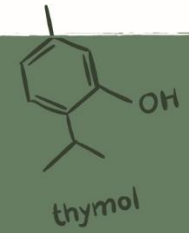
Universitat Autònoma de Barcelona

STUDYING THE EFFECTS OF PHYTOGENIC SUPPLEMENTATION AS FEEDING STRATEGIES TO OVERCOME PHYSIOLOGICAL AND PRODUCTIVE CHALLENGES IN HYPERPROLIFIC SOWS AND THEIR OFFSPRING

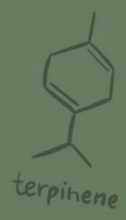
David Angel Reyes Camacho

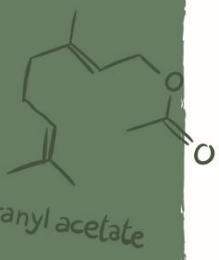


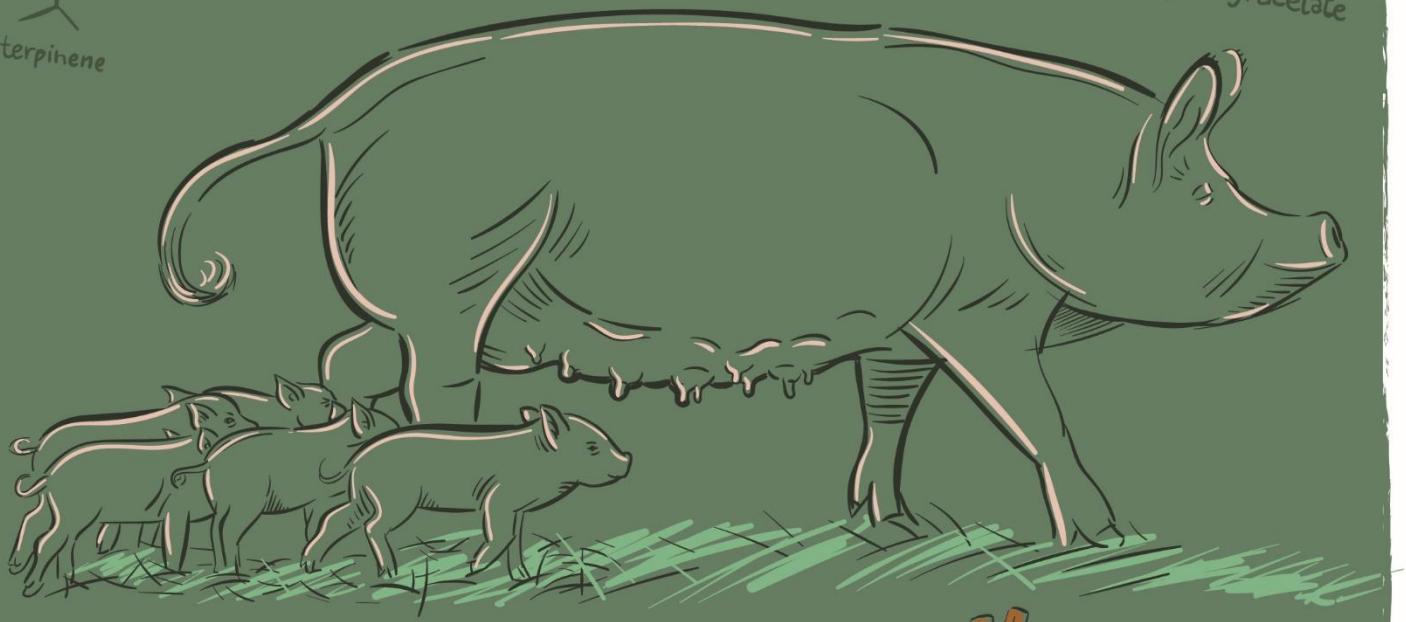
TESIS DOCTORAL · PRODUCCIÓN ANIMAL · 2021

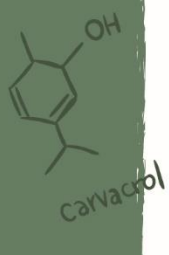
Studying the effects of phytogenic supplementation as feeding strategies to overcome physiological and productive challenges in hyperprolific sows and their offspring

DAVID ANGEL REYES CAMACHO









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Departament de Ciència Animal i dels Aliments

FACULTAT DE VETERINÀRIA

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STUDYING THE EFFECTS OF PHYTOGENIC SUPPLEMENTATION AS FEEDING STRATEGIES TO OVERCOME PHYSIOLOGICAL AND PRODUCTIVE CHALLENGES IN HYPERPROLIFIC SOWS AND THEIR OFFSPRING

TESIS DOCTORAL PRESENTADA POR:

David Angel Reyes Camacho

BAJO LA DIRECCIÓN DE LOS DOCTORES:

José Francisco Pérez Hernández y David Solà Oriol

PARA ACCEDER AL GRADO DE DOCTOR DENTRO DEL PROGRAMA DE DOCTORADO DE PRODUCCIÓN ANIMAL DEL DEPARTAMENTO DE CIENCIA ANIMAL Y DE LOS ALIMENTOS

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Ilustración de la portada creada por Gerard Forner i Gallardo. Representación gráfica de la síntesis del proyecto, ilustrando elementos como si estuvieran dibujados en una pizarra, en forma de bocetos o ideas.

José Francisco Pérez Hernández, Catedrático del Departamento de Ciencia Animal y de los Alimentos de la Facultad de Veterinaria de la Universidad Autónoma de Barcelona, y **David Solà Oriol,** investigador del Servei de Nutrició i Benestar Animal (SNIBA),

Certifican:

Que la memoria titulada **"Studying the effects of phytogenic supplementation as feeding strategies to overcome physiological and productive challenges in hyperprolific sows and their offspring",** presentada por David Angel Reyes Camacho con la finalidad de optar al grado de Doctor en Veterinaria, ha sido realizada bajo su dirección y, considerándola finalizada, autorizan su presentación para que sea juzgada por la comisión correspondiente.

Y para que conste a efectos oportunos, firman la presente en Bellaterra, 16 de septiembre de 2021.

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16 de Septiembre de 2021

Sala de Grados, Facultad de Veterinaria UAB

El presente proyecto de tesis ha sido posible gracias a un contrato (SNiBA 2016-30-DEL) de personal investigador en formación predoctoral concedido por el Departamento de Ciencia Animal y de los Alimentos de la Universidad Autónoma de Barcelona y el financiamiento proporcionado por Delacon Biotechnik GmbH.

Con profundo cariño y agradecimiento:

A mi familia

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A mis directores de tesis, **Francisco y David,** muchas gracias por la oportunidad, apoyo y dirección para hacer posible la realización de este estudio. Sin duda, vuestras aportaciones fueron no solo de formación académica, sino también personal.

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Compañeros Snibanos del **despacho V-315**, y aquellos fuera de este rincón, que sino los menciono es porque seguro me dejaría a más de alguno, a todos gracias. **Sergi López, Mireia, Nedra, Gerard, Agustina, Montse,** a vosotros gracias por acompañarme y compartir vuestra amistad de manera tan familiar durante mi andar por esta fase de vida.

"La dificultad atrae al hombre de carácter, porque es en la adversidad que el verdadero hombre se conoce a sí mismo. De modo que un hombre no se siente orgulloso de las alegrías y del placer. En el fondo lo único que da orgullo y alegría al espíritu son los esfuerzos superados con bravura y los sufrimientos soportados con paciencia."

"Conozca todas las teorías, domine todas las técnicas, pero al tocar un alma humana sea apenas otra alma humana". Carl Gustav Jung

"Es cierto que los sueños tienen un precio, pero no perseguir tus sueños también lo tiene... y es mucho mayor. Porque el precio es lo que se paga, pero el valor es lo que recibes." José Manuel Galán

Summary

Genetic selection for hyperprolific sow lines has increased litter size considerably in the last decades. The increased number of piglets is a major challenge for both sow and its offspring physiology and productivity during gestation, at parturition and during lactation. In addition, post-weaning is a period characterized by feed neophobia, where piglets commonly suffer the low feed intake and impaired growth. Thus, in modern swine industry, the establishment of proper feeding strategies applied for sows and piglets are a main goal to achieve a safe, efficient, and sustainable pig production. Phytogenic feed additives (PFAs) may be defined as standardized, science-based combinations of plant-derived bioactive compounds that promote livestock health and well-being and growth and production efficiency. The purpose of this PhD thesis is, to investigate the potential benefit of a dietary supplementation of PFAs as early feeding strategies to overcome the challenges that face hyperprolific sows and their offspring, but also weanling piglets.

In **Chapter 4**, a trial was conducted to evaluate the effects of a specific blend of phytogenic compounds (BPC1) supplemented in lactation (L) or during gestation and lactation (GL) of hyperprolific sows on placental and milk maternal transfer of these BPC1 and colostrum/milk features, sows and piglet's antioxidant status, reproductive performance (litter size), piglet's body weight (BW) and gut health-related gene expression changes. The results showed that several BPC1 were transferred into placental fluid and milk of supplemented sows. In addition, compared to Control unsupplemented sows, the supplementation of BPC1 in GL increased the number of piglets born alive, which showed lower mean birthweight, but this difference in BW disappeared during lactation and post-weaning period. Antioxidant status (CAT, SOD, and GSH-PX) of supplemented sows and their offspring was improved. Moreover, colostrum protein in GL and milk fat content in L and fat content and bactericide activity in GL were increased, which influenced on suckling piglet's jejunum mRNA overexpression of *MUC2*, *IDO*, *PPARGC-α, TNF-α, TGF-β1*, and *IL-10* genes.

Maternal nutrition plays a fundamental role on the fetal development in swine. Therefore, in **Chapter 5** effects of a specific blend of phytogenic compounds (BPC2) supplemented during gestation of hyperprolific sows on jejunum health-related gene expression and histomorphology changes were evaluated in neonate piglets. Compared to Control unsupplemented sows, the supplementation of BPC2 enhanced the number of

piglets born and live-born from 17.8 to 19.9 and from 14.7 to 17.2, while tended to decrease the mean birthweight. In addition, BPC2 supplementation during gestation of sows downregulated the neonate piglets' jejunal genes involved in oxidation *SOD2* and nutrient transport *SLC16A1/MCT1, SLC11A2/DMT1*, and *SLC39A/ZIP4*, while *IFNG* and *CLDN4* related to immune response and barrier function, respectively, were upregulated. Furthermore, the jejunal villus height (VH) and the ratio of the VH to crypt depth tended to increase, while goblet cell volume density was higher in neonate BPC2.

As PFAs have the potential to modulate feed palatability and, therefore, feed intake and growth performance, in **Chapter 6**, feed preference or aversion for prestarter diets supplemented with botanicals compounds (BCs) such as D-limonene, trans-anethole, or eucalyptol were evaluated by using a double-choice feeding test in one-week post-weaning piglets. The results showed that piglets preferred diets with D-limonene (53.8%) or trans-anethole (54.5%), while avoided diets with eucalyptol (41.6%). In addition, piglets preferred diets with D-limonene or trans-anethole showed better ADG when compared with aversive eucalyptol. In addition, based on piglets can learn about flavors added in the maternal diet, which may reduce neophobia and modulate feed intake, and, thus, growth performance, in **Chapter 6** was also studied the pre- and postnatal exposure (amniotic fluid and milk) to D-limonene, trans-anethole, and eucalyptol. The results indicated all above-mentioned BCs were transfer to amniotic fluid, while only D-limonene and trans-anethole into milk. In addition, weanling piglets exposed to BCs showed lower BW at day 7 when compared to unsupplemented Control group. Therefore, prenatal exposure to preferred D-limonene and eucalyptol, or familiarity to eucalyptol was not effective to overcome the innate aversion to eucalyptol, affecting post-weaning piglets' growth performance.

Resumen

La selección genética de líneas de cerdas hiperprolíficas ha incrementado considerablemente el tamaño de la camada en las últimas décadas. El aumento del número de lechones es un gran desafío para la fisiología y la productividad tanto de la cerda como de su descendencia durante la gestación, el parto y la lactancia. Además, el post-destete es un periodo caracterizado por la neofobia alimentaria, donde los lechones comúnmente sufren una baja de ingesta de alimento y un crecimiento deficiente. Así, en la industria porcina moderna, el establecimiento de estrategias de alimentación adecuadas aplicadas a cerdas y lechones es un objetivo primordial para lograr una producción porcina segura, eficiente y sostenible. Los aditivos fitogénicos para piensos (PFAs) pueden definirse como combinaciones estandarizadas y científicas de compuestos bioactivos derivados de plantas que promueven la salud y el bienestar del ganado y la eficiencia del crecimiento y la producción. El propósito de esta tesis doctoral es investigar el beneficio potencial de una suplementación dietética de PFAs como estrategias de alimentación temprana para superar los desafíos que enfrentan las cerdas hiperprolíficas y sus crías, pero también los lechones destetados.

En el **Capítulo 4**, se realizó un experimento para evaluar los efectos de una mezcla especifica de compuestos fitogénicos (BPC1) suplementada en la lactancia (L) o durante la gestación y la lactación (GL) de cerdas hiperprolíficas sobre la transferencia materna de esta BPC1 en la placenta y leche, las características del calostro/leche, estado antioxidante de las cerdas y lechones, rendimiento reproductivo (tamaño de la camada), peso corporal (BW) y cambios en la expresión génica relacionada con la salud intestinal de los lechones. Los resultados mostraron que diversos BPC1 se transfirieron al liquido placentario y leche de cerdas suplementadas. También, en comparación con las cerdas Control no suplementadas, la suplementación de BPC1 en GL aumentó el número de lechones nacidos vivos, los cuales mostraron un menor peso medio al nacer, sin embargo, esta diferencia en el peso corporal desapareció durante la lactancia y el periodo post-destete. Se mejoró el estado antioxidante de las cerdas suplementadas y su descendencia. Además, la proteína del calostro en GL y el contenido de grasa de la leche en L y el contenido de grasa y la actividad bactericida en leche GL aumentaron, lo cual influyó en la sobreexpresión de mRNA en yeyuno de lechones lactantes de *MUC2, IDO, PPARGC-α, TNF-α, TGF-β1,* y *IL-10* genes.

La nutrición materna juega un papel fundamental en el desarrollo fetal del cerdo. Por lo tanto, en el **Capítulo 5** se evaluaron los efectos de una mezcla especifica de compuestos fitogénicos (BPC2) suplementados durante la gestación de cerdas hiperprolíficas sobre la expresión génica relacionada con la salud y los cambios histomorfológicos en el yeyuno de lechones neonatos. En comparación con las cerdas Control no suplementadas, la suplementación de BPC2 incrementó el número de lechones nacidos y nacidos vivos de 17.8 a 19.9 y de 14.7 a 17.2, mientras que tendió a disminuir el peso medio al nacer. Además, la suplementación con BPC2 durante la gestación de las cerdas reguló a la baja los genes yeyunales en los lechones neonatos involucrados en la oxidación *SOD2* y el transporte de nutrientes *SLC16A1/MCT1, SLC11A2/DMT1* y *SLC39A/ZIP4,* mientras que *IFNG* y *CLDN4* relacionados con la respuesta inmune y la función de la barrera, respectivamente, fueron regulados a la alza. Además, la altura de la vellosidad yeyunal (VH) y el ratio VH y de profundidad de las criptas tendieron a aumentar, mientras que la densidad del volumen de células caliciformes fue mayor en los neonatos BPC2.

Como los PFAs tienen el potencial de modular la palatabilidad del alimento y, por lo tanto, la ingesta de alimento y el crecimiento, en el **Capítulo 6**, la preferencia o aversión por dietas de preinicio suplementadas con compuestos botánicos (BCs) como D-limoneno, *trans*-anetol o eucaliptol fueron evaluados mediante el uso de una prueba de alimentación de doble elección en lechones una semana después del destete. Los resultados mostraron que los lechones prefirieron dietas con D-limoneno (53.8%) o *trans*-anetol (54.5%), mientras que evitaron las dietas con eucaliptol (41.6%). También, los lechones que prefirieron dietas con D-limoneno o *trans*-anetol mostraron una mejor GMD en comparación con eucaliptol. Además, como los lechones pueden aprender sobre los sabores adicionados en la dieta materna, lo cual puede reducir la neofobia y modular la ingesta de alimento y, por lo tanto, el crecimiento, en el **Capítulo 6** también se estudió la exposición pre y posnatal a D-limoneno, *trans*-anetol y eucaliptol. Los resultados indicaron que todos los BCs mencionados se transfirieron al líquido amniótico, mientras que solo D-limoneno y *trans*-anetol en la leche. Además, los lechones destetados expuestos a BCs mostraron un menor peso corporal al día 7 en comparación con el grupo Control sin suplementar. Por lo tanto, la exposición prenatal al D-limoneno y *trans*-anetol preferidos, o la familiaridad con el eucaliptol, no superó la aversión innata al eucaliptol, lo cual afecto el crecimiento de los lechones después del destete.

Resum

La selecció genètica de línies de truges hiperprolífiques ha incrementat considerablement la mida de la ventrada en les últimes dècades. L'augment de nombre de garrins és un gran desafiament per a la fisiologia i la productivitat tant de la truja com de la seva descendència durant la gestació, el part i la lactància. A més, el post-deslletament és un període caracteritzat per la neofòbia alimentària, on els garrins comunament pateixen una baixa d'ingesta d'aliment i un creixement deficient. Així, en la indústria porcina moderna, l'establiment d'estratègies d'alimentació adequades aplicades a truges i garrins és un objectiu primordial per aconseguir una producció porcina segura, eficient i sostenible. Els additius fitogénicos per a pinsos (PFAs) poden definir-se com combinacions estandarditzades i científiques de compostos bioactius derivats de plantes que promouen la salut i el benestar de la ramaderia i l'eficiència de l'creixement i la producció. El propòsit d'aquesta tesi és explorar el benefici potencial d'una suplementació dietètica de PFAs com a estratègies d'alimentació primerenca per superar els desafiaments que enfronten les truges hiperprolífiques i les seves cries, però també els garrins deslletats.

En el **capítol 4**, es va realitzar un experiment per avaluar els efectes d'una barreja específica de compostos fitogénicos (BPC1) suplementada en la lactància (L) o durant la gestació i la lactació (GL) de truges hiperprolífiques sobre la transferència materna d'aquesta BPC1 a la placenta i llet, les característiques de l'calostre / llet, estat antioxidant de les truges i garrins, rendiment reproductiu (mida de la ventrada), pes corporal (BW) i canvis en l'expressió gènica relacionada amb la salut intestinal dels garrins. Els resultats van mostrar que diversos BPC1 es van transferir a el líquid placentari i llet de truges suplementades. També, en comparació amb les truges Control no suplementades, la suplementació de BPC1 en GL va augmentar el nombre de garrins nascuts vius, els quals van mostrar un menor pes mitjà a l'néixer, però, aquesta diferència en el pes corporal va desaparèixer durant la lactància i el període post-deslletament. Es va millorar l'estat antioxidant de les truges suplementades i la seva descendència. A més, la proteïna de l'calostre en GL i el contingut de greix de la llet en L i el contingut de greix i l'activitat bactericida en llet GL van augmentar, la qual cosa va influir en la sobreexpressió de mRNA en jejú de garrins lactants de *MUC2*, *ANAT*, *PPARGC-α, TNF-α, TGF-β1*, *i IL-10* gens.

La nutrició materna juga un paper fonamental en el desenvolupament fetal de l'porc. Per tant, en el **Capítol 5** es van avaluar els efectes d'una barreja específica de compostos fitogénicos (BPC2) suplementats durant la gestació de truges hiperprolífiques sobre l'expressió gènica relacionada amb la salut i els canvis histomorfológicos en el jejú de garrins nounats. En comparació amb les truges Control no suplementades, la suplementació de BPC2 va incrementar el nombre de garrins nascuts i nascuts vius de 17.8 a 19.9 i de 14.7 a 17.2, mentre que va tendir a disminuir el pes mitjà a l'néixer. A més, la suplementació amb BPC2 durant la gestació de les truges va regular a la baixa els gens yeyunales en els garrins nounats involucrats en l'oxidació SOD2 i el transport de nutrients SLC16A1/MCT1, SLC11A2/DMT1 i SLC39A/ZIP4, mentre que IFNG i CLDN4 relacionats amb la resposta immune i la funció de la barrera, respectivament, van ser regulats a l'alça. A més, l'altura de la vellositat jejunal (VH) i la ràtio VH i de profunditat de les criptes van tendir a augmentar, mentre que la densitat de l'volum de cèl·lules caliciformes va ser major en els nounats BPC2.

