# EFFECTS OF N-3 LONG-CHAIN FATTY ACIDS IN SOW AND PIGLET DIETS ON PERINATAL PIGLET PERFORMANCE, OXYLIPINS, IMMUNITY, AND MICROBIOTA

**Eudald Llauradó Calero**

DOCTORAL THESIS

# EFFECTS OF n-3 LONG-CHAIN FATTY ACIDS IN SOW AND PIGLET DIETS ON PERINATAL PIGLET PERFORMANCE, OXYLIPINS, IMMUNITY, AND MICROBIOTA.

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# Effects of n-3 long-chain fatty acids in sow and piglet diets on perinatal piglet performance, oxylipins, immunity, and microbiota

Doctoral Thesis

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FAIG CONSTAR que aquest treball, titulat “Effects of n-3 long-chain fatty acids in sow and piglet diets on perinatal piglet performance, oxylipins, immunity, and microbiota”, que presenta **Eudald Llauradó Calero** per a l’obtenció del títol de Doctor, ha estat realitzat sota la meva direcció al Programa de Nutrició Animal de IRTA i que compleix els requisits per a l’obtenció de la Menció Internacional de Doctorat.

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HAGO CONSTAR que el presente trabajo, titulado “Effects of n-3 long-chain fatty acids in sow and piglet diets on perinatal piglet performance, oxylipins, immunity, and microbiota”, que presenta **Eudald Llauradó Calero** para la obtención del título de Doctor, ha sido realizado bajo mi dirección en el Programa de Nutrició Animal de IRTA y que cumple los requisitos para la obtención de la Mención Internacional de Doctorado.

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I STATE that the present work, entitled “Effects of n-3 long-chain fatty acids in sow and piglet diets on perinatal piglet performance, oxylipins, immunity, and microbiota”, presented by **Eudald Llauradó Calero** to obtain the degree of Doctor, has been carried out under my supervision at IRTA Animal Nutrition Programme and that it is eligible to apply for the International Doctoral Mention.

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Tarragona, 09/06/2023

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## ABBREVIATIONS

**bBW:** Birth body weight

**BW:** Body weight

**DHA:** Docosahexaenoic acid

**EPA:** Eicosapentaenoic acid

**FA:** Fatty acid

**HBW:** High birth weight

**Ig:** Immunoglobulin

**IL:** Interleukin

**LPS:** Lipopolysaccaride

**LCFA:** Long-chain fatty acid

**LBW:** Low birth weight

**TLR:** Toll-like receptor

**TNFα:** Tumor necrosis factor α

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- Llauradó-Calero, E., Badiola, I., Delpino-Rius, A., Lizardo, R., Torrallardona, D., Esteve-Garcia, E., Tous, N., 2021. Fish oil rich in eicosapentaenoic acid and docosahexaenoic acid in sow diets modifies oxylipins and immune indicators in colostrum and milk. Animal 15: 100403.

- Llauradó-Calero, E., Badiola, I., Samarra, I., Lizardo, R., Torrallardona, D., Esteve-Garcia, E., Tous, N., 2022. Eicosapentaenoic acid- and docosahexaenoic acid-rich fish oil in sow and piglet diets modifies blood oxylipins and immune indicators in both, sows and suckling piglets. Animal 16: 100634.

- Llauradó-Calero, E., Climent, E., Chenoll, E., Ballester, M., Badiola, I., Lizardo, R., Torrallardona, D., Esteve-Garcia, E., Tous, N., 2022. Influence of dietary n-3 long-chain fatty acids on microbial diversity and composition of sows' feces, colostrum, milk, and suckling piglets' feces. Frontiers in Microbiology 13: 982712.


# SUMMARIES

Effects of n-3 long-chain fatty acids in sow and piglet diets on perinatal piglet performance, oxylipins, immunity, and microbiota.

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## SUMMARY

The genetic improvement in sow prolificity has increased litter size and the proportion of piglets born with low birth body weight. Consequently, low birth weight piglets born from large litters are weaker and less likely to survive due to increased difficulties to face early life events such as weaning. Weaning is one of the most critical steps in piglet's life, it represents an abrupt change of environment, and transition from maternal milk to solid feed associated with increased stress. In the past, antimicrobials had been used as growth promoters to address these challenges. However, the European Union banned their use to fight against antibiotic microbial resistance. Thus, research on nutritional strategies capable to improve sow reproductive performance, colostrum and milk composition, and piglet's development and health is on the rise. Fish oil is rich in eicosapentaenoic acid (**EPA**) and docosahexaenoic acid (**DHA**). These n-3 long-chain fatty acids (**n-3 LCFAs**), together with their derived oxygenated derivatives, also known as oxylipins, are associated with anti-inflammatory and inflammation resolving roles. n-3 LCFAs are also associated with increases in length of gestation, number of oocytes implanted, gut microbial diversity and potentially beneficial bacteria. Therefore, the global objective of this thesis was the study of fish oil in sow and piglet post-weaning diets to improve the productive efficiency of the sow, and the robustness and health status of the piglets during early life.

Thirty-six sows from four consecutive batches were randomly distributed between a control and an n-3 LCFA-rich dietary treatments, which were offered from insemination until the end of lactation. At birth, two piglets with lightest birth body weight (>800g) and two with the highest birth body weight were selected from each litter. At weaning, the selected piglets were distributed again between two post-weaning diets (control or n-3 LCFA diets), which resulted in a 2 x 2 x 2 factorial distribution of treatments (2 maternal diets x 2 piglet diets x 2 birth BW categories) until day 28 post-weaning. For the first batch of sows, at the end of the trial (day 28 post-weaning), piglets were humanely slaughtered and sampled.

Sow weight, litter characteristics, and piglet's growth were monitored during the whole trial. Average feed intake was also recorded for sows and weaned piglets. Colostrum and milk were sampled at farrowing and at weaning, respectively. Blood and faeces were sampled in gestating and lactating sows, and in suckling and weaned piglets. In addition, ileal mucosa and caecum contents were also sampled from the weaned piglets in the first batch.

The piglets from n-3 LCFA fed sows tended to be heavier at weaning than those from control sows. However, since the litter size of sows that were fed n-3 LCFA also tended to be smaller, this effect could not be directly associated to n-3 LCFAs. During post-weaning, the inclusion of n-3 LCFAs in maternal and piglet diets did not affect piglet's growth. Regarding FAs and oxylipin profiles, dietary fish oil increased the concentrations of EPA, DHA, and their derived oxylipins in sows' blood, colostrum, and milk. Moreover, this effect was also observed in the blood of the suckling piglets at weaning, suggesting certain transmission from sows to piglets. In post-weaned piglets, these parameters were only increased by the inclusion of n-3 LCFAs in the piglet diet, indicating that the effect of maternal diet does not last over time. Regarding the immune factors analysed, an increase in immunoglobulin M in blood from sows and weaned piglets and a decrease in tumor necrosis factor α in milk were observed. However, additional studies evaluating a broader range of immune parameters are necessary to clearly understand the immunomodulatory role of n-3 LCFAs. Finally, while the inclusion of fish oil in the maternal diet increased the microbial diversity in faeces of suckling piglets', it also decreased faecal diversity at 28 days post-weaning. Nevertheless, potentially beneficial bacterial populations such as the mucin-degraders genera *Akkermansia* and *Bacteroidetes* in suckling piglets' faeces and the short-chain FAs producer genus *Ruminococcus* in weaned piglets' faeces were increased.

All in all, this thesis suggests that the dietary inclusion of dietary n-3 LCFAs may improve the growth of suckling piglets by increasing the blood serum concentrations of n-3 LCFAs, in particular EPA and DHA, and their oxidated derivatives in sows, piglets, colostrum and milk. Furthermore, maternal dietary n-3

LCFAs are also able to shape the microbiota during the early stages of life of the pig and this effect could last up to 28 days post-weaning.

## RESUM

La millora genètica en la prolificitat de les truges ha augmentat la mida de les garrinades i la proporció de garrins nascuts amb un baix pes corporal. Conseqüentment, els garrins d'aquestes garrinades nascuts amb un baix pes són més febles i tenen menys probabilitats de sobreviure, fet que els hi dificulta fer front a esdeveniments durant les primeres etapes de vida com ara el deslletament. El deslletament és una dels moments més crítics en la vida dels garrins, representa un canvi brusc d'instal·lacions, a més de la transició de la llet materna a l'aliment sòlid, resultant en un augment de l'estrès. En el passat, els antimicrobians s'utilitzaven com a promotors de creixement per abordar aquest repte. No obstant, la Unió Europea va prohibir el seu ús per combatre les resistències antimicrobianes. Per aquests motius, s'està duent a terme recerca sobre diferents estratègies nutricionals amb capacitat de millorar el rendiment productiu de les truges, la composició del calostre i la llet, i el desenvolupament i l'estat de salut dels garrins. L'oli de peix és ric en àcid eicosapentaenoic (**EPA**) i àcid docosahexaenoic (**DHA**). Aquests àcids grassos n-3 de cadena llarga (**n-3 LCFAs**), juntament amb els seus derivats oxigenats, coneguts com a oxilipines, tenen un paper important en funcions antiinflamatòries i de resolució de la inflamació. No obstant, diferents estudis també han associat els n-3 LCFAs, en un augment de la llargada de la gestació, el número d'oòcits implantats, i un augment de la diversitat bacteriana i bactèries associades a possibles beneficis per la salut. Per tant, l'objectiu global d'aquesta tesi va ser incloure oli de peix en les dietes de les truges i dels garrins en transició per millorar tan l'eficiència productiva de les truges, com la robustesa i l'estat de salut dels garrins durant les primeres etapes de vida.

Trenta-sis truges de quatre bandes consecutives es van distribuir de manera aleatòria en una dieta de control o en una dieta rica en n-3 LCFAs des de la inseminació fins al final de la lactació. En el moment del naixement, es van seleccionar els dos garrins amb el pes corporal més lleuger (>800 g) i els dos amb el pes corporal més elevat de cada garrinada. Al deslletament, els garrins es van agrupar y distribuir entre dues dietes de transició (dietes control o n-3 LCFA)

resultant en una distribució factorial dels tractaments 2 x 2 x 2 (2 dietes maternals x 2 dietes garrí x 2 pes corporal al naixement) fins el dia 28 de transició. De la primera banda de truges, al acabar l'estudi de transició, els garrins es van sacrificar per tal de fer la recollida del diferent tipus de mostres.

El pes de les truges, les característiques de la garrinada i el creixement dels garrins es va controlar durant tot l'estudi. També es va enregistrar la ingesta de pinso mitjana de les truges, i dels garrins deslletats. El calostre i llet es van mostrejar al naixement i en el moment del deslletament, respectivament. També es van obtenir mostres de sang i femta de les truges gestants i lactants, així com dels garrins en fase de lactància i deslletats. A més a més, també es van prendre mostres de la mucosa ileal i del contingut del cec dels garrins deslletats de la primera tanda.

Pel que fa al rendiment productiu, els garrins de les truges alimentades amb una dieta rica en n-3 LCFAs tendien a pesar més en el moment del deslletament en comparació amb els garrins del grup de control. No obstant això, aquest efecte no es pot associar directament als n-3 LCFAs ja que les truges alimentades amb n-3 LCFAs tendien a tenir una mida de la garrinades més petita. La inclusió d'n-3 LCFAs en les dietes de les truges i els garrins no va afectar el creixement dels garrins durant la fase de transició. Pel que fa a la composició d'àcids grassos i oxilipines, l'oli de peix en la dieta augmenta les concentracions d'EPA, DHA i les seves oxilipines derivades a la sang de les truges, al calostre i a la llet. A més, aquest efecte també es va observar a la sang dels garrins lactants en el moment del deslletament, suggerint certa transmissió de la truja al garrí. No obstant, en els garrins deslletats, l'augment d'aquestes concentracions només es va deure a la inclusió d'n-3 LCFAs en la dieta dels garrins, indicant que l'efecte de la dieta maternal en aquests paràmetres no perdura en el temps. Referent als factors immunitaris analitzats, es va observar un augment d'immunoglobulina M a la sang de les truges i dels garrins deslletats i una disminució del factor de necrosi tumoral α en la llet. Tanmateix, són necessaris futurs estudis que avaluïn una gamma més àmplia de paràmetres immunològics per acabar de comprendre clarament el paper immunomodulador dels n-3 LCFAs. Finalment, mentre que la inclusió d'oli de peix

en la dieta de les truges va augmentar la diversitat microbiana a les femtes dels garrins en fase de lactància, va disminuir aquest paràmetre en les femtes 28 dies després del deslletament. No obstant, es van observar increments en poblacions bacterianes potencialment beneficioses com els gèneres *Akkermansia* i *Bacteroidetes,* degradadors de mucina, en les femtes dels garrins en fase de lactància i el gènere *Ruminococcus,* productor d'àcids grassos de cadena curta, en les femtes dels garrins deslletats.

Així doncs, aquesta tesi demostra com la inclusió d'àcids grassos n-3 de cadena llarga en la dieta pot millorar el creixement dels garrins durant la fase de lactació, augmenta les concentracions de n-3 LCFAs, en particular l'EPA i el DHA, i els seus derivats oxidatius en la sang de les truges i garrins, calostre i llet, augmentant molècules associades a rols antiinflamatoris i de resolució de la inflamació. A més a més, la suplementació maternal amb n-3 LCFAs pot modular la microbiota durant les primeres etapes de la vida del garrí i els seus efectes poden perdurar fins a 28 dies després del deslletament.

## RESUMEN

La mejora genética en la prolificidad de las cerdas ha aumentado el tamaño de las camadas y la proporción de lechones nacidos con un bajo peso corporal. En consecuencia, los lechones de estas camadas nacidos con un bajo peso son más débiles y tienen menos probabilidades de sobrevivir, lo que les dificulta enfrentarse a eventos en las primeras etapas de vida, como el destete. El destete es uno de los momentos más críticos en la vida de los lechones, representa un cambio brusco de instalaciones, además de lo que implica la transición de la leche materna a alimento sólido, resultando en un aumento del estrés. En el pasado, se utilizaban antimicrobianos como promotores de crecimiento para abordar este desafío. Sin embargo, la Unión Europea prohibió su uso para combatir la resistencia antimicrobiana. Por estos motivos, se está llevado a cabo investigaciones sobres diferentes estrategias nutricionales capaces de mejorar el rendimiento productivo de las cerdas, la composición del calostro y la leche, y el desarrollo y estado de salud de los lechones. El aceite de pescado es rico en ácido eicosapentaenoico (**EPA**) y ácido docosahexaenoico (**DHA**). Estos ácidos grasos n-3 de cadena larga (**n-3 LCFAs**), junto con sus derivados oxigenados, también conocidos como oxilipinas, desempeñan un papel importante en funciones antiinflamatorias y de resolución de la inflamación. Sin embargo, diferentes estudios también han asociado a los n-3 LCFAs con un aumento de la duración de la gestación y el número de oocitos implantados, y un aumento de la diversidad bacteriana y bacterias con posibles efectos beneficiosos para la salud. Por lo tanto, el objetivo global de esta tesis fue incluir aceite de pescado en las dietas de las cerdas y los lechones en transición para mejorar tanto la eficiencia productiva de las cerdas como la robustez y estado de salud de los lechones durante las primeras etapas de vida.

Treinta y seis cerdas de cuatro bandas consecutivas se distribuyeron al azar a una dieta control o a una dieta rica en n-3 LCFAs desde la inseminación hasta el final de la lactanción. Al nacer, se seleccionaron los dos lechones con el peso corporal más bajo (> 800 g) y los dos con el peso corporal más alto de cada camada. Al

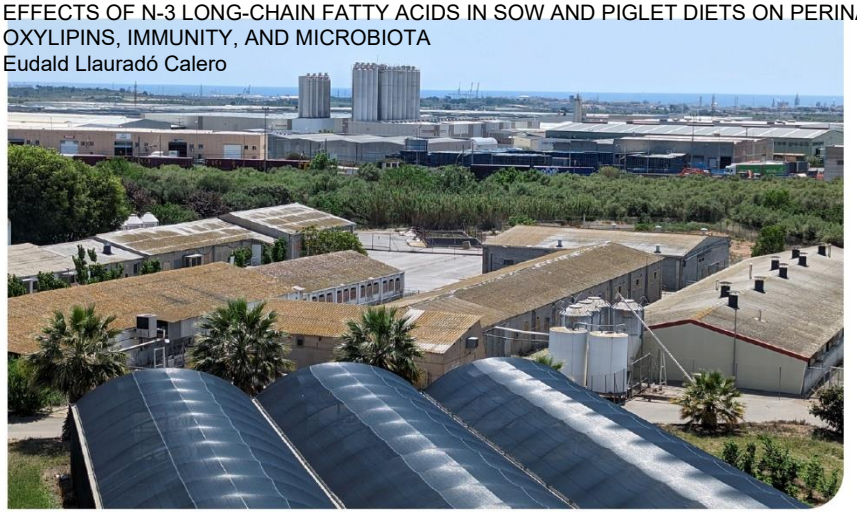
destete, los lechones seleccionados se agruparon y distribuyeron entre dos dietes de transición (dietas control o n-3 LCFA) resultando en una distribución factorial de los tratamientos 2 x 2 x 2 (2 dietas maternales x 2 dietas lechón x 2 pesos corporales al nacimiento) al nacimiento hasta el día 28 de transición. De la primera banda de cerdas, al terminar el estudio de transición se sacrificaron los lechones a fin de recoger diferentes tipos de muestras.

Se monitoreó el peso de las cerdas, las características de la camada y el crecimiento de los lechones durante todo el estudio. También se registró la ingesta promedio de pienso para las cerdas y los lechones destetados. Se recolectaron muestras de calostro y leche al nacer y al destete, respectivamente. Se recolectaron muestras de sangre y heces de las cerdas gestantes y lactantes, así como de los lechones lactantes y destetados. Además, también se tomaron muestras de la mucosa ileal y el contenido del ciego de los lechones destetados de la primera banda.

En cuanto al rendimiento productivo, los lechones de las cerdas alimentadas con una dieta rica en n-3 LCFAs tendieron a pesar más en el momento del destete en comparación con los lechones del grupo control. Sin embargo, este efecto no puede asociarse directamente a los n-3 LCFAs, ya que las cerdas alimentadas con n-3 LCFAs tendían a tener un tamaño de las camadas más pequeño. La inclusión de n-3 LCFAs en las dietas de las cerdas y los lechones no afectó el crecimiento de los lechones durante la fase de transición. En cuanto a la composición de ácidos grasos y oxilipinas, el aceite de pescado en la dieta aumenta las concentraciones de EPA, DHA y sus oxilipinas derivadas en la sangre de las cerdas, el calostro y la leche. Además, este efecto también se observó en la sangre de los lechones lactantes en el momento del destete, lo que sugiere cierta transmisión de la cerda al lechón. Sin embargo, en los lechones destetados, el aumento de estas concentraciones sólo se debió a la inclusión de n-3 LCFAs en la dieta de los lechones, lo que indica que el efecto de la dieta maternal sobre estos parámetros no perdura en el tiempo. En referencia a los factores inmunitarios, se observó un aumento de la inmunoglobulina M en la sangre de las cerdas y los lechones

destetados, y una disminución del factor de necrosis tumoral α en la leche. No obstante, se necesitan estudios futuros que evalúen una gama más amplia de parámetros inmunológicos para terminar de comprender claramente el papel inmunomodulador de los n-3 LCFAs. Finalmente, mientras que la inclusión de aceite de pescado en la dieta de las cerdas aumentó la diversidad microbiana en las heces de los lechones lactantes, este parámetro disminuyó en las heces 28 días después del destete. Sin embargo, se observaron aumentos en poblaciones bacterianas potencialmente beneficiosas como los géneros degradadores de mucina *Akkermansia* y *Bacteroidetes* en las heces de los lechones lactantes, y el género productor de ácidos grasos de cadena corta *Ruminococcus* en las heces de los lechones destetados.

Por lo tanto, esta tesis demuestra cómo la inclusión de ácidos grasos n-3 de cadena larga en la dieta puede mejorar el crecimiento de los lechones durante la fase de lactación, aumenta las concentraciones de n-3 LCFAs, en particular EPA y DHA, y sus derivados oxidativos en la sangre de las cerdas, lechones, calostro y leche, aumentando también moléculas asociadas a roles antiinflamatorios y de resolución de la inflamación. Además, la suplementación maternal con n-3 LCFAs puede modular la microbiota durante las primeras etapas de la vida de los lechones y sus efectos pueden perdurar hasta 28 días después del destete.





# GENERAL INTRODUCTION

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# GENERAL INTRODUCTION

This introduction, first aims to briefly contextualize the main problems that exist in the current pig production systems, mainly focusing on the first stages of the piglet's life. Then, it summarises the roles that lipids may exert in swine nutrition, and finally, revises in depth previous results on the effects of dietary n-3 LCFAs on productive parameters, colostrum and milk composition or FA profiles as well as the emerging research topics in relation to n-3 LCFAs oxygenated derivatives, and their effects on immune parameters, or microbiota.

## Root of the problem

The genetic improvement of sow prolificity over the last decades has resulted, nowadays, in a high proportion of hyperprolific sows (Rosero *et al.*, 2016; Farmer and Edwards, 2022). According to the Spanish Porcine Reference Database – BDporc (www.bdporc.irta.es) the litter size in Spain has increased from 12.1 in 2013 to 14.9 piglets born alive in 2022 (Figure 1). However, large litters have also been associated with a reduction in the average birth body weight (**bBW**) of the individual piglets and consequently, in an increased proportion of low bBW piglets (**LBW**; less than 1.0 kg) and a larger variability of bBW within the litter (Quiniou *et al.*, 2002; Pardo *et al.*, 2013). Low bBW piglets are characterised by being weaker and less likely to survive due to their poorer thermoregulatory ability (Herpin *et al.*, 2002), due to their higher surface-to-volume ratio, and their lower success in competing within the litter to achieve early and adequate colostrum intake (Edwards and Baxter, 2015). Despite efforts to improve management, cross-fostering in particular, piglet mortality has also increased by more than 5% in the last 10 years (www.bdporc.irta.es), reaching percentages of up to 17% (Figure 1).

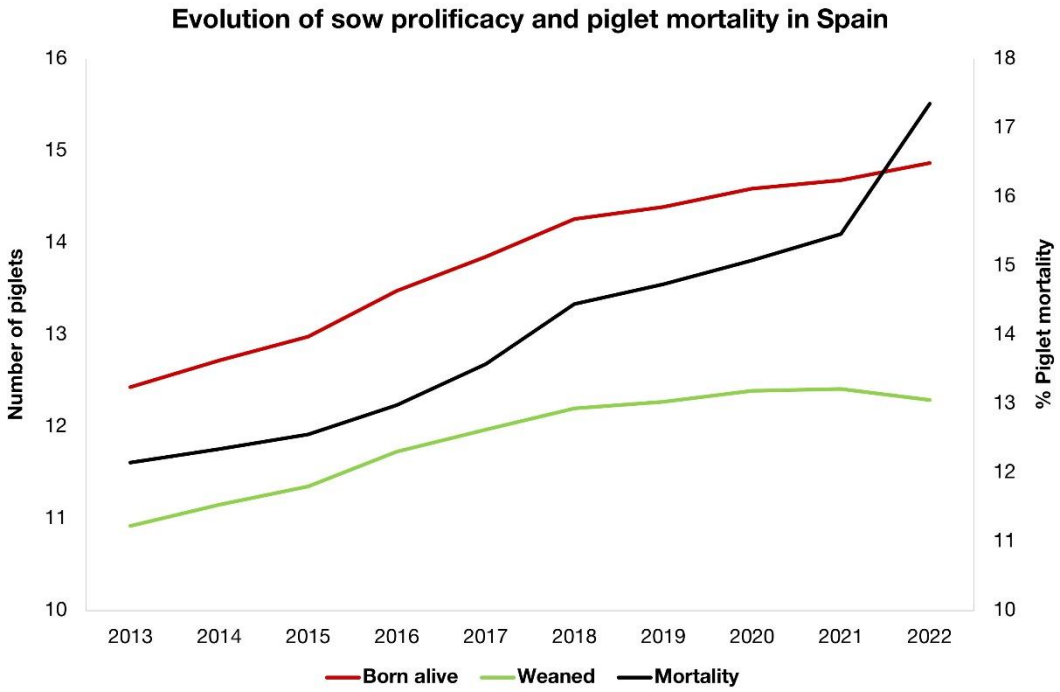


***Figure 1:*** *Number of piglets born alive and weaned per sow, and piglet mortality rate (%) from 2013 to 2022. Data collected by Spanish Porcine Reference Database - BDporc (www.bdporc.irta.es).*

Weaning is another critical step in the development and maintenance of an adequate health status of the piglets. The transition from maternal milk, which is characterized by being rich in protective factors, lactose, proteins, lipids, growth factors and immune cells, etc… (Koblasa *et al.*, 1987), to a solid diet mainly based on cereals and soya protein results in an abrupt change in feed source to which many piglets fail to adapt. This results in an inadequate feed intake after weaning that is associated with intestinal atrophy and reduced absorption of nutrients (Pluske *et al.*, 1997). This may also result in unspecific intestinal inflammatory response and epithelium disruption which facilitate the appearance of diarrhoea (McCracken *et al.*, 1999; Xu *et al.*, 2000; Pie *et al.*, 2004), and increase the risk of enteric infections (Lallès *et al*., 2007).

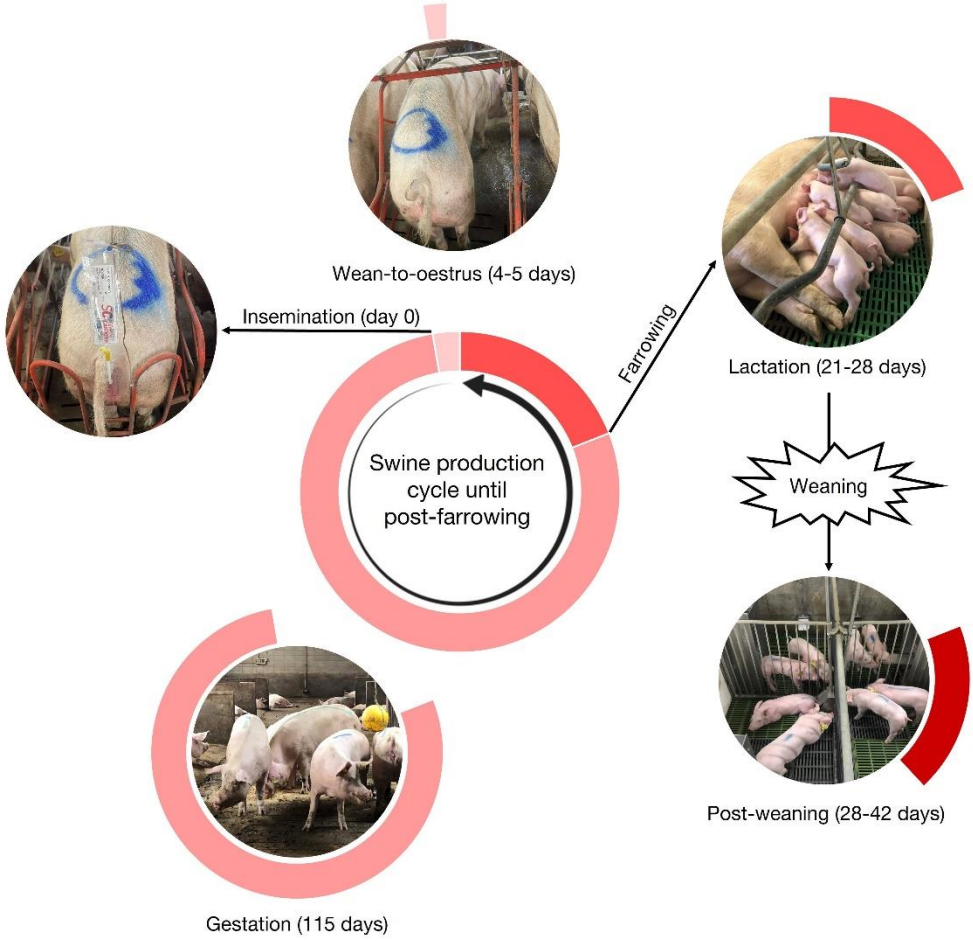


***Figure 2:*** Swine production cycle from insemination until the end of post-weaning period.

In the past, antibiotic growth promoters had been used in medicated feeds to prevent the mentioned enteric health problems associated with weaning. Until recently, zinc oxide in piglet feeds has also had a preventive role against post-weaning diarrhoea when used at pharmacological levels as established by Poulsen *et al*. (1989) and Hill *et al*. (1993). However, in their fight against the development of resistance to antibiotics, the European Union decided to ban the use of antibiotic growth promoters in 2006 (Regulation 1831/2003 on additives used in animal nutrition), and that of zinc oxide from 2022 (Article 35 of Directive 2001/82/EC). The first consequence of the ban was a huge increase on the therapeutic (not preventive or metaphylactic) use of medicated feeds containing antibiotics and/or zinc oxide.

To avoid this, the European Union decided to implement additional actions aiming to effectively reduce the use of antimicrobials in animal production. Consequently, the Spanish Agency of Medicines promoted the creation of a strategic action plan to reduce the risk of selection and dissemination of antibiotic resistance (*Plan Nacional frente a la Resistencia a los Antibióticos (PRAN)*) (www.resistenciaantibioticos.es). On the other hand, the use of high doses of zinc oxide was also cause of concern for the potential environmental risk of the long-term accumulation of this metal in soils (Long *et al.*, 2017).

Considering these challenges and to preserve the efficiency and effectiveness of antibiotics for public health, the development of early-life nutritional strategies has become essential for the piglets' development, health, and robustness.

**Lipids in swine nutrition**

Chemically lipids belong to an heterogeneous group of organic compounds present in the cells and tissues of plants and animals characterised by being soluble in organic or non-polar solvents such as benzene, chloroform, or ether, but insoluble in polar solvents such as water (Gurr *et al.*, 2002; McDonald *et al.*, 2011). Historically, lipids can be briefly classified into "fats" and "oils" (Wealleans *et al.*, 2021). Both have the same general structure but differ in physical and chemical properties (McDonald *et al.*, 2011). Concretely, due to their different melting point, fats are greasy and solid in texture, while oils are liquid at ambient temperature (McDonald *et al.*, 2011; Kerr *et al.*, 2015; Wealleans *et al.*, 2021). However, the term "fat" is frequently used to include both groups (McDonald *et al.*, 2011). Fats are composed predominantly by fatty acids (**FAs**) with the trihydric alcohol glycerol (McDonald *et al.*, 2011). When the three alcohols of the glycerol are found esterified by FAs, the resulting molecule is known as triacylglycerol, or more commonly as triglyceride (McDonald *et al.*, 2011). However, although triacylglycerols are predominant, monoacylglycerols and diacylglycerols can also occur naturally and in much smaller amounts (McDonald *et al.*, 2011). In addition, FAs can also be

found individually without being esterified to the glycerol molecule, which are known as free FAs.

In swine nutrition, dietary lipids are extensively used as a source of energy, being the triglycerides the category of lipid that became the major component of the lipids used in animal feeds (Rosero *et al.*, 2016; Wealleans *et al.*, 2021). Concretely, approximately an 80% of the extracted lipids from diets or body tissues are recovered as FAs by gas chromatography, with the remaining 20% being unidentified FAs, glycerol and the unsaponifiable fraction (Lizardo *et al*., 2002). FAs are carboxylic acids composed by hydrocarbon chains that can contain between 2 and 24 carbons with a methyl group (-CH3) at one end and a carboxyl group (-COOH) at the other (Leskanich and Noble, 1999). However, the most common are those containing between 12 and 24 carbons (Nelson and Cox, 2017). Depending on the length of the hydrocarbon chain, FAs can be distinguished in short-chain FAs, less than 6 carbons, medium-chain FAs, between 6 and 12 carbons, and long-chain FAs (**LCFAs**), more than 12 carbon. FAs can also be classified according to their degree of unsaturation as a consequence of the presence of double bounds between the carbon atoms. In this way, FAs can be grouped in saturated FAs if they do not present any double bond, monounsaturated FAs if they present one double bound, and polyunsaturated FAs if they present more than one double bound in the hydrocarbon chain (Wealleans *et al.*, 2021). In addition, depending on the double bond position relative to the methyl terminal of the FA, it is possible to differentiate between three main families, ω- or n-3, -6 and -9 FAs (Kerr *et al.*, 2015). In nutrition, the methyl carbon at the distal end of the hydrocarbon chain is commonly considered as carbon atom 1 and it is also known as “ω” or “n” (McDonald *et al.*, 2011). Therefore, “ω- or n-(3, 6 or 9)” indicates the location of the first double bound respect to the methyl carbon (Leskanich and Noble, 1999). The common FAs of fats and oils are presented in Table 1.

***Table 1:*** *Fatty acids of natural fats and oils (McDonald et al., 2011).*

| Common fatty acids | |
|---|---|
| Name | Lipid numbers |
| Saturated | |
| Caprylic acid | C8:0 |
| Capric acid | C10:0 |
| Lauric acid | C12:0 |
| Myristic acid | C14:0 |
| Palmitic acid | C16:0 |
| Stearic acid | C18:0 |
| Monounsaturated | |
| Palmitoleic acid | C16:1 n-7 |
| Oleic acid | C18:1 n-9 |
| Polyunsaturated | |
| Linoleic acid | C18:2 n-6 |
| α-linolenic acid | C18:3 n-6 |
| Arachidonic acid | C20:4 n-6 |
| Eicosapentaenoic acid | C20:5 n-3 |
| Docosahexaenoic acid | C22:6 n-3 |

Additionally to their use as source of energy, due to its nature, lipids may also have essential functions in the organisms. In pigs, the FA synthesis implies two collaborative systems. The *de novo* FA synthesis is cytoplasmatic, involves two enzyme complexes (Acetyl-CoA carboxylase and FA synthetase), and generates mainly palmitic acid from acetyl-CoA (Enser, 1884; Lizardo *et al*., 2002). The second system is mitochondrial or microsomal and concerns elongation adding two carbon atoms to the existing FA (Enser, 1884; Lizardo *et al*., 2002). Subsequently, FAs may be desaturated through activity of endogenous elongases and desaturase enzymes (Lemarchal, 1992; Lizardo *et al*., 2002). Although all polyunsaturated FAs are derived from SFAs by elongation and adding double bounds, pigs are not able to synthetize autonomously n-6 and n-3 LCFAs since they are unable to introduce double bonds distal to carbon 10 due to the absence of Δ-12 and Δ-15 desaturase

enzymes (Lemarchal, 1992; Rosero *et al.*, 2016). Therefore, they must be obtained by animals through the diet. Therefore, dietary lipids are the source to obtain essential FAs, such as linoleic (C18:2 n-6) and α-linolenic acid (C18:3 n-3) (Lizardo *et al*., 2002). Linoleic acid and α-linolenic acid are the precursors of n-6 and n-3 families of LCFAs, respectively, and their long derivatives synthesized by desaturation and elongation processes (Figure 3) (Tanghe and De Smet, 2013).

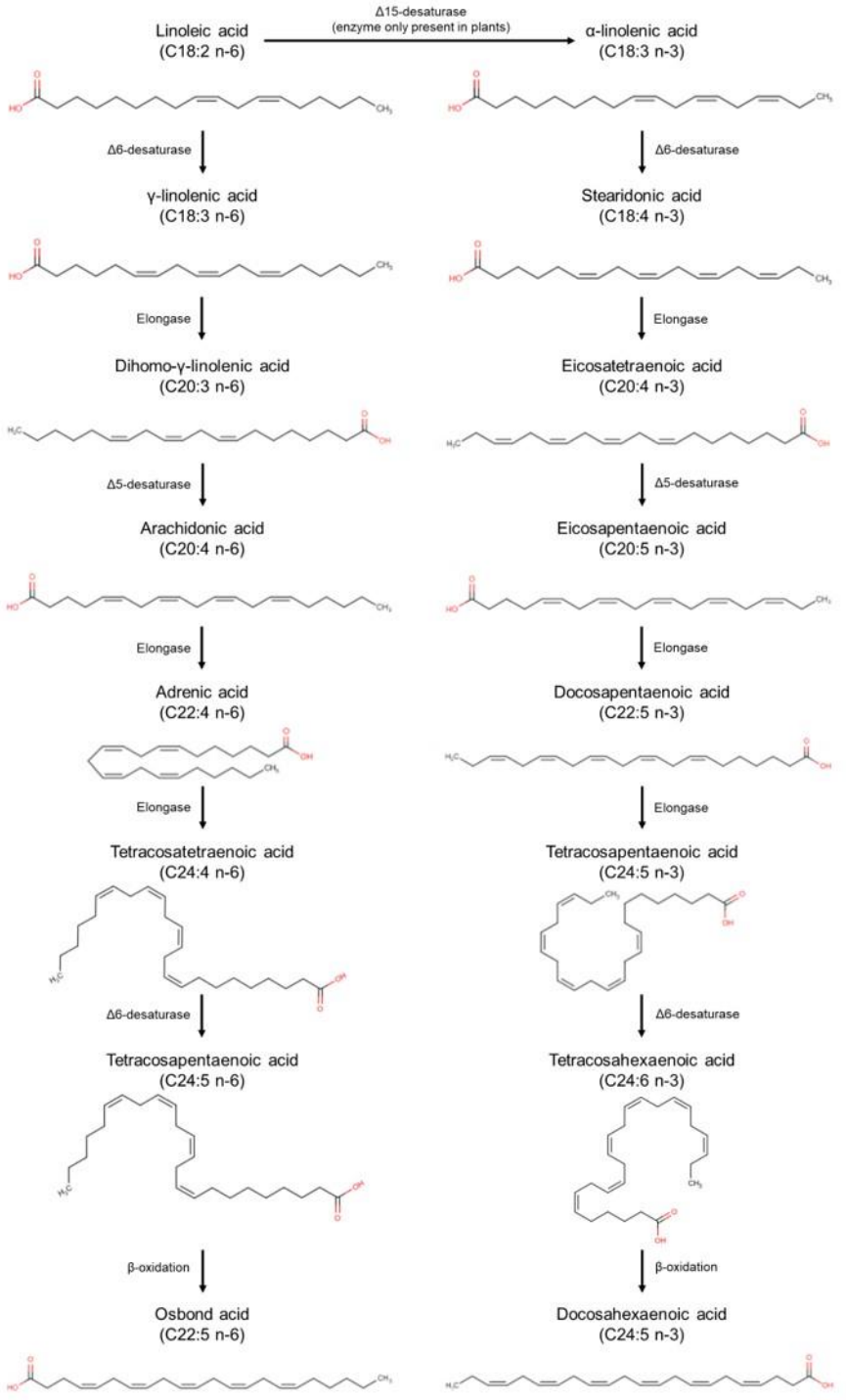


***Figure 3:*** *Pathways of n-6 and n-3 polyunsaturated fatty acids synthesis from the precursors linoleic acid (C18:2 n-6) and α-linolenic acid (C18:3 n-3), respectively. Adapted from Tanghe and Smet (2013). Structures of fatty acids were obtained from Human Metabolome Database Version 5.0.*

### *Lipids in feed*

Cereals are the primary ingredient in swine diets being the mix corn soybean meal the most used in the North America. Other cereal grains such as barley, wheat, sorghum, and oats are commonly employed in Europe. As can be observed in Table 2, their fat content varies from 1.40 to 4.90%, being wheat and oats the cereals with the lower and the higher percentage, respectively. In Table 2, it can be observed that the content of FAs differs between the different types of cereals. However, they are mainly characterized by being rich in n-6 polyunsaturated FA, concretely linoleic acid, followed by the monounsaturated FA oleic acid (C18:1 n-9) and/or the saturated FA palmitic acid (C16:0).

***Table 2:*** *Fat content and fatty acid profile of the main cereal grains used in swine feeds.*

| | Cereal grains and soybean[1] | | | | | |
|---|---|---|---|---|---|---|
| | Corn | Barley | Wheat | Sorghum | Oat | Rice |
| Ether extract (%)[2] | 3.30 | 1.70 | 1.40 | 3.00 | 4.90 | 1.00 |
| Percentage of fatty acids[3] | 90.0 | 70.0 | 70.0 | 90.0 | 90.0 | 85.0 |
| Fatty acid profile (%)[4] | | | | | | |
| Myristic acid (C14:0) | - | - | - | - | 0.30 | - |
| Palmitic acid (C16:0) | 11.0 | 23.0 | 19.0 | 17.0 | 19.0 | 17.0 |
| Palmitoleic acid (C16:1 n-7) | - | - | - | - | 0.40 | - |
| Stearic acid (C18:0) | 2.00 | - | 1.50 | - | 1.00 | 2.00 |
| Oleic acid (C18:1 n-9) | 27.0 | 13.0 | 15.0 | 31.0 | 35.0 | 40.0 |
| Linoleic acid (C18:2 n-6) | 56.0 | 56.0 | 57.0 | 45.0 | 39.0 | 37.0 |
| α-linolenic acid (C18:3 n-6) | 1.0 | 6.0 | 5.00 | 3.00 | 2.00 | 1.50 |
| C≥20 | - | - | - | - | 0.04 | 2.00 |

Information obtained from FEDNA (Federación Española para el Desarrollo de la Nutrición Animal) (de Blas *et al*., 2019).

[1]Cereals used to write the table: corn, two-row barley 11.3% crude protein, soft wheat 12.9% crude protein, white sorghum (low in tannins <0.4%), oats, and broken rice.

[2]Percentage of ether extract in the chemical composition.

[3]Percentage of fatty acids in the ether extract.

[4]Percentage of each fatty acids in the total fatty acid content.

Besides cereals, also oilseed meals were included into swine diets. However, most of these meals were a by-product after oil extraction so, in most of the cases, their fat content is negligible. The inclusion of fats and oils in swine diets is a common practice to improve their energy content. Based on their origin, fats can be from vegetable or animal origin, or a mixture of them. In Table 3, it is reported the FA profile of the vegetable oils most used in animal nutrition. Depending on their origin, some of the vegetable oils such as sunflower, corn or soybean contain high levels of n-6 unsaturated FAs, while others such as palm oil are richer in saturated FAs. Instead, linseed oil is characterized mainly to contain high percentages of the n-3 FA α-linolenic acid.

***Table 3:*** *Fatty acid profile of the main vegetable oils used in swine nutrition.*

| | Vegetable oil | | | | | |
|---|---|---|---|---|---|---|
| | Linseed | Sunflower | Corn | Soybean | Rapeseed | Palm |
| Fatty acids (%)[1] | | | | | | |
| C≤14 | - | - | - | - | - | - |
| Myristic acid (C14:0) | - | - | - | - | - | 1.00 |
| Palmitic acid (C16:0) | 6.00 | 6.40 | 10.7 | 9.50 | 5.00 | 43.0 |
| Palmitoleic acid (C16:1 n-7) | - | - | 0.50 | 10.5 | 0.30 | 0.30 |
| Stearic acid (C18:0) | 4.50 | 5.00 | 2.40 | 4.00 | 2.20 | 4.80 |
| Oleic acid (C18:1 n-9) | 19.0 | 22.6 | 26.0 | 22.0 | 57.0 | 40.0 |
| Linoleic acid (C18:2 n-6) | 16.0 | 63.0 | 56.0 | 54.0 | 20.5 | 10.0 |
| α-linolenic acid (C18:3 n-3) | 54.0 | <0.50 | 1.00 | 7.30 | 9.00 | - |
| C≥20 | 1.00 | 1.10 | 0.50 | 1.10 | 4.40 | - |

Information obtained from FEDNA (Federación Española para el Desarrollo de la Nutrición Animal) (de Blas *et al*., 2019).

[1]Percentage of each fatty acids in the total fatty acid content.

Animal fat sources used in animal feeds are presented in Table 4. Although they are characterised by containing mainly saturated FAs, some differences can also be observed among them. Poultry fat is characterized by containing a medium-

high amount of unsaturated FAs mainly due to a high percentage of oleic and linoleic acids, followed by lard, which is considered a fat source moderately unsaturated despite its also contain considerable amounts of saturated FAs. On the other hand, butter and tallow are considered as rich sources of saturated FAs despite their moderate level of oleic acid content.

***Table 4:*** *Fatty acid profile of the main animal fat sources used in swine nutrition.*

| | Animal fat source | | | |
|---|---|---|---|---|
| | Butter | Tallow | Lard | Poultry fat |
| Fatty acids (%)[1] | | | | |
| C≤14 | 12.5 | - | - | - |
| Myristic acid (C14:0) | 11.3 | 3.20 | 1.50 | 1.00 |
| Palmitic acid (C16:0) | 27.5 | 25.0 | 23.7 | 21.6 |
| Palmitoleic acid (C16:1 n-7) | 3.10 | 3.20 | 3.00 | 5.40 |
| Stearic acid (C18:0) | 10.6 | 21.1 | 13.0 | 7.40 |
| Oleic acid (C18:1 n-9) | 26.4 | 38.3 | 44.0 | 44.0 |
| Linoleic acid (C18:2 n-6) | 2.20 | 2.20 | 10.0 | 19.0 |
| α-linolenic acid (C18:3 n-3) | - | - | 0.8 | 1.00 |
| C≥20 | 2.00 | - | 1.3 | - |

Information obtained from FEDNA (Federación Española para el Desarrollo de la Nutrición Animal) (de Blas *et al*., 2019).

[1]Percentage of each fatty acids in the total fatty acid content.

Thus, cereal grains and additional fat sources used in swine nutrition tend to be rich in n-6 polyunsaturated FAs and/or saturated FAs, and their addition in feeds is mainly due to their value as an energy source. However, due to recommendations from human nutritionists regarding the increase in n-3 FAs in the diet, particularly α-linolenic acid and some of its derivatives such as eicosapentaenoic acid (**EPA**) (C20:5 n-3) and docosahexaenoic acid (**DHA**) (C22:6 n-3), in the first decade of the 21st century emerged the interest of using different n-3 FAs sources to enrich meat or other animal products (Vorin *et al*., 2003; Wilfart *et al*., 2004). In this way, sources of n-3 FAs such as linseed (rich in α-linolenic acid) and fish oils (rich in EPA and DHA) have been used in animal feed. Due to cost considerations, the most common

source was linseed or linseed oil. Additionally, the inclusion of fish oils in at high concentrations was related with fishy odours and flavours (Wood *et al*., 2008).

Moreover, studies on vegetable alternatives to the use of fish oil as a n-3 polyunsaturated FA source have been carried out more recently. Concretely, research has focused on evaluating the impact of the inclusion of marine algae and marine algae oils. However, these sources present certain drawbacks such as their high price and although they contain a suitable amount of DHA the amount of EPA is moderate in comparison to fish oil (Roszkos *et al.*, 2020). Fatty acid profile of the main fish oil sources rich in n-3 fatty acids used in swine nutrition is provided in Table 5.

***Table 5:*** *Fatty acid profile of the main fish oil sources rich in n-3 fatty acids used in swine nutrition.*

| | Animal fat sources rich in n-3 fatty acids | | |
|---|---|---|---|
| | Fish oil | | |
| | Chilean (anchovy) | Nordic (herring) | Spanish (tuna) |
| Fatty acids (%)[1] | | | |
| C≤14 | - | - | 0.10 |
| Myristic acid (C14:0) | 7.00 | 6.00 | 4.30 |
| Palmitic acid (C16:0) | 19.0 | 11.0 | 15.7 |
| Palmitoleic acid (C16:1 n-7) | 9.00 | 7.20 | 4.10 |
| Stearic acid (C18:0) | 4.90 | 1.20 | 4.30 |
| Oleic acid (C18:1 n-9) | 16.0 | 11.0 | 13.5 |
| Linoleic acid (C18:2 n-6) | 2.00 | 1.00 | 1.80 |
| α-linolenic acid (C18:3 n-3) | 0.90 | 0.50 | 1.10 |
| C≥20 | > 36.0 | > 45.0 | > 47.0 |
| Eicosapentaenoic acid (C20:5 n-3) | 10.0 | 8.10 | 11.0 |
| Docosahexaenoic acid (C22:6 n-3) | 11.0 | 7.80 | 11.0 |

Information obtained from FEDNA (Federación Española para el Desarrollo de la Nutrición Animal) (de Blas *et al*., 2019).

[1]Percentage of each fatty acids in the total fatty acid content.

The study of the inclusion of n-3 FAs in other species has also focused on other regulatory effects associated with their potentially functions during gestation (Allen and Harris, 2001), as constituents of brain cells and retina function (Birch *et al*., 1998), immune function (Miles and Calder, 1998), signal transduction (Bazan, 2003) or gene regulation (Jump and Clarke, 1999).

### *Digestion and absorption of fat*

In a nutshell, fat digestion consists of a three-step process: emulsification, hydrolysis and absorption (Wealleans *et al*., 2021). The fat digestion process starts in the mouth immediately after the consumption of feed, with the secretion of saliva and the mechanical process of chewing. Salivary lipases are mixed with feed by the action of chewing, initiating the release of triacylglycerols from the feed matrix and the process of emulsification (Wealleans *et al*., 2021). In the stomach, the shear forces continue with the early emulsification process by breaking the fat globules into fat droplets and facilitating triacylglycerols hydrolysis by gastric lipases. Despite the action of pre-duodenal lipases, in pigs, most of the fat is absorbed in the small intestine, with more than 70% of the fat entering the duodenum in form of triacylglycerols (Jones and Rideout, 2012). In addition, it is also worth considering that, upon entering the duodenum, most of the fat is present as large droplets that have not been fully emulsified yet (Wilfart et al., 2007). Thus, it is in the duodenum where bile salts together with other amphiphilic compounds coat the fat surface and complete their emulsification into smaller droplets (Wealleans *et al*., 2021). These fat droplets stimulate the secretion of pancreatic enzymes, including pancreatic lipase and colipase (Watanabe et al., 1988). Colipase act as a cofactor to allow the anchoring of pancreatic lipase on the oil/water interface of the emulsified droplet and facilitates the process of hydrolysis of triacylglycerols first into diacylglycerols and free FAs, and then the resulting diacylglycerols into monoacylglycerols and more free FAs (Wealleans *et al*., 2021). Short-chain FAs and medium-chain FAs are solubilized as individual components in the intestinal lumen, while the transport of LCFAs and monoacylglycerols requires polar lipids such as

bile salts and lysophospholipids (Wilde and Chu, 2011) capable of forming mixed bile salt micelles incorporating monoacylglycerols, free FAs and fat-soluble nutrients (Lairon, 2009). These mixed micellar structures are composed of an apolar core of LCFAs, fat-soluble vitamins and cholesteryl esters, and a cover consisting of a polar monolayer including lipids such as monoacylglycerols, free cholesterol, phospholipids and lysophospholipids (Lairon, 2009; Lo and Tso, 2009). Then, these mixed micelles together with short-chain FAs and medium-chain FAs are absorbed through the enterocytes of the small intestine. Although not all details of the lipid absorption process have been fully described, it is known that short-chain FAs and medium-chain FAs are taken up by simple diffusion across the enterocyte membrane, while LCFAs are taken up by an active protein-mediated process (Lairon, 2009; Lo and Tso, 2009).

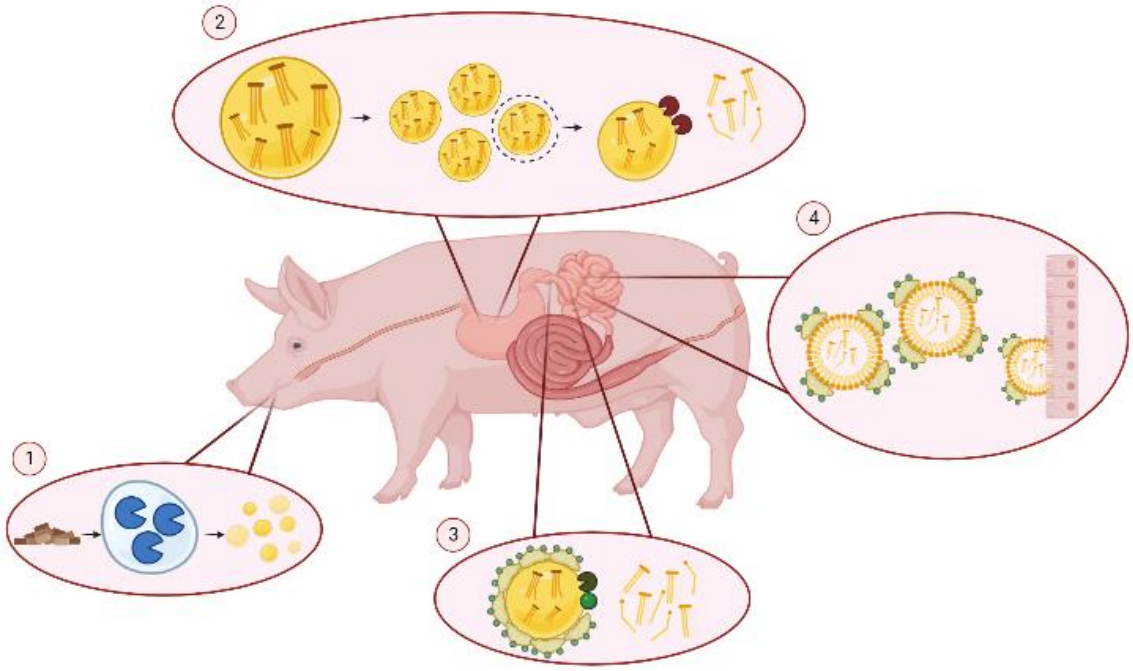


***Figure 4:*** *Simplified scheme of digestion and absorption of dietary fat in pigs. 1. The action of chewing releases the fat from the feed and makes it accessible to salivary lipase action; 2. In the stomach fat globules are reduced to fat droplets and then, the action of gastric lipases hydrolyses some of the triacylglycerols; 3. In the duodenum, bile salts allow fat droplets to emulsify and the action of the complex pancreatic lipase - colipase hydrolyses the triacylglycerols into diacylglycerols, monoacylglycerols and free fatty acids, and mixed*

*micelles are formed; 4. Mixed micelles are absorbed through enterocytes and bile salts are released to return to the gall bladder. Adapted from Wealleans et al. (2021) and Verge (2022).*

**Dietary n-3 LCFAs in swine**

Dietary polyunsaturated FAs can exert different effects on animal physiology depending on the family they come from. Concretely, n-3 LCFAs were initially introduced in growing and finishing pig diets to increase the its content in meat products (Vorin *et al*., 2003; Wilfart *et al*., 2004). Their effects as an energy source in sow diets were also evaluated on the sow reproductive performance and piglet growth (Tanghe and Smet, 2013; Rosero *et al.*, 2016; Roszkos *et al.*, 2020). Although the exact mechanisms of action have not been described yet, n-3 LCFAs are essential for the development of tissues like brain and retina and they could influence the reproductive performance through their incorporation into the cell membrane of oocytes and altering the eicosanoid production (Wathes *et al.*, 2007; Tanghe and Smet, 2013). More recently, their potential benefits on the immune system and gut microbiota are gaining importance (Liu, 2015; Lauridsen, 2020). Thus, the n-3 LCFAs are related with anti-inflammatory effects through the production of their oxygenated derivatives with weakly inflammatory, anti-inflammatory and/or inflammation resolving roles, and decreasing the production of pro-inflammatory cytokines (Calder, 2010). In addition, the increase of the microbial diversity has been described as another novel possible anti-inflammatory action of n-3 LCFAs (Calder, 2019) and increases of beneficial bacteria such the short-chain FAs producer phylum Bacteroidetes had been described in other animal species (Costantini et al., 2017).

In terms of n-3 LCFA sources, as mentioned above, the most used sources were linseed and fish oil. Moreover, there are other sources of n-3 LCFAs of interest such as echium, hempseed, rapeseed, and seaweed/microalgae. Table 6 also shows different n-3 LCFAs sources and their content in the specific FAs.

***Table 6:*** *Main n-3 fatty acids sources used in animal nutrition.*

| n-3 fatty acid source | Main fatty acid |
|---|---|
| Echium oil[1] | α-linolenic acid (C18:3 n-3) |
| Hempseed oil[2] | α-linolenic acid (C18:3 n-3) |
| Fish oil[1,3,4] | eicosapentaenoic (C20:5 n-3) and docosahexaenoic (C22:6 n-3) acids. |
| Linseed oil[1,3,4] | α-linolenic acid (C18:3 n-3) |
| Rapeseed oil[1,3,4] | α-linolenic acid (C18:3 n-3) |
| Seaweed/microalgae[1] | α-linolenic (C18:3 n-3), eicosapentaenoic (C20:5 n-3) and docosahexaenoic (C22:6 n-3) acids. |
| Soybean oil[1,3,4] | α-linolenic acid (C18:3 n-3) |

[1]Palmquist (2009).

[2]Vodolazska and Lauridsen (2020).

[3]de Blas et al. (2019).

[4]Tanghe and De Smet (2013).

Therefore, the present literature review discuss the effects of n-3 LCFAs as a nutritional strategy for sows and piglets existing results on performance, colostrum and milk composition, blood FA profile, FA oxygenated derivatives, immune indicators and microbiota.

### *Sow and piglet performance*

The effects of the addition of different fat sources to swine diets on performance has been studied for many years, with the main focus on sow nutrition due to their specific energy requirements during gestation and lactation (Azain, 2001). Thus, most of the studies conducted with dietary n-3 LCFAs have focused their research on the sow reproductive performance, litter characteristics and/or piglet growth in their first stages of life.

*Dietary n-3 LCFAs on sow performance*

The nutrition of the sow during gestation and lactation is of vital importance to prepare the animal for farrowing and its subsequent lactation. During gestation, the maintenance needs of the sow and the growth of the conceptus must be satisfied. Once these requirements are met, any "extra" nutrients are stored in the sow's tissues. However, it is important to consider that excessive weight gain and fattening of the sow can also have negative effects such as dystocia at farrowing, reduced feed intake during lactation, or reduced longevity (Trottier and Johnston, 2001). For these reasons, the research on the use of dietary n-3 LCFAs in sows is particularly focussed on the assessment of their effects on live weight, backfat thickness and feed intake.

Despite most of the studies that recorded these parameters in sows did not find differences due to n-3 LCFAs, Smit *et al*. (2013) observed that the dietary supplementation with 84g/day of a marine oil-based product from day 60 of gestation tended to increase the BW of sows at day one post-farrowing (179 kg in control vs 181 kg in marine oil). Similarly, McDermott *et al*. (2020), described that the inclusion of 1% salmon oil in the diets during gestation and lactation resulted in higher BW of the sows at weaning (230.9 kg in control vs 234.8 kg in salmon oil).

Estienne *et al*. (2006) observed that the dietary supplementation with 1% of a protected fish oil from day 35 before breeding increased the backfat thickness of gilts at day 27 of gestation (12.3 mm in control vs 14.0 mm in protected fish oil). In addition, Eastwood *et al*. (2014) reported that at day 7 of lactation, sows consuming (from day 80 of gestation) plant-based diets, where the source of n-3 FAs was linseed, with n-6:n-3 ratios of 5:1 and 1:1 presented larger backfat thicknesses (13.7 and 14.1 mm, respectively), than sows consuming a plant-based diet with a n-6:n-3 ratio of 9:1 or a fish oil-based diet with a n-6:n-3 ratio of 5:1 that had intermediate levels (both 13.4 mm), and sows consuming the basal diet presented the lowest thickness (12.5 mm). These differences were maintained in a similar way at weaning, when the sows fed the plant-based diets with 5:1 and 1:1 ratios presented backfat thicknesses of 13.1 and 13.2 mm, respectively, those of sows

fed with the 9:1 (plant based) and 5:1 (fish oil-based) ratios were 12.6 and 12.9 mm, and that of the control fed sows was 11.9 mm.

Some of the studies have also described differences in feed intake. Lauridsen and Danielsen (2004) observed that sows fed a control diet without addition of fat from day 108 of gestation until farrowing presented a significantly higher daily feed intake (2.74 kg) than sows that were fed diets with 8% rapeseed oil (2.34 kg) and 8% fish oil (2.34kg). Eastwood *et al*. (2014) described that sows fed a fish oil-based diet with a 5:1 n-6:n-3 ratio during two consecutive reproductive cycle, consumed 10% less feed than sows that were fed control or plant-based diets with the same n-6:n-3 ratio during the second reproductive cycle. Additionally, Lavery *et al*. (2019) reported that sows that were fed diets containing salmon oil from day 105 of gestation had a higher feed intake at the third week of lactation than sows that were offered diets containing soybean oil (salmon oil 61.6 kg in salmon oil vs 58.1kg in soybean oil).

*Dietary n-3 LCFAs on litter characteristics at birth*

The n-3 LCFAs can also influence gestation length and litter characteristics. Previously, it has been described that n-3 LCFAs may positively influence the number and quality of follicles and oocytes, but they can reduce the production of 2-series prostaglandins which play a role in the initiation of farrowing and alter the expression of key enzymes involved in the synthesis of prostaglandins and steroids (Whates *et al*., 2007; Tanghe and De Smet, 2013). The 2-series prostaglandins are derived from arachidonic acid (C20:4 n-6) and the dietary enrichment with n-3 LCFAs has typically been accompanied by a reduction in the content of this n-6 LCFA (Calder, 2010), delaying labour and, consequently, increasing the gestation length (Tanghe and De Smet, 2013). Moreover, n-3 LCFAs could also affect myometrial contractions through direct impacts on ion channels and cell signalling, which could be another reason for an elongated gestation period (Roszkoz *et al*., 2020).

Regarding litter characteristics, a distinction can be made between studies assessing the impact of dietary n-3 LCFAs on embryonic and/or foetal development (Farnworth and Kramer, 1988; Perez Rigau *et al*., 1995; Estienne *et al*., 2006; Brazle *et al*., 2009; Smit *et al*., 2013; Smits *et al*., 2013; Heras-Molina *et al*., 2021), and studies directly assessing effects on litter characteristics of born animals. As previously discussed in the review of Tanghe and De Smet (2013), no significant or conclusive differences due to n-3 LCFAs supplementation have been observed in the number, development, size, or survival of embryos and/or foetuses. However, Smits *et al*. 2013 described a trend towards a higher embryo survival in gilts that had been fed fish oil-rich diets from 6 weeks before breeding, with an optimal dietary supplementation of 3 g/kg of fish oil (81.8% in control vs 88.9% in 3 g/kg fish oil vs 84.3% in 10 g/kg fish oil). Moreover, Heras-Molina *et al*. (2021) also reported a higher mean litter size (8.42 in control vs 9.86 in linseed oil in terms of foetuses at day 100 of gestation/sow) and a higher proportion of large litters (19% in control vs 77% in linseed oil) in Iberian sows that had been fed 4% linseed oil-rich diet from day 35 of gestation. While some studies on the impact of different n-3 LCFAs sources in sow diets have observed no differences in litter characteristics, others have reported effects in terms of total piglets born, piglets born alive, stillborn piglets, litter weight and/or individual piglet weight.

In terms of litter size, Rooke *et al*. (2001c) described that as the amount of salmon oil in the diet from day 60 of gestation increased from 0% to 2%, the size of litters decreased from 14.0 to 10.3 piglets. Similar results were reported by Corson *et al*. (2008) and Smit *et al*. (2015), who noted a decrease in the number of total piglets born and born alive when the sows were fed a diet with 10% sunflower (rich in linoleic acid) from early to mid-gestation or diets supplemented with a 0.5% protected fish oil during gestation and lactation, respectively. Concretely, Corson *et al*. (2008) reported decreases of 0.8 piglets born and 2.2 piglets born alive, and Smit *et al*. (2015) decreases of 1.5 piglets born and born alive compared with their respective controls. On the other hand, Vodolazska and Lauridsen (2020) observed increases in the number of total piglets born and born alive (11.6 piglets for both)

in sows that were fed hemp seed oil from day 108 of gestation, compared with sows that were fed a soybean oil diet (9.63 piglets in hemp seed oil vs 9.13 piglets in soybean oil). Despite the differences in total number of piglets cannot be explained by dietary treatment, they also reported decreases of 0.27 or 0.37 stillborn piglets when the sows were fed with 5% hemp seed oil compared with 5% of a mixture of hemp seed and soybean oils (50:50) or 5% soybean oil, respectively. In another study, Webel *et al*. (2003) reported increases of 0.6 total piglets born and 0.5 piglets born alive at the subsequent farrowing when the sow diets were top-dressed with 85 g/day protected fish oil.

In reference to litter or individual piglet weight at birth, results are also discordant between different studies. Concretely, Rooke *et al*. (2001b) and Rooke *et al*. (2011) observed a decrease of 0.3 kg in piglet birth BW in commercial sows when they were fed, from day 58 of gestation, diets with 1.65% and 3.3% salmon oil, respectively. Similarly, Heras-Molina *et al*. (2020) observed a reduction of 0.1 kg in piglets from Iberian sows that were fed a diet with 4% linseed oil from day 35 of gestation. However, Rooke *et al*. (2001c), Mitre *et al*. (2005) and Posser *et al*. (2018) reported the opposite effect. Rooke *et al*. (2001c) and Mitre *et al*. (2005) used fish oil (1.65% salmon oil and 32 g/day shark-liver oil, respectively) from day 60 and day 80 of gestation, respectively, and both studies observed a decrease or a tendency towards a decrease in litter size of 2.6 to 3.7 piglets, accompanied by an increase in the individual weight of piglets of approximately 0.15 kg. Posser *et al*. (2018) used different doses of microalgae as a source of n-3 LCFAs in diets for the last 30 days of gestation, and observed a higher piglet bBW with the dose of 28 g/day (1.41 kg), than with the control diet or the dose of 3.5 g/day (1.26 kg). Moreover, it is also worth mentioning that studies such as those published by Papadopoulus *et al*. (2009) and Tanghe *et al*. (2014) also observed differences in piglet bBW between experimental treatments. Particularly, Papadopoulus *et al.* (2009) reported a higher piglet bBW when sows were fed diets with 2% fish oil or 1.5% sunflower oil from day 107 of gestation (1.41 and 1.43 kg, respectively) compared to sows that were fed diets with 2% fish oil or 1.5% sunflower oil from

day 111 of gestation (1.30 and 1.28 kg, respectively). Finally, Tanghe *et al.* (2014) described that the offspring from sows that were fed a diet containing 1% fish oil from day 73 of gestation had lower bBW than that from sows that were fed a diet with 1% linseed oil (1.41 vs 1.54 kg).

Some studies also looked at the impact of n-3 LCFAs in sow diets on the gestation length. Specifically, Rooke *et al*. (2001b), Boundary *et al*. (2009) and Rooke *et al*. (2011) observed an average increase in the gestation period of 0.5 days in sows that were fed diets with 1.65% or 3.3% salmon oil (Rooke *et al*. (2001b) and Rooke *et al*. (2011), respectively) or 1 day in sows that were fed 3% linseed oil (Boundary *et al*., 2009). Unfortunately, none of these studies analysed the concentration of series-2 prostaglandins or genes related to their formation pathways, and therefore, the association of these longer gestation periods with the dietary fat is speculative.

*Dietary n-3 LCFAs on the growth of suckling piglets*

The results on the effects of n-3 LCFAs on piglet growth and performance during lactation are discordant and remain controversial. No differences in piglet growth during the suckling period were observed for any of the following studies: Rooke *et al*. (2000) (1.75% tuna oil or 1.75% maize oil (42%) and linseed oil (58%), Boudry *et al*. (2009) (3% linseed oil), De Quelen *et al*. (2010) (1.5% and 5.5% linseed oil during gestation and lactation, respectively) and Farmer *et al*. (2010) (10% linseed, 6.5% linseed meal or 3.5% linseed oil) which included the corresponding n-3 LCFAs source from early or mid-gestation, or Fritsche *et al*. (1993a) (3.5% or 7% fish oil), Rooke *et al*. (1998) (3% soybean or tuna oils), Lauridsen and Danielsen (2004) (8% rapeseed or fish oil), Lauridsen and Jensen (2007) (8% rapeseed or fish oil), Laws *et al*. (2007b) (10% extra energy from fish oil), Papadopoulos *et al*. (2009) (2% fish oil), Leonard *et al*. (2011) (100 g/day fish oil, 1.8 g/day seaweed extract or 100 g/day fish oil + 1.8 g/day seaweed extract), Smits *et al*. (2011) (0.33% fish oil), Luo *et al*. (2020) (2.5% and 2.8% fish oil during gestation and lactation,

respectively), and Vodolazska and Lauridsen (2020) (5% hemp seed oil or 5% mix of hemp seed and soybean oils (50:50)) which included the n-3 LCFAs sources from mid to late or late gestation. On the contrary, Rooke *et al*. (2001a), described that piglets from sows that were fed with 1.75% tuna oil diets during late gestation were at least 0.10 kg heavier at days 14 and 28 of lactation than those from sows that were fed a control diet or a diet with 1.75% tuna oil between day 63 and 92 of gestation. Similarly, Mitre *et al*. (2005) reported an average increase of 0.39 kg at weaning in the piglets from sows that were fed a diet with 32 g/day of shark-liver oil from late gestation. Laws *et al*. (2007a) also reported an increase of 0.72 kg in the piglet's weaning BW after the inclusion of 10% extra energy from fish oil in sow diets between early and mid-gestation, and Mateo *et al*. (2009) found an increase of 0.52 kg when 0.2% protected fish oil was added in the diet of gilts after mid-gestation. In addition, Luo *et al*. (2013) observed an increase of 61 g in the average daily weight gain of piglets during lactation when sows were fed a diet with 7% fish oil. Nevertheless, Cools *et al*. (2011) reported a decreased weaning BW of the piglets from 8.5 to 7.0 kg as the percentage of fish oil in the sow's diet increased up to 4%.Finally, Eastwood *et al*. (2014), described a greater average weaning weight of piglets from sows consuming linseed as n-3 LCFA source, with n-6:n-3 ratios of 9:1 and 5:1 from day 80 of gestation (both 8.6 kg) compared to those from sows that were fed a linseed-based diet with a 1:1 ratio (8.0 kg) or a fish oil-based diet with a 5:1 ratio (7.8 kg). Moreover, they also observed a lower average daily gain of the piglets from the 5:1 fish oil-based diet (0.02 kg/day less) compared to the control piglets in the subsequent weaning period. On the other hand, Heras-Molina *et al*. (2020) observed that the piglets from Iberian sows that were fed a diet with 4% linseed oil diet had higher average daily weight gain and a fractional growth rate at weaning than the piglets from the control sows (approximately 170 vs 200 g/day and 55 vs 70 g/kg/day, respectively).

