IV, and Cluster C showed opposite sign correlations, results evidenced the relevant impact of weaning on the gut microbiota and piglet metabolic response.

The multivariate analysis performed to identify possible associations between metabolites and microbial groups showed several significant correlations (as shown in **Annex 2: Table S5.4.**). From those results, we could hypothesize that the significant increment of *Coriobacteriaceae* (P=0.0476) observed after weaning could be associated with the significant increment of serum 3-hydroxybutyrate and decrease of lipids (r=0.40 and r=-0.39, respectively), while the significant reduction of *Streptococcaceae* (P=0.0025) could be related with the significantly elevated levels of valine and adipate (r=-0.52 and r=-0.42, respectively) and the significantly lower levels of alanine (r=0.48). Regarding the remaining correlations, other authors have also described significant positive correlations between the bacterial genera *Ruminococcus* and *Coprococcus*, from *Ruminococcaceae* and *Lachnospiraceae* families respectively, with glucose (Sun, Su and Zhu, 2016). Moreover, it has been reported that Firmicutes genera such as *Streptococcus*, correlate positively with amino acids like valine, isoleucine, and alanine (Y. Li, Fu, *et al.*, 2018). Although the interplay between the gut microbiome and mammalian blood metabolites has been demonstrated (Wikoff *et al.*, 2009), it is difficult to establish if there is any causative effect in these correlations. There is still a long way to go to fully understand the interaction between the pig's intestinal microbiota and its impact on the serum metabolome.

5.6. Conclusion

The present study evidenced the great changes that are produced at a microbial, genetic, and metabolic level in the piglet shortly after weaning. Caecal microbiota showed remarkable structural differences just 3 days after, with increases in alpha diversity and significant changes in taxonomic groups. The higher relative abundances of *Bacteroides* and *Lactobacillus* in suckling piglets correlated with a milk-oriented microbiome, whereas the higher abundances of *Roseburia*, *Ruminococcus*, *Coprococcus*, *Dorea*, and *Lachnospira* genera in weaned piglets showed the quick adaptation of the piglets' gut microbiota to cope with diets rich in complex carbohydrates. From the taxonomic changes observed in the intestinal microbiota, we could also infer changes in functional metabolic pathways. Shortly after weaning the microbiota would give priority to pathways related to adaptation to the new environment rather than pathways related to different metabolic routes, as observed during lactation. At the jejunal level, a decrease was found in the gene expression of various barrier function genes (*OCLN*, *CLDN4*, *MUC2*, and *MUC13*) and nutrient transport genes (such as *SLC15A1* and *SLC13A1*) revealing the negative impact of weaning on intestinal functionality. The metabolomic approach evidenced the impact of weaning on the energy metabolism with increases in β-hydroxybutyrate levels and decreases in choline, LDL, triglycerides, fatty acids, alanine, and isoleucine, suggesting an incipient ketosis state. Furthermore, the hierarchical clustering of Pearson correlations identified four metabolite clusters corresponding to specific chemical or metabolic categories suggesting potential causal relationships between microbiota and animal metabolism. In this sense, *Coriobacteriaceae*, significantly reduced after weaning, appears positively correlated to serum 3-hydroxybutyrate and negatively with lipid, while *Streptococcaceae*, significantly increased after weaning, appears negatively correlated to valine and adipate and positively to alanine.

In summary, the results of this study highlight the huge changes that occur in piglets raised in commercial farms shortly after weaning and show how microbiome and animal metabolism respond to it in a coordinated and interdependent way evidencing the interplay between the gut microbiota and its host.

5.7. Declarations

5.7.1 Data availability

The raw sequencing data employed in this article has been submitted to the NCBI's sequence read archive (https://www.ncbi.nlm.nih.gov/sra); BioProject: PRJNA767391.

5.7.2. Ethics declarations

The housing, management, husbandry, and slaughtering conditions of this experiment conformed to the European Union Guidelines (Directive 2010/63/EU). All experimental procedures used in this study were approved by the Animal and Human Experimental Ethical Committee of Universitat Autònoma de Barcelona (UAB) (permit code CEEAH 3817) and designed in compliance with the ARRIVE guidelines.