Com els PFAs tenen el potencial de modular la palatabilitat de l'aliment i, per tant, la ingesta d'aliment i el creixement, en el **Capítol 6**, la preferència o aversió per dietes de preinici suplementades amb compostos botànics (BCS) com D-limonè , trans-anetol o eucaliptol van ser avaluats mitjançant l'ús d'una prova d'alimentació de doble elecció en garrins una setmana després de l'deslletament. Els resultats van mostrar que els garrins van preferir dietes amb D-limonè (53.8%) o trans-anetol (54.5%), mentre que van evitar les dietes amb eucaliptol (41.6%). També, els garrins que van preferir dietes amb D-limonè o trans-anetol van mostrar una millor GMD en comparació amb eucaliptol. A més, com els garrins poden aprendre sobre els sabors addicionats en la dieta materna, la qual cosa pot reduir la neofòbia i modular la ingesta d'aliment i, per tant, el creixement, en el **Capítol 6** també es va estudiar l'exposició pre i postnatal a D-limonè, trans-anetol i eucaliptol. Els resultats van indicar que tots els BCS esmentats es van transferir a el líquid amniòtic, mentre que solament D-limonè i trans-anetol en la llet. A més, els garrins deslletats exposats a BCS van mostrar un menor pes corporal a el dia 7 en comparació amb el grup Control sense suplementar. Per tant, l'exposició prenatal a l'D-limonè i trans-anetol preferits, o la familiaritat amb el eucaliptol, no va superar l'aversió innata a l'eucaliptol, la qual cosa afecte el creixement dels garrins després de l'deslletament.

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Abbreviations

ADFI	average daily feed intake
ADG	average daily gain
ALPI	intestinal alkaline phosphatase
ANPEP	aminopeptidase-N
ATTD	apparent total tract nutrient digestibility
BC	botanical compounds
BPC	blend of botanical compounds
BW	body weight
CAT	catalase
CCK	cholecystokinin
CD	crypt depth
CLDN4	claudin-4
DAO1	diamine oxidase
DCHT	double choice feeding test
EO	essential oils
FCR	feed conversion ration
FDR	false discovery rate
FEDNA	federación española para el desarrollo de la nutrición animal
GIT	gastrointestinal tract
GSH-PX	glutathione peroxidase
IDO1	indoleamine 2,3-dioxygenase
IFNG	interferon gamma
IL-10	interleukin-10
IUGR	intrauterine growth retardation
LBW	low birthweight
MUC2	mucin-2
NBW	normal birthweight
NO	nitric oxide
OCLN	occludin

PFAs	phytogenic feed additives
PPARGC1	peroxisome proliferative activated receptor gamma coactivator 1 alpha
SADFI	standardized average daily feed intake
SLC	solute carrier
SOD	superoxide dismutase
SOD2	superoxide dismutase-2
TAC	total antioxidant capacity
TGF-β1	transforming growth factor beta 1
TNF-α	tumor necrosis factor alpha
VH	villus height

CHAPTER 1

General introduction

Supplying protein to a growing world population in a safe, efficient, and sustainable way, is one of most pressing challenges for the Agri companies in the coming decades. Modern swine production is largely contributing to this effort as large improvements have been obtained in major pig (*Sus scrofa domesticus*) populations in terms of growth, feed efficiency, carcass composition (Tribout et al., 2010), and sow prolificacy (Silalahi et al., 2017). Genetic selection for highly prolific sows has led to a steady increase in litter size over the last decades (Kemp et al., 2018). However, it is well-known that selection for higher litter size has resulted in lower and more variable piglet birthweights (Moreira et al., 2020). The causes of low birthweight (LBW) in pigs are determined by two terms: intrauterine crowding and intrauterine growth retardation (IUGR) (Wu et al., 2006). Common life-long challenges in IUGR encompass, both mental, and physical, such as immaturity and disfunction of organs including gastrointestinal tract (GIT) and reduced weight gain (Matheson et al., 2018). Furthermore, it has been described the challenge of large litters on the immune (Oliviero et al., 2019), and oxidative system (Berchieri-Ronchi et al., 2011), which can affect not only physiology and health and well-being of sows, but also their offspring.

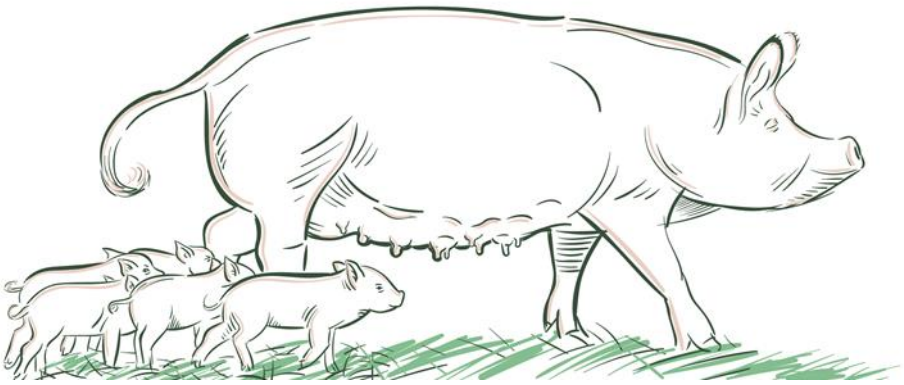
Porcine colostrum and milk also play an essential role in ensuring the survival, growth, and health of piglets. However, over the last 30 years, colostrum and milk nutrient composition have not increased significantly with litter size (Li et al., 2018). In particular, sow colostrum yield is independent of litter size and suckling intensity but is largely driven by sow-related factors (Vadmand et al., 2015). Moreover, currently it is common to see a greater number of piglets per litter than the available functional teats at the sow's udder (Quesnel and Farmer, 2019). Taken together, this scenario means that nutrient intake of piglets from large litters may be restricted. Greater within-litter variation, and the presence of a significant percentage of LBW piglets in the litters represent a considerable economic problem to modern pig producers because of increased competition for adequate colostrum intake (Declerck et al., 2017), morbidity and pre-weaning mortality (Kirkden et al., 2013), as well as impaired postnatal growth performance and lowest lean percentage at slaughter (López-Vergé et al., 2018a).

Weaning stress, due to physiological, environmental, social, and nutritional (i.e., neophobia) factors, may also contribute to intestinal and immune system dysfunctions, making newly-weaned piglets extremely vulnerable to post-weaning diseases and impaired growth performance (Campbell et al., 2013). In the past, nutritionists were able

to mask GIT infections by using in-feed antibiotics, pharmaceutical levels of zinc and/or high levels of copper in piglet diets. However, several countries have restricted or banned the use of in-feed antibiotics (Ma et al., 2021), while the use of therapeutic zinc oxide in pigs diets must be phased out in the EU by 2022 (Satessa et al., 2020). In recent years, the phytogenic feed additives (PFAs) have attracted increased attention from the husbandry industry. The PFAs may be defined as standardized science-based combinations of plant-derived bioactive compounds (phytochemicals) that promote livestock health and well-being and growth and production efficiency (Reyes-Camacho et al., 2020).

The bibliography has described widely the potential effects of PFAs on animal health status because of properties such as stimulation of nutrient absorption and gut morphology, antimicrobial, regulation of gut microflora, anti-inflammatory activities, and antioxidant properties, mainly in monogastric species, such as swine and poultry (Zeng et al., 2015); (Upadhaya and Kim, 2017). It has been also described other effects on growth performance by sensory properties of PFAs able to influence palatability and feed intake in pigs by dietary supplementation (Clouard and Val-Laillet, 2014) or sensory maternal learning (Blavi et al., 2016). In this sense, transfer of bioactive and critical nutritional components from maternal diet to offspring may start already before birth, through the amniotic fluid, but also after birth through mammary secretions. Therefore, maternal nutrition may play a vital role in fetal development, early neonatal development, and lactation which might regulates the lifetime productivity of offspring (Zhang et al., 2019).

In present thesis project we intend to explore the possibilities offered by the use of PFAs in swine feeding, with special interest in promoting early strategies aiming to modulate the negative impact of high prolificacy on the gestating and lactating sow physiology and improve piglet's response before and after weaning



CHAPTER 2

Literature review

2.1 Biological implications of high prolificacy on the sow and piglet's physiology

In swine, litter traits mainly include total number of piglets born, number of piglets born alive, number of weaned piglets, birth weight, gestation length, and number of stillborn piglets (Zheng et al., 2020). The total number of piglets born and born alive, as well as the number of pigs weaned per sow per year, are commonly used as benchmarking measurements to compare the productivity of breeding herds, either among herds in a country or among countries (Koketsu et al., 2017). In modern swine systems, genetic selection for highly prolific sows during the last decades have increased the target values for total number of piglets born and number of piglets born alive from 11.05 to 15.85 and from 10.25 to 14.69 respectively (Moreira et al., 2020).

In fact, with some hyperprolific sow lines coming from northern Europe, such as Topigs Norsvin 70 or Danbred breeds, it is not uncommon to have litters up to 18–20 total born pigs (Care and Gouessant, 2020). In addition, the target values for pigs weaned per sow per year have increased from 20 to 30 pigs over the last three decades, and it is likely that genetics and sow management will increase the number of pigs weaned per sow per year up to 30–40 pigs in the future (Koketsu et al., 2017). However, as litter size (total born) increases, the average litter birthweight decreases (Figure 2.1), and the within-litter variation of born alive piglet birthweight increases (Figure 2.2). According to Moreira et al. (2020), an average increase of 4.8 piglets reduced the average BW of born alive piglets by 12.4%, while increasing their variability by 4.54%. Lower average and more variable piglet birthweights are an issue because of the increased chances of mortality in LBW piglets (Kemp et al., 2018), but also affect their postnatal growth performance and carcass quality at slaughter (López-Vergé et al., 2018a).

Litter size in the pig is determined by underlying physiological processes, like ovulation rate, fertilization rate of oocytes, and embryo and fetal survival and development (Kemp et al., 2018). The latter is determined to a significant extent by the uterine capacity (Vallet et al., 2014). In fact, the causes of LBW in piglets are determined using two terms: intrauterine crowding and IUGR (Matheson et al., 2018). The latter is defined as impaired growth and development of the mammalian embryo/fetus or its organs during pregnancy (Wu et al., 2006). Thus, increasing litter size is a major challenge for the physiology of the gestating hyperprolific sow, but also for her offspring (Oliviero et al., 2019). The following review section will focus on the gestational physiology processes of the sow.

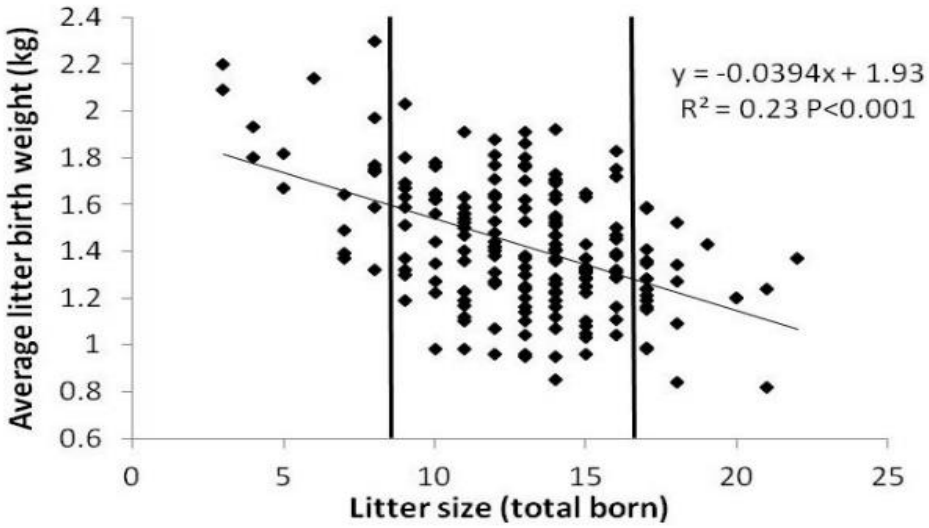


Figure 2.1. *Relationship between litter size (total born) and the average piglet birthweight by litter (Adapted from Smit et al., 2013).*

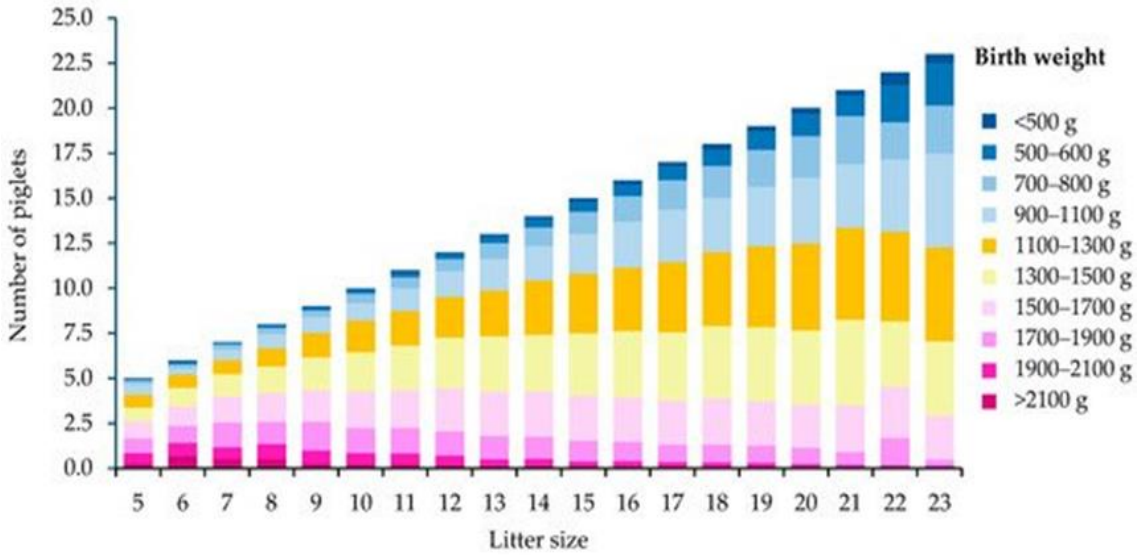


Figure 2.2. *Effect of litter size on birthweight distribution. The data were collected at Schothorst Feed Research B.V. (Lelystad, The Netherlands) from 2011 to 2019, based on 97,552 piglets born alive from 7888 litters (Adapted from Huting et al., 2021).*

2.1.1 Gestational physiology processes determining reproductive performance in hyperprolific sows

Physiologically, porcine pregnancy has been divided into three sub-phases: 1) embryo implantation and itself gestation are established during first weeks, 2) the intermediate period of growth and development of the placenta, fetus differentiation and sow adaptation, 3) finally, in last week's occurs the exponential growth of fetuses and the mammary preparation and development, including the synthesis of colostrum (Curso Fedna, 2018). Figure 2.3 shows the temporal evolution in percentage of placental and

fetal weight, and the amniotic fluid. Placenta grows exponentially between days 20 and 60-70 while fetus grow between days 60-70 and the end of gestation (Wu et al., 2017).

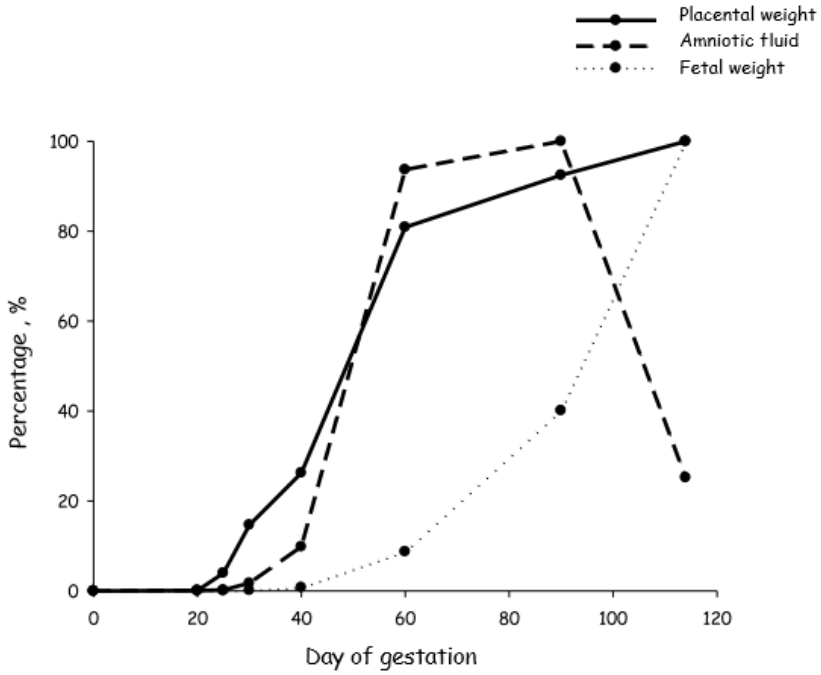


Figure 2.3. *Evolution in percentage (%) of placental and fetal weight, and amniotic fluid during porcine gestation (Adapted from Wu et al., 2017).*

The first month of gestation in pigs is critical to the success of pregnancy because it is in this period that pregnancy is either established successfully or, in the case of insufficient interaction between the conceptuses and the uterus, is not maintained. It is also in this period that the potential litter size is set, being determined by the number of embryos that survive (Langendijk, 2015), which is in turn affected by management (Koketsu et al., 2017) and nutritional strategies (Ji et al., 2017). Paramount for a successful pregnancy and maximizing embryo survival is the formation of sufficient luteal tissue once the luteinizing hormone surge has triggered luteinization of the pre-ovulatory follicles at oestrus and during the ensuing two weeks.

Meanwhile, processes like ovulation rate and embryonic and fetal survival are related to each other and high ovulation rates and consequent uterine crowding may not only negatively affect embryo and fetal survival, but also placental development and thereby embryo and fetal development (Kemp et al., 2018). In this sense, following the high genetic correlation with total number of piglets born, ovulation rate increased sharply in the last decades. An overview of published data on ovulation rate from 1980

up to 2017 described an increase of 0.2 ovulations per year in both gilts and sows, and averages ovulation rate of 25 up to 30 are relatively common nowadays (Da Silva, 2018).

Sharp increases in ovulation rate might negatively influence follicular and oocyte quality, compromising embryonic development and piglet birthweight (Da Silva, 2018). Moreover, an increase in ovulation rate leads to a higher number of embryos surviving to the post-implantation period (> 13 days of pregnancy), therefore, leading to a higher competition between littermates for adequate uterine space with an increase of intrauterine crowding (Foxcroft et al., 2006). For instance, in gilts, high ovulation rates were related to higher within-litter variation in weight (Da Silva et al., 2017). In sows, high ovulation rates were related with lower placental lengths, where each additional corpus luteum represented a decrease in placental length of 0.38 cm at day 35 of pregnancy (Da Silva et al., 2016).

In swine, these effects on the embryo-placental units were related to the level of late embryonic mortality, indicating that high ovulation rates combined with uterine crowding compromises the development of the remaining embryo-placental units (Da Silva et al., 2016). The Figure 2.4 shows the increased gap between ovulation rate and number of embryos at day 35 of pregnancy with an increase in ovulation rate, in both sows and gilts. As is shown, the difference between ovulation rate and total number of embryos is considered to be early mortality, and the difference between total and vital embryos is considered to be late embryonic mortality.

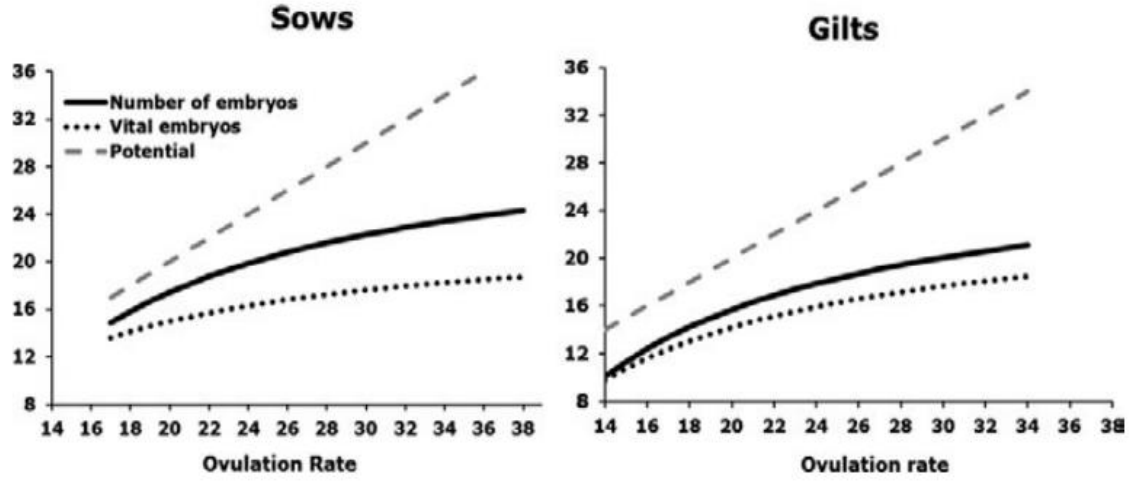


Figure 2.4. *Relation between ovulation rate and the predicted number of total (thick line) and vital (this line) embryos at day 35 of pregnancy in sows and gilts. The dashed line (- - -) represents the potential number of embryos (i.e. ovulation rate) (Adapted from Kemp et al., 2018).*

Placental efficiency. The placenta is located at the maternal-fetal interface and modulates the *in utero* environment to promote optimal fetal development. Thus, proper placental development and function are central to the health of both the mother and the fetus during pregnancy (Pereira et al., 2015). In pigs, the weight of the fetus/piglet per mass of placenta, has been suggested as a broad indicator of placental efficiency (Vallet et al., 2014). Previously, Vallet et al. (2003) suggested that fetal growth rate is less sensitive to intrauterine crowding than placental growth rate and, within certain limits an increase in placental efficiency may initially protect the developing fetus from a limitation in placental size. In fact, large within-litter variation in placental efficiency has been proposed as a significant contributor to the large within-litter variation in piglets birthweight (Wilson and Ford, 1999).