As for pre-weaning mortality, the results also remain inconsistent. While studies such as those by Mateo *et al*. (2009), Eastwood *et al*. (2014) and Smit *et al*. (2015) observed similar piglet mortalities regardless of dietary treatment, Cools *et al*.

(2011) reported an increase in mortality rate from 7.4% to 18.4% as the percentage of fish oil increased (from 0% to 4%). On the other hand, Rooke *et al*. (2001b) (1.65% salmon oil), Farmer *et al*. (2010) (10% linseed, 6.5% linseed meal, and 3.5% linseed oil) and Lavery *et al*. (2019) (flat diet with 1.79% salmon oil and phased diet with 5.98% salmon oil) described that the maternal supplementation with an n-3 FAs source reduced pre-weaning mortality rate within a range of 1.5% to 7.8%.

*Dietary n-3 LCFAs on the growth of weaned piglets*

The influence of dietary n-3 LCFAs on piglet growth during the post-weaning period has been less studied, but results still differ among trials. While Lauridsen and Jensen (2007) did not observe any effect of the inclusion of 8% rapeseed or fish oil in the maternal diet on piglet post-weaning weight gain, Rooke *et al*. (2000) reported that the dietary inclusion of 1.75% tuna oil in the sow diet during gestation had no significant effect on piglet weight at 7 days post-weaning (35 days of age), but its inclusion during lactation resulted in higher piglet BW (8.88 kg) compared to piglets from sows fed a diet with 1.75% of a mix of maize oil (42%) and linseed oil (58%) (8.56 kg). Conversely, Rooke *et al*. (2001a) observed a higher average piglet BW in sows that had been fed a diet with 1.75% tuna oil diet from either mid (10.25 kg) or late gestation (10.18 kg) than in control sows (10.09 kg). Other studies have also reported increases in the growth of the post-weaning piglets with the inclusion of n-3 LCFAs in the sow diets (Farmer *et al*. (2010), Leonard *et al*. (2011), McAfee *et al*. (2019) and Heras-Molina *et al*. (2020).) Farmer *et al*. (2010) observed a higher BW at 35 days post-weaning (56 days of age) in piglets from sows that had been fed a diet with 6.5% linseed meal (23.8 kg) compared to those from sows that had been fed diets with 10% linseed (22.4 kg) or 3.5% linseed oil (22.8 kg) from day 63 of gestation until weaning. Leonard *et al*. (2011) described that piglets weaned from sows fed a diet supplemented with a 1.8% seaweed extract from day 109 of gestation until weaning tended to have a higher average daily gain during the first 21 days post-weaning than the piglets from non-supplemented sows (0.308 vs 0.275 kg/day), as well as a higher average daily feed intake during the second week

of post-weaning (0.424 vs 0.366 kg/day). Moreover, they also described that, compared to piglets from non-supplemented sows, piglets weaned from sows fed a diet supplemented with 100 g/day of fish oil during the same period had a higher average daily gain (0.340 vs 0.278 kg/day) and gain to feed ratio (0.844 vs 0.699) during the second week post-weaning. McAfee *et al*. (2019) observed a weight gain between the last week of suckling and the day 3 post-weaning that was approximately 1 kg greater in the piglets from gilts fed diets supplemented with 1% protected fish oil than in the from control sows. Additionally, Heras-Molina *et al*. (2020) described that the piglets from Iberian sows fed a 4% linseed oil-rich diet from day 35 of gestation until farrowing were larger in terms of head size and corpulence than their control counterparts at 30 days post-weaning (60 days of age).Nguyen *et al*. (2020) revealed that at day 21 of post-weaning (47 days of age), piglets from control sows that were fed a diet supplemented with 4 g/kg of seaweed powder had higher BW (14.14 kg) and average daily gain (0.360 kg/day) than the piglets from the same sows that were fed a control diet (12.41 kg and 0.276 kg/day, respectively).

Other studies focus on the inclusion of n-3 FAs in the post-weaning diet rather than in the maternal diet. Li *et al*. (2014) did not report any improvement in performance with an inclusion of 3% marine n-3 LCFAs in the diet of weaned piglets, whereas Zhang *et al*. (2020) observed that, relative to piglets fed the control diet, piglets fed a diet supplemented with 5, 10, or 15 g/kg of coated n-3 LCFAs during the post-weaning period had greater BW (up to 0.43 kg more at week 6 post-weaning), improved average daily gain (at least 8 g/day on week one and three of post-weaning), and increased gain to feed ratio than control piglets (0.74 vs 0.75 ratio).

It can be summarised that the source of n-3 LCFAs, their doses of inclusion, and the period and duration of their application, as well as the genetic line or the physiological state of the animal are variables that could explain the wide variety of results obtained in the different studies in terms of sow reproductive performance, litter characteristics and piglet growth.

### *Colostrum and milk composition*

Colostrum and milk are the first sources of nutrients for the newborn piglets and their intake is essential to ensure the viability, growth, and survival until weaning. Both, colostrum and milk, are composed by a mixture of carbohydrates, lipids, proteins, minerals, vitamins, and cells (Hurley, 2015). Colostrum and milk differ in their time of secretion and composition. Colostrum is the first secretion of the mammary gland after farrowing, and it is largely synthesized before parturition. Specifically, colostrum secretion and intake by the piglets is considered to occur mainly during the first 24 hours after birth (Devillers *et al*., 2004; Quensel *et al*., 2015). During parturition its production is continuous and then becomes discontinuous, with lactation occurring at regular intervals of 40-60 min (Le Dividich *et al*., 2005). Colostrum is characterized by a complex mix of constituents that directly or indirectly influence the immune competence of piglets among which immunoglobulins (**Ig**) are the most important (Le Dividich *et al*., 2005). Among these, IgG are present at very high concentrations and IgA and IgM are present at lower concentrations (Klobasa *et al*., 1987). The composition of colostrum changes very rapidly after farrowing, with a decrease in the immunoglobulin contents and increases in the concentrations of fat and lactose. After colostrum, the next secretion is known as transitional milk that is already much richer in lipids, and it occurs until about day 4 of lactation. From day 10 onwards, the composition of the milk is much more stable and becomes what is known as mature milk (Klobasa *et al*., 1987; Quensel *et al*., 2015).

The content of fat in colostrum and milk is considered as the most variable component. As already mentioned, the amount of fat is lower in colostrum and increases in milk. Moreover, the main effect of sow diet on the composition of colostrum and milk is on their fat composition by the dietary inclusion of lipids. Particularly, the composition of the gestation diet may affect the composition of colostrum and possibly have a carry-over effect on milk during lactation, and the lactation diet could influence the composition of milk (Hurley, 2015). However, other factors, such as the breed and the parity number of the sow, may also

influence the amount of fat in the mammary secretions (Hurley, 2015; Rosero *et al*., 2016).

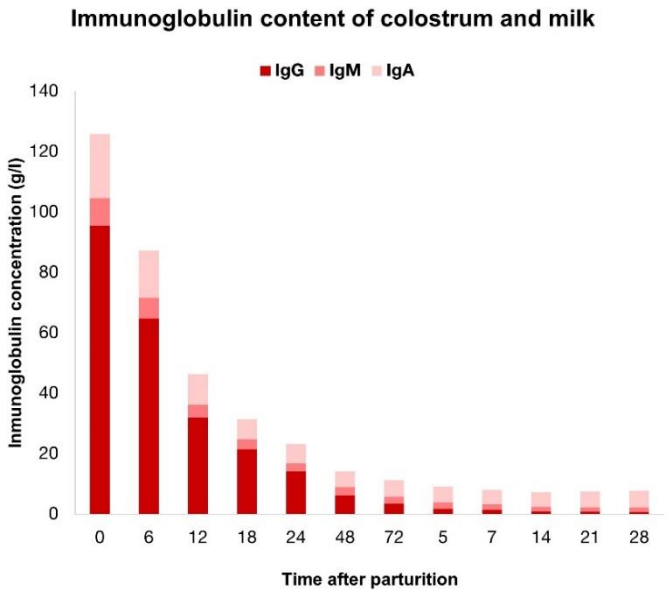


***Figure 5:*** *Evolution of immunoglobulin contents in sow's colostrum and milk during the first 72 hours and up to 28 days of lactation. Information obtained from Klobasa et al. (1987). Adapted from Darragh and Moughan (1998).*

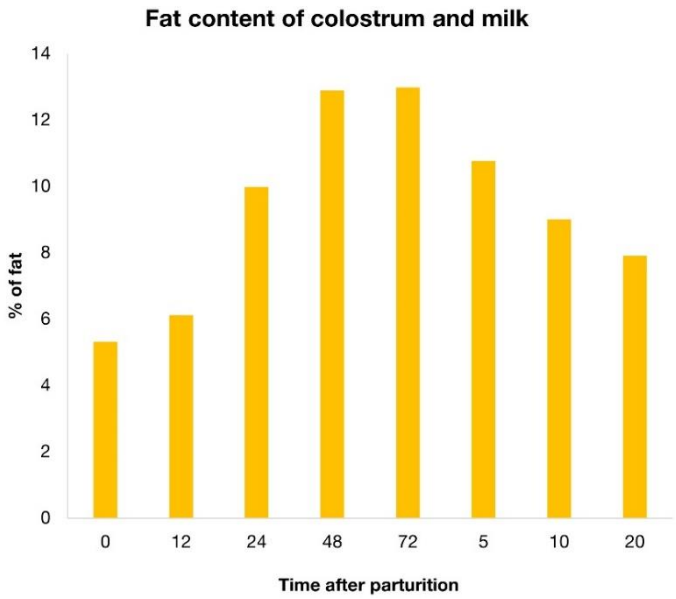


***Figure 6:*** *Evolution of fat content in sow colostrum and milk during the first 72 hours and up to 20 days of lactation. Information obtained from Csapó et al. (1996). Adapted from Darragh and Moughan (1998).*

The newborn piglets are considered as being very vulnerable since they are born with poor energy reserves and devoid of immune protection (Le Dividich *et al*., 2005). Therefore, both colostrum and milk, but especially colostrum immediately after farrowing, are essential in providing energy for thermoregulation and passive immunity to protect the piglets until its immune system becomes more mature (Le Dividich *et al*., 2005; Quensel *et al*., 2015).

*Dietary n-3 LCFAs on the FA composition of colostrum and milk*

Piglets can digest more than 90% of the lipids in colostrum and milk, which evidences them as very efficient vehicles for transferring FAs (Azain, 2001). Moreover, considering that 5% of the energy provided by triglycerides is attributed to glycerol, and the remaining 95% to FAs, lipid supply via colostrum and milk became important for the growth and development of piglets (Lauridsen, 2020).

As reviewed by Lauridsen (2020), the FAs commonly present in colostrum and milk are myristic acid (C14:0), palmitic acid, palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid and linoleic acid. The diet is one of the factors known to have an impact on the lipid composition of colostrum and milk, by changing the nature of the dietary fat source it may be possible to increase specific FA groups and their transfer to offspring (Lauridsen, 2020). However, the mechanisms of porcine mammary epithelial cells involved in the uptake of these FAs from bloodstream have not yet been well described (Zhang *et al*., 2018).

Several studies have evaluated the impact of including n-3 LCFAs in sow diets on the FA composition of their colostrum and milk. Therefore, depending on the source of n-3 LCFAs used, the individual FAs composition of colostrum and milk are slightly different. For example, plant-based sources such as linseed oil, rapeseed oil, soybean oil or hemp seed oil resulted in clear increases in the α-linolenic acid concentrations of colostrum and milk (Rooke *et al*., 1998 (3% soybean oil); Rooke *et al*., 2000 (1.75% maize oil (42%) and linseed oil (58%); Lauridsen and Danielsen, 2004 (8% rapeseed); Vodolazska and Lauridsen, 2020

(5% hemp seed oil or 5% mix of hemp seed and soybean oils (50:50))). Specifically, the values of α-linolenic acid ranged between 1 and 2% of total FA content in control diets or diets with fish oil, but these values could reach up to 5% in the case of rapeseed, while practically reaching 7% in the case of linseed. However, fish oils are rich in highly polyunsaturated FAs, so their inclusion as a source of n-3 LCFAs in sow diets results mostly in increases of EPA and DHA concentrations, but also of docosapentaenoic acid (C22:5 n-3) (Rooke *et al*., 1998 (3% tuna oil); Rooke *et al*., 2001b (1.65% salmon oil); Lauridsen and Danielsen, 2004 (8% fish oil)), with levels reaching approximately 8% of total FAs in milk for EPA and 10% for DHA. Moreover, in some of the studies where fish oil has been used, decreases of arachidonic acid content of approximately 0.5% in total FAs content were also observed (Rooke *et al*., 2001b (1.65% salmon oil); Mitre *et al*., 2005 (32 g/day shark-liver oil); Eastwood *et al*., 2014 (n-6:n-3 ratio of 5:1 adding fish oil)) .

*Dietary n-3 LCFAs on the immune parameters of colostrum and milk*

In addition to studying their influence on FA composition of colostrum and milk, research on dietary n-3 LCFAs has also focused on their impact on immune traits due to their important role in the transfer of passive immunity. Mitre *et al*. (2005) observed that the inclusion of 32 g/day of shark-liver oil in sow diets from day 80 of gestation until weaning resulted in larger levels of IgG in colostrum (control ≈50 g/ml vs shark-liver oil ≈100 g/ml) and milk at days 14 and 28 of lactation (control ≈1 g/ml vs shark-liver oil ≈1.5 g/ml). The same authors also detected an improvement of specific immunity in colostrum with an increase of anti-Aujeszky antibodies. Similar results were observed by Mateo *et al*. (2009) and Leonard *et al*. (2010) who described increases of approximately 5 mg/ml in colostral IgG concentrations with the use of n-3 FAs-rich diets. Specifically, Mateo *et al*. (2009) tested the addition of 0.2% protected fish oil from day 60 of gestation until weaning, and Leonard *et al*. (2010) fed sows with 10 g/day seaweed extract from day 109 of gestation until weaning. However, other studies did not detect any effects of the dietary n-3 FAs on immunoglobulins concentrations (Farmer *et al*.

(2010) (10% linseed, 6.5% linseed meal or 3.5% linseed oil) and Eastwood *et al*. (2014) (different n-6:n-3 ratios using linseed or fish oil as n-3 LCFA sources)). On the contrary, Yao *et al*. (2012) reported lower amounts of colostral IgG (27.0 vs 41.1 mg/ml) and milk IgM (0.95 vs 1.34 mg/ml) in diets with an n-6:n-3 ratio of 3:1 compared to diets with a ratio of 9:1, and Vodolazska and Lauridsen (2020) observed that, the average concentrations of IgG, IgA and IgM during the whole lactation period were lower in colostrum and mature milk when the sows were fed a diet with 5% hemp seed oil.

Therefore, the previous studies indicate that the FA composition of colostrum and milk is influenced by the source and the dose of dietary n-3 LCFAs. However, its effect on the amount of immunoglobulins differs among studies.

***Blood FA profile***

In pigs, dietary fat results in the digestion and absorption of different profiles of FAs inhibiting the *de novo* synthesis of FAs, which in turn may lead in changes in the FA composition of certain tissues of the animal reflecting the profile in the diet (Hausman *et al*., 2009). Specifically, in sows, the tissues that have been studied are blood (or some of its components such as red blood cells) (Rooke *et al*., 2000; Boudry *et al*., 2009; Smit *et al*., 2013), adipose tissue (Rooke *et al*., 2000), or reproduction related tissues such as conceptus (Pérez-Rigau *et al*., 1995; Brazle *et al*., 2009), endometrium (Brazle *et al*., 2009), placenta (De Quelen *et al*., 2010; Luo *et al*., 2020), corpora lutea (Smit *et al*., 2013), cord red blood cells (Luo *et al*., 2020), and even embryos (Smit *et al*., 2013). In piglets, the studied tissues have been: blood (or some of its components such as red blood cells) (Arbuckle and Innis, 1993; Fritsche *et al*., 1993b), adipose tissue (Lauridsen and Jensen, 2007), liver (Arbuckle and Innis, 1993; Fritsche *et al*., 1993b), brain (Arbuckle and Innis, 1993; Rooke *et al*., 1998), retina (Arbuckle and Innis, 1993; Rooke *et al*., 1998), spleen (Rooke *et al*., 1998; Rooke *et al*., 1999), thymus (Fritsche *et al*., 1993b), heart (Goustard-Langelier *et al*., 1999), sexual organs (Rooke *et al*., 1999), intestine

(Goustard-Langelier *et al*., 1999; Boudry *et al*., 2009) and muscular tissues (such as *Longissimus dorsi* (Luo *et al*., 2013; Tanghe *et al*., 2014) or *biceps femoris* (Heras-Molina *et al*., 2020)). In addition, some studies have also evaluated the influence of n-3 LCFAs on the FA composition of specific cell groups such as splenocytes or alveolar macrophages (Fritsche *et al*., 1993b).

*Dietary n-3 LCFAs on the FA composition of blood in sows and piglets*

In sows, incorporating different sources of n-3 LCFAs in the diet resulted in increased concentrations of this FA in their blood (serum or plasma). De Quelen *et al*. (2010) described that sows that were fed a diet with 1.5% linseed oil from day 28 of gestation showed a higher serum concentrations of α-linolenic acid (1.4 vs 9.3 g/100g serum), but also an increase in EPA (0.3 vs 1.2 g/100g serum) and a decrease in arachidonic acid (5.6 vs 3.4 g/100g serum). Similarly, Eastwood *et al*. (2014) reported increases of 0.31 mg/ml in α-linolenic acid in serum of gestating sows with a linseed-based diet (n-6:n-3 ratio of 1:1) and of 0.24 mg/ml of EPA with a fish oil-based diet (n-6:n-3 ratio of 5:1), supplementing diets from day 80 of gestation. Moreover, a decrease of at least 0.10 mg/ml in arachidonic acid was observed when the sows were fed a 1:1 linseed-based diet compared with the control or a 9:1 linseed-based diet. When fish oil has been used as the dietary n-3 LCFA source, the main reported influence has been an increased amount of highly polyunsaturated FAs of approximately 46 mg/ml for EPA and 15 mg/ml for DHA (Perez-Rigau *et al*., 1995; Rooke *et al*., 1998). In addition, while the study of Perez-Rigau *et al*. (1995) described a decrease of 29 mg/ml in arachidonic acid with the inclusion of 4% menhaden oil, Rooke *et al*. (1998) and Rooke *et al*. (1999) did not observe any changes for this FA using diets containing 3% of tuna oil.

In piglets, the results of the effects dietary n-3 LCFAs on blood FA composition are in line with those described so far in sows. Bazinet *et al*. (2003), De Quelen *et al*. (2010) and Yao *et al*. (2012) observed that a maternal diet rich in linseed products (rich in α-linolenic acid), from early and late gestation, respectively, resulted in

increased concentrations of this FA and those of highly unsaturated FAs in the blood of suckling piglets, reaching levels of around 12% for α-linolenic acid and between 0.5 and 1% for EPA and DHA. Nevertheless, while Bazinet *et al*. (2003) reported a decrease of 2.3 mg/ml in the amount of arachidonic acid with the supplementation of 20 g/kg of linseed oil from late gestation until day 14 of lactation, De Quelen *et al*. (2010) (1.5% and 5.5% linseed oil during gestation and lactation, respectively) and Yao *et al*. (2012) (different n-6:n-3 ratios using linseed oil as n-3 LCFA source) did not observe any effect. Similarly, different studies have shown increases in blood highly unsaturated FAs with the inclusion of fish oil as n-3 LCFAs source. These increments typically achieved levels of EPA ranging between 5 and 12%, and levels of DHA ranging between 5 and 8% of total FAs. These are usually accompanied by a decrease, of potentially up to 50%, in the arachidonic acid concentration (Fritsche *et al*., 1993a (3.5% or 7% fish oil); Rooke *et al*., 1998 (3% tuna oil); Eastwood *et al*., 2014 (different n-6:n-3 ratios using linseed or fish oil as n-3 LCFA sources).

To recap, similar to what was observed for colostrum and milk, the variety of the obtained results depends on the n-3 LCFA source, time of its inclusion and dosage. It is clear that an inclusion of dietary n-3 LCFAs increases their concentration in the blood of the animals. However, their impact on FAs from other families is confusing.

### *Polyunsaturated FA oxygenated derivatives*

In the organism, polyunsaturated FAs can be oxygenated through oxygen dependent reactions. The by-products of these oxygenations are known as oxylipins and they exert functional signalling roles, for which they are considered to be the main mediators for the effects of polyunsaturated FAs in the body (Gabbs *et al*., 2015; Shearer and Walker, 2018). Oxylipins can be classified as n-6 polyunsaturated FAs- and n-3 polyunsaturated FAs-derivatives. Among the most commonly studied oxylipins, there are those derived from linoleic and arachidonic acids as n-6 LCFAs and those derived from α-linolenic acid, EPA and DHA as n-3

LCFAs. Although the tissue profile of oxylipins does not necessarily mimic the dietary intake of polyunsaturated FAs, the FAs composition of the diet may influence the oxylipin profile, so diets rich in n-6 or n-3 polyunsaturated FAs may be associated with a higher concentrations of their respectively derived oxylipins (Gabbs *et al*., 2015).

*Synthesis of oxylipins*

Oxylipin synthesis usually begins with cell activation and the release of the polyunsaturated FAs in the middle position of the glycerol backbone (*sn*-2) of membrane phospholipids mainly by the action of cytosolic phospholipase $A_2$ (Gabbs *et al*., 2015; Christie and Harwood, 2020). In addition, it has also been reported that, to a lesser extent, FAs still esterified on phospholipid or cholesterol can be oxidized resulting in their derived oxylipins *in situ* (Gabbs *et al*., 2015). The oxygenation of polyunsaturated FAs into their derived oxylipins can occur by enzymatic or free radical-mediated reactions (Astarita *et al*., 2015). Three different enzymatic pathways are involved in oxylipin formation. Specifically, they are those catalysed by the enzymes cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450 (CYP) (Astarita *et al*., 2015; Gabbs *et al*., 2015). Briefly, the cyclooxygenase pathway converts polyunsaturated FAs such as arachidonic acid and EPA into prostanoids, including prostaglandins (PGs) and thromboxanes (TXs), or hydroxy-FAs; the lipoxygenase pathway catalyses the synthesis of hydroxy-FAs and derived metabolites such as arachidonic acid-derived leukotrienes (LTs) and lipoxins (LXs), or EPA- and DHA-derived resolvins (RVs) and protectins (Ps); and the cytochrome P450 pathway catalyses the hydroxylation and/or epoxygenation reactions of polyunsaturated FAs, such as arachidonic acid, EPA and DHA, resulting in the formation of epoxy-arachidonic acid, -EPA, and -DHA derived acids through epoxygenase activity, and hydroxy-arachidonic acid, -EPA, and -DHA derived acids through hydroxylation activity (Astarita *et al*., 2015; Gabbs *et al*., 2015; Christie and Harwood, 2020). At last, these oxylipins are exported from the

cell out and activates G-protein coupled receptors on nearby cells or mediate their own effects (Mokoena *et al*., 2020).

Figure 7 shows a schematic overview of the synthesis of oxylipins derived from linoleic acid, α-linolenic acid, arachidonic acid, EPA, and DHA, together with their corresponding enzymatic pathways.

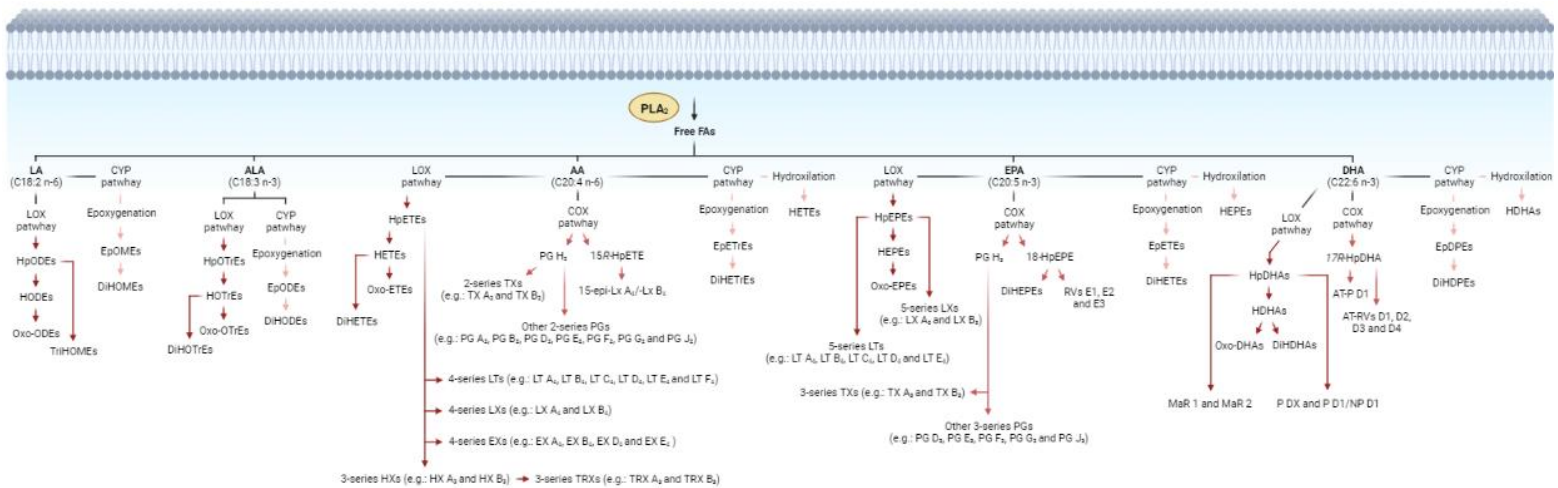


***Figure 7:*** *Schematic overview of the synthesis of oxylipins from the precursor FAs linoleic acid (LA), α-linolenic acid (ALA), arachidonic acid (AA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). AT-P, aspirin-triggered protectin; AT-RV, aspirin-triggered resolvin; COX, cyclooxygenase; CYP, cytochrome P450; DiHDHA, dihydroxy-docosahexaenoic acid; DiHDPE, dihydroxy-docosapentaenoic acid; DiHEPE, dihydroxy-eicosapentaenoic acid; DiHETE, dihydroxy-eicosatetraenoic acid; DiHETrE, dihydroxy-eicosatrienoic acid; DiHODE, dihydroxy-octadecadienoic acid; DiHOME, dihydroxy-octadecenoic acid; DiHOTrE, dihydroxy-octadecatrienoic acid; EpDPE, epoxy-docosapentaenoic acid; EpETE, epoxy-eicosatetraenoic acid; EpETrE, epoxy-eicosatrienoic acid; EpODE, epoxy-octadecadienoic acid; EpOME, epoxy-octadecenoic acid; EX, eoxin; FA, fatty acid; HDHA, hydroxy-docosahexaenoic acid; HEPE, hydroxy-eicosapentaenoic acid; HETE, hydroxy-eicosatetraenoic acid; HODE, hydroxy-octadecadienoic acid; HOTrE, hydroxy-octadecatrienoic acid; HpDHA, hydroperoxy-docosahexaenoic acid; HpEPE, hydroperoxy-eicosapentaenoic acid; HpETE, hydroperoxy-eicosatetraenoic acid; HpODE, hydroperoxy-octadecadienoic acid; HpOTrE, hydroperoxy-octadecatrienoic acid; HX, hepoxilin; LOX, lipoxygenase; LT, leukotriene; LX, lipoxin; MaR, maresin; Oxo-DHA, oxo-docosahexaenoic acid; Oxo-EPE, oxo-eicosapentaenoic acid; Oxo-ETE, oxo-eicosatetraenoic acid; Oxo-ODE, oxo-octadecadienoic acid; Oxo-OTrE, oxo-octadecatrienoic acid; P, protectin; PG, prostaglandin; PLA2, phospholipase A2; RV, resolvin; TriHOME, trihydroxy-octadecenoic acid; TRX, trioxilin; TX, thromboxane. Adapted from Gabbs et al. (2015) and Astarita et al. (2015).*

Oxylipins are found in all the tissues of the organism, including blood and even in urine. They have been classically described as non-storage metabolites that are synthesised *in situ* and have short half-life and local action (Gabbs *et al.,* 2015). However, certain oxylipins may have stable concentrations and may be found in a free and/or esterified form in tissues such as liver, adipose tissue, kidney, and ileum (Gabbs *et al*., 2015). Free oxylipins are considered to be the ones that have active biological functionality, while those in the esterified from could provide certain properties to the membrane and/or act as a storage depot Gabbs *et al.* (2015). More recently, it has been described that oxylipins follow a similar distribution to that of FAs in plasma, circulating in their free form, bound to plasma proteins such as albumin, or esterified in glycerolipids or cholesterol-esters (Shearer and Walker, 2018).

*Biological role of oxylipins*

Oxylipins are involved in a wide variety of functions such as apoptosis, tissue repair, blood clotting, blood vessel permeability, blood pressure regulation and cell proliferation, but the roles that are currently arousing more interest are those associated with the modulation of immunity and inflammatory processes (Gabbs *et al*., 2015). Considering their precursor, the oxylipins that are derived from n-6 polyunsaturated FAs have been associated with a pro-inflammatory potential, whereas those that are derived from n-3 polyunsaturated FAs have been associated with a less inflammatory, anti-inflammatory or inflammation resolving activities (Calder, 2010). Even so, Shearer and Walker (2018) suggested in their review that the association of n-6 polyunsaturated FAs with pathology is not as clear as previously thought, and, in some cases, is even erroneous.

In this way, the oxygenation of linoleic and arachidonic acids can lead to the formation of oxylipins that are associated with a pro-inflammatory role such as the linoleic acid-derived hydroxy-octadecadienoic acids (HODEs) and dihydroxy-octadecenoic acids (DiHOMEs), and the arachidonic acid-derived 2-series

prostaglandins, 2-series thromboxanes and hydroxy-eicosatetraenoic acids (HETEs). However, other products derived from the oxygenation of these PUFAs, such as the linoleic acid derivative 13-Oxo-octadecadienoic acid (13-Oxo-ODE) or the arachidonic acid derivatives 4-series lipoxins, have been linked with anti-inflammatory and inflammation-resolving properties (Liput *et al*., 2021). In reference to n-3 polyunsaturated FAs-derived oxylipins, certain α-linolenic acid-derived octadecanoids such as 9- and 13-hydroxy-octadecatrienoic acids (HOTrEs) have been shown to have anti-inflammatory and immunomodulatory effects in mice by reducing tissue inflammation and the secretion of pro-inflammatory cytokines (Cambiaggi *et al*., 2023). In terms of EPA and DHA oxygenated derivatives, end products such as 3-series prostaglandins, 3-series thromboxanes and 5-series leukotrienes, or hydroxy-FAs, have been reported to be less potent than their analogous derivatives from n-6 polyunsaturated FAs. Therefore, they are described as less efficient and potent oxylipins, with a biological role that tends to be less inflammatory or anti-inflammatory (Gabbs *et al*., 2015). Thus, an increase in n-3 FAs derived oxylipins not only compromises their intrinsic functionalities but may also reduce their biological activity by decreasing the amount n-6 polyunsaturated FAs-derived oxylipins (Gabbs *et al*. 2015). EPA and DHA also give rise to anti-inflammatory and inflammation resolving mediators. Within this group, compounds such as resolvins, protectins, lipoxins and maresins stand out, with roles in the prevention of neutrophilic infiltration or the inhibition of pro-inflammatory cytokine production, as mainly described in cell cultures and rodents feeding studies (Serhan *et al*., 2008; Calder, 2010). These last inflammation resolving mediators gained interest due to their important roles in the resolution of inflammation and the shutting off of ongoing inflammatory processes (Calder, 2010).

*Dietary n-3 LCFAs and the oxylipin profile*

Dietary inclusion of n-3 LCFAs is an effective strategy to shift the polyunsaturated FA pools, and thus the nature of the oxylipin precursors, towards n-3 derivatives (Shearer and Walker, 2018). This commonly implies a decrease in n-6 LCFAs-

derived oxylipins, but this change does not always occur, and in some cases even an increase in those may be observed (Shearer and Walker, 2018).

To the authors' knowledge, there are no studies evaluating the impact of dietary n-3 LCFAs on the circulating oxylipin profile in pigs, although there are a few studies that have assessed the concentration of specific oxylipins in pigs. Fritsche *et al*. (1993b) described a reduction of approximately 5 pg/0.1 ml of prostaglandin E, 55 pg/0.1 ml of thromboxane B and 750 pg/0.1 ml of leukotriene B in alveolar macrophages from three to four weeks old suckling piglets with the inclusion of 7% fish oil in the sow diet after day 107 of gestation. Lauridsen *et al*. (2007) also reported decreases in the synthesis of prostaglandin $E_2$ and thromboxane $B_2$ of 1250 and 800 pg/$10^6$ cells, respectively, in the same type of cells in 25 day-old suckling piglets, when comparing sow diets offered from day 108 of gestation with 8% fish oil or 8% sunflower oil. In mice, Fan *et al*. (2020) reported that animals fed with an α-linolenic acid-enriched butter presented higher plasmatic contents of α-linolenic acid, EPA, and their oxygenated derivatives in comparison to mice fed with conventional butter. These animals also presented a decrease in the plasmatic contents of arachidonic acid and its derivate oxylipins. In humans, Schebb *et al*. (2014) also described an increase in EPA-derived oxylipins, a decrease arachidonic acid oxylipins, and no effect on the oxygenated derivatives of DHA with the dietary supplementation of n-3 polyunsaturated FA capsules rich in EPA and DHA for 12 weeks. Differently, Marchix *et al*. (2020) reported an increase in oxygen derivatives of linoleic and arachidonic acids, and a decrease in oxylipins from EPA and DHA in plasma from rats fed a diet supplemented with linoleic acid.

The above results are in line with those suggesting that the intake of a diet rich in n-3 LCFAs may promote the incorporation of FAs such as EPA and DHA into the membrane phospholipids at the expense of arachidonic acid (Grimm *et al*., 2002). Moreover, n-6 and n-3 LCFAs compete for the same enzymes (ciclooxygenase, lipoxygenase and cytochrome P450) for their oxygenation, and therefore the dietary increase in n-3 LCFAs would result in an increase of their derived oxylipins, but a reduction of the oxylipins derived from n-6 LCFAs.

### *Immune parameters*

In addition to the immunomodulatory effects that the oxygenated products of polyunsaturated FAs can exert, polyunsaturated FAs may also have an immunomodulatory role and influence inflammatory processes through other mechanisms. Concretely, different authors describe the influence of n-3 polyunsaturated FA on immune cells leukocyte chemotaxis, adhesion molecules and adhesion interactions, transcription factors involved in the regulation of inflammatory gene expression and modulation of inflammatory cytokine production (Calder, 2010; Calder, 2013; Gutierrez *et al*., 2019).

*Dietary n-3 LCFAs on immunoglobulin and cytokine production in sows and piglets*

As for colostrum and milk, research on the effects of dietary n-3 LCFAs on the immune parameters in blood of swine has mainly focused on the production of Igs and cytokines. Mitre *et al*. (2005) reported no variations in the concentrations of total IgG and IgM in sow serum at parturition and at day 14 of lactation after including 32 g/day of shark-liver from day 80 of gestation. However, they did note an overall significant increase (of between 20-50%) in the IgG concentration in piglets from sows that had been fed fish oil fed sows, at days 2, 21 and 36 of age. These results are in agreement with those reported by Smit *et al*. (2015), who also observed no modification on serum IgG concentration in gestating sows fed a diet supplemented with a 0.5% protected fish oil from the previous weaning, and those observed by Leonard *et al*. (2010), who noted ≈3 mg/ml greater serum IgG concentrations in suckling piglets at days 5 and 12 of lactation when the sows had been fed a diet with 10 g/day of seaweed from day 109 of gestation. The later piglets also presented a ≈1 mg/ml greater serum IgA concentration at day 5 of lactation. However, the same authors observed a 0.6 mg/ml lower IgA concentration in piglet serum at day 5 of lactation when the sows had been fed a diet with 100 g/day of fish oil from day 109 of gestation. On the contrary, both Lavery *et al*. (2019) and Zhang *et al*. (2020) did not detect any effect of dietary n-3

LCFAs on the serum IgG concentrations of piglets while suckling, at weaning or after weaning.

Regarding the influence of n-3 LCFAs on the production of cytokines, there are few studies that have evaluated the impact of dietary n-3 LCFAs on these parameters in sows and piglets. Papadopoulus *et al*. (2009) did not observe any effects of dietary n-3 LCFAs in the serum concentrations of tumor necrosis factor α (**TNFα**) and interleukin (**IL**) 6 in lactating sows that had been fed a diet with 2% fish oil from late gestation. These results are in agreement with those of Yong *et al*. (2015) who also did not detect differences in serum TNFα, IL6 and IL1β in lactating sows or suckling piglets when sows had been fed diets with fish oil (2% from day 84 of gestation and 4.8% in lactation). On the other hand, Luo *et al*. (2013) studied the effects of dietary n-3 LCFAs on the production of cytokines in spleen and muscle tissue. Specifically, they observed 0.2 and 0.3 lower relative gene expressions for TNFα and IL6, respectively, in the *longissimus dorsi* muscle of weaned piglets from sows that had been fed diets with 7% of fish oil instead of lard during lactation. Additionally, the same authors reported that the piglets from the fish oil fed sows presented a 0.75 higher relative expression for TNFα in spleen.

In brief, as also summarized for the impact of dietary n-3 LCFAs on the Ig composition of milk and colostrum, the results are unclear and differ depending on the analysed tissue.

### *Microbiota*

The microbiota of the mammalian gut is estimated to contain 100 trillion bacteria and acts as a major regulator of animal physiology and health (Holman *et al*., 2017; Beaumont *et al*., 2021). In brief, microbiota provides health benefits by producing short-chain FAs and vitamins, by preventing the gut colonization by potential pathogens, and by contributing to the development and maintenance of the immune system of the host (Holman *et al*., 2017). For these reasons, its correct

acquisition and establishment is a key factor for the piglet's development and survival.

*Establishment and acquisition of gut microbiota in newborn piglets*

The precise timing of the initial colonization of pig microbiota remains unknown. So far, no studies have described *in utero* colonization in piglets and the results in other species are rather contradictory (Nowland *et al*., 2021). Therefore, it is unclear whether the development of the microbiota in the piglet could start during the gestation period. However, it is reasonable to hypothesize that microbiota of sows may also play a substantial role in shaping the microbiota of their offspring prior to and during parturition, being the microbiota of the vagina of sow the one that plays an important role at farrowing (Nowland *et al*., 2021).

Once born, the microbiota of piglets is affected by various external factors. During the early postnatal period, the main determinants shaping the initial microbial composition of newborn piglets are likely to be colostrum, milk, feed, oral-faecal transmission, and the environment (Nowland *et al*., 2019; Lauridsen, 2020). As previously described, colostrum and milk are sources of energy and immunity for piglets in the early stages of life, but it is also worth noting that both contain a diverse range of bacteria and prebiotic compounds that support the development of the gut microbiota. Therefore, the optimal consumption and quality of colostrum and milk during lactation are important factors for the colonization of the gut microbiota (Nowland *et al*., 2021). Another factor that contributes in the establishment of the microbiota of piglets is the microbiota of the faeces of sows during lactation (Nowland *et al*., 2021). In reference to oral-faecal transmission, Chen *et al*. (2018) observed that as the lactation period progressed, the microbiota of the piglet's faeces became more similar to that of their mother. Finally, environmental factors such as husbandry hygiene may also have a clear effect on the acquisition of the microbiota as documented by Schmidt *et al*. (2011) who

described that piglets require continuous microbial exposure during the early post-natal stages for the development of their "adult-like" microbiota.

At weaning, the introduction of feed is another item to consider for the establishment of the microbiota. In this way, it has been documented that the influence that the sow can have on the microbiota of the piglet is diminished when feed is offered (Bian *et al*., 2016). For this and other reasons, weaning is generally associated with a disrupted state of the microbiota that can be referred as *dysbiosis* characterized as a gut microbial imbalance (Gresse *et al*., 2017). In addition, social and environmental factors such as the separation from the mother, mixing of litters, handling, transport, and change of physical environments can generate stress in the piglet, which consequently can also contribute to this *dysbiosis* state (Gresse *et al*., 2017). Therefore, the acquisition and establishment of the microbiota is a factor that may play an essential role on the health of piglets during the suckling-to-weaning transition (Beaumont *et al*., 2021).

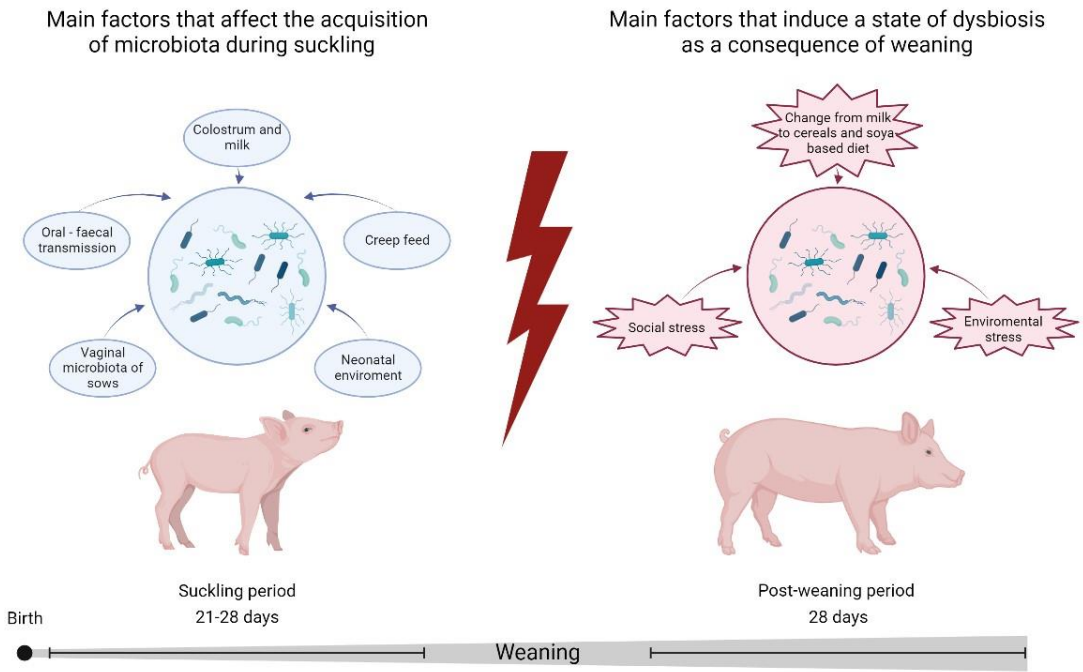


***Figure 8:*** *Main factors affecting the acquisition and establishment of microbiota in piglets during suckling and main factors that may induce a state of dysbiosis as a consequence of weaning. Information obtained from Gresse et al. (2017).*

*Dietary n-3 LCFAs on microbiota in sows and piglets*

Diet is considered as one of the most important factors affecting the intestinal microbiota colonization (Nowland *et al*., 2019). Thus, dietary interventions have become an appealing strategy to modulate microbiota to improve animal health and production outcomes due to their significant impact on the health and productivity of food-producing animals, such as in swine (Holman *et al*., 2017). Research on the influence of n-3 LCFAs on gut microbiota is still on relative early stages (Costantini *et al*., 2017). In the review by Fu *et al*. (2021), described that n-3 LCFAs can influence the gut microbiota mainly in three different ways: by modulating the abundance and composition of the microbiota, by altering the concentration of proinflammatory mediators such as lipopolysaccharides (**LPS**), or by regulating the levels of short-chain FAs. This review also described that n-3 LCFAs can promote a decrease in the Fimicutes/Bacteroidetes ratio, which is associated with different human diseases such as obesity, and increase the abundance of beneficial bacteria such as the genera *Akkermansia* (Santoru *et al*., 2017; Warner *et al*., 2019). On the other hand, Costantini *et al*., 2017 described that dietary n-3 LCFAs can potentially promote a state of *eubiosis* through improving the Firmicutes/Bacteroidetes ratio and also enhancing health-promoting bacteria.

The few studies that have assessed in any way the impact of dietary n-3 LCFAs on the microbiota of sows and piglets have looked at very specific populations and not to the whole microbiome. In terms of sow microbiota, Yin *et al*. (2017) observed that sows fed diets with 10:1 and 15:1 n-6:n-3 ratios, in which linseed oil was used as the source of n-3 LCFAs, showed a decrease in faecal *E. coli* counts compared to those that were fed a diet with a 20:1 ratio. In addition, the sows fed diets with 10:1 and 15:1 ratios also had increased faecal counts of *Lactobacillus* compared to sows that had been fed a diet with 20:1 ratio. Regarding piglets, Leonard *et al*. (2011) described that weaned piglets from sows fed diets with 1.8 g/day seaweed had a numerically reduced population of *Escherichia coli* in the caecum compared with piglets from non-supplemented sows (3.67 vs 4.89 colony-forming units/g digesta). In addition, the same piglets also presented lower populations of

*Bifidobacterium* (8.32 vs 9.12 $log_{10}$ colony-forming units/g) and tended to have reduced *Escherichia coli* (4.50 vs 5.34 $log_{10}$ colony-forming units/g) and *Lactobacillus* (8·17 v. 8·75 $log_{10}$ colony-forming units/g). Additionally, Zhang *et al*. (2020) reported a tendency to increase *Lactobacillus* in piglets that were fed a diet containing 15 g/kg of coated n-3 FAs at weeks 3 and 6 post-weaning (7.58 vs 7.46 and 7.52 vs 7.40 $log_{10}$ colony-forming units/g, respectively).

Therefore, knowledge about the impact of n-3 LCFAs in the diets of sows and piglets on their microbiota is limited to a very few studies that focused on very specific populations rather than providing a comprehensive examination of the overall impact. Furthermore, these studies also differed in the source of n-3 LCFAs used, their period of inclusion, and their dosage.

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# HYPHOTESES AND OBJECTIVES

Effects of n-3 long-chain fatty acids in sow and piglet diets on perinatal piglet performance, oxylipins, immunity, and microbiota.

UNIVERSITAT ROVIRA I VIRGILI
EFFECTS OF N-3 LONG-CHAIN FATTY ACIDS IN SOW AND PIGLET DIETS ON PERINATAL PIGLET PERFORMANCE, OXYLIPINS, IMMUNITY, AND MICROBIOTA
Eudald Llauradó Calero

## HYPOTHESES AND OBJECTIVES

Modern swine production systems face new challenges resulting from the increases in litter size and proportion of piglets born with low birth weight and compromised vitality. In addition, the recent restrictions on the use of antibiotics and zinc oxide have resulted in increased mortality during the early stages of the piglet's life. Consequently, exploring alternative strategies to improve the piglet's vitality has become crucial.

In this context, nutritional approaches, such as the inclusion of n-3 LCFAs in diets of sows and piglets, can be a valuable tool to address these concerns. As already noted in the above literature review, the focus of study of n-3 LCFAs has changed over time. The early studies mainly focussed on their effect on the sow's reproductive performance, piglet growth and FA composition in different tissues from sows and piglets, while more recently the emphasis is on the study of the anti-inflammatory and inflammation-resolving effects that they can exert in the animal.

The effects of n-3 LCFAs on sow performance, litter characteristics and piglet growth remain controversial and new information is needed to understand the realrole of n-3 LCFAs on these issues. In addition, to the authors' knowledge, there are not studies in which the effects of dietary n-3 LCFAs on the FA profile have been accompanied with the study of their oxygenated derivatives. Moreover, little is known about how the dietary inclusion of n-3 LCFAs in the diets of sows and piglets impact on the piglet's immune response and its microbial diversity and composition, which are considered key factors for the piglet development and survival during the early stages of life.

**Hypotheses**

For all the above-mentioned reasons this Doctoral Thesis proposes the following hypotheses:

**Hypothesis 1** The inclusion of fish oil in the diets for sows and weaned piglets will improve the productive efficiency of the sow increasing the number and weight of the piglets during the early stages of life.

**Hypothesis 2** The inclusion of a fish oil in the diets for sows will increase the concentrations of EPA and DHA in blood, colostrum and milk, as well as those of their derived oxylipins. These will confer anti-inflammatory properties with inflammation resolving actions to the piglets during lactation and their effects will persist after weaning.

**Hypothesis 3** The inclusion of a fish oil in the diets for piglets will also increase their blood concentrations of EPA and DHA, and those of their related oxylipins. Both, n-3 LCFAs and their derived oxylipins will improve piglet's immune status and survival, particularly for piglets born with LBW.

**Hypothesis 4** In addition to their immunomodulatory actions, the inclusion of a fish oil in the diets for sows and piglets will also promote the acquisition and establishment of a beneficial microbiota.

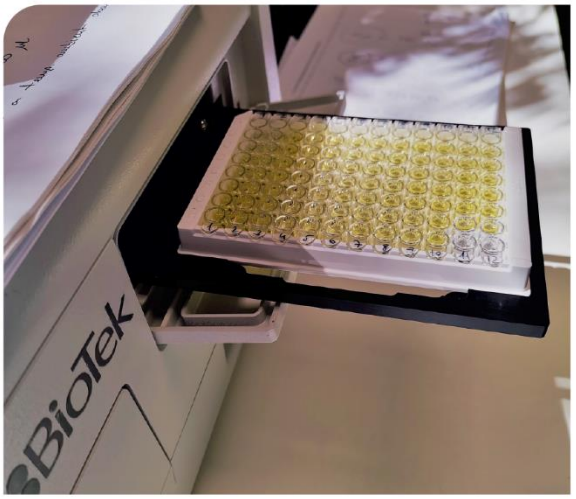
## Objectives

Globally, the objectives of this thesis are to determine the influence of the inclusion of a fish oil source rich in n-3 LCFA (specifically EPA and DHA) in the gestation and lactation diets of sows and in the post-weaning diets of piglets to:

- Improve the productive efficiency of the sow increasing the number and weight of piglets per litter both at birth and at weaning.
- Improve piglet health increasing pre- and post-weaning survival, and, consequently, reducing the need for the preventive use of antibiotics or other antimicrobials during the post-weaning phase.

The achievement of these global objectives can be split into different specific objectives. Therefore, this thesis aims to evaluate the effects of dietary n-3 LCFAs on:

- The productive parameters and fat deposition of sows.
- Colostrum and milk concentrations of FAs, oxylipins and immune indicators.
- Productive parameters of piglets at birth, at weaning and at day 28 post-weaning.
- FA and oxylipin profile in blood of sows during gestation and lactation, and of piglets at weaning and 28 days post-weaning.
- Systemic immune indicators of sows during gestation and lactation, and of piglets at weaning and 28 days post-weaning, as well as the immune indicators in the ileal mucosa of piglets at day 28 post-weaning.
- Microbiota diversity and composition in colostrum, milk and faeces of gestating and lactating sows and piglets at weaning, and days 7 and 28 post-weaning, and in caecum contents of piglets at 28 days post-weaning.
- To determine whether the effects of n-3 LCFA are greater in piglets born with LBW compared to piglets born with high BW (**HBW**).




# CHAPTER 1

## Fish oil rich in eicosapentaenoic acid and docosahexaenoic acid in sow diets modifies oxylipins and immune indicators in colostrum and milk.

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## CHAPTER 1

# Fish oil rich in eicosapentaenoic acid and docosahexaenoic acid in sow diets modifies oxylipins and immune indicators in colostrum and milk

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This chapter describes and discusses the effect of the inclusion of the fish oil rich in n-3 LCFAs in gestation a lactation diets for sows on:

- Weight, backfat and feed intake of sows.
- Litter characteristics, and weight and feed intake of piglets during lactation.
- Fatty acid composition, oxylipin profile and immune indicators of colostrum and milk.

**Fish oil rich in eicosapentaenoic acid and docosahexaenoic acid in sow diets modifies oxylipins and immune indicators in colostrum and milk**

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**Abstract**

Colostrum and milk are the first nutrient sources for newborn piglets. In addition, n-3 fatty acids (**FAs**) and their oxygenated derivatives (oxylipins) have the capacity to modulate immune components. The aim of the current study was to include a fish oil rich in eicosapentaenoic acid (**EPA**) and docosahexaenoic acid (**DHA**) in sow diets to promote an increase of anti-inflammatory molecules in colostrum and milk to benefit piglets. Thirty-six sows were randomly assigned from insemination to the end of lactation to either a control diet with animal fat (15 g/kg in gestation and 30 g/kg in lactation) or an n-3 diet in which animal fat was totally (gestation) or half (lactation) replaced by an equivalent amount of solid fish oil. Performance of sows and piglets was monitored during the study. Colostrum and milk samples were obtained after the birth of the first piglet and at weaning, respectively. From all samples (n = 18 per treatment), FAs were quantified by gas chromatography and immunoglobulins and cytokines by ELISA. Three samples per treatment were randomly selected to analyse oxylipin composition by liquid chromatography-tandem mass spectrometry. In colostrum and in milk, the n-3 FA ($P = 0.020$ and $P < 0.001$), particularly EPA ($P < 0.001$ and $P < 0.001$) and DHA ($P < 0.001$ and $P < 0.001$), and also their oxygenated derivatives were increased in samples from sows fed n-3 diet. Fish oil had no effect on immunoglobulin concentrations, but reduced tumour necrosis factor a (**TNFα**) ($P = 0.011$) and a tendency to reduce interleukin 10 (**IL10**) ($P = 0.059$) were observed in milk. In conclusion, fish oil in sow diets increased n-3 FA, particularly EP A and DHA, and their oxygenated derivatives in colostrum and milk, reducing TNFα and IL10 in milk.

**Keywords**

Lactation, n-3 long-chain fatty acids, Maternal passive immunity, Oxygenated lipid mediators, Swine.

**Implications**

This study shows that the inclusion of fish oil in sow diets during gestation and lactation may reduce litter size without affecting total litter weight during lactation and increases the concentration of n-3 fatty acids and their oxygenated derivatives in colostrum and milk. Further studies with larger number of animals are needed to assess the effects of n-3 fatty acids on litter size and piglet's growth performance. On the other hand, the transfer of these anti-inflammatory molecules to suckling piglets could result in an improved immune status of newborn piglets.

**Introduction**

Colostrum and milk are the earliest nutrient sources for the newborn piglets. They contain a mixture of constituents such as lactose, proteins (mainly casein and immunoglobulins), fatty acids (**FAs**), growth factors and immune cells that are crucial for the transfer of energy and maternal passive immunity (Klobasa *et al*., 1987; Darragh and Moughan, 1998). However, they differ in time of secretion and in composition. Colostrum is the first secretion of the mammary glands, and it is produced within the first 24 h after farrowing. It is mainly composed of immunoglobulins (**Ig**) (concretely IgG) and it is the main energy source for piglets immediately after birth (Devillers *et al*., 2011). Its composition rapidly changes to that of mature milk as fat and lactose concentrations increase and protein and Ig decrease (Quesnel *et al*., 2015). Considering that piglets are born with low body fat and energy reserves, and devoid of immune protection (Le Dividich *et al*., 2005), colostrum and milk play a critical role for piglet survival.

Several studies grouped in the review of Rosero *et al*. (2016) have shown that lipid supplementation to lactation sow diets slightly reduce sow BW loss during lactation and increased fat content in milk resulting in a higher energy intake for piglets which could positively improve their growth. However, the role of individual or families of FA has only recently been evaluated (Rosero *et al*., 2016). For this reason, the nature of lipids used in sow diets needs to be examined in detail (Bontempo and

Jiang, 2015). Different studies found that the different sources of dietary polyunsaturated FAs increase their concentration in milk (Smit *et al*., 2015; Gessner *et al*., 2016). It is well established that these polyunsaturated FAs have an impact on the immune status (Lauridsen, 2020) through the modification of the oxylipin profile (Balvers *et al*., 2012) and its subsequent effect on the synthesis of cytokines (Calder, 2010). Oxylipins are oxygenated lipid mediators and are the major mediators for polyunsaturated FA effects in the body (Gabbs *et al*., 2015). Because they are considered as bioactive lipid mediators critically involved in neonatal physiology (Wu *et al*., 2016), their presence in human milk (Robinson *et al*., 2017) suggests that they may have relevant effects on the immune status of the newborn after colostrum and milk intake. There are differences between n-6 and n-3 polyunsaturated FAs in their effects on immune status, as n-6 polyunsaturated FAs are precursors of proinflammatory oxylipins, while n-3 polyunsaturated FAs are precursors of anti-inflammatory oxylipins (Calder, 2010). To our knowledge, no prior studies have analysed oxylipin concentration in colostrum in any species and there are only some studies in bovine (Kuhn *et al*., 2017) and human (Robinson *et al*., 2017) milk, but not in sows.

In the current study, it is hypothesised that fish oil in sow diets promotes the increase of n-3 polyunsaturated FA and n-3 polyunsaturated FA-derived oxylipins in colostrum and milk, which could have an impact on their immunological properties. Therefore, the aim was to evaluate the impact of a solid fish oil rich in eicosapentaenoic acid (**EPA**) (C20:5 n-3) and docosahexaenoic acid (**DHA**) (C22:6 n-3) in sow diets on the nutritional composition of colostrum and milk (FA content, oxylipins, Ig and cytokines) which will be transferred to the newborn piglet and could affect their growth and immune status. Preliminary results have been published in an abstract form (Llauradó-Calero *et al*., 2020).

## Material and methods

*Animals, housing and experimental design*

The study was performed with thirty-six sows in four consecutive batches (12, 9, 5 and 10 sows in the 1st, 2nd, 3rd and 4th batch, respectively). The different number of sows differs between batches due to the availability of sows that met the selection criteria of being between the 3rd parity and 6th parity. In each batch, sows were divided into groups of two as similar as possible regarding parity number and BW. Within each group, sows were randomly assigned to a control diet or an n-3 long-chain FAs (**LCFA**) rich diet. Sows were involved in the trial from insemination until the end of lactation (±28 days after farrowing). Cross fostering of piglets was only performed during the first 24 h postfarrowing to standardise litter size (12 piglets per sow), and exclusively among sows belonging to the same experimental treatment. At day eleven of lactation, a control or an n-3 LCFA prestarter creep feed for piglets was introduced in accordance with the maternal diet.

Sows were allocated to individual stalls from insemination to pregnancy confirmation, afterwards till one week before farrowing sows were group-housed in a gestation barn and one week before farrowing, they were moved to individual farrowing crates (0.7 x 2 m) equipped with partially slatted floor and a heated floor panel for piglets (set at 32–34 ºC). The room was lit via skylight and artificial light (non-programmable), and its ventilation was via single, variable-speed fans linked to temperature sensors. The temperature inside the building was automatically controlled. The target temperature of the rooms was set at 24 ºC at farrowing, and it was reduced by 0.5 ºC per week until weaning. Sows were fed via hoppers and piglets from round feeders on the ground. Water was provided ad libitum from nipple drinkers.

*Experimental diets*

Gestation and lactation diets were formulated according to FEDNA specifications (de Blas *et al*., 2013). Sows were fed either a control diet with animal fat (15 and 30 g/kg for gestation and lactation specifications, respectively) or an n-3 LCFA diet in

which animal fat was totally (gestation) or half (lactation) replaced by an equivalent amount of a solid fish oil (Lipomega®; V&S Asociados, Madrid, Spain). For piglets, the control prestarter creep feed was formulated to contain 30 g/kg of animal fat and in the n-3 LCFA diet, it was totally replaced by an equivalent amount of solid fish oil. The diets were formulated to contain the same level of the main nutrients (metabolisable energy, CP, digestive lysine, and ether extract) (Table 1) except for the FA composition (Table 2). Feed intake was restricted to a maximum of 3 kg/day during gestation, and gradually increased after farrowing until reaching ad libitum feed intake.

***Table 1:*** *Ingredient and nutrient composition of the gestation and lactation sow diets and piglets creep feed (as fed basis).*

| | Gestation | | Lactation | | Creep feed | |
|---|---|---|---|---|---|---|
| | Control | n-3 LCFA | Control | n-3 LCFA | Control | n-3 LCFA |
| Ingredient (g/kg) | | | | | | |
| Barley | 443 | 435 | - | - | 226 | 220 |
| Corn | 200 | 200 | 508 | 499 | 314 | 315 |
| Sunflower seed 37% | 100 | 100 | 40.0 | 40.0 | - | - |
| Soybean hulls | - | - | 82.3 | 84.3 | - | - |
| Wheat middlings | 80.0 | 80.0 | - | - | - | - |
| Sugar-beet pulp | 80.0 | 80.0 | 60.0 | 60.0 | - | - |
| Soybean 48% | 40.6 | 42.7 | 240 | 241 | 150 | 150 |
| Whey, sweet, skim (dehydrated) | - | - | - | - | 110 | 110 |
| Dicalcium phosphate | 15.4 | 15.4 | 19.2 | 19.3 | 18.2 | 18.2 |
| Animal fat (5 Sysfeed)[1] | 15.0 | - | 30.0 | 15.0 | 30.0 | - |
| Fish oil (Lipomega®)[2] | - | 21.5 | - | 21.5 | - | 48.6 |
| L-lysine HCL | 1.30 | 1.30 | 1.37 | 1.34 | 5.50 | 5.50 |
| L-threonine | 0.20 | 0.20 | 0.46 | 0.45 | 2.50 | 2.50 |
| DL-methionine | - | - | 0.24 | 0.24 | 2.70 | 2.70 |
| L-tryptophan | - | - | 0.17 | 0.17 | 0.80 | 0.80 |
| L-Valine | - | - | - | - | 1.30 | 1.30 |
| Calcium carbonate | 10.5 | 10.4 | 6.92 | 6.83 | 2.00 | 2.70 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Sodium bicarbonate | 8.50 | 8.50 | - | - | 4.50 | 4.50 |
| Sodium chloride | 0.80 | 0.80 | 4.63 | 4.62 | 0.80 | 0.80 |
| Sodium caseinate | - | - | - | - | 20.0 | 20.0 |
| Celite | - | - | - | - | 15.0 | - |
| HP 300[3] | - | - | - | - | 88.8 | 89.3 |
| Vitamin-Mineral premix[4,5] | 4.00 | 4.00 | 4.00 | 4.00 | 6.00 | 6.00 |
| Antioxidant (Noxyfeed 56P)[6] | 0.30 | 0.30 | 2.50 | 2.50 | 2.50 | 2.50 |
| Analysed nutrient composition[7] (g/kg) | | | | | | |
| ME (MJ/kg) | 12.4 | 12.5 | 13.7 | 13.6 | 14.1 | 13.8 |
| Dry matter | 903 | 905 | 887 | 888 | 875 | 876 |
| Crude fibre | 57.9 | 60.4 | 54.3 | 54.5 | 19.0 | 18.7 |
| Ether extract | 38.6 | 37.2 | 56.7 | 59.2 | 49.8 | 52.4 |
| Crude protein | 131 | 132 | 182 | 179 | 204 | 205 |
| Lysine | 5.60 | 5.60 | 9.20 | 9.20 | 1.33 | 1.33 |

LCFA, long chain fatty acid; ME, metabolizable energy.

[1]Product of Sysfeed SLU (Granollers, Spain). It contains myristic acid (C14:0) 1.50%, palmitic acid (C16:0) 18.0%, palmitoleic acid (C16:1 n-7) 2.00%, stearic acid (C18:0) 14.0%, oleic acid (C18:1 n-9 cis) 28.0%, linoleic acid (C18:2 n-6 cis) 12.0%, α-linolenic acid (C18:3 n-3 cis) 6.00%, saturated-unsaturated 0.7%.

[2]Product of V&S Asociados (Madrid, Spain). It contains 63.36% of fat, myristic acid (C14:0) 4.79%, palmitic acid (C16:0) 14.9%, stearic acid (C18:0) 3.77%, oleic acid (C18:1 n-9 cis) 12.3%, linoleic acid (C18:2 n-6 cis) 2.71%, α-linolenic acid (C18:3 n-3 cis) 1.21%, arachidonic acid (C20:4 n-6 cis) 0.75%, eicosapentaenoic acid (C20:5 n-3 cis) 7.92%, docosahexaenoic acid (C22:6 n-3 cis) 6.91% and 36.64% of the inert excipient Tixosil® silica (Solvay, Brussels, Belgium).

[3]Product of Hamlet Protein (Horsens, Denmark). It contains 56.0% of protein, 23.2% of carbohydrates, 8.0% of H2O, 6.8% of ash, 3.5% of crude fibre and 2.5% of fat. Essential amino acids (g/16g of N): lysine 6.1, methionine 1.3, cysteine 1.4, threonine 3.9, tryptophan 1.35, leucine 7.7, isoleucine 4.6, phenylalanine 5.0, tyrosine 3.7, valine 4.8, histidine 2.6 and arginine 7.2.

[4]Vitamin-Mineral premix (sows): Product of TecnoVit S.L. (Alforja, Spain). Supplied per kilogram of feed: vitamin A (E-672) 10000 UI; vitamin D3 (E-671) 1600 UI; vitamin E

(alfa-tocopherol) 15 mg; vitamin B1 1 mg; vitamin B2 2.7 mg; vitamin B6 1.8 mg; vitamin B12 15 µg; vitamin K3 1 mg; calcium panthotenate 11 mg; nicotinic acid 15 mg; folic acid 1 mg; biotin 100 µg; choline 200 mg; Fe (E-1) (from FeSO4·7H2O) 150 mg; I (E-2) (from Ca(IO3)2) 0.5 mg; Co (E-3) (from 2CoCO3·3Co(OH)2·H2O) 0.5 mg; Cu ( E-4) (from CuSO4·5H2O) 10 mg; Mn (E-5) (from MnO) 40 mg; Zn (E-6) (from ZnO) 100 mg; Se (E-8) (from Na2SeO3) 0.25 mg.

[5]Vitamin-Mineral premix (piglets): Product of TecnoVit S.L. (Alforja, Spain). Supplied per kilogram of feed: vitamin A (E-672) 10000 UI; vitamin D3 (E-671) 2000 UI; vitamin E (alfa-tocopherol) 25 mg; vitamin B1 1.5 mg; vitamin B2 3.5 mg; vitamin B6 2.4 mg; vitamin B12 20 µg; vitamin K3 1.5 mg; calcium panthotenate 14 mg; nicotinic acid 20 mg; folic acid 0.5 mg; biotin 50 µg; Fe (E-1) (from FeSO4·H2O) 120 mg; I (E-2) (from Ca(IO3)2) 0.75 mg; Co (E-3) (from 2CoCO3·3Co(OH)2·H2O) 0.6 mg; Cu ( E-4) (from CuSO4·5H2O) 6 mg; Mn (E-5) (from MnO) 60 mg; Zn (E-6) (from ZnO) 110 mg; Se (E-8) (from Na2SeO3) 0.37 mg.

[6]Product of Itpsa (Barcelona, Spain). It contains 56% of antioxidants substances (butylated hydroxytoluene + propyl gallate) and synergistic (Citric acid 14% + authorized support).

[7]Nutrient composition values correspond to the analysed values except for ME and lysine which were estimated according to INRA tables (Sauvant *et al*., 2004).

***Table 2:*** *Fatty acid composition of the gestation and lactation sow diets and piglets creep feed.*[1]

| | Gestation | | Lactation | | Creep feed | |
|---|---|---|---|---|---|---|
| | Control | n-3 LCFA | Control | n-3 LCFA | Control | n-3 LCFA |
| Fat (g/kg feed) | 35.3 | 34.6 | 54.4 | 55.0 | 45.3 | 52.1 |
| Fatty Acids (mg FA/g fat) | | | | | | |
| C14:0 | 4.23 | 16.8 | 5.12 | 12.8 | 8.34 | 26.1 |
| C15:0 | 0.55 | 1.61 | 0.38 | 1.05 | 0.63 | 2.10 |
| C15:1 | ND | ND | ND | ND | ND | ND |
| C16:0 | 146 | 130 | 136 | 127 | 143 | 130 |
| C16:1 | 11.4 | 18.7 | 11.2 | 17.4 | 11.7 | 26.5 |

| | | | | | | |
|---|---|---|---|---|---|---|
| C17:0 | 1.15 | 2.50 | 1.52 | 2.29 | 1.60 | 3.17 |
| C18:0 | 37.9 | 22.3 | 47.7 | 39.1 | 47.8 | 30.1 |
| C18:1 n-7 | 10.6 | 13.4 | 13.5 | 14.8 | 13.4 | 17.1 |
| C18:1 n-9 *cis* | 200 | 130 | 242 | 199 | 228 | 139 |
| C18:1 n-9 *trans* | 0.70 | 0.36 | 1.00 | 0.64 | 1.29 | 0.66 |
| C18:1 n-11 *cis* | 0.13 | 0.37 | 0.40 | 0.88 | 0.71 | 1.41 |
| C18:2 n-6 *cis* | 293 | 256 | 237 | 214 | 214 | 167 |
| C18:3 n-3 *cis* | 20.1 | 21.3 | 14.5 | 15.3 | 15.1 | 17.2 |
| C18:4 n-3 | ND | 0.56 | ND | 0.42 | ND | 0.86 |
| C20:1 n-9 *cis* | 4.18 | 8.00 | 3.90 | 5.85 | 13.4 | 17.1 |
| C20:2 n-6 *cis* | 1.74 | 0.98 | 2.33 | 1.63 | 2.58 | 1.04 |
| C20:3 n-3 *cis* | 0.23 | 0.33 | 0.40 | 0.55 | 0.49 | 0.56 |
| C20:4 n-6 | 0.92 | 2.64 | 1.54 | 2.47 | 1.76 | 3.85 |
| C20:5 n-3 | ND | 26.7 | ND | 18.8 | ND | 41.3 |
| C22:4 n-6 | ND | 1.60 | ND | 1.23 | ND | 1.89 |
| C22:5 n-3 | 0.49 | 3.49 | 0.63 | 2.24 | 0.56 | 5.77 |
| C22:6 n-3 | 0.49 | 23.2 | 0.49 | 16.5 | 0.53 | 36.0 |
| C23:0 | 0.48 | 0.48 | 0.54 | 0.38 | 0.51 | 1.80 |
| C24:0 | 1.56 | 1.50 | 1.40 | 1.33 | 1.11 | 1.20 |
| C24:1 n-9 *cis* | 0.76 | 1.44 | 0.50 | 0.98 | 4.31 | 8.50 |
| Minor FA[2] | 5.28 | 8.46 | 5.36 | 6.67 | 6.61 | 10.6 |
| SFA | 197 | 182 | 197 | 192 | 209 | 203 |
| MUFA | 227 | 173 | 272 | 243 | 260 | 196 |
| PUFA | 317 | 338 | 258 | 277 | 236 | 276 |
| n-3 | 21.3 | 75.7 | 16.0 | 54.0 | 16.7 | 102 |
| n-6 | 296 | 263 | 242 | 223 | 219 | 175 |
| n-6:n-3 | 13.9 | 3.47 | 15.1 | 4.13 | 13.2 | 1.72 |

FA, fatty acid; LCFA, long chain fatty acid; MUFA, monounsaturated fatty acid; ND, non-detected; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.

[1]FA quantification results are reported from C12:0.

[2]Minor FAs include: C12:0, C14:1 n-9 *cis*, C18:2 n-6 *trans*, C18:3 n-6 *cis*, C19:0, C20:0, C20:3 n-6, C21:0, C22:0, C22:1, C22:2 n-6 *cis*, and C22:3 n-3.

*Growth measurements and sampling*

Sows were weighed at insemination, at entering the farrowing unit (day 107 of gestation), the day after farrowing and at weaning. Daily feed intake was monitored individually throughout the study and recorded for each period (gestation and lactation). Average daily gain during gestation was calculated from insemination to day 107 of gestation, and average daily gain during lactation from the day after farrowing to weaning. The number of piglets at birth, piglets born alive/death, mummies and their individual weight were monitored at birth. For lactation, piglets were weighted at 24 h, 20 days after birth and at weaning. All adoptions were completed within 24 h after birth, and the 24 h recordings were considered as the initial values for the litter characteristics and growth performance of suckling piglets. Thus, litter and piglet's average daily gain were calculated between 24 h and 20 days after birth or weaning. Creep feed disappearance was monitored from day eleven after birth until weaning.

Colostrum samples from each sow were obtained immediately after birth of the first piglet, and milk samples were collected at the end of lactation after the piglet's removal. Sows were milked from all mammary glands after i.v. injection of 1.0 mL of oxytocin (20 IU/mL) (Super's Diana S.L., Parets del Vallès, Spain). Samples from different nipples in each sow were pooled and aliquots for FA, oxylipins (with tubs containing 0.005% butylated hydroxytoluene (Merck, Darmstadt, Germany) as antioxidant), Ig and cytokines were stored at 80 C until analysis.

One sow offered n-3 LCFA diet farrowed out of the scheduled time, without supervision, and litter characteristics at birth and colostrum sampling were not possible. Moreover, two sows from the same n-3 LCFA group gave birth to less than six piglets and were excluded for the analysis of litter characteristics at birth.

*Quantitative analysis of fatty acids*

Fat was extracted from all colostrum and milk samples with chloroform (PanReac AppliChem, Barcelona, Spain) – methanol (Honeywell, Charlotte, NC, USA) according to Folch *et al*. (1957) and transmethylated with boron trifluoride (Sigma Aldrich, St. Louis, MO, USA) and potassium hydroxide 0.5 M (PanReac, Barcelona, Spain) in methanol according to Morrison and Smith (1964). Fatty acids were determined by gas chromatography (Agilent 6890N, Boston, MA, USA) using a capillary column (0.25 mm x 0.25 µm x 30 m; DB23, Agilent, Bellefonte, PA, USA) and a flame ionisation detector. A temperature gradient with an initial temperature of 170 ºC increased at a rate of 2.5 C/min until 210 ºC followed by another increase at a rate of 5 ºC/min to 240 ºC, where it remained for 5 min. Injector and detector temperatures were set at 250 ºC. Injection was in split mode at a ratio of 100.6:1. The carrier gas was helium with a flux of 55.8 mL/min at the column head (Tous *et al*., 2014). Internal standards were not used due to the presence of C17:0 and C19:0 in the analysed samples.

The external standards used were fatty acid methyl esters (FAMEs) Mix C4-C24 (Supelco, Bellefonte, PA, USA), Nonadecanoic acid methyl ester (Sigma Aldrich, St. Louis, MO, USA), cis-7,10,13,16- Docosatetranoic acid methyl ester (Sigma Aldrich, St. Louis, MO, USA), cis-4,7,10,13,16,19-Docosahexaenoic acid (Sigma Aldrich, St. Louis, MO, USA) and methyl all-cis-5,8,11,14,17-eicosapentaeo nate (Sigma Aldrich, St. Louis, MO, USA). Colostrum and milk FA contents were quantified from C12:0 and expressed as mg of FA per g of extracted fat.

*Metabolomic analysis of oxylipins*

Three colostrum and three milk samples from each treatment were chosen at random, and sixty-five oxylipins were quantified. Aliquots of 1 mL of colostrum or milk were mixed with 0.1 mL of internal standard mixture prepared in methanol (butylated hydroxytoluene 0.001 M). A volume of 0.9 mL of methanol (butylated hydroxytoluene 0.001 M) was added to the samples and incubated 30 min at 20

ºC. Samples were centrifuged, and the supernatant was recovered and diluted with 10 mL of a 0.1% of formic acid (Merck, Darmstadt, Germany) in Milli-Q water (Millipore, Burlington, MA, USA). A clean-up was applied using Oasis PRIME HLB cartridges (1 cc Vac Cartridge, 30 mg sorbent; Waters Corporation, Milford, MA, USA) eluting twice with 0.6 mL of acetonitrile (Merck, Darmstadt, Germany): methanol (9:1, v/v). The elute was evaporated to dryness under a stream of nitrogen and reconstituted in 0.1 mL of Milli-Q water:methanol (1:1, v/v) (Ostermann, 2017).

An Ultra HPLC 1290 Series coupled to a triple quadrupole mass spectrometer 6490 series instrument (Agilent, Santa Clara, CA, USA) with an analytical column Eclipse XDB C18 1.8 µL (2.1 x 100 mm) (Agilent, Santa Clara, CA, USA) was used to analyse the extracts. The chromatographic separation was performed with a gradient elution using Milli-Q water (0.01% acetic acid (Merck, Darmstadt, Germany)) and acetonitrile:methanol (85:15, v/v) as a mobile phase at a flow rate of 0.4 mL/min and 45 C. The injection volume was 10 µL (4 ºC). The electrospray source ionisation was in negative mode, and the acquisition was performed in dynamic Multiple Reaction Monitoring.

Identification of oxylipins with the corresponding standard was limited because these standards are not commercially available. Therefore, for tentative identification, published data about chromatographic behaviour on C18 columns together with mass spectrometry confirmed oxylipins providing the molecular ion [M–H] and fragmentation patterns using electrospray source ionisation in negative mode were used (Astarita *et al*., 2015; Zhang *et al*., 2015; Ostermann, 2017). Also published data in relation to the main oxylipin in the studied matrices were used for identification purposes (Bruins *et al*., 2013; Mavangira *et al*., 2015). The linear calibration curves used to quantify oxylipins were constructed from available commercial standards using internal standard correction. Internal standard was selected based on chromatographic behaviour criteria. For the compounds with non-available commercial standard, a calibration curve of similar compound was used to perform a tentative identification (Serhan *et al*., 2006; Isobe, *et al*., 2012). The available oxylipin analytical standards used were SPM D-series LC-MS

Mixture, Lipoxin LC-MS Mixture, EPA Oxylipin LC-MS Mixture, Primary COX and LOX LC-MS Mixture, Leukotriene B4 Pathway LC-MS Mixture, Linoleic Acid Oxylipins LC-MS Mixture, SPM E-series LC-MS Mixture, ALA and GLA Oxylipin LC-MS Mixture, 10(s),17(s)-DiHDHA, 20-HETE, (±)11(12)-DiHET and 8-iso Prostaglandin F2a (Cayman chemicals, Ann Arbor, MI, USA). The stable isotope labelled standards were Resolvin D1-d5, Lipoxin A4-d5, Deuterated Linoleic Acid Oxylipins LC-MS Mixture, Deuterated Primary COX and LOX LC-MS Mixture, Leukotriene B4-d4 (Cayman chemicals, Ann Arbor, MI, USA).

*Immune indicators*

Different sandwich ELISA kits were employed for the quantitative measurement of Ig and cytokines in all colostrum and milk samples according to the manufacturer's instructions. IgG, IgA and IgM were analysed through Pig IgG ELISA Kit (E101-104; Bethyl Laboratories, Montgomery, Tx, USA), Pig IgA ELISA Kit (ab190536; Abcam, Cambridge, UK) and Pig IgM ELISA Kit (ab190537; Abcam, Cambridge, UK). The cytokine interleukin 1β (**IL1β**), interleukin 6 (**IL6**), interleukin 10 (**IL10**) and tumour necrosis factor α (**TNFα**) were quantified through Pig IL-1β ELISA Kit (ab100754; Abcam, Cambridge, UK), Pig IL-6 ELISA Kit (ab100755; Abcam, Cambridge, UK), Swine IL-10 ELISA Kit (KSC0101/KSC0102; Invitrogen, Carlsbad, CA, USA) and Pig TNF-α ELISA Kit (ab100756; Abcam, Cambridge, UK), respectively. In all kits, the colorimetric reaction was performed with a specific biotinylated secondary antibody and the addition of a streptavidin-conjugated horseradish peroxidase that catalyses the chromogenic substrate 3,30,5,50-tetramethylbenzidine. The absorbance was measured at 450 nm. The sample concentrations were determined by comparing the optical density of the samples to a standard curve. Precision values of all kits are given in Supplementary Table S1.

*Statistical analysis*

The MIXED procedure of SAS software (SAS/STAT 14.1; SAS Institute Inc., Cary, NC, USA) was used to perform the analysis of variance of the different continuous variables and the GLIMMIX procedure of SAS software was used for discrete variables (number of piglets born, number of piglets alive/death or mummies). The model included dietary treatment as fixed effect and batch as random effect. Sow BW at the beginning of the experiment and parity were initially introduced in the model as covariates. However, parity had no significant effect and it was removed from the statistical analysis. For all data at weaning, the variable days of lactation was included in the model as covariate. A square root transformation ($\sqrt{X + 0.5}$) of stillborn piglets, deaths and mummies was performed to normalise the data, but least squares means of the original data are presented in tables. Similarly, a logarithmic transformation ($\log_{10}(X + 1)$) of the oxylipin concentration values was performed and means of original data are presented in supplementary tables. When the limit of detection was not reached, the missing values were replaced by 1/5 of the minimum positive value of each variable. Data suspected to be outliers were tested using Kolmogrov-Smirnov test, and values were excluded if $P < 0.01$. Results were expressed as least squares means ± RMSE, except oxylipins that were expressed as means ± RMSE. Differences were considered significant at $P < 0.05$, while those at $P < 0.1$ are reported as tendencies.

MetaboAnalyst 5.0 (http://www.metaboanalyst.ca, Alberta, CA, USA) was used to perform principal component analysis of FA and oxylipins and the heatmap of oxylipins.

## Results

*Sow's weight and feed intake*

The growth and feed intake of sows are presented in Table 3. Sow's BW at insemination ($P = 0.159$), 107 days of gestation ($P = 0.314$) and at weaning ($P =$

0.830), and the average daily gain during gestation ($P$ = 0.315) and lactation ($P$ = 0.559) did not differ between treatments. While sows fed with control diet gained 66. 8 ± 2.8 kg during gestation and lost 19.5 ± 3.1 kg during lactation, sows fed with n-3 LCFA diet gained 63.2 ± 2.7 kg during gestation and lost 20.4 ± 3.2 kg during lactation. Finally, average daily feed intake also did not differ between treatments during gestation ($P$ = 0.694) or lactation ($P$ = 0.621).

***Table 3:*** *Effect of dietary fish oil on the growth and feed intake of gestating and lactating sows.*[1]

| | Control (n=18) | n-3 LCFA (n=18) | RMSE | *P* value |
|---|---|---|---|---|
| Days of gestation | 116 | 116 | 1.05 | 0.612 |
| Days of lactation | 26.8 | 27.0 | 1.02 | 0.681 |
| Average BW (kg) | | | | |
| Insemination | 213 | 225 | 24.7 | 0.159 |
| End of gestation | 286 | 282 | 10.2 | 0.314 |
| Day after farrowing | 264 | 265 | 10.2 | 0.703 |
| At weaning | 245 | 246 | 13.5 | 0.830 |
| Average daily gain (kg) | | | | |
| Gestation | 0.57 | 0.54 | 0.09 | 0.315 |
| Lactation | -0.79 | -0.89 | 0.47 | 0.559 |
| Total | 0.19 | 0.19 | 0.10 | 0.835 |
| Average daily feed intake (kg) | | | | |
| Gestation | 2.78 | 2.78 | 0.05 | 0.694 |
| Lactation | 5.59 | 5.45 | 0.81 | 0.621 |

LCFA, long chain fatty acid.

[1]Values are least squares means ± RMSE.

*Litter characteristics and piglet's weight and feed intake during lactation*

The number of piglets born, piglets born alive/death, mummies, and piglet's weight and feed intake are presented in Table 4. Litter characteristics at birth did not show

any significant difference between treatments, and only a tendency to reduce mummies in n-3 LCFA sows was observed (tendency at $P = 0.093$). At 24 h after birth, the average number of piglets still alive per litter decreased in the n-3 LCFA group ($P = 0.003$) and this difference was maintained at day 20 of lactation and at weaning (tendency at $P = 0.052$ and $P = 0.093$, respectively). In terms of the average litter weight, no difference was observed between treatments. However, the average of individual piglet's BW at day 20 after birth ($P = 0.013$) and at weaning (tendency at $P = 0.058$), and piglet's average daily gain between 24 h and day 20 after birth ($P = 0.010$) and between 24 h and weaning (tendency at $P = 0.072$) were increased in piglets from n-3 LCFA sows compared to piglets from control sows. Piglet creep feed disappearance did not differ between treatments ($P = 0.471$).

***Table 4:*** *Effect of dietary fish oil on the litter characteristics and growth performance of suckling piglets.*[1]

| | Control (n=18) | n-3 LCFA (n=16) | RMSE | *P* value |
|---|---|---|---|---|
| At birth[2] | | | | |
| Average total born | 15.2 | 14.3 | 3.92 | 0.533 |
| Born alive | 14.8 | 13.8 | 3.67 | 0.442 |
| Stillborn | 0.30 | 0.46 | 0.28 | 0.468 |
| Mummies | 0.41 | 0.10 | 0.24 | 0.093 |
| Average litter weight (kg) | 19.6 | 18.9 | 3.63 | 0.596 |
| Average piglet BW (kg) | 1.37 | 1.37 | 0.24 | 0.953 |
| SD piglet BW (kg) | 0.25 | 0.26 | 0.06 | 0.716 |
| 24h after birth[3] | | | | |
| Average still alive | 13.5 | 11.8 | 1.54 | 0.003 |
| Average of deaths 24h | 0.39 | 0.90 | 0.41 | 0.189 |
| Average litter weight (kg) | 19.5 | 18.2 | 2.78 | 0.207 |
| Average piglet BW (kg) | 1.42 | 1.54 | 0.23 | 0.161 |
| SD piglet BW (kg) | 0.31 | 0.32 | 0.09 | 0.574 |
| 20 days after birth | | | | |
| Average still alive | 11.7 | 10.9 | 1.13 | 0.052 |

| | | | | |
|---|---|---|---|---|
| Average litter weight (kg) | 71.4 | 72.7 | 8.41 | 0.669 |
| Litter ADG (24h → 20d) (kg) | 2.57 | 2.68 | 0.35 | 0.392 |
| Average piglet BW (kg) | 6.06 | 6.68 | 0.68 | 0.013 |
| SD piglet BW (kg) | 1.22 | 1.19 | 0.38 | 0.797 |
| Piglet ADG (24h → 20d) (kg) | 0.24 | 0.27 | 0.03 | 0.010 |
| At weaning | | | | |
| Average still alive | 11.6 | 10.9 | 1.17 | 0.093 |
| Average of deaths lactation | 2.37 | 1.80 | 0.58 | 0.420 |
| Average litter weight (kg) | 92.5 | 93.5 | 10.2 | 0.794 |
| Litter ADG (24h → W) (kg) | 2.77 | 2.85 | 0.33 | 0.472 |
| Average piglet BW (kg) | 7.91 | 8.50 | 0.87 | 0.058 |
| SD piglet BW (kg) | 1.48 | 1.46 | 0.47 | 0.913 |
| Piglet ADG (24h → W) (kg) | 0.25 | 0.27 | 0.03 | 0.072 |
| Piglet creep feed intake (kg) | 0.29 | 0.31 | 0.07 | 0.471 |

ADG, average daily gain; LCFA, long chain fatty acid; W, weaning.

[1]Values are least squares means ± RMSE.

[2]One sow from n-3 LCFA diet farrowed out of the scheduled time, without supervision, and litter characteristics at birth was not possible. Two sows from n-3 LCFA diet gave birth less than 6 piglets and were excluded for the data analysis of litter characteristics at birth.

[3]Adoptions were completed within 24 hours after birth and the 24 h recordings were considered as the initial values for the litter characteristics and growth performance of suckling piglets.

*Fatty acid profile*

*The changes caused by sow diet's* fat source on colostrum and milk FA profile are shown in Table 5. In colostrum, the fish oil did not change the fat content compared to control diet. In terms of FAs, no changes by dietary treatment were observed in total saturated FAs or monounsaturated FAs. However, some saturated FAs such as C14:0 ($P < 0.001$), C15:0 ($P < 0.001$), C17:0 ($P = 0.049$), C23:0 ($P < 0.001$), and

C24:0 ($P$ = 0.040), and some monounsaturated FAs such as C15:1 ($P$ < 0.001), C16:1 ($P$ = 0.014) and C18:1 n-11 cis ($P$ = 0.034) were increased and only a reduction of the monounsaturated FA C18:1 n-9 trans ($P$ < 0.001) was observed in n-3 LCFA-treated sows. In reference to polyunsaturated FAs, an increase in total n-3 FAs ($P$ < 0.001), α-linolenic acid (C18:3 n-3 cis) ($P$ = 0.003), stearidonic acid (C18:4 n-3) ($P$ < 0.001), eicosatrienoic acid (C20:3 n-3 cis) ($P$ < 0.001), EPA ($P$ < 0.001), docosapentaenoic acid (C22:5 n-3) ($P$ < 0.001), and DHA ($P$ < 0.001) was observed in n-3 LCFA-treated sows compared to control. In contrast, the n-6 family was barely affected by dietary treatment since only a reduction of arachidonic acid (C20:4 n-6) was observed in fish oil-treated sows ($P$ = 0.003). Consequently, total polyunsaturated FAs ($P$ = 0.020) were increased and the n-6:n-3 ratio was significantly reduced ($P$ < 0.001) in colostrum from n-3 LCFA fed sows.

In milk, fat content, total saturated FAs and monounsaturated FAs were not affected by dietary treatments, and only trends to increase C15:0 ($P$ = 0.065) and C23:0 ($P$ = 0.061), an increase of C15:1 ($P$ < 0.001) and C24:1 n-9 ($P$ = 0.050) and a reduction of C18:1 n-9 trans ($P$ = 0.005) were observed in samples from the n- 3 LCFA sows. In addition, sows fed the n-3 LCFA diet had a higher concentration of n-3 polyunsaturated FAs ($P$ < 0.001) mainly due to increases in stearidonic acid ($P$ < 0.001), EPA ($P$ < 0.001), docosapentaenoic acid ($P$ = 0.002), and DHA ($P$ < 0.001). The n-6 FAs were not changed and only arachidonic acid concentration was reduced ($P$ = 0.019) and the adrenic acid (C22:4 n-6) ($P$ = 0.027) concentration increased by dietary fish oil, which resulted in a reduction of the n-6:n-3 ratio ($P$ < 0.001) without an increase of total polyunsaturated FAs.

***Table 5:*** *Colostrum and milk fatty acid profile from sows fed control or n-3 LCFA diet.*[1,2]

| | Colostrum | | | | Milk | | | |
|---|---|---|---|---|---|---|---|---|
| | Control (n=18) | n-3 LCFA (n=17) | RMSE | *P* value | Control (n=18) | n-3 LCFA (n=18) | RMSE | *P* value |
| Fat (g/kg sample) | 64.2 | 56.6 | 18.8 | 0.244 | 73.8 | 71.1 | 19.0 | 0.672 |
| Fatty acid (mg FA/g fat) | | | | | | | | |
| C14:0 | 8.42 | 13.1 | 3.44 | <0.001 | 18.1 | 19.3 | 5.63 | 0.545 |
| C15:0 | 0.71 | 1.14 | 0.33 | <0.001 | 0.44 | 0.57 | 0.21 | 0.065 |
| C15:1 | 0.35 | 0.57 | 0.13 | <0.001 | 0.17 | 0.33 | 0.11 | <0.001 |
| C16:0 | 135 | 153 | 40.3 | 0.212 | 152 | 152 | 45.5 | 0.970 |
| C16:1 | 15.9 | 20.4 | 4.96 | 0.014 | 47.1 | 49.0 | 17.6 | 0.758 |
| C17:0 | 1.75 | 2.21 | 0.64 | 0.049 | 0.87 | 0.94 | 0.33 | 0.506 |
| C18:0 | 35.4 | 39.2 | 12.1 | 0.369 | 21.0 | 19.8 | 6.70 | 0.588 |
| C18:1 n-7 | 15.7 | 17.9 | 4.89 | 0.196 | 12.2 | 12.5 | 4.45 | 0.843 |
| C18:1 n-9 *cis* | 181 | 188 | 70.0 | 0.741 | 186 | 178 | 63.0 | 0.723 |
| C18:1 n-9 *trans* | 1.26 | 0.76 | 0.39 | <0.001 | 1.16 | 0.75 | 0.40 | 0.005 |
| C18:1 n-11 *cis* | 0.87 | 1.24 | 0.48 | 0.034 | 0.84 | 0.97 | 0.27 | 0.175 |
| C18:2 n-6 *cis* | 132 | 148 | 41.2 | 0.282 | 76.2 | 74.5 | 24.4 | 0.841 |
| C18:3 n-3 *cis* | 6.96 | 9.79 | 2.57 | 0.003 | 3.91 | 4.32 | 1.53 | 0.427 |
| C18:4 n-3 | 0.05 | 0.90 | 0.55 | <0.001 | 0.03 | 0.38 | 0.10 | <0.001 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| C20:1 n-9 *cis* | 1.38 | 1.80 | 0.96 | 0.219 | 2.21 | 2.17 | 1.06 | 0.925 |
| C20:2 n-6 *cis* | 3.04 | 2.61 | 1.30 | 0.346 | 2.19 | 1.84 | 0.80 | 0.211 |
| C20:3 n-3 *cis* | 0.87 | 1.26 | 0.27 | <0.001 | 0.58 | 0.65 | 0.22 | 0.375 |
| C20:4 n-6 | 7.47 | 4.98 | 2.22 | 0.003 | 2.70 | 1.85 | 1.02 | 0.019 |
| C20:5 n-3 | 0.08 | 8.22 | 1.60 | <0.001 | 0.14 | 3.22 | 0.83 | <0.001 |
| C22:4 n-6 | 0.76 | 0.83 | 0.15 | 0.182 | 0.17 | 0.36 | 0.23 | 0.027 |
| C22:5 n-3 | 2.34 | 9.93 | 2.23 | <0.001 | 0.60 | 1.55 | 0.84 | 0.002 |
| C22:6 n-3 | 0.70 | 11.7 | 2.74 | <0.001 | 0.37 | 3.83 | 0.95 | <0.001 |
| C23:0 | 0.09 | 0.74 | 0.18 | <0.001 | 0.06 | 0.13 | 0.11 | 0.061 |
| C24:0 | 0.95 | 1.06 | 0.15 | 0.040 | 0.92 | 1.32 | 0.93 | 0.206 |
| C24:1 n-9 *cis* | 0.76 | 0.75 | 0.17 | 0.876 | 0.72 | 0.56 | 0.23 | 0.050 |
| Minor FA[3] | 6.59 | 6.56 | 1.67 | 0.958 | 6.35 | 6.30 | 1.74 | 0.932 |
| SFA | 184 | 212 | 56.0 | 0.163 | 197 | 196 | 57.4 | 0.991 |
| MUFA | 218 | 232 | 69.2 | 0.556 | 252 | 246 | 80.1 | 0.839 |
| PUFA | 159 | 202 | 51.5 | 0.020 | 88.6 | 94.3 | 29.1 | 0.569 |
| n-3 | 11.0 | 41.8 | 8.52 | <0.001 | 6.36 | 14.7 | 3.46 | <0.001 |
| n-6 | 148 | 161 | 44.3 | 0.406 | 83.2 | 83.8 | 26.9 | 0.940 |
| n-6:n-3 | 13.3 | 3.88 | 0.58 | <0.001 | 13.0 | 5.70 | 0.79 | <0.001 |

FA, fatty acid; LCFA, long chain fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.

[1]Values are least squares means ± RMSE.

[2]FA quantification results are reported from C12:0.

[3]Minor FAs include: C12:0, C13:0, C14:1 n-9 *cis*, C18:2 n-6 *trans*, C18:3 n-6 *cis*, C19:0, C20:0, C20:3 n-6, C21:0, C22:0, C22:1, C22:2 n-6 *cis*, and C22:3 n-3. C21:0 and C22:3 n-3 have not been detected in colostrum.

Principal component analysis allowed to observe a different distribution of the samples according to sample type (colostrum or milk) and diet (control or n-3 LCFA) (Figure 1, A). EPA was the FA with the highest contribution in explaining the principal component analysis distribution.

*Oxylipin profile*

In colostrum, most of the differences observed between treatments were for oxylipins derived from EPA or DHA, and their concentrations were increased when sows were fed the fish oil diet (Supplementary Table S2). The EPA-derived oxylipins that increased by the n-3 LCFA diet were 17,18-dihydroxy-EPA ($P = 0.004$); 5(s)-, 8-, 11-, 12(s)-, 15(s)- and 18-hydroxy-EPA ($P < 0.001$, $P = 0.002$, $P = 0.013$, $P = 0.030$, $P = 0.005$ and $P = 0.006$, respectively) and Resolvin E2/E3 (P = 0.002). In addition, the DHA-derived oxylipins increased by n-3 LCFA are mainly hydroxyl-DHA metabolites such as 10-, 16- and 20-hydroxy-DHA ($P < 0.001$, $P = 0.036$ and $P = 0.012$, respectively); 4,5-dihydroxy-docosapentaenoic acid ($P = 0.013$); Resolvin D5 ($P = 0.009$) and Protectin D1 (tendency at $P = 0.076$). Prostaglandin D2 (tendency at $P = 0.058$) derived from arachidonic acid was also increased when fish oil was included in the sow diet.

For the sows fed the n-3 LCFA diet, 24 oxylipin concentrations were increased ($P < 0.05$) and 6 tended to increase ($P < 0.1$) in milk (Supplementary Table S3). Among these, the EPA-derived oxylipins that increased were as follows: 8, 9-, 14,15-/11,15- and 17,18-dihyoxy-eicosatetraenoic acid ($P = 0.002$, $P = 0.003$ and $P = 0.006$, respectively); 5(s)-, 8-, 9-, 11-, 12(s)-, 15(s)- and 18-hydroxy-EPA ($P = 0.001$, $P = 0.005$, $P = 0.002$, $P = 0.003$, $P = 0.005$, $P = 0.007$ and $P = 0.003$, respectively) and Resolvin E2/3 ($P = 0.050$). The DHA-derived oxylipins that increased were as follows: 10(s),17 (s)-dihydroxy-DHA (also known as Neuroprotectin D1; tendency at $P = 0.069$); 4,5-dihydroxy-docosapentaenoic ($P = 0.002$); 4-, 8-, 10-, 11-, 13-, 14-, 16-, 17- and 20-hydroxy-DHA ($P < 0.001$, $P = 0.002$, $P < 0.001$, $P = 0.012$, $P < 0.001$, $P = 0.001$, $P = 0.002$, $P = 0.002$ and $P < 0.001$, respectively) and Protectin

D1/Protectin D1- (*P* = 0.016 and *P* = 0.024). In addition to the changes in EPA and DHA-derived oxylipins, 9(10)-epoxy-octadecadienoic acid (tendency at *P* = 0.064) and 9(s)-hydroxy-octadecatrienoic acid (tendency at *P* = 0.058), which are derived from a-linolenic acid, and 9(10)- and 12(13)-epoxy-octadecenoic acid (tendency at *P* = 0.056 and *P* = 0.038, respectively) and 9(s)-hydroxy-octadecadienoic acid (tendency at *P* = 0.054), which are derived from linoleic acid (C18:2 n-6) were also increased.

Even though the oxylipin profile of the n-3 LCFA sows is clearly differentiated in the 2D plots obtained through principal component analysis for both, colostrum and milk (Figure 1, B), these differences could not be explained by changes in any particular oxylipin, rather they were consequence of the overall changes observed in the whole oxylipin profile. Taken together, the colostrum and milk n-3 FA-derived oxylipins were strongly increased by dietary fish oil, and a higher number of oxylipins were modified in milk than in colostrum (Figure 2). Finally, a schematic overview of the oxylipins increased by the n-3 LCFA diet and their FA precursor is summarised in Supplementary Figure S1.

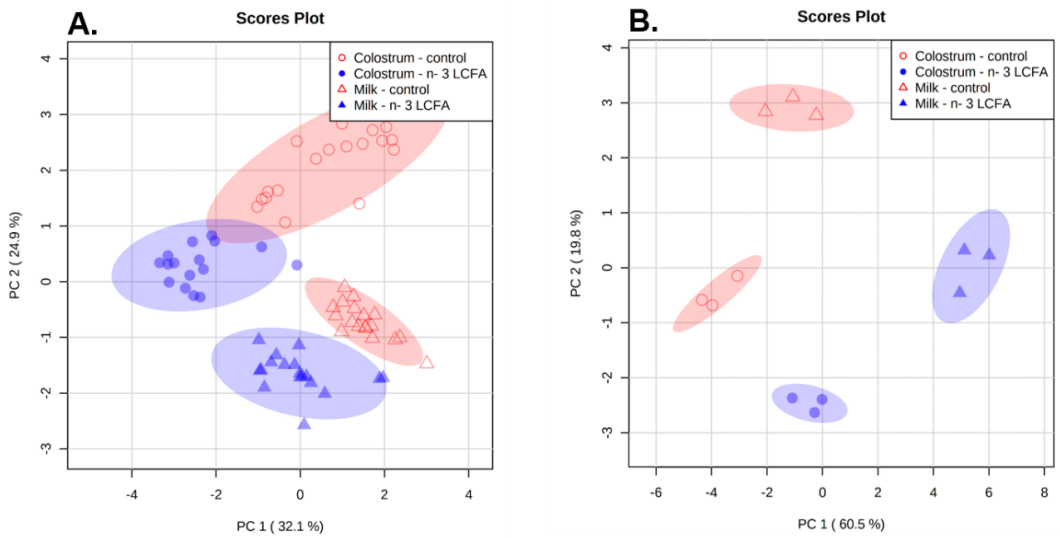


***Figure 1:*** *Fish oil source rich in eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in sow diets modifies fatty acid (FA) and oxylipins in colostrum and milk. Principal component analysis 2 dimensions score plot of FA profile (A) and oxylipins (B) of colostrum and milk samples. In terms of FA profile (A) values are means of 18 samples per treatment, except for colostrum n-3 Long Chain Fatty Acids (n-3 LCFA) diet (n = 16). In terms of oxylipins profile (B) values are means of 3 samples per treatment.*

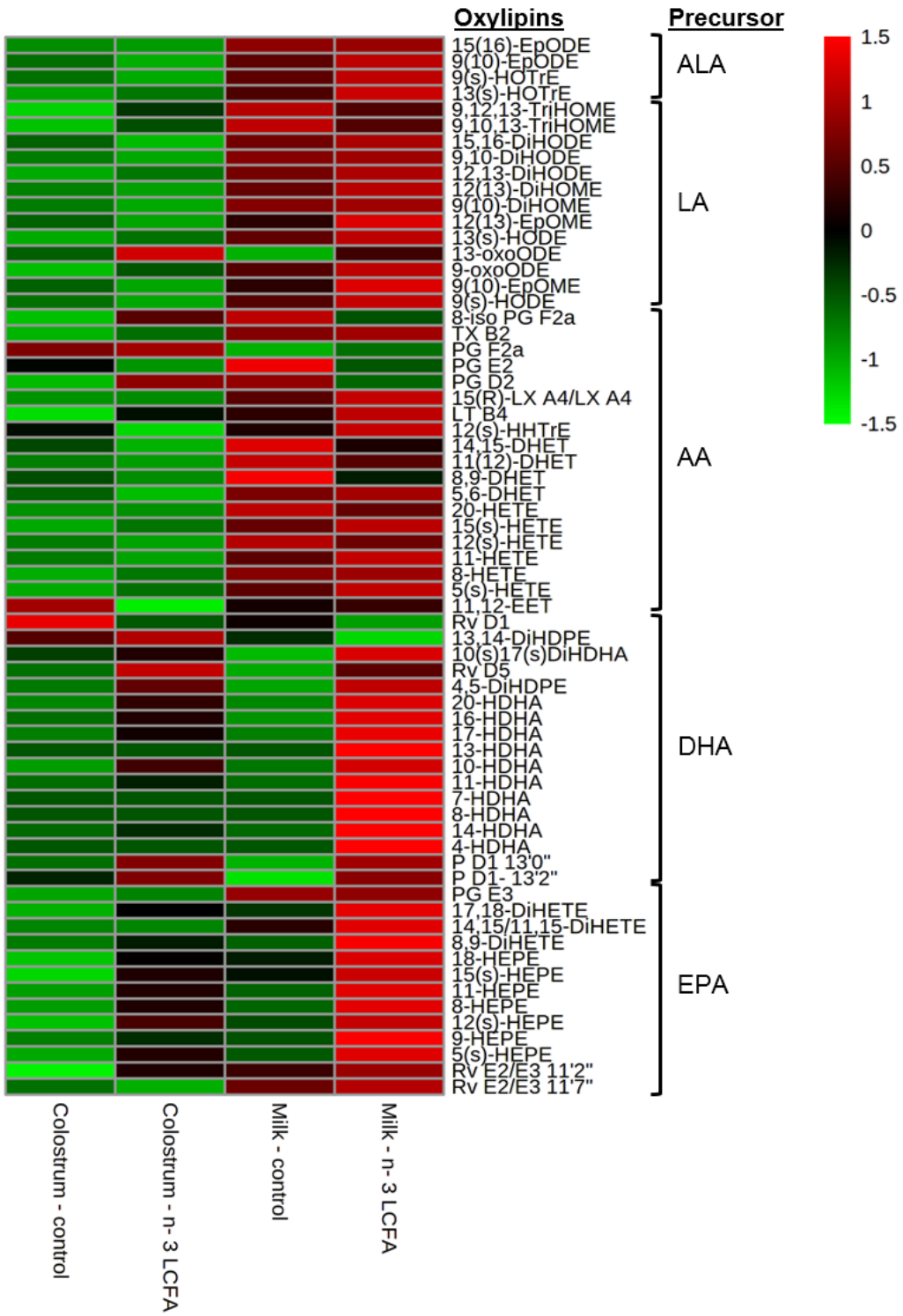


***Figure 2:*** *Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)-derived oxylipins are increased in colostrum and milk from fish oil-fed sows. Values are means of 3 samples per treatment. AA, arachidonic acid; ALA, α-linolenic acid; DiHDPE, dihydroxy-*

*docosapentaenoic acid; DiHDHA, dihydroxy-docosahexaenoic acid; DiHETE, dihydroxy-eicosatetraenoic acid; DiHODE, dihydroxy-octadecadienoic acid; DiHOME, dihydroxy-octadecenoic acid; DHET, dihydroxy-eicosatrienoic acid; EET, epoxy-eicosatrienoic acid; EpODE, epoxy-octadecadienoic acid; EpOME, epoxy-octadecenoic acid; HDHA, hydroxy-docosahexaenoic acid; HEPE, hydroxy-eicosapentaenoic acid; HETE, hydroxy-eicosatetraenoic acid; HHTrE, hydroxy-heptadecatrienoic acid; HODE, hydroxy-octadecadienoic acid; HOTrE, hydroxy-octadecatrienoic acid; LA, linoleic acid; LCFA, long chain fatty acid; LT, Leukotriene; oxoODE, oxo-octadecadienoic acid; PG, Prostaglandin; TX, Thromboxane; TriHOME, trihydroxy-octadecenoic acid.*

*Immunological analysis*

Colostrum and milk Ig were not significantly affected by dietary treatment (Table 6). IgG concentrations were higher in colostrum than in milk and ranged between 185–212 mg/mL and 0.649– 0.737 mg/mL, respectively. However, IgA and IgM concentrations were similar in both, colostrum and milk. IgA and IgM concentrations ranged between 2.01–4.48 mg/mL and 1.03–1.42 mg/mL in colostrum, and between 3.20–4.42 mg/mL and 1.54–1.55 mg/mL in milk, respectively.

Cytokine concentrations in colostrum were not affected by dietary treatment despite being larger than those of milk samples (Table 6). In milk, no changes were observed for IL1β and IL6. However, a trend for reduced IL10 (tendency at $P$ = 0.059) and a reduced TNFα ($P$ = 0.011) was observed in milk from sows fed the n-3 LCFA diet.

***Table 6**: Colostrum and milk immune indicator from sows fed control or n-3 LCFA diet.*[1]

| | Colostrum | | | | Milk | | | |
|---|---|---|---|---|---|---|---|---|
| | Control (n=18) | n-3 LCFA (n=17) | RMSE | *P* value | Control (n=18) | n-3 LCFA (n=18) | RMSE | *P* value |
| Immunoglobulins (mg/mL) | | | | | | | | |
| IgG | 186 | 149 | 141 | 0.461 | 0.65 | 0.74 | 0.39 | 0.504 |
| IgA | 4.48 | 2.01 | 7.12 | 0.316 | 3.20 | 4.42 | 2.21 | 0.111 |
| IgM | 1.42 | 1.03 | 1.22 | 0.354 | 1.54 | 1.55 | 0.66 | 0.966 |
| Cytokines (ng/mL) | | | | | | | | |
| IL1β | 25.1 | 31.1 | 13.0 | 0.197 | 6.12 | 7.09 | 8.25 | 0.729 |
| IL6 | 137 | 187 | 190 | 0.448 | 7.00 | 6.19 | 1.90 | 0.243 |
| IL10 | 3.70 | 11.4 | 20.0 | 0.298 | 0.10 | 0.09 | 0.01 | 0.059 |
| TNFα | 1.15 | 1.92 | 2.25 | 0.334 | 0.16 | 0.15 | <0.01 | 0.011 |

IgA, immunoglobulin A; IgG, Immunoglobulin G; IgM, immunoglobulin M; IL1β, interleukin 1β; IL6, interleukin 6; IL10, interleukin 10; TNFα, tumor necrosis factor α.

[1]Values are least squares means ± RMSE.

## Discussion

Colostrum and milk are the first energy and nutrient sources for newborn piglets, which have low energy reserves. In addition, they also play a key role in the transfer of passive immunity (Darragh and Moughan, 1998). For these reasons, colostrum and milk are essential for the preweaning growth of piglets. The reduction of litter size observed in this study is in line with the results reported by Rooke *et al*. (2001) feeding sows with salmon oil from day 60 of gestation. However, and as summarised in the revision of Tanghe and De Smet (2013), previous studies reported no effect of dietary n-3 polyunsaturated FAs on embryo number, development, size or survival, and the reason for the reduced litter size is still unclear. On the other hand, Rooke *et al*. (2001) and Laws *et al*. (2007) found that fish oil supplementation lowered piglet birth weight contrary to the increase of birth weight of piglets from treated litter observed in the current study. This could be

related to the fact that smaller litters in n-3 LCFA sows allow their piglets to have more access to colostrum and milk though litter weight was not different throughout lactation.

Piglets are able to digest more than 90% of the lipids present in colostrum and milk (Azain, 2001), thus becoming efficient vehicles to transfer FA (Lauridsen, 2020). Under commercial conditions, sow diets are composed of ingredients that contain considerable amounts of n-6 FAs, the fat source is rich in saturated FAs, and fish oil is not commonly used. Several studies have already observed an increased n-3 FAs concentration in colostrum and milk when an n- 3 rich source is used in the maternal diet (Rooke *et al*., 2001; Eastwood *et al*., 2014). In the current study, apart from EPA and DHA, increases for α-linolenic acid, stearidonic acid, eicosatrienoic acid and docosapentaenoic acid in colostrum and stearidonic acid and docosapentaenoic acid in milk were also observed when the sows were fed the fish oil diet, which is in contrast with previous studies that reported no differences for these FAs (Eastwood *et al*., 2014). Regarding the n-6 polyunsaturated FA family, we observed a decline of arachidonic acid concentration in both, milk and colostrum, when fish oil was added to the sow diet. These results confirm those of Eastwood *et al*. (2014) in colostrum. This decline in arachidonic acid concentration could be explained by the fact that an n-3 FA diet enrichment is typically accompanied by a decrease in the content of arachidonic acid in different cell types (Calder, 2010). In our study, total n-3 FA increased but saturated FAs, monounsaturated FAs and total n-6 FAs were not affected by dietary treatment in colostrum or milk. We also observed that the n-3 LCFA diet had more impact in colostrum than in milk since a higher number of FAs were altered and the magnitude of the changes was also larger. Regarding EPA and DHA, we observed concentrations of 0.006 mg and 0.045 mg per gram of colostrum in the control, and 0.450 mg and 0.629 mg in the n-3 LCFA samples, respectively. In milk, the concentrations ranged between 0.003 mg and 0.030 mg per gram in the control, and 0.225 mg and 0.265 mg per gram in the n-3 LCFA samples, respectively.

It is well established that polyunsaturated FAs can influence inflammatory processes through a variety of mechanisms, one of them being their oxidation by enzymatic or non-enzymatic pathways and the consequent formation of oxylipins (Calder, 2010). To our knowledge, the presence of these compounds has not been studied in colostrum or sow's milk. Fish oil in sow diets has an impact on FA profile and consequently on oxylipin profile. In addition, the detection of these lipid mediators in colostrum and milk implies a transfer of oxylipins from sow to piglet. In fact, it has been described that these oxylipins influence the coordination of a balanced inflammatory response and that each oxylipin possesses proinflammatory and/or anti-inflammatory functions (Gabbs *et al*., 2015), which could affect the immune status of piglets. Furthermore, it is known that n-6 polyunsaturated FA-derivate oxylipins tend to have proinflammatory activity whereas n-3 polyunsaturated FA-derivate oxylipins present a low proinflammatory potential or/and anti-inflammatory potential (Calder, 2010). The enhanced concentration of EPA and DHA in colostrum and milk of fish oil-fed sows resulted in increases of hydroxy-EPA or hydroxy-DHA that are directly derived from these LCFAs through the enzymatic pathway involving lipoxygenase (Astarita *et al*., 2015). Most of these intermediate hydroxy-FAs currently do not have a defined function except those described in specific murine or human cell lines by Gabbs *et al*. (2015). EPA and DHA also give rise to resolvins and related compounds as protectins through the lipoxygenase or cyclooxygenase pathways (Serhan *et al*., 2002; Serhan *et al*., 2008). In the current study, E-series Resolvin E2/3 and D-series Resolvin D5 were increased in the colostrum and milk samples from the n-3 LCFA diet fed sows. In addition, Protectin D1 and Neuroprotectin D1 in milk, and Protectin D1 in colostrum were also increased. The biological effects of resolvins and protectins have been widely examined in several cell cultures and animal models of inflammation, and they have been shown to possess potent anti-inflammatory and inflammation resolving activity (Calder, 2010). Some studies suggest that this increment of EPA- and DHA-derived oxylipins should be accompanied by a decrease in arachidonic acid-derived oxylipins (Calder, 2010).

However, the review of Shearer and Walker (2018) mentions that an inclusion of n-3 polyunsaturated FA can increase the availability of some n-6 polyunsaturated FA-derived oxylipins. In the present study, no significant reduction of arachidonic acid-derived oxylipins or any other n-6 FA-derived oxylipins was observed in colostrum or milk from the fish oil-fed animals. In fact, there was a trend to increase Prostaglandin D2 in colostrum, which is an arachidonic acid-derived eicosanoid. Prostaglandin D2 plays an important role in reproduction, especially in the implantation and maintenance of pregnancy (Saito, *et al*., 2002), which could explain this increase in colostrum (produced during the late stages of gestation) but not in milk. Tanghe and De Smet (2013) suggest that EPA and DHA may regulate gestation length decreasing the synthesis of 2-series Prostaglandins such as Prostaglandin E2 and Prostaglandin F2a. However, no differences in the concentration of these last two Prostaglandins or the gestation length were observed with the fish oil inclusion in the current study. In milk, 9(10) epoxy-octadecadienoic acid and 9(s)-hydroxy-octadecatrienoic acid derived from a-linolenic acid and 9(10)-, 12(13)-epoxy-octadecenoic, 9(s)-hydroxy-octadecadienoic acid derived from linoleic acid were also increased in the n-3 LCFA diet, although no differences in the precursor FAs were detected. Contrary to FA profile, the major changes in oxylipins were observed in milk rather than in colostrum. This could be explained by the high amounts of EPA- and DHA-derived oxylipins found in milk, suggesting a larger oxygenation process of n-3 LCFA.

In addition to the effect that n-3 polyunsaturated FAs can exert on inflammation via changes in the pattern of oxylipins, they also have an impact on the production of cytokines and Ig (Mitre *et al*., 2005; Calder, 2010; Yao, *et al*., 2012). In the current study, IgG concentrations were higher than those reported in the literature (Mitre *et al*., 2005; Leonard *et al*., 2010; Yao *et al*., 2012) but no differences between treatments were observed. However, although some studies reported increases in IgG concentration in colostrum and milk with dietary fish oil (Mitre *et al*., 2005), others have not observed this effect (Leonard *et al*., 2010). In addition, Yao *et al*. (2012) reported an increase in colostrum IgG and milk IgM for sow dietary n-6:n-3

ratios of 9:1 in comparison to a 3:1 ratio. These ratios are similar to those used in the present study; however, in the present case, Ig concentrations in colostrum and milk were not altered by the n-6:n-3 ratio of the maternal diet. Such discrepancies among studies deserve to be further investigated. In terms of cytokine production, the fish oil diet did not cause changes in colostrum. However, there is a trend to reduce IL10 and a reduction of TNFα in the milk of animals fed the n-3 LCFA diet. These effects may be due to the greater effect of n-3 LCFA diet on the oxylipin profile in milk than in colostrum. IL10 plays an important anti-inflammatory role inhibiting the production of proinflammatory cytokines (Walter, 2014), and TNFα plays a proinflammatory role since it is an inducer of inflammatory response (Idriss and Naismith, 2000). In the review of Calder (2010) data from in vitro (cell culture) and in vivo (mice and humans) studies showed a lowering effect of proinflammatory cytokine production such as IL1β, IL6 and TNFα by dietary fish oil. However, other studies do not confirm this effect and they refer to a possible dose effect, to technical factors, to the relative contributions of EPA and DHA and even to effects of polymorphisms of certain genes. Moreover, the studies in the review did not focus on the effects of n-3 LCFA in colostrum or milk.

As already mentioned, resolvins and protectins possess potent anti-inflammatory and inflammation resolving activities (Calder, 2010). Resolvin E2/3 is related with the inhibition of proinflammatory cytokine production and the production of anti-inflammatory cytokines in peritonitis studies with human and murine models (see Gabbs *et al*., (2015) for references). Previous in vitro reports show that protectins inhibit IL1β and TNFα production in human cell lines (see Serhan *et al*., (2008) for references). Moreover, it has been described that 18-hydroxy-eicosapentaenoic acid, an hydroxy-EPA, and 13- and 17-hydroxy-docosahexaenoic acid, hydroxy-DHA derivates decrease or inhibit TNFα secretion in murine or human cell lines (see Gabbs *et al*., (2015) for references) (Serhan *et al*., 2002). The decline of TNFα observed in milk is in line with the reported information. In the current study, although not differentially significant, IL1β levels in milk and colostrum were numerically higher for the n-3 LCFA diet than for the control diet. Future studies

evaluating a wider range of cytokines and transcription factors that play a role in inflammation could provide a more complete overview of the effects of n-3 LCFA on the immunological profile of colostrum and milk.

## Conclusion

Finally, this study provides a complete picture of precursors, intermediate molecules, and final mediators in colostrum and milk when sows are fed control or n-3 LCFA diets, which allows us to conclude that the inclusion of fish oil rich in EPA and DHA in sow diets during gestation and lactation may reduce litter size without affecting total litter weight during lactation, promotes an increase of EPA, DHA and their oxygenated derivatives with anti-inflammatory and inflammation resolving activity in colostrum and milk, and reduces TNFα and IL10 in milk. The implications of the changes observed in the colostrum and milk of sows fed n-3 LCFA for the immune status of the piglets remain to be analysed.

## Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.animal.2021.100403.

## Ethics approval

IRTA's Ethical Committee on Animal Experimentation approved the use of animals for this experiment in accordance with the Directive 2010/63/EU of 22 September 2010 and according to the recommendation of the European Commission 2007/526/CE, the Spanish guidelines for the care and use of animals in research (B. O.E. number 34, Real Decreto 53/2013) and the regional regulations on the use and handling of experimental animals (Decree 214/97, Generalitat de Catalunya) (project number: 10294).

## Data and model availability statement

None of the data were deposited in an official repository. The data that support the study findings are available upon request.

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## Declaration of interest

The authors report no conflict of interests.

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## Supplementary materials

***Supplementary Table S1:*** *Precision values of the ELISA kits employed for the quantitative measurement of immunoglobulins and cytokines in colostrum and milk from sows fed control or n-3 LCFA diet.*

| | %CV | |
|---|---|---|
| | Intra-Assay | Inter-Assay |
| Pig IgG ELISA Kit (E101-104; Bethyl Laboratories, Montgomery, Tx, USA) | <10% | <10% |
| Pig IgA ELISA Kit (ab190536; Abcam, Cambridge, UK) | <10% | <10% |
| Pig IgM ELISA Kit (ab190537, Abcam, Cambridge, UK) | <10% | <10% |
| Pig IL-1β ELISA Kit (ab100754; Abcam, Cambridge, UK) | <10% | <12% |
| Pig IL-6 ELISA Kit (ab100755; Abcam, Cambridge, UK) | <10% | <12% |
| Swine IL-10 ELISA Kit (KSC0101/KSC0102; Invitrogen, Carlsbad, CA, USA) | <6.3% | <9.4% |
| Pig TNF-α ELISA Kit (ab100756; Abcam, Cambridge, UK) | <10% | <12% |

IgA, immunoglobulin A; IgG, immunoglobulin G; IgM, Immunoglobulin M; IL-1β, interleukin 1β; IL-6, interleukin 6; IL-10, interleukin 10; TNF-α, tumor necrosis factor α.

***Supplementary Table S2:*** *Colostrum oxylipin quantification from sows fed control or n-3 LCFA diet.*[1]

| | Precursor | Control (n=3) | n-3 LCFA (n=3) | RMSE | *P* value |
|---|---|---|---|---|---|
| Oxylipin (pg/mL) | | | | | |
| 15(16)-EpODE | ALA | 83.7 | 89.2 | 0.21 | 0.847 |
| 9(10)-EpODE | ALA | 47.8 | 33.0 | 0.12 | 0.188 |
| 9(s)-HOTrE | ALA | 40.6 | 28.9 | 0.14 | 0.287 |
| 13(s)-HOTrE | ALA | 137 | 179 | 0.19 | 0.598 |
| 9,12,13-TriHOME | LA | 302 | 446 | 0.34 | 0.443 |
| 9,10,13-TriHOME | LA | 348 | 477 | 0.37 | 0.587 |
| 15,16-DiHODE | LA | 19.6 | 18.3 | 0.32 | 0.614 |
| 9,10-DiHODE | LA | 4.60 | 2.73 | 0.17 | 0.332 |
| 12,13-DiHODE | LA | 3.76 | 5.84 | 0.15 | 0.279 |
| 12(13)-DiHOME | LA | 223 | 220 | 0.25 | 0.706 |
| 9(10)-DiHOME | LA | 189 | 136 | 0.30 | 0.503 |
| 12(13)-EpOME | LA | 268 | 194 | 0.13 | 0.298 |
| 13(s)-HODE | LA | 1064 | 1430 | 0.19 | 0.444 |
| 13-oxoODE | LA | 337 | 371 | 0.40 | 0.584 |
| 9-oxoODE | LA | 197 | 248 | 0.15 | 0.460 |
| 9(10)-EpOME | LA | 392 | 270 | 0.17 | 0.374 |
| 9(s)-HODE | LA | 1115 | 861 | 0.08 | 0.180 |
| 8-iso Prostaglandin F2a | AA | 12.2 | 14.5 | 0.28 | 0.841 |
| Thromboxane B2 | AA | 8.04 | 11.2 | 0.21 | 0.629 |
| Prostaglandin F2a | AA | 213 | 222 | 0.28 | 0.767 |
| Prostaglandin E2 | AA | 8.30 | 5.52 | 0.19 | 0.473 |

| | | | | | |
|---|---|---|---|---|---|
| Prostaglandin D2 | AA | 1.15 | 3.63 | 0.15 | 0.058 |
| 15(R)-Lipoxin A4/Lipoxin A4 | AA | 0.64 | 0.74 | 0.17 | 0.905 |
| Leukotriene B4 | AA | 0.22 | 0.38 | 0.07 | 0.397 |
| 12(s)-HHTrE | AA | 5.22 | 3.21 | 0.16 | 0.303 |
| 14,15-DHET | AA | 70.1 | 61.1 | 0.19 | 0.636 |
| 11(12)-DHET | AA | 21.8 | 19.8 | 0.17 | 0.913 |
| 8,9-DHET | AA | 2.41 | 1.33 | 0.31 | 0.746 |
| 5,6-DHET | AA | 2.17 | ND | 0.19 | 0.374 |
| 20-HETE | AA | ND | ND | - | - |
| 15(s)-HETE | AA | 135 | 151 | 0.27 | 0.776 |
| 12(s)-HETE | AA | 164 | 81.1 | 0.41 | 0.865 |
| 11-HETE | AA | 140 | 116 | 0.21 | 0.853 |
| 8-HETE | AA | 11.8 | 8.60 | 0.47 | 0.872 |
| 5(s)-HETE | AA | 5.67 | 12.1 | 0.38 | 0.728 |
| 11,12-EET | AA | 9.77 | 4.24 | 0.24 | 0.245 |
| 14,15-EET | AA | ND | ND | - | - |
| Resolvin D1 | DHA | 4.95 | 4.23 | 0.03 | 0.108 |
| 13,14-DiHDPE | DHA | 19.8 | 22.2 | 0.12 | 0.532 |
| 10(s),17(s)-DiHDHA | DHA | 0.29 | 0.49 | 0.05 | 0.207 |
| Resolvin D5 | DHA | 0.78 | 11.8 | 0.21 | 0.009 |
| 4,5-DiHDPE | DHA | 0.83 | 10.6 | 0.22 | 0.013 |
| 20-HDHA | DHA | 2.12 | 28.4 | 0.29 | 0.012 |
| 16-HDHA | DHA | 6.77 | 29.5 | 0.26 | 0.036 |
| 17-HDHA | DHA | ND | 41.8 | 0.42 | 0.150 |
| 13-HDHA | DHA | ND | ND | - | - |

| | | | | | |
|---|---|---|---|---|---|
| 10-HDHA | DHA | ND | 35.1 | 0.08 | <0.001 |
| 11-HDHA | DHA | ND | 5.84 | 0.24 | 0.374 |
| 7-HDHA | DHA | ND | ND | - | - |
| 8-HDHA | DHA | ND | ND | - | - |
| 14-HDHA | DHA | ND | 7.31 | 0.25 | 0.374 |
| 4-HDHA | DHA | ND | ND | - | - |
| Protectin D1 | DHA | 0.37 | 1.49 | 0.15 | 0.125 |
| Protectin D1- | DHA | 0.29 | 1.91 | 0.16 | 0.076 |
| Prostaglandin E3 | EPA | 35.0 | 54.3 | 0.21 | 0.379 |
| 17,18-DiHETE | EPA | 1.59 | 6.47 | 0.10 | 0.004 |
| 14,15-DiHETE/11,15-DiHETE | EPA | ND | ND | - | - |
| 8,9-DiHETE | EPA | 0.95 | 3.17 | 0.29 | 0.305 |
| 18-HEPE | EPA | 1.41 | 13.0 | 0.18 | 0.006 |
| 15(s)-HEPE | EPA | ND | 175 | 0.22 | 0.005 |
| 11-HEPE | EPA | 4.06 | 34.0 | 0.26 | 0.013 |
| 8-HEPE | EPA | ND | 15.3 | 0.12 | 0.002 |
| 12(s)-HEPE | EPA | 10.4 | 78.3 | 0.39 | 0.030 |
| 9-HEPE | EPA | ND | 12.0 | 0.26 | 0.374 |
| 5(s)-HEPE | EPA | ND | 21.4 | 0.07 | <0.001 |
| Resolvin E2/E3 (11.2min) | EPA | ND | 5.81 | 0.10 | 0.002 |
| Resolvin E2/E3 (11.7min) | EPA | 1.39 | 1.54 | 0.13 | 0.971 |
| Resolvin E2/E3 (12.4min) | EPA | ND | ND | - | - |

AA, arachidonic acid; ALA, α-linolenic acid; DHA, docosahexaenoic acid; DiHDPE, dihydroxy-docosapentaenoic acid; DiHDHA, dihydroxy-docosahexaenoic acid; DiHETE, dihydroxy-eicosatetraenoic acid; DiHODE, dihydroxy-octadecadienoic acid; DiHOME, dihydroxy-octadecenoic acid; DHET, dihydroxy-eicosatrienoic acid; EET, epoxy-eicosatrienoic acid; EPA, eicosapentaenoic acid; EpODE, epoxy-octadecadienoic acid;

EpOME, epoxy-octadecenoic acid; HDHA, hydroxy-docosahexaenoic acid; HEPE, hydroxy-eicosapentaenoic acid; HETE, hydroxy-eicosatetraenoic acid; HHTrE, hydroxy-heptadecatrienoic acid; HODE, hydroxy-octadecadienoic acid; HOTrE, hydroxy-octadecatrienoic acid; LA, linoleic acid; LCFA, long chain fatty acid; ND, non-detected; oxoODE, oxo-octadecadienoic acid; TriHOME, trihydroxy-octadecenoic acid.

[1]Values are the mean ± RMSE

***Supplementary Table S3:*** *Milk oxylipin quantification from sows fed control or n-3 LCFA diet.*[1]

| | Precursor | Control (n=3) | n-3 LCFA (n=3) | RMSE | *P* value |
|---|---|---|---|---|---|
| Oxylipin (pg/mL) | | | | | |
| 15(16)-EpODE | ALA | 473 | 530 | 0.15 | 0.843 |
| 9(10)-EpODE | ALA | 163 | 274 | 0.12 | 0.064 |
| 9(s)-HOTrE | ALA | 136 | 233 | 0.11 | 0.058 |
| 13(s)-HOTrE | ALA | 413 | 792 | 0.16 | 0.118 |
| 9,12,13-TriHOME | LA | 962 | 738 | 0.24 | 0.474 |
| 9,10,13-TriHOME | LA | 1101 | 831 | 0.25 | 0.450 |
| 15,16-DiHODE | LA | 50.1 | 71.9 | 0.24 | 0.612 |
| 9,10-DiHODE | LA | 66.0 | 76.9 | 0.22 | 0.589 |
| 12,13-DiHODE | LA | 37.8 | 64.3 | 0.17 | 0.258 |
| 12(13)-DiHOME | LA | 915 | 1707 | 0.22 | 0.300 |
| 9(10)-DiHOME | LA | 2169 | 2796 | 0.15 | 0.424 |
| 12(13)-EpOME | LA | 491 | 1088 | 0.14 | 0.038 |
| 13(s)-HODE | LA | 4210 | 7434 | 0.16 | 0.183 |
| 13-oxoODE | LA | 201 | 288 | 0.18 | 0.363 |
| 9-oxoODE | LA | 356 | 436 | 0.12 | 0.390 |

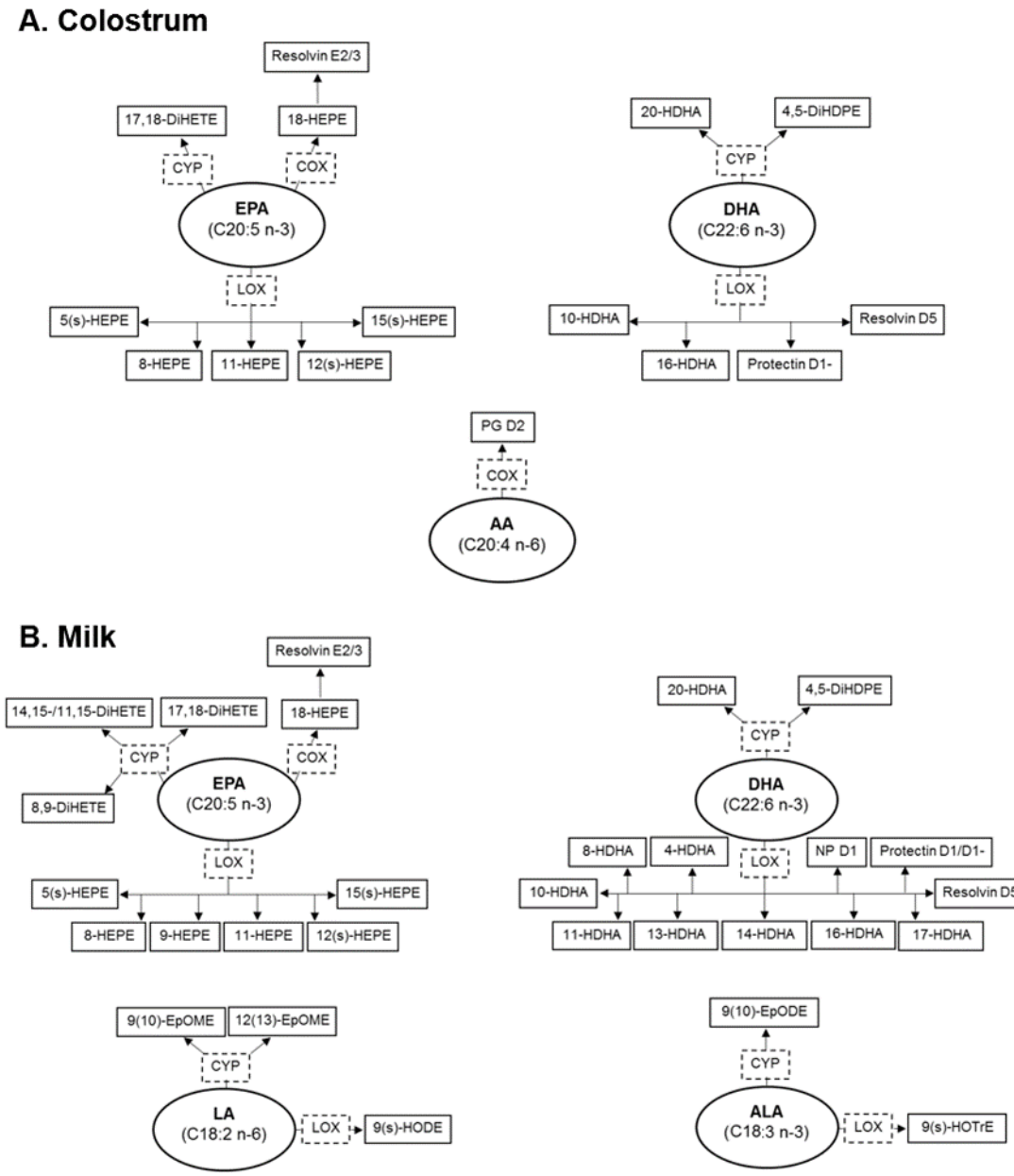
| | | | | | |
|---|---|---|---|---|---|
| 9(10)-EpOME | LA | 734 | 1584 | 0.16 | 0.056 |
| 9(s)-HODE | LA | 2678 | 4324 | 0.10 | 0.054 |
| 8-iso Prostaglandin F2a | AA | 12.6 | 11.3 | 0.05 | 0.353 |
| Thromboxane B2 | AA | 22.0 | 23.3 | 0.16 | 0.785 |
| Prostaglandin F2a | AA | 41.6 | 60.3 | 0.21 | 0.473 |
| Prostaglandin E2 | AA | 16.2 | 8.93 | 0.40 | 0.457 |
| Prostaglandin D2 | AA | 4.11 | 3.53 | 0.32 | 0.741 |
| 15(R)-Lipoxin A4/Lipoxin A4 | AA | 3.05 | 6.93 | 0.16 | 0.103 |
| Leukotriene B4 | AA | 1.19 | 1.32 | 0.25 | 0.953 |
| 12(s)-HHTrE | AA | 5.48 | 7.76 | 0.14 | 0.316 |
| 14,15-DHET | AA | 114 | 83.8 | 0.15 | 0.317 |
| 11(12)-DHET | AA | 28.9 | 26.8 | 0.13 | 0.654 |
| 8,9-DHET | AA | 4.74 | 4.12 | 0.37 | 0.458 |
| 5,6-DHET | AA | 6.17 | 7.76 | 0.11 | 0.327 |
| 20-HETE | AA | 179 | 92.2 | 0.24 | 0.151 |
| 15(s)-HETE | AA | 291 | 430 | 0.30 | 0.665 |
| 12(s)-HETE | AA | 277 | 219 | 0.13 | 0.365 |
| 11-HETE | AA | 191 | 241 | 0.12 | 0.435 |
| 8-HETE | AA | 21.1 | 25.8 | 0.24 | 0.854 |
| 5(s)-HETE | AA | 17.3 | 27.6 | 0.15 | 0.187 |
| 11,12-EET | AA | 7.06 | 8.18 | 0.26 | 0.903 |
| 14,15-EET | AA | ND | ND | - | - |
| Resolvin D1 | DHA | 4.45 | 4.08 | 0.02 | 0.183 |
| 13,14-DiHDPE | DHA | 14.9 | 10.9 | 0.08 | 0.114 |
| 10(s),17(s)-DiHDHA | DHA | 0.33 | 1.18 | 0.11 | 0.069 |

| | | | | | |
|---|---|---|---|---|---|
| Resolvin D5 | DHA | ND | 4.10 | 0.07 | 0.001 |
| 4,5-DiHDPE | DHA | ND | 32.0 | 0.13 | 0.002 |
| 20-HDHA | DHA | ND | 220 | 0.11 | <0.001 |
| 16-HDHA | DHA | 6.55 | 290 | 0.28 | 0.002 |
| 17-HDHA | DHA | ND | 239 | 0.14 | 0.002 |
| 13-HDHA | DHA | ND | 182 | 0.06 | <0.001 |
| 10-HDHA | DHA | 3.47 | 309 | 0.30 | <0.001 |
| 11-HDHA | DHA | ND | 37.4 | 0.25 | 0.012 |
| 7-HDHA | DHA | ND | 23.6 | 0.42 | 0.116 |
| 8-HDHA | DHA | ND | 84.4 | 0.15 | 0.002 |
| 14-HDHA | DHA | ND | 78.3 | 0.12 | 0.001 |
| 4-HDHA | DHA | ND | 291 | 0.10 | <0.001 |
| Protectin D1 | DHA | 0.34 | 1.37 | 0.08 | 0.016 |
| Protectin D1- | DHA | ND | 1.99 | 0.14 | 0.024 |
| Prostaglandin E3 | EPA | 2825 | 2613 | 0.77 | 0.945 |
| 17,18-DiHETE | EPA | 4.32 | 44.7 | 0.20 | 0.006 |
| 14,15-DiHETE/11,15-DiHETE | EPA | 4.68 | 48.6 | 0.18 | 0.003 |
| 8,9-DiHETE | EPA | 2.16 | 38.9 | 0.19 | 0.002 |
| 18-HEPE | EPA | 40.6 | 175 | 0.24 | 0.003 |
| 15(s)-HEPE | EPA | 102 | 1473 | 0.94 | 0.007 |
| 11-HEPE | EPA | 8.89 | 381 | 0.31 | 0.003 |
| 8-HEPE | EPA | 5.69 | 107 | 0.27 | 0.005 |
| 12(s)-HEPE | EPA | 22.0 | 290 | 0.25 | 0.005 |
| 9-HEPE | EPA | 3.34 | 132 | 0.26 | 0.002 |
| 5(s)-HEPE | EPA | 4.43 | 355 | 0.24 | <0.001 |

| | | | | | |
|---|---|---|---|---|---|
| Resolvin E2/E3 (11.2min) | EPA | 7.15 | 14.0 | 0.12 | 0.050 |
| Resolvin E2/E3 (11.7min) | EPA | 2.45 | 2.80 | 0.28 | 0.930 |
| Resolvin E2/E3 (12.4min) | EPA | ND | ND | - | - |

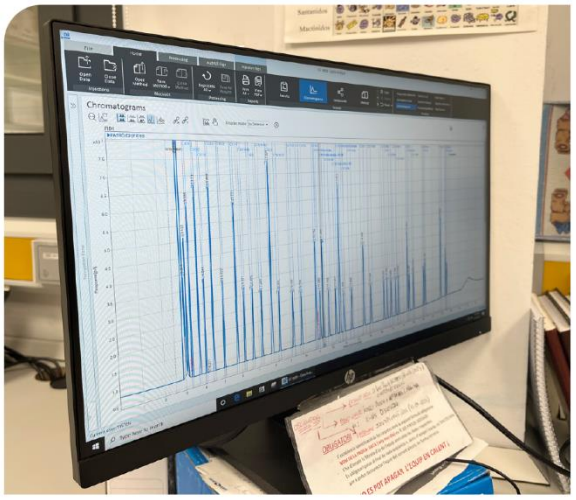
AA, arachidonic acid; ALA, α-linolenic acid; DHA, docosahexaenoic acid; DiHDPE, dihydroxy-docosapentaenoic acid; DiHDHA, dihydroxy-docosahexaenoic acid; DiHETE, dihydroxy-eicosatetraenoic acid; DiHODE, dihydroxy-octadecadienoic acid; DiHOME, dihydroxy-octadecenoic acid; DHET, dihydroxy-eicosatrienoic acid; EET, epoxy-eicosatrienoic acid; EPA, eicosapentaenoic acid; EpODE, epoxy-octadecadienoic acid; EpOME, epoxy-octadecenoic acid; HDHA, hydroxy-docosahexaenoic acid; HEPE, hydroxy-eicosapentaenoic acid; HETE, hydroxy-eicosatetraenoic acid; HHTrE, hydroxy-heptadecatrienoic acid; HODE, hydroxy-octadecadienoic acid; HOTrE, hydroxy-octadecatrienoic acid; LA, linoleic acid; LCFA, long chain fatty acid; ND, non-detected; oxoODE, oxo-octadecadienoic acid; TriHOME, trihydroxy-octadecenoic acid.