Abnormal development of the placental vasculature leads to placental insufficiency, which can result in a decrease in the exchange of nutrients and wastes between maternal and fetal circulations (Pereira et al., 2015). The major determinant of intrauterine growth is the placental nutrient supply, which, in turn, depends on the size, morphology, blood supply and transporter abundance of the placenta, and on synthesis and metabolism of nutrients and hormones by the uteroplacental tissues (Fowden et al., 2006). Thus, the exponential fetal growth and development is dependent of the maternal capacity to facilitates efficient exchange of nutrients (Reynolds et al., 2005). The efficiency of this process is influenced by the placental blood flow rates, which are dependent on appropriate development of the placental vascular network, including vascularization by angiogenesis (creation of new blood vessel networks by branching and elongating previously existing vessels), and vasodilatation by extravillous trophoblast that mediated arterial remodeling factors (Pereira et al., 2015); (Reynolds et al., 2006).

Studies in animal models of placental angiogenesis, described that placental produces regulating placental angiogenesis factors, including vascular endothelial growth factor, basic fibroblast growth factor, the angiopoietins, and their receptors. In fact, expression of these factors, and their receptors have been correlated with normal placental vascular development (Reynolds et al., 2005). It has been also suggested that impaired placental syntheses of nitric oxide (NO) (a major vasodilator and angiogenesis factor) and polyamines (key regulators of DNA and protein synthesis) may provide a unified explanation for IUGR in response to the 2 extremes of maternal nutritional problems (undernutrition and overnutrition). Impaired nitric oxide synthesis may impair placental

development and utero-placental blood flows, and therefore reduced transfer of nutrients and O_2 from mother to fetus, and thus fetal growth restriction (Wu et al., 2004) (Figure 2.5). Recently, Hu et al. (2020) mentioned that decreased angiogenesis in the pig placentae may be attributed to the increased oxidative stress level. They also demonstrated increased oxidative damage, decreased mitochondrial function, impaired angiogenesis, and downregulated glucose transporters in placentae for LBW piglets.

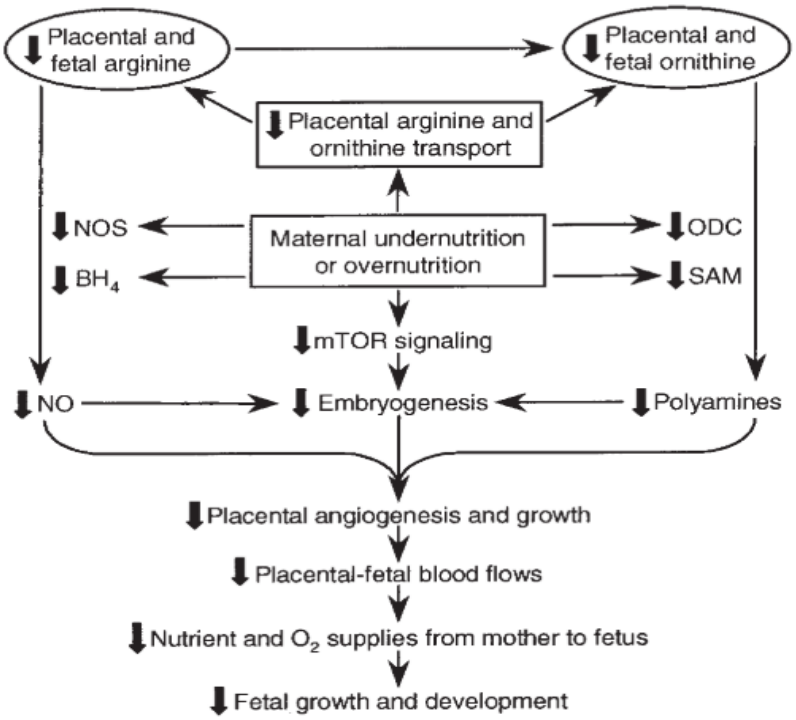


Figure 2.5. *Proposed mechanisms for fetal growth restriction in underfed and overfed dams. Both maternal undernutrition and overnutrition may impair placental syntheses of NO and polyamines, and therefore placental development and utero-placental blood flows. This may result in reduced transfer of nutrients and O_2 from mother to fetus, and thus fetal growth restriction. mTOR, mammalian target of rapamycin. The symbol "↓" denotes reduction (Adapted from Wu et al., 2004).*

In general, poor vascularization of the placenta not only can lead to fetal growth restriction, but also preeclampsia, and in some cases fetal death (Pereira et al., 2015). **In the present thesis project, we will explore if supplementation of PFAs to gestating hyperprolific sows may promote changes on potential factors determining reproductive performance outcomes.**

2.1.2 Effects of increased litter size on the oxidative status of gestating hyperprolific sows

As pregnancy progresses and the metabolic demands of the fetus rise, there is an increase in both placental mitochondria mass and mitochondrial electron chain enzyme activity. This increase is characterized by the elevated placental production of reactive oxygen species (ROS) including superoxide ($O_2^{-}\cdot$), hydroxide ($OH^{-}\cdot$), and hydrogen peroxide (H_2O_2) and increased oxidative stress (Pereira et al., 2015). They are primarily formed from mitochondrial oxidative phosphorylation, where electrons are transferred across respiratory chain enzymes and leak onto molecular oxygen (Fridavich, 1995). Consequently, oxidative stress is described as an imbalance in the production of ROS and the ability of antioxidant defenses (enzymes), such as superoxide dismutase (SOD), glutathione peroxidase (GSH-PX), glutathione reductase, catalase, and other nonenzymatic anti-oxidants (i.e., glutathione, vitamins C and D) (Yin et al., 2013) to scavenge them (Myatt and Cui, 2004).

As is shown in Figure 2.6, the oxidative stress has been proposed as a contributory pathway for placental dysfunction by its influence on the expression of a number of transcription factors important in mediating angiogenesis and trophoblast invasion. Mitochondria within the placenta (depicted in pale pink) are a major producer of ROS, such as $O_2^{-}\cdot$ which can cause a state of oxidative stress (illustrated in yellow). Oxidative stress within the placenta can act as a signaling pathway to influence the expression of transcription factors, such as Krüppel-like factor 8 (KLF8), E26 transformation specific oncogene homolog 1 (Ets-1), nuclear factor kappa-light- chain-enhancer of activated B (NF-kB), specificity protein 1 (Sp1) and specificity protein 3 (Sp3), signal transducer and activator of transcription 3 (STAT-3), and NF-E2-related factor 2 (Nrf2). These transcription factors regulate the expression and activity of proteins related to angiogenesis and trophoblast invasion (as shown by the lines linking to the invasion and angiogenesis panels) (Pereira et al., 2015).

Therefore, although angiogenesis is favorable for tissue growth and regeneration, an alteration in the net balance between pro- and anti-angiogenic molecules by stimuli like oxidative stress, mechanical and metabolic stress (low pO_2), immune or inflammatory response and genetic mutations, results in several pathological complications (e.g., DNA damage and cancer) (Carmeliet and Jain, 2011). In this sense, it has been suggested that highly prolific sows may be under systemic oxidative stress,

which can affect not only fertility and well-being of sows, but also affect the fate of the offspring. Berchieri-Ronchi et al. (2011) described that endogenous DNA damage in hyperprolific sows because of elevated oxidative stress is significantly augmented in the second quarter of gestational period (G 60); maintaining elevated damage throughout the lactational period (Figure 2.7).

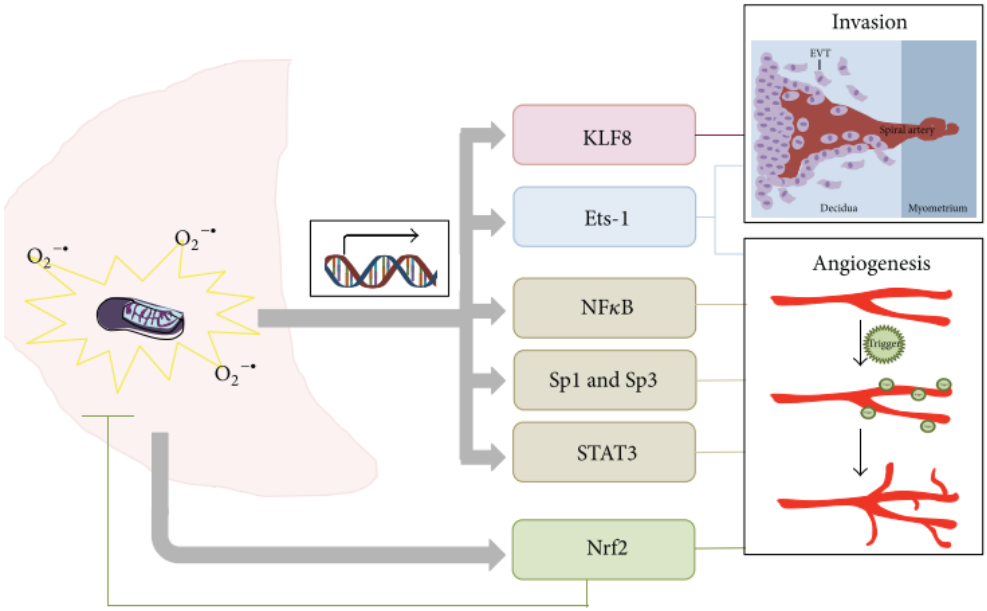


Figure 2.6. *Placental oxidative stress triggers the expression of transcription factors to regulate angiogenesis and trophoblast invasion (Adapted from Pereira et al., 2015).*

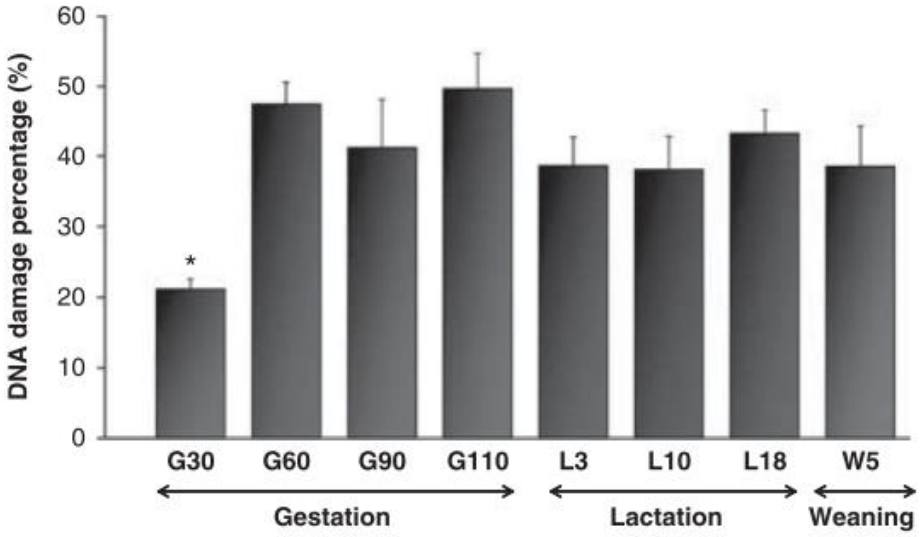


Figure 2.7. *Lymphocyte DNA damage in multiparous sows during gestational, lactational and weaning periods; with lower ($p < 0.05$) endogenous DNA damage at day 30 of gestation (G30; 21%) as compared with those of the other time points (38% to 47% lesion) such as G60, G90, G110, L3, L10 and day 5 of postweaning (W5) (Adapted from Berchieri-Ronchi et al., 2011).*

Several humans' preclinical studies reported the efficiency of phytochemicals in modulating angiogenesis pathways. In general, they were found to modulate angiogenesis through down-regulating the secretion of angiogenic factors (VEGF, FGF, Ang, MMPs), blocking receptors phosphorylation or by inhibiting receptor mediated signaling pathways (PI3K/Akt, ERK pathways) (Rajasekar et al., 2019). **One of the objectives of this thesis project was to study the effects of some phytochemicals on the oxidative status of hyperprolific gestating and lactating sows, and their offspring, and their likely relation with the performance response of the animals.**

2.1.3 The challenge of increasing litter size for lactating hyperprolific sows

Because of the epitheliochorial nature of the porcine placenta, pig placenta conformation does not allow exchange of immunoglobulins between maternal and fetal blood. Therefore, the newborn piglet must acquire maternal immunoglobulins from adequate amount of ingested colostrum and milk to absorb passively IgG and IgM for passive immune protection until the immune system of the piglet becomes fully developed (Rooke and Bland, 2002). It has been suggested that increased in litter size also poses a serious challenge to the immunity of the sow and piglets, due to large litters imply longer duration of farrowing. Due to IgG content in the colostrum declines rapidly after the start of parturition, and sows producing low colostrum yield tended to have a longer birth interval during the early process of parturition, thus, indirectly this can affect the sow's passive immunity output (Oliviero et al., 2019).

In addition, with the use of hyperprolific sows, there is a tendency to see a greater number of piglets born than the available teats at the sow's udder. In these high-competitive conditions, underprivileged piglets, with LBW or with signs of IUGR, should have additional support in order to get access to adequate amount of high- quality colostrum (Oliviero et al., 2019). It has been also described that piglets' intake of colostrum and milk are dependent on the production of the sow, which is a good marker for the maternal quality of the sow (Le Dividich et al., 2017). Nevertheless, milk macronutrient components do not significantly increase with enhanced reproductive performance in sows as depicted in Figure 2.8.Current concentrations of protein, fat and lactose in colostrum are similar to those of 30 years ago (Li et al., 2018).

According to Vadmand et al., (2015) litter size is independent related with both, sows colostrum yield and milk yield in lactation weeks 1 to 4. In addition, milk protein

concentration was negatively correlated with milk yield in all 4 weeks, which indicated that high yielding sows were unable to maintain milk protein synthesis during lactation. It was also demonstrated that litter size does not regulate total sow milk yield using 'weigh–suckle–weigh' estimates of milk yield (Devillers et al., 2007). This indicates that nutrient intake of piglets from large litters can be restricted, as they receive less milk with similar quality. In fact, litter size turned out to be a determining factor for sow productivity in the colostrum period, at onset of lactation, and throughout lactation (Vadmand et al., 2015). Consequently, litter size should always be considered when feeding lactating sows.

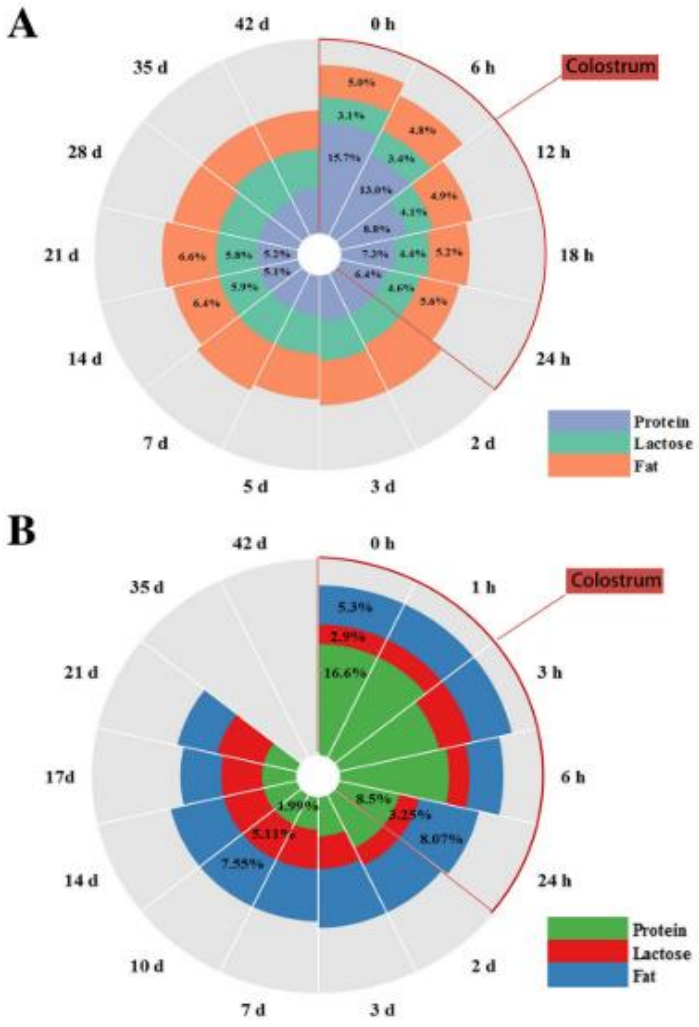


Figure 2.8. *Composition of sow milk throughout lactation.* ***A:*** *Porcine milk composition in 1980s.* ***B:*** *Porcine milk composition in 2010s (Adapted from Li et al., 2018).*

As the only nutritional source for newborn piglets, porcine colostrum and milk contain critical nutritional and immunological components including carbohydrates, lipids, and proteins (immunoglobulins). However, porcine milk composition is more

complex than these three components. Recently, have been identified additional and novel components of sow colostrum and milk, including exosomes, oligosaccharides, and bacteria, which possibly act as biological signals and modulate the intestinal environment and immune status in piglets and later in life (Li et al., 2018). In that regard, Hurley, (2015) mentioned that composition of sow colostrum and milk including concentrations of protein, fat, vitamins and minerals are affected by diet. For instance, increasing the feed allowances for sows in mid-pregnancy could be favorable to improve chemical composition and fatty acid profile of colostrum and milk (Wiecek et al., 2018). Moreover, influences of PFAs on lactating process in swine have been also documented. In this sense, the supplementation of lactating sows' diet with 0.5% of powder star anise (*Illicium verum* Hook f) improved the ADFI and milk yield of sows, as well as increased the IGF-1 in milk and prolactin in serum of sows (Wang et al., 2015).

Modern lactating sows are very productive and show a reduced feed intake capacity as a result of the genetic selection for higher feed efficiency. Therefore, hyperprolific lactating sows may suffer from excessive BW mobilization which leads to intense catabolism of the body tissues of sows, negatively affecting their milk production, and the litter weight gain, and subsequent weaning to estrus interval, but also possibly number of piglets born in the next litter (De Bettio et al., 2015). These means a major challenge for the physiology of the lactating hyperprolific sow, but also for her offspring. Therefore, improvement of nutrient transfer and synthesis mechanisms of nutritional and non-nutritional components in porcine colostrum and milk is critical to ensuring the survival, growth, and health of piglets. As shown, nutritional interventions including supplementation PFAs around prenatal and lactation period can be valuable tools that should be considered. **Present thesis project will also focus the influence of the dietary supplementation of gestating and lactating hyperprolific sows with PFAs on pre- and postnatal (placental fluid and milk) maternal transfer of these PFAs, but also changes in the nutritional profiles of colostrum and milk, and bacteriostatic activity in sows' milk.**

2.1.4 The impact of large litter size on neonatal programming of postnatal growth and gut health of piglets

The high incidence of LBW (<1.0 kg) piglets in the litter of hyperprolific sows (Matheson et al., 2018) represent a considerable economic problem to pig producers as a result of increased morbidity and mortality. This is illustrated in Figure 2.9, which shows

that both individual piglet birthweight and litter birthweight variation affect piglet mortality during lactation. In this review section, the biological consequences of large litter size in piglets can be divided into outcomes that are causally related to a crowded gestation environment and outcomes that are related to experiencing post-natal life in a large litter.