[1]Values are the mean ± RMSE




***Supplementary Figure S1:*** *Schematic overview of increased oxylipins, the enzymatic pathway involved in their generation and their fatty acid precursor in colostrum and milk from fish oil-fed sows. AA, arachidonic acid; ALA, α-linolenic acid; COX, cyclooxygenase; CYP, cytochrome P450; DHA, docosahexaenoic acid; DiHDPE, dihydroxy-docosapentaenoic acid; DiHETE, dihydroxy-eicosatetraenoic acid; EPA, eicosapentaenoic acid; EpODE, epoxy-octadecadienoic acid; EpOME, epoxy-octadecenoic acid; HDHA, hydroxy-docosahexaenoic acid; HEPE, hydroxy-eicosapentaenoic acid; HODE, hydroxy-*

*octadecadienoic acid; HOTrE, hydroxy-octadecatrienoic acid; LA, linoleic acid; LOX, lipoxygenase; NP, neuroprotectin; PG, prostaglandin.*




# CHAPTER 2

## Eicosapentaenoic acid- and docosahexaenoic acid-rich fish oil in sow and piglet diets modifies blood oxylipins and immune indicators in both, sows and suckling piglets.

UNIVERSITAT ROVIRA I VIRGILI
EFFECTS OF N-3 LONG-CHAIN FATTY ACIDS IN SOW AND PIGLET DIETS ON PERINATAL PIGLET PERFORMANCE, OXYLIPINS, IMMUNITY, AND MICROBIOTA
Eudald Llauradó Calero

## CHAPTER 2:

# Eicosapentaenoic acid- and docosahexaenoic acid-rich fish oil in sow and piglet diets modifies blood oxylipins and immune indicators in both, sows and suckling piglets

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This chapter describes and discusses the effect of the inclusion of the fish oil rich in n-3 LCFAs in gestation a lactation diets for sows and creep feed for suckling piglets on:

- Fatty acid composition, oxylipin profile and immune indicators of blood from gestating and lactating sows, and suckling piglets. In addition, in this chapter it has also been studied whether the effect of dietary inclusion of n-3 LCFA differs according to the body weight of the piglets at birth (LBW vs HBW piglets).
- Correlations between FA or oxylipins and immune indicators.

# Eicosapentaenoic acid- and docosahexaenoic acid-rich fish oil in sow and piglet diets modifies blood oxylipins and immune indicators in both, sows and suckling piglets

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**Abstract**

Over the last decades, genetic selection has increased sows' litter size. Consequently, there is a high proportion of piglets born with low weight which are vulnerable. Their viability may potentially be enhanced through early nutrition. The aim of the current study was to evaluate whether including a fish oil rich in eicosapentaenoic acid (**EPA**) and docosahexaenoic acid (**DHA**) in the diets of the sow and piglets was able to increase concentrations of anti-inflammatory molecules in their blood. Thirty-six sows, in four consecutive batches, were randomly assigned to either a control diet with animal fat (15 g/kg in gestation and 30 g/kg in lactation) or an n-3 long-chain fatty acid (**n-3 LCFA**) diet from insemination until the end of lactation. From day 11 of lactation, piglets were also offered a diet containing 30 g/kg of animal fat or n-3 LCFA. To prepare the n-3 LCFA diet, 15 g/kg or 30 g/kg of animal fat in the control diet were replaced by an equivalent amount of solid fish oil for sows and piglets, respectively. All the sows were sampled for serum and plasma at day 108 of gestation and at weaning. Additionally, only for the first batch of sows, blood samples were also obtained at weaning from the two lightest (>800 g) and the two heaviest birth weight piglets in each litter. Serum fatty acids (**FAs**) were quantified by gas chromatography, plasma oxylipins by ultra-HPLC-MS and plasma immunoglobulins (**Ig**) and cytokines by ELISA. The n-3 LCFA diet increased the concentrations of n-3 FAs in gestating and lactating sows and in piglets ($P < 0.001$, $P < 0.001$ and $P = 0.011$, respectively), particularly EPA ($P < 0.001$, $P < 0.001$ and $P < 0.001$, respectively) and DHA ($P < 0.001$, $P < 0.001$ and $P < 0.001$, respectively), and also their oxygenated derivatives. In addition, fish oil increased plasma IgM in gestating and lactating sows ($P = 0.014$ and $P = 0.008$, respectively), interleukin (**IL**) 6 in sows at weaning ($P = 0.012$), and IL1β in piglets ($P = 0.018$). Birth BW of piglets, regardless of diet, slightly influenced some of the n-6-derived oxylipins. In conclusion, fish oil addition in diets increased the blood concentrations of n-3 FAs and their oxygenated derivatives, some of which have anti-inflammatory activity, in gestating and

lactating sows and piglets, IgM in gestating and lactating sows, IL6 in lactating sows and IL1β in piglets.

**Keywords**

Early feeding, n-3 long-chain fatty acids, Oxygenated lipid mediators, Plasma immune indicators, Preweaning.

**Implications**

This study shows that the inclusion of fish oil in sow diets during gestation and lactation and in creep feed influences blood serum fatty acid and plasma oxylipin profiles in sows at late gestation and weaning and piglets at weaning. The observed increases in n-3 fatty acids and their oxygenated derivatives have been described to play an anti-inflammatory role. Therefore, fish oil in the diets of sows could improve the immune status of piglets, particularly during the postweaning period which, from an immunocompetence point of view, is the most critical period in swine production.

**Introduction**

In the current swine production system, genetic selection of sows has focused in increasing their litter size, and this has resulted in a reduction of the average birth weight of piglets and an increase in the proportion of piglets born with low weight (<1.0 kg birth weight) (Blavi *et al*., 2021). Low birth weight (**LBW**) piglets are characterised by a particularly poor thermoregulation ability and generally have problems to achieve and adequate colostrum intake (Edwards and Baxter, 2015). For these reasons, LBW piglets are most vulnerable and have a greater incidence of mortality than their heavier littermates (Farmer and Edwards, 2022).

A potential approach to improve LBW piglets' viability is through sow nutrition. Dietary polyunsaturated fatty acids (**FAs**) are commonly present in swine diets and are used as an energy source (Rosero *et al*., 2016). However, they also play important roles as structural components of the cell membrane, metabolic substrates in biochemical pathways, cell-signalling molecules and immune modulators (Liu, 2015). It is well established that polyunsaturated FAs can influence the immune status through a variety of mechanisms (Lauridsen, 2020; Cader, 2012). One of them is through the formation of oxygenated derivatives (oxylipins) (Balvers *et al*., 2012) and their subsequent effect on cytokines synthesis (Calder, 2010). Concretely, oxylipins exert multiple functions in the modulation of health and disease in mammals (Gabbs *et al*., 2015; Christie and Harwood, 2020). They are important mediators of the PUFA effects in the body, and their formation is associated with the nature of dietary FAs.

Under commercial conditions, dietary n-6:n-3 FA ratios in sow and piglet diets are very high, as their main ingredients are commonly rich in n-6 FAs and contain supplemental fat sources that are rich in saturated FAs and have very low levels of n-3 FAs. Dietary n-6 and n-3 polyunsaturated FAs have different effects on the immune system; while the n-6 family are precursors of oxylipins with proinflammatory potential, the n-3 family are precursors of anti-inflammatory and inflammation resolving oxylipins (Calder, 2010). Oxylipins are the major lipid mediators for the polyunsaturated FA effects in the body (Gabbs *et al*., 2015) and are considered to be critically involved in neonatal physiology (Wu *et al*., 2016).
Considering the anti-inflammatory effects associated with n-3 FAs, it was hypothesised that n-3 long-chain FAs (**n-3 LCFA**) in sow and piglet diets would increase n-3 polyunsaturated FAs and their oxygenated derivatives, which in turn would influence immune indicators in different biological matrices from sows and piglets in different points of the productive swine cycle. In the scope of this project, we previously reported that the inclusion of fish oil in sow diets promoted an increase of n-3 polyunsaturated FAs and their oxygenated derivatives in colostrum and milk, and reduced tumour necrosis factor a (**TNF**α) and interleukin (IL) 10 in

milk (Llauradó-Calero *et al*., 2021). This suggests that dietary fish oil may also influence the FA content, oxylipin profile and immune status of suckling piglets. This is particularly relevant since piglets are born with poor energy reserves and are devoid of immune protection (Le Dividich *et al*., 2005).

Therefore, we analysed blood samples from sows and piglets in our previous study with the aim of determining how the inclusion of fish oil, rich in eicosapentaenoic acid (**EPA**) (C20:5n-3) and docosahexaenoic acid (**DHA**) (C22:6n-3), in sow and piglet diets influence FA and oxylipin profiles, and systemic immune indicators of sows at the end of gestation and at weaning and particularly in piglets at weaning.

## Material and methods

*Animals, housing and experimental design*

Thirty-six sows from four consecutive batches (same animals and experimental set-up as Llauradó-Calero *et al*. (2021) were used. The sows were randomly assigned to either a control diet or an n-3 LCFA-rich diet from insemination (day 0) until the end of lactation (±28 days of lactation). In the first batch of sows (12 sows), the two piglets with lightest birth weight (>800 g) and the two piglets with highest birth weight in each litter were selected for blood sampling. Cross fostering (to standardise the litter size to 12 piglets) was exclusively conducted among sows belonging to the same experimental treatment and within the first 24 h postfarrowing. At day 11 of lactation, creep feed (with or without n-3 LCFA) was also offered to piglets in accordance with the maternal diet.

Sows were allocated to individual stalls from insemination to pregnancy confirmation, followed by group housing in a gestation barn until 1 week before farrowing, when they were moved to individual farrowing crates (0.7 x 2 m) equipped with partially slatted floor and a heated floor panel for piglets (set at 32–34 ºC). The temperature inside the building was automatically set at 24 ºC at farrowing and reduced by 0.5 ºC per week until weaning. Ventilation was via single,

variable-speed fans linked to temperature sensors. Sows were fed via individual feed hoppers and piglets from snap-in round feeders. Water was provided ad libitum from independent nipple drinkers for sows and piglets.

*Experimental diets*

Gestation and lactation diets were formulated (iso-nutritive between dietary treatments) according to FEDNA specifications (de Blas *et al*., 2013). In the control diets, animal fat was included at 15 g/kg (gestation phase) and 30 g/kg (lactation phase). In the n- 3 LCFA diets, animal fat was totally (gestation phase) or half replaced (lactation phase) by solid fish oil (Lipomega; V&S Asociados, Madrid, Spain). Creep feed contained 30 g/kg of animal fat in the control diet, and this was totally replaced by solid fish oil in the n-3 LCFA diet. The ingredient and nutrient composition of the diets has already been described in Llauradó-Calero *et al*. (2021).

Sows were feed-restricted during gestation at 3 kg/day and after farrowing, feed intake was gradually increased until reaching ad libitum consumption. From day 11 of lactation, creep feed was offered to piglets *ad libitum*.

*Blood sampling*

The 36 sows were sampled for blood at day 108 of gestation (gestating sows) and after weaning (lactating sows). In addition, blood samples from the 48 selected piglets (two lightest and two heaviest birth weights in each litter) of the first batch of 12 sows were also obtained at weaning. Blood was collected by jugular venepuncture in non-heparinised tubes and in tubes with ethylenediaminetetraacetic acid (EDTA) for serum and plasma separation, respectively. Non-heparinised tubes were held at ambient temperature until centrifugation (3000 rpm, 10 min), and EDTA samples were kept at 4 ºC (maximum 120 min) until centrifugation (3000 rpm, 10 min). Aliquots of serum for FA, and

aliquots of plasma for oxylipins (in tubes containing 0.005 % butylated hydroxytoluene (Merck, Darmstadt, Germany) as antioxidant), Ig and cytokines were obtained and quickly stored at 80 ºC (maximum 30 min from centrifugation to storage).

*Quantitative analysis of fatty acids*

Fat from serum of sows and piglets was extracted with chloroform (PanReac AppliChem, Barcelona, Spain) – methanol (Honeywell, Charlotte, NC, USA) according to Folch *et al*. (1957) and transmethylated with boron trifluoride (Sigma Aldrich, St. Louis, MO, USA) and methanolic potassium hydroxide 0.5 M (PanReac, Barcelona, Spain) according to Morrison and Smith (1964). Fatty acids were determined by gas chromatography (Agilent 6890N, Boston, MA, USA) according to the procedure previously described in Llauradó-Calero *et al*. (2021). FAs were quantified from C12:0, and results were expressed as mg of FA per g of serum.

*Metabolomic analysis of oxylipins*

Fifty-three oxylipins were quantified from the plasma of sows and piglets. Preparation of samples was performed as previously described in Llauradó-Calero *et al*. (2021) for colostrum and milk samples but optimised for plasma samples. In brief, aliquots of 0.25 ml of plasma were mixed with 0.1 ml of internal standard mixture prepared in methanol (internal standard concentrations: Deuterated Primary COX and LOX LC-MS Mixture (10 µg/l), Deuterated Linoleic Acid Oxylipins LC-MS Mixture (5 µg/l), Leukotriene B4-d4 (50 µg/l), Lipoxin A4-d5 (50 µg/l), Arachidonic acid-d8 (1 mg/l), Resolvin D1-d5 (5 µg/l), 8-isoProstaglandin F2a-d4 (100 µg/l) (Cayman chemicals, Ann Arbor, MI, USA)). Thereafter, 0.5 ml of methanol (butylated hydroxytoluene 0.001 M) was added and samples were incubated for 30 min at 20 ºC. After centrifugation, the supernatant was diluted with 4 ml of a 0.1 % of formic acid (Sigma Aldrich, St. Louis, MO, USA) in Milli-Q water (Millipore,

Burlington, MA, USA). A clean-up using Oasis PRIME HLB cartridges (1 cc Vac Cartridge, 30 mg sorbent; Waters Corporation, Milford, MA, USA), the evaporation to dryness and the reconstitution of the samples were performed according to Ostermann (2017).

Oxylipin concentrations were determined using an ultra-HPLC 1290 Series coupled to a triple quadrupole MS 6490 series instrument (Agilent, Santa Clara, CA, USA) with an analytical column Eclipse XDB C18 1.8 µl (2.1 x 150 mm) (Agilent, Santa Clara, CA, USA). Gradient elution was performed using LC-MS water (Scharlab, Barcelona, Spain) (0.01 % acetic acid (Sigma Aldrich, St. Louis, MO, USA)) and acetonitrile:methanol (85:15, v/v) as a mobile phase at a flow rate of 0.4 ml/min and 45 ºC. The injection volume was 10 µl (4 ºC). The electrospray source ionisation was in negative mode, and the acquisition was performed in dynamic Multiple Reaction Monitoring (MRM). Identification with standards or tentative identification of oxylipins was previously described in Llauradó-Calero *et al*. (2021).

*Immune indicators*

The concentration of plasmatic IgG, IgA and IgM, and IL1β, IL6, IL10 and TNFαcytokines were quantitatively measured using sandwich ELISA kits as previously described in Llauradó-Calero *et al*. (2021).

*Statistical analysis*

The GLIMMIX procedure of SAS software (SAS/STAT 14.1; SAS Institute Inc., Cary, NC, USA) was used to perform the ANOVA. For gestating and lactating sows, the model included dietary treatment as fixed effect and batch as random effect, and for suckling piglets, the model included dietary treatment, birth body weight (**bBW**) category and the interaction between them as fixed effects and sow as random effect. When the limit of detection was not reached, the missing values were replaced by 1/5 of the minimum positive value of each variable. Data suspected to

be outliers were tested using Kolmogorov-Smirnov test, and values were excluded if $P < 0.01$. Although a logarithmic transformation ($\log_{10}(X + 1)$) was performed to compare the oxylipin concentration between treatments, original means are presented in supplementary tables. Therefore, results are expressed as least squares means ± RMSE, except for oxylipins that are expressed as means ± RMSE (from transformed data). Differences were considered significant at $P < 0.05$, while those at $P < 0.1$ are reported as tendencies.

The PROC CORR procedure of SAS software was used to obtain the Pearson correlation coefficient between the concentrations of FA or oxylipins and the immune indicators that were significantly modified by the n-3 LCFA diet or by bBW. The significant correlation level was set at $P < 0.05$.

MetaboAnalyst 5.0 (https://www.metaboanalyst.ca, Alberta, CA, USA) was used to perform Principal Component Analysis (PCA) of FAs and oxylipins and the heatmap of oxylipins.

## Results

*Fatty acid profile*

The changes caused by the inclusion of dietary fish oil on the FA profile of serum from gestating and lactating sows, and from piglets are shown in Tables 1 and 2, respectively.

Dietary n-3 LCFA did not change total saturated FAs in gestating and lactating sows but tended to decrease monounsaturated FAs ($P = 0.088$) in gestating sows. In gestating sows, the n-3 LCFA diet tended to increase myristic acid (C14:0) ($P = 0.093$), decreased heneicosylic acid (C21:0) ($P < 0.001$), and tended to decrease oleic acid (C18:1n-9 cis) ($P = 0.061$) compared to sows fed the control diet. However, in lactating sows, the only saturated or monounsaturated FA that was reduced by the n-3 LCFA diet was heneicosylic acid ($P = 0.002$). In terms of polyunsaturated FAs, n-3 LCFA diet increased total n-3 FAs, both at gestation and

lactation ($P < 0.001$ and $P < 0.001$, respectively), EPA ($P < 0.001$ and $P < 0.001$, respectively), docosapentaenoic acid (C22:5n-3) ($P < 0.001$ and $P < 0.001$, respectively), and DHA ($P < 0.001$ and $P < 0.001$, respectively) compared to control diet. In contrast, total n-6 FA content did not differ between the two dietary treatments in gestating nor lactating sows. However, the n-3 LCFA diet reduced the concentrations of γ-linolenic acid (C18:3n-6 cis) ($P = 0.038$, $P < 0.001$, respectively), eicosadienoic acid (C20:2n-6 cis) ($P = 0.017$ and $P = 0.002$, respectively) and arachidonic acid (C20:4n-6) ($P < 0.001$ and $P < 0.001$, respectively) in gestating and lactating sows. Consequently, while total polyunsaturated FAs were not affected by dietary treatment, the n-6:n-3 ratio was reduced in the plasma of gestating ($P < 0.001$) and lactating ($P < 0.001$) sows that were fed the n-3 LCFA diet.

***Table 1:*** *Serum fatty acid profiles of gestating and lactating sows fed control or n-3 LCFA diets.*[1,2]

| | Gestating sows | | | | Lactating sows | | | |
|---|---|---|---|---|---|---|---|---|
| | Control (n=18) | n-3 LCFA (n=18) | RMSE | *P* value | Control (n=18) | n-3 LCFA (n=18) | RMSE | *P* value |
| Fatty acid (mg FA/g serum) | | | | | | | | |
| C12:0 | 0.033 | 0.037 | <0.01 | 0.572 | 0.028 | 0.028 | <0.01 | 0.996 |
| C14:0 | 0.027 | 0.034 | <0.01 | 0.093 | 0.002 | 0.003 | <0.01 | 0.645 |
| C16:0 | 0.75 | 0.71 | 0.03 | 0.455 | 0.65 | 0.65 | 0.02 | 0.956 |
| C16:1 | 0.042 | 0.046 | <0.01 | 0.499 | 0.044 | 0.043 | <0.01 | 0.862 |
| C18:0 | 0.57 | 0.51 | 0.02 | 0.133 | 0.54 | 0.52 | 0.01 | 0.512 |
| C18:1 n-9 *cis* | 0.98 | 0.78 | 0.02 | 0.061 | 0.91 | 0.85 | 0.08 | 0.492 |
| C18:1 n-7 | 0.063 | 0.067 | <0.01 | 0.567 | 0.078 | 0.074 | <0.01 | 0.520 |
| C18:2 n-6 *cis* | 1.04 | 1.11 | 0.11 | 0.567 | 1.02 | 0.89 | 0.11 | 0.285 |
| C18:3 n-6 *cis* | 0.014 | 0.007 | <0.01 | 0.038 | 0.026 | 0.022 | <0.01 | <0.001 |
| C18:3 n-3 *cis* | 0.052 | 0.056 | <0.01 | 0.384 | 0.036 | 0.039 | <0.01 | 0.362 |
| C20:2 n-6 *cis* | 0.015 | 0.011 | <0.01 | 0.017 | 0.015 | 0.010 | <0.01 | 0.002 |
| C21:0 | 0.030 | 0.007 | <0.01 | <0.001 | 0.025 | 0.013 | <0.01 | 0.002 |
| C20:3 n-6 | 0.012 | 0.013 | <0.01 | 0.673 | 0.012 | 0.010 | <0.01 | 0.431 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| C20:4 n-6 | 0.26 | 0.12 | <0.01 | <0.001 | 0.27 | 0.18 | <0.01 | <0.001 |
| C20:5 n-3 | 0.003 | 0.16 | <0.01 | <0.001 | 0.001 | 0.14 | <0.01 | <0.001 |
| C24:0 | 0.022 | 0.019 | <0.01 | 0.144 | 0.018 | 0.018 | <0.01 | 0.610 |
| C22:5 n-3 | 0.051 | 0.10 | <0.01 | <0.001 | 0.037 | 0.064 | <0.01 | <0.001 |
| C22:6 n-3 | 0.019 | 0.13 | <0.01 | <0.001 | 0.012 | 0.084 | <0.01 | <0.001 |
| Minor FA[3] | 0.27 | 0.25 | <0.01 | 0.206 | 0.27 | 0.30 | <0.01 | 0.118 |
| SFA | 1.52 | 1.40 | 0.11 | 0.331 | 1.38 | 1.37 | 0.07 | 0.884 |
| MUFA | 1.17 | 0.97 | 0.12 | 0.088 | 1.11 | 1.05 | 0.09 | 0.536 |
| PUFA | 1.56 | 1.77 | 0.21 | 0.180 | 1.50 | 1.52 | 0.21 | 0.898 |
| n-3 | 0.19 | 0.48 | <0.01 | <0.001 | 0.14 | 0.38 | <0.01 | <0.001 |
| n-6 | 1.37 | 1.29 | 0.16 | 0.533 | 1.36 | 1.15 | 0.16 | 0.113 |
| n-6:n-3 | 7.51 | 2.68 | 2.21 | <0.001 | 9.66 | 3.01 | 1.28 | <0.001 |

FA, fatty acid; LCFA, long chain fatty acid; ND, non-detected; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.

[1]Values are least squares means ± RMSE.

[2]FA quantification results are reported from C12:0.

[3]Minor FAs include: C13:0, C14:1 n-9 *cis*, C15:0, C15:1, C17:0, C17:1, C18:1 n-9 *trans*, C18:1 n-11 *cis*, C18:2 n-6 *trans*, C19:0, C18:4 n-3, C20:0, C20:1 n-9 *cis*, C20:3 n-3 *cis*, C22:0, C22:1, C22:2 n-6 *cis*, C23:0, C22:4 n-6, C22:3 n-3, and C24:1 n-9 *cis*. C13:0 and C14:1 n-9 *cis* have not been detected in plasma from lactating and gestating sows, respectively.

In piglets, no differences in the concentrations of total saturated and monounsaturated FAs were observed due to dietary treatment, bBW category or their interaction. However, regarding saturated FA, myristic acid (C14:0) was increased ($P = 0.003$) and lignoceric acid (C24:0) was decreased in the n-3 LCFA piglets ($P < 0.001$). In addition, heneicosylic acid (C21:0) was decreased in n-3 LCFA compared to control piglets ($P < 0.001$) and increased in LBW compared to high birth weight (**HBW**) piglets ($P = 0.039$). In terms of polyunsaturated FAs, on the one hand, total n-3 FAs were increased in the n-3 LCFA piglets ($P = 0.011$). Particularly, dietary n-3 LCFA increased α-linolenic acid (C18:3n-3 cis) ($P = 0.047$), EPA ($P < 0.001$), docosapentaenoic acid ($P < 0.001$) and DHA ($P < 0.001$), while no

changes were observed in total n-6 FAs. On the other hand, the n-3 LCFA diet decreased γ-linolenic acid ($P = 0.009$), and an interaction between dietary treatment and body birth weight was observed for arachidonic acid ($P = 0.025$) where n-3 LCFA reduced the concentration of this FA only in the LBW piglets.

***Table 2:*** *Serum fatty acid profiles of suckling piglets from sows fed control or n-3 LCFA diets, born with low or high body weight.*[1,2,3]

| | Suckling piglets | | | | | | |
|---|---|---|---|---|---|---|---|
| | MDiet | | bBW | | | | |
| | Control (n=28) | n-3 LCFA (n=20) | HBW (n=24) | LBW (n=24) | RMSE | *P* value MDiet | *P* value bBW |
| Fatty acid (mg FA/g serum) | | | | | | | |
| C12:0 | 0.018 | 0.019 | 0.019 | 0.018 | <0.01 | 0.662 | 0.883 |
| C14:0 | 0.061 | 0.086 | 0.071 | 0.076 | <0.01 | 0.003 | 0.560 |
| C16:0 | 1.68 | 1.90 | 1.73 | 1.85 | 0.18 | 0.114 | 0.354 |
| C16:1 | 0.25 | 0.30 | 0.27 | 0.28 | 0.01 | 0.195 | 0.833 |
| C18:0 | 0.76 | 0.79 | 0.74 | 0.80 | 0.02 | 0.742 | 0.127 |
| C18:1 n-9 *cis* | 1.31 | 1.35 | 1.26 | 1.40 | 0.24 | 0.819 | 0.332 |
| C18:1 n-7 | 0.14 | 0.14 | 0.14 | 0.15 | <0.01 | 0.906 | 0.438 |
| C18:2 n-6 *cis* | 1.54 | 1.51 | 1.55 | 1.51 | 0.24 | 0.835 | 0.766 |
| C18:3 n-6 *cis* | 0.035 | 0.028 | 0.031 | 0.032 | <0.01 | 0.009 | 0.794 |
| C18:3 n-3 *cis* | 0.030 | 0.040 | 0.035 | 0.036 | <0.01 | 0.047 | 0.856 |
| C20:2 n-6 *cis* | 0.031 | 0.025 | 0.026 | 0.030 | <0.01 | 0.128 | 0.151 |
| C21:0 | 0.027 | 0.013 | 0.018 | 0.021 | <0.01 | <0.001 | 0.039 |
| C20:3 n-6 | 0.025 | 0.025 | 0.023 | 0.027 | <0.01 | 0.827 | 0.174 |
| C20:4 n-6* | 0.52 | 0.33 | 0.42 | 0.44 | <0.01 | 0.006 | 0.507 |
| C20:5 n-3 | 0.009 | 0.18 | 0.10 | 0.088 | <0.01 | <0.001 | 0.375 |
| C24:0 | 0.034 | 0.017 | 0.025 | 0.026 | <0.01 | <0.001 | 0.912 |
| C22:5 n-3 | 0.065 | 0.11 | 0.083 | 0.088 | <0.01 | <0.001 | 0.550 |
| C22:6 n-3 | 0.20 | 0.46 | 0.26 | 0.40 | 0.09 | <0.001 | 0.171 |
| Minor FA[4] | 0.31 | 0.25 | 0.28 | 0.28 | <0.01 | 0.328 | 0.908 |
| SFA | 2.65 | 2.91 | 2.68 | 2.88 | 0.33 | 0.268 | 0.236 |
| MUFA | 1.87 | 1.91 | 1.82 | 1.96 | 0.37 | 0.819 | 0.448 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| PUFA | 2.54 | 2.75 | 2.58 | 2.71 | 0.65 | 0.454 | 0.323 |
| n-3 | 0.37 | 0.81 | 0.52 | 0.66 | 0.43 | 0.011 | 0.268 |
| n-6 | 2.17 | 1.94 | 2.06 | 2.05 | 0.35 | 0.240 | 0.929 |
| n-6:n-3 | 7.72 | 2.67 | 5.52 | 4.87 | 1.23 | <0.001 | 0.767 |

bBW, birth weight; FA, fatty acid; HBW, high birth weight piglets; LBW, low birth weight piglets; LCFA, long chain fatty acid; MDiet, maternal diet; ND, non-detected; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.

[1]Values are least squares means ± RMSE.

[2]FA quantification results are reported from C12:0.

[3]The *P* value of the interaction MDiet*bBW is not reported in the table since only a significant *P* values were observed for C20:4 n-6 concentration.

[4]Minor FAs include: C14:1 n-9 *cis*, C15:0, C15:1, C17:0, C17:1, C18:1 n-9 *trans*, C18:1 n-11 *cis*, C18:2 n-6 *trans*, C19:0, C18:4 n-3, C20:0, C20:1 n-9 *cis*, C22:0, C22:1, C22:2 n-6 *cis*, C23:0, C22:4 n-6, C22:3 n-3, and C24:1 n-9 *cis*. C13:0 and C20:3 n-3 *cis* have not been detected.

**P* value of the interaction MDiet*bBW for C20:4 n-6 was *P* = 0.025 where the concentrations in mg FA/g serum were $0.56^{a}$ for control-LBW, $0.48^{b}$ for control-HBW, $0.31^{c}$ for n-3 LCFA-LBW, and $0.36^{bc}$ for n-3 LCFA-HBW.

Principal component analysis shows the distribution of samples according to the type of sample (gestating sows, lactating sows or suckling piglets) and the dietary treatment (control or n-3 LCFA) (Figure 1, A). EPA was the FA with the highest contribution in explaining the distribution of the principal component analysis. Another principal component analysis according to bBW category indicates that there is not a different distribution of FA profiles of suckling piglets in terms of this variable (Supplementary Figure S1, A).

*Oxylipin profile*

Dietary n-3 LCFA inclusion modified the plasma concentrations of 25 oxylipins in gestating sows and 28 oxylipins in lactating sows. Concretely, differences between treatments are mainly due to increases in the concentrations of oxylipins derived from EPA and DHA in the sows that were fed the fish oil diet (Supplementary Table S1 and S2). In gestating sows, the EPA-derived oxylipins that were increased by n-3 LCFA were 5(s)-, 8-, 9-, 11-, 12(s)-, 15(s)- and 18-hydroxy-EPA ($P < 0.001$). In terms of DHA-derived oxylipins, n-3 LCFA increased 4-, 7-, 8-, 10-, 11-, 13-, 14-, 16-, 17- and 20-hydroxy-DHA ($P < 0.001$). In addition, fish oil also modified some oxylipins that are derived from other LCFAs. Concretely, n-3 LCFA tended to increase 15(16)-epoxy-octadecadienoic acid ($P = 0.096$) that is derived from α-linolenic acid and 15,16-dihydroxy-octadecenoic acid ($P = 0.069$) that is derived from linoleic acid. It also increased 15(R)-Lipoxin A4/Lipoxin A4 ($P = 0.012$), decreased 8,9-, 11(12)- and 14,15-dihydroxy-eicosatrienoic acid ($P < 0.001$, $P = 0.014$ and $P = 0.010$, respectively), 20-hydroxy-eicosatetraenoic acid ($P = 0.008$) and tended to reduce 11,12-epoxy-eicosatrienoic acid ($P = 0.076$) that are derived from arachidonic acid. In lactating sows, n-3 LCFA also increased the EPA-derived oxylipins 5(s)-, 8-, 9-, 11-, 12(s)-, 15(s)- and 18-hydroxy-EPA ($P < 0.001$), and the DHA-derived oxylipins 4-, 7-, 8-, 10-, 11-, 13-, 14-, 16-, 17- and 20-hydroxy-DHA ($P < 0.001$). In addition to these modifications, lactating sows fed n-3 LCFA presented increased concentrations of 15(R)-Lipoxin A4/Lipoxin A4 ($P < 0.001$) and 8-hydroxy-eicosatetraenoic acid ($P = 0.004$), which are derived from arachidonic acid. They also had decreased concentrations (or tendencies to decrease) of 9(10)-epoxy-octadecadienoic acid ($P = 0.072$) and 9(s)-hydroxy-octadecatrienoic acid ($P = 0.044$), which are derived from α-linolenic acid; 9(10)- and 12(13)-epoxy-octadecenoic acid ($P = 0.012$ and $P = 0.071$, respectively), derived from linoleic acid; and 8-, 9-, 11(12)- and 14,15-dihydroxy-eicosatrienoic acid ($P = 0.021$, $P = 0.043$ and $P = 0.021$, respectively), 12(s)-hydroxy-eicosatetraenoic acid ($P = 0.097$) and 14,15-epoxy-eicosatrienoic acid ($P = 0.014$) derived from arachidonic acid.

The piglets' plasma oxylipin concentrations according to dietary treatment and birth BW category are reported in Supplementary Table S3. The inclusion of fish oil in the diet modified a total of 26 oxylipins. Concretely, dietary n-3 LCFA increased the EPA-derived oxylipins 5(s)-, 8-, 9-, 11-, 12(s)-, 15(s)- and 18-hydroxy-EPA ($P < 0.001$); the DHA-derived oxylipins 4-, 7-, 8-, 10-, 11-, 13-, 14-, 16-, 17- and 20-hydroxy-DHA ($P < 0.001$); the α-linolenic acid-derived oxylipin 15(16)-epoxy-octadecadienoic acid ($P = 0.014$); and the linoleic acid-derived oxylipin 9,10-dihydroxyoctadecadienoic acid ($P = 0.036$). On the other hand, n-3 LCFA also decreased (or tended to decrease) the DHA-derived oxylipin 13,14- dihydroxy-docosapentaenoic acid ($P = 0.006$); and the arachidonic acid-derived oxylipin thromboxane B2 ($P = 0.052$), 8,9- and 11(12)-dihydroxy-eicosatrienoic acid ($P = 0.051$ and $P = 0.085$, respectively), 8- and 20-hydroxy-eicosatetraenoic acid ($P = 0.018$ and $P = 0.021$, respectively), and 11,12-epoxy-eicosatrienoic acid ($P = 0.099$). Moreover, the plasma concentrations of 17 oxylipins also differed between LBW and HBW piglets. The effect of bBW was observed mainly for oxylipins that were derived from n-6 FAs. Compared to HBW piglets, the LBW piglets had (or tended to have) higher concentrations of 9,10- and 15,16-dihydroxy-octadecadienoic acid ($P = 0.072$ and $P = 0.048$, respectively), 9(10)- and 12(13)-epoxy-octadecenoic acid ($P = 0.087$ and $P = 0.082$, respectively), 9(s)- and 13(s)-hydroxy-octadecadienoic ($P = 0.006$ and $P = 0.050$, respectively), and 9- and 13-oxo-octadecadienoic ($P = 0.047$ and $P = 0.056$, respectively) that are derived from linoleic acid; and of 5,6-dihydroxy-eicosatrienoic acid ($P = 0.015$), and 5(s)-, 11-, 12(s)-, 15(s)- and 20-hydroxy-eicosatetraenoic acid ($P = 0.007$, $P = 0.025$, $P < 0.001$, $P = 0.017$ and $P = 0.042$, respectively) that are derived from arachidonic acid. In addition, 5(s) and 15(s)-hydroxy-EPA ($P = 0.053$ and $P = 0.003$, respectively) and 13-hydroxy-DHA ($P = 0.059$), which are derived from n-3 LCFA were also increased in the LBW piglets.

The 2D plots obtained through principal component analysis shows clearly differentiated plasma oxylipin profiles for gestating and lactating sows and piglets (Figure 1, B). However, the differences observed between sample types could not

be explained by changes in any particular oxylipin and they were due to overall changes in the whole oxylipin profile. The heatmap represented in Figure 2 illustrates how the plasma concentrations of n-3 FA-derived oxylipins in gestating and lactating sows and piglets were clearly increased in the fish oil group. Moreover, it can also be observed that, independently of the dietary treatment, sows presented higher concentrations of oxylipins derived from a-linolenic acid and linolenic acid, while piglets presented higher concentrations of arachidonic acid-derived metabolites. Finally, schematic overviews of the oxylipins from different FA precursors that were modified by dietary n-3 LCFA inclusion or by bBW are summarised in Supplementary Figures S2 and S3.

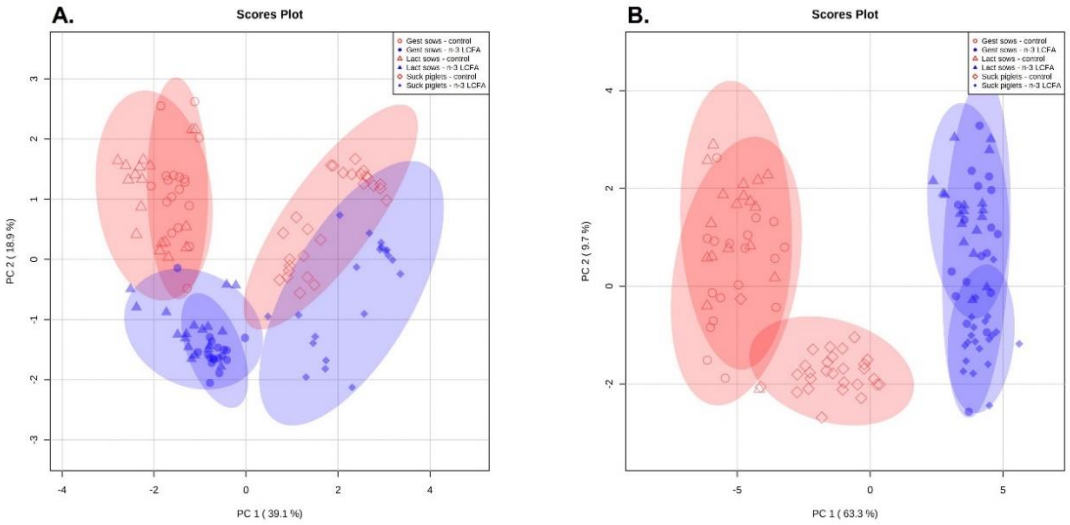


***Figure 1:*** *Principal component analysis 2 dimensions score plot showing the effect of dietary fish oil on fatty acids (FA) in serum (A) and oxylipins in plasma (B) of gestating and lactating sows and suckling piglets. Values are means of 18 samples per treatment in gestating and lactating sows, and means of 28 control samples and 20 n-3 LCFA samples in suckling piglets. Gest sows, gestating sows; Lact sows, lactating sows; LCFA, long chain fatty acid; Suck piglets, suckling piglets.*

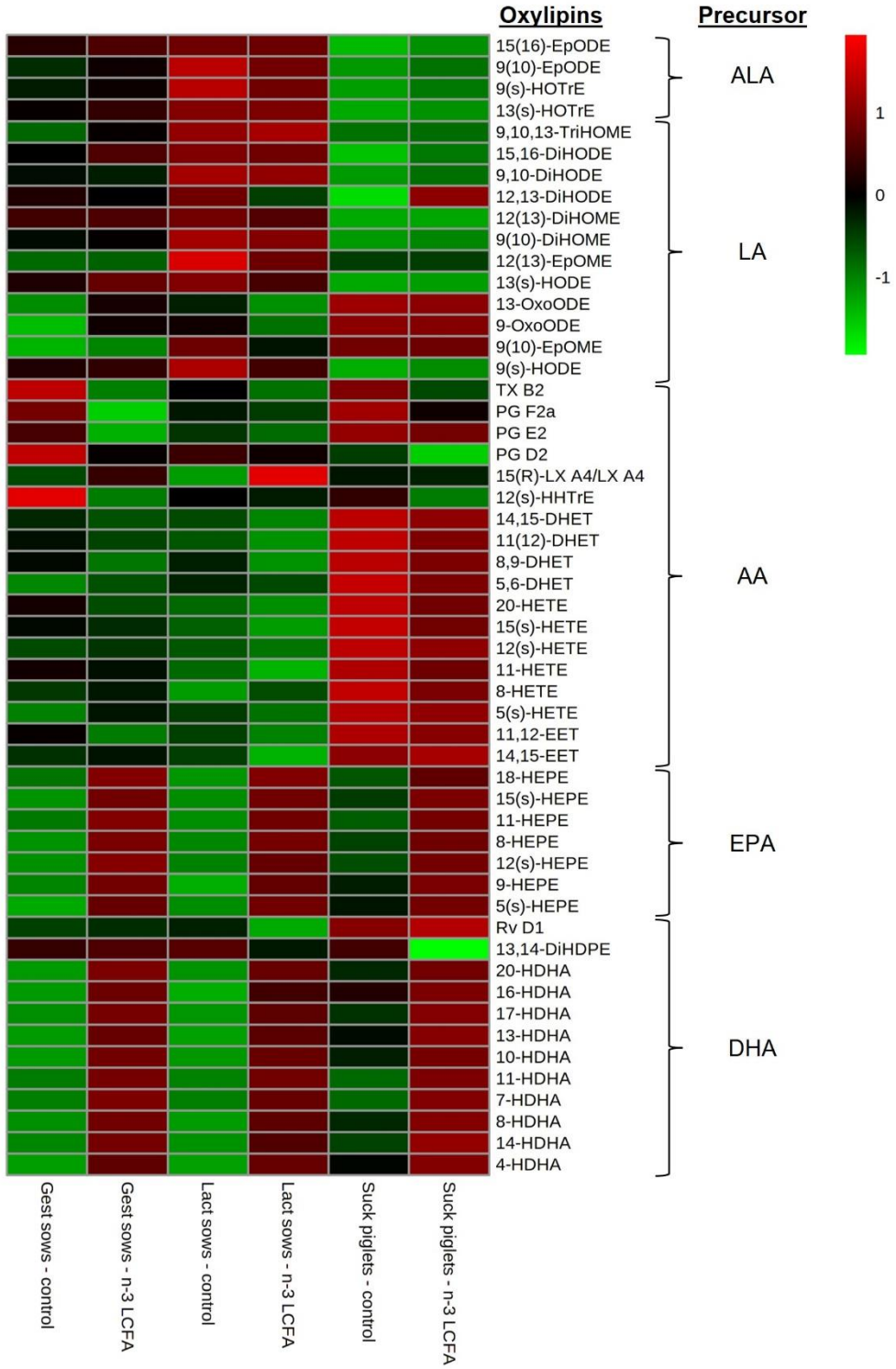


***Figure 2:*** *Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)-derived oxylipins are increased in plasma of gestating and lactating fish oil-fed sows, and suckling piglets also from fish oil-fed sows. Each coloured cell on the map corresponds to a concentration value*

*being green lower concentrations and red higher concentrations. Values are means of 18 samples per treatment in gestating and lactating sows, and means of 28 control samples and 20 n-3 LCFA samples in suckling piglets. AA, arachidonic acid; ALA, α-linolenic acid; DiHDPE, dihydroxy-docosapentaenoic acid; DiHODE, dihydroxy-octadecadienoic acid; DiHOME, dihydroxy-octadecenoic acid; DHET, dihydroxy-eicosatrienoic acid; EET, epoxy-eicosatrienoic acid; EpODE, epoxy-octadecadienoic acid; EpOME, epoxy-octadecenoic acid; Gest sows, gestating sows; HDHA, hydroxy-docosahexaenoic acid; HEPE, hydroxy-eicosapentaenoic acid; HETE, hydroxy-eicosatetraenoic acid; HHTrE, hydroxy-heptadecatrienoic acid; HODE, hydroxy-octadecadienoic acid; HOTrE, hydroxy-octadecatrienoic acid; LA, linoleic acid; Lact sows, lactating sows; LCFA, long chain fatty acid; LT, Leukotriene; LX, Lipoxin; OxoODE, oxo-octadecadienoic acid; PG, Prostaglandin; Suck piglets, suckling piglets; TriHOME, trihydroxy-octadecenoic acid; TX, Thromboxane.*

*Immunological analysis*

The plasma concentrations of Ig and cytokines in gestating and lactating sows are shown in Table 3. Dietary fish oil increased IgM in both gestating and lactating sows ($P = 0.014$ and $P = 0.008$, respectively) and IL6 in lactating sows.

***Table 3**: Plasma immune indicators in gestating and lactating sows fed control or n-3 LCFA diets.*[1]

| | Gestating sows | | | | Lactating sows[2] | | | |
|---|---|---|---|---|---|---|---|---|
| | Control (n=18) | n-3 LCFA (n=17) | RMSE | *P* value | Control (n=13) | n-3 LCFA (n=14) | RMSE | *P* value |
| Immunoglobulins (mg/mL) | | | | | | | | |
| IgG | 130 | 159 | 52.2 | 0.396 | 5.27 | 5.08 | 1.51 | 0.864 |
| IgA | 2.55 | 3.14 | 0.54 | 0.108 | 1.44 | 1.62 | 0.49 | 0.614 |
| IgM | 3.95 | 4.86 | 0.53 | 0.014 | 4.43 | 6.34 | 0.91 | 0.008 |
| Cytokines (ng/mL) | | | | | | | | |
| IL1β | 17.4 | 26.0 | 12.5 | 0.314 | 145 | 204 | 80.8 | 0.322 |
| IL6 | 34.8 | 83.1 | 45.7 | 0.116 | 136 | 421 | 147 | 0.012 |
| IL10 | 1.89 | 11.2 | 10.6 | 0.198 | 0.11 | 0.68 | 0.64 | 0.240 |
| TNFα | 1.01 | 1.44 | 0.84 | 0.444 | 1.40 | 0.91 | 1.20 | 0.579 |

IgA, immunoglobulin A; IgG, Immunoglobulin G; IgM, immunoglobulin M; IL1β, interleukin 1β; IL6, interleukin 6; IL10, interleukin 10; TNFα, tumor necrosis factor α.

[1]Values are least squares means ± RMSE.

[2] Samples from the second batch of sows (5 from control diet and 4 from n-3 LCFA diet) could not be analysed due to a conservation issue.

The plasma immune indicators in piglets are reported in Table 4. An interaction between dietary treatment and bBW was observed for IgA ($P$ = 0.043). The plasmatic concentration of IgA was highest in the HBW piglets of the control group, which differed from those of control-LBW and n-3 LCFA-HBW piglets. Regarding the cytokines, both dietary treatment and bBW group had significant effects on IL1β concentrations, which were higher in the n-3 LCFA than the control piglets ($P$ = 0.018) and in the HBW than the LBW piglets ($P$ = 0.002).

***Table 4:*** *Plasma immune indicators of suckling piglets from sows fed control or n-3 LCFA diet, born with low or high body weight.*[1,2]

| | Suckling piglets | | | | | | |
|---|---|---|---|---|---|---|---|
| | MDiet | | bBW | | | | |
| | Control (n=28) | n-3 LCFA (n=20) | HBW (n=24) | LBW (n=24) | RMSE | *P* value Diet | *P* value bBW |
| Immunoglobulins (mg/mL) | | | | | | | |
| IgG | 7.46 | 12.0 | 10.6 | 8.88 | 1.80 | 0.110 | 0.112 |
| IgA* | 0.42 | 0.37 | 0.41 | 0.38 | 0.07 | 0.373 | 0.431 |
| IgM | 0.62 | 0.68 | 0.67 | 0.63 | 0.31 | 0.723 | 0.622 |
| Cytokines (ng/mL) | | | | | | | |
| IL1β | 6.08 | 22.1 | 15.1 | 13.1 | 2.11 | 0.018 | 0.002 |
| IL6 | 13.5 | 17.0 | 17.7 | 12.8 | 19.0 | 0.802 | 0.389 |
| IL10 | 0.24 | 0.54 | 0.39 | 0.39 | 0.32 | 0.132 | 0.897 |
| TNFα | 0.22 | 0.29 | 0.28 | 0.23 | 0.18 | 0.580 | 0.375 |

bBW, birth weight; HBW, high birth weight piglets; IgA, immunoglobulin A; IgG, Immunoglobulin G; IgM, immunoglobulin M; IL1β, interleukin 1β; IL6, interleukin 6; IL10, interleukin 10; LBW, low birth weight piglets; LCFA, long chain fatty acid; MDiet, maternal diet; TNFα, tumor necrosis factor α.

[1]Values are least squares means ± RMSE.

[2]The *P* value of the interaction MDiet*bBW is not reported in the table since only a significant *P* values were observed for IgA and n-3 concentrations.

**P* value of the interaction MDiet*bBW for IgA was *P* = 0.043 where the concentrations in mg/mL were $0.37^{b}$ for control-LBW, $0.48^{a}$ for control-HBW, $0.40^{ab}$ for n-3 LCFA-LBW, and $0.35^{b}$ for n-3 LCFA-HBW.

*Correlations between fatty acids or oxylipins and immune indicators*

In gestating sows, the IgM concentration was negatively correlated with eicosadienoic acid (*r* = -0.36, *P* = 0.030; Figure 3, A) and positively correlated with 5(s)-, 9-, 11- and 12(s)-hydroxy-EPA (*r* = 0.41, *P* = 0.013; *r* = 0.38, *P* = 0.030; *r* =

0.37, $P = 0.027$ and $r = 0.36$, $P = 0.030$, respectively), and 8- and 11-hydroxy-DHA ($r = 0.34$, $P = 0.042$ and $r = 0.35$, $P = 0.039$, respectively; Figure 3, B).

In lactating sows, the concentrations of IgM and IL6 were negatively correlated with those of the n-6 family FAs γ-linolenic acid ($r = -0.42$, $P = 0.028$ and $r = -0.54$, $P = 0.004$, respectively) and arachidonic acid ($r = -0.44$, $P = 0.021$ and $r = -0.39$, $P = 0.044$, respectively) (Figure 3, C). Moreover, IgM was positively correlated with the arachidonic acid-derived oxylipins 15(R)-Lipoxin A4/ Lipoxin A4 ($r = 0.39$, $P = 0.042$) and 8-hydroxy-eicosatetraenoic acid ($r = 0.41$, $P = 0.035$), and all the detected hydroxy-EPAs and hydroxy-DHAs (all $r \geq 0.39$, $P \leq 0.017$), particularly with 14-hydroxy-DHA for which a relatively high positive correlation was observed ($r = 0.64$, $P < 0.001$). IL6 was also positively correlated with most of the hydroxy-EPA and hydroxy-DHA oxylipins (all $r \geq 0.39$, $P \leq 0.046$; Figure 3, D).

In piglets, IL1β was positively correlated with the n-3 family FAs: α-linolenic acid ($r = 0.34$, $P = 0.021$), EPA ($r = 0.57$, $P < 0.001$) and docosapentaenoic ($r = 0.49$, $P < 0.001$) acid (Figure 3, E). Moreover, IL1β was also positively correlated with 15(16)-epoxy-octadecadienoic acid which is derived from α-linolenic acid ($r = 0.38$, $P = 0.007$); 9,10-dihydroxy-octadecadienoic acid ($r = 0.32$, $P = 0.026$) and 9(s)-hydroxy-octadecadienoic acid ($r = 0.45$, $P = 0.001$) derived from linoleic acid; all hydroxy-EPA detected, derived from EPA; and most hydroxy-DHA detected, derived from DHA (all $r \geq 0.49$, $P < 0.001$; Figure 3, F). Among the positive correlations observed, those with 4- and 14-hydroxy-DHA stand out ($r = 0.70$, $P < 0.001$ and $r = 0.72$, $P < 0.001$, respectively). Finally, a negative correlation was also observed between IL1β and the DHA-derived oxylipin 13,14-dihydroxy-docosapentaenoic acid ($r = -0.44$, $P = 0.002$).

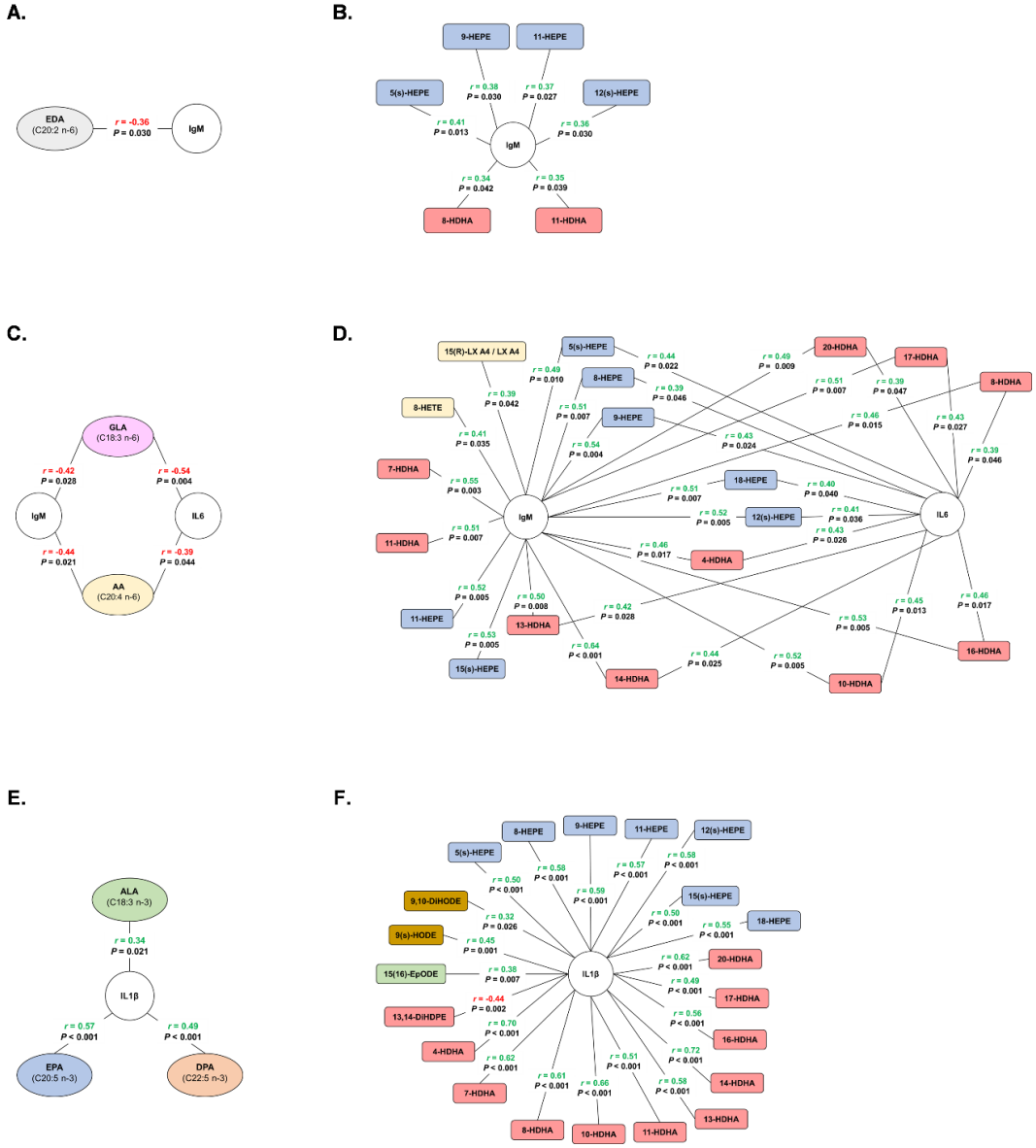


***Figure 3:*** *Correlations between FA and oxylipins and immune indicators modified by n-3 LCFA diet for gestating (A and B) and lactating sows (C and D), and suckling piglets (E and F). Pearson correlation coefficient (r) in red and in green indicates negative and positive correlation, respectively. Significant correlation level was set at P < 0.05. Fatty acids and their derived oxylipins are represented by the same colour. AA, arachidonic acid; ALA, α-linolenic acid; DiHDPE, dihydroxy-docosapentaenoic acid; DiHODE, dihydroxy-octadecadienoic acid; EDA, eicosadienoic acid; EPA, eicosapentaenoic acid; EpODE, epoxy-octadecadienoic acid; DPA, docosapentaenoic acid; GLA, γ-linolenic acid; HDHA, hydroxy-docosahexaenoic acid; HEPE, hydroxy-eicosapentaenoic acid; HETE, hydroxy-*

*eicosatetraenoic acid; HODE, hydroxy-octadecadienoic acid; IgM, immunoglobulin M; IL1β, interleukin 1β; IL6, interleukin 6; LX, Lipoxin.*

## Discussion

Piglets are born with low energy stores and without an effective immune system (Le Dividich *et al*., 2005), and recently, this has been further aggravated by the increasing proportion of LBW piglets born from hyperprolific sows, which makes them even more vulnerable (Farmer and Edwards, 2022). Changing the source of fat in sow (and the consequent changes in colostrum and milk composition) and piglet diets could have a positive impact on the physiology of newborn piglets, particularly as it has been described that they are able to digest more than 90 % of the lipids present in colostrum and milk (Azain, 2001). In the current study, the inclusion of fish oil in sow diets increased the total amount of n-3 polyunsaturated FAs in serum from gestating and lactating sows mainly by increasing the amounts of EPA, docosapentaenoic acid and DHA. Moreover, the n-3 LCFA diet also decreased the concentration of some minor saturated or n-6 polyunsaturated FAs such as heneicosylic acid, γ-linolenic acid, eicosadienoic acid and arachidonic acid, without modifying the amounts of total saturated or n-6 polyunsaturated FAs. In addition, the changes in the FA profile of piglets' serum observed with the n-3 LCFA-rich diet are in line with those observed in gestating and lactating sows and with those recently reported by our research group in the colostrum and milk of the same sows (Llauradó-Calero *et al*., 2021). Furthermore, the changes observed in the serum of suckling piglets were also in good agreement with studies that evaluated different dietary n-6: n-3 ratios in serum of presuckling piglets (Eastwood *et al*., 2014) and plasma of piglets at day 21 of lactation (Yao *et al*., 2012).

It is well established that polyunsaturated FAs can influence the coordination of a balanced inflammatory response. One of the mechanisms of action could be through oxidation processes via enzymatic or non-enzymatic pathways and the consequent formation of oxylipins (Calder, 2010). Each oxygenated FA derivative

can exert a proinflammatory and/or anti-inflammatory activity (Gabbs *et al*., 2015), which could influence not only the immune system of sows, but also that of piglets, which may be particularly relevant as they are born with an absolute absence of immune protection (Le Dividich *et al*., 2005). Moreover, oxylipins derived from n-3 FAs tend to have weak proinflammatory, anti-inflammatory or inflammation resolving properties, whereas those derived from n-6 FA are usually associated with proinflammatory activities (Calder, 2010). The enhanced concentrations of EPA and DHA in the serum of gestating and lactating sows and of piglets resulted in increases of their hydroxy-derivatives in plasma, which are mainly generated through the lipoxygenase enzymatic pathway, in some specific cases through the cytochrome P450 enzymatic pathway, or also via non-enzymatic pathways (Astarita *et al*., 2015). Although, for most of these oxylipins, precise functions have only been described in specific murine or human cell lines, some of them, such as 18- hydroxy-EPA, 13-hydroxy-DHA or 17-hydroxy-DHA, are related with an anti-inflammatory role and with the inhibition of the production of proinflammatory cytokines such as TNFα (Gabbs *et al*., 2015). In addition, these results are also in line with those reported by our group in colostrum and milk (Llauradó-Calero *et al*., 2021), which suggests a direct influence of including fish oil in the sow diet on the oxylipin profile of the suckling piglets by increasing the concentration of oxylipins with an anti-inflammatory role. In addition, the n-3 LCFA diet also changed the concentration of oxylipins derived from other FAs. In the review of Shearer and Walker (2018), it is described that a major increase in circulating n-3 oxylipins commonly leads to a decrease in n-6 FA-derived oxylipins, which was also observed in this study, particularly for those derived from arachidonic acid. However, Shearer and Walker (2018) also report that in some cases, oxylipins from n-6 FAs can be increased with n-3 FA supplementation, which could explain the increases observed for certain oxylipins derived from linoleic acid or arachidonic acid. Among the changes caused by n-3 LCFA diet in the piglets' plasma concentrations of n-6 FA-derived oxylipins, the reduced levels of 20-hydroxy-eicosatetraenoic acid stand out, and this oxylipin has been described to stimulate proinflammatory cytokine production in human endothelial cells (Gabbs *et al*.,

2015). In addition, two final oxidation products were modified by fish oil: concretely, lipoxin A4 was increased in gestating and lactating sows and thromboxane B2 tended to decrease in suckling piglets. On the one hand, lipoxin A4 is the main physiological lipoxin during inflammation in mammalian systems and has been proven to have a powerful anti-inflammatory role under many pathological conditions that trigger inflammation (Shi *et al*., 2017). In contrast, thromboxane B2 is related with vascular resistance, vasoconstriction activity and platelet aggregation (Gabbs *et al*., 2015).

To our knowledge, this is the first study assessing the effect of bBW on plasma oxylipin concentrations. It was observed that some oxylipins derived from the n-6 FA linoleic acid and arachidonic acid were increased in LBW piglets. However, for most of them, a well-defined function is not known yet, which makes it difficult to understand the role that bBW may have on oxylipin-mediated activities.

Moreover, both, n-3 FAs and their oxygenated derivates, can influence the production of certain Ig (Mitre *et al*., 2005; Calder, 2010; Yao *et al*., 2012). In the current study, n-3 LCFA increased IgM concentrations in the blood of gestating and lactating sows, although no changes in IgM concentration were reported in the colostrum and milk of the same sows (Llauradó-Calero *et al*., 2021). Other studies evaluating the effect of n-3 FAs in sow diets describe changes in the concentration of immunoglobulins in plasma of suckling piglets at different times during lactation (Mitre *et al*., 2005; Leonard *et al*., 2010; Yao *et al*., 2012), yet immunoglobulin concentrations in suckling piglets were not affected in the current study. However, in all the mentioned reports, the effect of increasing the concentration of the different types of immunoglobulins fades as lactation progresses. Leonard *et al*. (2010) observed differences in IgM concentration in the serum of piglets from sows that were supplemented with shark-oil at the beginning of lactation but not at the time of weaning, which could explain why we did not observe any changes at the end of lactation in our study. In addition, we showed that IgM was positively correlated with the increase of oxylipins derived from n-3 FAs, but negatively correlated with FAs from the n-6 family such as eicosadienoic acid, γ-linolenic acid

and arachidonic acid. In terms of cytokine production, increases in the plasmatic concentrations of IL6 in lactating sows and IL1β in suckling piglets were observed with the n-3 LCFA diet, whereas cytokine IL1β was also higher in HBW than LBW piglets. On the one hand, IL6 is a soluble mediator with a pleiotropic effect on inflammation and immune response (Tanaka *et al*., 2014), and the role that its increase may play, without modifications on other cytokines concentrations, is not clear. Furthermore, in line with our results for plasmatic IgM, IL6 concentration was negatively correlated with n-6 FAs and positively correlated with most of the n-3 FA-derived oxylipins. On the other hand, IL1β is considered to be one of the major proinflammatory cytokines (Nordgreen *et al*., 2020). The observed increase in the concentration of IL1β has also been reported by Yao *et al*., 2012 in suckling piglets from sows that were fed a diet with an n-6:n-3 ratio of 3:1, which is similar to the ratio used in this experiment, in comparison to ratios of 9:1 or 13:1. Therefore, and considering that the inclusion of n-3 LCFA in the diets resulted in increases of oxylipins with anti-inflammatory roles, and that the concentration of IL1β was positively correlated with both, the n- 3 FAs and their oxygenated derivatives, future studies evaluating a wider range of cytokines and their relationship with n-3 FAs in piglets may help to better understand the results obtained in this study.

## Conclusion

This study provides a picture of precursors, intermediate molecules, and final mediators in serum and plasma of gestating sows, lactating sows and suckling piglets that had been fed diets with or without supplementation with n-3 LCFA, which complement our previous study in which these parameters were described in colostrum and milk. It can be concluded that the dietary inclusion of fish oil rich in EPA and DHA offered to sows during gestation and lactation increases the blood concentrations of EPA, DHA and their oxygenated derivatives with anti-inflammatory role in sows and suckling piglets. In addition, dietary n-3 LCFA also had an impact on immune indicators such as IgM, which was increased in gestating and lactating sows, and IL6 and IL1β that were increased in lactating sows and

suckling piglets, respectively. In addition, although the bBW of the piglets had little effect on FA composition, it was observed that LBW piglets had higher concentrations of oxylipins derived from n-6 FA (i.e. linoleic acid or arachidonic acid) and reduced IL1β compared to HBW piglets. Overall, further research is required to confirm the feasibility of improving piglets' vitality through the inclusion of n-3 LCFA in sow and piglet diets.

## Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.animal.2022.100634.

## Ethics approval

IRTA's Ethical Committee on Animal Experimentation approved the use of animals for this experiment in accordance with the Directive 2010/63/EU of 22 September 2010 and according to the recommendation of the European Commission 2007/526/CE, the Spanish guidelines for the care and use of animals in research (B. O.E. number 34, Real Decreto 53/2013) and the regional regulations on the use and handling of experimental animals (Decree 214/97, Generalitat de Catalunya) (project number: 10294).

## Data and model availability statement

None of the data were deposited in an official repository. The data that support the study findings are available upon request.

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## Declaration of interest

The authors report no conflict of interests.

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## Supplementary materials

***Supplementary Table S1:*** *Plasma oxylipin quantification from gestating sows fed control or n-3 LCFA diet.*[1]

| | | Gestating sows | | | |
|---|---|---|---|---|---|
| | Precursor | Control (n=18) | n-3 LCFA (n=18) | RMSE | *P* value |
| Oxylipin (pg/mL) | | | | | |
| 15(16)-EpODE | ALA | 14 222 | 19 734 | 0.22 | 0.096 |
| 9(10)-EpODE | ALA | 565 | 630 | 0.21 | 0.178 |
| 9(s)-HOTrE | ALA | 465 | 496 | 0.18 | 0.265 |
| 13(s)-HOTrE | ALA | 1 599 | 1 779 | 0.15 | 0.103 |
| 9,10,13-TriHOME | LA | 551 | 1 835 | 0.39 | 0.348 |
| 15,16-DiHODE | LA | 167 | 248 | 0.20 | 0.069 |
| 9,10-DiHODE | LA | 213 | 191 | 0.26 | 0.559 |
| 12,13-DiHODE | LA | 244 | 276 | 0.31 | 0.237 |
| 12(13)-DiHOME | LA | 4 841 | 5 007 | 0.16 | 0.603 |
| 9(10)-DiHOME | LA | 6 105 | 6 548 | 0.18 | 0.491 |
| 12(13)-EpOME | LA | 3 851 | 3 504 | 0.23 | 0.914 |
| 13(s)-HODE | LA | 10 318 | 12 332 | 0.15 | 0.110 |
| 13-OxoODE | LA | 379 | 692 | 0.35 | 0.253 |
| 9-OxoODE | LA | 537 | 1 011 | 0.34 | 0.325 |
| 9(10)-EpOME | LA | 641 | 728 | 0.24 | 0.642 |
| 9(s)-HODE | LA | 24 711 | 25 648 | 0.19 | 0.626 |
| Thromboxane B2 | AA | 1 131 | 1 373 | 0.72 | 0.176 |
| Prostaglandin F2a | AA | 600 | 794 | 0.97 | 0.239 |
| Prostaglandin E2 | AA | 142 | 269 | 0.72 | 0.192 |

| | | | | | |
|---|---|---|---|---|---|
| Prostaglandin D2 | AA | 190 | 230 | 0.66 | 0.337 |
| 15(R)-Lipoxin A4/Lipoxin A4 | AA | 0.63 | 3.60 | 0.34 | 0.012 |
| 12(s)-HHTrE | AA | 1 271 | 1 659 | 0.77 | 0.156 |
| 14,15-DHET | AA | 200 | 161 | 0.10 | 0.010 |
| 11(12)-DHET | AA | 242 | 177 | 0.14 | 0.014 |
| 8,9-DHET | AA | 9.92 | 6.15 | 0.12 | <0.001 |
| 5,6-DHET | AA | 8.69 | 9.10 | 0.14 | 0.521 |
| 20-HETE | AA | 378 | 221 | 0.36 | 0.008 |
| 15(s)-HETE | AA | 225 | 291 | 0.34 | 0.772 |
| 12(s)-HETE | AA | 128 | 150 | 0.15 | 0.281 |
| 11-HETE | AA | 217 | 255 | 0.27 | 0.611 |
| 8-HETE | AA | 46.1 | 55.7 | 0.13 | 0.380 |
| 5(s)-HETE | AA | 52.9 | 229 | 0.34 | 0.228 |
| 11,12-EET | AA | 14.0 | 10.1 | 0.37 | 0.076 |
| 14,15-EET | AA | 3.66 | 4.65 | 0.39 | 0.550 |
| 18-HEPE | EPA | 17.4 | 581 | 0.17 | <0.001 |
| 15(s)-HEPE | EPA | 0.81 | 339 | 0.16 | <0.001 |
| 11-HEPE | EPA | 0.94 | 247 | 0.20 | <0.001 |
| 8-HEPE | EPA | ND | 560 | 0.09 | <0.001 |
| 12(s)-HEPE | EPA | 2.58 | 247 | 0.21 | <0.001 |
| 9-HEPE | EPA | 11.6 | 249 | 0.35 | <0.001 |
| 5(s)-HEPE | EPA | 2.71 | 391 | 0.27 | <0.001 |
| Resolvin D1 | DHA | 23.3 | 24.8 | 0.14 | 0.159 |
| 13,14-DiHDPE | DHA | 144 | 151 | 0.16 | 0.787 |
| 20-HDHA | DHA | ND | 204 | 0.12 | <0.001 |

| | | | | | |
|---|---|---|---|---|---|
| 16-HDHA | DHA | 1.20 | 135 | 0.20 | <0.001 |
| 17-HDHA | DHA | 2.11 | 146 | 0.21 | <0.001 |
| 13-HDHA | DHA | 4.22 | 177 | 0.20 | <0.001 |
| 10-HDHA | DHA | 3.35 | 304 | 0.24 | <0.001 |
| 11-HDHA | DHA | 0.47 | 42.0 | 0.17 | <0.001 |
| 7-HDHA | DHA | ND | 96.7 | 0.11 | <0.001 |
| 8-HDHA | DHA | 1.15 | 117 | 0.18 | <0.001 |
| 14-HDHA | DHA | 0.43 | 20.7 | 0.23 | <0.001 |
| 4-HDHA | DHA | 1.28 | 90.4 | 0.19 | <0.001 |

AA, arachidonic acid; ALA, α-linolenic acid; DHA, docosahexaenoic acid; DiHDPE, dihydroxy-docosapentaenoic acid; DiHODE, dihydroxy-octadecadienoic acid; DiHOME, dihydroxy-octadecenoic acid; DHET, dihydroxy-eicosatrienoic acid; EET, epoxy-eicosatrienoic acid; EPA, eicosapentaenoic acid; EpODE, epoxy-octadecadienoic acid; EpOME, epoxy-octadecenoic acid; HDHA, hydroxy-docosahexaenoic acid; HEPE, hydroxy-eicosapentaenoic acid; HETE, hydroxy-eicosatetraenoic acid; HHTrE, hydroxy-heptadecatrienoic acid; HODE, hydroxy-octadecadienoic acid; HOTrE, hydroxy-octadecatrienoic acid; LA, linoleic acid; LCFA, long chain fatty acid; ND, non-detected; OxoODE, oxo-octadecadienoic acid; TriHOME, trihydroxy-octadecenoic acid.

[1]Values are the mean ± RMSE (RMSE was obtained from transformed data).

***Supplementary Table S2:*** *Plasma oxylipin quantification from lactating sows fed control or n-3 LCFA diet.*[1]

| | | Lactating sows | | | |
|---|---|---|---|---|---|
| | Precursor | Control (n=18) | n-3 LCFA (n=18) | RMSE | *P* value |
| Oxylipin (pg/mL) | | | | | |
| 15(16)-EpODE | ALA | 23 093 | 21 729 | 0.20 | 0.946 |
| 9(10)-EpODE | ALA | 1 283 | 950 | 0.19 | 0.072 |
| 9(s)-HOTrE | ALA | 1 037 | 788 | 0.18 | 0.044 |

| | | | | | |
|---|---|---|---|---|---|
| 13(s)-HOTrE | ALA | 2 467 | 2 445 | 0.17 | 0.689 |
| 9,10,13-TriHOME | LA | 884 | 950 | 0.14 | 0.588 |
| 15,16-DiHODE | LA | 256 | 244 | 0.16 | 0.944 |
| 9,10-DiHODE | LA | 1 086 | 935 | 0.28 | 0.473 |
| 12,13-DiHODE | LA | 294 | 286 | 0.47 | 0.777 |
| 12(13)-DiHOME | LA | 5 797 | 5 410 | 0.15 | 0.219 |
| 9(10)-DiHOME | LA | 16 769 | 13 941 | 0.17 | 0.198 |
| 12(13)-EpOME | LA | 7 606 | 6 550 | 0.20 | 0.071 |
| 13(s)-HODE | LA | 12 822 | 11 424 | 0.13 | 0.126 |
| 13-OxoODE | LA | 296 | 272 | 0.23 | 0.224 |
| 9-OxoODE | LA | 501 | 465 | 0.18 | 0.244 |
| 9(10)-EpOME | LA | 1 310 | 861 | 0.18 | 0.012 |
| 9(s)-HODE | LA | 34 892 | 28 624 | 0.17 | 0.105 |
| Thromboxane B2 | AA | 887 | 294 | 0.54 | 0.562 |
| Prostaglandin F2a | AA | 617 | 316 | 0.79 | 0.855 |
| Prostaglandin E2 | AA | 227 | 54.5 | 0.70 | 0.740 |
| Prostaglandin D2 | AA | 176 | 72.1 | 0.55 | 0.886 |
| 15(R)-Lipoxin A4/Lipoxin A4 | AA | 0.08 | 9.55 | 0.28 | <0.001 |
| 12(s)-HHTrE | AA | 1 222 | 410 | 0.62 | 0.904 |
| 14,15-DHET | AA | 173 | 128 | 0.15 | 0.021 |
| 11(12)-DHET | AA | 176 | 125 | 0.19 | 0.043 |
| 8,9-DHET | AA | 9.47 | 6.32 | 0.20 | 0.021 |
| 5,6-DHET | AA | 10.1 | 9.22 | 0.13 | 0.550 |
| 20-HETE | AA | 200 | 171 | 0.54 | 0.459 |
| 15(s)-HETE | AA | 212 | 144 | 0.25 | 0.516 |

| | | | | | |
|---|---|---|---|---|---|
| 12(s)-HETE | AA | 119 | 102 | 0.13 | 0.097 |
| 11-HETE | AA | 178 | 118 | 0.24 | 0.330 |
| 8-HETE | AA | 30.7 | 42.0 | 0.13 | 0.004 |
| 5(s)-HETE | AA | 65.4 | 58.1 | 0.20 | 0.281 |
| 11,12-EET | AA | 12.0 | 7.28 | 0.34 | 0.219 |
| 14,15-EET | AA | 3.07 | 0.76 | 0.27 | 0.014 |
| 18-HEPE | EPA | 12.2 | 586 | 0.31 | <0.001 |
| 15(s)-HEPE | EPA | 1.87 | 308 | 0.24 | <0.001 |
| 11-HEPE | EPA | ND | 165 | 0.12 | <0.001 |
| 8-HEPE | EPA | 0.64 | 545 | 0.19 | <0.001 |
| 12(s)-HEPE | EPA | 4.05 | 149 | 0.24 | <0.001 |
| 9-HEPE | EPA | 6.63 | 206 | 0.27 | <0.001 |
| 5(s)-HEPE | EPA | 3.77 | 513 | 0.32 | <0.001 |
| Resolvin D1 | DHA | 28.2 | 22.1 | 0.21 | 0.209 |
| 13,14-DiHDPE | DHA | 152 | 139 | 0.23 | 0.406 |
| 20-HDHA | DHA | 0.66 | 158 | 0.19 | <0.001 |
| 16-HDHA | DHA | 0.07 | 64.9 | 0.31 | <0.001 |
| 17-HDHA | DHA | 1.74 | 91.9 | 0.22 | <0.001 |
| 13-HDHA | DHA | 2.13 | 141 | 0.19 | <0.001 |
| 10-HDHA | DHA | 4.39 | 261 | 0.27 | <0.001 |
| 11-HDHA | DHA | 0.30 | 40.1 | 0.18 | <0.001 |
| 7-HDHA | DHA | ND | 73.6 | 0.10 | <0.001 |
| 8-HDHA | DHA | 0.43 | 82.3 | 0.11 | <0.001 |
| 14-HDHA | DHA | ND | 13.7 | 0.18 | <0.001 |
| 4-HDHA | DHA | 1.00 | 107 | 0.16 | <0.001 |

AA, arachidonic acid; ALA, α-linolenic acid; DHA, docosahexaenoic acid; DiHDPE, dihydroxy-docosapentaenoic acid; DiHODE, dihydroxy-octadecadienoic acid; DiHOME, dihydroxy-octadecenoic acid; DHET, dihydroxy-eicosatrienoic acid; EET, epoxy-eicosatrienoic acid; EPA, eicosapentaenoic acid; EpODE, epoxy-octadecadienoic acid; EpOME, epoxy-octadecenoic acid; HDHA, hydroxy-docosahexaenoic acid; HEPE, hydroxy-eicosapentaenoic acid; HETE, hydroxy-eicosatetraenoic acid; HHTrE, hydroxy-heptadecatrienoic acid; HODE, hydroxy-octadecadienoic acid; HOTrE, hydroxy-octadecatrienoic acid; LA, linoleic acid; LCFA, long chain fatty acid; ND, non-detected; OxoODE, oxo-octadecadienoic acid; TriHOME, trihydroxy-octadecenoic acid.

[1]Values are the mean ± RMSE (RMSE was obtained from transformed data).

***Supplementary Table S3:*** *Plasma oxylipin quantification from suckling piglets from sows fed control or n-3 LCFA diet.*[1,2]

| | | Suckling piglets | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | MDiet | | bBW | | | | |
| | Precursor | Control (n=28) | n-3 LCFA (n=20) | HBW (n=24) | LBW (n=24) | RMSE | *P* value MDiet | *P* value bBW |
| Oxylipin (pg/mL) | | | | | | | | |
| 15(16)-EpODE | ALA | 2 872 | 3 799 | 3 091 | 3 426 | 0.16 | 0.014 | 0.296 |
| 9(10)-EpODE | ALA | 327 | 375 | 329 | 364 | 0.15 | 0.275 | 0.321 |
| 9(s)-HOTrE | ALA | 249 | 284 | 246 | 282 | 0.12 | 0.304 | 0.108 |
| 13(s)-HOTrE | ALA | 831 | 906 | 827 | 897 | 0.13 | 0.763 | 0.343 |
| 9,10,13-TriHOME | LA | 402 | 407 | 383 | 425 | 0.10 | 0.850 | 0.177 |
| 15,16-DiHODE | LA | 95.8 | 117 | 91.6 | 118 | 0.16 | 0.182 | 0.048 |
| 9,10-DiHODE | LA | 53.7 | 79.2 | 59.2 | 69.5 | 0.15 | 0.036 | 0.072 |
| 12,13-DiHODE | LA | 206 | 309 | 229 | 269 | 0.48 | 0.322 | 0.878 |
| 12(13)-DiHOME | LA | 1 660 | 1 685 | 1 576 | 1 765 | 0.13 | 0.905 | 0.175 |
| 9(10)-DiHOME | LA | 2 475 | 2 890 | 2 559 | 2 737 | 0.13 | 0.501 | 0.340 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 12(13)-EpOME | LA | 3 641 | 3 643 | 3 366 | 3 918 | 0.10 | 0.976 | 0.082 |
| 13(s)-HODE | LA | 5 865 | 5 811 | 5 336 | 6 349 | 0.12 | 0.857 | 0.050 |
| 13-OxoODE | LA | 452 | 441 | 410 | 484 | 0.15 | 0.807 | 0.056 |
| 9-OxoODE | LA | 628 | 614 | 571 | 673 | 0.14 | 0.949 | 0.047 |
| 9(10)-EpOME | LA | 1 248 | 1 236 | 1 143 | 1 343 | 0.12 | 0.898 | 0.087 |
| 9(s)-HODE | LA | 12 975 | 14 454 | 12 017 | 15 165 | 0.11 | 0.616 | 0.006 |
| Thromboxane B2 | AA | 358 | 301 | 272 | 396 | 0.33 | 0.052 | 0.194 |
| Prostaglandin F2a | AA | 330 | 302 | 363 | 274 | 0.47 | 0.484 | 0.328 |
| Prostaglandin E2 | AA | 61.0 | 73.5 | 50.9 | 81.5 | 0.31 | 0.747 | 0.120 |
| Prostaglandin D2 | AA | 32.8 | 33.1 | 27.9 | 38.0 | 0.29 | 0.346 | 0.604 |
| 15(R)-Lipoxin A4 / Lipoxin A4 | AA | 2.06 | 2.30 | 2.20 | 2.12 | 0.32 | 0.959 | 0.817 |
| 12(s)-HHTrE | AA | 356 | 333 | 285 | 407 | 0.42 | 0.190 | 0.418 |
| 14,15-DHET | AA | 661 | 563 | 597 | 644 | 0.14 | 0.205 | 0.468 |
| 11(12)-DHET | AA | 715 | 508 | 605 | 653 | 0.18 | 0.085 | 0.592 |
| 8,9-DHET | AA | 24.0 | 18.6 | 20.5 | 23.1 | 0.14 | 0.051 | 0.307 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 5,6-DHET | AA | 15.4 | 14.1 | 13.1 | 16.6 | 0.14 | 0.558 | 0.015 |
| 20-HETE | AA | 1 623 | 928 | 1 196 | 1 471 | 0.19 | 0.021 | 0.042 |
| 15(s)-HETE | AA | 311 | 259 | 257 | 321 | 0.13 | 0.115 | 0.007 |
| 12(s)-HETE | AA | 566 | 442 | 443 | 586 | 0.09 | 0.147 | <0.001 |
| 11-HETE | AA | 320 | 265 | 273 | 321 | 0.10 | 0.111 | 0.025 |
| 8-HETE | AA | 126 | 95.7 | 107 | 120 | 0.11 | 0.018 | 0.169 |
| 5(s)-HETE | AA | 131 | 123 | 112 | 144 | 0.13 | 0.489 | 0.007 |
| 11,12-EET | AA | 29.9 | 24.7 | 27.2 | 28.3 | 0.17 | 0.099 | 0.842 |
| 14,15-EET | AA | 12.0 | 15.9 | 12.4 | 14.9 | 0.23 | 0.264 | 0.201 |
| 18-HEPE | EPA | 25.0 | 346 | 139 | 178 | 0.11 | <0.001 | 0.105 |
| 15(s)-HEPE | EPA | 16.2 | 389 | 142 | 201 | 0.39 | <0.001 | 0.053 |
| 11-HEPE | EPA | 5.24 | 188 | 69.6 | 93.2 | 0.33 | <0.001 | 0.743 |
| 8-HEPE | EPA | 6.40 | 429 | 154 | 211 | 0.29 | <0.001 | 0.862 |
| 12(s)-HEPE | EPA | 12.4 | 190 | 73.7 | 99.1 | 0.30 | <0.001 | 0.469 |
| 9-HEPE | EPA | 41.8 | 269 | 122 | 150 | 0.33 | <0.001 | 0.264 |
| 5(s)-HEPE | EPA | 15.6 | 602 | 219 | 301 | 0.29 | <0.001 | 0.003 |

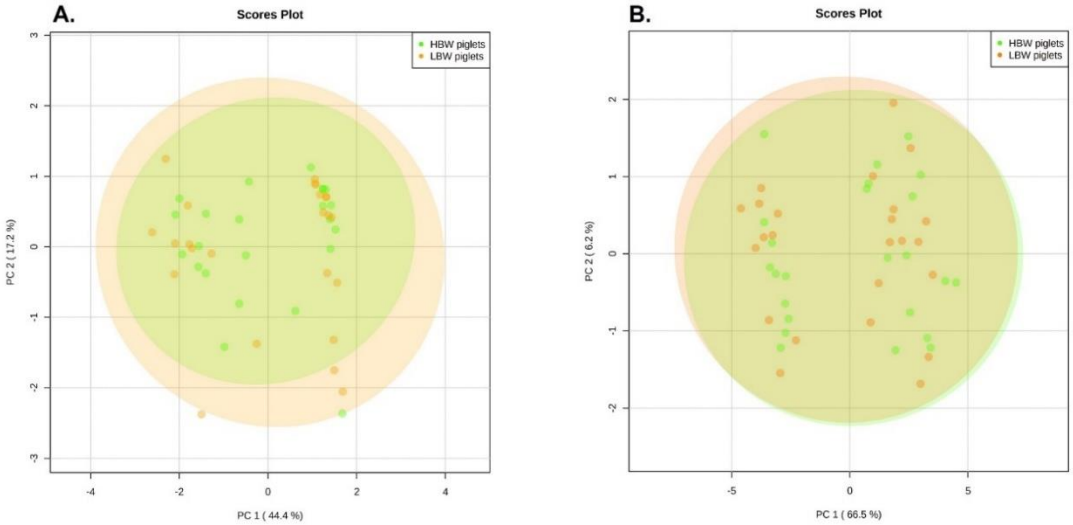
| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Resolvin D1 | DHA | 39.2 | 41.4 | 39.7 | 40.6 | 0.12 | 0.759 | 0.242 |
| 13,14-DiHDPE | DHA | 145 | 101 | 134 | 119 | 0.16 | 0.006 | 0.108 |
| 20-HDHA | DHA | 18.1 | 164 | 73.4 | 84.0 | 0.18 | <0.001 | 0.709 |
| 16-HDHA | DHA | 27.4 | 187 | 83.7 | 104 | 0.13 | <0.001 | 0.152 |
| 17-HDHA | DHA | 17.2 | 178 | 74.5 | 93.9 | 0.39 | <0.001 | 0.701 |
| 13-HDHA | DHA | 38.9 | 270 | 121 | 150 | 0.18 | <0.001 | 0.059 |
| 10-HDHA | DHA | 23.2 | 332 | 141 | 162 | 0.17 | <0.001 | 0.690 |
| 11-HDHA | DHA | 1.88 | 55.4 | 16.1 | 32.2 | 0.33 | <0.001 | 0.183 |
| 7-HDHA | DHA | 3.89 | 111 | 43.3 | 53.6 | 0.28 | <0.001 | 0.280 |
| 8-HDHA | DHA | 10.2 | 155 | 63.8 | 77.0 | 0.22 | <0.001 | 0.345 |
| 14-HDHA | DHA | 2.89 | 28.2 | 13.7 | 13.2 | 0.27 | <0.001 | 0.157 |
| 4-HDHA | DHA | 28.3 | 177 | 83.8 | 97.0 | 0.27 | <0.001 | 0.275 |

AA, arachidonic acid; ALA, α-linolenic acid; bBW, birth body weight; DHA, docosahexaenoic acid; DiHDPE, dihydroxy-docosapentaenoic acid; DiHODE, dihydroxy-octadecadienoic acid; DiHOME, dihydroxy-octadecenoic acid; DHET, dihydroxy-eicosatrienoic acid; EET, epoxy-eicosatrienoic acid; EPA, eicosapentaenoic acid; EpODE, epoxy-octadecadienoic acid; EpOME, epoxy-octadecenoic acid; HBW, high birth weight; HDHA, hydroxy-docosahexaenoic acid; HEPE, hydroxy-eicosapentaenoic acid; HETE, hydroxy-eicosatetraenoic acid; HHTrE, hydroxy-heptadecatrienoic acid; HODE, hydroxy-octadecadienoic acid; HOTrE, hydroxy-octadecatrienoic acid; LA, linoleic acid; LBW, low birth weight;

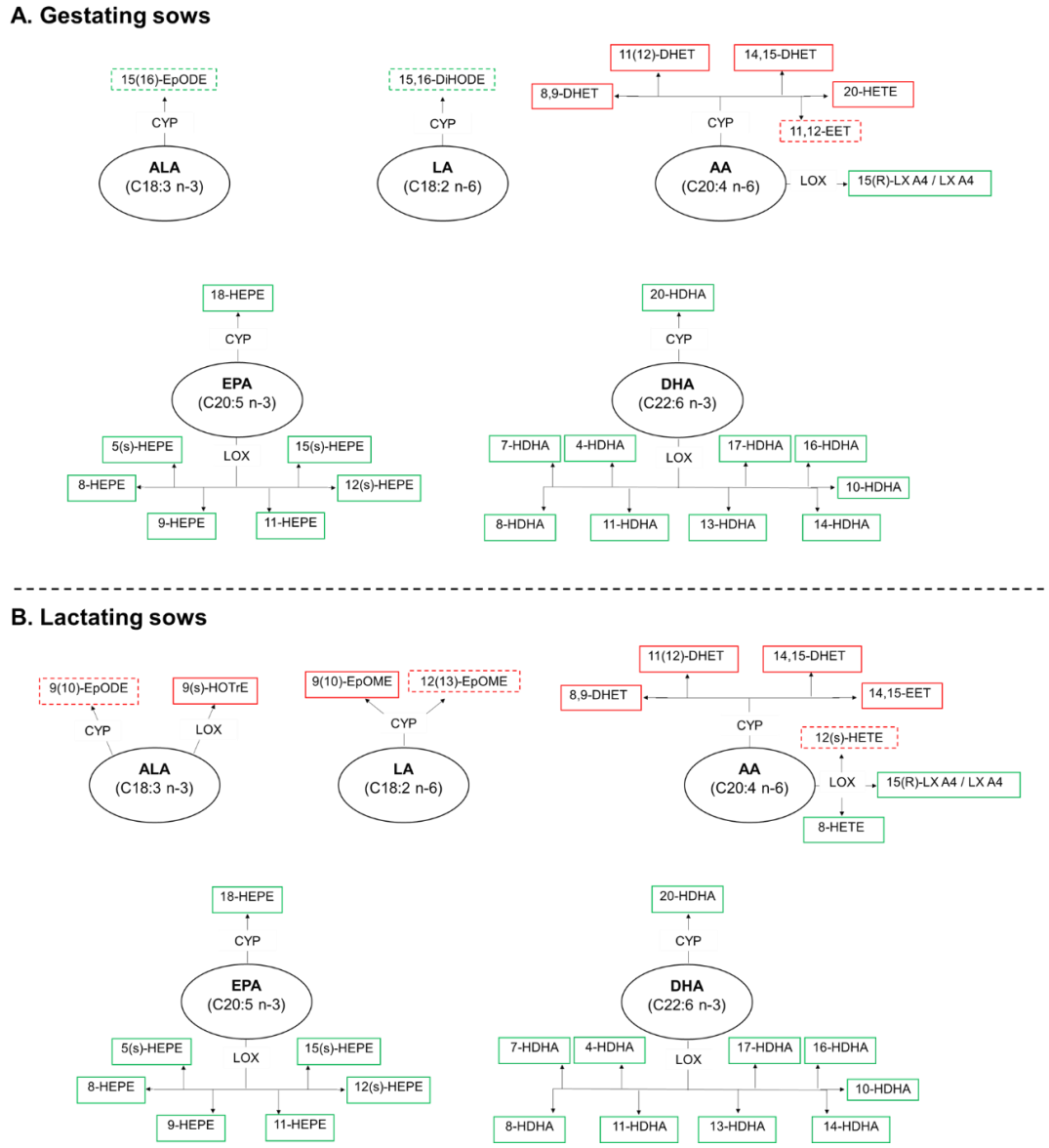
LCFA, long chain fatty acid; Mdiet, maternal diet; ND, non-detected; OxoODE, oxo-octadecadienoic acid; TriHOME, trihydroxy-octadecenoic acid.

[1]Values are the mean ± RMSE (RMSE was obtained from transformed data).

[2]The *P* value of the interaction MDiet*bBW is not reported in the table since no significant *P* values were observed in any of the oxylipins analysed.

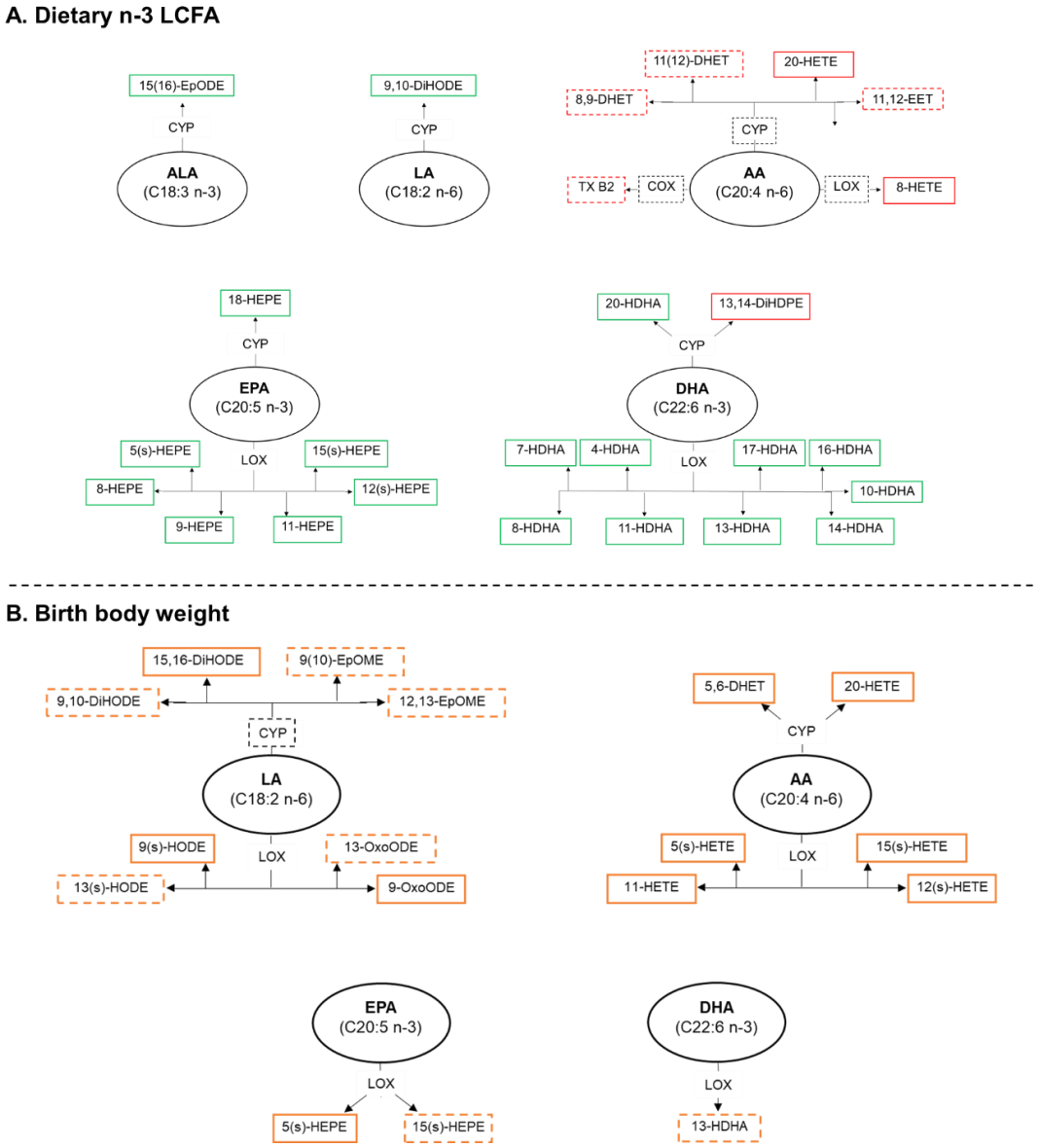



***Supplementary Figure S1:*** *Principal component analysis 2 dimensions score plot showing the influence of birth body weight of piglets (low body weight (LBW) or high body weight (HBW)) on fatty acids (FA) composition in serum (A) and oxylipins in plasma (B) of suckling piglets. Values are means of 24 samples for each birth body weight category.*



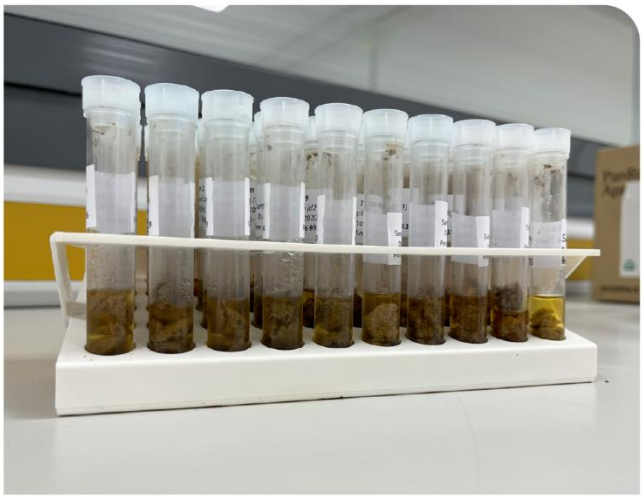

***Supplementary Figure S2:*** *Schematic overview of modified oxylipins, the enzymatic pathway involved in their generation and their fatty acid precursor in plasma from gestating (A) and lactating (B) fish oil-fed sows. Green boxes mean increased oxylipins and red boxes decreased oxylipins by the influence of dietary n-3 long chain fatty acids (n-3 LCFA). Boxes in dashes indicates P < 0.1, otherwise P < 0.05. AA, arachidonic acid; ALA, α-linolenic acid; CYP, cytochrome P450; DHA, docosahexaenoic acid; DiHODE, dihydroxy-octadecadienoic acid; DHET, dihydroxy-eicosatrienoic acid; EET, epoxy-eicosatrienoic acid; EPA, eicosapentaenoic acid; EpODE, epoxy-octadecadienoic acid; EpOME, epoxy-octadecenoic acid; HDHA, hydroxy-docosahexaenoic acid; HEPE, hydroxy-eicosapentaenoic acid; HETE,*

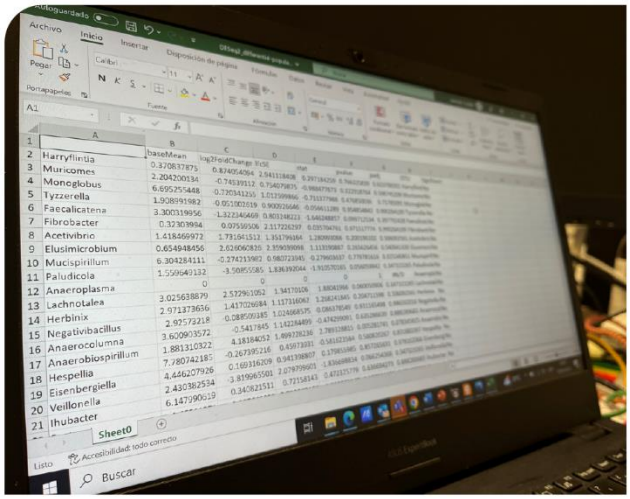
*hydroxy-eicosatetraenoic acid; HOTrE, hydroxy-octadecatrienoic acid; LA, linoleic acid; LOX, lipoxygenase; LX, lipoxin.*




***Supplementary Figure S3:*** *Schematic overview of modified oxylipins, the enzymatic pathway involved in their generation and their fatty acid precursor in plasma from suckling piglets. (A) Modified oxylipins by dietary n-3 long chain fatty acids (n-3 LCFA) and (B) modified oxylipins by birth body weight of piglets. Green boxes mean increased oxylipins and red boxes decreased oxylipins by the influence of dietary n-3 long chain fatty acids (n-3 LCFA). Orange boxes mean oxylipins increased in low birth weight piglets in comparison*

*with high birth weight piglets. Boxes in dashes indicates P < 0.1, otherwise P < 0.05. AA, arachidonic acid; ALA, α-linolenic acid; COX, cyclooxygenase; CYP, cytochrome P450; DHA, docosahexaenoic acid; DiHDPE, dihydroxy-docosapentaenoic acid; DiHODE, dihydroxy-octadecadienoic acid; DHET, dihydroxy-eicosatrienoic acid; EET, epoxy-eicosatrienoic acid; EPA, eicosapentaenoic acid; EpODE, epoxy-octadecadienoic acid; EpOME, epoxy-octadecenoic acid; HDHA, hydroxy-docosahexaenoic acid; HEPE, hydroxy-eicosapentaenoic acid; HETE, hydroxy-eicosatetraenoic acid; HODE, hydroxy-octadecadienoic acid; LA, linoleic acid; LCFA, long chain fatty acid; LOX, lipoxygenase; OxoODE, oxo-octadecadienoic acid; TX, thromboxane.*






# CHAPTER 3

## Influence of dietary n-3 long-chain fatty acids on microbial diversity and composition of sows' feces, colostrum, milk, and suckling piglets' feces.

Effects of n-3 long-chain fatty acids in sow and piglet diets on perinatal piglet performance, oxylipins, immunity, and microbiota.

UNIVERSITAT ROVIRA I VIRGILI
EFFECTS OF N-3 LONG-CHAIN FATTY ACIDS IN SOW AND PIGLET DIETS ON PERINATAL PIGLET PERFORMANCE, OXYLIPINS, IMMUNITY, AND MICROBIOTA
Eudald Llauradó Calero

## CHAPTER 3:

# Influence of dietary n-3 long-chain fatty acids on microbial diversity and composition of sows' feces, colostrum, milk, and suckling piglets' feces

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This chapter describes and discusses the effect of the inclusion of the fish oil rich in n-3 LCFAs in gestation a lactation diets for sows and creep feed for suckling piglets on:

- Weight, backfat and feed intake of sows, litter characteristics, and weight and feed intake of piglets during lactation.
- Microbiota diversity, composition, and differential microbial communities of feaces from gestating and lactating sows, colostrum, milk, and faeces from suckling piglets.
- Concentration of lipopolysaccharides in plasma of suckling piglets.
- Correlations of differential microbial populations between sample types.

**Influence of dietary n-3 long-chain fatty acids on microbial diversity and composition of sows' feces, colostrum, milk, and suckling piglets' feces**

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**Abstract**

Very little is known about the impact of n-3 long-chain fatty acids (**n-3 LCFAs**) on the microbiota of sows and their piglets. The aim of this study was to evaluate the effect of n-3 LCFA in sow diets on the microbiota composition of sows' feces, colostrum, and milk as well as that of piglets' feces. Twenty-two sows were randomly assigned to either a control or an n-3 LCFA diet from service to weaning. Sows' and piglets' performance was monitored. The gestating and lactating sows' microbiomes in feces, colostrum, and milk were characterized by 16s ribosomal RNA gene sequencing. The fecal microbiome from the two lowest (>800 g) and the two highest birth weight piglets per litter was also characterized, and the LPS levels in plasma were analyzed at weaning. n-3 LCFA increased microbiota alpha diversity in suckling piglets' and gestating sows' feces. However, no effects were observed in colostrum, milk, or lactating sows' feces. Dietary n-3 LCFA modified the microbiota composition of gestating sows' feces, milk, and suckling piglets' feces, without affecting lactating sows' feces or colostrum. In gestating sows' feces and milk, the decrease in genus *Succinivibrio* and the increase of Proteobacteria phylum, due to the increased genera *Brenneria* and *Escherichia*, respectively, stand out. In the feces of suckling piglets, the higher abundance of the beneficial genus *Akkermansia* and *Bacteroides*, and different species of *Lactobacillus* are highlighted. In addition, positive correlations for families and genera were found between lactating sows' feces and milk, milk and suckling piglets' feces, and lactating sows' feces and suckling piglets' feces. To conclude, dietary n-3 LCFA had a positive impact on the microbiome of suckling piglet's feces by increasing microbial diversity and some beneficial bacteria populations, had a few minor modifications on the microbiome of milk and gestating sows' feces and did not change the microbiome in lactating sows' feces or colostrum. Therefore, this study shows the effect of dietary n-3 LCFA on the microbiota of sows, colostrum, milk, and suckling piglets during the lactation period providing crucial information on the microbiota status at the early stages of life, which have an impact on the post-weaning.

**Keywords**

Gestating and lactating sows, Suckling piglets, n-3 long-chain fatty acids, Microbial communities, colostrum, milk, microbial transference.

## Introduction

The microbiota of pigs is composed of hundreds of different microorganisms and their acquisition and establishment are influenced by different external factors. Concretely, the predominant immediate postnatal factors that determine initial microbial definition in newborn piglets are likely colostrum, milk, feed, oral-fecal transmission, and the neonatal environment (Nowland *et al*., 2019; Lauridsen, 2020). However, the exact time of initial colonization in pigs has not yet been determined, and some previous reports described prenatal microbial colonization driven by the mother, so it is reasonable to assume that sows' microbiota would also significantly influence the microbiota of their offspring before or during parturition (Nowland *et al*., 2021). Moreover, the establishment of the gut microbiota has become a key factor in piglet survival, as suggested by previous studies in humans showing that an appropriate intestinal microbiota resulting from optimal colonization may improve health and limit diseases (Nowland *et al*., 2019). After all, the microbiota is an important regulator of mammal physiology and could exert several beneficial roles in digestion, protection against pathogens, maintenance of normal function of intestinal villi, and regulation of the immune response (Gresse *et al*., 2017). In addition, optimal colonization during lactation could become a critical factor considering that the change from maternal milk to solid food during weaning entails microbial modifications that coincide with the morphological and functional maturation of the gut barrier, and with important changes in the gut immune system (Hooper, 2004; Beaumont *et al*., 2021).

Genetic selection for hyperprolific sows has resulted in a substantial increase in litter size, and this increase in the sows' prolificacy has resulted in an increased

proportion of piglets born with a low weight (< 1.0 kg birth weight) (Quiniou *et al*., 2002). Low birth weight piglets present a greater energy requirement per kg of body weight (Noblet and Etienne, 1987), and a poorer thermoregulatory ability, which is combined with a limited colostrum intake (Edwards and Baxter, 2015) due to being less vigorous when competing for the limited number of teats in hyperprolific sows. As consequence, these animals present reduced growth rates (Lopez-Verge *et al*., 2018) and they are less likely to survive. Nutritional strategies applied to the sow may become an effective tool to shape the microbiota establishment of piglets to improve their growth and immune development.

Although under commercial conditions the ingredients in sow diets contain considerable amounts of n-6 long-chain fatty acids (**LCFAs**) and the fat sources used are rich in saturated fatty acids, over the last few years the study of the inclusion of n-3 LCFA sources in sow diets has gained interest (Tanghe and De Smet, 2013). Concretely, research on n-3 LCFA in sow nutrition has mainly focused on their influence on milk composition and offspring performance (Lauridsen, 2020; Llauradó-Calero *et al*., 2021). Moreover, due to their anti-inflammatory effects (Calder, 2010) and capability to influence the epithelial barrier functions (Liu, 2015), the impact of n-3 LCFA on immune status (Huber *et al*., 2018) and intestinal epithelium function (Liu *et al*., 2012) has also been evaluated. However, little is known about the influence of n-3 LCFA on microbiota diversity and composition in sows' feces, colostrum, milk, and piglets' feces.

Considering the sow as the first and most relevant factor for the establishment of an optimal microbiota in the newborn piglet, the aim of the current study was to evaluate the inclusion of n-3 LCFA, concretely a solid fish oil rich in eicosapentaenoic acid (**EPA**) (C20:5 n-3) and docosahexaenoic acid (**DHA**) (C22:6 n-3), in sow diets and their impact on the microbiota of gestating and lactating sows' feces, colostrum, milk and the feces of suckling piglets. The impact of n-3 LCFA on the sow to piglet microbial transference was also analyzed.

## Material and methods

*Ethics statement*

Institute for Food and Agricultural Research and Technology's (IRTA) Ethical Committee on Animal Experimentation approved the use of animals for this experiment in accordance with Directive 2010/63/EU of 22 September 2010 and according to the recommendation of the European Commission 2007/526/CE, the Spanish guidelines for the care and use of animals in research (B.O.E. number 34, Real Decreto 53/2013) and the regional regulations on the use and handling of experimental animals (Decree 214/97, Generalitat de Catalunya) (project number: 10294).

*Animals, experimental design, and housing*

Twenty-two sows in two batches (12 and 10 sows met the selection criteria of being between the third and the sixth parity, respectively) were fed one of the two experimental diets from service until the end of lactation (c.a. 28 days post-farrowing). Within each batch, sows were grouped regarding their body weight and their parity number into pairs, as similar as possible, and sows in each pair were randomly assigned to either a control or an n-3 long-chain fatty acid (n-3 LCFA) diet. At birth, the two piglets with the lowest (>800 g) (LBW) and the two piglets with the heaviest (HBW) body weight in each litter were selected for future microbiome analyses. Cross-fostering of piglets was performed only during the first 24 h of life to standardize litter size to 12 piglets per sow, whenever possible, solely among sows belonging to the same experimental treatment, and without involving selected piglets. Sow feeds were provided *ad libitum* in self-dispensing feeding hoppers. At 11 days of age, piglets were offered either a control or an n-3 LCFA creep feed according to the corresponding maternal diet, using floor-attached round feeders. Water was provided *ad libitum* from nipple drinkers.

Sows were allocated in individual stalls from service until pregnancy confirmation. Once confirmed, they were grouphoused in a gestation barn until 1 week before farrowing. Sows were then relocated to individual farrowing crates (0.7 x 2 m) in

pens equipped with partially slatted floors and a heated floor panel for piglets (set at 32–34ºC). The farrowing room was lit with natural light from a window and with fluorescent artificial light (manually operated), and ventilated *via* single, variable-speed fans linked to temperature sensors. The inside temperature of the building was automatically controlled, and the target temperature of the stables at farrowing was set at 24ºC and it was reduced by 0.5ºC per week during the lactation period. Unfortunately, one sow from the second batch (n-3 LCFA treatment) farrowed out of the scheduled time, without supervision, and it was not possible to record the litter characteristics at birth and sample colostrum, so the sow was removed from the trial.

*Experimental diets*

Barley–corn-based gestation and lactation diets for sows and the creep feed for piglets were formulated in accordance with FEDNA specifications (de Blas *et al*., 2013), and their ingredient and nutrient composition were already described by Llauradó- Calero *et al*. (2021). Diets were formulated to contain the same level of nutrients (metabolizable energy, crude protein, digestive lysine, and ether extract) except for the fat content. In control diets, dietary fat was included using a common animal fat source (5 Sysfeed®; Sysfeed SLU, Granollers, Spain) at 15 and 30 g/kg in the gestation and lactation phases, respectively. In the test diets (n-3 LCFA), 15 g/kg of fat was replaced (totally during gestation and one-half during lactation) by solid fish oil (Lipomega®; V&S Asociados, Madrid, Spain). Creep feed diets for piglets contained 30 g/kg of animal fat or an equivalent amount of solid fish oil in the control or n-3 LCFA diet, respectively.

Feed intake of sows was restricted to a maximum of 3 kg/day during the gestation period and progressively increased after farrowing until reaching *ad libitum* feed intake. Piglets' creep feed was offered *ad libitum* from day 11 post-farrowing.

*Growth measurements and sampling*

Sows were weighed at service, 1 week before farrowing (when moved to the farrowing crates), the day after farrowing, and at weaning. In addition, backfat thickness in the P2 position of sows was also measured at the same time points (except the day after farrowing) through ultrasound scanning (Piglog 105®; Frontmatec, Kolding, Denmark). Daily feed intake was monitored and recorded individually during gestation and lactation. The average daily gain of gestation period was calculated from service to day 107 of gestation (1 week before farrowing), and the average daily gain of lactation period from 1 day after farrowing to weaning (c.a. 28 days postfarrowing). At birth, the total number of piglets born, piglets born alive/dead, mummies, and their individual weights were recorded for each sow. During lactation, the weight of piglets was monitored at 24 h, at 20 days of age, and weaning. Cross-fostering was performed within 24 h after birth and the 24 h recordings were considered as the initial values for litter characteristics and growth performance of suckling piglets during lactation. In the same way, the average daily gain of litters and piglets was calculated from 24 h post-farrowing to each weighing time. Creep feed disappearance was monitored from day 11 of lactation until weaning. One sow from the n-3 LCFA group gave birth to less than six piglets and the corresponding data for litter characteristics at birth (obtained before performing cross-fostering) were excluded from the analysis.

Individual animals were selected for the microbiome studies. Fecal samples from all the sows were collected 1 week before parturition (day 107 of gestation) and at weaning after removing the piglets (c.a. day 28 post-farrowing). At birth, the two lowest (> 800 g) and the two highest birth-weight piglets in each litter were selected and their feces were sampled at weaning. In all cases, samples were individually preserved with Real stock buffer (Durviz, Paterna, Spain) and stored at -80ºC until analysis.

Colostrum samples from each sow were obtained immediately after the birth of the first piglet and milk samples were collected at weaning after the piglet's removal. Sows were milked from all mammary glands following i.v. injection of 1.0 ml of oxytocin (20 IU/ml) (Super's Diana S.L., Parets del Vallès, Spain). The samples from

the different nipples in each sow were pooled and aliquots of at least 3 ml were immediately frozen and stored at -80ºC until analysis for microbiome determination.

Blood samples from selected piglets were collected at weaning. Blood was obtained by jugular venipuncture in tubes with ethylenediaminetetraacetic acid (EDTA) and was kept at 4ºC a maximum of 120 min until centrifugation (3,000 rpm, 10 min). Plasma aliquots for LPS measurement were obtained and stored at -80ºC for a maximum of 30 min after centrifugation.

*Deoxyribonucleic acid extraction and bacterial 16S gene amplification and sequencing*

For microbiome analysis, DNA from feces was isolated with the aid of QIAamp Power Fecal Pro DNA Kit (Qiagen, Hilden, Germany), with bead beating and enzymatic lysis steps prior to extraction to avoid bias in DNA purification toward the misrepresentation of gram-positive bacteria. For milk and colostrum, samples were processed with bead beating and enzymatic lysis steps followed by the Blood & Tissue Kit (Qiagen) and further DNA concentration. To evaluate the bacterial composition, massive genome sequencing of the hypervariable region V3–V4 of the bacterial 16s rRNA gene was conducted. Samples were amplified using key-tagged eubacterial primers (Klindworth *et al*., 2013) and sequenced on a MiSeq Illumina Platform, using a 2 x 300nt paired-end strategy, following Illumina Library preparation and sequencing for metagenomic studies protocol (Zhang *et al*., 2014; Marcel, 2011).

*Analysisi of sequencing data*

The resulting sequences were split considering the barcode introduced during the PCR reaction. PEAR program version 0.9.1 was used to overlap R1 and R2 reads (overlap of 50 nt and quality of the overlap with a minimum of Q20 (Zhang *et al*.,

2014), providing a single FASTQ file for each of the samples. Cutadapt v2.6 (Marcel, 2011) was used to trim 16S rRNA PCR primers and sequences were treated with a quality filter, removing low-quality fragments (under Q20 in Phred scale) and short sequences (under 200nt). Chimeric sequences that potentially arise during the PCR amplification step were identified de novo using CD-HIT software v4.8.1 (Li *et al*., 2012) and removed. CD-HIT software was also used to create OTUs at 99.7% of identity. The BLAST tool was used to taxonomically identify each OTU against the National Center for Biotechnology Information (NCBI) 16S rRNA database (20th December 2020) using BLASTn version 2.10.0+.

*Lipopolysaccharides measurement in plasma of suckling piglets*

The sandwich ELISA kit Porcine Lipopolysaccharides (LPSs) ELISA Kit (MBS269464; MyBioSource, San Diego, CA, USA) was employed according to the manufacturer's instructions for the quantitative measurement of LPS in all plasma samples from weaned piglets. Intra-assay precision and inter-assay precision of the kit were ≤ 8% and ≤ 12%, respectively.

*Statistical analysis*

The analysis of variance of growth and performance data of gestating and lactating sows and suckling piglets and LPS concentration data of plasma from suckling piglets were performed through the GLIMMIX procedure of SAS software (SAS/STAT 14.1; SAS Institute Inc., Cary, NC, USA). For growth and performance measurements, dietary treatment was included in the model as the fixed effect and batch as the random effect. Sow body weight at the beginning of the trial and the parity number were initially introduced into the model as covariates. However, only the initial sow body weight was included in the statistical analysis since parity had no significant effect. In addition, the variable days of lactation were included in the model as a covariate for all the data at weaning. In terms of stillborn piglets, deaths, and mummies, data were normalized using a square root transformation ($\sqrt{X + 0.5}$)

but the means of the original data are presented in tables. For LPS concentrations, the model included dietary treatment as the fixed effect and sow as the random effect. All data were tested using Kolmogorov-Smirnov test to identify possible outliers and values were excluded if $P < 0.01$. Results were expressed as means ± SD. Significant differences were set at $P < 0.05$.

Microbiome analyses were done using R software (R Core Team, 2012). Alpha and beta diversity indexes were obtained using the vegan package, as implemented for R version 3.2.3 (Oksanen *et al*., 2020). Bray–Curtis distances were selected to analyze beta diversity, and their significance was studied with PERMANOVA tests. DESeq2 package from R (Love *et al*., 2014) was used to generate a generalized linear model with fixed effects (control vs. n-3 LCFA diet) with negative binomial family to compare operational taxonomic unit (OTU) counts between groups and select the potential bacterial biomarkers. *P*-values were corrected for multiple testing with Benjamini and Hochberg method, and the statistical significance cutoff was set at $P < 0.05$. The ratios Firmicutes/Bacteroidetes and *Lactobacillus*/Proteobacteria were calculated through relative abundances and adjusted to a maximum value of 100. Higher values were considered outliers and were removed from the analysis. Significant differences were set at $P < 0.05$, while tendencies at $P < 0.1$ using Mann–Whitney-Wilcoxon test.

The PROC CORR procedure of SAS software was used to obtain the Pearson correlation coefficient of the relative abundances of differential microbial populations regardless of the dietary treatment between gestating sows' feces and colostrum, lactating sows' feces and milk, milk and sucking piglets' feces, and lactating sows' feces and suckling piglets' feces to study the sow-piglet microbial transference and between suckling piglets' feces and growth measurements of suckling piglets to identify possible microbial populations affecting the animal growth. A significant correlation level was set at $r > 0.5$ and $P < 0.05$.

## Results

Sows' *weight, feed intake, litter characteristics, and piglets' weight*

The body weight, backfat thickness, and feed intake of sows during gestation and lactation are presented in Supplementary Table S1. Sow's body weight at service ($P$ = 0.494), 107 days of gestation ($P$ = 0.207), one day after farrowing ($P$ = 0.336), and at weaning ($P$ = 0.812), and sow's average daily weight gain during gestation ($P$ = 0.202) and lactation ($P$ = 0.278) did not differ between dietary treatments. In the same way, backfat thickness in the P2 position at service ($P$ = 0.696), 107 days of gestation ($P$ = 0.795), and at weaning ($P$ = 0.502), and the average daily feed intake during gestation ($P$ = 0.787) or lactation ($P$ = 0.683) did also not differ either between treatments.

The number of piglets born, piglets born alive/dead, mummies, piglet's weight, average daily gain during lactation, and creep feed intake are presented in Supplementary Table S2. Litter characteristics and the average piglet's body weight did not show any significant differences between treatments at birth, 24 h after birth, 20 days after birth, or at weaning (all $P \geq 0.166$). In the same way, no effects of dietary fish oil were observed for litter or piglet average daily weight gain during the first 20 days ($P$ = 0.339 and $P$ = 0.553, respectively) or during the whole lactation period ($P$ = 0.974 and $P$ = 0.714, respectively). Piglet creep feed disappearance did not differ between control and n-3 LCFA diets ($P$ = 0.599) either.

*Microbiota diversity*

Table 1 shows richness and diversity values for each experimental group and type of sample at phylum, family, genera, and species levels. The largest differences in terms of alpha diversity were observed in the feces of suckling piglets. An increase in microbiota diversity calculated through Simpson and Shannon indices in all studied levels was detected in piglets from the n-3 LCFA sows compared with piglets from the control sows. In addition, microbiota diversity was significantly increased by dietary fish oil through the Simpson index in feces from n-3 LCFA gestating sows.

Alpha-diversity in terms of Richness, Simpson, and Shannon indices in colostrum, lactating sow's feces, or milk were not significantly affected by fish oil in the sows' diet (Table 1).

***Table 1:*** *Microbiota alpha diversity indices, calculated at phylum, family, genera and species level, of feces of gestating and lactating sows, colostrum, milk, and feces of suckling piglets from animals fed either control or n-3 LCFA diets.*[1]

| | Richness | | | Simpson index | | | Shannon index | | |
|---|---|---|---|---|---|---|---|---|---|
| Taxonomy Level | control | n-3 LCFA | *P* value | control | n-3 LCFA | *P* value | control | n-3 LCFA | *P* value |
| Gestating sow's faeces | | | | | | | | | |
| Phylum | 7.65 ± 0.63 | 7.83 ± 0.55 | 0.48 | 0.57 ± 0.02 | 0.55 ± 0.02 | 0.11 | 1.01 ± 0.08 | 0.96 ± 0.09 | 0.21 |
| Family | 27.0 ± 1.28 | 27.1 ± 1.33 | 0.83 | 0.74 ± 0.04 | 0.77 ± 0.04 | 0.073 | 1.90 ± 0.10 | 1.94 ± 0.15 | 0.47 |
| Genera | 50.1 ± 1.44 | 49.6 ± 2.59 | 0.55 | 0.74 ± 0.04 | 0.77 ± 0.04 | 0.068 | 2.08 ± 0.11 | 2.12 ± 0.19 | 0.49 |
| Species | 80.1 ± 3.14 | 81.3 ± 4.96 | 0.52 | 0.75 ± 0.04 | 0.79 ± 0.05 | 0.037 | 2.31 ± 0.14 | 2.45 ± 0.25 | 0.14 |
| Colostrum | | | | | | | | | |
| Phylum | 8.04 ± 0.78 | 8.19 ± 0.35 | 0.58 | 0.54 ± 0.14 | 0.54 ± 0.08 | 0.93 | 1.08 ± 0.24 | 1.08 ± 0.14 | 0.94 |
| Family | 52.1 ± 4.98 | 52.8 ± 3.36 | 0.69 | 0.82 ± 0.17 | 0.83 ± 0.09 | 0.83 | 2.50 ± 0.53 | 2.47 ± 0.41 | 0.91 |
| Genera | 102 ± 19.5 | 102 ± 9.93 | 0.92 | 0.84 ± 0.16 | 0.85 ± 0.10 | 0.78 | 2.81 ± 0.60 | 2.81 ± 0.49 | 0.99 |
| Species | 206 ± 43.8 | 200 ± 29.4 | 0.72 | 0.91 ± 0.07 | 0.91 ± 0.09 | 0.99 | 3.58 ± 0.54 | 3.58 ± 0.56 | 0.97 |
| Lactating sow's faeces | | | | | | | | | |
| Phylum | 8.18 ± 0.40 | 8.06 ± 0.35 | 0.46 | 0.59 ± 0.04 | 0.54 ± 0.07 | 0.064 | 1.06 ± 0.10 | 0.97 ± 0.15 | 0.091 |
| Family | 21.9 ± 0.76 | 21.9 ± 1.16 | 0.97 | 0.75 ± 0.04 | 0.77 ± 0.03 | 0.51 | 1.85 ± 0.13 | 1.84 ± 0.13 | 0.80 |
| Genera | 48.0 ± 1.77 | 47.6 ± 2.42 | 0.61 | 0.77 ± 0.05 | 0.78 ± 0.04 | 0.42 | 2.10 ± 0.19 | 2.10 ± 0.13 | 0.96 |
| Species | 76. 4 ± 2.86 | 77.9 ± 4.23 | 0.36 | 0.78 ± 0.05 | 0.80 ± 0.05 | 0.34 | 2.36 ± 0.23 | 2.38 ± 0.14 | 0.77 |
| Milk | | | | | | | | | |
| Phylum | 8.00 ± <0.01 | 7.82 ± 0.60 | 0.33 | 0.56 ± 0.09 | 0.58 ± 0.11 | 0.58 | 1.12 ± 0.17 | 1.17 ± 0.21 | 0.57 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Family | 45.6 ± 3.45 | 46.6 ± 1.81 | 0.49 | 0.86 ± 0.05 | 0.88 ± 0.04 | 0.34 | 2.49 ± 0.16 | 2.62 ± 0.21 | 0.11 |
| Genera | 97.1 ± 7.81 | 97.1 ± 7.88 | 0.99 | 0.87 ± 0.05 | 0.89 ± 0.04 | 0.36 | 2.80 ± 0.20 | 2.93 ± 0.24 | 0.16 |
| Species | 208 ± 23.6 | 207 ± 25.0 | 0.92 | 0.90 ± 0.06 | 0.92 ± 0.04 | 0.34 | 3.43 ± 0.29 | 3.63 ± 0.30 | 0.14 |
| Suckling piglet's faeces | | | | | | | | | |
| Phylum | 9.83 ± 0.89 | 9.50 ± 0.74 | 0.064 | 0.68 ± 0.06 | 0.71 ± 0.07 | 0.036 | 1.36 ± 0.18 | 1.46 ± 0.21 | 0.026 |
| Family | 35.8 ± 2.76 | 35.3 ± 2.77 | 0.45 | 0.78 ± 0.07 | 0.83 ± 0.07 | 0.006 | 2.09 ± 0.21 | 2.28 ± 0.29 | 0.001 |
| Genera | 72.7 ± 5.87 | 70.0 ± 7.18 | 0.065 | 0.81 ± 0.07 | 0.84 ± 0.06 | 0.021 | 2.45 ± 0.23 | 2.56 ± 0.25 | 0.054 |
| Species | 118 ± 10.5 | 117 ± 12.9 | 0.62 | 0.82 ± 0.07 | 0.86 ± 0.07 | 0.009 | 2.69 ± 0.28 | 2.89 ± 0.34 | 0.005 |

LCFA, long chain fatty acid.

[1]Values are means ± SD of 11 control and 10 n-3 LCFA samples from sows (gestating and lactating sows' feces, colostrum and milk) or 44 control and 40 n-3 LCFA samples from piglets (suckling piglets' feces).

*Microbiota composition and differential microbial communities*

The PCoA analysis of sows and piglets' feces, colostrum, and milk regardless of experimental dietary treatments is presented in Figure 1. PERMANOVA revealed distinct clusters for each sample type ($R^2$ = 0.32; $P$ = 0.001). Beta-dispersion analysis showed differences among all the different types of samples analyzed (all $P \leq 0.037$) except between feces of gestating and lactating sows ($P$ = 0.235).

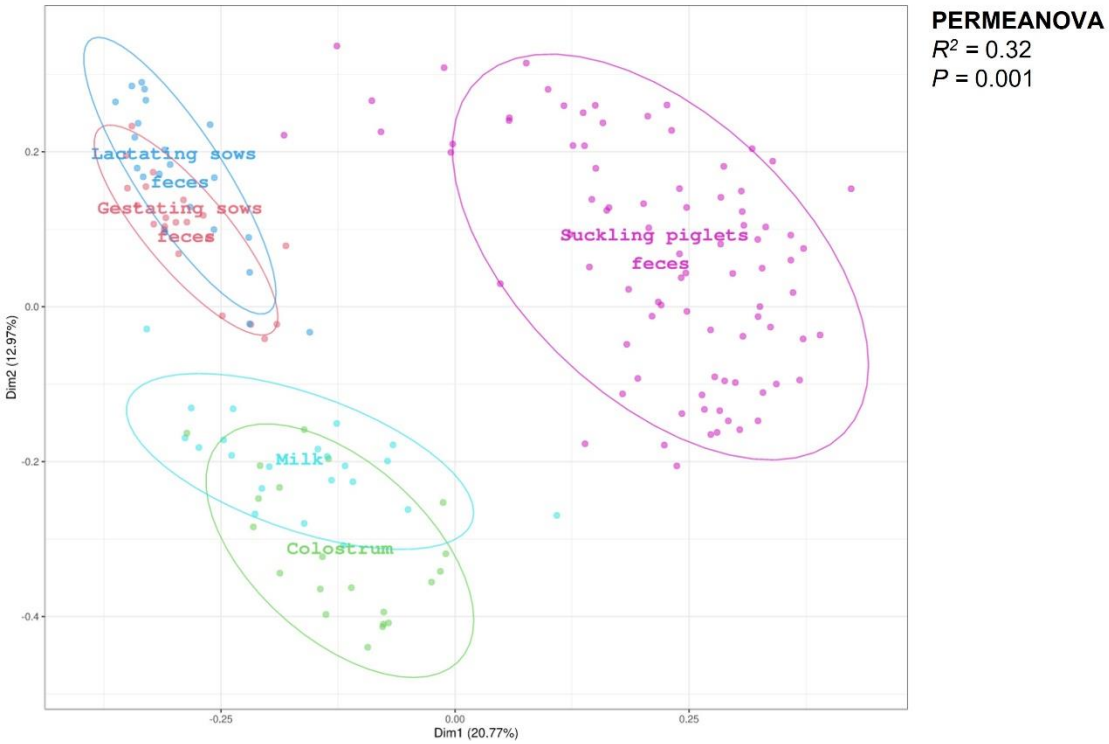


***Figure 1:*** *Principal coordinates analysis (PCoA) plot of gestating and lactating sows' feces (n =21, respectively), colostrum (n =21), milk (n =21), and suckling piglets' feces (n =84), regardless of experimental dietary treatments, revealed distinct clusters for each sample type ($R^2$ = 0.32; P = 0.001). PERMANOVA test performed using Bray-curtis distances to analyze beta-dispersion showed differences between all the different types of samples analyzed (all P ≤ 0.037) except among gestating and lactating sows' feces (P = 0.235).*

The fecal microbiome of gestating sows was composed mainly of phylum Firmicutes, Families *Clostridiaceae*, *Peptostreptococcaceae*, *Lactobacillaceae*, *Streptococcaceae*, *Erysipelotrichaceae*, and *Lachnospiraceae,* and genera *Clostridium*, *Lactobacillus, Streptococcus, Terrisporobacter*, and *Turicibacter*

(Figure 2). Lower abundances of the family *Succinivibrionaceae* ($P$ = 0.020) and genus *Succinivibrio* ($P$ = 0.031) were observed in the feces of sows from the n-3 LCFA group than those from the control group (Figure 3).

The core microbiome obtained in colostrum samples was represented by phyla Firmicutes, Actinobacteria, and Proteobacteria. *Staphylococcaceae*, *Lactobacillaceae*, *Clostridiaceae*, and *Moraxellaceae* were the dominant Families and *Staphylococcus*, *Lactobacillus*, *Clostridium*, and *Streptococcus* represented the most abundant genera (Figure 2). No differential bacterial communities were detected between control and n-3 LCFA treatments at any taxonomical level studied (Figure 3).

In the feces of lactating sows, Firmicutes was also the most abundant phylum. Families *Peptostreptococcaceae*, *Clostridiaceae*, *Erysipelotrichaceae*, *Lactobacillaceae*, and *Lachnospiraceae* and genera *Clostridium*, *Terrisporobacter*, *Turicibacter*, and *Lactobacillus* had the highest abundance (Figure 2). As for colostrum, no significant differences between the control and n-3 LCFA groups were observed for any taxa at any taxonomical level (Figure 3).

In milk, Firmicutes, Proteobacteria, and Actinobacteria were the dominant bacterial phyla followed by Bacteroidetes. At the family and genus level, the dominant taxa were *Lactobacillaceae*, *Streptococcaceae*, *Peptostreptococcaceae*, *Staphylococcaceae*, and *Lachnospiraceae,* and *Lactobacillus*, *Streptococcus*, *Staphylococcus*, and *Clostridium*, respectively (Figure 2). Differences between the control and the n-3 LCFA treatments were observed for specific groups (Figure 3). Concretely, Phylum Proteobacteria ($P$ = 0.017) was increased by dietary n-3 LCFA due to the increased families *Pectobacteriaceae* ($P$ = 0.020) and *Enterobacteriaceae* ($P$ = 0.020) and the main genera belonging to these families, *Brenneria* ($P$ = 0.026) and *Escherichia* ($P$ = 0.026), respectively. In addition, other changes by the inclusion of dietary fish oil were detected such as decreases in the family *Ruminococcaceae* ($P$ = 0.023) and genera *Ruminococcus* ($P$ = 0.026), increases in family *Propionibacteriaceae* ($P$ = 0.020) and genera *Cutibacterium* ($P$ = 0.026), and decreases in family *Spirochaetaceae* ($P$ = 0.023) and genera *Treponema* ($P$ = 0.028).

Microbiota composition in the feces of suckling piglets was dominated by phyla Firmicutes, Bacteroidetes, Proteobacteria, Euryarchaeota, and Actinobacteria. *Lachnospiraceae*, *Bacteroidaceae*, *Lactobacillaceae*, *Ruminococcaceae*, and *Clostridiaceae* were the most abundant families, and *Bacteroides*, *Lactobacillus*, *Gemmiger*, *Clostridium*, and *Methanobrevibacter* the most abundant genera (Figure 2). Piglet feces were the type of sample that presented a larger number of differences in microbial communities between treatments. Concretely, one phylum, 15 families, and 27 genera differed between treatments (Figure 3). Phylum Verrucomicrobia ($P = 0.045$) and genera *Akkermansia* ($P = 0.041$) were increased in the feces of piglets from n-3 LCFA-fed sows. In addition, and within Firmicutes, dietary n-3 LCFA reduced the family *Ruminococcaceae* ($P < 0.001$) due to reductions in the genera *Gemmiger* ($P = 0.032$) and *Ruminococcus* ($P = 0.036$). Within the same phylum, the family *Clostridiaceae* ($P = 0.002$) and genera *Clostridium* ($P = 0.001$) were increased by dietary n-3 LCFA. Family *Enterococcaceae* ($P = 0.010$) and genera *Enterococcus* ($P = 0.011$) were also increased by fish oil. It should also be pointed out that the feces from piglets in the n-3 LCFA group had a reduced abundance of genus *Holdemanella* ($P = 0.032$), *Flintibacter* ($P = 0.024$), and *Anaerotaenia* ($P = 0.009$), and an increased abundance of genus *Streptococcus* ($P = 0.023$) and *Erysipelatoclostridium* ($P < 0.001$). Regarding phylum Bacteroidetes, dietary fish oil increased in piglets' feces in the families *Tannerellaceae* ($P < 0.001$) and *Odoribacteraceae* ($P = 0.042$) due to the increase in their respective genera *Parabacteroides* ($P < 0.001$) and *Butyricimonas* ($P = 0.002$). Within the same phylum also stands out the increased abundance of genera *Bacteroides* ($P = 0.009$) by n-3 LCFA. In terms of phylum Proteobacteria, an increase of families *Pectobacteriaceae* ($P = 0.047$) and *Enterobacteriaceae* ($P = 0.023$), due to the increase in their respective genera *Brenneria* ($P < 0.001$) and *Escherichia* ($P < 0.001$), and a reduction of family *Desulfovibrionaceae* ($P = 0.006$) were observed in piglets from sows fed the n-3 LCFA. In the phylum of Actinobacteria, n-3 LCFA reduced family *Coriobacteriaceae* ($P < 0.001$) and genera *Collinsella* ($P < 0.001$), and increased family *Actinomycetaceae* ($P < 0.001$) and genera *Schaalia* ($P = 0.007$), and family *Atopobiaceae* ($P < 0.001$), including genus

*Olsenella* ($P < 0.001$) and *Atopobium* ($P < 0.001$). In addition, n-3 LCFA also reduced genera *Synergistes* ($P = 0.002$) but not modifying phylum *Synergistetes* or family *Synergistaceae*.

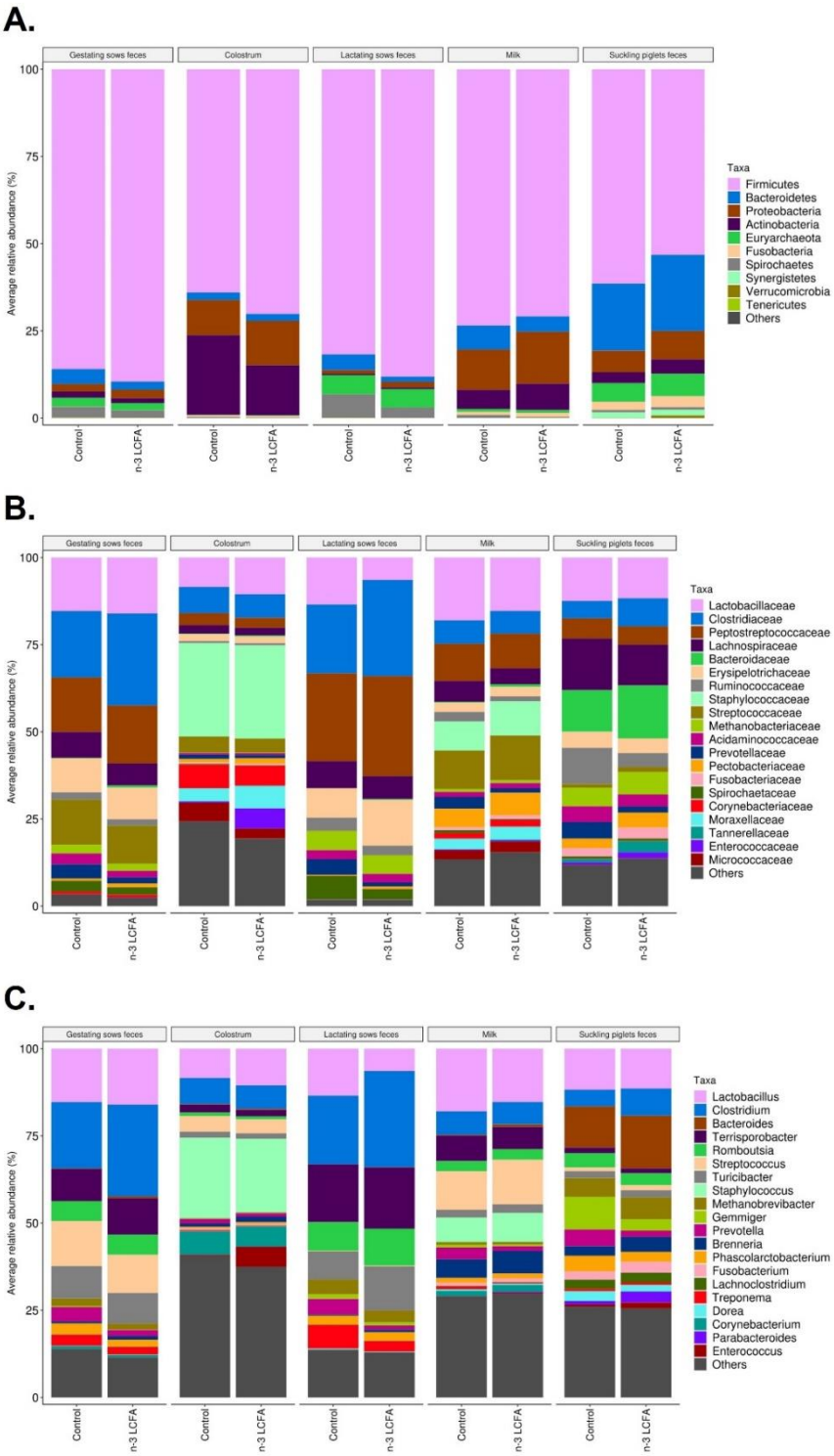


***Figure 2:*** *Microbiota composition by the relative abundance of the top 10 phyla (A), top 20 families (B) and top 20 genera (C) of feces of gestating and lactating sows (*n *=21), colostrum (*n *=21), milk (*n *=21), and feces of suckling piglets (*n *=84) according to dietary treatment (control vs n-3 LCFA). LCFA, long chain fatty acids.*

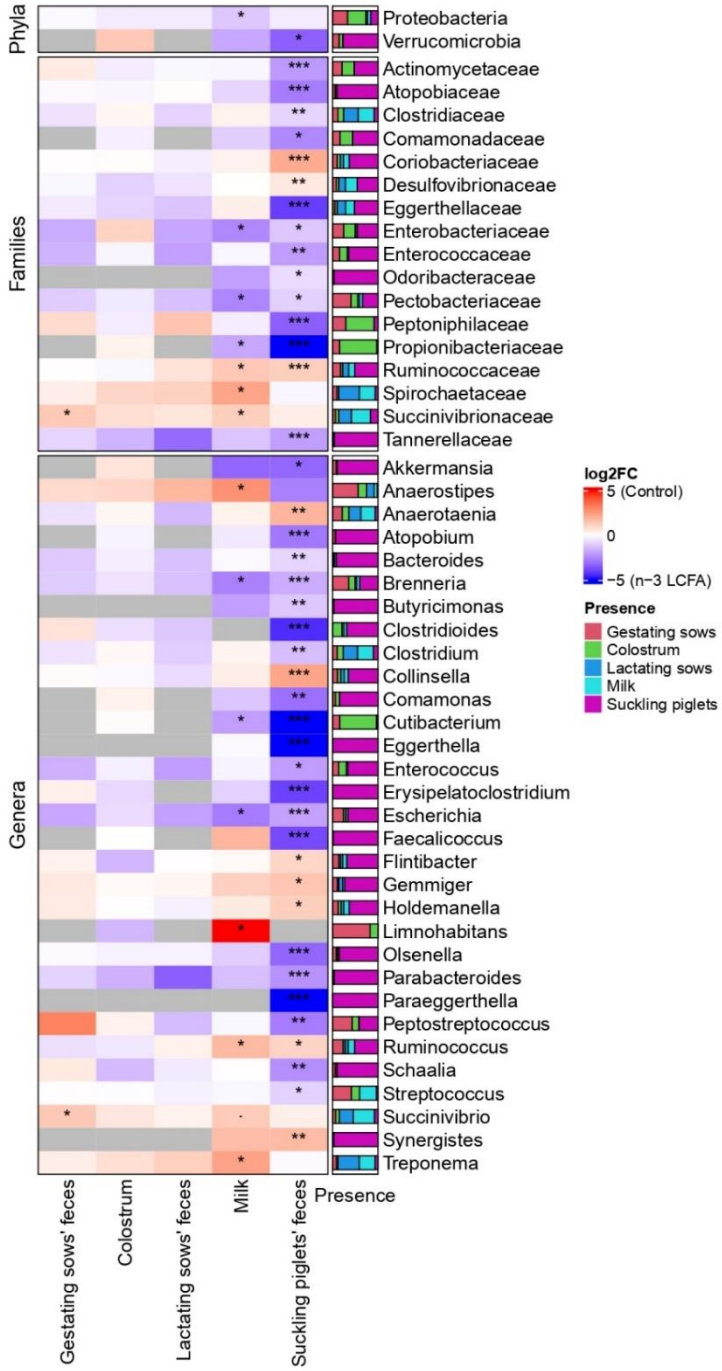


***Figure 3:*** *Heatmap representing the differentially abundant phyla, families and genera between control and n-3 LCFA dietary treatments in feces of gestating and lactating sows, colostrum, milk, and feces of suckling piglets. For feces of gestating and lactating sows, colostrum and milk; n=11 for control and n=10 for n-3 LCFA. For feces of suckling piglets; n=44 for control and n=40 for n-3 LCFA. Significant differences between treatments were set at P < 0.05 (*), P < 0.01 (**) and P < 0.001 (***).*

Finally, the ratios Firmicutes/Bacteroidetes and *Lactobacillus*/Proteobacteria were also compared between treatments for all types of samples, and this is reported in Figure 4. In the feces of lactating sows, an increase in the ratio Firmicutes/Bacteroidetes ($P$ = 0.040) and a tendency to decrease the ratio *Lactobacillus*/Proteobacteria were observed ($P$ = 0.072) with the n-3 LCFA inclusion, while a tendency to reduce the ratio Firmicutes/Bacteroidetes ($P$ = 0.070) was observed in feces of suckling piglets of n-3 LCFA group. For feces of gestating sows, colostrum, and milk, no differences between treatments were observed for either, Firmicutes/Bacteroidetes ratio (all $P \geq 0.22$) or *Lactobacillus*/Proteobacteria ratio (all $P \geq 0.19$).