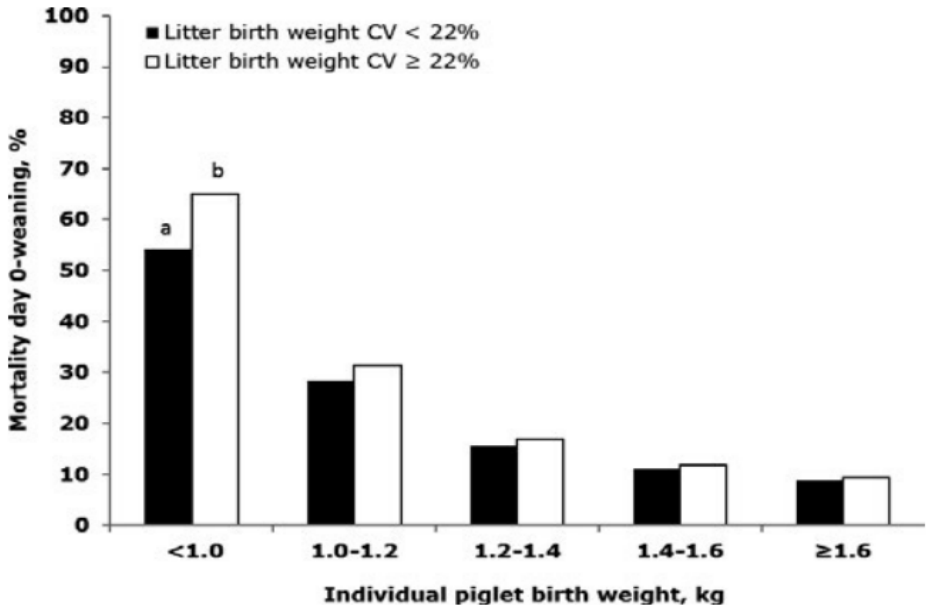


Figure 2.9. *Mortality of live born piglets during lactation for piglets of different birthweight classes (in % of number of live born piglets) and for litters with low (<22%) or high (>22%) birthweight CV (based on live born piglets in the litter).* [ab]*bars with different superscripts differ p < 0.10 (corrected for the random effect of birth litter) (Adapted from Wientjes, 2013).*

The first point at which selection for litter size could be expected to affect piglet biology is in the uterus because of variation in birthweight due to compromised fetal-placental units as a result of uterine crowding (Kemp et al., 2018). The IUGR fetuses prioritize brain and heart development over other organs, such as the liver, GIT, and the development of muscle fiber (Rehfeldt and Kuhn, 2006). Among the late consequences of these effects, LBW piglets have been described to develop enteric health problems, poorer feed efficiency as well as carcass yield and quality with increased adiposity; (L. Zhang et al., 2018). It has been described that IUGR affects intestinal growth and morphology in association with altered gene expression of growth-related proteins (D'Inca et al., 2011). In fact, the microstructure of stomach and intestine is significantly changed in IUGR piglets with hyperplasia around the gastric pits and decreased intestinal microvillus (T. Wang et al., 2005). Therefore, efficiency of nutrient utilization is reduced

in neonates with IUGR compared with those with a normal birth weight (NBW) (Wang et al., 2010).

For instance, Wang et al. (2010) described interesting evidence for an alteration of the proteome in the small intestine of IUGR offspring (d 1 to d 21). Their findings reveal that the villus height (VH) and width of jejunum at birth were lower in IUGR than in NBW piglets; and these difference in jejunal VH between IUGR and NBW piglets was even more pronounced at d 21. In addition, IUGR piglets showed increased levels of proteins related to oxidative stress and apoptosis, as well as decreased abundance of proteins required for digestion, absorption and metabolism of nutrients (including glucose, lipids, amino acids, vitamins and minerals). Ayuso et al. (2021) also described a delayed development of intestinal villi and crypts, as well as transcriptomic alterations as lower expression of genes involved in nutrient digestion (ANPEP and SI) and barrier function (OCLN and CLDN4) in LBW than NBW piglets. Collectively, these changes may be the major mechanisms responsible for intestinal growth impaired, atrophy and dysfunction, which may contribute to both the short- and long- term failure of LBW neonates. In that regard, it has been also described that phytochemicals including capsicum and turmeric oleoresin, and garlic botanical can regulated the intestinal genes expression including nutrient transport and immune responses, perhaps providing benefits by enhancing the gut mucosa health and stimulating the immune system in young pigs (Liu et al., 2014).

Since maternal nutrition may have transgenerational impacts on organ structure and pre- and postnatal growth and development (Ji et al., 2017), in this thesis project **we propose to explore that supplementation of PFAs to the diets of gestating and lactating hyperprolific sows and piglets may affect the gut functions and morphology of the neonate, suckling, and weaned piglets.**

2.2 Nutritional interventions to overcome the post-weaning challenge in piglets

Weaning also causes morphological and functional changes and dysfunctions of the small intestine of pigs (Zheng et al., 2021). Weaning under modern-day commercial conditions inflicts stress (i.e., environmental, nutritional, psychological, and social) on pigs and is associated with marked changes in GIT physiology, microbiology and immunology (Lallès et al., 2007). Consequently, the period following weaning is generally characterized by increased intestinal permeability, epithelial barrier function

and morphology disruption, and upregulation of proinflammatory cytokines indicating a robust activation of the GIT immune system (Figure 2.10) (Hu et al., 2013). These changes are associated to sub-optimal growth (e.g., low feed intake, body weight loss) (Pluske, 2013), deteriorated feed efficiency, and a high incidence of intestinal disturbances with diarrhea (of bacterial and (or) dietary origin) often occurring that, in turn, can cause morbidity and/or mortality (Heo et al., 2013).

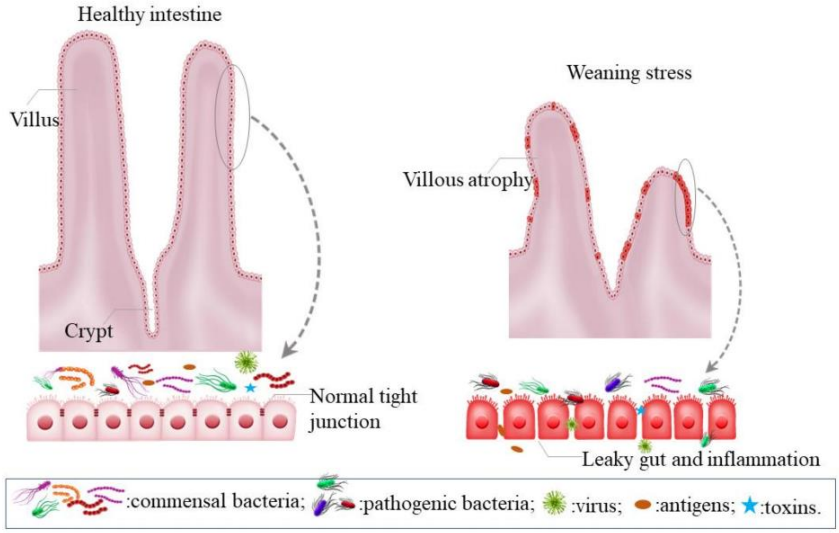


Figure 2.10. *A generalized overview of the impacts of weaning stressors on the developmental trajectory of gastrointestinal tract in pigs.*

2.2.1 Improving food palatability and sensory maternal learning on innate preference in weanling piglets

Common anorexia and undernutrition at post-weaning has been related with adverse feeding behaviors i.e.; with feed neophobia being an important factor for feed refusal that persist until piglets are completely familiarized with their novel feed and environmental conditions (Roura et al., 2008). Improving the palatability and, thus, innate preference by familiarity for a certain type of food or specific flavor compounds and their use as feed additive may be helpful strategy to stimuli shorth-term feed appetence and the initiation of feed consumption (Dong and Pluske, 2007). In that regard, PFAs, for instance, *Stevia rebaudiana, Citrus sinensis,* and extracts of hot-flavored spices (Clouard et al., 2012), as well as cinnamon and star anise (Clouard and Val-Laillet, 2014) are reported to act as sensory feed additives able to influence feed preference, intake and weight gain in pigs. However, among many potential sensory feed additives, the palatability of the ingredient and the optimum supplemental level are important, and these should be considered when applying into nursery pig diets.

Moreover, flavor exposure through the maternal diet that appear in the amniotic fluid and mother's milk (sensory maternal learning) is known to reduce neophobia and modulate preference for similarly flavored food types around weaning in many species (Oostindjer et al., 2011). In swine, flavors of PFAs such as anethol, cinnamaldehyde, eugenol (Blavi et al., 2016b) limonene, menthol, and carvone (Val-Laillet et al., 2018) provided to gestating and lactating sows increased the progeny's feed intake and growth, suggesting nutritional programming and/or sensory conditioning during the perinatal period. However, yet still little is known about the mechanism underlying perinatal flavor learning. Thus, this thesis project will focus the likely effects of PFAs on innate feed preference of weanling piglets by supplementation of prestarter diets and sensorial maternal learning.

2.3 Phytogenic feed additives in animal nutrition: special emphasis in swine

As above mentioned, phytogenic feed additives (PFAs) may be defined as standardized, science-based combinations of plant-derived bioactive compounds (phytochemicals) that promote livestock health and well-being and growth and production efficiency. Basically, PFAs comprise a wide range of phytochemicals from many different groups of natural bioactive substances, e.g., flavonoids, tannins, saponins, mucilages, alkaloids, phenolics, and essential oils (EOs) obtained from plant material (flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits, and roots) (Figure 2.11), which are categorized as plant secondary metabolites based on their enzymology that are often specific to a family or genus and confer defense mechanism or functional properties (Jones and Faas, 2020).

In PFAs, phytochemicals can be used in solid, dried, and ground form or as extracts (crude or concentrated), but also can be classified as EOs (volatile and aromatic oily liquids containing complex mixtures of low-boiling-phenylpropenes and terpenes obtained by cold extraction or steam/alcohol distillation) and oleoresins (extracts derived by non-aqueous solvents) depending on the process used to derive the active ingredients. In fact, EOs, represent a major group of PFAs (Gadde et al., 2017). Major components of EOs are characterized by two or three components at fairly high concentrations (up to 85%) compared to others components present in trace amounts, which reflect the biophysical and biological features of the EOs from which they were isolated (Stevanović et al., 2018). In Table 2.1 are described the major components of a number of EOs with potential in animal nutrition.

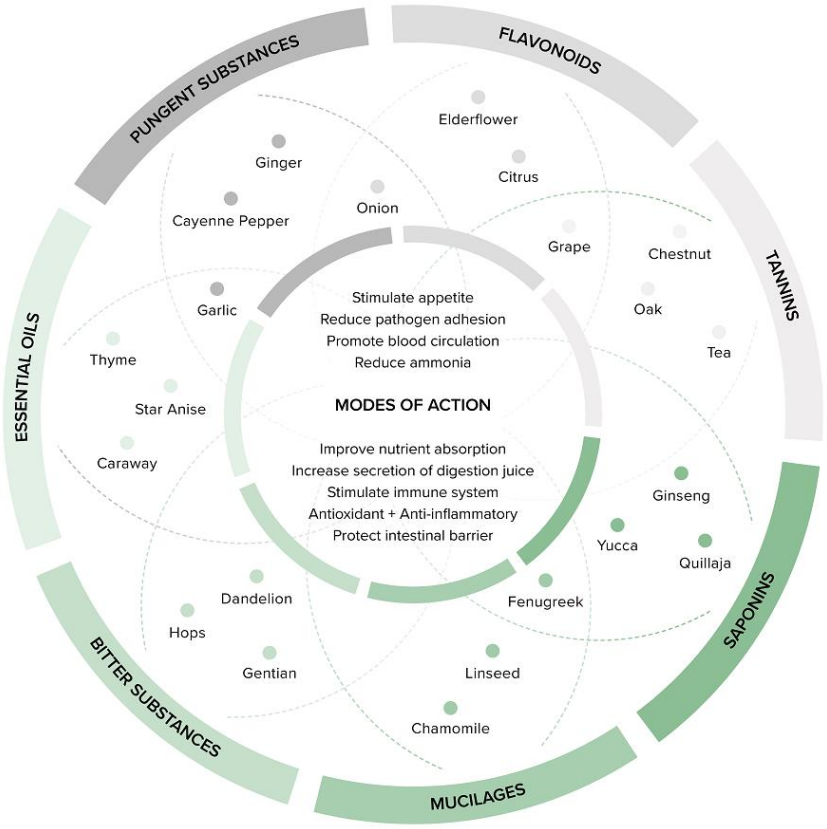


Figure 2.11. *Some phytochemical groups included in the PFAs and their modes of action on animal functions and health. Source: Delacon Biotechnik GmbH.*

The PFAs are considered as a first line alternative to in-feed antibiotics based on their complex bioactivity, mainly due to antimicrobial, antioxidant, and anti-inflammatory properties (Yang et al., 2015); (Stevanović et al., 2018). In addition, literature have described biological activities of PFAs on feed palatability and GIT functions, influence on digestion, feed efficiency and growth-promoting efficacy in swine and poultry (Windisch et al., 2008); (Brenes and Roura, 2010), but also immunomodulation, and ruminal function (Durmic and Blache, 2012). In this context, in Table 2.2 is summarized some effects of plant bioactive compounds on selected animal functions and health. However, the mechanisms underlying functions and the fully potential of PFAs in the swine industry are still largely unclear. Some of the main suggested effects of PFAs are summarized follow.

Table 2.1. *Some essential oils and their major components (Adapted from Brenes and Roura, 2010).*

Essential oil	Main components	Total (%)	Essential oil	Main components	Total (%)
Angelica root	α-Pinene	24.7	Mandarine	Limonene	79.5
	δ-3-carene	10.5		γ-Terpinene	9.7
	α-Phellandrene +myrcene	10.8	Nutmeg	α-Pinene	26.0
	Limonene	12.9		β-Pinene	15.0
	β-Phellandrene	10.4		Sabinene	27.1
	p-Cymene	7.7		Myristicin	5.9
Bergamot	β-Pinene	7.7	Orange	α-Pinene	9.0
	Limonene + β-phellandrene	39.4		β-Pinene	10.4
	γ-Terpinene	8.6		Sabinene	19.4
	Linalool	11.1		δ-3-carene	5.4
	Lynalil acetate	28.0		Limonene	17.5
Cynnamon bark	(E)-Cinnamaldehide	77.1		β-Caryophyllene	14.7
	Eugenol	7.2	Pine (Scotch)	α-Pinene	9.0
Coryander	p-Cymene	6.1		β-Pinene	10.4
	Linalool	72.0		δ-3-carene	21.6
Dill (Indian)	Limonene	50.9		Myrcene + α-terpin	5.8
	Trans-Dihydrocarvone	10.4	Rosemary	α-Pinene	7.4
	Carvone	20.3		β-Pinene	5.0

	Dillapiole	36.3		1,8-Cineole	43.6
Eucalyptus	Citronellal	72.8		Camphor	12.3
	Citronellol	14.5	Rosewood	Linalool	80.0
Geranium	Isomenthone	6.4	Sage	1,8-Cineole	8.4
	Citronellol	42.0		α-Thujone	31.8
	Geraniol	5.5		β-Thujone	33.2
	Cytronellyl formate	14.2	Savory	γ-Terpinene	7.4
Ginger	Camphene	14.1		p-Cymene	25.9
	Neral	4.9		Carvacrol	37.7
	Geranial + bornyl acetate	8.1	Tarragon	(Z)-β-Ocimene	7.3
	β-Bisabolene	22.1		(E)-β-Ocimene	6.9
	Ar-Curcumene	14.5		Methyl chavicol	77.6
	B-Eudesmol	5.4			
Juniper berry	α-Pinene	33.7			
	Sabinene	27.6			
	Myrcene	5.5			
Lime	Geranial	6.0			
	Limonene	55.5			
	α-Pinene	11.0			
	γ-Terpinene	14.5			

Table 2.2. *Summary of effects of bioactive plants on biologically functions or systems which have consequences on the health and welfare of animals (Adapted from Durmic and Blache, 2012).*

Organ system or function	**Positive**	**Negative**
Digestive function		
Food consumption	Appetizer	Aversion
	Increase intake	Reduce intake
	Self-medication	
Stomach	Prevent gastric damage	Gastroenteritis
Intestine	Improve digestion	Reduce activity of enzymes
	Improve feed efficiency	Reduce feed efficiency
	Maintain microflora balance	Impair nutrient utilization
	Inhibit/destroy gut pathogens	Alter anatomy and physiology of the gut wall
	Reduce shedding of foodborne bacteria	Reduce viscosity of intestinal content
	Reduce of gastro-intestinal spasms or motility	
	Prevent/cure diarrhea and constipation	
Rumen	Improve microbial activity	Reduce microbial activity
	Reduce methane	Reduce fiber utilization
	Improve fiber digestion	Reduce VFA
	Increase VFA	Ruminal atony
	Increase propionate	

	Protein binding	
	Reduce proteolysis, aminolysis	
	Increase microbial protein synthesis	
	Control rumen disorders	
	Reduce biohydrogenation	
Blood parameters	Maintain blood parameters	Alter blood parameter
	Decrease blood parameters	Increase blood parameters
	Short term increase in blood insulin	Reduce blood insulin
	Antihyperlipidemic	Reduced/increased hematocrit
	Hypocholesterolemia	Hemolysis
	Anticoagulants	Anemia
Immune function		
Overall	Anti-inflammatory	Inflammation
	Enhanced humoral and cellular immunity	Reduce cell count and activity
	Enhanced innate and specific immunity	Reduce Ig
	Modulation of specific receptors, enzymes, and immune molecules	
	Increase in immune cell count and activity	
	Increase Ig	

2.3.1 Impact of PFAs on antimicrobial and immune responses

The primary mode of action of PFAs is the modulation of beneficial intestinal microbiota by controlling potential pathogens. They act as antimicrobials by disintegration of cell membrane, leakage of critical intracellular materials including ions and eventually cell death. In addition, the active components in phytochemicals disturb the enzyme system of bacteria and block the development of virulence structures in bacteria, such as flagella, which critical for bacterial adhesion (Lillehoj et al., 2018). Figure 2.12 describes some potential mechanisms of action of the EOs and/or their components and shows the potential cell targets of their antimicrobial activity. However, each of these actions cannot be considered separate events but instead may be a consequence of the other activities. In that regard, EOs including linalool, carvacrol, eugenol, thymol, p-Cymene, 1,8-cineole, α-Pinene, β-Pinene, etc., exhibit a wide spectrum of antimicrobial activities against Gram-negative (G^-) and Gram-positive (G^+) bacteria (Burt, 2004). In addition, their antimicrobial action highly depends on the hydroxyl group of the phenolic terpenoids and the presence of delocalized electrons which often determine the level of their antimicrobial activity on different bacteria (Ultee et al., 2002).

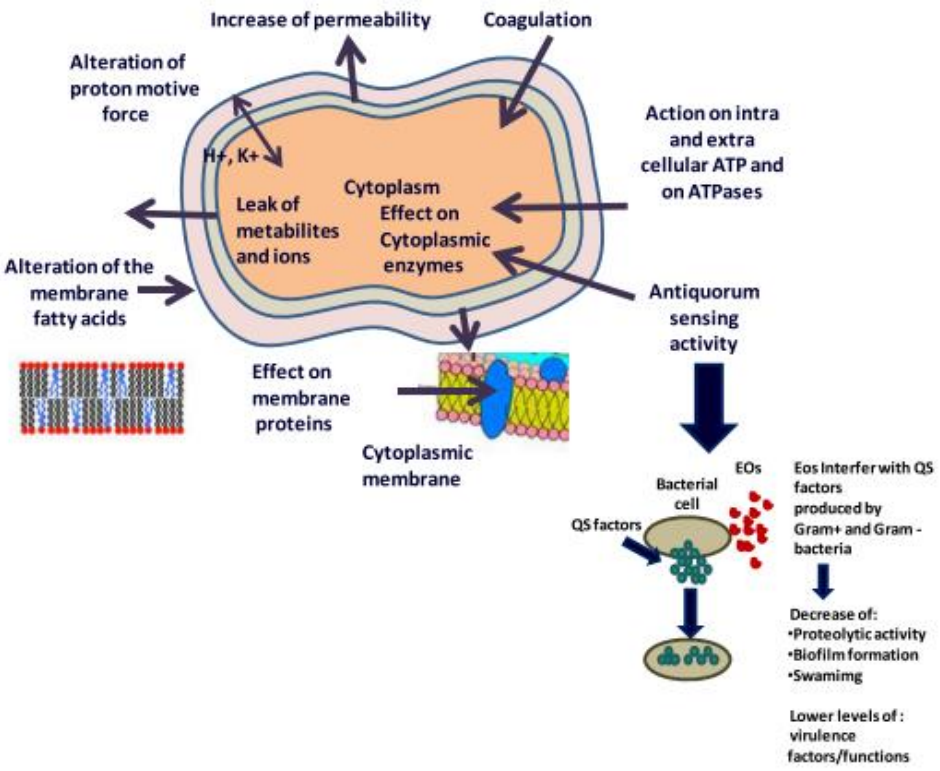


Figure 2.12. *Mechanism of action and target sites of the phytochemicals such as essential oils on microbial cells (Adapted from Nazzaro et al., 2013).*

For instance, although carvacrol and thymol have several target sites in bacterial cells, the biosynthetic machinery of bacterial cell walls is their main target site (Yap et al., 2014). First, carvacrol and thymol can sensitize the cell walls (including membranes) and cause significant membrane damages, leading to integrity collapse of the bacterial cytoplasmic membrane, leakage of vital intracellular contents and eventually death of the bacterial cells. The leakage often happens through cell wall damage, cytoplasmic membrane damage, cytoplasm coagulation and membrane protein destruction as well as reduction of proton motive force (Nazzaro et al., 2013). Secondly, with their lipophilic structure, carvacrol and thymol can easily get into the bacterial membranes among the fatty acid chains and cause the membranes to expand and become more fluid (Lambert et al., 2001).

Studies in swine, clearly demonstrated the effects of PFAs on immune responses and gut integrity. For instance, Xi et al. (2017) indicated that dietary supplementation of 200 mg/kg of Ginseng polysaccharides at late gestation and during lactation, improved immunity-related bio-molecular levels in sow serum and milk (increased the GSH-px, and the IgG, IL-2, IL-6, TNF-α, IFN-γ), while IL-2 and TNF-α in piglets. Li et al. (2012) described that supplementation of weaning pigs with 18 mg/kg of thymol and cinnamaldehyde EOs decreased the diarrhea incidence rate, while improved the immune status (IGF-I), and the numbers of *E. coli* in the cecum, colon and rectum were reduced, as well as the villus height to crypt depth ratio (V:C) in the jejunum were improved. Recently, Dong et al. (2019) described that weaned pigs supplemented with dosages of 50 to 150 mg of Tea tree oil, showed an improved in the jejunum morphology (greater VH) and the ratio V:C of all small intestine segments; likewise, increased the gene expression of intestinal mucosal immunity.

Furthermore, the supplementation with 0.025% of a blend containing cinnamaldehyde (4.5%) and thymol (13.5%) EOs increased the immune status (IgA, IgG, and albumin), intestinal VH, and apparent total tract nutrient digestibility (ATTD), as well as decreased the *E. coli* and total anaerobes in weaned piglets (Zeng et al., 2015). Ahmed et al. (2013) described that resveratrol and EOs of oregano, anise, orange peel, and chicory decreased bacterial counts and pathogen loads via an antibacterial effect, and developed antioxidant and anti-inflammatory activities in the intestinal lumen. Recently, Chen et al. (2019) documented that supplementation of weaned pigs with 12.5 g/kg of Macleaya cordata extract, a medicinal plant rich in isoquinoline alkaloids, increased

immune (IgG), as well as total antioxidant capacity (TAC), and GSH-PX, and SOD serum levels. In addition, decreased the fecal *Salmonella spp.* and diarrhea, whereas increase fecal *Lactobacillus spp.* and improved the intestinal histomorphology (V:C).

2.3.2 Effect of PFAs on oxidative stress and intestinal inflammation

In swine, the oxidative stress might be associated with a drop in performance, compromised immunity, muscle degeneration, increased risk of stroke in fast growing pigs, mulberry heart disease, reduced appetite, diarrhea, destruction of liver tissue, and increased risk of abortion of gestating sows (Omonijo et al., 2018). The antioxidant system, which is able to prevent oxidative stress through reducing the formation and/or scavenging of oxidants, is comprised of three components: (i) antioxidant enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GSH-PX) and catalase (CAT); (ii) low molecular mass antioxidants, such as tocopherols (vitamin E), ascorbic acid (vitamin C), carotenoids including b-carotene, uric acid, glutathione, and polyphenols; and (iii) proteins that are able to sequestrate free transition metals (Halliwell, 1996).

Some common non-phenolic components of EOs and plant extracts such as limonene, linalool and citral are well known by their anti-oxidative properties (Baschieri et al., 2017). In addition, Zou et al. (2016) investigated the antioxidative effects of oregano EO in pig small intestinal epithelial cells (IPEC-J2) and demonstrated that ROS and malondialdehyde induced by H_2O_2 were dramatically suppressed by inducing nuclear factor-erythroid 2-related factor-2 (Nrf2) and several antioxidant enzymes (SOD1 and g-glutamylcysteine ligase). On the other hand, Fan et al. (2015) supplemented 200 mg/kg of Catechins, a type of natural phenol and antioxidant during early gestation, describing an enhanced of serum SOD and CAT levels in sows at farrowing, with and increment in the number of piglets born alive and piglets born healthy, as well as a decrease in stillborn. Moreover, Meng et al. (2018) reported that supplementation of 300 mg/kg of resveratrol in gestating and lactating sows improved the activities of SOD and CAT in the milk and plasma of sows and piglets. Moreover, it regulated placental antioxidant gene expression by Keap1-Nrf2 pathway and Sirt1 in placenta.

Oxidative stress is directly linked with inflammation due to the fact that the abovementioned oxidants are activators of nuclear factor kappa B (NF-$_K$B), the key regulator of inflammation (Gessner et al., 2017). After activated by several inducers, such as pro-inflammatory cytokines, ROS, and lipo-polysaccharides, NF-$_K$B is translocated from the cytoplasm to the nucleus and then induces the expression of numerous pro-

inflammatory proteins including cytokines, chemokines, adhesion molecules and enzymes that are involved in inflammation, cell apoptosis and proliferation (Barnes and Karin, 1997). It has been evidenced that there is potential cross-talks between the Nrf2 and NF-κB pathways and Nrf2 gene dysfunction could lead to increased susceptibility to inflammatory stresses (Khor et al., 2006).

The aim of inflammation is to induce immunological processes to eliminate invading pathogens and toxins and to repair damaged tissue. In fact, when immune response is initiated, macrophages are recruited in the tissues to produce an inflammatory reaction, and then T cells are also involved in the inflammation in later stages of the innate immune response (Gessner et al., 2017). Gut inflammation is associated with compromised gut growth and development and reduced efficiency of nutrient utilization. It has been reported that gut acute and chronic inflammatory diseases often lead to gut morphological changes, mucosa damage, increased mucosal permeability, compromised gut development and poor nutrient absorption capacity (Strober et al., 2002).

Several studies have demonstrated that EOs including cinnamaldehyde (Wondrak et al., 2010) and oregano oil (Zou et al., 2016) increased the expression and translocation of Nrf2 and prevented the activation of NF-κB. However, there are other examples of EOs (eucalyptus, rosemary, lavender, millefolia) along with other plants (pine, clove and myrrh) that have been used as mixed formulations as anti-inflammatory agents (Darshan and Doreswamy, 2004). In addition, polyphenols are able to block activation of NF-κB directly by inhibition of the phosphorylation of IκB and by scavenging ROS. Moreover, polyphenols are able to inhibit activation of NF-κB in an indirect manner, by activation of PPARγ which causes transrepression of NF-κB and by activating Nrf2. Nrf2, a redox-sensitive transcription factor, stimulates the transcription of antioxidative enzymes which are helpful to eliminate ROS (Figure 2.13) (Gessner et al., 2017).

Studies in swine also described the potential of PFAs to modulate metabolic inflammation. For instance, diets with 1% of polyphenols (GSGME; grape seed and grape marc extract) suppresses the activity of NF-κB by transactivation of Nrf2 in the duodenal mucosa of piglets (Gessner et al., 2013). Similarly, Fiesel et al. (2014) described that supplementation of diets with 1% of polyphenols (GSGME) improved gut morphology (V:C ratio) and, downregulated intestinal pro-inflammatory (ICAM1, IL1B, IL8 and TNF), and nutrient transports (SLC2A2 and SLC15A1) genes in piglet jejunum when compared with control group.

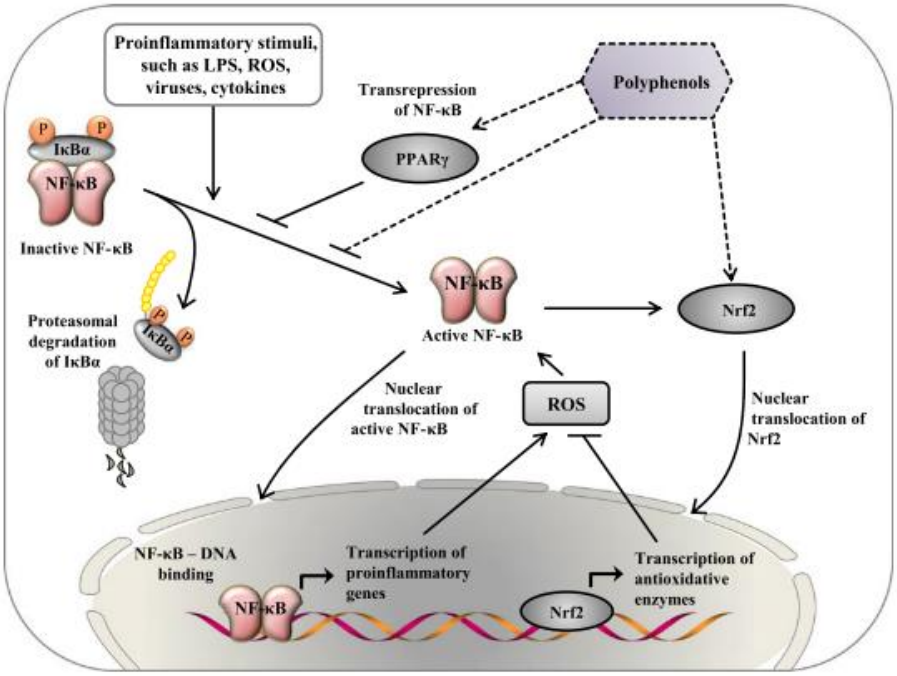


Figure 2.13. *A simplified overview of key mechanisms by which polyphenols prevent inflammation (Adapted from Gessner et al., 2017).*

2.3.3 Influence of PFAs on nutrient metabolism and digestibility and performance

The intestinal physiology (e.g., gut chemosensory system) has received a lot of attention due to the fact that the system can regulate digestion, absorption, and metabolism, with potential nutritional and pharmacological applications to improve gut growth, development, and health. Approximately 90% cells in the gut epithelium are absorptive epithelial cells and the cells express various nutrient transporters (Mace and Marshall, 2013). In fact, the intestine continuously monitors the composition of its contents to optimize assimilation of nutrients and to ward off threats to its integrity. Thus, the intestine is endowed with a range of sensory receptors that activate four major effector systems—the enteroendocrine system, the nervous system, the gut immune system, and the nonimmune defense systems of the gut (see Figure 2.14) (Furness et al., 2013).

The intestinal chemosensors transduce information regarding the nutrient profile and concentration of the lumen to regulate intestinal gene expression (e.g., transporters), digestive enzyme and gut peptide secretions, eventually to control feed intake, digestion, absorption, and metabolism. For instance, the sweet (T1R1 + T1R2), umami (T1R1 + T1R3), while bitter (T2Rs) taste receptors are coupled through the G protein-coupled receptors (GPCRs), which are characterized in the oral cavity but also in the intestine (Furness et al., 2013), and are co-expressed with enteroendocrine cells that can secret gut

hormone peptides, such as glucoinsulinotropic polypeptide, glucagon-like peptides 1 and 2 (GLP-1 and 2) and peptide YY (Mace and Marshall, 2013).

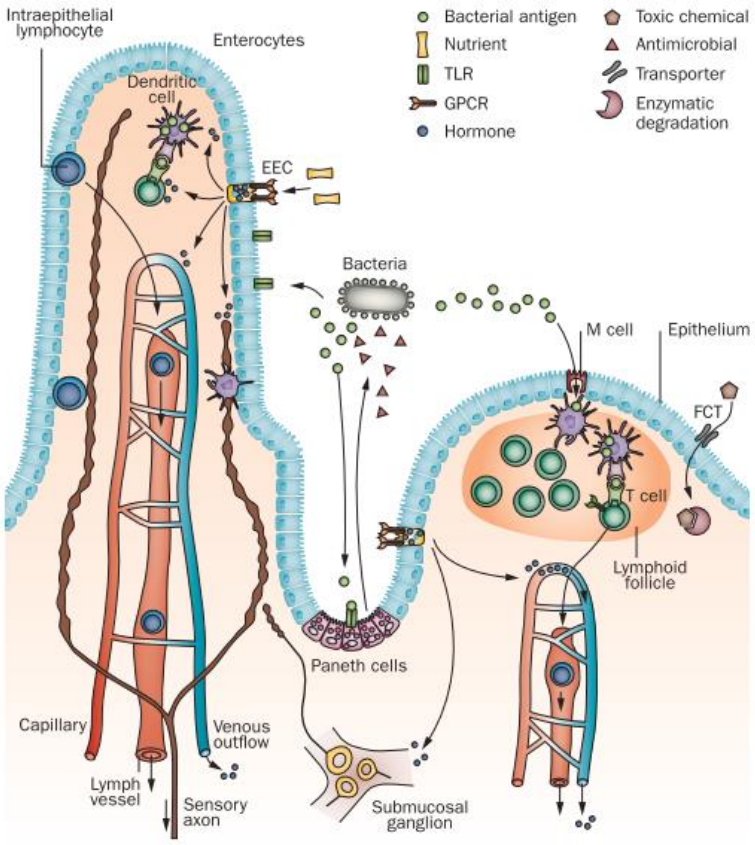


Figure 2.14. *Sensory systems at the luminal interface. Description: the intestinal lining epithelium is exposed to nutrients, which are primarily detected by receptors, most of which are GPCRs, on enteroendocrine cells. These cells release hormones that signal to the epithelium, lymphocytes, arteries, and neurons locally, and which enter the circulation to act at remote sites (Adapted from Furness et al., 2013).*

Several studies have been shown that phytochemicals can modulate the activation of the transient receptor potential (TRP) channels and the taste receptor cells located in taste buds but also in the gut (Premkumar, 2014). Moreover, in pigs, it has been described that PFAs exhibit various biological activities that are related to the functions of the GIT, such digestive secretions and nutrient absorption. For instance, Costa et al. (2013) and Maenner et al. (2011) reported the biological activities of clove, thyme, and oregano extracts, as well as menthol and cinnamon EOs, respectively, to stimulate digestive secretions (saliva, digestive enzymes, bile, and mucus) for improving nutrient digestibility. For their part, Diao et al. (2015) using benzoic acid and thymol reported an improved of gut morphology which could enhance feed efficiency, diarrhea, and microbiota profile. However, the mechanisms regulating gene expression relating to

immune and digestive functions are still not fully clear. It is very important to identify specific receptors for PFAs, which will help us to understand of the underlying mechanisms.

Finally, as above mentioned, the low feed intake immediately after weaning is responsible for morphology atrophy and reduced growth rate in piglets. Thus, it is extremely important to increase feed intake in newly-weaned pigs in order to reduce post-weaning diarrhea and to improve growth performance (Dong and Pluske, 2007). In fact, the growth-promoting features of PFAs including production parameters such as feed intake, weight gain, and feed conversion ratio (FCR) are associated mainly with effects on the GIT to: increase the feed palatability, stimulate secretion of digestive fluids, improve intestinal morphology, stabilize intestinal microbiome, and reduce inflammation (Hashemi and Davoodi, 2011). Therefore, based on the assumption that a preferred feed is consumed in greater quantity, sensory additives including PFAs may turn out to be a useful method to improve the feedstuffs palatability, and, therefore, feed intake and growth performance in pigs (Clouard et al., 2012); (Michiels et al., 2012).

In that regard, PFAs were mainly classified as sensory additives (flavors, colorants, etc., affecting the sensoric properties of feed quality and animal products); however, due to the potential effects of PFAs on animal health and performance they have been also included in some cases among zootechnical additives (anti-oxidants, immunomodulators, digestive stimulants, growth promoters of non-microbial origin, substances increasing performance or quality of animal products, etc.) (European Union, 2016). In fact, Table 2.3 presents an overview of the effects of PFAs on the performance and several physiological responses in sows and piglets, i.e., gestation and lactation, and their litters with connecting effects on weaned pigs. Thus, taking together PFAs effects, in present thesis project **we also propose that dietary supplementation of PFAs may influence physiological and health status, but also productive performance of sows and piglets**.

Table 2.3. *Some effects of phytogenic feed additives (PFAs) on productive performance and physiological responses of sows and piglets.*

Animals	Phytogenic compounds	Dosage - mode	Effects respect to control		References
			Performance	**Other responses**	
Sows (G) + (L)	Oregano (0.7-2.5% thymol and carvacrol 80-82.5%)	250 mg/kg - EO	↑energy intake ≈ increase ADG and milk intake in piglets on d 1 to 5	↑T lymphocytes in milk at d 14 ↓ milk fat at d 7	Ariza-Nieto et al. (2011)
Sows (late G) + (L)	Carvacrol 5.4% Cinnamaldehyde 3.2% Capsicum oleoresin 2.2%	100 mg/kg - extract	↓ BF loss in lactation ↑ newborn BW ↑weaning piglets BW ↓pre-weaning mortality	↑milk lactose	Matysiak et al. (2012)
Sows (late G) + (L)	Ginger	0.5% - extract	↑newborn BW	↑antioxidants, IgG and phenolic compounds in plasma of sows and piglets ↑IgG and protein in colostrum	Lee et al. (2013)
Sows (G) + (L)	Oregano (5% *Origanum vulgare subsp. hirtum*)	15 mg/kg - EO	≈ increase ADFI of sows at third week of lactation ↑ suckling piglets ADG	↓ serum 8-OHdG and TBARS of lactating sows at d 1 ↑ fecal *lactobacillus* ↓ fecal *E. coli*	Tan et al. (2015)
Sows (L)	Star anise (*Illicium verum* Hook f)	0.5% - powder	↑ ADFI and milk yield of lactating sows ↑ piglets ADG	↑ IGF-1 in milk and prolactin in serum of sows	Wang et al. (2015)
Sows (late G) + (L)	Fenugreek (*Trigonella foenum-graecum L.*)	0.2% - extract	↑ADG in suckling piglets from week 2 to weaning	↑ IgG in plasma of piglets and sows at weaning ↑ ATTD of N and gross energy in sows	Hossain et al. (2015)

				↓ fecal *E. coli* and ammonia in sows at weaning	
Sows (early G)	Flavonoid (Catechins)	200 mg/kg -	↑ litter born alive ↑ piglets born healthy ↓ stillborn	↑ sow serum SOD and CAT at farrowing ↓ serum MDA and H_2O_2 of sow at farrowing	Fan et al. (2015)
Sows (late G) + (L)	>25% Anethole and cinnamaldehyde and > 10% eugenol	375 mg/kg	↑ creep feed intake ↑ piglet consumption and growth after weaning	→ transfer of anethole, cinnamaldehyde, and eugenol into amniotic fluid and milk	Blavi et al. (2016b)
Sows (late G) + (L)	Ginseng polysaccharides	200 mg/kg -	↑weaning piglets BW	↑ IgG in milk and serum of sows ↑ IL-2, IL-6, TNF-α, IFN-γ in milk and serum of sows, while IL-2 and TNF-α in piglets ↑ GSH-PX in colostrum-milk	Xi et al. (2017)
Sows (G) + (L)	Resveratrol	300 mg/kg -	↑weaning piglets BW	↑ antioxidant profile in milk, plasma and plasma of sows and piglets → regulates antioxidant gene expression in placenta by modulation of Keap1-Nrf2 pathway and Sirt1	Meng et al. (2018)
Sows (late G) + (L)	Limonene, cinnamaldehyde, menthol, carvone and anethole	0.1% -	↑ piglets BW, ADG and ADFI	→ transfer of limonene, carvone and anethole into colostrum/milk → modified colostrum/milk sensory profile	Val-laillet et al. (2018)
Suckling piglets	*Taraxaci mongolia, Viola yedoensis*	60 mg/mL (milk replacer) –	↑ ADG	→ ameliorate intestinal lesion	Kim et al. (2015)

(challenged with virulent PEDV)	*Makino, Rhizoma coptidis* and *Radix isatidis* (1:1:1:2)	powdered extracts		↑ villus height ↓ crypt depth	
Weaned piglets	Allicin 25%	0.10g/kg to 0.25g7kg - oil	↑ ADG linearly ↓ FCR linearly	↓ incidence of diarrhea ↓ attractiveness of faces to flies	Huang et al. (2011)
Weaned piglets	Thymol and cinnamaldehyde 18%	18mg/kg - EO	↑ ADG	↑ DM and CP digestibility ↑ immune status ↑ intestine ecology ↓ diarrhea	Li et al. (2012)
Weaned piglets	*Stevia rebaudiana* 10 to 20% + high-saponin 5 to 10% (1) *Citrus sinensis* 60 to 80% (2) Hot-flavored spices 5 to 15% (3)	0.4% (1) 0.0031% (2) 0.0405% (3)	-	↑ palatability at short-term during feed transition (2) ↑ palatability at long-term (1) and (3)	Clouard and Val-Laillet. (2014)
Weaned piglets (challenged with *E. coli*)	Capsicum 6% or Garlic 40%	10 ppm/kg – oleoresin/extract	-	↑ gene expression related to gut mucosa health ↑ clinical immune response	Liu et al. (2014)
Weaned piglets	Grape seed and grape marc meal 8.5%	1.0% - extract	↑ FCR	↑ intestinal V:C ratio ↑ ATTD ↑ fecal pH ↓ VFA and *Streptococcus spp.* and *Clostridium C. XIVa* ↓intestinal pro-inflammatory *ICAM1, IL1B, IL8* and *TNF*, as well as nutrient transport *SLC2A2, SLC2A5,* and *SLC15A1* genes	Fiesel et al. (2014)

Weaned piglets (challenged with *E. coli* K88)	Clove 5% Cinnamon 3% Fenugreek 16%	0.05% - powder	↑ ADG	↑ ATTD of ash and phosphorus	Devi et al. (2015)
Weaned piglets	Cinnamaldehyde 4.5% and thymol 13.5%	0.025% - EO	↑ ADG	↑ ATTD of DM, CP and energy ↑ villus height ↓ *E. coli* and total anaerobes ↑ IgA, IgG and albumin ↑ T-AOAC	Zhikai Zeng et al. (2015)
Weaned piglets	Macleaya cordata 1.5%	12.5 g/kg - extract	↑ ADG and ADFI ↓ FCR	↑ serum IgG ↑ T-AOAC, GSH-PX, SOD ↑ *Lactobacillus spp.* ↓ *Salmonella spp.* and diarrhea ↑ intestinal histomorphology	Chen et al. (2019)
Weaned piglets	Tea tree 20%	50mg to 150mg - oil	↑ ADFI ≈ increase ADG	↑ small intestine morphology ↑ gene expression related to intestinal mucosal immunity	Dong et al. (2019)
Weaned piglets	Cinnamaldehyde 15% and thymol 5% + organic acids (OA)	1 kg/t – EO + OA	↑ final BW and ADG	↑ fecal concentration of isovaleric acid → modulated microflora	Yang et al. (2019)
Weaned piglets	Carvacrol 2.2% and thymol 1.1%	1 g/kg - EO	↑ ADG	↓ fecal ammonia emission, blood urea nitrogen and diarrhea	Tan et al. (2020)

CHAPTER 3

Hypothesis and objectives

Present PhD dissertation is part of the project SNiBA 2016-30-DEL in agreement with Delacon Biotechnik GmbH focused on improving the pre- and postnatal physiological and health status of the hyperprolific sow and their offspring (Chapter 4, and 5). Meanwhile, Chapter 6 will also be focused post-weaning feed consumption in piglets. Under commercial farm conditions, a series of three studies related to dietary supplementation with phytogenic compounds on gestating and lactating hyperprolific sows, and weanling piglets were performed.