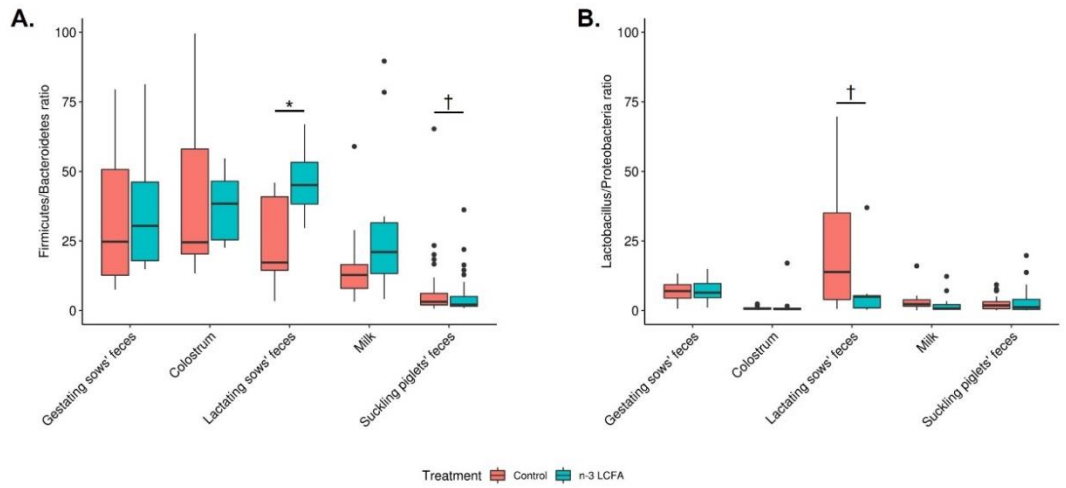


***Figure 4:*** *Firmicutes/Bacteroidetes (A) and Lactobacillus/Proteobacteria (B) ratios calculated between treatments in all sample types. For feces of gestating and lactating sows, colostrum and milk; n=11 for control and n=10 for n-3 LCFA. For feces of suckling piglets; n=44 for control and n=40 for n-3 LCFA. Significant differences between treatments were set at P < 0.05 (*), while tendencies were set at P < 0.10 (†). Ratios were adjusted at a maximum value of 100 and higher values were removed by being considered outliers.*

*The concentration of lipopolysaccharides in plasma of suckling piglets*

As shown in Figure 5, no differences in plasma LPS concentration were observed between samples from piglets of the control group and piglets of the n-3 LCFA group (181 ± 98.4 and 162 ± 77.8 pg/ml, respectively; $P = 0.700$).

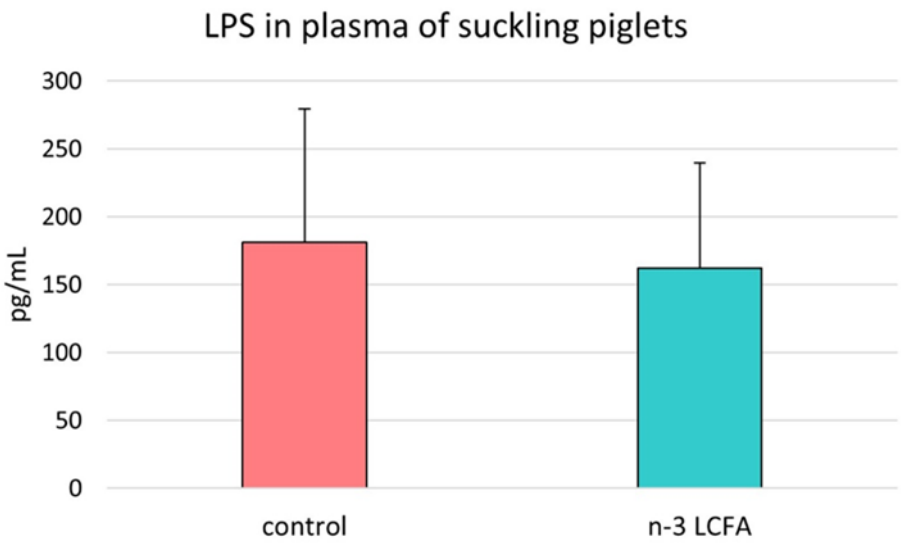


***Figure 5:*** *Lipopolysaccharide (LPS) concentration in plasma of suckling piglets from control and n-3 LCFA fed sows.*

*Correlations of differential microbial populations between sample types*

The comparison of feces from lactating sows and their milk rendered positive correlations at the family level, *Ruminococcaceae* in feces with *Ruminococcaceae* and *Spirochaetaceae* in milk, and four positive correlations at the genus level, *Anaerostipes* in feces with *Anaerostipes* in milk, and *Ruminococcus* in feces with *Anaerostipes*, *Ruminococcus,* and *Treponema* in milk (Figure 6).

Between milk and feces of suckling piglets, two positive correlations were observed at the family level and five positive correlations at the genus level (Figure 6). Concretely, at the family level, *Odoribacteraceae* and *Tannerellaceae* in milk

were positively correlated with *Tannerellaceae* in piglets' feces. At the genus level, *Akkermansia*, *Bacteroides*, *Flintibacter*, and *Parabacteroides* in milk were also positively correlated with *Parabacteroides* in feces and *Erysipelatoclostridium* in milk with *Olsenella* in feces.

One positive correlation at family and genus levels was observed between feces from lactating sows and feces from suckling piglets (Figure 6). Specifically, the correlations were between the family *Atopobiaceae* and genus *Atopobium* in lactating sows' feces with the family *Peptoniphilaceae* and genus *Akkermansia* in suckling piglets' feces, respectively.

Finally, no significant correlations between microbial populations were observed at any taxonomical level between gestating sow's feces and colostrum, and between bacterial populations in suckling piglets' feces and the growth measurements of suckling piglets during lactation.

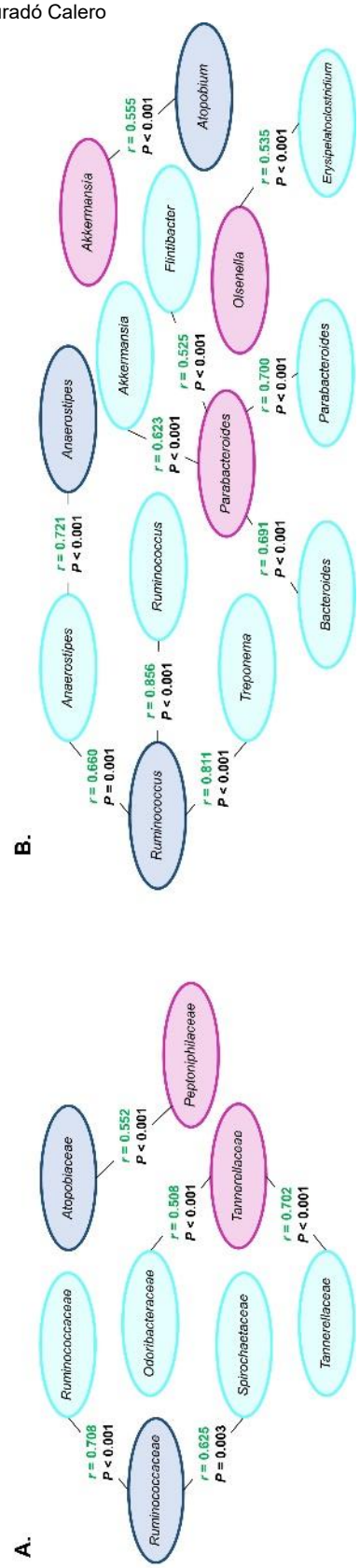


***Figure 6:*** *Correlations between modified microbial families (A) and genera (B) from feces of lactating sows (navy blue, n = 21), milk (light blue, n = 21) and feces of suckling piglets (pink, n = 84). Pearson correlation coefficient (r) in green indicates positive correlation. Significant correlation level was set at r > 0.5 and P < 0.05.*

## Discussion

The microbiota plays an integral role in influencing host metabolism, its immune system, and the development of a healthy gastrointestinal tract. In addition, the health of offspring is strongly linked to microbial exposure throughout life (Nowland *et al*., 2019). Considering that weaning is a critical part of pig life characterized by being a complex and stressful event due to the dietary, social and environmental changes (Gresse *et al*., 2017), the acquisition of an optimal microbiota during the suckling period becomes crucial. Few studies on mice and humans have studied the effect of n-3 fatty acids on the gut microbiota; however, it is described as a poorly understood topic (Costantini *et al*., 2017). To our knowledge, no prior studies have evaluated the impact of n-3 LCFA on the microbial diversity and composition of sows' feces, colostrum, and milk and the feces of their suckling piglets, and therefore research on the impact of n-3 LCFA on swine microbiota deserves further attention.

The effects of the inclusion of n-3 LCFA in sows' diets on sow's weight, litter characteristics, and growth performance of piglets during lactation have been previously reported (Tanghe and De Smet, 2013). The animals selected for the current study are part of a larger trial studying the impact of n-3 LCFA inclusion in the diets of gestating and lactating diets of sows on different parameters including performance (Llauradó-Calero *et al*., 2021), and a trend to increase average piglet body weight at weaning is reported, which was in the line with the results of Rooke *et al*. (2001). However, with the group of sows selected for the current study, no differences in performance between treatments were observed, which would also be consistent with the results of Lauridsen and Danielsen (2004) and Leonard *et al*. (2010).

Despite diet being one of the main factors affecting intestinal microbiota (Duan *et al*., 2019), in the current study, the inclusion of fish oil as a source of n-3 LCFA in sow diets cause minor changes in microbial populations in the feces of the sows. This may be due to the fact that sows, as adult animals, are characterized by presenting a stable and well-developed microbiota (Simpson *et al*., 2000; Niu *et al*.,

2019). However, in gestating sows, microbial diversity increased when they were fed with the n-3 LCFA diet. In addition, the decrease of family *Succinivibrionaceae* due to the decrease of genus *Succinivibrio* stands out, which is considered a core microbiome of the proximal colon or cecum of swine and is related to propionate formation and decarboxylation process (Bergamaschi *et al*., 2020). However, in lactating sows, no differences in diversity or populations at family and genus levels were found between control and n-3 LCFA-fed animals. Even so, in lactating sows, an increase in the ratio of Firmicutes/Bacteroidetes was observed for the n-3 LCFA group. More Firmicutes and fewer Bacteroidetes have been suggested as a characteristic of fat pigs and have been related to fat deposition (Zhao *et al*., 2015), although we found no differences between treatments for the weight of the sows or their backfat thickness on P2 position at weaning.

Both colostrum and milk play a critical role in piglets' development since they are the first sources of nutrients for newborn piglets which are characterized as having low energy reserves and being immunologically naive. In addition, colostrum and milk not only provide nutrients, energy, and immunity, but they also enable the establishment of commensal microbes (Nowland *et al*., 2019). Given that optimal colostrum and milk supply are crucial for intestinal microbiota colonization and development (Nowland *et al*., 2021), the impact of n-3 LCFA on colostrum and milk microbiota composition becomes very relevant. In the current study, the bacterial compositions of colostrum and milk were shown to be different, regardless of dietary treatment. On the one hand, the microbiota of colostrum was dominated mainly by phyla Firmicutes, Actinobacteria, and Proteobacteria and families *Staphylococcaceae*, *Lactobacillaceae*, *Clostridiaceae*, and *Moraxellaceae*. On the other hand, the microbiota of milk was dominated by phyla Firmicutes, Proteobacteria, and Actinobacteria and *Lactobacillaceae*, *Streptococcaceae*, *Peptostreptococcaceae*, *Staphylococcaceae*, and *Lachnospiraceae* at the family level. Interestingly, dietary n-3 LCFA impacted differently on the microbial populations from colostrum and milk. In colostrum, no changes were observed in microbial diversity nor differential populations due to n-3 LCFA supplementation. In milk, however, one phylum, six families, and seven genera were modified by the

n-3 LCFA diet, although no changes in microbial diversity were observed. Among these changes, the increase in Proteobacteria should be highlighted since it is one of the most dominant phyla in milk microbiota. This increase was mainly due to the higher abundance of families *Pectobacteriaceae* and *Enterobacteriaceae* and the increase in genera *Brenneria* and *Escherichia*, respectively. While no effects of *Brenneria* have been described on animal microbiota, *Escherichia* is a common inhabitant in swine gut microbiota although some of its species could be pathogenic (Schierack *et al*., 2007). In addition, the decrease of the family *Ruminococcaceae* due to the decrease of the genus *Ruminococcus* stands out. *Ruminococcaceae* has been reported to play a role in the degradation of complex carbohydrates (Crost *et al*., 2018) and the production of butyrate, and it is associated with anti-inflammatory effects (Liu *et al*., 2019). Particularly, *Ruminococcus* OTU was identified as *R. flavefaciens,* which plays an important role in the digestion of hemicellulose and cellulose plant cell walls (Fontes and Gilbert, 2010), and their degradation-derived products may act as prebiotics to gut microbiota (Rajan *et al*., 2021).

Compared to sows, suckling piglets experienced larger modifications in the diversity and bacterial populations of their fecal microbiota due to n-3 LCFA. However, we cannot be sure whether the higher diversity in the fecal samples from n-3 LCFA piglets is due to the direct effect of dietary n-3 LCFA in creep feed, vertical transmission from the sow at birth, or a combination of both. In addition, no effects of birth weight category (low vs. high birth weight piglets) or interactions between dietary treatment and piglets' birth weight were observed for either diversity or bacterial populations. This contrasts with the results reported by Li *et al*. (2018), Li N. *et al*. (2019) who described differences between LBW and normal birth weight piglets during the suckling period for the microbiota of feces and the microbiota of digesta in the ileum and colon. Regarding dietary treatment, bacterial alpha diversity according to Simpson and Shannon indices was increased in suckling piglets on the n-3 LCFA diet. In a previous study, Djuric *et al*. (2019) described an increase of colonic bacterial diversity in healthy human adults following dietary fish oil supplementation which they related to the anti-

inflammatory effects of n-3 LCFA, agreeing with Calder (2019), who suggested a new mechanism by which n-3 LCFA dampen intestinal inflammation. In terms of differential microbial populations, n- 3 LCFA modified one phylum, 15 families, and 27 genera in feces from suckling piglets.

Among the observed changes in the fecal microbiota of suckling piglets, the phylum Verrucomicrobia increased due to the increase in the *Akkermansia* genus with its tentative species *A. muciniphila*. A previous study with mice also reported an increase in *A. muciniphila* after fish oil supplementation (Caesar *et al*., 2015). Moreover, another study in healthy humans described an increase in the family *Akkermansiaceae* in an n- 3 LCFA-treated group (Watson *et al*., 2018). *A. muciniphila* is the most common species of Verrucomicrobia and colonizes the mucus layer acting as a mucin degrader (Ottman *et al*., 2017). It is well established that it plays a crucial role in supplying mucin-derived nutrients to other members of the gut microbiota that are unable to degrade the mucin layer by themselves (Tailford *et al*., 2015). This mucin-degrading capacity makes *A. muciniphila* a modulator for gut homeostasis improving and regulating the gut barrier function (Guo *et al*., 2017). In addition, its presence in the feces of highly feed-efficient animals (Gardiner *et al*., 2020) and its possible role as the host immune system modulator (Crespo-Piazuelo *et al*., 2019) have also been previously described. Other studies propose that excessive mucin degradation may facilitate the access of pathogens to the mucosa (Ganesh *et al*., 2013). However, in the current study, no differences in LPS concentration in the plasma of suckling piglets at weaning, as a gut barrier integrity marker, were observed between n-3 LCFA and control diets.

Within the important bacterial populations of the Firmicutes phylum that were modified by n-3 LCFA in the feces of suckling piglets, we observed a decrease in the family *Ruminococcaceae* and the increase of family *Clostridiaceae*, which are two of the core families in the swine gastrointestinal tract (Holman *et al*., 2017). Decreased *Ruminococcaceae* was due to the decrease of genera *Ruminococcus* (tentatively identified as *R. gnavus*) and *Gemmiger*, and increased *Clostridiaceae*

due to the increase of the genus *Clostridium* and, specifically, the increases of OTUs identified as *C. innocuum*, *C. cadaveris*, and *C. perfringens*. On the one hand, as already described, *Ruminococcaceae* is associated with anti-inflammatory effects (Liu *et al*., 2019). Conversely, *R. gnavus* has been associated with a pro-inflammatory role (Hall *et al*., 2017; Henke *et al*., 2019). On the other hand, members of the family *Clostridiaceae* are also associated with the production of butyrate, so they can also contribute to decreasing inflammation in the gut of the host (Holman *et al*., 2017). However, species *C. innocuum*, *C. cadaveris*, and *C. perfringens* can become pathogenic causing infections (Crum-Cianflone, 2009; Gupta *et al*., 2020; Posthaus *et al*., 2020). Concretely, *C. perfringens* can cause severe, acute, and necrotic enteritis in humans and livestock, particularly in neonatal pigs (Posthaus *et al*., 2020). The pathogenicity of *C. perfringens* is given by toxin-a and toxin-b, which are generated by *C. perfringens* type A and C (Baker *et al*., 2010; Posthaus *et al*., 2020). Although *C. perfringens* infection causes diarrhea, low weaning weights, and pre-weaning mortality, no differences in body weight nor mortality were observed between treatments for suckling piglets at weaning. Within the same phylum, we also observed an increase in the abundance of *Streptococcus,* which contains some species described as probiotics and has been related to the improvement of colostrum quality, milk quality and quantity, litter size, and piglet vitality, and body weight (Knecht *et al*., 2020). In addition, *Lactobacillus* species (OTUs tentatively identified as *L. delbrueckii* and *Lactobacillus mucosae*) were also increased in the feces of n-3 LCFA suckling piglets. Previous reports have described an immunomodulatory effect of *L. mucosae* (Ryan *et al*., 2019), and that the oral administration of *L. delbrueckii* improves intestinal integrity, stimulates the intestinal immune response, and alleviates intestinal oxidative damage in piglets (Li Y. *et al*., 2019; Chen *et al*., 2020). Moreover, Yang *et al*. (2017) described that many beneficial bacteria belonging to the Firmicutes phylum, such as *Enterococcus*, *Streptococcus*, *Lactobacillus*, and *Clostridium,* were reduced in diarrheic piglets. In the current study, these beneficial genera and pertaining species were increased by dietary n-3 LCFA.

Relevant modifications of bacterial populations belonging to phyla Bacteroidetes and Proteobacteria by dietary n-3 LCFA should also be noted in the feces suckling piglets. Regarding Bacteroidetes, n-3 LCFA increased the *Bacteroides* genus, which is one of the core bacterial genera of pigs' microbiota and is reported to be found in more than 90% of healthy pigs of different ages (Luo *et al*., 2022). Moreover, as described above for *A. muciniphila*, genus *Bacteroides* is also considered to be a mucin glycan degrader (Bell and Juge, 2021) and its low abundance has been associated with post-weaning diarrhea (Ren *et al*., 2022). It should be mentioned that n-3 LCFA also decreased *Prevotella copri* species, which are present during lactation at low abundances but increase drastically upon weaning (Amat *et al*., 2020). A high abundance of *P. copri* has recently been related to hosting chronic inflammation responses resulting in excessive fat accumulation in pigs (Chen *et al*., 2021). In terms of Proteobacteria, n-3 LCFA increased the *Pectobacteriaceae* and *Enterobacteriaceae* families mainly due to the increases of genus *Brenneria* and *Escherichia*, respectively, modifications that match those observed in milk. *Enterobacteriaceae* consists of a set of genera that colonize the intestinal microbiota and includes commensal microbiota as well as pathogens (Schierack *et al*., 2007). This family contains LPS-producing bacteria and *Escherichia coli* species, which can cause diarrhea and infections in both humans and animals (Schierack *et al*., 2007; Costantini *et al*., 2017). However, in the present study, despite *Escherichia* being increased in the feces of n-3 LCFA suckling piglets, no increase in *E. coli* was detected and as already mentioned, there were no differences in piglets' LPS plasma levels. According to Lauridsen (2020), colostrum, milk and oralfecal transmission play a crucial role in the acquisition and establishment of the microbiota of newborn piglets. Some correlations were observed between lactating sows' feces and milk, milk and suckling piglets' feces, and lactating sows' feces and suckling piglets' feces showing that the changes observed in the former due to the inclusion of dietary n-3 LCFA may be transferred and have an impact on the latter. First, between lactating sows' feces and milk, the family *Ruminococcaceae* and genus *Ruminococcus* in lactating sows were positively correlated with the same family and genus in milk, but also with the family

*Spirochaetaceae*, and genera *Anaerostipes* and *Treponema*. Second, the genera *Akkermansia*, *Bacteroides*, *Flintibacter*, and *Parabacteroides* in milk correlate positively with the genus *Parabacteroides* in suckling piglets' feces. Concretely, different OTUs belonging to these four previous genera in milk were positively correlated with *Parabacteroides distasonis* in suckling piglets' feces, which is described as immunomodulatory health-promoting bacteria (Kverka *et al*., 2011). Moreover, positive correlations between lactating sows' feces and suckling piglets' feces were also observed. OTUs such as *Olsenella scatoligenes*, *Erysipelatoclostridium ramosum*, *Peptostreptococcus stomatis*, *A. muciniphila*, and *L. delbrueckii* on feces from lactating sows were positively correlated with the *Bacteroides* species *Bacteroides stercoris* and *Bacteroides fluxus* in feces from suckling piglets. Correlations between species are reported in Supplementary Figure 5.1. Therefore, milk appears to be the type of sample with more bacterial populations positively correlated with those in the feces of lactating sows or suckling piglets, suggesting its key role in microbial transfer from sow to piglets. Finally, it is important to mention that the study of correlations was performed using samples that were collected at the same time and although the transference of microbiota is not an immediate process, sampling was carried out when both, sows and piglets, had been eating for several days the same diet and it could be assumed that results are representative of the correlations among the different types of samples. Nevertheless, a future study evaluating microbial transference considering a time window between samplings may be of interest.

## Conclusions

The inclusion of fish oil as a source of n-3 LCFA in sow diets influences the microbiota of the feces of gestating sows, their milk, and the feces of suckling piglets, while no effects were observed in colostrum or the feces of lactating sows. The largest impact of n-3 LCFA supplementation is observed in the feces of suckling piglets, which are young animals that are in the process of acquiring microbiota. Concretely, n-3 LCFA increased piglets' fecal microbial diversity and

the relative abundance of beneficial bacteria such as the mucin-degraders genera *Akkermansia* and *Bacteroides*, and different species of *Lactobacillus*, which may contribute to the achievement of a gut anti-inflammatory microbiota. In addition, it can also be concluded that some of these modifications were positively correlated among the feces of lactating sows, their milk, and the feces of the suckling piglets. Milk stands out as the factor with more bacterial populations correlated with both, lactating sows' and suckling piglets' feces.

**Data availability statement**

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ebi. ac.uk/ena, PRJEB53326.

**Ethics statement**

This animal study was reviewed and approved by IRTA's Ethical Committee on Animal Experimentation and Generalitat de Catalunya (project no: 10294).

**Author contributions**

IB, RL, DT, EE-G, and NT contributed to conception and design of the study. EL-C, ErC, EmC, MB, and NT performed the methodology and statistical analysis. EL-C wrote the first draft of the manuscript. ErC and EmC wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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## Conflict of interest

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

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## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.982712/full#supplementary-material

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## Supplementary materials

***Supplementary Table S1:*** *Influence of dietary fish oil rich in n-3 LCFA on the body weight, backfat thickness, and feed intake of gestating and lactating sows.*[1]

| | Control (*n*=11) | n-3 LCFA (*n*=10) | *P* value |
|---|---|---|---|
| Days of gestation | 116 ± 1.10 | 116 ± 1.23 | 0.531 |
| Days of lactation | 25.1 ± 0.94 | 24.9 ± 1.10 | 0.929 |
| Average BW (kg) | | | |
| Service | 212 ± 31.0 | 221 ± 28.0 | 0.494 |
| End of gestation (day 107 of gestation) | 283 ± 23.5 | 286 ± 27.7 | 0.207 |
| Day after farrowing | 259 ± 27.2 | 271 ± 24.7 | 0.336 |
| At weaning (c.a. 28 days post-farrowing) | 238 ± 29.5 | 247 ± 25.0 | 0.812 |
| Average backfat thickness in P2 (mm) | | | |
| Service | 15.4 ± 4.51 | 15.3 ± 5.13 | 0.696 |
| End of gestation (day 107 of gestation) | 15.1 ± 4.37 | 15.0 ± 4.47 | 0.795 |
| At weaning (c.a. 28 days post-farrowing) | 11.3 ± 3.45 | 12.7 ± 4.31 | 0.502 |
| Average daily gain (kg) | | | |
| Gestation | 0.62 ± 0.11 | 0.55 ± 0.06 | 0.202 |
| Lactation | -0.83 ± 0.36 | -1.02 ± 0.36 | 0.278 |
| Total | 0.19 ± 0.11 | 0.18 ± 0.10 | 0.818 |
| Average daily feed intake (kg) | | | |
| Gestation | 2.78 ± 0.02 | 2.79 ± 0.03 | 0.787 |
| Lactation | 5.62 ± 1.05 | 5.45 ± 0.71 | 0.683 |

BW, body weight; LCFA, long chain fatty acid.

[1]Values are means ± SD.

***Supplementary Table S2:*** *Influence of dietary fish oil rich in n-3 LCFA on the litter characteristics at birth and growth performance of suckling piglets.*[1]

| | Control (*n*=11) | n-3 LCFA (*n*=10) | *P* value |
|---|---|---|---|
| At birth[2] | | | |
| Average total born | 15.2 ± 3.82 | 15.4 ± 3.50 | 0.908 |
| Born alive | 14.7 ± 3.41 | 14.8 ± 3.11 | 0.984 |
| Stillborn | 0.46 ± 0.69 | 0.67 ± 0.87 | 0.573 |
| Mummies | 0.55 ± 0.69 | 0.22 ± 0.44 | 0.166 |
| Average litter weight (kg) | 19.7 ± 3.13 | 19.6 ± 3.66 | 0.898 |
| Average piglet BW (kg) | 1.39 ± 0.28 | 1.34 ± 0.19 | 0.665 |
| SD piglet BW (kg) | 0.25 ± 0.05 | 0.25 ± 0.09 | 0.850 |
| 24h after birth[3] | | | |
| Average still alive | 13.7 ± 1.49 | 12.8 ± 1.23 | 0.169 |
| Average of deaths 24h | 0.64 ± 1.03 | 1.00 ± 1.63 | 0.388 |
| Average litter weight (kg) | 19.4 ± 2.89 | 19.1 ± 2.89 | 0.898 |
| Average piglet BW (kg) | 1.43 ± 0.25 | 1.50 ± 0.20 | 0.476 |
| SD piglet BW (kg) | 0.31 ± 0.05 | 0.29 ± 0.09 | 0.238 |
| 20 days after birth | | | |
| Average still alive | 12.0 ± 1.10 | 11.7 ± 1.25 | 0.752 |
| Average litter weight (kg) | 74.1 ± 9.82 | 74.0 ± 7.04 | 0.785 |
| Litter average daily gain (24h → 20d) (kg) | 2.73 ± 0.40 | 2.80 ± 0.30 | 0.339 |
| Average piglet BW (kg) | 6.19 ± 0.72 | 6.34 ± 0.49 | 0.563 |
| SD piglet BW (kg) | 1.23 ± 0.31 | 1.66 ± 0.53 | 0.879 |
| Piglet average daily gain (24h → 20d) (kg) | 0.25 ± 0.03 | 0.26 ± 0.03 | 0.553 |
| At weaning (c.a. 28 days post-farrowing) | | | |
| Average still alive | 11.9 ± 1.14 | 11.7 ± 1.25 | 0.945 |
| Average of deaths lactation | 1.82 ± 1.54 | 1.10 ± 0.99 | 0.920 |
| Average litter weight (kg) | 95.6 ± 11.2 | 95.0 ± 11.4 | 0.608 |
| Litter average daily gain (24h → W) (kg) | 2.83 ± 0.39 | 2.84 ± 0.31 | 0.974 |

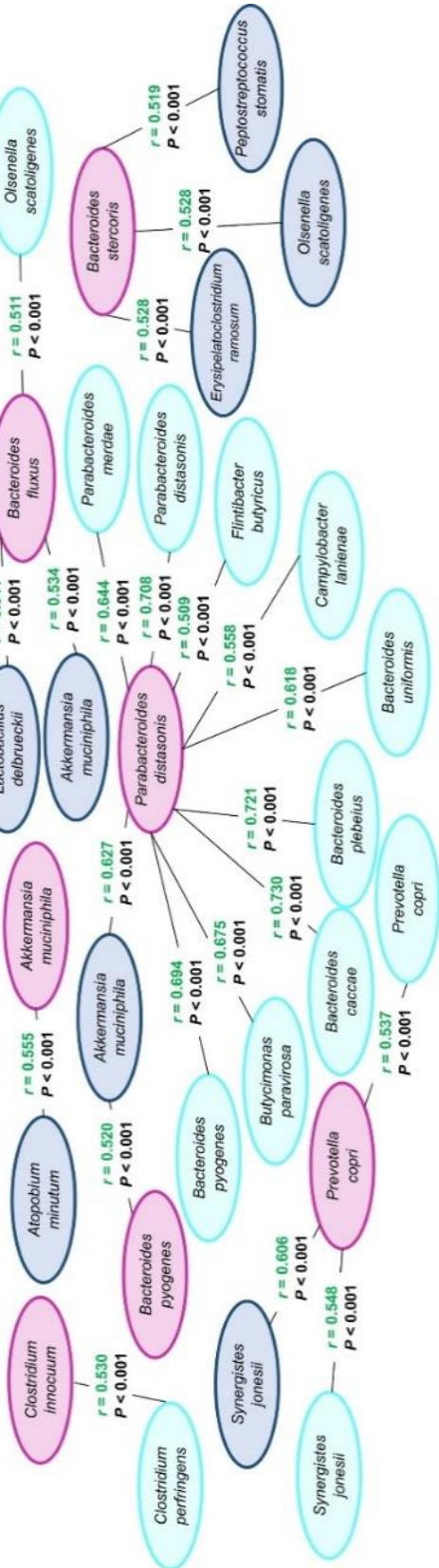
| | | | |
|---|---|---|---|
| Average piglet BW (kg) | 8.07 ± 0.98 | 8.13 ± 0.62 | 0.738 |
| SD piglet BW (kg) | 1.51 ± 0.38 | 1.66 ± 0.53 | 0.482 |
| Piglet average daily gain (24h → W) (kg) | 0.25 ± 0.03 | 0.25 ± 0.02 | 0.714 |
| Piglet creep feed intake (kg) | 0.33 ± 0.06 | 0.30 ± 0.06 | 0.599 |

BW, body weight; LCFA, long chain fatty acid; SD, standard deviation; W, weaning.

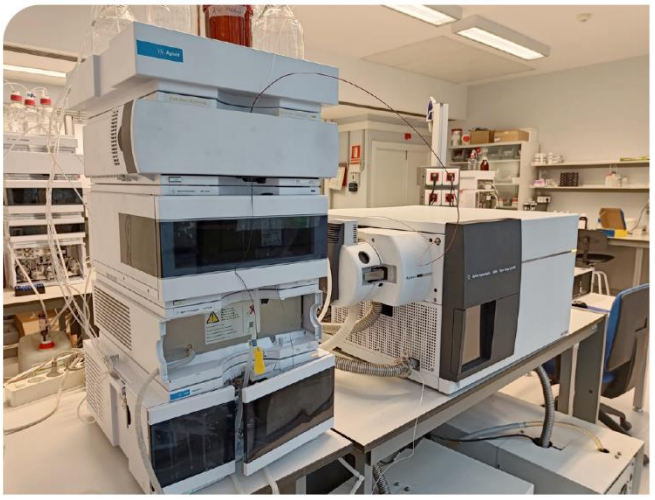
[1]Values are means ± SD.

[2]One sow from n-3 LCFA diet gave birth less than 6 piglets and were excluded for the data analysis of litter characteristics at birth.

[3]Adoptions were completed within 24 hours after birth and the 24 h recordings were considered as the initial values for the litter characteristics and growth performance of suckling piglets.




***Supplementary Figure S1:*** *Correlations between modified microbial species from lactating sows' feces (navy blue, n = 21), milk (light blue, n = 21) and suckling piglets' feces (pink, n = 84). Pearson correlation coefficient (r) in green indicates positive correlation. Significant correlation level was set at r > 0.5 and P < 0.05.*



# CHAPTER 4

## Impact of adding eicosapentaenoic and docosahexaenoic acids-rich fish oil in sow and piglet diets on blood oxylipins and immune indicators of weaned piglet.

**CHAPTER 4:**

# Impact of adding eicosapentaenoic and docosahexaenoic acids-rich fish oil in sow and piglet diets on blood oxylipins and immune indicators of weaned piglets

This chapter has not been published yet

It will be submitted to ***Animal: The International Journal of Animal Science***.

This chapter describes and discusses the effect of adding fish oil rich in n-3 LCFAs in diets for gestating and lactating sows and post-weaning diets for piglets on:

- Weight, growth, and feed intake of piglets 28 days post-weaning.
- Fatty acid composition, oxylipin profile and immune indicators in blood from piglets 28 days post-weaning.
- The effect of dietary n-3 LCFA on these parameters according to the birth BW of the piglets (LBW vs HBW piglets).

# Impact of adding eicosapentaenoic and docosahexaenoic acids-rich fish oil in sow and piglet diets on blood oxylipins and immune indicators of weaned piglets

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**Abstract**

Weaning is considered as a decisive event in the life of piglets and their criticality has been accentuated because of the increasing proportion of piglets born with low weight due to the current hyperprolificacy of the sows. The post-weaning viability of piglets may potentially be enhanced with nutritional strategies. The aim of this study was to evaluate whether the inclusion of fish oil, rich in eicosapentaenoic and docosahexaenoic acids (**EPA** and **DHA**, respectively), in sow and piglet post-weaning diets increased the concentration of anti-inflammatory molecules in the blood of weaned piglets. In addition, it also studied the impact of piglet birth body weight (**bBW**) on these parameters. Thirty-six sows in four consecutive batches were randomly distributed between two diets from service until weaning (ca. 28 days). Treatments consisted of a control diet with animal fat (15 g/kg in gestation and 30 g/kg in lactation) and a diet with n-3 long-chain fatty acid (**LCFA;** 21 g/kg to totally replace (gestation diet) or half replace (lactation diet) animal fat). At birth, the two piglets with the lowest (**LBW**) and the two with the heaviest (**HBW**) bBW in each litter were identified, and at weaning they were grouped in 4 categories according to maternal diet and bBW. Pens in each category were further distributed into two piglet diets: control diet with animal fat (30 g/kg) and LCFA diet with n-3 LCFA (48.6 g/kg) until the end of post-weaning (ca. 28 days). Post-weaning growth and feed intake of piglets was monitored and at day 28 blood and ileal mucosa samples were collected from the 48 selected piglets from the first batch. Serum FA were quantified by GC, plasma oxylipins by ultra-HPLC-MS, plasma immune indicators by ELISA, and ileal mucosa immune indicators by Real-Time-PCR. The largest effect was exerted by the inclusion of fish oil in the piglet diet where increased concentrations of total n-3 FA ($P < 0.001$), particularly EPA ($P < 0.001$) and DHA ($P < 0.001$), and their derived oxylipins were detected in blood of weaned piglets. In addition, immunoglobulin (**Ig**) M tended to increase also in n-3 LCFA fed piglets ($P = 0.067$). While the inclusion of fish oil in maternal diet did not affect FA composition and immune indicators, and only increased two DHA-derived oxylipins, Finally, an increased expression of Toll-like

receptor (**TLR**) 4 in ileal mucosa ($P < 0.001$) and a trend for increased tumor necrosis factor α (**TNFα**) in plasma ($P = 0.083$) were observed in the LBW piglets compared to HBW. It is concluded that the inclusion of fish oil in post-weaning diets increased the blood concentration of n-3 FAs and their derived oxylipins, which are related with anti-inflammatory effects, and tended to increase IgM in blood of weaned piglets. Compared to HBW piglets, LBW piglets presented a higher expression of TLR4 in ileal mucosa and a higher concentration of TNFα in blood, which are related with inflammation.

**Keywords**

n-3 long chain fatty acids, oxygenated lipid mediators, immune indicators, post-weaning, weaned piglets

**Implications**

The current study reveals that the inclusion of fish oil in piglet post-weaning diets increases serum n-3 fatty acids and their derived oxylipins with an anti-inflammatory role and tend to increase immunoglobulin M in plasma, while its inclusion has no effect on the growth and feed intake of piglets between days 0-28 post-weaning. These changes could help to modulate the immune status of piglets during the weaning transition, which is one of the most critical points in the swine production cycle.

**Introduction**

Weaning is considered as one of the most critical steps in swine production. The transition from the maternal milk to a diet based on cereals and soya is an abrupt change. Many piglets do not adapt to the new solid feed source and enter into a low feed intake state or directly stop eating (de Vries and Smidt, 2020). This is

commonly associated with an intestinal atrophy and a consequent reduction in the digestibility and absorption of nutrients (Pluske *et al*., 1997). Moreover, weaning is a very stressful period (social and environmental changes) related with other problems, such as unspecific intestinal inflammatory response and epithelium disruption, which are associated with the appearance of diarrhoea (McCracken *et al*., 1999; Xu *et al*., 2000; Pie *et al*., 2004). To address these challenges, antibiotics were used as growth promoters until 2006, when the European Union banned their use with this purpose (Regulation O: No 1831/2003 of the European Parliament and council of 22 September 2003 on additives for the use in animal nutrition) and recommended the elimination of their preventive and/or metaphylactic use to minimise the risk of developing antibiotic-resistant bacteria. On the other hand, the increased prolificity of sows increased the number of piglets born with a low birth weight (**LBW**) which are more likely to be less robust at weaning, accentuating this critical step in the swine production cycle. For these reasons, studies on perinatal and early nutrition strategies that may impact on piglet development and immune status during the post-weaning period are nowadays a focus of interest.

Polyunsaturated fatty acids (**FAs**) in sow and post-weaning diets have commonly been used for their role as an energy source (Rosero *et al*., 2016), but they can also play an important role as immune modulators (Calder, 2012). Their effects on the immune system vary depending on their nature. In this way, n-6 polyunsaturated FAs and their oxygenated derivatives, also known as oxylipins, are associated with more proinflammatory effects, while n-3 polyunsaturated FAs and their derived oxylipins are related with a less proinflammatory, anti-inflammatory and/or inflammation resolving role (Calder, 2010).

This study is part of a larger project that evaluated the influence of including a fish oil source rich in n-3 polyunsaturated FAs (mainly eicosapentaenoic acid (**EPA**) (C20:5 n-3) and docosahexaenoic acid (**DHA**) (C22:6 n-3)) in sow diets on the improvement of the robustness and health status of their piglets. Previous results showed that the inclusion of n-3 polyunsaturated FAs in sow diets increases total n-3 FAs and their oxygenated derivatives in colostrum and milk, while decreasing

proinflammatory cytokines such as tumour necrosis factor α (**TNFα**) in milk (Llauradó-Calero *et al*., 2021). Similar results in terms of FAs and oxylipins were observed in plasma of sows and suckling piglets, but in that case, an increase of plasma immunoglobulin (**Ig**) M in n-3 FAs fed sows was also observed (Llauradó-Calero *et al*., 2022).

The current study hypothesized that the dietary inclusion of n-3 polyunsaturated FAs could similarly increase total n-3 FAs and their derived oxylipins and modify immune indicators in blood of piglets, also in the postweaning period. The aim of the study was to evaluate the impact of sow and post-weaning diets supplementation with n-3 polyunsaturated FAs on growth, FAs and oxylipin blood profiles, and certain immune indicators in blood and ileal mucosa of piglets at day 28 post-weaning. Understanding the influence that this type of dietary fat may have on the selected parameters could contribute to the development of nutritional strategies based on n-3 polyunsaturated FAs to increase molecules with anti-inflammatory immune modulation capabilities.

## Material and methods

### *Animals, housing, and experimental design*

Thirty-six sows from four consecutive batches (same sows and experimental set-up as those described in Llauradó-Calero *et al*. (2021) and Llauradó-Calero *et al*. (2022)) were grouped in pairs, within each batch, and were randomly assigned to either a control or a n-3 long chain FA (**LCFA**) diet. Sows were fed the dietary treatments from service until weaning (ca. 28 days post-farrowing). All farrowings were supervised and according to their birth body weight (**bBW**) the two lightest (>800 g) (low bBW (**LBW**)) and the two heaviest (high bBW (**HBW**)) piglets from each sow were selected. Pre-starter creep feed (treatment in accordance with maternal diet) was offered to piglets from day eleven of lactation until weaning.
At twenty-eight days of life, the selected piglets from the first batch of sows were weaned and moved to the post-weaning facilities (two piglets per pen) where they

were grouped in 4 categories according to their maternal dietary treatment and their birth BW category. Pens in each category were further distributed between two post-weaning dietary treatments (control or n-3 LCFA diets), resulting in a 2 x 2 x 2 factorial distribution of treatments (2 maternal diets x 2 piglet diets x 2 birth BW categories) until the end of post-weaning period (28 days post-weaning and ± 56 days of age). The pre-starter diet was offered from weaning until day 14 post-weaning and the starter diet between day 14 and day 28 post-weaning.

Post-weaning facilities consisted of a room with 24 slatted pens of 1.7 $m^2$ (1.8 x 0.95 m). The inside of the building was lit through skylight and non-programmable artificial light and ventilated through single variable-speed fans linked to temperature sensors. The temperature was adjusted according to the standard program used at the farm, with a gradual decrease from 30°C to 24°C during the first 21 days post-weaning, and from 24°C to 23°C from day 21 to day 28 post-weaning. Piglets were fed via hoppers and water availability was *ad libitum* consumption via one nipple drinker per pen. At the end of the post-weaning period, piglets were humanely slaughtered in compliance with European Union ethical and welfare regulations.

One sow from the n-3 LCFA treatment in the third batch farrowed outside the expected period and unsupervised, and another two sows in the fourth batch, also from the n-3 LCFA treatment, had very small litter sizes (less than six piglets). For these reasons, piglets' selection according to the BW category was not possible for these sows, and they were therefore removed from the trial.

*Experimental diets*

Gestation and lactation diets for sows (maternal diets) and post-weaning diets for piglets (piglet diets) were formulated according to FEDNA specifications (de Blas *et al*., 2013). For sows, the control diets were formulated to contain 15 and 30 g/kg of animal fat during gestation and lactation, respectively. In the n-3 LCFA diets, 21.5 g/kg of solid fish oil (Lipomega®; V&S Asociados, Madrid, Spain) were used to totally replace (gestation diet) or half replace (lactation diet) animal fat in the

control diets. For the post-weaning diets of piglets, the control diets were formulated to contain 30 g/kg of animal fat (both pre-starter and starter specifications), and in the n-3 LCFA diets animal fat was totally replaced by an equivalent amount of solid fish oil (48.6 g/kg). Pre-starter diets in mash form were also offered as a creep feed from day eleven of lactation until weaning. For all periods, diets were provided *ad libitum*.

Sows' and piglets' diets were formulated to contain the same level of the main nutrients between dietary treatments with the exception of FA composition. Ingredient and nutrient, as well as the FA composition of pre-starter and starter diets are described in Table 1 and Table 2, respectively. All the information about sow diets has been previously described in Llauradó-Calero *et al*. (2021).

***Table 1:*** *Ingredient and nutrient composition of the control and n-3 LCFA pre-starter and starter diets for weaned piglets (as fed basis).*

| | Pre-starter | | Starter | |
|---|---|---|---|---|
| | Control | n-3 LCFA | Control | n-3 LCFA |
| Ingredients (g/Kg) | | | | |
| Barley | 226 | 220 | 150 | 150 |
| Corn | 314 | 315 | 514 | 509 |
| Soybean 48% | 150 | 150 | 235 | 236 |
| Sweet whey (dehydrated) | 110 | 110 | - | - |
| Dicalcium phosphate | 18.2 | 18.2 | 17.0 | 17.0 |
| Animal fat (5 Sysfeed)[1] | 30.0 | - | 30.0 | - |
| Fish oil (Lipomega®)[2] | - | 48.6 | - | 48.6 |
| L-lysine HCL | 5.50 | 5.50 | 5.20 | 5.20 |
| L-threonine | 2.50 | 2.50 | 2.30 | 2.30 |
| DL-methionine | 2.70 | 2.70 | 2.10 | 2.10 |
| L-tryptophan | 0.80 | 0.80 | 0.70 | 0.70 |
| L-Valine | 1.30 | 1.30 | 1.00 | 1.00 |
| Calcium carbonate | 2.00 | 2.70 | 2.60 | 3.30 |
| Sodium bicarbonate | 4.50 | 4.50 | 4.50 | 4.50 |
| Sodium chloride | 0.80 | 0.80 | 1.70 | 1.70 |

| | | | | |
|---|---|---|---|---|
| Sodium caseinate | 20.0 | 20.0 | - | - |
| Celite | 15.0 | - | 15.5 | - |
| HP 300[3] | 88.8 | 89.3 | 12.1 | 12.4 |
| Vitamin-Mineral Premix[4] | 6.00 | 6.00 | 6.00 | 6.00 |
| Antioxidant (Noxyfeed 56P)[5] | 2.50 | 2.50 | 0.20 | 0.20 |
| Analysed nutrient composition[6] (g/Kg) | | | | |
| ME (MJ/Kg) | 14.1 | 13.8 | 13.5 | 13.6 |
| Crude fibre | 19.0 | 18.7 | 20.9 | 20.1 |
| Ether extract | 49.8 | 52.4 | 62.0 | 54.5 |
| Crude protein | 204 | 205 | 177 | 176 |
| Lysine | 13.3 | 13.3 | 12.0 | 12.0 |

LCFA, long chain fatty acid; ME, metabolizable energy; ND, non-detected.

[1]Product of Sysfeed SLU (Granollers, Spain). It contains myristic acid (C14:0) 1.50%, palmitic acid (C16:0) 18.0%, palmitoleic acid (C16:1 n-7) 2.00%, stearic acid (C18:0) 14.0%, oleic acid (C18:1 n-9 cis) 28.0%, linoleic acid (C18:2 n-6 cis) 12.0%, α-linolenic acid (C18:3 n-3 cis) 6.00%, saturated-unsaturated 0.7%.

[2]Product of V&S Asociados (Madrid, Spain). It contains 63.36% of fat, myristic acid (C14:0) 4.79%, palmitic acid (C16:0) 14.9%, stearic acid (C18:0) 3.77%, oleic acid (C18:1 n-9 cis) 12.3%, linoleic acid (C18:2 n-6 cis) 2.71%, α-linolenic acid (C18:3 n-3 cis) 1.21%, arachidonic acid (C20:4 n-6 cis) 0.75%, eicosapentaenoic acid (C20:5 n-3 cis) 7.92%, docosahexaenoic acid (C22:6 n-3 cis) 6.91% and 36.64% of inert excipients.

[3]Product of Hamlet Protein (Horsens, Denmark). It contains 56.0% of protein, 23.2% of carbohydrates, 8.0% of $H_2O$, 6.8% of ash, 3.5% of crude fibre and 2.5% of fat. Essential amino acids (g/16g of N): lysine 6.1, methionine 1.3, cysteine 1.4, threonine 3.9, tryptophan 1.35, leucine 7.7, isoleucine 4.6, phenylalanine 5.0, tyrosine 3.7, valine 4.8, histidine 2.6 and arginine 7.2.

[4]Product of TecnoVit S.L. (Alforja, Spain). Supplied per kilogram of feed: vitamin A 10,000 UI, vitamin $D_3$ 2,000 UI, vitamin E 25.0 mg, vitamin $B_1$ 1.50 mg, vitamin $B_2$ 3.50 mg, vitamin $B_6$ 2.40 mg, vitamin $B_{12}$ 0.04 mg, vitamin $K_3$ 1.50 mg, calcium D-pantothenate 14.0 mg, nicotinamide 20.0 mg, folic acid 0.50 mg, biotin 0.05 mg, Fe (as $FeSO_4 \cdot H_2O$) 120 mg, I (as $Ca(IO_3)_2$) 0.75 mg, Mn (as MnO) 60.0 mg, Se (as $Na_2SeO_3$) 0.37 mg.

[5]Product of Itpsa (Barcelona, Spain). It contains 56% of antioxidants substances (BHT + propyl gallate) and synergistic (Citric acid 14% + authorized support).

[6]Nutrient composition values correspond to the analysed values except for ME and lysine which were estimated according to INRA tables (Sauvant *et al*., 2004).

***Table 2:*** *Fatty acid composition of the control and n-3 LCFA pre-starter and starter diets for weaned piglets.*[1]

| | Pre-Starter | | Starter | |
|---|---|---|---|---|
| | Control | n-3 LCFA | Control | n-3 LCFA |
| Fat (%) | 4.53 | 5.21 | 5.38 | 5.55 |
| Fatty Acids (mg FA/g fat) | | | | |
| C14:0 | 8.34 | 26.1 | 5.27 | 21.5 |
| C15:0 | 0.63 | 2.10 | 0.33 | 1.68 |
| C16:0 | 143 | 130 | 139 | 120 |
| C16:1 | 11.7 | 26.5 | 10.9 | 24.6 |
| C17:0 | 1.60 | 3.17 | 1.46 | 2.77 |
| C18:0 | 47.8 | 30.1 | 47.0 | 26.6 |
| C18:1 n-9 *cis* | 228 | 139 | 242 | 149 |
| C18:1 n-9 *trans* | 1.29 | 0.66 | 0.96 | 0.47 |
| C18:2 n-6 *cis* | 214 | 167 | 239 | 185 |
| C18:3 n-6 *cis* | ND | ND | ND | ND |
| C20:2 n-6 *cis* | 2.58 | 1.04 | 2.46 | 0.96 |
| C21:0 | ND | ND | ND | ND |
| C20:4 n-6 | 1.76 | 3.85 | 1.77 | 3.55 |
| C20:5 n-3 | ND | 41.3 | 0.20 | 39.7 |
| C22:3 n-3 | ND | ND | ND | ND |
| C24:0 | 1.11 | 1.20 | 1.18 | 1.07 |
| C22:5 n-3 | 0.56 | 5.77 | 0.57 | 5.48 |
| C22:6 n-3 | 0.53 | 36.0 | 0.72 | 34.9 |
| Minor FA[3] | 41.8 | 61.9 | 39.6 | 56.4 |
| SFA | 209 | 203 | 200 | 182 |
| MUFA | 260 | 196 | 272 | 202 |

| | | | | |
|---|---|---|---|---|
| PUFA | 236 | 276 | 260 | 289 |
| n-3 | 16.7 | 102 | 16.6 | 97.2 |
| n-6 | 219 | 175 | 243 | 192 |
| n-6:n-3 | 13.2 | 1.72 | 14.6 | 1.97 |

FA, fatty acid; LCFA, long chain fatty acid; MUFA, monounsaturated fatty acid; ND, non-detected; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.

[1]FA quantification results are reported from C12:0.

[2]Minor FAs include: C12:0, C14:1 n-9 *cis*, C17:1, C18:1 n-11 *cis*, C18:1 n-7, C18:2 n-6 *trans*, C19:0, C18:3 n-3 *cis*, C18:4 n-3, C20:0, C20:1 n-9 *cis*, C20:3 n3 *cis,* C20:3 n-6, C22:0, C22:1, C23:0, C22:4 n-6 and C24:1 n-9 *cis*. C13:0, C15:1 and C22:2 n-6 *cis* have not been detected.

*Growth measurements*

Piglets were weighted at weaning, at days 8 and 15 after weaning, and the end of the trial day 28 post-weaning. Daily feed intake was monitored for each pen and recorded on the same days of weighing. Piglets' BWs were analysed individually, and average daily gain was calculated using the piglet as experimental unit. Average daily feed intake and gain to feed ratio was calculated per pen (experimental unit).

Due to piglet availability, four of the pens (one pen from control diet and three from n-3 LCFA diet) could not be formed using pigs with the same birth category, and these were not used for the feed intake and gain to feed calculations and analyses.

*Sampling description*

For the first batch of sows, blood and ileal mucosa samples were collected at the end of post-weaning period from the selected piglets. Blood was obtained by jugular venepuncture in non-heparinised tubes for serum and in ethylenediaminetetraacetic acid (EDTA) tubes for plasma. Non-heparinised and

EDTA tubes were kept at ambient temperature and at 4ºC for a maximum of 120 min until centrifugation (300 rpm, 10 min). Aliquots of serum for FA analysis, and of plasma for oxylipins (in tubs containing 0.005% butylated hydroxytoluene as antioxidant (Merck, Darmstadt, Germany)), Ig and cytokines analyses were collected and quickly stored at -80ºC (maximum 30 min from centrifugation to storage). Mucosa samples from ileum were collected with a scraper and stored at -80ºC in 2.5 mL RNA cryogenic tubs containing 1 mL of RNAlater (Sigma Aldrich, St. Louis, MO, USA) until the analysis of determined mucosal immune indicators.

*Quantitative analysis of fatty acids*

The serum collected from weaned piglets was used to extract the fat with chloroform (PanReac AppliChem, Barcelona, Spain) - methanol (Honeywell, Charlotte, NC, USA) according to Folch *et al*. (1957). Afterwards, extracted fat was transmethylated with boron trifluoride (Sigma Aldrich, St. Louis, MO, USA) and methanolic potassium hydroxide 0.5M (PanReac, Barcelona, Spain) according to Morrison and Smith (1964). Individual FAs were determined by GC (Agilent 6890N, Boston, MA, USA) using the analytical procedure formerly described in Llauradó-Calero *et al*. (2021). FAs quantifications were performed from C12:0 and results are reported as mg of FA per g of serum.

*Metabolomic analysis of oxylipins*

A total of fifty-three oxylipins were quantified from the collected plasma of weaned piglets. Aliquots of 0.25 mL of plasma were set up according to the previously described optimized process (Llauradó-Calero *et al*., 2022). The oxylipins concentrations were determined using an Ultra High Performance LC (UHPLC) 1290 Series coupled to a triple quadrupole mass spectrometer 6490 series instrument (Agilent, Santa Clara, CA, USA) with an analytical column Eclipse XDB C18 1.8 µL (2.1 x 150 mm) (Agilent, Santa Clara, CA, USA). The identification with

standards or tentative identification of oxylipins was previously described in Llauradó-Calero *et al*. (2021).

*Immune indicators*

Plasmatic concentrations of different immunoglobulins (immunoglobulin (**Ig**) G, A and M) and cytokines (interleukins (**IL**) 1β, 6, 10 and tumor necrosis factor α (**TNFα**)) were quantitatively determined throughout the sandwich ELISA kits used and previously reported in Llauradó-Calero *et al*. (2021).

The mucosal immune indicators IL2 and IL10, interferon γ, tumor necrosis factor α, toll-like receptors (**TLR**) 2 and 4, and major histocompatibility complex class II were quantitatively measured from ileal mucosa. RNA was extracted from mucosa employing the RNeasy Mini Kit (Qiagen,Hilden, Germany) and all immune indicators were measured by Real-Time PCR (RT-PCR) using Invitrogen Express One-Step Sybr GreenER Kit Universal (11780200; Invitrogen, Waltham, MA, USA). Samples in Real Time-PCR are analysed per duplicate and the program was 50ºC/5'; 95ºC/20''; (95ºC/3''; 60ºC/30'') x 40 cycles. Results from Real-Time PCR were calculated with the method $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001), using the Glyceraldehyde 3 phosphate dehydrogenase as housekeeping gene. All primers used for Real-Time PCR analysis are described in Supplementary Table S1.

*Statistical analysis*

The GLIMMIX procedure of SAS software (SAS/STAT 14.1; SAS Institute INC., Cary, NC, USA) was applied to analyse the results with an ANOVA test. The piglet was used as the experimental unit for the weight of piglets and their growth, FAs and oxylipins concentrations and for the immune indicators, and the model included maternal diet, piglet diet and bBW category as fixed effects and the pen as random effect. For feed intake and gain to feed ratio, the pen was used as experimental unit and the model included piglet diet and bBW category as fixed

effects and batch as random effect. Interactions between fixed effects were also calculated.

When the limit of detection was not reached in the analysis of FAs, oxylipins or immune indicators, missing values were replaced by 1/5 of the minimum positive value for each variable. Data suspected of being outliers were examined by means of a Kolmogorov-Smirnov test excluding the values if $P < 0.01$. To compare the oxylipins concentrations between treatments a logarithmic transformation ($\log_{10}(X + 1)$) of the data was performed. The original means are presented in the supplementary tables. The results of growth and feed intake, FAs and immune indicators are presented as least squares means ± RMSE, and the results of oxylipins are expressed as means ± RMSE (from transformed data). Significant differences and tendencies were considered at $P < 0.05$ and $P < 0.1$, respectively. The Principal Component Analyses (PCAs) for FAs and oxylipins and the heatmap for oxylipins were performed using MetaboAnalyst 5.0 (https://www.metaboanalyst.ca, Alberta, CA, USA).

## Results

*Piglets' weight, growth, and feed intake*

BW, average daily weight gain, average daily feed intake, and gain to feed ratio of piglets during the post-weaning period are reported in Table 3. The interactions between diets (maternal and piglet diets) and diets with bBW category are reported as footnotes. Effects of n-3 LCFA inclusion in maternal or piglet diets were not observed on the piglets' BW and average daily gain during the post-weaning period. However, as expected, there were significant differences in BW between LBW and HBW animals (all $P < 0.001$). In addition, a trend ($P = 0.058$) towards a higher average daily weight gain in the HBW compared with LBW piglets was also observed during the last period of the trial (between 15 and 28 days). Significant effects due to piglet diet or BW category were not observed for feed intake nor for gain to feed ratio.

Interactions between maternal diet or piglet diet and bBW indicated a maternal diet effect until day 15, while the effect of the piglet diet persists throughout the post-weaning phase.

***Table 3:*** *Effect of dietary fish oil in maternal or post-weaning diets and piglet birth body weight on growth performance parameters of weaned piglets.*[1,2]

| | Weaned piglets | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | MDiet | | PDiet[3] | | bBW[3] | | | *P* | *P* | *P* |
| | Control (n=72) | n-3 LCFA (n=60) | Control (n=66/32) | n-3 LCFA (n=66/30) | LBW (n=66/31) | HBW (n=66/31) | RMSE | value MDiet | value PDiet | value bBW |
| Average BW (kg)[4] | | | | | | | | | | |
| At weaning | 8.04 | 8.48 | 8.20 | 8.32 | 7.21 | 9.31 | 1.19 | 0.145 | 0.583 | <0.001 |
| 8 days post-weaning | 8.80 | 9.31 | 8.94 | 9.18 | 7.99 | 10.1 | 1.17 | 0.134 | 0.296 | <0.001 |
| 15 days post-weaning | 10.7 | 11.4 | 11.0 | 11.1 | 9.90 | 12.2 | 1.49 | 0.154 | 0.816 | <0.001 |
| 28 days post-weaning | 17.2 | 17.8 | 17.3 | 17.6 | 16.0 | 19.0 | 2.85 | 0.522 | 0.681 | <0.001 |
| Average daily gain (kg)[4] | | | | | | | | | | |
| Weaning → 8 days pw | 0.094 | 0.11 | 0.093 | 0.11 | 0.097 | 0.105 | 0.05 | 0.650 | 0.349 | 0.593 |
| Weaning → 15 days pw* | 0.18 | 0.19 | 0.19 | 0.19 | 0.18 | 0.20 | 0.05 | 0.491 | 0.915 | 0.290 |
| 8 days pw → 15 days pw** | 0.28 | 0.29 | 0.30 | 0.27 | 0.27 | 0.30 | 0.08 | 0.513 | 0.397 | 0.345 |
| Weaning → 28 days pw*** | 0.33 | 0.33 | 0.33 | 0.33 | 0.31 | 0.35 | 0.08 | 0.847 | 0.799 | 0.106 |
| 15 days pw → 28 days pw | 0.50 | 0.49 | 0.49 | 0.50 | 0.47 | 0.52 | 0.14 | 0.877 | 0.636 | 0.058 |
| Average daily feed intake (kg)[5] | | | | | | | | | | |
| Weaning → 8 days pw | - | - | 0.17 | 0.17 | 0.17 | 0.17 | 0.04 | - | 0.887 | 0.436 |
| Weaning → 15 days pw | - | - | 0.25 | 0.24 | 0.24 | 0.25 | 0.05 | - | 0.499 | 0.297 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 8 days pw → 15 days pw | - | - | 0.68 | 0.64 | 0.639 | 0.68 | 0.17 | - | 0.424 | 0.349 |
| Weaning → 28 days pw**** | - | - | 0.45 | 0.44 | 0.427 | 0.50 | 0.08 | - | 0.869 | 0.121 |
| 15 days pw → 28 days pw***** | - | - | 1.34 | 1.35 | 1.29 | 1.40 | 0.26 | - | 0.938 | 0.108 |
| Gain to feed ratio[5] | | | | | | | | | | |
| Weaning → 8 days pw | - | - | 0.54 | 0.58 | 0.563 | 0.55 | 0.24 | - | 0.534 | 0.817 |
| Weaning → 15 days pw | - | - | 0.75 | 0.74 | 0.747 | 0.74 | 0.21 | - | 0.780 | 0.872 |
| 8 days pw → 15 days pw | - | - | 0.44 | 0.44 | 0.437 | 0.44 | 0.33 | - | 0.983 | 0.981 |
| Weaning → 28 days pw | - | - | 0.73 | 0.72 | 0.722 | 0.73 | 0.14 | - | 0.829 | 0.895 |
| 15 days pw → 28 days pw | - | - | 0.36 | 0.40 | 0.370 | 0.39 | 0.25 | - | 0.570 | 0.713 |

bBW, birth weight; FA, HBW, high birth weight piglets; LBW, low birth weight piglets; LCFA, long chain fatty acid; MDiet, maternal diet; PDiet, piglet diet; pw, post-weaning.

[1]Values are least squares means ± RMSE.

[2]*P* values of the significantly different interactions are reported in the footnotes.

[3]For PDiet i bBW, the first *n* corresponds to piglets and the second to pens.

[4]Piglet was considered the experimental unit for calculations.

[5]Pen was considered the experimental unit for calculations.

**P* value of the interaction MDiet*bBW was *P* = 0.022 were 0.158[b] for control-LBW, 0.200[a] for control-HBW, 0.198[ab] for n-3 LCFA-LBW, and 0.188[ab] for n-3 LCFA-HBW; *P* value of the interaction PDiet*bBW was *P* = 0.031 were 0.162[b] for control-LBW, 0.212[a] for control-HBW, 0.194[ab] for n-3 LCFA-LBW, and 0.175[ab] for n-3 LCFA-HBW. Values are expressed in kg.

**$P$ value of the interaction MDiet*bBW was $P$ = 0.030 were $0.245^{b}$ for control-LBW, $0.304^{a}$ for control-HBW, $0.302^{ab}$ for n-3 LCFA-LBW, and $0.284^{ab}$ for n-3 LCFA-HBW; $P$ value of the interaction PDiet*bBW was $P$ = 0.020 were $0.257^{b}$ for control-LBW, $0.334^{a}$ for control-HBW, $0.290^{ab}$ for n-3 LCFA-LBW, and $0.255^{b}$ for n-3 LCFA-HBW. Values are expressed in kg.

***$P$ value of the interaction PDiet*bBW was $P$ = 0.049 where the values in kg were $0.289^{b}$ for control-LBW, $0.364^{a}$ for control-HBW, $0.336^{ab}$ for n-3 LCFA-LBW, and $0.328^{ab}$ for n-3 LCFA-HBW.

****$P$ value of the interaction PDiet*bBW was $P$ = 0.036 where the values in kg were $0.405^{b}$ for control-LBW, $0.485^{a}$ for control-HBW, $0.451^{ab}$ for n-3 LCFA-LBW, and $0.432^{ab}$ for n-3 LCFA-HBW.

*****$P$ value of the interaction PDiet*bBW was $P$ = 0.047 where the values in kg were $1.21^{b}$ for control-LBW, $1.47^{a}$ for control-HBW, $1.37^{ab}$ for n-3 LCFA-LBW, and $1.32^{ab}$ for n-3 LCFA-HBW.

*Fatty acid composition*

The influence of fish oil in maternal or piglet diets and piglet bBW on the weaned piglet's FAs concentrations in blood serum is shown in Table 4. The interactions between diets (maternal and piglet diets) and diets with bBW category are reported as footnotes. The addition of fish oil in the maternal diet did not change any of the individual FAs analysed. On the contrary, the inclusion of n-3 LCFAs in the piglet diets decreased or tended to decrease the saturated FAs pentadecylic acid (C15:0) ($P$ = 0.007), palmitic acid (C16:0) ($P$ = 0.020), margaric acid (C17:0) ($P$ < 0.001), stearic acid (C18:0) ($P$ = 0.084), heneicocylic acid (C21:0) ($P$ = 0.010) and lignoceric acid (C24:0) ($P$ = 0.024), the monounsaturated FA elaidic acid (C18:1 n-9 *trans*) ($P$ = 0.026), the n-6 polyunsaturated FAs γ-linolenic acid (C18:3 n-6 *cis*) ($P$ = 0.063), eicosadienoic acid (C20:2 n-6 *cis*) ($P$ = 0.003) and arachidonic acid (C20:4 n-6) ($P$ = 0.031), and the n-3 polyunsaturated FA docosatrienoic acid (C22:3 n-3) ($P$ = 0.018). On the other hand, n-3 LCFAs in piglet diets increased or tended to increase the saturated FA myristic acid (C14:0) ($P$ < 0.001), the monounsaturated FA palmitoleic acid (C16:1) ($P$ = 0.074), and the n-3 polyunsaturated FAs EPA ($P$ < 0.001) and DHA ($P$ < 0.001). In addition, despite not detecting changes in the total amount of polyunsaturated FAs or the n-6 family, piglets offered the n-3 LCFA diet had a higher concentration of total n-3 FAs ($P$ < 0.001) and a lower n-6:n-3 ratio ($P$ < 0.001) than the piglets from the control diet. Regarding piglet bBW, LBW piglets tended to decrease the concentrations of the n-6 polyunsaturated FAs: γ-linolenic acid ($P$ = 0.058) and eicosadienoic acid ($P$ = 0.062).