The hypotheses of this PhD dissertation are:

1) Dietary specific blends of phytogenic compounds supplemented to hyperprolific sows may influence on the oxidative status and performance of sows and offspring. The effect maybe higher if supplementation starts from mating and continues throughout gestation.
2) The BPC in the feed of gestating and lactating sows may be transferred to the offspring through pre-, peri- and post-natal fluids (amniotic fluid, colostrum, and milk).
3) Both colostrum and milk composition might be modified by dietary supplementation of sows with BPC during gestation and lactation.
4) The pre- and postnatal exposure of the offspring to BPC may affect the piglets gut health-related functions and histomorphology.
5) Prestarter diets supplemented with phytogenic compounds may improve feed palatability and, therefore, promote feed preference of weanling diets by familiarity.
6) Sensory maternal learning to BPC might influence feed intake and growth of newly-weaned piglets.

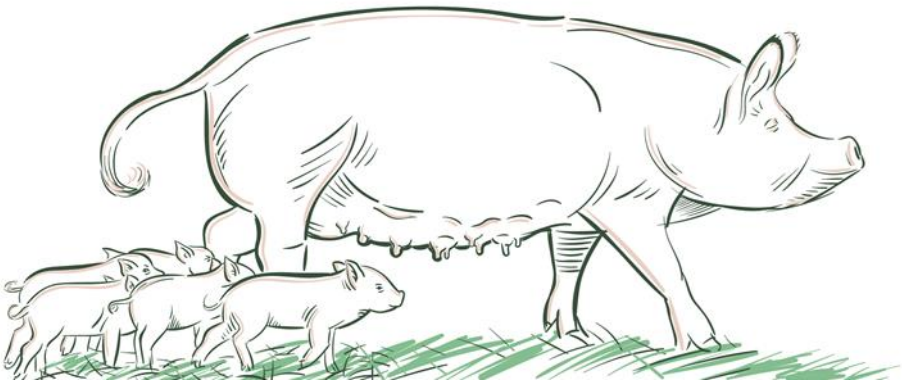
The main objective of present thesis project is, therefore, to **investigate the pre- and postnatal dietary supplementation of hyperprolific sows and piglets with phytogenic compounds as strategies to improve physiology, health, and performance during the gestation, lactation, and weanling period**. Thus, the specific objectives are:

1) To determinate the effects of the BPC1 supplementation to sow diets during gestation and lactation, or lactation only, on 1) the sow's reproductive performance, and oxidative status of sows and offspring; 2) the maternal transfer of BPC into placental fluid and milk, and colostrum-milk composition; and 3) the

piglet performance and jejunal gene expression-related intestinal functions (**Chapter 4 and 5**).

2) To explore whether the BPC2 supplemented to the diets of gestating hyperprolific sows affects the jejunal histological responses and gene expression of jejunal health-related functions in neonate piglets (**Chapter 5**).
3) To investigate by using a double-choice feeding test (DCHT) the effects of prestarter diets supplemented with D-limonene, *trans*-anethole, or eucalyptol versus unsupplemented standard diet on innate feed preference in one-week post-weaning piglets (**Chapter 6**).
4) To study the pre- and postnatal transfer (presence or absence) of a blend of botanical compounds (BPC) containing abovementioned compounds from feed diets into the placental fluid and milk of sows and their influence on weanling piglet growth performance (**Chapter 4 and 6**).

To assess these four objectives, 3 different trials were performed. Results were included in Chapters 4 to 6.



CHAPTER 4

Phytogenic actives supplemented in hyperprolific sows: effects on maternal transfer of phytogenic compounds, colostrum and milk features, performance and antioxidant status of sows and their offspring, and piglet intestinal gene expression

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Phytogenic actives supplemented in hyperprolific sows: effects on maternal transfer of phytogenic compounds, colostrum and milk features, performance and antioxidant status of sows and their offspring, and piglet intestinal gene expression

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Abstract

Phytogenic actives (PA) are plant-derived natural bioactive compounds that may promote livestock health and well-being, as well as improve growth performance and production efficiency. The current study aims to evaluate their effects on sows and their offspring. Eighty-one hyperprolific sows (up to parity 7) were assigned to 3 experimental treatments. Control sows were offered a nonsupplemented diet during gestation and lactation, and treated sows were fed the control diet supplemented with 1 g/kg of a blend of PA (BPA) in lactation (L) or during gestation and lactation (GL). An evaluation was made of placental and milk maternal transfer of these BPA and colostrum-milk features, sows and piglets antioxidant status, reproductive performance (litter size), body weight (BW) changes, weaning-estrus interval, and litter performance. Finally, piglet´s jejunum gene expression was measured. The BPA supplementation during gestation (GL) increased the number of piglets born alive ($P = 0.020$) and reduced ($P < 0.05$) the newborn piglets BW, while there were no differences among treatments on the suckling (day 20) and weaned (day 7) piglets BW ($P > 0.05$). Dietary phytogenic volatile compounds reached GL placental fluid, and milk of L and GL sows ($P < 0.05$). Moreover, colostrum protein in GL and milk fat content in L and GL were increased ($P < 0.05$). Milk of GL showed inhibitory activity against *Bacillus subtilis* and *Staphylococcus aureus* ($P < 0.05$). Antioxidant status of GL sows showed an enhanced ($P < 0.05$) of catalase (CAT) and total antioxidant capacity levels at early gestation (day 35), whereas higher levels of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) enzymes at late gestation (day 110). Likewise, GL newborn piglets showed higher CAT levels, whereas both CAT and SOD

levels in suckling piglets, as well as CAT, SOD, and GSH-Px in weaned piglets, were increased in L and GL ($P < 0.05$). Jejunum messenger ribonucleic acid abundance of suckling piglets in L and GL groups showed overexpression of barrier function *MUC2*, digestive enzyme *IDO*, and immune response *PPARGC-α, TNF-α, TGF-β1*, and *IL-10* genes ($P < 0.05$). In conclusion, dietary BPA supplementation in hyperprolific sows increased the litter size (born alive) and improved the composition and bioactivity of colostrum and milk, besides, modified the antioxidant status of sows and their offspring, as well as the suckling piglets gut health gene expression. Several BPA volatile compounds were prenatal and postnatal maternally transferred (placental fluid and milk).

Key words: antioxidant status, colostrum-milk, hyperprolific sows, maternal transfer, phytogenic actives, piglet gene expression

Abbreviations

ANPEP	aminopeptidase-N
BFT	back-fat thickness
BPA	blend of phytogenic actives
CAT	catalase
CLDN4	claudin-4
FEDNA	Spanish Federation for the Development of Animal Nutrition
GSH-Px	glutathione peroxidase
IDO	indoleamine 2, 3- dioxygenase
IL-10	interleukin 10
MUC2	mucin 2
NO	nitric oxide
PA	phytogenic actives
PPARGC1-α	peroxisome proliferative activated receptor gamma coactivator 1 alpha
ROS	reactive oxygen species
SOD	superoxide dismutase
TAC	total antioxidant capacity
TBARS	thiobarbituric acid-reactive substances
TGF-β1	transforming growth factor beta 1
TNF-α	tumor necrosis factor alpha
WOI	weaning-to-estrus interval

Introduction

Genetic selection for commercial hyperprolific sows during last decades has stimulated average litter size (total born) but at the expense of an increased number of stillborn piglets, a decreased mean piglet birth weight, reduced preweaning survival (Foxcroft et al., 2006), and increased within-litter weight variability at birth. Moreover, a high prevalence of low birth weight piglets may lead to long-term effects, with some animals showing poor growth performance and detrimental effects on carcass and meat quality at slaughter (López-Vergé et al., 2018). Major constraints on birth weight are due to a limited uterine capacity, which leads to the combined effects on uterine, placental, and embryo/fetal functions (Burton and Fowden, 2012). During their first days of life, piglets rely on colostrum and milk, which not only contain macronutrients but also various types of bioactive substances, including immune compounds, enzymes, hormones, and growth factors. However, colostrum and milk composition of sows have not significantly changed during the last 30 yr (Zhang et al., 2018). Additionally, litter size is positively related to the colostrum and milk yield, while the milk composition has been negatively correlated with milk yield (Vadmand et al., 2015). This may suggest that milk composition in high-yielding sows may be affected, restricting the nutrient intake and, consequently, the early development of piglets from large litters. It is well accepted that high fetal development during late pregnancy, and the synthesis of colostrum and milk during lactation lead to the catabolic status of sows (Berchieri-Ronchi et al., 2011), the production of reactive oxygen species (ROS), and the induction of oxidative stress (Kim et al., 2013). Different studies have described that mothers may experience increased oxidative stress and inflammation during these periods, which probably determine reproductive disorders, such as embryonic reabsorption, or intrauterine growth retardation, and fetal death (Agarwal et al., 2005). Oxidative stress indicators and cytokines can also be transferred from mothers to the colostrum and milk, significantly affecting the oxidative stress and the health of their offspring (Wang et al., 2010).

Appropriate dosage of dietary antioxidant nutrients or feed additives may be considered during pregnancy and lactation. Phytogenic actives (PA) is a term used to describe plant-derived natural bioactive compounds that promote livestock health and well-being and improve growth and production efficiency. These actives represent a source of various phytochemical compound groups, such as terpenes, phenols, glycosides, saccharides, aldehydes, esters, and alcohols. Although the modes of action of many phytogenic compounds are not fully understood, their benefits to the overall health of animals have been noted. The literature describes some of their effects, such as stimulation of digestive secretions, immune stimulation and anti-inflammatory activities, intestinal microflora modulation and antioxidant effects (Durmic and Blache, 2012), as well as estrogenic and hyperprolactinaemic properties (Farmer, 2018), and effects on colostrum and milk porcine sensory profiles (Val-Laillet et al., 2018). They represent interesting antibiotic alternatives in swine production (Omonijo et al., 2018). There are also references that indicate that herbal extracts may improve intrauterine growth of fetuses in rats by an elevation of fetal blood glucose and growth hormone levels (Takei et al., 2007).

In the present study, we hypothesized that dietary PA supplementation of hyperprolific sows during the lactation, or the whole gestation and lactation period, may influence the reproductive performance and oxidative status of sows and offspring, including changes in bioactivity and composition of colostrum and milk. Therefore, the objectives of this study were to determine the effects of a blend of PA (BPA) supplementation to sow diets during gestation and lactation, or lactation only, on: 1) the reproductive performance and oxidative status of the sows; 2) the maternal transfer of BPA to placental fluid and milk and milk composition; and 3) the piglet performance and jejunal gene expression-related intestinal functions (digestive enzymes, intestinal integrity, and local immune response).

Materials and Methods

The protocol and all experimental procedures used were approved by the Animal Ethics Committee of the Autonomous University of Barcelona and were performed according to the directive of the European Parliament, 2010/63/EU, on the protection of animals used for scientific purposes.

Experimental design, animals, and housing

Eighty-one hyperprolific gilts and sows (up to parity 7) DanBred hybrid line (Landrace × Yorkshire) were randomly distributed by parity number and body weight (BW) into 3 dietary treatment groups (*n = 27*). After breeding, sows were fed unsupplemented control diets (C) during gestation and lactation or the control diets supplemented with 1 kg /MT of a BPA (Delacon Biotechnik GmbH, Steyregg, Austria) either during lactation (L) or during the whole gestation and lactation period (GL). In addition, piglets received experimental treatments in the creep feed and prestarter diets. At the start of the experiment, sows were assigned to individual cages (1.8 × 0.8 m) and kept in those from mating (0 d) until confirmed gestation (35 d). Thereafter, sows were allocated by parity and BW into group pens (4.5 × 5.0 m; 9 sows/pen) until day 110 of gestation. On day 110, sows were moved to the farrowing unit, where they were placed in individual farrowing pens (2.6 × 1.8 m). The number of sows allotted per treatment at farrowing and during lactation was *n* = 19 for C, *n* = 19 for L, and *n* = 20 for GL. Difference with the initial number of sows before breeding corresponds to sows that showed physiological issues (heat failure [gilts], repeated estrous, or abortion). Farrowing pens were mounted over a partially slatted floor with a heated floor pad for piglets and equipped with individual feeders and nipple drinkers for sows and piglets. The temperature in the farrowing room was automatically controlled. Parturitions were monitored as much as possible to interfere opportunely in the farrowing process. Within 24-48 h after birth, piglets were cross-fostered within the respective treatment group of sows (C, L, or GL) in order to standardize litter size to 15 piglets/litter but not among treatments. After weaning, piglets were moved to the nursery unit on the same farm in order to evaluate BW at weaning and growth performance during the prestarter phase (day 7) postweaning.

Experimental diets and feeding system

Control diets for each experimental period were formulated to meet or exceed nutrient requirements for DanBred sows (Tybirk et al., 2015), with adaptations based on Spanish recommendations for gestating and lactating sows (FEDNA, 2013; Table 1) and for prestarter piglets (same specification and formula than for creep feed; Table 2). Experimental diets were control diets plus 1 kg/MT BPA supplement containing a blend of eucalyptol, p-cymene, linalool, anethole, and thymol added as essential oils from the Fabaceae, Laminaceae, Schisandraceae, and Zingiberaceae plant families. Sows were fed 2.1 kg/d from weaning to service, a mean of 2.9 kg/d from service to 35 d of gestation, based on individual body condition, and 2.5 kg/d from 35 to 110 d of gestation of the gestation diet of their corresponding treatments (flat line). Each gestating pen was equipped with enough mechanical free access self-closing semi-cage without pneumatic actuators (Rotecna, Lleida, Spain) to keep animals individually monitored during feeding. From 110 d of gestation and during lactation, sows were fed ad libitum. Control creep feed diet was unsupplemented, whereas experimental diets were creep feed formula than for the control group but supplemented with 1 g/kg of BPA, which were offered to each respective litter as mash from day 7 of lactation to weaning. At weaning, all piglets from the same experimental treatment were allotted in a single large pen (all together, mixed litters within each treatment); this resulted in 3 large pens (1 per treatment) of 350-400 piglets each. Each pen was equipped with complete slatted floor, 3 ad libitum pan hoppers (Swing Feeder R3 Wet WTF, Rotecna, Spain) in the middle of the pen and free access to fresh water with nipple drinkers on the wall. The corresponding pelleted creep feed fed to the piglets during lactation for each treatment was used as prestarter diet until day 7 postweaning. Weaned piglets were individually weighted, and each piglet was considered as experimental unit for either BW, as well as blood and tissue sampling.

Table 1. Ingredients and calculated nutrient compositions of gestation and lactation diets (as-fed basis)

Item	Gestation	Lactation
Ingredient composition, %		
Barley	35.00	10.00
Maize	22.70	27.01
Wheat middling's	15.00	7.00
Wheat	9.00	25.55
Sunflower meal	5.65	4.50
Sugar beet pulp	3.10	2.50
Soybean meal, 47 % CP	2.50	13.50
Rapeseed meal	2.50	4.50
Calcium carbonate	0.99	1.25
Lard	1.05	1.00
Dicalcium phosphate	0.99	1.25
Salt	0.40	0.50
L-lysine HCl	0.31	0.63
L-threonine	0.10	0.18
Mycofix plus 3.E	0.10	0.10
Vit-min premix[1]	0.50	0.50
Calculated nutrient composition		
Net energy, kcal/kg	2,261	2,455
CP, %	13.0	16.7
Calcium, %	0.85	0.91
Total phosphorus, %	0.56	0.57
Dig. phosphorus, %	0.35	0.37
SID lysine, %	0.60	1.00

[1]Supplied the following per kg of diet: vitamin A (retinyl acetate), 10,000 IU; vitamin D3 (cholecalciferol), 2,000 IU; vitamin E (acetate de tot-rac-3-tocopheryl), 45 mg; vitamin K3 (menadione nicotinamide bisulphite), 3 mg; vitamin B1 (thiamine mononitrate), 3 mg; vitamin B2 (riboflavin), 9 mg; vitamin B6 (pyridoxine hydrochloride), 4.5 mg; vitamin B12 (cyanocobalamin), 0.04 mg; nicotinamide, 51 mg; pantothenic acid (calcium D-pantothenate), 16.5 mg; biotin (D-(+)-biotin), 0.15 mg; folic acid, 1.8 mg; choline chloride, 350 mg; iron (as iron sulphate monohydrate), 54 mg; zinc (as zinc oxide), 66 mg; manganese (as manganese oxide), 90 mg; iodine (as calcium iodine anhydrous), 1.2 mg; selenium (as sodium selenate), 0.18 mg; copper (as copper sulphate pentahydrate), 12 mg; ethoxyquin, 4 mg; D,L-malic acid, 60 mg; fumaric acid, 75 mg; sepiolite, 907 mg; vermiculite 2001 mg; colloidal silica 45 mg.

Data recording and sampling

Sow BW and back-fat thickness (BFT) were measured at day 0, day 110 of gestation, and at weaning. BFT was measured by digital B-ultrasound (model WED-3000V, Welld, Shenzhen, China) at P2 position (left side of the midline at last rib and 7.5 cm to the spine). Average daily feed intake (ADFI) was controlled per sow during gestation by weighing the feed

Table 2. Ingredients and nutrient compositions of control basal diet used from lactation day 7 to weaning (as creep feed in mash form) and as prestarter diet (pelleted form) from weaning to postweaning day 7 (as-fed basis)

Item	Creep feed/prestarter diet
Ingredient composition, %	
Maize + extruded barley	44.00
Sweet milk whey	10.26
Wheat	10.00
Barley	15.00
HP 300	5.00
Maize flour	1.01
Extruded soybean	5.13
Soybean meal, 47% CP	5.00
Plasma	1.50
L-lysine	0.81
Dicalcium phosphate	0.87
Methionine	0.23
Salt	0.25
Threonine solid	0.21
L-valine	0.09
L-tryptophan	0.03
Vit-min premix[1]	0.60
Calculated nutrient composition	
Net energy, kcal/kg	2,438
CP, %	17.0
Calcium, %	0.30
Total phosphorus, %	0.40
Dig. phosphorus, %	0.30
SID lysine, %	1.11

[1]Supplied the following per kg of diet: vitamin A (retinyl acetate), 10,000 IU; vitamin D3 (cholecalciferol), 2,000 IU; vitamin E (all-rac α-tocopheryl-acetate) 100 ppm; choline chloride, 187 ppm; iron (as iron sulphate monohydrate), 100 ppm; iodine (potassium iodide), 100 ppm; copper (as copper sulphate pentahydrate), 149 ppm; manganese (as manganese oxide), 58 ppm; zinc (as zinc oxide), 120 ppm; selenium (as sodium selenate), 0.30 ppm; selenomethionine (produced by *Saccharomyces cerevisiae*), 0.1 ppm; butyl-hydroxytoluene (BHT), 63 ppm; citric acid, 8 ppm.

offered and checking for refusals (mechanical free access self-closing semi-cage; Rotecna, Spain). During lactation, sows were fed ad libitum by using a feeding ball system (ad libitum pan with ball mechanism for farrowing; Rotecna, Spain) and ADFI was recorded by weighing feed offered and refusals (feeding ball hopper was filled twice a day at 800 and 1600 hours to ensure that ad libitum feed availability and feed refusals were weighed the day after, before the morning hopper filling). Reproductive and production performance parameters included the litter size at farrowing, taking into account the total number of live, dead, or mummified piglets, as well as the individual piglet BW at birth, cross-fostering, day 20 of lactation, weaning, and day 7 postweaning. The number of days from weaning to estrus (weaning-to-estrus interval [WOI]) was also recorded.

Blood samples (6 mL per sow) were collected from sows by caudal venepuncture on day 0 (day of service), day 35, and day 110 of gestation (n = 27 per treatment) and day 2 (140 d) postweaning (n = 12 per treatment). Placental fluid samples (60 mL per sow) were collected at farrowing from the same subset of sows in order to determine maternal transfer of compounds (n = 12 per treatment). Colostrum samples (30 mL per sow) were collected within 12 h of farrowing (n = 12 per treatment) and milk samples (30 mL per sow) were collected at day 20 of lactation from all functional mammary glands, after injecting 2 mL oxytocin, both for chemical composition and maternal transfer of compounds (n = 12 per treatment). Samples were not filtered and were immediately chilled on farm and stored at −20 °C until analysis. From the same subset of sows, 8 piglets per treatment (1 piglet per litter with a medium BW) were euthanized at farrowing, at day 20 of lactation, and at day 7 postweaning in order to obtain both a blood sample (6 mL per treatment) and jejunum tissue (n = 8 per treatment) for gene expression analysis.

The newborn piglets selected to be euthanized at farrowing were removed immediately after birth without sucking colostrum. Piglet blood samples were collected by jugular vein puncture. Before being euthanized, piglets were anesthetized by intramuscular injection of 1 mL/22 kg BW of the final combination containing 100 mg telazol, 50 mg ketamine, and 50 mg xylazine in 1 mL. Piglets were subsequently euthanized with sodium pentobarbital 0.5 mL/kg of BW by jugular vein injection and a sample of jejunum tissue (1 cm^2) was collected immediately and preserved into 1 mL of aqueous RNAlater (Applied Biosystems, Foster City, CA) in order to stabilize and protect RNA, with immediate RNase inactivation for messenger RNA (mRNA) analysis. RNA samples were stored for 24 h at room temperature (25 °C) and, then, stored at −80 °C until analysis. The blood samples were centrifuged at 3,500 rpm for 15 min to obtain plasma or serum and stored at -20 °C until analysis.

Determination of maternal transfer of volatile compounds

The volatile profile of PA compounds maternally transferred through the placental fluid and milk were determined by solid-phase microextraction, gas chromatography-mass spectrometry (Servei d'Anàlisi Química, Autonomous University of Barcelona [UAB], Bellaterra, Spain) based on the volatile PAs profile characterized in the tested BPA. Results of the relative peak abundance were estimated based on the ratio (abundance/retention time) for each compound and expressed as proportional increased concentrations in relation to values in control sows.

Colostrum and milk chemical analyses

Colostrum and milk composition were determined by standardized methods as follows: crude protein (CP) by the Kjeldahl method AOAC 991.22; crude fat by the Röse-Gottlieb method AOAC 905.02; lactose by the Luff-Schoorl volumetry method B.O.E. num. 52 R.D. 2257/ 1994, and ash content was measured by difference method B.O.E. num. 52 R.D. 71/250/ CEE.

Evaluation of antimicrobial activity of milk

The antimicrobial growth inhibition of milk against *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus*, *Lactobacillus plantarum*, and *Candida albicans* was determined using a modification of the Kirby-Bauer test (Hudzicki, 2009). In our study, the antibiotic discs were replaced by sterile discs impregnated with each one of the milk samples under study. The inhibition values (IVs) were calculated using the equation (IV = [inhibition diameter of zone − diameter of sterile disc impregnated with milk]/2) and were expressed as millimeters of inhibition.

Measurement of oxidative status in sows and offspring

Oxidative status of sows and their offspring was determined by measuring the antioxidant enzyme activities in plasma or serum

of catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px). Lipid peroxidation was measured using thiobarbituric acid-reactive substances (TBARS), nitric oxide (NO), and total antioxidant capacity (TAC) values in plasma or serum. Antioxidant enzyme activities were determined using standardized Cayman Chemical Kits (Cayman Chemical, Ann Arbor, USA) according to the manufacturer's protocol applied on a Tecan microplate reader (SunRise, Austria, Tecan, Männedorf, Switzerland). Catalase activity was assayed at 540 nm and results were expressed as nanomoles per minute per milliliter. SOD activity was measured using the cytochrome c and xanthine oxidase coupled assay at 440-460 nm and results expressed as units per milliliter. One unit was defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. GSH-Px activity was measured every minute at 340 nm for 10 min, and the results were expressed as nanomoles per minute milliliter.

Plasma lipid peroxidation was analyzed based on the measurement of TBARS present in the sample as a byproduct of lipid peroxidation. In short, plasma or serum was mixed with deionized water, 0.5 N HCL, and thiobarbituric acid and incubated at 95 ºC for 15 min. The measurement was made in fluorescence mode (maximum excitation 515 nm; emission range 548 nm) using a Tecan Infinite M200 microplate reader. The results were expressed as nanomoles of malondialdehyde per milliliter of plasma using 1,1,3,3-tetramethoxypropane as standard. NO levels were evaluated using the Nitrate/Nitrite Colorimetric Assay Kit (Cayman Chemical, MI) by following the manufacturer's instructions. Absorbance was read at 550 nm using a microplate reader (Tecan Infinite M200), with results measured using a $NaNO_2$ standard curve ranging from 0 to 100 µM. The TAC in plasma was assayed using a QuantiChrom kit (BioAssay Systems, Hayward, USA) as follows: 20 µL undiluted plasma or serum or Trolox standard solution along with 100 µM working reagent were added to a 96-well microplate, mixed by tapping, and incubated at room temperature for 10 min according to manufacturer's protocol. The absorbance of the reaction was read at 570 nm using a Tecan Infinite M200 microplate reader. Results were expressed as micromolar Trolox equivalents.

Piglet intestinal gene expression reverse transcription polymerase chain reaction

Total RNA was extracted from 50 mg of jejunum tissue using the Ambion RiboPure Kit (Life Technologies, Carlsbad, CA) by following the manufacturer's protocol. RNA concentration was measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop products), and RNA quality was checked using Agilent Bioanalyzer-2100 equipment (Agilet Technologies). Around 1 µg of total RNA in a final volume of 20 µL was used for cDNA synthesis with random primers using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). The following temperature profile was applied: 25 °C 10 min; 37 °C 120 min; 85 °C 5 min; 4 °C hold. A 25-ng cDNA sample was preamplified, using a TaqMan PreAmp Master Mix (Life Technologies, Foster City, CA) and a Pooled Taqman Gene Expression custom assay following the manufacturer's protocol. A total of 56 genes were previously selected based on the bibliography, including 4 reference genes. Primers were designed by spanning exon-exon boundaries using PrimerExpress 2.0 software (Applied Biosystems), and genomic DNA amplification and primer dimer formation were controlled. One replicate per sample was run in a Taqman Open Array gene expression custom plate format for gene expression with 56 assays of 48 samples per plate (OpenArray plate) in a QuantStudio 12K Flex Real-Time PCR System (Applied Biosystems, Foster City, CA). Data was collected and analyzed using the ThermoFisher Cloud software 1.0 (Applied Biosystems) applying the appropriate standard curve method for relative quantification.

Calculations and statistical analyses

Different procedures of the statistical package SAS 9.4 (SAS Inst. Inc., Cary, NC) were used to analyze all of the data. During whole gestation, the sows were individually controlled; therefore, the individual sows were considered as experimental unit at service and gestation period, while pen was included as a random effect. During lactation, the sow with her litter was considered as experimental unit. The performance and oxidative status of sows and piglets, colostrum-milk composition, milk maternal transfer, and gene expression were analyzed with ANOVA by using the GLIMMIX procedure, defining the model:

$$Y_{ij} = \mu + \text{treat}_i + \text{parity}_j + \varepsilon_{ij}$$

where Y_{ij} relates to each observation of the outcome variable, µ is the global mean, treat_i is the main effect of treatment, parity_j is the covariate effect for sow parity number and, finally, ε_{ij} is the experimental error term.

Therefore, all data were analyzed considering the treatment as main effect, and results are presented as least square (LS) means with their corresponding SEM. Regarding placental maternal transfer, data from 2 groups (C vs. GL) were analyzed by using the TTEST procedure. Data corresponding to milk bacteriostatic activity was analyzed using Fisher exact test. All data were checked for outliers before the statistical analysis *t* with outliers defined by a deviation of ≥2.5 times the SD of the mean. Normality and equal variances were verified in all continuous variables using the Shapiro-Wilk test by using the UNIVARIATE procedure. Tukey adjust test was considered for all multiple comparisons between treatments. Finally, mean significant differences were declared at $P < 0.05$, while $0.05 < P < 0.10$ were considered near-significant trends.

Results

Reproductive sow and litter performances

Sow BW weight and BFT parameters during gestation and lactation periods (Table 3) were not affected ($P > 0.05$) by the dietary treatments. Likewise, no differences were observed ($P > 0.05$) between treatments either on ADFI or on WOI. Sow reproductive and litter performance during lactation and early postweaning are shown in Table 4. Litter birth weight and litter size were not affected ($P > 0.05$) by BPA supplementation during gestation. However, BPA supplementation (GL group) increased ($P = 0.020$) the number of piglets born alive but reduced ($P = 0.024$) piglet weight at birth. There was a mean of 2.02 piglets per litter below 1 kg birth weight in the GL group and 1.55 in the C and L groups (data not shown). Cross-fostering homogenized the mean number of piglets per litter between 1 and 2 d of life, with no significant differences being observed among treatments ($P > 0.05$) on piglets BW (at lactation day 20 and at 7 d postweaning), as well as the days of lactation length and the preweaning mortality rate.

Table 3. Effects of dietary BPA supplementation during lactation or gestation and lactation on performance of sows

Item	Treatment			SEM	*P*-value[5]
	Control	L	GL		
No. of sows[1]	27	27	27	—	—
Parity, *n*	2.78	2.72	2.70	—	—
Sow BW, kg					
Breeding (day 0)	209.22	214.00	218.62	5.490	0.415
Gestation (day 110)	269.21	267.71	273.17	8.171	0.882
Farrowing standardized[2]	246.76	244.94	251.24	7.610	0.826
Weaning	237.87	231.52	245.33	7.501	0.340
Loss lactation	−8.89	−13.42	−5.91	3.871	0.291
Sow BFT, mm					
Breeding (day 0)	14.04	13.89	15.00	0.905	0.341
Gestation (day 110)	13.83	14.17	12.68	1.079	0.584
Weaning	12.14	12.18	11.81	0.892	0.642
Loss lactation	−1.69	−1.99	−0.87	0.498	0.270
Feed intake, kg					
Lactation ADFI (day 20)[3]	6.13	5.82	6.40	0.370	0.537
Total feed intake[4]	428.39	425.92	434.64	5.535	0.491
WOI, d	3.43	4.44	3.84	0.313	0.176

[1]Total number of sows allocated per treatment at the beginning of trial.
[2]Sow BW at day 110 less litter birth weight.
[3]Lactation ADFI at day 20.
[4]Standardized total feed intake considered both the gestation and lactation at day 20 feed intake.
[5]Sow parity was considered as covariable for the statistical analysis.

Table 4. Effects of dietary BPA supplementation during lactation or gestation and lactation on reproductive sows and litter performances

Item	Treatment			SEM	*P*-value[4]
	Control	L	GL		
No. of sows[1]	19	19	20	—	—
Parity, *n*	2.84	2.83	2.75	—	—
Sow reproductive performance					
Litter birth weight, kg	22.01	21.85	21.90	1.350	0.996
Total born piglets, n	17.01	17.67	19.13	0.941	0.238
Piglets born alive, n	14.47[b]	15.28[b]	17.53[a]	0.811	0.020
Born alive piglet weight, kg	1.41[a]	1.24[ab]	1.16[b]	0.070	0.024
Litter performance					
Litter size at cross-fostering (CF), *n*[2]	14.39	14.90	14.93	0.173	0.057
Piglet CF weight, kg	1.48	1.44	1.33	0.072	0.299
Piglet lactation weight day 20, kg	4.94	5.04	4.70	0.247	0.585
Piglet postweaning weight day 7, kg	5.41	5.58	5.51	0.288	0.701
Piglet weight gain from CF to day 7 postweaning, kg	3.93	4.14	4.18	0.203	0.497
Lactation length, d	23.45	22.86	23.91	0.575	0.403
Preweaning mortality rate,[3] %	2.19	1.27	3.68	0.199	0.281

[a,b]Means within a row with different superscripts indicate significant differences ($P < 0.05$).
[1]Data from number of sows allotted per treatment at farrowing and during lactation. Difference with the initial number of sows before breeding corresponds to sows that showed physiological issues (heat failure [gilts], repeated estrous, or abortion).
[2]Litter sizes were adjusted by cross-fostering within treatment between days 1 and 2 after farrowing.
[3]Piglets preweaning mortality rate was estimated from cross-fostering to weaning.
[4]Sow parity was considered as covariable for the statistical analysis.

Placental and milk maternal transfer of phytogenic compounds

Thymol, anethole, linalool, and p-cymene were detected in placental fluid and milk, while eucalyptol was detected in placental fluid but not in milk. Dietary BPA supplementation during gestation (GL) increased ($P < 0.05$) the concentration of some tested volatile compounds in the placental fluid (Fig. 1), such as thymol, anethole, linalool, and eucalyptol. Supplementation of BPA during the lactation period increased ($P < 0.05$) the concentrations of thymol, anethole and p-cymene in milk compared to C in both L and GL (Fig. 2). Concentrations of p-cymene in placental fluid and linalool in milk were not significantly changed ($P > 0.05$).

Colostrum and milk composition

The chemical composition of colostrum and milk is described in Table 5. Results showed that BPA supplementation during the gestation period (GL) increased the CP content in colostrum ($P \leq 0.001$). No significant changes ($P > 0.05$)

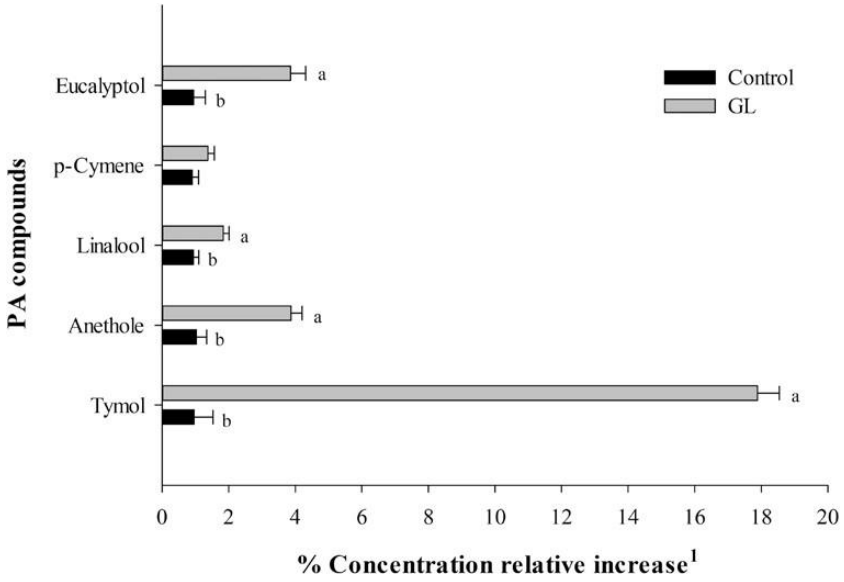

Figure 1. Proportional increase of PA volatile compounds in placental fluid by maternal transfer of PA dietary supplementation of sows during gestation against not supplemented. Control, not supplemented; GL, PA supplemented during gestation. Values are expressed as percentage of concentration (proportion = 1:100%) ± SEM, n = 12. [a,b]Means with different superscripts indicate significant differences ($P < 0.05$). [1]Percentage of concentrations (abundance/retention time) relative increase respect to control. Data were analyzed by t-test procedure.

were observed in colostrum lactose, fat, or ash content. Supplementation of BPA during gestation and lactation (GL) or lactation (L) increased ($P = 0.028$) the crude fat content in milk, but no significant changes ($P > 0.05$) were observed in CP, lactose, or ash content.

Milk bacterial inhibition activity

The antimicrobial activity of milk is shown in Table 6. Milk from the C and L groups did not show any antimicrobial effects against the studied microorganisms, while milk from the GL sows exhibited inhibitory activity against *B. subtilis* ($P = 0.015$) and *S. aureus* ($P = 0.001$) growth in 6 and 7 samples out of 7 analyzed samples, respectively.

Sows and piglets plasma antioxidant status

Oxidation and antioxidant status in the plasma or serum of sows (Fig. 3) showed changes during the gestation and lactation period and also between experimental treatments. GSH-Px increased in sows during both the gestation and lactation period, showing the highest values at the end of the lactation period. In contrast, TAC was clearly reduced during the first weeks of pregnancy and NO was temporarily increased at the end of pregnancy. Among treatments, dietary BPA supplementation during gestation increased ($P < 0.05$) CAT, NO, TBARS, and TAC on day 35, while SOD activity was lower ($P < 0.05$) than C group. At the end of the gestation period (day 110), BPA increased ($P < 0.05$) GSH-Px and SOD activity. During the lactation period, supplementation of BPA increased ($P < 0.05$) CAT activity at day 140 in L, while it also decreased the TAC in blood of L and GL groups.

As regards the antioxidant status of the offspring (Fig. 4), results show that plasma CAT activity and TBARS concentrations were higher ($P < 0.05$) in newborn piglets from GL than from L and C groups. At day 20 of lactation and after weaning, suckling piglets from GL showed higher ($P < 0.05$) CAT and SOD activities than C piglets. In contrast, piglets from L group showed higher ($P < 0.05$) TAC than the GL group at day 20 of lactation. In the postweaning period, higher ($P < 0.05$) GSH activity was observed in the BPA groups (L and GL) compared to the C group.

Piglets intestinal gene expression

Jejunum gene expression of newborn and postweaned piglets was not significantly affected ($P > 0.05$) by the dietary treatments (data not shown). Jejunum gene expression of suckling piglets at day 20 is described in Fig. 5. As compared to the C group, BPA supplementation decreased ($P < 0.05$) barrier function claudin-4 (CLDN4) and digestive enzyme aminopeptidase-N (ANPEP) genes (L and GL) and increased immune response tumor necrosis factor alpha (TNFα) and transforming growth factor beta 1 (TGF-β1; L), digestive enzyme indoleamine 2, 3-dioxygenase (IDO; in GL group), and cytokine interleukin 10 (IL-10) genes (L and GL).

Discussion

Effects on sow metabolism and performance

In hyperprolific sows, the high metabolic energy demand during pregnancy and lactation may increase the oxygen requirement and the production of ROS and, consequently, the DNA damage (Berchieri-Ronchi et al., 2011). Oxidative stress refers to the imbalance due to excess ROS or oxidants over the capacity of the cell to mount an effective antioxidant response. TBARS are formed by degradation of the initial products of free radical attacks, and these can be measured as indicators of the damage produced by oxidative stress (Pryor, 1991). In response to oxidants, the organism can counteract their negative impact using a serial of antioxidant enzymes, such as those mentioned in the present study, SOD, CAT, and GSH-Px. In contrast to antioxidant enzymes, the TAC predominantly measures the low molecular weight chain-breaking antioxidants, excluding the contribution of the antioxidant enzymes mentioned above, and metal-binding proteins. Consequently, TAC is decreased under oxidative stress (Woodford and Whitehead, 1998).