As shown in the principal component analysis, maternal diet (Figure 1, A) and piglet birth BW (Figure 1, C) had not affected the distribution of the samples according to their plasma FAs concentration. On the contrary, it can be observed a differential distribution of the samples according to the piglet's experimental diets (control vs. n-3 LCFA diet) (Figure 1, B).

***Table 4:*** *Influence of dietary fish oil in maternal or post-weaning diets and piglet birth body weight on fatty acid profile of blood serum from weaned piglets.*[1,2,3,4]

| | 28 days weaned piglets (ca. 56 days of age) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | MDiet | | PDiet | | bBW | | | | | |
| | Control (n=27) | n-3 LCFA (n=20) | Control (n=24) | n-3 LCFA (n=23) | HBW (n=24) | LBW (n=23) | RMSE | *P* value MDiet | *P* value PDiet | *P* value bBW |
| Fatty acid (mg FA/g serum) | | | | | | | | | | |
| C14:0 | 0.024 | 0.024 | 0.018 | 0.030 | 0.023 | 0.025 | 0.01 | 0.934 | <0.001 | 0.490 |
| C15:0 | 0.013 | 0.013 | 0.015 | 0.011 | 0.012 | 0.014 | <0.01 | 0.767 | 0.007 | 0.501 |
| C16:0 | 0.63 | 0.60 | 0.70 | 0.52 | 0.63 | 0.59 | 0.21 | 0.671 | 0.020 | 0.571 |
| C16:1 | 0.048 | 0.050 | 0.044 | 0.054 | 0.050 | 0.048 | 0.02 | 0.803 | 0.074 | 0.820 |
| C17:0 | 0.054 | 0.047 | 0.067 | 0.034 | 0.051 | 0.050 | 0.02 | 0.468 | <0.001 | 0.944 |
| C18:0 | 0.40 | 0.38 | 0.43 | 0.35 | 0.41 | 0.37 | 0.12 | 0.812 | 0.084 | 0.470 |
| C18:1 n-9 *cis* | 0.65 | 0.67 | 0.74 | 0.60 | 0.70 | 0.63 | 0.21 | 0.767 | 0.161 | 0.530 |
| C18:1 n-9 *trans* | 0.020 | 0.012 | 0.023 | 0.009 | 0.015 | 0.017 | 0.02 | 0.187 | 0.026 | 0.672 |
| C18:2 n-6 *cis* | 0.73 | 0.85 | 0.82 | 0.76 | 0.86 | 0.72 | 0.22 | 0.466 | 0.621 | 0.248 |
| C18:3 n-6 *cis* | 0.021 | 0.022 | 0.023 | 0.020 | 0.023 | 0.020 | <0.01 | 0.736 | 0.063 | 0.058 |
| C20:2 n-6 *cis* | 0.010 | 0.011 | 0.015 | 0.006 | 0.013 | 0.007 | <0.01 | 0.626 | 0.003 | 0.062 |
| C20:4 n-6 | 0.17 | 0.17 | 0.20 | 0.14 | 0.19 | 0.14 | 0.05 | 0.840 | 0.031 | 0.253 |
| C20:5 n-3 | 0.12 | 0.12 | 0.024 | 0.21 | 0.11 | 0.12 | 0.10 | 0.993 | <0.001 | 0.752 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| C21:0 | 0.011 | 0.013 | 0.015 | 0.009 | 0.014 | 0.011 | <0.01 | 0.402 | 0.010 | 0.383 |
| C22:3 n-3 | 0.016 | 0.023 | 0.024 | 0.015 | 0.022 | 0.017 | 0.01 | 0.302 | 0.018 | 0.264 |
| C22:5 n-3* | 0.054 | 0.081 | 0.062 | 0.073 | 0.075 | 0.060 | 0.10 | 0.351 | 0.681 | 0.610 |
| C22:6 n-3 | 0.076 | 0.087 | 0.050 | 0.11 | 0.080 | 0.083 | 0.04 | 0.434 | <0.001 | 0.763 |
| C24:0 | 0.017 | 0.014 | 0.019 | 0.013 | 0.018 | 0.014 | <0.01 | 0.149 | 0.025 | 0.169 |
| Minor FA[5] | 0.23 | 0.27 | 0.26 | 0.24 | 0.27 | 0.23 | 0.11 | 0.473 | 0.645 | 0.246 |
| SFA | 1.18 | 1.15 | 1.28 | 1.06 | 1.21 | 1.13 | 0.30 | 0.806 | 0.105 | 0.575 |
| MUFA | 0.83 | 0.90 | 0.89 | 0.85 | 0.92 | 0.82 | 0.16 | 0.574 | 0.640 | 0.457 |
| PUFA | 1.27 | 1.41 | 1.33 | 1.34 | 1.44 | 1.23 | 0.34 | 0.495 | 0.950 | 0.172 |
| n-3 | 0.30 | 0.34 | 0.19 | 0.45 | 0.32 | 0.32 | 0.18 | 0.381 | <0.001 | 0.953 |
| n-6 | 0.96 | 1.08 | 1.06 | 0.97 | 1.12 | 0.92 | 0.23 | 0.527 | 0.465 | 0.234 |
| n-6:n-3 | 5.87 | 4.91 | 8.63 | 2.15 | 5.99 | 4.78 | 3.72 | 0.386 | <0.001 | 0.277 |

bBW, birth weight; FA, fatty acid; HBW, high birth weight piglets; LBW, low birth weight piglets; LCFA, long chain fatty acid; MDiet, maternal diet; MUFA, monounsaturated fatty acid; PDiet, piglet diet; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.

[1]Values are least squares means ± RMSE.

[2]FA quantification results are reported from C12:0.

[3]*P* values of the significantly different interactions are reported in the footnotes.

[4]During the analysis process, a sample corresponding to the control maternal diet, n-3 LCFA piglet diet, and LBW was lost.

5Minor FAs include: C12:0, C15:1, C17:1, C18:1 n-11 *cis*, C18:1 n-7, C18:2 n-6 *trans*, C19:0, C18:3 n-3 *cis*, C18:4 n-3, C20:0, C20:1 n-9 *cis*, C20:3 n-6, C22:0, C22:1, C22:2 n-6 *cis*, C23:0, C22:4 n-6 and C24:1 n-9 *cis*. C13:0, C14:1 n-9 *cis* and C20:3 n3 *cis* have not been detected.

*$P$ value of the interaction MDiet*bBW was $P = 0.038$ were $0.042^{b}$ for control-LBW, $0.071^{ab}$ for control-HBW, $0.13^{a}$ for n-3 LCFA-LBW, and $0.048^{b}$ for n-3 LCFA-HBW; $P$ value of the interaction PDiet*bBW was $P = 0.027$ were $0.12^{a}$ for control-LBW, $0.030^{b}$ for control-HBW, $0.056^{ab}$ for n-3 LCFA-LBW, and $0.089^{ab}$ for n-3 LCFA-HBW; $P$ value of the interaction MDiet*PDiet was $P = 0.042$ were $0.030^{b}$ for control-control, $0.083^{ab}$ for control-n-3 LCFA, $0.12^{a}$ for n-3 LCFA-control, and $0.061^{ab}$ for n-3 LCFA-n-3 LCFA. Concentrations are expressed in mg FA/g serum.

*Oxylipin profile*

The changes in the plasmatic oxylipin profile of weaned piglets as affected by the inclusion of fish oil in the maternal or piglet diets, and by the piglet bBW are shown in the Supplementary Table S2. The effects of the interactions between maternal/piglet diet and bBW, maternal and piglet diet, and between maternal diet, piglet diet and bBW are described as footnotes. Similar to what it was observed for FA composition, the inclusion of n-3 LCFA on the maternal diet had little impact on the oxylipin profile. Piglets from n-3 LCFA fed sows presented increased concentrations of 7- and 16-hydroxy-DHA ($P = 0.054$ and $P = 0.022$, respectively) compared to piglets from the control sows. Also, as for FAs, the inclusion of fish oil in the piglet diets was the factor causing most of the changes on the oxylipin concentrations. Up to a total of 27 oxylipins differed (or tended to differ) between the two piglet dietary treatments, where dietary n-3 LCFAs decreased the concentration of 5 of them and increased the concentration of another 22. Among the oxylipins that were decreased by the addition of n-3 LCFAs in the piglet diets there were the linoleic acid (C18:2 n-6 *cis*) derived oxylipins 9,10-dihydroxy-octadecadienoic acid ($P < 0.001$) and 9(10)-dihydroxy-octadecenoic acid ($P = 0.077$), and the arachidonic acid derived oxylipins thromboxane B2 ($P = 0.065$), prostaglandin E2 ($P = 0.091$) and 20-hydroxy-eicosatetraenoic acid ($P = 0.012$). Contrarily, the plasma concentrations of 15(R)-Lipoxin A4/Lipoxin A4 ($P < 0.001$), 5,6-dihydroxy-eicosatrienoic acid ($P = 0.002$), 8- and 15(s)-hydroxy-eicosatetraenoic acids ($P < 0.001$ and $P = 0.074$, respectively) and 14,15-epoxy-eicosatrienoic acids ($P = 0.017$), which are derived from arachidonic acid; 5(s)-, 8-, 9-, 11-, 12(s)-, 15(s)- and 18-hydroxy-eicosapentaenoic acids (all $P < 0.001$), which are derived from EPA; and 4-, 7-, 8-, 10-, 11-, 13-, 14-, 16-, 17- and 20-hydroxy-DHA (all $P < 0.001$), which are derived from DHA, were increased or tended to be increased in the n-3 LCFA diet fed piglets. Little effect piglet bBW was observed on plasma oxylipin concentration profile, only higher concentrations of 7- and 8-hydroxy-DHA ($P = 0.025$ and $P = 0.010$, respectively) were detected in the LBW piglets compared with those from the HBW category. At last, an interaction

between piglet diet and bBW was observed for 14-hydroxy-DHA, increasing their concentration in both, LBW and HBW piglets.

The principal component analysis also revealed that maternal diet (Figure 1, D) or piglets' bBW category (Figure 1, F) had not effects on the sample distribution according to their plasma oxylipin concentration. However, it can be observed a clear separation between samples from piglets fed control and n-3 LCFA (Figure 1, E). The heatmap presented in Figure 2 schematically shows that piglet diet is the main effect causing a substantial difference in terms of plasmatic oxylipin concentrations as a result of n-3 LCFAs dietary supplementation. Finally, an overview of all the oxylipins modified by the inclusion of fish oil in sow or piglet diets, or affected by piglet bBW category are summarized in Supplementary Figure S1 together with FAs precursors and the enzymatic pathways involved in the generation of each oxylipin.

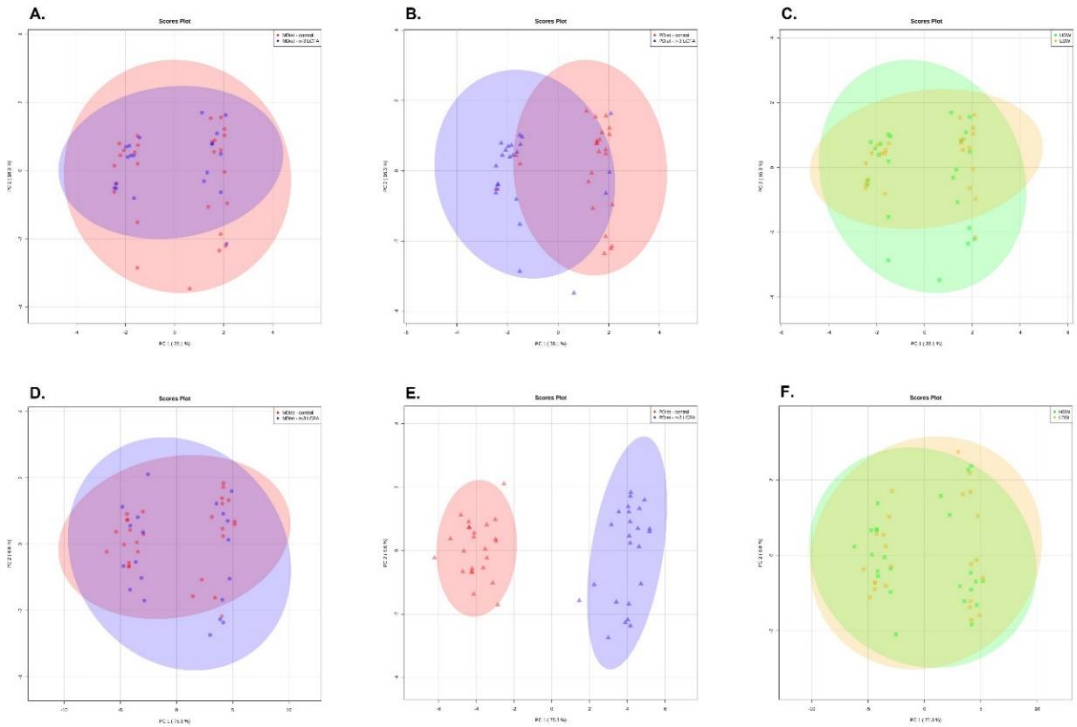


***Figure 1:*** *Principal component analysis 2-dimension plot expressing the effect of the inclusion of fish oil in the maternal diet and in the piglet diet, or the effect of the birth body weight of piglets on fatty acids (FAs) in serum and oxylipins in plasma from piglets at 28*

*days post-weaning. The influence of fish oil in the maternal diet and piglet diet on FAs and oxylipins is represented in A and D, and in B and E, respectively. The impact of birth body weight in FAs and oxylipins is represented in C and F, respectively. Values are means of 28 control and 20 n-3 LCFA samples in the study of maternal diet as main effect, 24 control and 24 n-3 LCFA samples in the study of piglet diet as main effect, and 24 LBW and 24 HBW samples in the study of birth body weight category as main effect. HBW, high birth weight; LBW, low birth weight; LCFA, long chain fatty acid; MDiet, maternal diet; PDiet, piglet diet.*

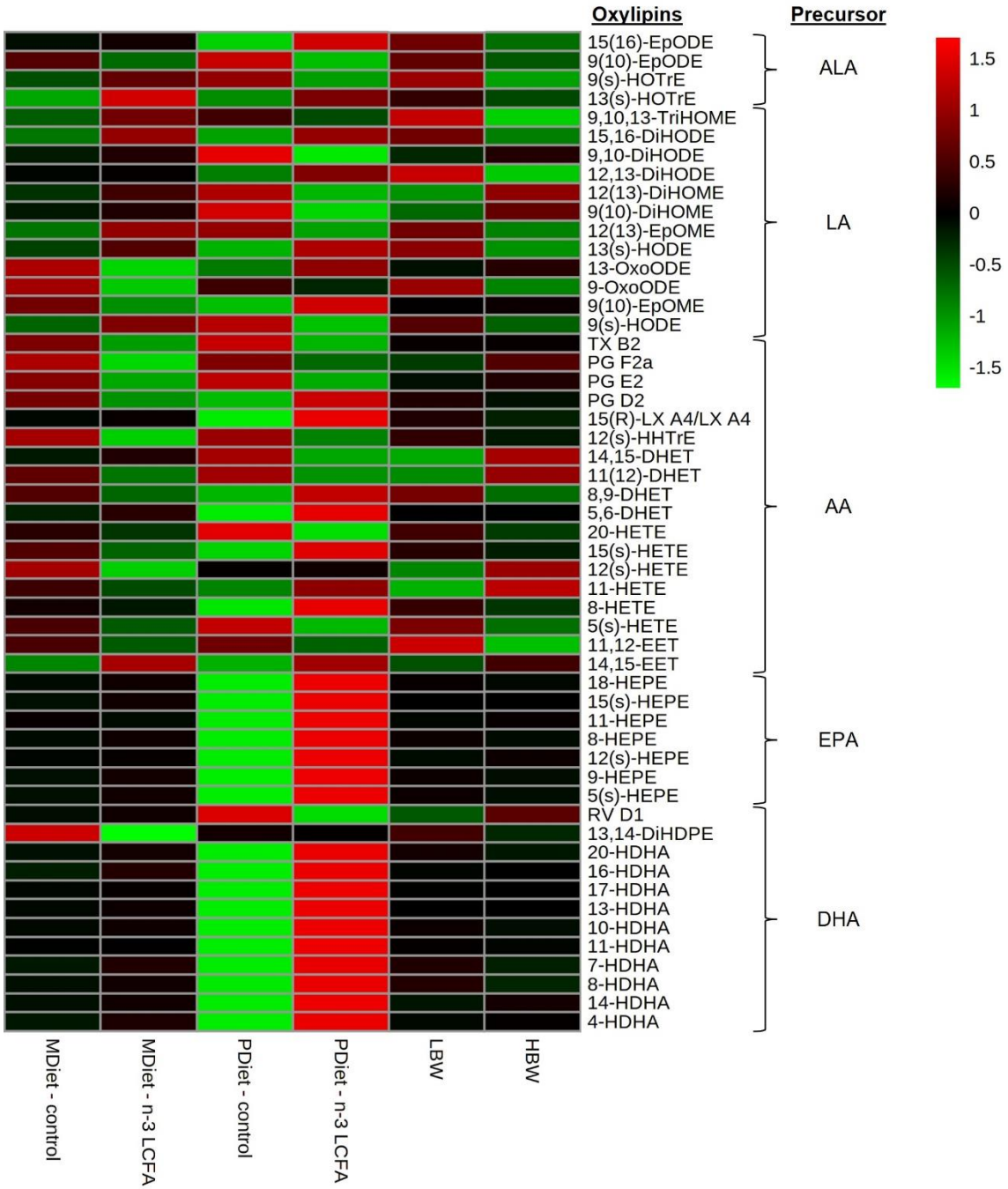


***Figure 2:*** *Differential concentrations of plasma oxylipins caused by the effect of the inclusion of fish oil rich in eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in sow and piglet diets or piglet body birth weight. Each coloured cell on the map corresponds to a concentration value being green lower concentrations and red higher concentrations. Values are means of 28 control and 20 n-3 LCFA samples for maternal diet, 24 control and 24 n-3 LCFA samples for piglet diet, and 24 LBW and 24 HBW samples for birth body weight*

*category. AA, arachidonic acid; ALA, α-linolenic acid; DiHDPE, dihydroxy-docosapentaenoic acid; DiHODE, dihydroxy-octadecadienoic acid; DiHOME, dihydroxy-octadecenoic acid; DHET, dihydroxy-eicosatrienoic acid; EET, epoxy-eicosatrienoic acid; EpODE, epoxy-octadecadienoic acid; EpOME, epoxy-octadecenoic acid; HBW, high birth weight; HDHA, hydroxy-docosahexaenoic acid; HEPE, hydroxy-eicosapentaenoic acid; HETE, hydroxy-eicosatetraenoic acid; HHTrE, hydroxy-heptadecatrienoic acid; HODE, hydroxy-octadecadienoic acid; HOTrE, hydroxy-octadecatrienoic acid; LA, linoleic acid; LBW, low birth weight; LCFA, long chain fatty acid; LT, Leukotriene; LX, Lipoxin; MDiet, maternal diet; OxoODE, oxo-octadecadienoic acid; PDiet, piglet diet; PG, Prostaglandin; TriHOME, trihydroxy-octadecenoic acid; TX, Thromboxane.*

*Immunological analysis*

Plasmatic immunoglobulin and cytokine concentration together with the concentration of ileal mucosa immune indicators are presented in Table 5. The effects of the interactions between maternal/piglet diet and bBW, maternal and piglet diet, and between maternal diet, piglet diet and bBW are described as footnotes.

Inclusion of n-3 LCFA in the maternal diet did not affect the immune parameters analysed in plasma or the ileal mucosa. However, their inclusion in piglet diets tended to increase IgM ($P$ = 0.067) in plasma without affecting the immune indicators in the mucosa. Piglets with a LBW tended to have a higher TNFα concentration ($P$ = 0.083) in plasma an increased concentration of TLR4 in ileal mucosa ($P$ = 0.001) than HBW piglets.

***Table 5:*** *Influence of dietary fish oil in maternal or post-weaning diets and piglet birth body weight on plasma and ileal mucosa immune indicators from weaned piglets.*[1,2]

| | 28 days weaned piglets (ca. 56 days of age) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | MDiet | | PDiet | | bBW | | | | | |
| | Control (n=28) | n-3 LCFA (n=20) | Control (n=24) | n-3 LCFA (n=24) | HBW (n=24) | LBW (n=24) | RMSE | *P* value MDiet | *P* value PDiet | *P* value bBW |
| Plasma immune indicators | | | | | | | | | | |
| Immunoglobulins (mg/mL) | | | | | | | | | | |
| IgG | 5.09 | 4.34 | 4.46 | 4.97 | 4.71 | 4.73 | 2.36 | 0.593 | 0.539 | 0.974 |
| IgA | 1.53 | 1.83 | 1.55 | 1.81 | 1.67 | 1.69 | 0.58 | 0.170 | 0.185 | 0.939 |
| IgM | 1.26 | 1.50 | 1.22 | 1.54 | 1.41 | 1.35 | 0.49 | 0.301 | 0.067 | 0.748 |
| Cytokines (ng/mL) | | | | | | | | | | |
| IL1β | 14.4 | 24.9 | 19.0 | 20.4 | 18.6 | 20.8 | 8.01 | 0.287 | 0.575 | 0.375 |
| IL6 | 8.50 | 64.4 | 36.2 | 36.7 | 30.9 | 42.0 | 33.8 | 0.171 | 0.973 | 0.388 |
| IL10 | 0.068 | 0.067 | 0.068 | 0.067 | 0.067 | 0.068 | <0.01 | 0.636 | 0.550 | 0.799 |
| TNFα | 0.13 | 0.16 | 0.13 | 0.17 | 0.12 | 0.17 | 0.10 | 0.692 | 0.171 | 0.083 |
| Ileal mucosa immune indicators | | | | | | | | | | |
| $2^{-\Delta\Delta Ct}$ | | | | | | | | | | |
| IL2 | 1.16 | 1.25 | 1.27 | 1.14 | 1.03 | 1.38 | 0.68 | 0.714 | 0.572 | 0.151 |
| IL10 | 1.08 | 0.83 | 1.02 | 0.89 | 1.03 | 0.88 | 0.29 | 0.136 | 0.440 | 0.587 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| INFγ* | 1.17 | 0.97 | 1.11 | 1.02 | 1.24 | 0.89 | 0.58 | 0.398 | 0.732 | 0.245 |
| TNFα** | 1.18 | 0.89 | 1.06 | 1.00 | 0.98 | 1.09 | 0.47 | 0.192 | 0.749 | 0.616 |
| TLR2*** | 1.13 | 0.98 | 1.01 | 1.10 | 1.05 | 1.06 | 0.70 | 0.490 | 0.674 | 0.992 |
| TLR4**** | 1.31 | 1.47 | 1.59 | 1.18 | 0.76 | 2.02 | 0.71 | 0.518 | 0.178 | 0.001 |
| MHC-II | 1.46 | 1.56 | 1.76 | 1.26 | 1.50 | 1.52 | 1.16 | 0.774 | 0.149 | 0.970 |

bBW, birth weight; HBW, high birth weight piglets; IgA, immunoglobulin A; IgG, immunoglobulin G; IgM, immunoglobulin M; IL1β, interleukin 1β; IL2, interleukin 2; IL6, interleukin 6; IL10, interleukin 10; INFγ, interferon γ; MHC-II, major histocompatibility complex class II; TLR2, Toll-like receptor 2; TLR4, Toll-like receptor 4; TNFα, tumor necrosis factor α; LBW, low birth weight piglets; LCFA, long chain fatty acid; MDiet, maternal diet; PDiet, piglet diet.

[1]Values are least squares means ± RMSE.

[2]*P* values of the significantly different interactions are reported in the footnotes.

**P* value of the interaction MDiet*PDiet*bBW was $P = 0.045$ where the $2^{-\Delta\Delta Ct}$ values were $1.67^{a}$ for control-control-LBW, $0.745^{bc}$ for control-control-HBW, $0.76^{bc}$ control-n-3 LCFA-LBW, $1.40^{ab}$ for control-n-3 LCFA-HBW, $1.18^{abc}$ n-3 LCFA-control-LBW, $0.73^{bc}$ for n-3 LCFA-control-HBW, $1.27^{abc}$ n-3 LCFA-n-3 LCFA-LBW, and $0.55^{c}$ for n-3 LCFA-n-3 LCFA-HBW.

***P* value of the interaction PDiet*bBW was $P = 0.043$ where the $2^{-\Delta\Delta Ct}$ values were 1.22 for control-LBW, 0.887 for control-HBW, 0.75 for n-3 LCFA-LBW, and 1.29 for n-3 LCFA-HBW, while differences between means were not observed.

*** *P* value of the interaction PDiet*bBW was $P = 0.040$ where the $2^{-\Delta\Delta Ct}$ values were 0.80 for control-LBW, 1.26 for control-HBW, 1.31 for n-3 LCFA-LBW, and 0.85 for n-3 LCFA-HBW, while differences between means were not observed.****P* value of the interaction MDiet*PDiet*bBW was $P = 0.013$ where the $2^{-\Delta\Delta Ct}$ values were $0.86^{cd}$ for control-control-LBW, $1.54^{bc}$ for control-control-HBW, $0.94^{bcd}$ control-n-3 LCFA-LBW, $1.75^{b}$ for control-n-3 LCFA-HBW, $0.41^{d}$ n-3 LCFA-control-LBW, $3.29^{a}$ for n-3 LCFA-control-HBW, $0.60^{d}$ n-3 LCFA-n-3 LCFA-LBW, and $1.41^{bcd}$ for n-3 LCFA-n-3 LCFA-HBW.

## Discussion

The increased prolificity of sows with the consequent increased percentage of LBW piglets, combined with the recent regulations on the use of antibiotics and other antimicrobials like zinc oxide is compromising the health and survival of piglets during critical phases such as weaning and post-weaning. For this reason, nutritional strategies, such as the dietary supplementation with n-3 LCFAs, that could improve the robustness and the health status of piglets are on the rise. In the current study, inclusion of n-3 LCFAs in the sow or piglet diets did not affected piglets' weight and growth. Tanghe and De Smet (2013) reported that previous studies evaluating the effect of the inclusion of n-3 PUFAs in maternal diets on piglet performance are inconsistent. Rooke *et al*. (2001) reported higher piglets' BW at 7 days post-weaning when the sow diets were supplemented with tuna oil during either the second-half or late gestation. In addition, Rooke *et al*. (2000) also described higher piglets' BW at the same age when the sow diets were supplemented with maize oil or tuna oil during lactation compared with those supplemented with maize oil plus linseed oil. Instead, other studies do not describe any effects of linseed, linseed meal or linseed oil offered to sows on the piglets' BW at 7 days post-weaning but show higher BW at 35 days post-weaning in piglets from sows that had been fed linseed meal (Farmer *et al*., 2010). Previous studies evaluating the inclusion of n-3 LCFAs in piglet diets are in line with the results obtained in the present study. Concretely, Eastwood *et al*. (2009), Li *et al*. (2014), and Lee *et al*. (2019) who tested n-3 LCFAs rich diets via the inclusion of linseed meal, marine oil, or fish oil and microalgae, respectively, also did not observe differences in BW, average daily weight gain, average daily feed intake or gain to feed ratio of the piglets during the post-weaning phase. Thus, the source of n-3 LCFAs, the initiation and duration of the dietary treatment are likely to play a key role in assessing outcomes. For these reason, future studies focused on standardising and controlling these parameters would be helpful.

Previous results within the same project of the current study, clearly describe that the inclusion of fish oil in the sow diets changes the FAs concentrations in blood

from gestating and lactating sows, colostrum, milk, and blood from suckling piglets (Llauradó-Calero *et al*., 2021, Llauradó-Calero *et al*., 2022). Thus, this should also be expected in the blood of piglets at the end of the postweaning phase. However, it is also important to determine at what extent the changes in blood FA composition of weaned piglets are induced by the fish oil in the sow or in the piglet diet. In this study, fish oil in the maternal diet had not affected the blood FA composition of weaned piglets, instead, the observed changes were due to the presence of fish oil in the piglet diet. Specifically, fish oil in the piglets' post-weaning diets increased the total amount of n-3 FAs, mainly EPA and DHA, without affecting the total amount of polyunsaturated FAs. Moreover, although changes in total saturated FAs, monounsaturated FAs and n-6 polyunsaturated FAs were not observed, some of the FAs in these groups also differed between dietary treatments. Among these, the decreases of palmitic acid, stearic acid and arachidonic acid concentrations are noteworthy. These results are in line with those previously reported for colostrum and milk (Llauradó-Calero *et al*., 2021) and blood from sows and suckling piglets (Llauradó-Calero *et al*., 2022), where increases in total n-3 FAs, due to EPA and DHA, and decreases in arachidonic acid are also observed. Regarding the effect of bBW, LBW piglets presented lower abundances of the n-6 polyunsaturated FAs γ-linolenic acid and eicosadienoic acid. However, both of them were present in a very low concentration.

Polyunsaturated FAs play a role in the coordination of inflammatory processes through different mechanisms. One of them is through their enzymatic or non-enzymatic oxidation and the formation of their derived oxylipins (Calder, 2010). Each oxylipin has its own immunomodulatory activity and those derived from n-6 FAs are commonly associated with pro-inflammatory activities while those derived from n-3 FAs are associated with less pro-inflammatory, anti-inflammatory and inflammation resolving roles (Calder, 2010; Gabbs *et al*., 2015). As for FA composition, the addition of fish oil in the maternal diets had virtually no effect on the oxylipin blood profile of piglets at day 28 postweaning, whereas all observed changes are due to the presence of fish oil in the post-weaning diets. This makes

sense since the oxylipins are classically described as short half-life mediators that are not stored by de cells (Gabbs *et al*., 2015). Bearing in mind that the precise activity exerted by most of oxylipins is not yet known, the increases in EPA and DHA concentrations have been reflected in increases of almost all their oxygenated derivatives. It is worth mentioning the increases of 12(s)-hydroxy-EPA, 15(s)-hydroxy-EPA, 18-hydroxy-EPA, 13- hydroxy-DHA and 17-hydroxy-DHA, all of which are formed via the lipoxygenase enzymatic pathway (Gabbs *et al*., 2015). Concretely, 12(s)-hydroxy-EPA might contribute to the anti-inflammatory potential of dietary n-3 FAs through platelet-neutrophil interaction (von Schacky *et al*., 1990), 15(s)-hydroxy-EPA plays and important role in the resolution phase of inflammation (Miller *et al*., 1990), and 18-hydroxy-EPA, 13- hydroxy-DHA and 17-hydroxy-DHA are related with different anti-inflammatory roles such as the inhibition of the pro-inflammatory cytokine TNFα production in murine or human cell lines (Astarita *et al*., 2015; Gabbs *et al*., 2015). Regarding the oxylipins derived from arachidonic acid, the decreases of thromboxane B2 and 20-hydroxy-eicosatetraenoic acid and the trend to decrease of prostaglandin E2 are in line with the reduction of the arachidonic acid concentration in serum resulted from the inclusion of dietary n-3 LCFA in post-weaning diets. The 20-hydroxy-eicosatetraenoic acid, formed via the cytochrome P450 enzymatic pathway, is defined as a potent vasoconstrictor and stimulator of proinflammatory cytokines production (Gabbs *et al*., 2015). Thromboxane B2 and prostaglandin E2 are final oxidative products formed via the cyclooxygenase enzymatic pathway (Astarita *et al*., 2015; Gabbs *et al*., 2015). Thromboxane B2 is a potent vasoconstrictor and platelet aggregating agent (Gabbs *et al*., 2015), while prostaglandin E2, which is the most common and biologically active prostaglandin in mammals, can exert a variety of functions such as inducing fever, decreasing T-cell proliferation and lymphocyte migration, and promoting the secretion of interleukins related with inflammatory processes (Harizi and Gualde, 2006). Moreover, the reduction of prostaglandin E2 after an EPA and DHA rich diet is in line with previous results as reviewed by Calder (2010). However, although the reduced serum concentration of arachidonic acid, the blood concentrations of some derived oxylipins such 15(R)-Lipoxin A4/Lipoxin A4, 5,6-

dihydroxy-eicosatrienoic acid), 8- and 15(s)-hydroxy-eicosatetraenoic acids and 14,15-epoxy-eicosatrienoic acids were increased. Shearer and Walker (2018) described that an increase in n-3 FAs derived oxylipins is commonly linked to a decrease in those derived from n-6 FAs. However, they also reported that in some cases n-6 oxylipins could be also increased, which could be in line with the results for oxygenated derivatives of arachidonic acid in the current study. Lipoxin A4, as prominent increased arachidonic acid oxygenated derivate, is defined as the main physiological lipoxin during inflammation in mammalian systems with a powerful anti-inflammatory role under many pathological conditions that trigger inflammation (Shi *et al*., 2017). In terms of differences between bBW categories, the concentration of the 7- and 8-hydroxy-DHA were lower in LBW piglets. It is known that both, 7- and 8-hydroxy DHA, are formed via lipoxygenase pathway. However, no functions have been described for 8-hydroxy-DHA and only an association with activating peroxisome proliferator activated receptor γ in an specific primate cell population have been reported for 7-hydroxy-DHA.

As mentioned above, polyunsaturated FAs together with their oxygenated derivatives may modulate the immune system. Therefore, blood changes in FA composition and in the oxylipin profile, such as those observed in the current study, could lead to changes in markers of immune response. Our group has previously reported an increased blood IgM concentration in gestating and lactating sows that were fed diets with n-3 LCFA, but differences were not found in the Ig concentration in colostrum, milk, or the blood of suckling piglets (Llauradó-Calero *et al*., 2021; Llauradó-Calero *et al*., 2022). In the current study, this same Ig tended to increase in the blood from piglets fed with the post-weaning diet with n-3 LCFAs at day 28 post-weaning. Although the impact of dietary n-3 LCFAs on different immune indicators in suckling piglets has been a topic of interest, there is not much literature available on its effects during the post-weaning period. Zhang *et al*. (2020) studied the effect of coated n-3 LCFA in post-weaning diets on serum IgG concentrations and did not report any effect. On the other hand, Luo *et al*. (2013) observed a significantly decreased in TNFα and IL6 genes expression in

*longissimus dorsi* muscle and a higher gene expression of TNFα in the spleen of weaned piglets from sows fed fish oil compared to piglets from sows fed lard during lactation. In the current study, fish oil did not affect the concentrations of blood cytokines nor ileal mucosa immune indicators. Finally, LBW piglets presented a higher expression of TLR4. TLRs are an ancient and conserved family of pattern recognition receptors that play a key role in the recognition of microbial pathogens and the modulation of host antimicrobial defence (Sabroe *et al*., 2008). Within this family, TLR4 is the most characterised member, and it is responsible for recognising endotoxin or lipopolisaccarydes from Gram-negative bacteria and initiating the inflammatory response, which may ultimately result in the activation of genes responsible for the production of proinflammatory cytokines (Liu *et al*., 2012). However, although no effects were detected on the proinflammatory cytokines expression in ileal mucosa, a tendency to increase TNFα was detected in the plasma of LBW piglets. These results may indicate a higher proinflammatory environment in the LBW piglets compared to HBW.

## Conclusion

This study attempted to provide a complete picture of the impact of n-3 LCFAs in the diets of sows and piglets on the blood concentrations of precursor FA, intermediate oxygenated molecules derived from them, and final derivatives in piglets at day 28 post-weaning. This was to complement previous studies from our group describing the influence of n-3 LCFAs in sow diets on the same parameters in colostrum, milk, and blood from sows and suckling piglets. From this study, it can be concluded that the inclusion of fish oil in either the sow or piglet diets did not affect the piglet's growth and feed intake during the post-weaning period. Moreover, the inclusion of fish oil in the piglet's diets increased EPA, DHA and total n-3 FAs, together with their derived oxylipins related with anti-inflammatory and inflammation resolving roles, and tended to increase IgM. Finally, the effect of fish oil in the sow diets is not seen at 28 days post-weaning, while a higher expression

of ileal TLR4 and a tendency to increase TNFα were observed in LBW piglets compared to HBW.

## Ethics approval

IRTA's Ethical Committee on Animal Experimentation approved the use of animals for this experiment in accordance with the Directive 2010/63/EU of 22 September 2010 and according to the recommendation of the European Commission 2007/526/CE, the Spanish guidelines for the care and use of animals in research (B.O.E. number 34, Real Decreto 53/2013) and the regional regulations on the use and handling of experimental animals (Decree 214/97, Generalitat de Catalunya) (project number: 10294).

## Data and model availability statement

None of the data were deposited in an official repository. The data that support the study findings are available upon request.

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**Declaration of interest**

The Authors report no conflict of interests.

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## Supplementary materials

***Supplementary Table S1:*** *Primers used for Real-Time PCR analyses.*

| Gene | Primer |
|---|---|
| INFγ | CAAAGCCATCAGTGAACTCATGA (forward)[1] |
| INFγ | TCTCTGGCCTTGGAACATAGTCT (reverse)[1] |
| TNFα | CCTCTTCTCCTTCCTCCTG (forward)[1] |
| TNFα | CCTCGGCTTTGACATTGG (reverse)[1] |
| IL2 | AACGGTGCACCTACTTCAAGCTCTAC (forward)[2] |
| IL2 | GTCAGTGTTGAGTAGATGCTTTGAC (reverse)[2] |
| IL10 | TGAGAACAGCTGCATCCACTTC (forward)[3] |
| IL10 | TCTGGTCCTTCGTTTGAAAGAAA (reverse)[3] |
| TLR2 | ACATGAAGATGATGTGGGCC (forward)[4] |
| TLR2 | TAGGAGTCCTGCTCACTGTA (reverse)[4] |
| TLR4 | CAGATAAGCGAGGCCGTCATT (forward)[5] |
| TLR4 | TTGCAGCCCACAAAAAGCA (reverse)[5] |
| MHC-II | TGGAACAGCCAGAAGGAC (forward)[6] |
| MHC-II | TCACAGAGCAGACCAGGAG (reverse)[6] |
| GAPDH | CAAATGGGGTGATGCTGGTG (forward)[6] |
| GAPDH | GAAGGGGCAGAGATGATGAC (reverse)[6] |

IL2, interleukin 2; IL10, interleukin 10; INFγ, interferon γ; MHC-II, major histocompatibility complex class II; TLR2, Toll-like receptor 2; TLR4, Toll-like receptor 4; TNFα, tumor necrosis factor α.

[1]Collado-Romero et al. (2010)

[2]Pappaterra et al. (2002)

[3]Flores-Mendoza et al. (2008)

[4]Tohno et al. (2005)

[5]Wang et al. (2013)

[6]Own design.

***Supplementary Table S2:*** *Influence of dietary fish oil in maternal or post-weaning diets and piglet birth weight on the plasmatic oxylipin concentrations of piglets at day 28 post-weaninig.*[1,2]

| | 28 days weaned piglets (ca. 56 days of age) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | MDiet | | PDiet | | bBW | | | *P* | *P* | *P* |
| | Precursor | Control (n=28) | n-3 LCFA (n=20) | Control (n=24) | n-3 LCFA (n=24) | HBW (n=24) | LBW (n=24) | RMSE | value MDiet | value PDiet | value bBW |
| Oxylipin (pg/mL) | | | | | | | | | | | |
| 15(16)-EpODE | ALA | 13485 | 13362 | 11959 | 14908 | 13012 | 13855 | 0.17 | 0.916 | 0.162 | 0.465 |
| 9(10)-EpODE | ALA | 535 | 507 | 539 | 507 | 515 | 530 | 0.17 | 0.745 | 0.505 | 0.764 |
| 9(s)-HOTrE | ALA | 469 | 476 | 474 | 470 | 463 | 480 | 0.17 | 0.808 | 0.720 | 0.697 |
| 13(s)-HOTrE | ALA | 1802 | 1986 | 1814 | 1944 | 1864 | 1894 | 0.12 | 0.662 | 0.382 | 0.700 |
| 9,10,13-TriHOME | LA | 928 | 959 | 959 | 923 | 910 | 972 | 0.14 | 0.593 | 0.729 | 0.350 |
| 15,16-DiHODE | LA | 144 | 164 | 135 | 169 | 142 | 163 | 0.18 | 0.188 | 0.118 | 0.225 |
| 9,10-DiHODE | LA | 396 | 478 | 631 | 230 | 469 | 392 | 0.24 | 0.610 | <0.001 | 0.404 |
| 12,13-DiHODE | LA | 251 | 316 | 256 | 301 | 240 | 317 | 0.43 | 0.869 | 0.531 | 0.331 |
| 12(13)-DiHOME | LA | 4465 | 4581 | 4791 | 4235 | 4868 | 4158 | 0.20 | 0.789 | 0.461 | 0.537 |
| 9(10)-DiHOME | LA | 7302 | 7692 | 8402 | 6527 | 8251 | 6678 | 0.20 | 0.851 | 0.077 | 0.377 |
| 12(13)-EpOME | LA | 4931 | 5508 | 5437 | 4906 | 4966 | 5377 | 0.17 | 0.275 | 0.218 | 0.320 |
| 13(s)-HODE | LA | 7997 | 7858 | 7711 | 8167 | 7884 | 7994 | 0.16 | 0.883 | 0.745 | 0.819 |
| 13-OxoODE | LA | 194 | 169 | 170 | 196 | 186 | 180 | 0.16 | 0.414 | 0.377 | 0.846 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 9-OxoODE | LA | 304 | 271 | 291 | 290 | 282 | 298 | 0.21 | 0.646 | 0.850 | 0.630 |
| 9(10)-EpOME | LA | 700 | 638 | 628 | 719 | 679 | 669 | 0.19 | 0.487 | 0.282 | 0.964 |
| 9(s)-HODE | LA | 19782 | 20511 | 21309 | 18863 | 19838 | 20333 | 0.16 | 0.621 | 0.364 | 0.658 |
| Thromboxane B2 | AA | 364 | 343 | 394 | 317 | 371 | 340 | 0.40 | 0.173 | 0.065 | 0.986 |
| Prostaglandin F2a | AA | 234 | 221 | 169 | 289 | 249 | 208 | 0.73 | 0.119 | 0.487 | 0.606 |
| Prostaglandin E2 | AA | 35.8 | 33.4 | 41.3 | 28.4 | 36.1 | 33.6 | 0.54 | 0.171 | 0.091 | 0.855 |
| Prostaglandin D2 | AA | 37.8 | 33.3 | 27.7 | 44.1 | 34.8 | 37.0 | 0.37 | 0.431 | 0.156 | 0.868 |
| 15(R)-Lipoxin A4/ Lipoxin A4 | AA | 19.4 | 20.9 | 1.00 | 39.1 | 18.4 | 21.6 | 0.32 | 0.690 | <0.001 | 0.130 |
| 12(s)-HHTrE | AA | 369 | 335 | 383 | 327 | 356 | 354 | 0.45 | 0.147 | 0.280 | 0.776 |
| 14,15-DHET | AA | 198 | 200 | 223 | 175 | 226 | 171 | 0.19 | 0.873 | 0.275 | 0.255 |
| 11(12)-DHET* | AA | 213 | 179 | 228 | 170 | 234 | 164 | 0.21 | 0.532 | 0.243 | 0.279 |
| 8,9-DHET | AA | 7.48 | 6.76 | 6.57 | 7.79 | 7.09 | 7.26 | 0.17 | 0.500 | 0.347 | 0.790 |
| 5,6-DHET | AA | 8.99 | 9.42 | 7.39 | 11.0 | 9.21 | 9.13 | 0.17 | 0.649 | 0.002 | 0.993 |
| 20-HETE | AA | 203 | 222 | 238 | 184 | 192 | 230 | 0.38 | 0.522 | 0.012 | 0.458 |
| 15(s)-HETE | AA | 145 | 131 | 123 | 156 | 138 | 140 | 0.14 | 0.378 | 0.074 | 0.752 |
| 12(s)-HETE | AA | 149 | 117 | 138 | 134 | 148 | 124 | 0.34 | 0.323 | 0.989 | 0.309 |
| 11-HETE | AA | 132 | 126 | 127 | 132 | 135 | 124 | 0.14 | 0.880 | 0.689 | 0.636 |
| 8-HETE | AA | 56.7 | 55.6 | 37.3 | 75.2 | 51.7 | 60.9 | 0.14 | 0.521 | <0.001 | 0.102 |
| 5(s)-HETE | AA | 38.1 | 34.4 | 36.8 | 36.3 | 36.6 | 36.5 | 0.18 | 0.787 | 0.490 | 0.682 |
| 11,12-EET | AA | 11.8 | 11.0 | 12.0 | 10.9 | 10.1 | 12.8 | 0.22 | 0.375 | 0.618 | 0.277 |
| 14,15-EET | AA | 7.83 | 9.26 | 6.05 | 10.8 | 8.96 | 7.90 | 0.29 | 0.180 | 0.017 | 0.386 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 18-HEPE | EPA | 720 | 771 | 30.1 | 1453 | 68 | 802 | 0.20 | 0.154 | <0.001 | 0.299 |
| 15(s)-HEPE | EPA | 296 | 320 | 7.42 | 604 | 288 | 323 | 0.48 | 0.970 | <0.001 | 0.426 |
| 11-HEPE | EPA | 228 | 238 | 2.64 | 462 | 242 | 223 | 0.30 | 0.402 | <0.001 | 0.403 |
| 8-HEPE | EPA | 802 | 856 | 11.7 | 1638 | 759 | 890 | 0.21 | 0.533 | <0.001 | 0.430 |
| 12(s)-HEPE | EPA | 209 | 224 | 12.2 | 418 | 223 | 207 | 0.27 | 0.722 | <0.001 | 0.435 |
| 9-HEPE | EPA | 257 | 277 | 11.6 | 520 | 237 | 294 | 0.35 | 0.258 | <0.001 | 0.345 |
| 5(s)-HEPE | EPA | 294 | 293 | 2.6 | 585 | 282 | 305 | 0.30 | 0.261 | <0.001 | 0.655 |
| Resolvin D1 | DHA | 30.2 | 30.7 | 32.6 | 28.3 | 29.9 | 30.9 | 0.27 | 0.933 | 0.189 | 0.642 |
| 13,14-DiHDPE | DHA | 151 | 154 | 157 | 148 | 159 | 146 | 0.22 | 0.645 | 0.996 | 0.938 |
| 20-HDHA | DHA | 198 | 206 | 11.0 | 392 | 184 | 219 | 0.25 | 0.340 | <0.001 | 0.112 |
| 16-HDHA | DHA | 138 | 150 | 12.8 | 272 | 133 | 153 | 0.24 | 0.022 | <0.001 | 0.880 |
| 17-HDHA | DHA | 137 | 149 | 5.96 | 277 | 133 | 151 | 0.29 | 0.679 | <0.001 | 0.917 |
| 13-HDHA | DHA | 195 | 201 | 11.2 | 384 | 188 | 207 | 0.36 | 0.556 | <0.001 | 0.972 |
| 10-HDHA | DHA | 291 | 307 | 22.9 | 572 | 270 | 325 | 0.16 | 0.192 | <0.001 | 0.151 |
| 11-HDHA** | DHA | 46.2 | 53.5 | 2.19 | 96.3 | 41.6 | 56.9 | 0.22 | 0.863 | <0.001 | 0.630 |
| 7-HDHA | DHA | 115 | 123 | 4.70 | 232 | 103 | 134 | 0.34 | 0.054 | <0.001 | 0.025 |
| 8-HDHA | DHA | 102 | 108 | 5.75 | 203 | 92.0 | 117 | 0.28 | 0.225 | <0.001 | 0.010 |
| 14-HDHA*** | DHA | 20.9 | 21.5 | 1.45 | 40.8 | 20.2 | 22.0 | 0.20 | 0.254 | <0.001 | 0.279 |
| 4-HDHA | DHA | 110 | 126 | 10.3 | 223 | 114 | 119 | 0.32 | 0.143 | <0.001 | 0.708 |

AA, arachidonic acid; ALA, α-linolenic acid; DHA, docosahexaenoic acid; DiHDPE, dihydroxy-docosapentaenoic acid; DiHODE, dihydroxy-octadecadienoic acid; DiHOME, dihydroxy-octadecenoic acid; DHET, dihydroxy-eicosatrienoic acid; EET, epoxy-eicosatrienoic acid; EPA, eicosapentaenoic acid; EpODE, epoxy-octadecadienoic acid; EpOME, epoxy-octadecenoic acid; HDHA, hydroxy-docosahexaenoic acid;

HEPE, hydroxy-eicosapentaenoic acid; HETE, hydroxy-eicosatetraenoic acid; HHTrE, hydroxy-heptadecatrienoic acid; HODE, hydroxy-octadecadienoic acid; HOTrE, hydroxy-octadecatrienoic acid; LA, linoleic acid; LCFA, long chain fatty acid; OxoODE, oxo-octadecadienoic acid; TriHOME, trihydroxy-octadecenoic acid.

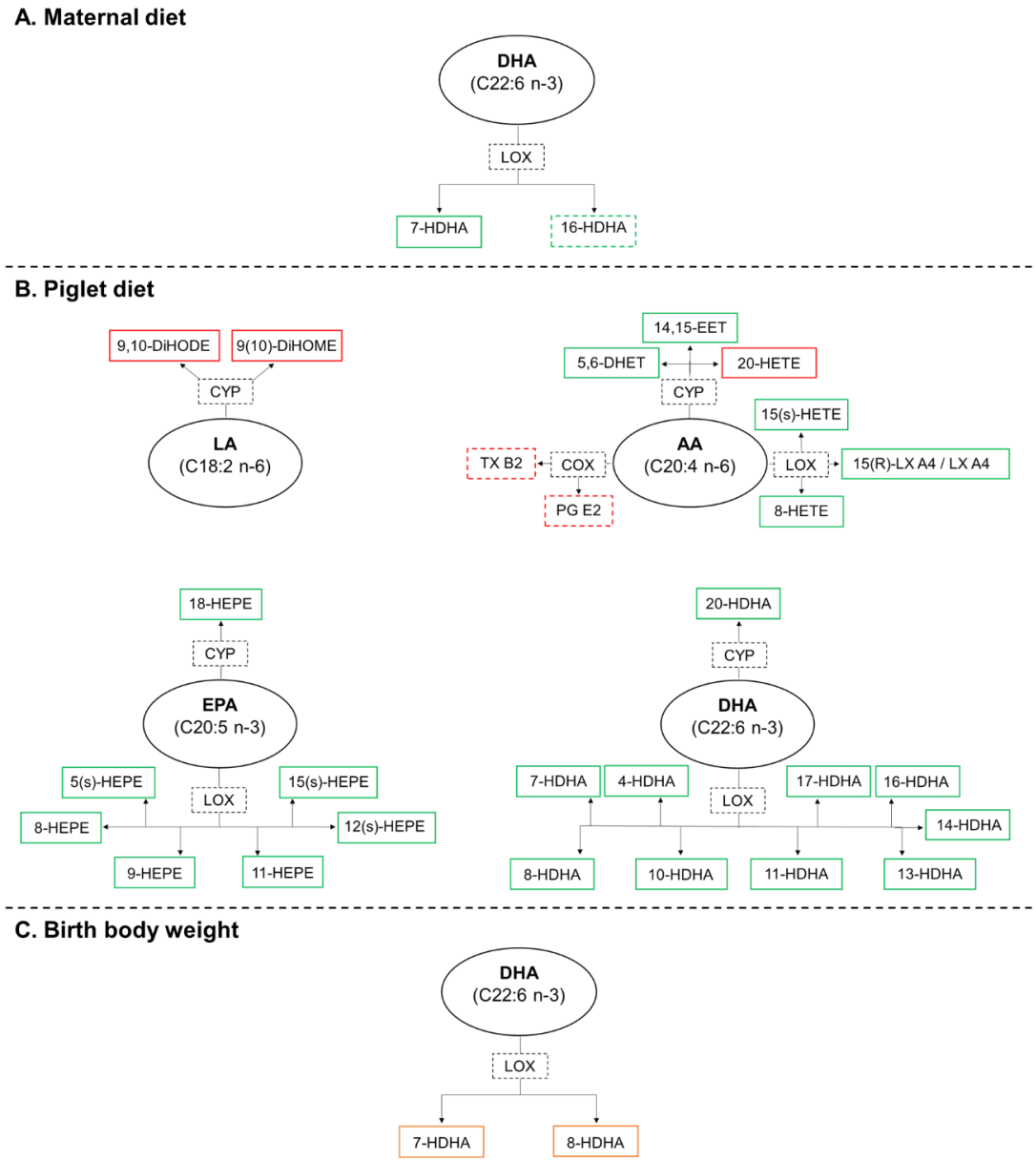
[1]Values are the mean ± RMSE

[2] *P* values of the significantly different interactions are reported in the footnotes.

**P* value of the interaction PDiet*bBW was *P* = 0.043 where the concentrations in pg/mL of plasma were $2.17^{b}$ for control-LBW, $2.37^{a}$ for control-HBW, $2.22^{ab}$ for n-3 LCFA-LBW, and $2.17^{b}$ for n-3 LCFA-HBW.

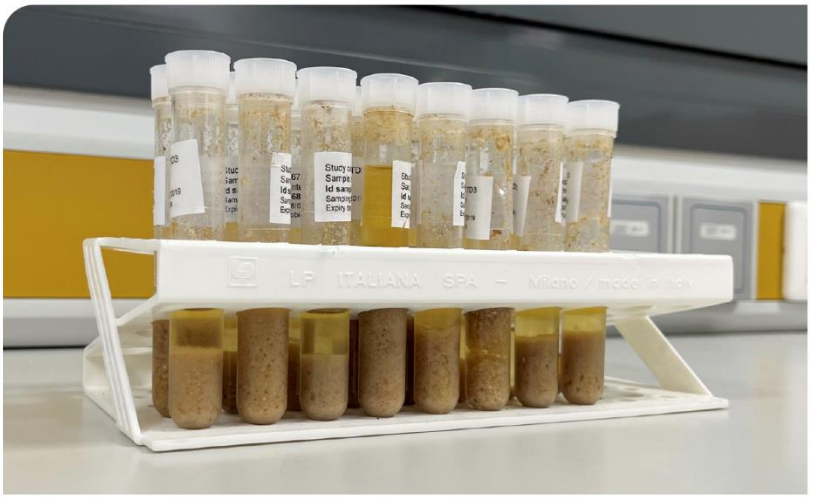
***P* value of the interaction MDiet*PDiet*bBW was *P* = 0.012 where the concentrations in pg/mL of plasma were $0.40^{c}$ for control-control-LBW, $0.72^{b}$ for control-control-HBW, $1.98^{a}$ control-n-3 LCFA-LBW, $1.78^{a}$ for control-n-3 LCFA-HBW, $0.64^{bc}$ n-3 LCFA-control-LBW, $0.38^{c}$ for n-3 LCFA-control-HBW, $2.01^{a}$ n-3 LCFA-n-3 LCFA-LBW, and $1.90^{a}$ for n-3 LCFA-n-3 LCFA-HBW.

****P* value of the interaction PDiet*bBW was *P* = 0.015 where the concentrations in pg/mL of plasma were $0.32^{c}$ for control-LBW, $0.58^{b}$ for control-HBW, $1.62^{a}$ for n-3 LCFA-LBW, and $1.55^{a}$ for n-3 LCFA-HB




***Supplementary Figure S1:*** *Schematic overview of modified oxylipins in plasma from piglets at 28 days post-weaning by the inclusion of fish oil rich in n-3 long chain fatty acids (n-3 LCFA) in maternal (A), piglet diet (B) or by the effect of piglet birth body weight category (C) including their fatty acid precursors and the enzymatic pathways involved in their synthesis (black boxes in dashes). Green boxes mean increased oxylipins and red boxes decreased oxylipins by the influence of n-3 LCFA. Orange boxes mean oxylipins increased in low birth weight piglets in comparison with high birth weight piglets. Boxes in dashes*

*indicate P < 0.1, otherwise P < 0.05. AA, arachidonic acid; COX, cyclooxygenase; CYP, cytochrome P450; DHA, docosahexaenoic acid; DiHODE, dihydroxy-octadecadienoic acid; DiHOME, dihydroxy-octadecenoic acid; DHET, dihydroxy-eicosatrienoic acid; EET, epoxy-eicosatrienoic acid; EPA, eicosapentaenoic acid; HDHA, hydroxy-docosahexaenoic acid; HEPE, hydroxy-eicosapentaenoic acid; HETE, hydroxy-eicosatetraenoic acid; LA, linoleic acid; LOX, lipoxygenase; LX, lipoxin; PG, prostaglandin; TX, thromboxane.*



# CHAPTER 5

## Influence of perinatal dietary n-3 long-chain fatty acids on microbial diversity and composition of intestinal content and faeces of weaned piglet.

UNIVERSITAT ROVIRA I VIRGILI
EFFECTS OF N-3 LONG-CHAIN FATTY ACIDS IN SOW AND PIGLET DIETS ON PERINATAL PIGLET PERFORMANCE, OXYLIPINS, IMMUNITY, AND MICROBIOTA
Eudald Llauradó Calero

# CHAPTER 5:

## Influence of perinatal dietary n-3 long-chain fatty acids on microbial diversity and composition of intestinal content and faeces of weaned piglets

This chapter has not been published yet.

It will be submitted to ***Frontiers in Microbiology***.

This chapter describes and discusses the effect of the inclusion of the fish oil rich in n-3 LCFAs in gestating, lactating and post-weaning diets on:

- Weight, growth, and feed intake of piglets during 28 days post-weaning.
- Microbiota diversity, composition, and differential microbial communities of feaces and caecum content from piglets at 7 and 28 days post-weaning.

**Influence of perinatal dietary n-3 long-chain fatty acids on microbial diversity and composition of intestinal content and faeces of weaned piglets**

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**Abstract**

Dietary n-3 long-chain fatty acids (**n-3 LCFAs**) are associated with an increase of microbial biodiversity which may dampen weaning effects on piglets such microbial dysbiosis. However, very little is known about their impact on the microbiota of weaned piglets. The aim of this study was to evaluate the impact of an inclusion of n-3 LCFAs in sow and post-weaning diets on microbial diversity and composition of faeces and caecum content of piglets weaned at 28 days. In addition, it is also studied the impact of piglet birth body weight (**bBW**) on these parameters. Twelve sows were randomly assigned to a control or an n-3 LCFA diets from service until weaning. At birth, the two piglets with the lowest (**LBW**) and the two with the heaviest (**HBW**) body weight (**BW**) for each litter were selected, and at weaning (ca. 28 days) they were grouped in blocks of two regarding their maternal diet and bBW. Pens in each category were further distributed into a control (30 g animal fat /kg) or an n-3 LCFA (48.6 g fish oil/kg) diet until the end of post-weaning (28 days). During the post-weaning period, piglets' growth and feed intake was monitored. Faeces from each piglet at day 7 and at day 28, and caecum content at day 28 were collected. Microbiomes were characterized by 16S ribosomal RNA gene sequencing. The n-3 LCFAs in the maternal diet decreased microbial diversity in faeces at day 28, while the n-3 LCFAs in the piglet diet decreased richness at phylum level in the same samples. In addition, n-3 LCFA-rich maternal diet decreased the abundance of phyla Actinobacteria and Bacteroides in faeces and, among others, increased the abundance of genera *Ruminococcus* and *Treponema*, and decreased genera *Streptococcus* and *Succinibivrio* in caecum content. On the other hand, also in caecum content, LBW piglets presented higher abundances of the family *Pectobacteriaceae* and the genus *Anaerostipes*, while HBW piglets presented higher abundances of the family *Streptococcaceae*. Thus, this study shows that maternal dietary n-3 LCFAs can influence the microbial communities of piglets 28 days post-weaning. Finally, the beta-dispersion analysis did not detect differences between faeces and caecum content at 28 days post-weaning

indicating that faeces may be a representative sample for studies of caecal microbiota in pigs.

**Keywords**

Weaned piglets, n-3 long-chain fatty acids, Maternal diet, Microbial diversity, Differential microbial communities, Faeces, Caecum content.

## Introduction

The microbiota of pigs is comprised of a diverse set of hundreds of microorganisms which could confer health benefits, protect against potential pathogens, and possess immune modulatory properties (Holman *et al*., 2017). For these reasons, it is considered as one of the major regulators of physiology and health (Beaumont *et al*., 2021), and its acquisition and establishment is crucial for the development and survival of piglets. Although the exact onset of gut bacterial colonization in the piglet is not known, the microbiota of sow's vagina, colostrum and milk, feed, oral-fecal transmission and the environment are considered key factors in the acquisition of the microbiota during the post-natal period (Nowland *et al*., 2019; Lauridsen, 2020). Thus, the suckling period becomes a crucial phase of microbial establishment (Gresse *et al*., 2017).

The transition from sow milk to a cereals and soya-based diet, among other factors such as social and environmental changes, makes weaning one of the most stressful and critical point in the piglet's life (Lallès *et al*., 2007). Thereby, weaning has been related to a state of microbiota disturbance and imbalance, which is commonly known as *dysbiosis* (Lallès *et al*., 2007). To tackle the challenges derived from weaning, antibiotics were used as growth promoters. However, the European Union (EU) banned the use for this purpose in 2006 (Regulation O: No 1831/2003 of the European Parliament and Council dated 22 September 2003 on additives used in animal nutrition) and also recommended the elimination of their

preventative and/or metaphylactic use to mitigate the risk of the emerging antibiotic-resistant bacterial strains. As mentioned above, diet could be considered as one of the most crucial factors in the establishment of piglets' microbiota (Nowland *et al*., 2019). Reason why dietary interventions could be used as strategy to modulate the microbiota and to improve both animal health and production (Holman *et al*., 2017).

According to Costantini *et al.* (2017), research on the impact of n-3 long-chain fatty acids (**n-3 LCFA**) on gut microbiota is still in its early stages. However, reviews such as the one published by Fu *et al. (*2021) describes some evidence suggesting a relationship between n-3 LCFAs and the gut microbiota. As far as the authors are aware, in reference to the influence that dietary n-3 LCFAs can exert on the microbial diversity and composition in weaned piglets is limited. The few studies carried out have only focused on specific populations such as genus *Lactobacillus* Zhang *et al*. (2020). However, this work is part of a larger study where the impact of n-3 LCFAs in sow diets influenced the microbiota of gestating sows, milk, and suckling piglets (Llauradó-Calero *et al*., 2022). Specifically, it highlighted an increase in microbial diversity in the faeces of suckling piglets, along with higher abundances of potentially beneficial bacteria such as the mucin-degraders genera *Akkermansia* and *Bacteroides*, and different species of *Lactobacillus*. Furthermore, milk became the factor with more bacterial populations correlated with lactating sows' and suckling piglets' faeces, suggesting that the changes observed in the sow due to the inclusion of dietary n-3 LCFAs may be transferred and have an impact on their offspring.

Therefore, the present work aims to complement the previous publication and help to provide a complete picture of the effect of dietary n-3 LCFAs on the microbiota during the early life stages of the piglet. For this reason, the aim was to evaluate the influence of the inclusion of a fish oil-rich in eicosapentaenoic acid (**EPA**) and docosahexaenoic acid (**DHA**) in sow and post-weaning diets on microbial diversity and composition of faeces and cecum content of weaned piglets, and, in addition, to assess whether the birth body weight (**bBW**) of the piglet, can also be a differential factor in the establishment of the microbiota.

## Material and methods

*Ethics statement*

Institute for Food and Agricultural Research and Technology's (IRTA) Ethical Committee on Animal Experimentation approved the use of animals for this experiment in accordance with Directive 2010/63/EU of 22 September 2010 and according to the recommendation of the European Commission 2007/526/CE, the Spanish guidelines for the care and use of animals in research (B.O.E. number 34, Real Decreto 53/2013) and the regional regulations on the use and handling of experimental animals (Decree 214/97, Generalitat de Catalunya) (project number: 10294).

*Animals, housing, and experimental design*

Twelve sows (same sows from the first batch and experimental set-up as described in Llauradó-Calero *et al*. (2022)) were group in pairs as similar as possible according to their body weight and parity number, and they were randomly distributed to either a control or a n-3 LCFA diet. Sows were fed the experimental diets from service until weaning (ca. 28 days post-farrowing). All farrowings were supervised and the two lightest (>800 g; **LBW**) and the two heaviest piglets per litter (**HBW**) were selected to perform the post-weaning study. Pre-starter creep feed according to their maternal diet was offered to piglets from day eleven of lactation until weaning.

At weaning, the selected piglets were moved to the post-weaning facilities and grouped in two piglets per pen based on their maternal diet and their bBW category. Pens in each category were also distributed to either control or n-3 LCFA diets until 28 days post-weaning (ca. 56 days of age), resulting in a 2 x 2 x 2 factorial design. Piglets were offered a pre-starter feed from weaning until day 14 post-weaning, and a starter feed from day 14 until day 28 post-weaning.

Post-weaning facilities consisted of a room with 24 slatted pens of 1.7 $m^2$ (1.8 x 0.95 m) with 2 piglets per pen. The inside of the building lit through skylight and non-programmable artificial light and ventilated through single variable-speed fans linked to temperature sensors. The temperature was adjusted according to the standard program used at the farm, with a gradual decrease from 30°C to 24°C during the first 21 days post-weaning. Piglets were fed via hoppers, and water was provided *ad libitum* via one nipple drinker per pen. At the end of the trial, piglets were humanely slaughtered under compliance with European Union ethical and welfare regulations.

*Experimental diets*

Gestation, lactation and post-weaning diets were formulated based on FEDNA specifications (de Blas *et al*, 2013). For sows, the control diet included animal fat was at rate of 15 g/kg or 30 g/kg for gestation and lactation, respectively. In the n-3 LCFA diet, animal fat was totally or half replaced for gestation and lactation diets, respectively, by an equivalent amount of a solid fish oil (Lipomega®; V&S Asociados, Madrid, Spain). In post-weaning diets, the control diet was formulated to contain 30 g/kg animal fat (for both, pre-starter and starter specifications), and in the n-3 LCFA diet, animal fat was totally replaced by an equivalent amount of the same solid fish oil. Pre-starter diets in mash form were also used as a creep feed from day eleven of lactation until weaning. Diets were provided *ad libitum* for all periods.

Diets were formulated to contain the same level of the main nutrients in both, control and n-3 LCFA diets, except for the FA composition. The composition for sows' diets has been previously described in Llauradó-Calero *et al*. (2021) and for piglets' diets in Chapter 4.

*Growth measurements*

The body weight (**BW**) of piglets was individually registered at weaning and at day 8,15 and 28 (end of the trial) post-weaning. Daily feed intake was recorded for each pen on the same days post-weaning. Average daily gain was calculed using the piglet as experimental unit. Average daily feed intake and gain to feed ratio using pen were calculated per pen (experimental unit).

*Sampling description*

Faeces were sampled at day 7 and 28 post-weaning (end of the trial). From the same piglets, caecum content was also collected *postmortem*. All samples were individually preserved with Real stock buffer (Durviz, Paterna, Spain) and stored at -80ºC until microbiota analysis.

*DNA extraction, amplification and sequencing of bacterial 16S gene, and analysis of sequencing data*

DNA extraction, bacterial 16S gene amplification and sequencing, and the analysis of sequencing data was previously described in detail by Llauradó-Calero, *et al*., 2022. DNA from faeces and caecum samples was isolated using the QIAmp Power Fecal Pro DNA Kit (Qiagen, Hilden, Germany). A massive genome sequencing of the hypervariable region V3-V4 of the bacterial 16s rRNA gene was performed to determine the bacterial composition. Samples were amplified through key-tagged eubacterial primers (Klindworth *et al*., 2013) and sequenced on a MiSeq Illumina Platform, employing a 2 x 300nt paired-end strategy, using the Illumina Library preparation and sequencing for metagenomic studies protocol (Zhang *et al*., 2014; Marcel, 2011).

The resulting sequences were split considering the barcode introduced during the PCR reaction. PEAR program version 0.9.1 was used to overlap R1 and R2 reads (overlap of 50 nt and a quality of the overlap with a minimum of Q20 (Zhang *et al*., 2014)), and a single FASTQ file was provided for each of the samples. Through the

Cutadapt v2.6 (Marcel, 2011) 16S rRNA PCR primers were trimmed and the sequences were treated with a quality filter to remove the low-quality fragments (Under Q20 in Phred scale) and the short sequences (Under 200nt). Chimeric sequences that may occur during PCR amplification were recognized *de novo* through the CD-HIT software v4.8.1 (Li *et al*., 2012) and eliminated. The same software was also applied to create OTUs at 99.7% of identity. Finally, using the BLAST tool each OTU was taxonomically identified against the National Center for Biotechnology Information (NCBI) 16S rRNA database (20th December 2020) using BLASTn version 2.10.0+.

### *Statistical analysis*

The GLIMMIX procedure of SAS software (SAS/STAT 14.1; SAS Institute INC., Cary, NC, USA) was applied to analyse the results of growth and feed intake with an ANOVA test. For the piglets' BWs and average daily gain the experimental unit was the piglet and the model included maternal diet, piglet diet and piglet bBW as fixed effects and the pen as random effect. For feed intake and the gain to feed ratio, the experimental unit was the pen and the model included the piglet diet and piglet bBW as fixed effects and batch as random effect. Interactions between fixed effects were also calculated. The results of growth and feed intake are presented as means ± SD. Significant differences and tendencies were considered at $P < 0.05$ and $P < 0.1$, respectively.

The statistical analyses for microbial data were performed using R software (R Core Team, 2012). The vegan package, implemented for R version 3.2.3 (Oksanen *et al*., 2020) was used to obtain the indices of alpha and beta diversity. Beta diversity was analysed using the selected Bray–Curtis distances, and their statistical significance was determined via PERMANOVA tests. DESeq2 package, also from R (Love *et al*., 2014), was applied to produce a generalized linear model with fixed effects (control vs n-3 LCFA diet to study the effect of maternal and piglet diet, or HBW vs LBW piglets to study the effect of bBW category) with negative binomial family to

compare operational taxonomic unit (OTU) counts between groups and select the potential bacterial biomarkers. The correction of *P*-values was carried out for multiple testing with Benjamini and Hochberg method, and the significance difference was set at $P < 0.05$. The calculation of the ratios Firmicutes/Bacteroidetes and *Lactobacillus*/Proteobacteria were performed using Mann-Whitney-Wilcoxon test through relative abundances and adjusted to a maximum value of 100. Higher values were considered outliers and were removed from the analysis. Significant differences were set at $P < 0.05$, while tendencies at $P < 0.1$.

## Results

### *Piglets' weight, growth, and feed intake*

Supplementary Table S1 shows the results of BW, average daily gain, average daily feed intake, and the gain to feed ratio of post-weaned piglets. The inclusion of fish oil in maternal or piglets' diets did not affect the BW and average daily gain of the piglets during this period. Nonetheless, the BW of piglets was different comparing LBW and HBW animals (in all cases $P = 0.001$). No differences in feed intake and gain to feed ratio were observed as result of the inclusion of n-3 LCFA in the maternal or piglet diets, or for the piglets' bBW category.

### *Microbiota Diversity of faeces and caecal content*

The influence of maternal or piglet diets, and the piglets' bBW on microbial richness and diversity at phylum, family, genus, and species level is shown in Table 1. At day 7 post-weaning, the different factors studied did not affect the alpha-diversity. In faeces at day 28 post-weaning, n-3 LCFAs in maternal diet tended to decrease piglet microbial diversity at phylum level according to Simpson ($P \leq 0.08$) and Shannon ($P \leq 0.08$) indices, and decreased piglet microbial diversity at family, genera, and species level according to the same indices $P \leq 0.024$ and $P \leq 0.027$

for Simpson and Shannon indices, respectively). In addition, the inclusion of fish oil in the post-weaning diet also resulted in a decrease in richness at phylum level in faeces at day 28 post-weaning ($P$ = 0.024). Piglet bBW did not influence the alpha-diversity of faeces.

Regarding caecum content, a tendency to decrease microbial diversity at family level was also observed due to an effect of maternal diet according to Shannon incex ($P$ = 0.010).

***Table 1:*** *Influence of the inclusion of dietary fish oil in maternal or post-weaning diets and piglets' birth body weight on the microbial diversity of faeces and caecum content from weaned piglets.*[1]

| | 28 days weaned piglets (ca. 56 days of age) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | MDiet | | PDiet | | bBW | | *P* value MDiet | *P* value PDiet | *P* value bBW |
| | Control (n=28) | n-3 LCFA (n=20) | Control (n=24) | n-3 LCFA (n=24) | HBW (n=24) | LBW (n=24) | | | |
| Faeces from day 7 post-weaning | | | | | | | | | |
| Richness | | | | | | | | | |
| Phylum | 6.78 ± 0.94 | 6.98 ± 0.67 | 6.97 ± 0.83 | 6.65 ± 0.85 | 6.97 ± 0.81 | 6.74 ± 0.87 | 1.00 | 1.00 | 1.00 |
| Family | 23.8 ± 2.50 | 24.8 ± 2.06 | 24.1 ± 2.19 | 24.0 ± 2.48 | 24.1 ± 2.21 | 23.9 ± 2.45 | 1.00 | 1.00 | 1.00 |
| Genera | 56.9 ± 5.99 | 58.4 ± 4.99 | 56.6 ± 6.32 | 58.0 ± 4.66 | 58.8 ± 4.26 | 56.9 ± 6.62 | 1.00 | 1.00 | 1.00 |
| Species | 97.7 ± 12.1 | 99.2 ± 9.54 | 97.7 ± 12.4 | 98.5 ± 9.67 | 98.4 ± 8.35 | 98.6 ± 13.3 | 1.00 | 1.00 | 1.00 |
| Simpson index | | | | | | | | | |
| Phylum | 0.59 ± 0.09 | 0.59 ± 0.10 | 0.60 ± 0.10 | 0.60 ± 0.09 | 0.60 ± 0.08 | 0.60 ± 0.11 | 1.00 | 1.00 | 1.00 |
| Family | 0.83 ± 0.06 | 0.82 ± 0.05 | 0.82 ± 0.07 | 0.83 ± 0.05 | 0.81 ± 0.05 | 0.84 ± 0.06 | 1.00 | 1.00 | 0.99 |
| Genera | 0.84 ± 0.07 | 0.84 ± 0.05 | 0.83 ± 0.07 | 0.84 ± 0.05 | 0.82 ± 0.05 | 0.85 ± 0.07 | 1.00 | 1.00 | 0.86 |
| Species | 0.90 ± 0.02 | 0.90 ± 0.02 | 0.90 ± 0.02 | 0.90 ± 0.02 | 0.90 ± 0.02 | 0.90 ± 0.02 | 0.98 | 1.00 | 1.00 |
| Shannon index | | | | | | | | | |
| Phylum | 1.13 ± 0.17 | 1.11 ± 0.19 | 1.13 ± 0.18 | 1.12 ± 0.18 | 1.10 ± 0.15 | 1.13 ± 0.20 | 1.00 | 1.00 | 1.00 |
| Family | 2.08 ± 0.27 | 2.04 ± 0.24 | 2.07 ± 0.27 | 2.08 ± 0.24 | 1.97 ± 0.25 | 2.13 ± 0.25 | 1.00 | 1.00 | 1.00 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Genera | 2.42 ± 0.32 | 2.38 ± 0.25 | 2.37 ± 0.31 | 2.40 ± 0.27 | 2.31 ± 0.27 | 2.49 ± 0.31 | 1.00 | 1.00 | 1.00 |
| Species | 2.91 ± 0.21 | 2.88 ± 0.16 | 2.85 ± 0.18 | 2.91 ± 0.19 | 2.87 ± 0.20 | 2.95 ± 0.18 | 0.29 | 1.00 | 1.00 |
| Faeces from day 28 post-weaning | | | | | | | | | |
| Richness | | | | | | | | | |
| Phylum | 8.00 ± 0.72 | 8.00 ± 0.74 | 8.52 ± 0.69 | 8.00 ± 0.66 | 8.30 ± 0.86 | 8.00 ± 0.57 | 1.00 | 0.024 | 1.00 |
| Family | 24.9 ± 1.67 | 24.5 ± 2.15 | 25.1 ± 1.61 | 24.2 ± 2.06 | 24.8 ± 2.09 | 24.7 ± 1.79 | 0.70 | 0.08 | 1.00 |
| Genera | 61.1 ± 3.42 | 59.6 ± 3.98 | 61.0 ± 3.51 | 60.5 ± 3.91 | 61.7 ± 3.57 | 59.5 ± 3.64 | 1.00 | 1.00 | 0.22 |
| Species | 103 ± 6.89 | 97.5 ± 2.29 | 100 ± 7.55 | 100 ± 7.76 | 103 ± 7.84 | 99.9 ± 7.35 | 1.00 | 1.00 | 0.95 |
| Simpson index | | | | | | | | | |
| Phylum | 0.61 ± 0.04 | 0.58 ± 0.04 | 0.59 ±0.04 | 0.59 ± 0.04 | 0.60 ± 0.04 | 0.59 ± 0.04 | 0.08 | 1.00 | 1.00 |
| Family | 0.79 ± 0.09 | 0.68 ± 0.09 | 0.73 ± 0.09 | 0.76 ± 0.10 | 0.73 ± 0.10 | 0.74 ± 0.09 | 0.024 | 1.00 | 1.00 |
| Genera | 0.81 ± 0.09 | 0.70 ± 0.09 | 0.74 ± 0.09 | 0.77 ± 0.10 | 0.74 ± 0.10 | 0.75 ± 0.09 | 0.021 | 1.00 | 1.00 |
| Species | 0.85 ± 0.10 | 0.72 ± 0.10 | 0.75 ± 0.10 | 0.79 ± 0.11 | 0.75 ± 0.11 | 0.78 ± 0.10 | 0.018 | 1.00 | 1.00 |
| Shannon index | | | | | | | | | |
| Phylum | 1.11 ± 0.09 | 1.05 ± 0.08 | 1.08 ± 0.10 | 1.09 ± 0.08 | 1.09 ± 0.09 | 1.08 ± 0.09 | 0.07 | 1.00 | 1.00 |
| Family | 1.92 ± 0.22 | 1.73 ± 0.20 | 1.81 ± 0.21 | 1.85 ± 0.25 | 1.85 ± 0.25 | 1.81 ± 0.21 | 0.021 | 1.00 | 1.00 |
| Genera | 2.21 ± 0.27 | 2.10 ± 0.21 | 2.17 ± 0.23 | 2.25 ± 0.29 | 2.23 ± 0.28 | 2.13 ± 0.24 | 0.027 | 1.00 | 1.00 |
| Species | 2.65 ± 0.32 | 2.32 ± 0.27 | 2.40 ± 0.33 | 2.51 ± 0.32 | 2.46 ± 0.36 | 2.41 ± 0.30 | 0.006 | 1.00 | 1.00 |
| Caecum content from day 28 post-weaning | | | | | | | | | |
| Richness | | | | | | | | | |
| Phylum | 7.00 ± 0.57 | 7.00 ± 0.51 | 7.00 ± 0.57 | 7.00 ± 0.50 | 7.00 ± 0.50 | 7.00 ± 0.57 | 1.00 | 1.00 | 1.00 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Family | 24.9 ± 1.81 | 24.0 ± 1.55 | 24.5 ± 1.74 | 24.4 ± 1.56 | 24.5 ± 1.64 | 24.3 ± 1.69 | 1.00 | 1.00 | 1.00 |
| Genera | 60.9 ± 4.15 | 61.1 ± 3.90 | 60.5 ± 4.29 | 61.2 ± 3.66 | 61.1 ± 3.65 | 60.8 ± 4.34 | 1.00 | 1.00 | 1.00 |
| Species | 101 ± 8.74 | 102 ± 7.29 | 101 ± 8.91 | 102 ± 6.75 | 102 ± 6.49 | 100 ± 9.14 | 1.00 | 1.00 | 1.00 |
| Simpson index | | | | | | | | | |
| Phylum | 0.61 ± 0.06 | 0.60 ± 0.04 | 0.60 ± 0.06 | 0.59 ± 0.04 | 0.60 ± 0.05 | 0.60 ± 0.05 | 1.00 | 1.00 | 1.00 |
| Family | 0.82 ± 0.06 | 0.81 ± 0.07 | 0.81 ± 0.07 | 0.82 ± 0.05 | 0.82 ± 0.06 | 0.81 ± 0.06 | 0.40 | 0.50 | 1.00 |
| Genera | 0.84 ± 0.06 | 0.82 ± 0.07 | 0.82 ± 0.07 | 0.83 ± 0.05 | 0.82 ± 0.06 | 0.83 ± 0.07 | 0.38 | 0.48 | 1.00 |
| Species | 0.90 ± 0.07 | 0.87 ± 0.07 | 0.88 ± 0.09 | 0.88 ± 0.05 | 0.88 ± 0.07 | 0.88 ± 0.07 | 0.74 | 0.59 | 1.00 |
| Shannon index | | | | | | | | | |
| Phylum | 1.09 ± 0.10 | 1.04 ± 0.09 | 1.07 ± 0.10 | 1.07 ± 0.09 | 1.05 ± 0.09 | 1.08 ± 0.10 | 0.20 | 1.00 | 1.00 |
| Family | 2.10 ± 0.19 | 1.95 ± 0.20 | 1.96 ± 0.20 | 2.03 ± 0.20 | 2.01 ± 0.21 | 1.97 ± 0.20 | 0.10 | 0.20 | 1.00 |
| Genera | 2.40 ± 0.22 | 2.30 ± 0.24 | 2.32 ± 0.23 | 2.42 ± 0.24 | 2.35 ± 0.22 | 2.34 ± 0.25 | 0.17 | 0.20 | 0.99 |
| Species | 2.83 ± 0.25 | 2.77 ± 0.28 | 2.74 ± 0.31 | 2.85 ± 0.22 | 2.85 ± 0.27 | 2.75 ± 0.27 | 0.55 | 0.51 | 1.00 |

bBW, birth body weight; HBW, high birth weight piglets; LBW, low birth weight piglets; LCFA, long chain fatty acid; MDiet, maternal diet; PDiet, piglet diet.

[1]Values are means ± SD.

*Microbiota composition and differential microbial communities in faeces and caecum content*

The PCoA analysis of faeces at day 7 and 28, and the caecum content at day 28 post-weaning is presented in Figure 1. PERMANOVA test indicated different clusters for each sample type ($R^2$ = 0.20; $P$ = 0.001). Beta-dispersion analysis revealed differences between faeces at day 7 and faeces and caecal content at day 28 post-weaning (both $P < 0.001$). In contrast, differences were not observed between faeces and caecum content collected at day 28 post-weaning.

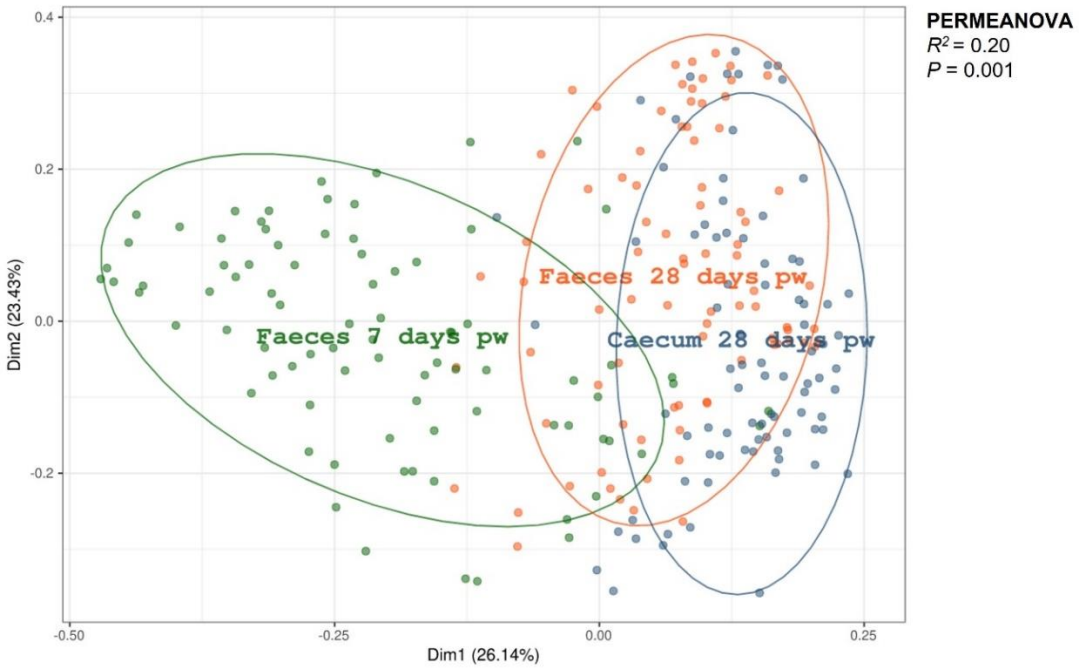


***Figure 1:*** *Principal coordinates analysis (PCoA) plot of weaned piglets' faeces at day 7 of post-weaning (pw) (n =48), faeces at day 28 of pw (n =48), and caecum content at day 28 of pw (n =48), regardless main effects (maternal diet, piglet diet, and birth body weight) revealed distinct clusters for each sample type ($R^2$ = 0.20; P = 0.001). PERMANOVA test performed using Bray-curtis distances to analyse beta-dispersion showed differences between faeces at day 7 and faeces and caecum content at day 28 pw (both P < 0.001). Differences were not observed between faeces and caecum content at day 28 pw.*

Relative abundance of microbial composition of faeces at day 7 and 28, and caecal content at 28 of post-weaning was presented as the top 10 phyla (A), top 20

families (B), and top 20 genera (C) is shown in Figure 2. The microbiome of faeces at day 7 post-weaning was mainly composed of the phyla Firmicutes, Bacteroidetes and Proteobacteria, families *Lachnospiraceae*, *Lactobacillaceae*, *Prevotellaceae*, *Ruminococcaceae* and *Succinivibrionaceae,* and genera *Lactobacillus*, *Prevotella*, *Succinivibrio*, and *Blautia* (Figure 2, A). Modification of bacterial communities was not detected by the inclusion of n-3 LCFAs in the maternal or piglet diets, or by the piglets' bBW.

In faeces from day 28 post-weaning, the most abundant phyla were Firmicutes, Bacteroidetes and Proteobacteria. The families more abundant were *Lactobacillaceae*, *Prevotellaceae*, *Lachnospiraceae*, *Ruminococcaceae*, and *Clostridiaceae*, and *Lactobacillus*, *Prevotella*, and *Blautia* were the genera with the highest abundance (Figure 2, B). In reference to differential bacterial communities, lower abundances of the phyla *Actinobacteria* and *Bacteroidetes* ($P = 0.046$ and $P = 0.047$, respectively) were observed by the inclusion of fish oil in the maternal diet (Figure 3. B), while no differences were observed due to piglet diet or piglets' bBW.

The core microbiome of caecum content at day 28 post-weaning was represented again by phyla Firmicutes, Bacteroidetes and Proteobacteria. The most abundant families were *Lactobacillaceae*, *Prevotellaceae*, *Lachnospiraceae, Ruminococcaceae* and *Veillonellaceae*, and the dominant genera were *Lactobacillus*, *Prevotella*, and *Megasphera* (Figure 2, C). Caecum content was the sample type with the greatest number of differential bacterial populations. Maternal diet modified the abundances of one phylum, two families, and four genera (Figure 3. B). Accurately, higher abundances of the phylum Spirochaetes ($P = 0.003$), the family *Spirochaetaceae* ($P = 0.015$), and de genera *Ruminococcus* ($P = 0.009$) and *Treponema* ($P = 0.022$) were detected in the caecum contents of piglets from sows fed fish oil. While the same treatment lowered the abundances of the family *Streptococcaceae* ($P = 0.044$), and the genera *Streptococcus* ($P = 0.046$) and *Succinivibrio* ($P = 0.022$). In terms of piglets' bBW, LBW piglets presented lower abundances of the families *Pectobacteriaceae* ($P < 0.001$), and genera

*Anaerostipes* ($P = 0.018$) and *Brenneria* ($P < 0.001$), and higher abundances of the family *Streptococcaceae* ($P = 0.018$) (Figure 3, A).

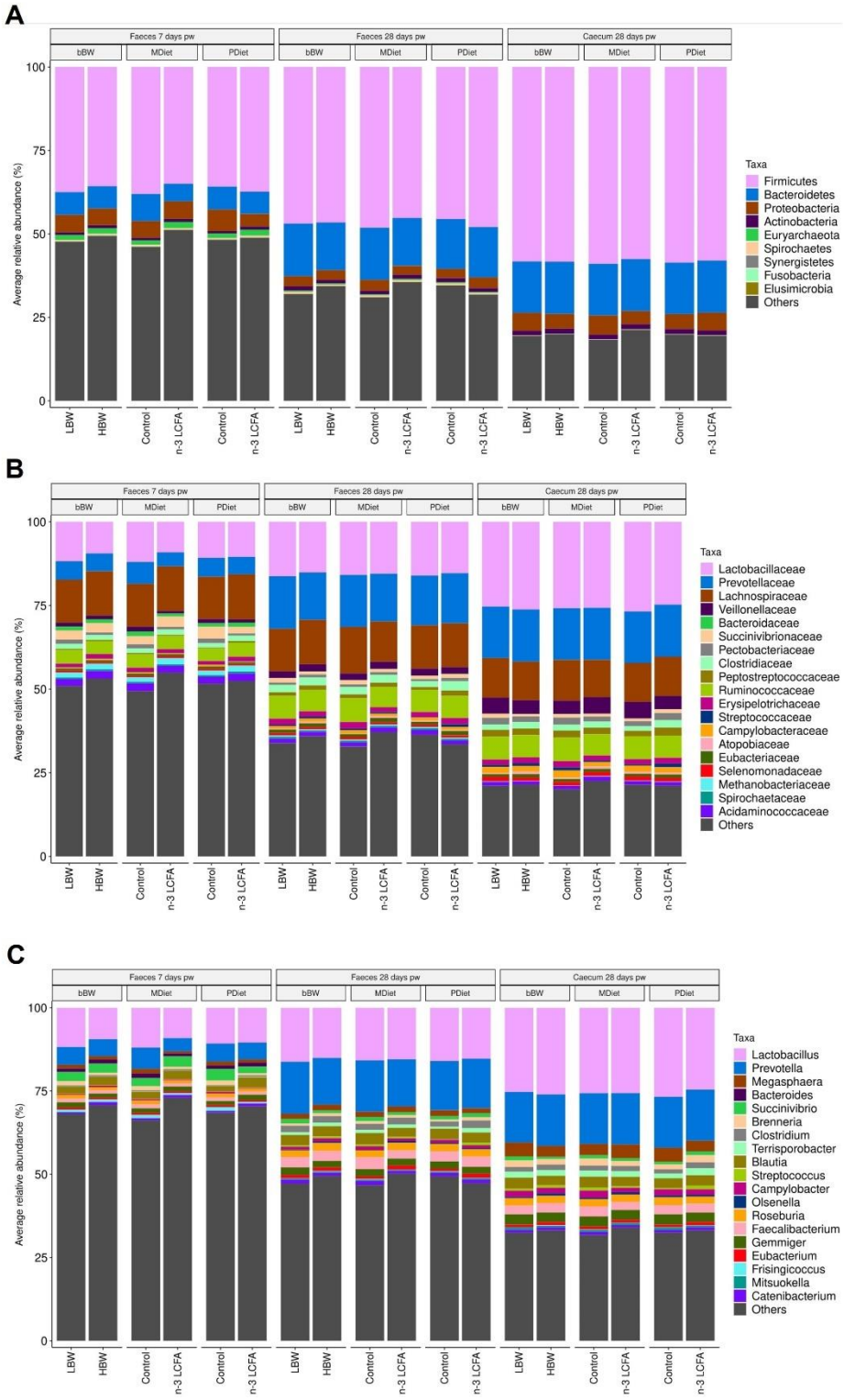


***Figure 2:*** *Microbiota composition presented as relative abundance of the top 10 phyla (A), top 20 families (B), and top 20 genera (C) of faeces at day 7 and 28, and caecum content at*

*28 days post-weaning. Results are shown by the main effects: piglet birth BW (n = 48), maternal diet (n = 48), or piglet diet (n = 48). BW, body weight; HBW, high body weight; LBW, low birth weight; LCFA, long-chain fatty acids; pw, post-weaning.*

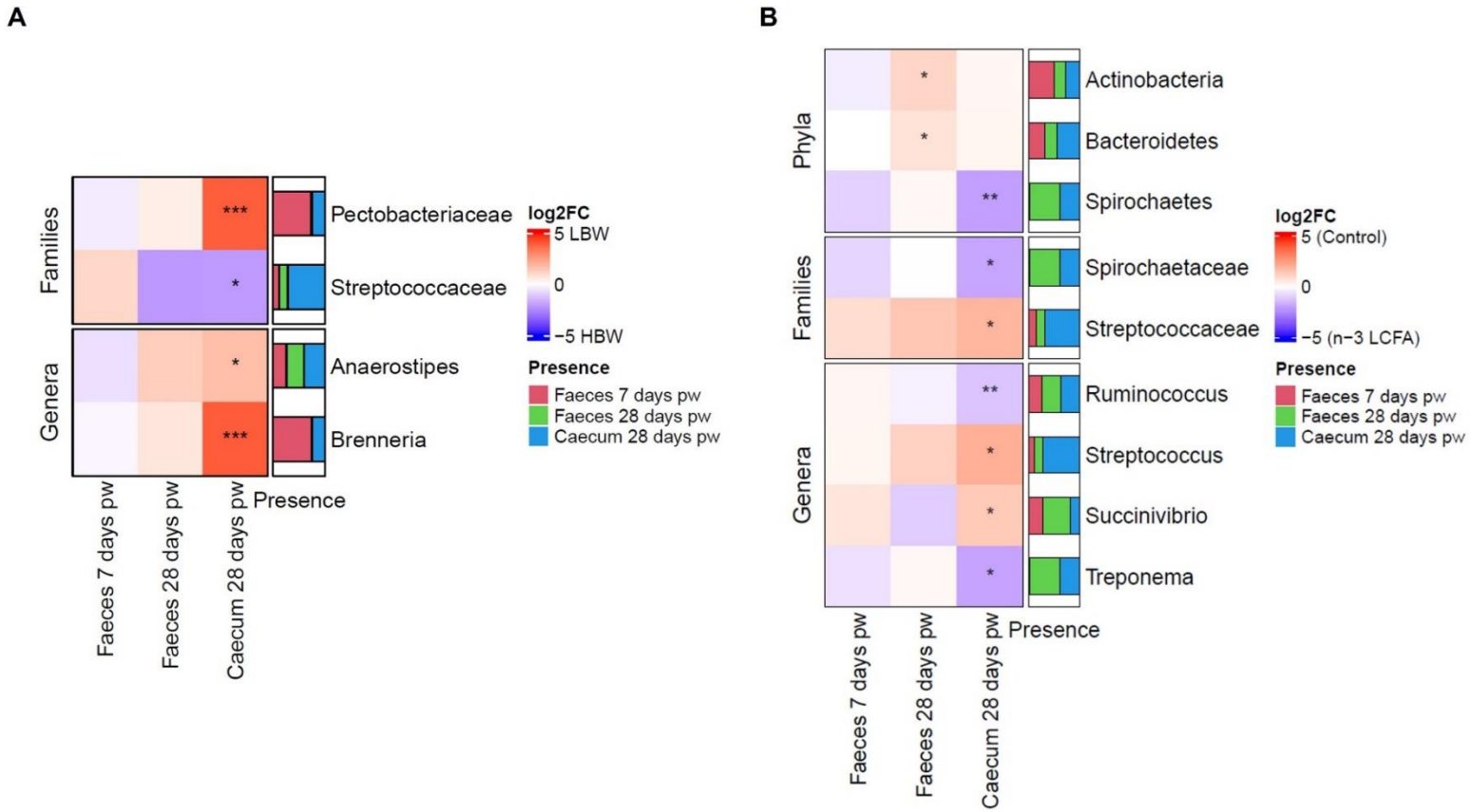


***Figure 3:*** *Heatmap representing the differentially abundant phyla, families, and genera between low birth weight (LBW) and high birth weight (HBW) piglets (A) and between control and n-3 long-chain fatty acids (n-3 LCFA) maternal diets (B) in faeces from piglets at day 7 and 28, and caecum content at day 28 post-weaning. For piglets' birth body weight: n = 24 for LBW and HBW. For maternal diets: n = 28 for control and n =20 for n-3 LCFA. Significant differences between treatments were set at P < 0.05 (*), P < 0.01 (**), and P < 0.001 (***). pw, post-weaning.*

The ratios Firmicutes/Bacteroidetes and *Lactobacillus*/Proteobacteria for each sample type, assessing the effect of maternal or piglet diets, or piglets' bBW are presented in Figure 4, but differences for the main factors were not observed for any of the calculated ratios (maternal diet ($P \geq 0.29$), piglet diet ($P \geq 0.60$), or piglet bBW (all $P \geq 0.42$).

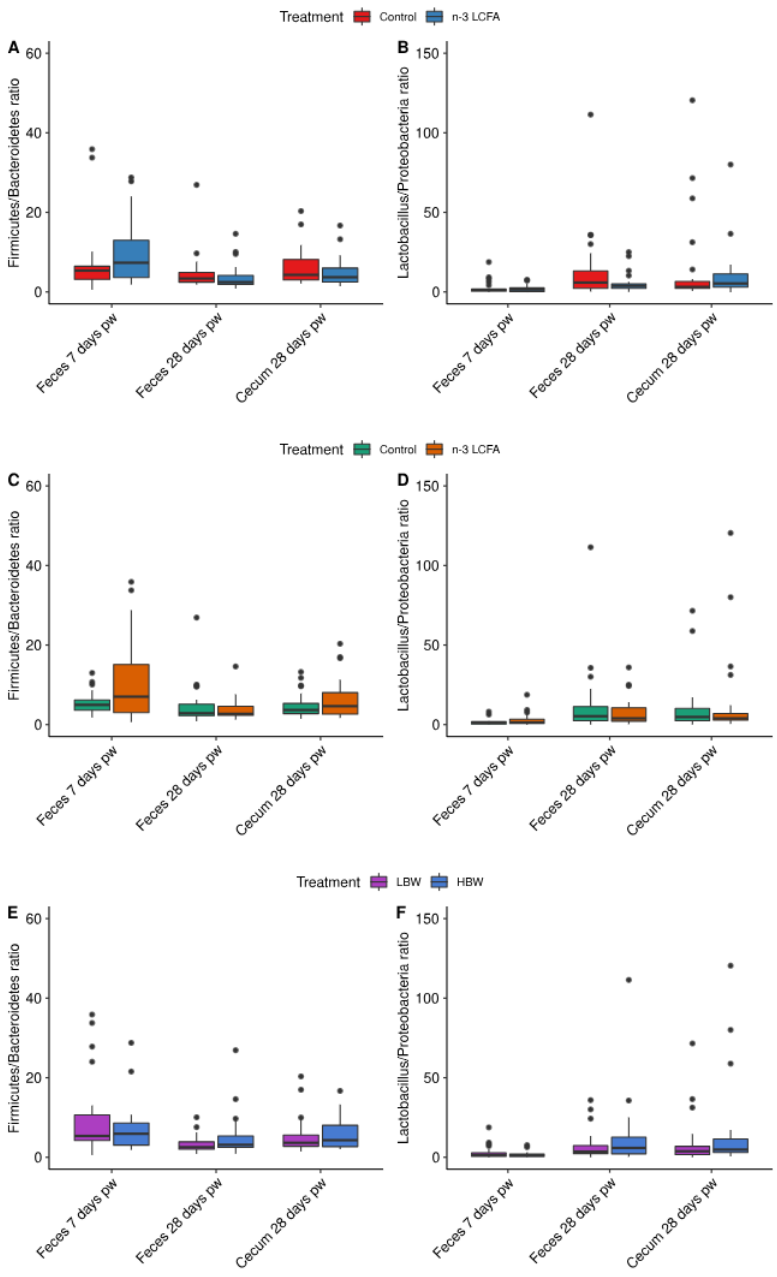


***Figure 4:*** *Firmicutes/Bacteroidetes and Lactobacillus/Proteobacteria ratios calculated by the effect of n-3 LCFA in maternal diet (A and B), in piglet diet (C and D), and by piglets'*

*birth BW (E and F) for all sample types. For maternal diet: n = 28 for control and n =20 for n-3 LCFA. Significant differences between treatments were set at P < 0.05, while tendencies were set at P < 0.10. Ratios were adjusted at a maximum value of 100 and higher values were removed by being considered outliers. HBW, high body weight; LBW, low birth weight; LCFA, long-chain fatty acids; pw, post-weaning.*

## Discussion

Weaning is probably considered as the most critical event in the swine production. This is due to the sudden dietary, social and environmental changes that it supposes for the piglets. Many of the piglets fail to adapt to the new solid food and environmental conditions and enter into a state of low food intake or cease eating (Gresse *et al*., 2017). This can affect both the growth of the animal and its microbiota. Concretely, the stresses associated to weaning result in a disruptive state of gut microbiota composition, known as *dysbiosis*, that can lead to an increase of potentially pathogenic bacteria and, as a consequence, it can give rise to episodes of post-weaning diarrhea Considering that diet is a key factor in the modulation of the microbiota, nutritional strategies are trending upward. In terms of piglets' growth and feed intake, no differential effect due to the inclusion of n-3 LCFAs in the sow or piglet diets was observed in comparison with control. The piglets used in this work are part of a larger trial studying the influence of n-3 LCFA in maternal and piglet diets on different parameters including performance (Chapter 4), and although the previous study included a higher number of animals, maternal or piglet dietary n-3- LCFA also did not modify piglet growth or feed intake. As mentioned in the review of Tanghe and De Smet (2013), studies such as those reported by Rooke *et al*. (2000) and Rooke *et al*. (2001) observed an increase in the BW of piglets one week after weaning with a maternal diet rich in n-3 LCFAs. However, most of the studies evaluating the effects of a dietary n-3 LCFAs source in sow diets did not describe any effect on piglet growth during the post-weaning phase (Tanghe and De Smet, 2013). In terms of the effect of n-3 LCFAs in piglet diets during post-weaning, most of the previous results are in concordance with

the current study (Eastwood *et al*., 2009; Li *et al*., 2014; Lee *et al*., 2019). However, more recently, Zhang *et al*. (2020) observed an increased BW at one, three, and six weeks post-weaning for piglets fed a diet with a coated n-3 LCFAs.

It has been previously described that the inclusion of fish oil in the sows' resulted in a higher diversity and an increase in potentially beneficial bacterial populations in suckling piglets' faeces (Llauradó-Calero *et al*., 2022). Contrary, the inclusion of n-3 LCFAs in sow diets resulted in a decreased bacterial diversity at all levels studied in faeces of piglets 28 days post-weaning, while differences were not observed in faeces at 7 days post-weaning. Nevertheless, this decrease in the faecal microbial diversity has not been reflected in the modification of any phenotypic parameter related to growth performance. Those results are in line with those described by Noriega *et al*. (2016), who observed a decreased species diversity in faeces from adult humans consuming 600 mg of n-3 FAs daily for 14 days. However, a decrease in bacterial diversity with a diet rich in n-3 LCFAs, an increase in microbial diversity has been suggested as a possible new anti-inflammatory mechanism of dietary n-3 LCFAs through which they could dampen inflammation (Calder, 2019).

Regarding the differences in specific bacterial populations, the greater number of changes were due to the maternal diet, some differences were also detected between LBW and HBW piglets, but the inclusion of fish oil in the post-weaning diets did not presented any influence. Caecum content was the sample type with the highest number of modifications, some changes were also observed in the faeces at 28 days post-weaning, while faeces at 7 days post-weaning remained unaffected. The inclusion of n-3 LCFAs in maternal diet resulted in lower abundances of the phyla Actinobacteria and Bacteroidetes in faecal samples at day 28 of post-weaning. In addition, both Actinobacteria and Bacteroidetes are two of the most dominant phyla in terms of microbiota composition and are defined as a common colonizers of gut microbiota of piglets (Hermann-Bank *et al*., 2015). Although a significant depletion of members from phylum Actinobacteria had been described in the intestinal content of piglets affected with neonatal diarrhea

(Hermann-Bank *et al*., 2015), an increased abundance of Actinobacteria was defined as a marker of diarrhea predisposed piglets (Karasova *et al*., 2021). On the other hand, although the decrease in the abundance of Bacteroidetes observed, no changes were observed in the Firmicutes/Bacteroides ratio, an indicator of normal intestinal homeostasis (Stojanov *et al*., 2020).

In caecum content, the inclusion of fish oil in the sow diets increased the phylum Spirochaetes mainly due to the increase of the family *Spirochaetaceae* and in turn the genus *Treponema*, and it also increased the abundance of the *Ruminococcus* genus. Contrary, the abundance of the family *Streptococcaceae* mainly due to the genus *Streptococcus*, and the genus *Succinivibrio* were found decreased due to the n-3 LCFA maternal diet. Firstly, it is worth mentioning that these four genera have been reported to be abundant in the low gastrointestinal tract in swine, which includes caecum samples (Holman et al., 2017) . The genus *Treponema* contains species classified as pathogenic in pigs (Cole, 1990). However, in this study the abundance of specific *Treponema* species was not differentially increased in the caecum content of piglets from sows fed the n-3 LCFA diet. The genus *Ruminococcus* is associated with butyrate production. In this study, the *Ruminococcus* species with a higher abundance due to the maternal n-3 LCFA diet was tentatively identified as *Ruminococcus champanellensis*. Concretely, this species was characterised as a cellulose degrader, and consequently a producer of short-chain FAs such as acetate or succinate (Chessard *et al*., 2012). The degradation of complex carbohydrates and the production of short-chain FAs has been associated as anti-inflammatory roles exerted by microbiota (Crost *et al*., 2018; Liu *et al*., 2019). These results are also in concordance with those previously described by Noriega *et al*. (2016) in humans, in which, together with the previously mentioned decrease in diversity, they also observed an increase in short-chain fatty acid producing bacteria. However, they differ from those previously described in the same piglets during lactation (Llauradó-Calero *et al*., 2022). In suckling piglets, the inclusion of fish oil in sow diets increased microbial diversity and decreased *Ruminococcus*, but in that case, *Ruminococcus* was associated tentatively with

*Ruminococcus gnavus* species which is linked with pro-inflammatory roles (Hall *et al*., 2017; Henke *et al*., 2019). The decreased abundance of genus *Streptococcus* by the inclusion of n-3 LCFA in maternal diets is contrary to what was observed in suckling piglets (Llauradó-Calero *et al*., 2022). It is known that certain species of this genus can act as probiotics and are related with different improvements in terms of colostrum and milk quality, litter size, and piglet vitality and BW (Knech *et al*., 2020). Instead, other species such as *Streptococcus suis*, are described as important bacterial pathogens affecting weaned piglets, causing mainly meningitis, arthritis, and sudden death (Obradovic *et al*., 2021). In the same way described for the genus *Treponema*, the abundance of specific species of *Streptococcus* did not differ in the caecum content of piglets from the two studied maternal diets. In reference to the decreased *Succinivibrio*, which has been reported to play a role also in the production of short-chain FAs (Bergamaschi, *et al*., 2020). At species level, higher and lower abundances of the *Lactobacillus pontis* and *Mitsuokella jalaludinii* (OTUs tentatively identified), respectively, were also detected in caecum content of piglets from n-3 LCFAs fed sows. Both *Lactobacillus pontis* and *Mitsuokella jalaludinii* are associated with carbohydrate fermentation and production of lactic acid (Vogel *et al*., 1994; Lan *et al*., 2002). As reviewed by Gresse *et al*. (2017), different studies evaluating the effects of weaning transition reported reductions of the *Lactobacillus* group. However, our results indicate that the inclusion of n-3 LCFA in sows' diets can increase the abundance of certain species of this genus.

In reference piglet bBW, LBW piglets presented higher abundance of the family *Pectobacteriaceae* mainly by the increases in the genus *Brenneria* and *Anaerostipes*, and a lower abundance of the family *Streptococcaceae*. While effects of *Brenneria* have not been described on animal microbiota, *Anaerostipes* is defined as a butyrate-producer bacteria (Van de Vliet and Joossens, 2022). On the other hand, the possible roles of the family *Streptococcaceae* were described above and, in this case different tentative species were not identified between groups. At species level, LBW piglets presented lower abundance of the tentatively

identified species *Lactobacillus salivarius*, which is considered as probiotic (Chaves et al., 2017).

Finally, the fact that no differences were observed between faeces and caecum content samples at 28 days post-weaning in the beta-dispersion analysis, is an indicator that faeces may be a representative sample for piglet caecum microbiota allowing to study the microbiome without the need to more invasive procedures.

## Conclusion

Fish oil as a n-3 LCFAs source in sow reduces microbial diversity in faeces of piglets at 28 days post-weaning, while the inclusion of n-3 LCFAs in the piglet diet or the bBW of piglets had not effect. In reference to differential microbial communities, the greatest impact was observed by the inclusion of n-3 LCFAs in the maternal diet in the caecum content where a higher relative abundance of the genera *Ruminococcus* and *Treponema*, and a lower relative abundance of the genera *Streptococcus* and *Succinibivrio* stand out. In addition, bBW of piglet also influenced few bacterial populations. Concretely, higher abundances of the family *Pectobacteriaceae* and the genera *Anaerostipes* and lower abundances of the family *Streptococcaceae* in LBW were found. Finally, beta-dispersion analysis did not detect differences between faeces and caecum content at 28 days post-weaning, which indicates faeces as a representative sample of the microbiota in the caecum.

## Data availability statement

The datasets presented in this study will be found in online repositories.

## Ethics statement

This animal study was reviewed and approved by IRTA's Ethical Committee on Animal Experimentation and Generalitat de Catalunya (project no: 10294).

## Author contributions

IB, RL, DT, EE-G, and NT contributed to conception and design of the study. EL-C, ErC, EmC, and NT performed the methodology and statistical analysis. EL-C wrote the first draft of the manuscript. ErC and EmC wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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## Conflict of interest

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

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## Supplementary materials

***Supplementary Table S1:*** *Effect of including fish oil in maternal or post-weaning diets and piglets' birth body weight on the growth performance parameters of weaned piglets.*[1,2]

| | Weaned piglets | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | MDiet | | PDiet[3] | | bBW[3] | | *P* value MDiet | *P* value PDiet | *P* value bBW |
| | Control (n=28) | n-3 LCFA (n=20) | Control (n=24/12) | n-3 LCFA (n=24/12) | LBW (n=24/12) | HBW (n=24/12) | | | |
| Average BW (kg)[4] | | | | | | | | | |
| At weaning* | 8.26 ± 1.70 | 8.05 ± 1.19 | 8.26 ± 1.17 | 8.08 ± 1.79 | 7.19 ± 1.28 | 9.14 ± 0.98 | 0.646 | 0.601 | <0.001 |
| 8 days post-weaning | 8.58 ± 1.51 | 8.21 ± 1.20 | 8.40 ± 1.20 | 8.39 ± 1.57 | 7.45 ± 1.05 | 9.34 ± 0.97 | 0.381 | 0.976 | <0.001 |
| 15 days post-weaning | 9.92 ± 1.69 | 10.1 ± 1.53 | 10.1 ± 1.77 | 9.95 ± 1.47 | 9.07 ± 1.26 | 10.9 ± 1.39 | 0.737 | 0.764 | <0.001 |
| 28 days post-weaning | 15.4 ± 3.30 | 15.6 ± 3.12 | 15.4 ± 3.49 | 15.6 ± 2.94 | 14.2 ± 2.41 | 16.8 ± 3.41 | 0.894 | 0.794 | 0.008 |
| Average daily gain (kg)[4] | | | | | | | | | |
| Weaning → 8 days pw | 0.03 ± 0.08 | 0.02 ± 0.08 | 0.02 ± 0.08 | 0.04 ± 0.08 | 0.03 ± 0.07 | 0.02 ± 0.09 | 0.913 | 0.410 | 0.751 |
| Weaning → 15 days pw** | 0.11 ± 0.08 | 0.14 ± 0.09 | 0.12 ± 0.09 | 0.12 ± 0.07 | 0.12 ± 0.06 | 0.12 ± 0.10 | 0.407 | 0.837 | 0.837 |
| 8 days pw → 15 days pw*** | 0.20 ± 0.11 | 0.27 ± 0.14 | 0.24 ± 0.13 | 0.22 ± 0.13 | 0.23 ± 0.10 | 0.23 ± 0.15 | 0.217 | 0.797 | 0.927 |
| Weaning → 28 days pw | 0.26 ± 0.09 | 0.27 ± 0.11 | 0.26 ± 0.11 | 0.27 ± 0.08 | 0.25 ± 0.08 | 0.27 ± 0.12 | 0.858 | 0.657 | 0.501 |
| 15 days pw → 28 days pw | 0.42 ± 0.15 | 0.42 ± 0.16 | 0.41 ± 0.15 | 0.44 ± 0.16 | 0.40 ± 0.13 | 0.45 ± 0.17 | 0.945 | 0.612 | 0.246 |
| Average daily feed intake (kg)[5] | | | | | | | | | |
| Weaning → 8 days pw | - | - | 0.12 ± 0.02 | 0.12 ± 0.03 | 0.12 ± 0.03 | 0.12 ± 0.02 | - | 0.444 | 0.660 |
| Weaning → 15 days pw | - | - | 0.19 ± 0.04 | 0.21 ± 0.06 | 0.20 ± 0.06 | 0.20 ± 0.04 | - | 0.360 | 0.933 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 8 days pw → 15 days pw | - | - | 0.54 ± 0.13 | 0.60 ± 0.22 | 0.57 ± 0.22 | 0.57 ± 0.14 | - | 0.427 | 0.963 |
| Weaning → 28 days pw | - | - | 0.36 ± 0.07 | 0.38 ± 0.07 | 0.36 ± 0.07 | 0.38 ± 0.70 | - | 0.370 | 0.639 |
| 15 days pw → 28 days pw | - | - | 1.11 ± 0.23 | 1.18 ± 0.21 | 1.12 ± 0.21 | 1.17 ± 0.24 | - | 0.470 | 0.567 |
| Gain to feed ratio[5] | | | | | | | | | |
| Weaning → 8 days pw | - | - | 0.25 ± 0.28 | 0.35 ± 0.27 | 0.30 ± 0.29 | 0.30 ± 0.28 | - | 0.434 | 0.961 |
| Weaning → 15 days pw | - | - | 0.63 ± 0.29 | 0.61 ± 0.28 | 0.64 ± 0.21 | 0.60 ± 0.34 | - | 0.868 | 0.679 |
| 8 days pw → 15 days pw | - | - | 0.65 ± 0.43 | 0.56 ± 0.49 | 0.61 ± 0.48 | 0.60 ± 0.45 | - | 0.641 | 0.938 |
| Weaning → 28 days pw | - | - | 0.69 ± 0.16 | 0.70 ± 0.10 | 0.69 ± 0.07 | 0.70 ± 0.18 | - | 0.842 | 0.850 |
| 15 days pw → 28 days pw | - | - | 0.40 ± 0.21 | 0.41 ± 0.11 | 0.43 ± 0.11 | 0.38 ± 0.21 | - | 0.953 | 0.430 |

bBW, birth weight; FA, HBW, high birth weight piglets; LBW, low birth weight piglets; LCFA, long chain fatty acid; MDiet, maternal diet; PDiet, piglet diet; pw, post-weaning.

[1]Values are least squares means ± SD.

[2]*P* values of the significantly different interactions are reported in the footnotes.

[3]For PDiet i bBW, the first *n* corresponds to piglets and the second to pens.

[4]Piglet was considered the experimental unit for calculations.

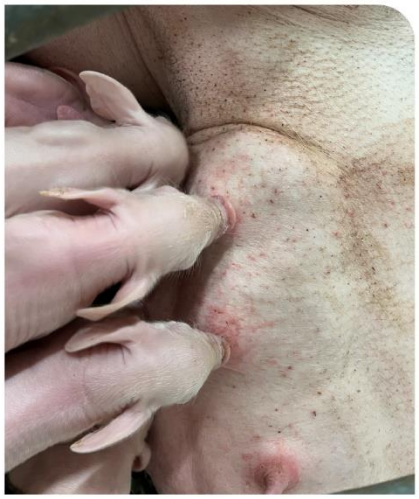
[5]Pen was considered the experimental unit for calculations.

**P* value of the interaction PDiet*bBW was *P* = 0.010 (7.69[b] for control-LBW, 8.80[a] for control-HBW, 6.71[c] for n-3 LCFA-LBW, and 9.40[a] for n-3 LCFA-HBW. Values are expressed in kg).

***P* value of the interaction MDiet*PDiet was *P* = 0.045 (0.12 for control-control, 0.11 for control-n-3 LCFA, 0.11 for n-3 LCFA-control, and 0.15 for n-3 LCFA-n-3LCFA. Values are expressed in kg).

***P* value of the interaction MDiet*PDiet was *P* = 0.039 (0.19 for control-LBW, 0.28 for control-HBW, 0.28 for n-3 LCFA-LBW, and 0.18 for n-3 LCFA-HBW. Values are expressed in kg).

# CHAPTER 3




# GENERAL DISCUSSION

Effects of n-3 long-chain fatty acids in sow and piglet diets on perinatal piglet performance, oxylipins, immunity, and microbiota.

UNIVERSITAT ROVIRA I VIRGILI
EFFECTS OF N-3 LONG-CHAIN FATTY ACIDS IN SOW AND PIGLET DIETS ON PERINATAL PIGLET PERFORMANCE, OXYLIPINS, IMMUNITY, AND MICROBIOTA
Eudald Llauradó Calero

# GENERAL DISCUSSION

The objectives of this PhD Thesis were twofold: to enhance the productive efficiency of sows by increasing the number and weight of piglets per litter at birth and weaning, and to improve piglet health by increasing pre- and post-weaning survival rates. These objectives were set in response to the current needs of swine industry. In this context, the early stages of piglet life, including birth, suckling, weaning, and the post-weaning period, become critical points due to the high prolificacy of the modern sow and the consequent increased proportion of piglets born with a LBW, and the ban on the use of antimicrobials such as antibiotics and zinc oxide during the post-weaning stage. Due to the multiple benefits associated with dietary n-3 LCFAs mentioned previously in the introduction, these main objectives were broken down into more specific goals such as determining the effect of their inclusion in diets for sows and piglets on sow productivity parameters and piglet growth; the composition of colostrum, milk, sows' and piglets' blood in terms of FAs, oxylipins and immune indicators; the expression of immune indicators in the weaned piglets' ileal mucosa; and on the diversity and composition of microbiota in colostrum, milk, sows' and piglets' faeces, and caecum content. In addition, due to the current issues in the swine industry, the impact of piglet birth BW on the aforementioned parameters is also intended to be studied.

Thus, the present discussion aims to jointly discuss the results presented in the different chapters to draw a more complete picture of the effects of including n-3 LCFAs in the sows' and piglet's diets. The discussion will first focus on the results obtained regarding sow productivity and piglet growth. Secondly, it will address the influence of n-3 LCFAs inclusion on the FA composition, oxylipin profile, and immune markers in the different types of analysed samples. Thirdly, it will be discussed the impact on the microbiota of the faeces, colostrum, milk, and caecum content. Finally, it will be deliberated whether piglet bBW has any effect on the analysed parameters.

**Influence of the inclusion of n-3 LCFAs in sow and piglet diets on sows' productivity and piglets' growth**

The results obtained in relation to sow productivity and piglet growth during suckling and post-weaning periods are presented in the Chapters 1 and 4.

In the review of Tanghe and De Smet (2013), it was suggested that n-3 LCFAs may regulate gestation length by influencing the arachidonic acid-derived 2-series prostaglandins, concretely prostaglandins $E_2$ and $F2_{\alpha}$, although stressing that the exact mechanism of action is not very clear. Chapter 3 presents the results of the FA composition in the blood of gestating sows, and it was observed a decrease in arachidonic acid concentration (0.026 mg/g serum in control vs 0.12 mg/ g serum in n-3 LCFA, $P$ <0.001) due to the dietary inclusion of n-3 LCFAs. However, this decrease in arachidonic acid content was not translated into a decrease in the concentration of the 2-series prostaglandins ($P$ = 0.192 and $P$ = 0.239 for prostaglandins $E_2$ and $F_{2\alpha}$, respectively). In the same review, it is suggested that most of the previous studies evaluating the effect of n-3 LCFA-rich diets on gestation length may have reported no significant changes because either the n-3 LCFA concentration in the sow diets was too low or their inclusion too short, although in none of these previous trials were studied the concentrations of 2-series prostaglandins (Tanghe and De Smet, 2013). The results of the current thesis show that a replacement of the animal fat included in the gestation diet (15 g/kg) by an equivalent amount of fish oil (21.5 g/kg) throughout gestation did not lead to an increase in the total gestation length (116 ± 1.05 days in both control and n-3 LCFA groups) and that 2-series prostaglandins were not affected by the inclusion of n-3 LCFAs.

In the review by Leroy *et al*. (2008), it was suggested that n-3 LCFAs maternal supplementation can reduce the secretion of prostaglandin production in the endometrium reducing the expression of the ciclooxygenase enzyme. Thus, it was proposed a possible positive effect of n-3 LCFAs on oocyte quality and early embryo survival by reducing prostaglandin $F_{2\alpha}$ secretion in the endometrium. However, as mentioned above, postaglandin $F_{2\alpha}$ concentration was not affected in

our study in gestating sows fed a diet rich in n-3 LCFAs and the results in terms of litter characteristics did not show an increase in litter size, rather the opposite. Differences in litter characteristics at birth and during suckling are described in the Chapter 1. At birth, although not significant, a lower number of piglets born alive was detected in sows fed the n-3 LCFA diet (15.2 piglets in control vs 14.3 piglets in n-3 LCFA, $P$ = 0.533). After cross-fostering (first 24h post-farrowing), this difference resulted significant (13.5 piglets in control vs 11.8 piglets in n-3 LCFA, $P$ = 0.003), and was maintained as a trend until day 20 of lactation (11.7 piglets in control vs 10.9 piglets in n-3 LCFA, $P$ = 0.052) and at weaning (11.6 piglets in control vs 10.9 piglets in n-3 LCFA, $P$ = 0.093). This difference in litter size did not result into differences in total litter weight, which remained similar between treatments throughout lactation. This is explained because piglets had a similar BW between treatments at birth (1.37 kg in control vs 1.37 in n-3 LCFA, $P$ = 0.953) and after cross-fostering (1.42 kg in control vs 1.54 kg in n-3 LCFA, $P$ = 0.161), but at day 20 and at weaning piglets from sows fed the n-3 LCFA diet had a higher BW (6.06 kg in control vs 6.68 kg in n-3 LCFA, $P$ = 0.013 at day 20; 7.91 kg in control vs 8.50 kg in n-3 LCFA, $P$ = 0.058 at weaning, respectively). Thus, indicating that piglets in the n-3 LCFA group had a higher daily weight gain between 24 h post-farrowing and day 20 (0.24 kg in control vs 0.27 kg/day in n-3 LCFA, $P$ = 0.010), and between 24 h and weaning (0.25 kg in control vs 0.27 kg/day in n-3 LCFA, $P$ = 0.072). Therefore, heavier piglets may be more robust at weaning, which could be a benefit long-term. However, from this study it cannot be concluded whether the increase of piglet BW during lactation is a direct effect due to the dietary n-3 LCFAs or to the greater accessibility they have to colostrum and milk resulting from the lower litter size.

Applying the equations to calculate colostrum intake and colostrum yield described by Devillers *et al*. (2004) or those described by Theil *et al*. (2014) to the data from the current study, it can be observed that piglets from the n-3 LCFA group showed higher colostrum intake with no differences in colostrum yield (Table 1). This suggests that weight gain would be due to this increase in colostrum intake.

***Table 1:*** *Colostrum intake and colostrum yield calculated according to the equations described by Devillers et al. (2004) and Theil et al. (2014).*

| | Devillers *et al.* (2004) | | | Theil *et al*. (2014) | | |
|---|---|---|---|---|---|---|
| | Control | n-3 LCFA | *P* value | Control | n-3 LCFA | *P* value |
| Colostrum intake (g) | 308 | 375 | 0.035 | 443 | 520 | 0.045 |
| Colostrum yield (kg) | 3.96 | 4.15 | 0.630 | 5.77 | 5.76 | 0.979 |

Results from the studies in the scope of the same project (RTA2017-00086-C02) in Iberian pigs carried out in collaboration with the *Centro de Investigaciones Científicas y Tecnológicas de Extremadura* (CICYTEX) showed that the inclusion of n-3 LCFAs in sow diets during gestation and lactation in the same proportion than the current study resulted in a similar litter size at birth (6.96 vs 7.08 born alive piglets in control and n-3 LCFA, $P = 0.899$) (unpublished data) and an increased piglets' daily weight gain during lactation (0.137 kg/day in control vs 0.155 kg/day in n-3 LCFA, $P = 0.007$) (unpublished data). While sows from n-3 LCFA treatment had piglets with a lower BW at birth (1.32 kg in control vs 1.23 kg in n-3 LCFA, $P = 0.001$) (unpublished data), these piglets were heavier at weaning (5.58 kg in control vs 6.07 kg in n-3 LCFA, $P = 0.028$) (unpublished data). It must be mentioned that the difference between litter sizes between the two studies is due to the difference in prolificacy between breeds.

An interaction between piglet diet and bBW indicates that LBW piglets fed n-3 LCFA-rich diet had a numerically higher growth compared to LBW piglets fed control diet (0.289 for control vs 0.336 g/day for n-3 LCFA) during post-weaning phase. However, the presented results in terms of the inclusion of n-3 LCFAs in the maternal or piglet diets showed no effect on piglet's growth or daily feed intake during the post-weaning phase.

Altogether, the results obtained so far suggest that an inclusion of n-3 LCFAs in sow diets from service to weaning could positively influence piglet weight gain during lactation. However, this trial was conducted on an experimental farm and

the total number of sows is limited. For this reason, and considering the potential benefit observed by the inclusion of fish oil in sows' diets, it would be convenient to replicate the study in a commercial farm with a higher number of sows.

**Influence of the inclusion of dietary n-3 LCFAs on FA composition and oxylipins profile in colostrum, milk, and sows' and piglets' blood**

Results in terms of FAs composition, oxylipin profile and immune indicators in colostrum and milk have been described in Chapter 1, in blood from sows and suckling piglets in Chapter 2, and in blood from weaned piglets in Chapter 4.

Dietary n-3 LCFAs increase total n-3 LCFAs, and in particular EPA and DHA in the different biological samples studied. These increases were higher in colostrum than in milk (colostrum reaching concentrations of 41.8 mg/g of fat for total n-3 FAs, 8.22 mg/g of fat for EPA and 11.7 mg/g of fat for DHA, and milk reaching concentrations of 14.7 mg/g of fat for total n-3 FAs, 3.22 mg/g of fat for EPA and 3.83 mg/g of fat for DHA). In blood, while the increases in total n-3 FA, EPA and DHA were similar between gestating and lactating sows, the concertation of DHA differed between suckling and weaned piglets regardless dietary treatment.

Based in the literature review presented above, the increases of n-3 FAs in blood result as its dietary supplementation is an expected result. Furthermore, the decrease in content of arachidonic acid observed in all sample types is also in line with what was previously discussed in the review by Calder (2010) by the dietary enrichment with marine n-3 LCFAs.

As it was expected by FAs, the concentration of their oxygenated derivatives is in resemblance to the dietary fat source. In this way, the results obtained in terms of n-3 LCFAs-derived oxylipins are in accordance with those reported by Shearer and Walker (2018) in which oxylipins follow a similar distribution to that of FAs in plasma. Since n-6 LCFAs and n-3 LCFAs-derived oxylipins compete for the same biosynthesis enzymes (Gabbs *et al*, 2015), a higher concentration of n-3 LCFAs

could be associated with a decrease in n-6 LCFAs-derived oxylipins. However, an increase in the intake of n-3 LCFAs does not always imply a reduction in oxylipins derived from n-6 LCFAs, rather an increase or no influence may be observed (Shearer and Walker, 2018). In this thesis, the inclusion of dietary n-3 LCFAs did not result in a global decrease in n-6 oxylipins.

Research done on the role of oxylipins is recent, and for this reason their actions on the immune system are still under study. From the precursor (polyunsaturated FA) to the final oxygenated metabolite, there are intermediate oxygenated mediators for which their function is still unknown for many of them. For this reason, this thesis draws a picture of the modifications of different oxylipin synthesis pathways by dietary n-3 LCFAs in colostrum, milk, and the blood of sows and piglets. Moreover, to author's knowledge this is the first thesis that evaluates the profile of oxylipins in these types of swine samples. Therefore, this not only allows us to gather information on how n-3 LCFAs can influence the concentration of certain well-known oxylipins, but also enables us to discuss the results based on the impact they have on many mediators within each oxygenation pathway. We have also to consider that oxylipins are compounds with a short half-life (Gabbs *et al*, 2015), this fact implies that we do not know the time that an intermediate metabolite in the oxygenation cascade may have before being converted into the next one. Therefore, the analyses conducted provide a specific snapshot of the oxylipin profile at the specific moment of sample collection. For a more comprehensive understanding of the metabolism of these oxylipins, serial samplings would allow to trace the concentration of these oxylipins over time.

The results of this thesis have also allowed us to visualize that the inclusion of n-3 LCFAs in the diet of sows could modify not only the FA composition and oxylipin profile in the blood of sows, colostrum, and milk but also in the blood of suckling piglets, indicating that these modifications have a real impact on the offspring. Since this was a prospective study, the limited number of samples analysed for colostrum and milk ($n$ = 3 for each experimental diet) did not allow us to establish possible correlations between affected oxylipins in the blood of gestating sows and

colostrum, blood of lactating sows and milk, and finally milk and blood of suckling piglets. Therefore, a future consideration would be to increase the samples analysed and try to establish the previous mentioned correlations to further understand the FAs and oxylipins transfer sow-offspring.

This thesis, apart from studying the direct application and the transference from sow to piglets of dietary n-3 LCFAs, studied the effect that maternal n-3 LCFAs could exert on the FA and oxylipin composition in post-weaning piglets. Findings reported in Chapter 4 indicate that maternal diet had little influence on the FA composition of post-weaned piglets and indeed on the oxylipin profile. However, the inclusion of fish oil in post-weaning diets increased serum total n-3 LCFAs, in particular EPA, docosapentaenoic acid, and DHA, and their oxidation derivatives. These results suggest that FA and oxylipin profile are modified by diet consumed close to the moment of sampling and that dietary n-3 LCFAs do not have a long-term influence on the blood FA and oxylipin composition.

**Influence of the inclusion of dietary n-3 LCFAs on immune indicators in colostrum, milk, and sows' and piglets' blood**

Newborn piglets are devoid of immune system (Le Dividich *et al*., 2005), therefore, the transfer of passive immunity from sows is crucial. For this reason, one of the purposes of the thesis was to determine whether the inclusion of fish oil in sow diets had the capacity to increase the concentration of Igs in colostrum and milk. Although an increase in the concentration of IgM was observed in blood from sows during both gestation (2.55 mg/mL in control vs 4.86 mg/mL in n-3 LCFA) and lactation (4.34 mg/mL in control vs 6.43 mg/mL in n-3 LCFA), only a tendency towards an increase in this Ig was detected in blood from weaned piglets (1.22 mg/mL in control vs 1.54 mg/mL in n-3 LCFA) due to the effect of n-3 LCFA in piglet's diet. Results within the project RTA2017-00086-C02 in Iberian breed found increased concentrations of IgG (30.1 mg/ml in control vs 50.7 mg/ml in n-3 LCFA) and IgA (6.64 mg/ml in control vs 12.4 mg/ml in n-3 LCFA) in colostrum from sows

fed the aforementioned n-3 LCFA-rich diet, which represents a clear improvement in the composition of colostrum and transfer of maternal-passive immunity from mother to offspring. Unfortunately, the results of Ig concentration in the blood of lactating piglets have not been analysed yet, and therefore, the transfer to piglets cannot be confirmed.

As described in the introduction, the inclusion of dietary n-3 LCFAs can modify the concentration of certain cytokines. In this way, in Chapter 1 was reported a decrease in TNFα and IL10 concentrations in milk which could have an anti-inflammatory connotation. However, as previously stated, the low number of samples analyzed did not allow to correlate these changes with changes in the FA composition and oxylipin profile. On the other hand, as reported in Chapter 3, the increased IL1β in the blood of piglets at weaning could be associated with inflammatory processes. Therefore, the results of the analyzed parameters are not sufficient to draw clear conclusions on how dietary n-3 LCFAs modulate the immune system. For this reason, future research on the modulation that dietary n-3 LCFAs can exert on a broader range of cytokines, transcription factors, and even the expression of genes related to inflammation in specific cell types, would further elucidate the immunomodulatory role that these n-3 LCFA can play. These types of studies would help to profile a complete picture of the up or down-regulated mechanisms and thus better understand the real significance of an increase or decrease in a particular cytokine.

**Influence of the inclusion of dietary n-3 LCFAs on microbial diversity and composition of colostrum, milk, sows' and piglets' faeces, and piglets' caecum content**

Results in terms of microbial diversity and composition in faeces of gestating and lactating sows, colostrum, milk, and faeces of suckling piglets have been described in Chapter 3, and in faeces and caecum content of weaned piglets in Chapter 5.

Although there is published information in humans and rodents, the impact of the dietary inclusion of n-3 LCFAs is not deeply understood (Costantini *et al*., 2017; Fu *et al*., 2021). Moreover, the studies conducted in pigs only evaluated certain populations of interest (Leonard *et al*., 2011; Yin *et al*., 2017; Zhang *et al*., 2020). Therefore, this thesis aimed to characterize the changes in bacterial diversity and composition induced by the inclusion of n-3 LCFAs in the diet. Dietary n-3 LCFAs had little impact on the faecal microbiota of sows. This could be explained because they are mature animals with an already well-established microbiota. In milk n-3 LCFAs increased the phylum Proteobacteria, one of the most dominant phyla, and decreased the family *Ruminococcaceae*, which is related with the degradation of complex carbohydrates and the production of short-chain FAs (Liu *et al*., 2019). In addition, microbial composition of milk was highly correlated with that of faeces of lactating sows and faeces of suckling piglets, underlying the crucial role of milk in the transfer of microbiota from sow to piglets. This fact further highlights the strong impact that milk has on the development and establishment of the piglet's microbiota, aligning with what was described by Frese *et al*. (2015), since they suggested that early consumption of milk by piglets results in a milk-oriented gut microbiome. In addition, the faeces of piglets at the end of lactation were the sample type and time point with the most differences in bacterial diversity and composition by the inclusion of dietary n-3 LCFAs. This could be due to newborn piglets acquire and establish their microbiota quickly, and the feeding during the suckling clearly influences its composition.

Another important point of the establishment of the microbiota of piglets is weaning (Gresse *et al*., 2017). In the Chapter 5, the factor with the greatest influence on the composition of faecal and caecal microbiota of piglets 28 days post-weaning is still the maternal diet. Thus, these results continue to underscore the importance of maternal diet, as its impact is not only reflected in piglets during lactation but also remains as a key element in the modulation of the microbiota 28 days post-weaning. However, although the effect of maternal diet could be detected 28 days post-weaning, the effect of including these n-3 LCFAs in the sow’s diet was not

observed seven days after weaning. One of the factors that may influence the absence of differences one week after weaning is the change in the diet, specifically the transition from the maternal milk to a solid feed, which could have resulted in a significantly reduced or absent intake during this period (de Vries and Smidt, 2020). In addition, social and environmental stresses associated to weaning such as the separation from the mother, handling, transport, changing physical environment and the mixture of litters affect the microbiota development during the post-weaning period (Campbell *et al*., 2013; de Vries and Smidt, 2020). In this thesis, at the time of weaning, piglets from sows fed with an n-3 LCFA diet exhibited higher bacterial diversity and among others, a lower abundance of the genus *Ruminococcus*. However, at 28 days post-weaning, the same piglets presented lower diversity and a higher abundance of *Ruminococcus*. Other consequences of weaning are the shift from a milk-derived glycan metabolism to a plant-derived glycan metabolism (Frese *et al*., 2015) and an increase in the relative abundance of anaerobic bacteria (Konstantinov *et al*., 2006). *Ruminococcus* is considered a strictly anaerobic bacteria and a fibre degrader (La Reau and Suen, 2018). In this way, n-3 LCFAs could enhance the relative abundance of this genera related with the production of short-chain FA.

Results on microbial composition have emerged other future points of emphasis, such is the analysis of short-chain FAs in the intestine. Short-chain FAs are considered as one of the most important microbiota products and are formed mainly by the fermentation of non-digestible carbohydrates (Morrison and Preston, 2016). Recently, there has been a growing interest in the research of the immunomodulatory role of short-chain FAs due to their potential anti-inflammatory properties. However, it remains unclear whether SCFAs primarily act as signals to induce tolerance to the host-associated microbiome or directly reduce inflammatory responses (Morrison and Preston, 2016).

It is also important to consider that in commercial farms post-weaning ranges between 28 and 42 days. For this reason, the analysis of microbiota later than 28

days post-weaning would provide a more complete understanding of the long-term influence of n-3 LCFAs on the establishment of the microbiota after weaning.

Altogether, this thesis provides novel information in understanding the potential tole of dietary n-3 LCFAs as nutritional strategy to improve the development and health of piglets during early life. This work gives data about the capacity of dietary n-3 LCFAs to influence the FA and oxylipins profile in blood of sows and piglets, colostrum and milk, together with their impact on immune parameters and microbiota. Therefore, the present results contribute to the knowledge on the use of this family of FAs as a new nutrition strategy in swine nutrition,

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# CONCLUSIONS

Effects of n-3 long-chain fatty acids in sow and piglet diets on perinatal piglet performance, oxylipins, immunity, and microbiota.

## CONCLUSIONS

The conclusions of this Doctoral Thesis in line with the initial hypotheses raised regarding the effects of LCFAs in the diets of sows and weaned piglets are:

- The inclusion of fish oil in diets for sows tended to increase the piglets BW at weaning. However, sows fed n-3 LCFAs also tended to have a smaller litter size, which could also be an explanation for the increased average BW of the piglets (**Hypothesis 1**).
- The inclusion of fish oil in sow or piglet diets does not affect piglet's growth and feed intake during the post-weaning phase (**Hypothesis 1**).
- The inclusion of fish oil in the diets for sows increases the concentrations of EPA, DHA, and their derived oxylipins in sows' blood, colostrum, and milk. This effect is also observed in piglets' blood at weaning (indicating a certain capacity for their transmission) but not at 28 days post-weaning (transmission does not persist overtime) (**Hypothesis 2**).
- The inclusion of fish oil in post-weaning piglet diets increases the concentrations of EPA, DHA, and their derived oxylipins in blood, but the immunomodulation that they exerted is not clear (**Hypothesis 3**).
- The inclusion of fish oil in sow diets increases microbial diversity and the relative abundance of potentially beneficial bacteria such as the mucin-degraders genera *Akkermansia* and *Bacteroides*, and different species of *Lactobacillus* in faeces from suckling piglets (**Hypothesis 4**).
- Milk bacterial populations are correlated with both those of the faeces of lactating sows and those of faeces of suckling piglets, suggesting key role of milk in the transmission of microbiota from sow to piglet during suckling (**Hypothesis 4**).
- The inclusion of fish oil in sow diets reduces microbial diversity in faeces of piglets at day 28 post-weaning, contrary to what had been observed when they were suckling. Furthermore, increases in relative abundance of potentially beneficial bacteria populations such as the short-chain FAs-producers *Ruminococcus* and a spieces of *Lactobacillus* were observed the caecum content of the post-weaned piglets (**Hypothesis 4**).

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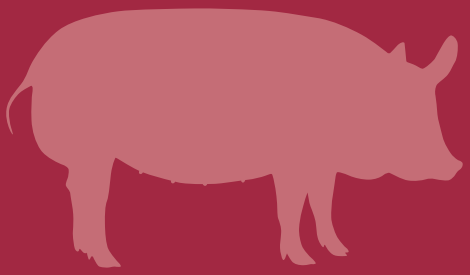

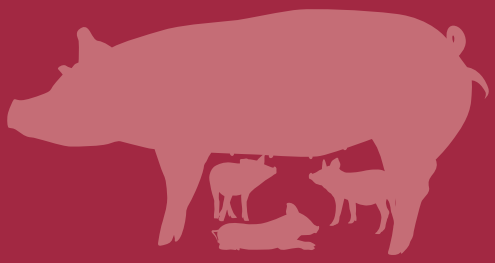

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