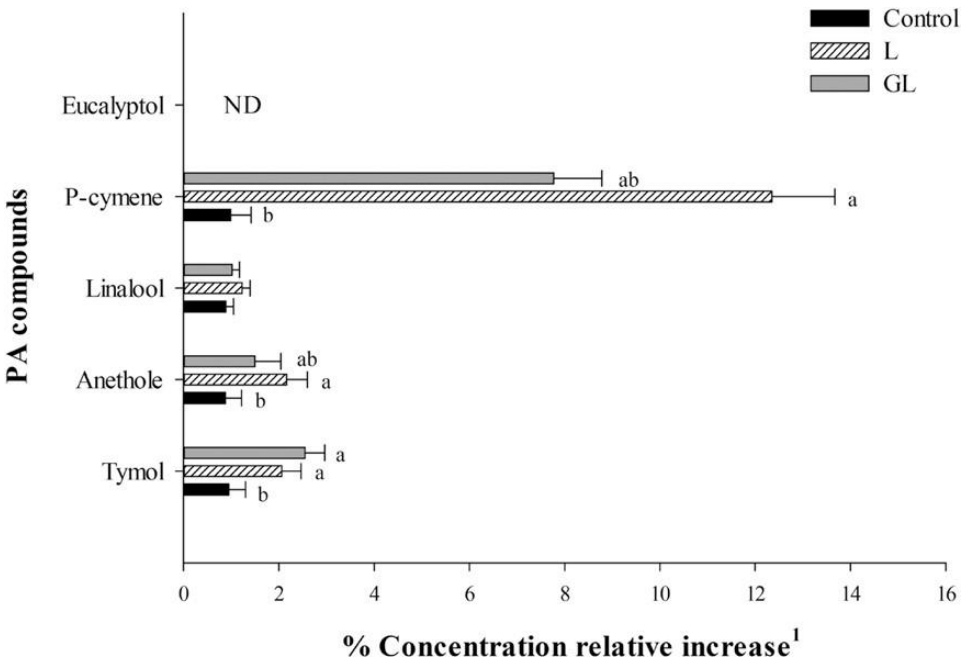

Figure 2. Proportional increase of PA volatile compounds in milk at 20 d by maternal transfer of PA dietary supplementation of sows during gestation and/or lactation against not supplemented. Control, not supplemented; L, PA supplemented during lactation; GL, PA supplemented during gestation and lactation. Values are expressed as percentage of concentration (proportion = 1:100%) ± SEM, n = 12. [a,b]Means with different superscripts indicate significant differences ($P < 0.05$). [1]Percentage of concentrations (abundance/retention time) relative increase respect to control.

Table 5. Effects of dietary BPA supplementation during lactation or gestation and lactation on colostrum and milk composition of sows

	Treatment				
Item	Control	L	GL	SEM	*P*-value[1]
Analyzed nutrient content, %					
Colostrum					
Protein	15.71[b]	15.74[b]	20.80[a]	0.397	<0.001
Fat	5.57	5.55	6.10	0.200	0.098
Lactose	3.05	3.13	2.48	0.070	0.061
Ash	0.67	0.67	0.71	0.013	0.134
Milk					
Protein	6.85	6.61	6.16	0.240	0.152
Fat	7.21[b]	8.94[a]	9.12[a]	0.414	0.028
Lactose	4.89	4.57	4.75	0.051	0.085
Ash	0.79	0.79	0.78	0.019	0.773

[a,b]Means within a row with different superscripts indicate significant differences ($P < 0.05$).
[1]Sow parity was considered as covariable for the statistical analysis.

Based on our results, the oxidant status in sows were associated with a stepwise increase in the enzymatic antioxidant activities during gestation, with the highest values found at day 35 for SOD and GSH-Px activity at final gestation and lactation, as well a rapid decrease in TAC during the first third of gestation, which confirmed the induced oxidative stress during gestation. Similarly, Meng et al. (2018) described increase in oxidative stress markers during gestation. Among antioxidant enzymes, SOD destroys the free radical superoxide by converting it into peroxide, which, in turn, can be destroyed by CAT or GSH-Px reactions (Matés, 2000). Therefore, increases in SOD enzyme activity corresponds to enhanced resistance to oxidative stress. In the present study, the C and L groups showed higher SOD levels in early gestation (day 35), with simultaneously lowering values of TBARS, and a similar tendency was shown in GL sows at late gestation (day 110).

The catalase is one of the most efficient antioxidant enzymes known; it cannot be saturated by H_2O_2 at any concentration, protecting the cells from endogenous hydrogen peroxide by breaking down H_2O_2 to O_2 and 2 molecules of water (Aruoma et al., 2006). Thereby, it plays an important role in the acquisition of tolerance to oxidative stress in the adaptive response of cells (Hunt et al., 1998). According to our results, short-term responses on CAT were observed with BPA dietary treatments on sows (day 35) and on piglets. Similar to SOD and CAT, GSH-Px is located in the mitochondria and the cytosol, where it serves as an important cellular protectant against low levels of oxidant stress, whereas CAT becomes more significant in protecting against severe oxidant stress (Yan and Harding, 1997). Despite that GSH-Px shares the substrate H_2O_2 with CAT, it alone can react effectively with lipid and other organic hydroperoxides. In this study, BPA supplementation contributed to increase

the GSH-Px levels in gestating sows (day 110) and postweaned piglets (day 7).

Oxidative stress may exert major effects on embryonic development. During early gestation, the embryo is more susceptible to oxidative stress and antioxidant defenses are important in modulating oxidative stress-mediated events (Dennery, 2007). In the present study, supplementation of BPA during gestation (GL group) increased CAT activity in early gestation (day 35), as well as NO levels, associated with a greater litter size and piglets survival at farrowing. It is known that NO can exert a role in the endothelial cells, resulting in increased blood flow and vasodilation (Kim et al., 2013). Besides, it is known that high ovulation rates (>30) in commercial hyperprolific sows, as well as deficits in fetal or placental NO production, are associated with the number of surviving embryos, resulting in likely uterine crowding in the early postimplantation period (Foxcroft et al., 2006; Takei et al., 2007). Nevertheless, volatile phytogenic compounds were also detected in amniotic fluid of sows fed BPA supplemented diets, which likely contributed to placental angiogenesis process. Therefore, changes in placental functionality, such as vascularization and nutrient transport due to BPA, as a major mediator and determinant of fetal growth and viability deserves to be studied.

Recently, Su et al. (2017) described that placental antioxidant system of sows may have an adaptive response to oxidative stress, which is normalized by antioxidant supplementation. They also reported that feeding oxidized corn oil to sows markedly decreased the contents of protein and fat in colostrum and milk during 21 d of lactation. Therefore, sow colostrum-milk composition may depend on the oxidation status of the animal; due to higher dietary fat sources, stability may promote higher availability of those energy sources for final milk yield and quality. We described a possible galactagogue (lactogenic activity) effect of BPA, with significant increases in protein and fat content in sow colostrum and milk. There are limited reports about changes promoted by phytogenic compounds on milk composition in sows and none for colostrum. For example, reductions in the fat percentage in milk on day 7 (6.6% vs. 8.3%, $P < 0.05$) and day 14 ($P = 0.07$) were observed in sows supplemented with oregano essential oils during lactation compared with those fed a plain diet (Ariza-Nieto et al., 2011).

It was reported in humans that a mixture of ginger, a spice that is believed to increase blood circulation, and fenugreek, a spice known to enhance prolactin levels by stimulating the anterior pituitary gland, may improve milk yield by around 49% (Bumrungpert et al., 2018). Farmer et al. (2014) have reported that silymarin increased prolactin levels in sows. Not many reports are available on sows that describe changes in mammary secretion (colostrum-milk) composition associated with dietary changes or BPA supplementation. In our study, we observed that PAs, such as thymol, anethole, and p-cymene, but not linalool and eucalyptol, were significantly transferred to supplemented

Table 6. Bactericide capacity in sow milk by dietary BPA supplementation during lactation or gestation and lactation of sows

Item	Control n = 7[1]	L n = 8	GL n = 7	*P*-value[3]
Bactericide capacity against				
Bacillus subtilis, n[2]	0[b]	0[b]	6[a]	0.015
Escherichia coli, n	0	0	0	—
Staphylococcus aureus, n	0[b]	0[b]	7[a]	0.001
Lactobacillus plantarum, n	0	0	0	—
Candida albicans, n	0	0	0	—

[a,b]Values within a row with different superscripts indicate significant differences ($P < 0.05$).
[1]Total number of milk samples analyzed per treatment.
[2]Number of milk samples with bactericide capacity.
[3]Data were analyzed using Fisher test.

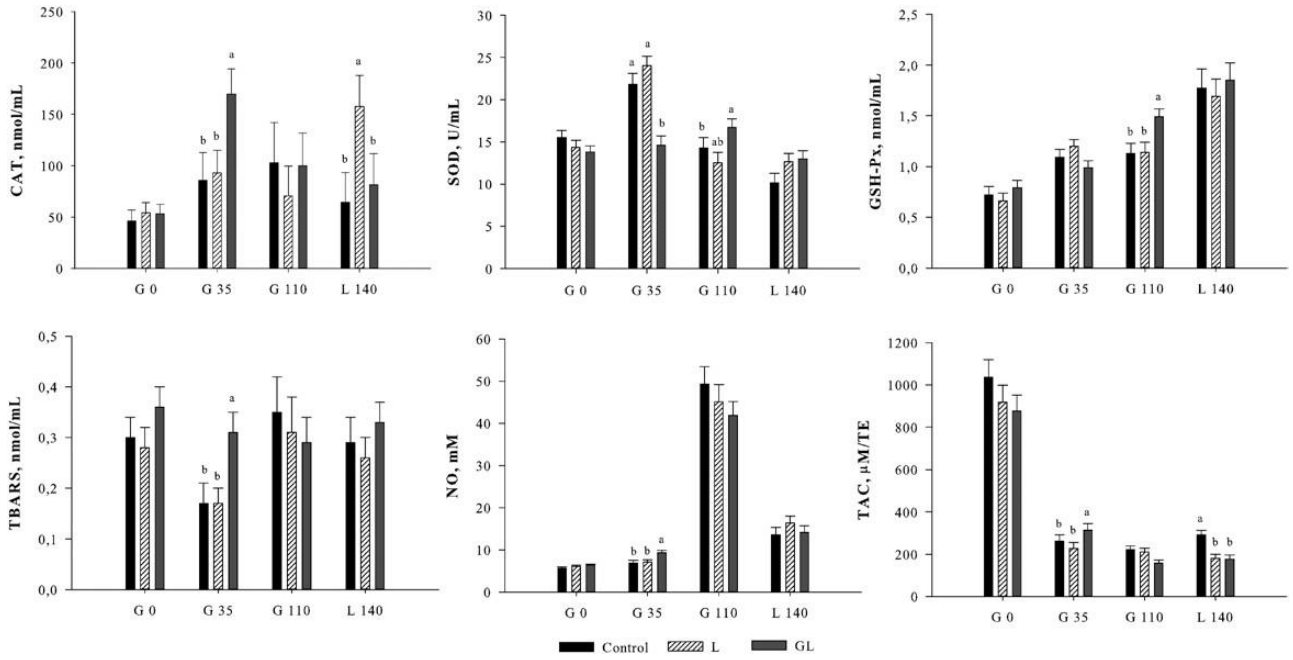

Figure 3. Antioxidant status in plasma or serum of supplemented sows with BPA during gestation and/or lactation against not supplemented. Control, not supplemented; L, PA supplemented during lactation; GL, PA supplemented during gestation and lactation. G 0, gestation day 0 ($n = 27$); G 35 = gestation day 35 ($n = 22$); G 110, gestation day 110 ($n = 20$); L 140, postlactation day 2 ($n = 19$). Values are expressed as LS means ± SEM. [a,b]Means with different superscripts indicate significant differences ($P < 0.05$).

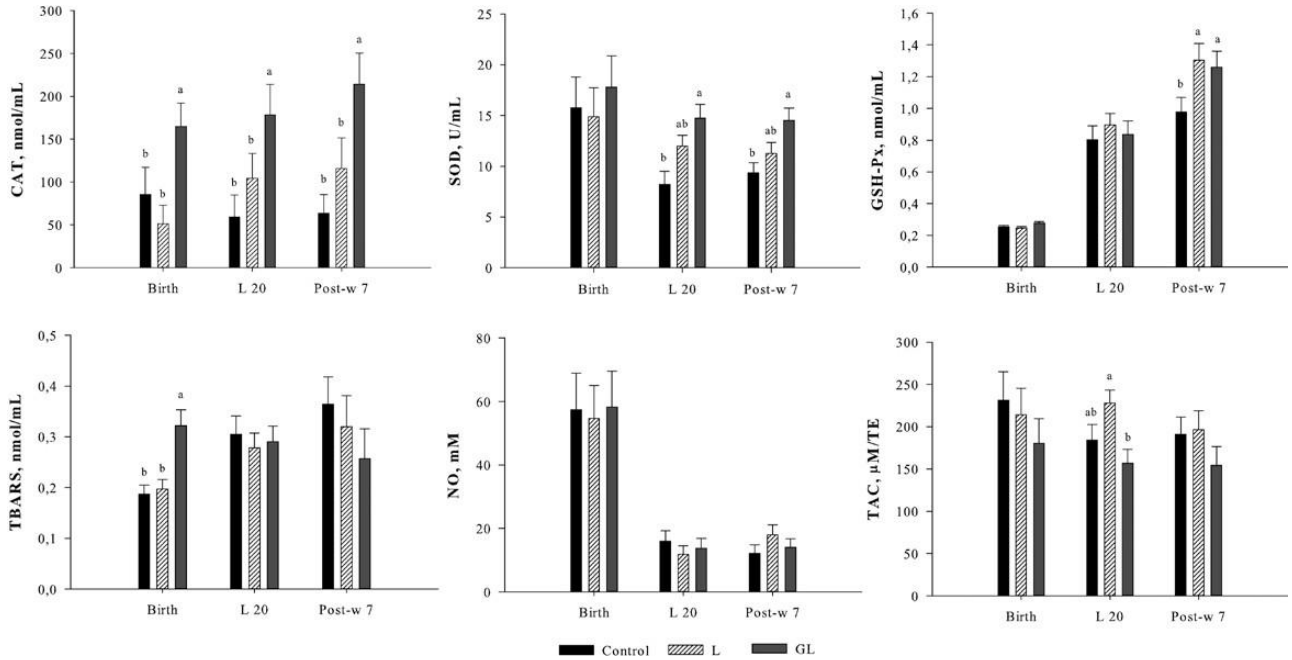

Figure 4. Antioxidant status in plasma or serum of piglets from supplemented sows with BPA during gestation and/or lactation against not supplemented. Control, not supplemented; L, PA supplemented during lactation; GL, PA supplemented during gestation and lactation. Birth, newborn piglets without suckling; L 20, lactation day 20; Post-w 7, postweaning day 7. Values are expressed as LS means ± SEM, $n = 8$. ^{ab}Means with different superscripts indicate significant differences ($P < 0.05$).

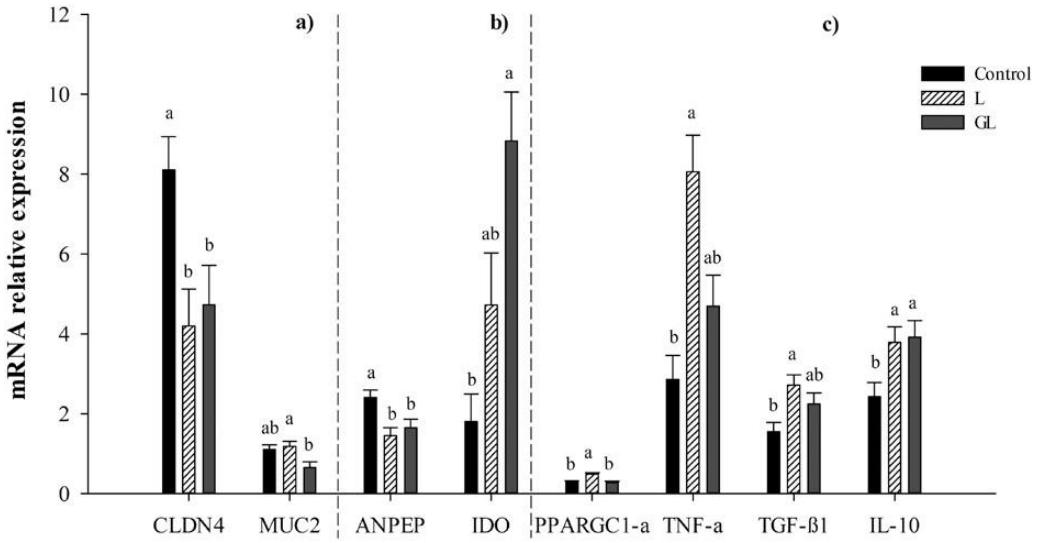

Figure 5. Effects of dietary BPA supplementation during gestation and/or lactation of sows on mRNA relative expression of jejunum genes in suckling piglets at 20 d. Control, not supplemented; L, PA supplemented during lactation; GL, PA supplemented during gestation and lactation. (a) Barrier function genes: *CLDN4, claudin-4; MUC2, mucin 2.* (b) Digestive enzyme genes: *ANPEP, aminopeptidase-N*; *IDO, indoleamine 2, 3-dioxygenase.* (c) Immune response genes: *PPARGC1-α, peroxisome proliferative activated receptor gamma coactivator 1 alpha*; *TNF-α, tumor necrosis factor alpha*; *TGF-β1, transforming growth factor beta 1*; *IL-10, interleukin 10.* All values are expressed as LS means ± SEM, $n = 8$. ^{ab}Means with different superscripts indicate significant differences ($P < 0.05$).

sows milk, which may be related to the enhanced composition of colostrum (protein) and milk (fat). Val-Laillet et al. (2018) also referred the transfer of limonene, carvone, and anethole into sow colostrum and milk. Flavor compounds appear to reach the milk differentially from the mother's diet (Hausner et al., 2008), with lipophilic compounds ingested by the mother showing a higher probability of being detected in the milk as compared to hydrophilic compounds.

Our results also showed an inhibitory effect of milk from GL sows against *B. subtilis* and *S. aureus*, which reflect the presence of PA compounds in milk. For example, p-cymene is a naturally occurring aromatic organic compound, which is a constituent of several essential oils, most commonly the oil of cumin and thyme. Different studies have reported an antimicrobial effect of thyme essential oil against foodborne pathogens and spoilage microorganisms, such as *E. coli, S. aureus, Listeria monocytogenes,*

and *Salmonella typhimurium* (Kang et al., 2018). Thyme essential oil had a marked effect on whole-cell proteins of *Bacillus cereus* by either inhibiting their synthesis or destroying them after synthesis, with evident consequences on the bacterial cell lives. Anethole also has potent antimicrobial properties against bacteria, yeasts, and fungi (De et al., 2002). Likewise, our results indicated that supplementation of BPA was able to influence the total enzymatic activity in milk of sows (data not shown). When a pooled milk sample per treatment was analyzed, there was an increase in enzyme activities, such as alkaline phosphatase, acid phosphatases, and β-glucuronides, among others, in GL (235 nmol) and L (175 nmol) compared to C (20 nmol) sows. In this sense, some reports suggested that adenosine triphosphate dephosphorylation in alkaline phosphatase may reduce intestinal inflammation, regulate calcium absorption, and modulate intestinal bacterial growth (Hashem et al., 2016). However, the likely contribution of essential oils transferred into sow colostrum or milk to control gut microbiota has hardly been explored.

Effects on piglet's metabolism and performance

The relationship between the sow and the fetus during pregnancy is exhibited through the placental function, which could be perceived as an important factor modulating the programming of the progeny. During the lactation period, sows exert their influence on piglets via colostrum and milk. Therefore, early stages in the piglets life (including maternal environment) may play a key role in setting the offspring's short- and long-term metabolism and health status (Chavatte-Palmer et al., 2016). Our results showed that TBARS values and CAT activity in newborn piglets were higher in GL compared to C and L sows, with similar changes to those observed in gestating sows during the early period of BPA supplementation (day 35). The results also demonstrated that L and GL piglets also increased CAT, SOD, and GSH-Px at day 20 of lactation and after weaning (7 d postweaning). Moreover, Hu et al. (2015) described that dietary supplementation with glycitein (soy isoflavone) in sows during late pregnancy and lactation increased the antioxidant factors (CAT, SOD, GSH-Px, and TAC). In addition, these isoflavones decreased the malondialdehyde content in sow´s plasma and milk and improved the milk protein and fat contents, resulting in enhanced growth performance of the suckling piglets. Meng et al. (2018) reported that improving the dietary antioxidant intake of sows might prevent or alleviate oxidative stress by increasing the antioxidant status, with beneficial implications for piglets' weight at weaning.

It is interesting to highlight that suckling piglets were also offered creep feed from lactation day 7, including BPA for the GL and L groups. Although it is possible that a confounding effect could be stated with the effect of dietary feed provided to the sows, creep feed intake was very low (<300g/litter during the whole lactation). However, this is not the case for newborn piglets to whom the prenatal maternal effect is clear. Suckling piglets from the BPA treatments showed decreases in genes related to the barrier function group, such as CLDN4, and digestive enzymes, such as the ANPEP involved in the digestion of protein. They also showed higher gene expression for proinflammatory cytokines, such as TNF-α, and their counterpart anti-inflammatory responses, with significant increases in the gene expression for IDO, the peroxisome proliferative activated receptor gamma, coactivator 1 alpha (PPARGC1-α), TGF-β1, and IL-10. In porcine small intestine, *PPARD* and related genes, such as *PPARGC1*-α, play a critical role in glucose homeostasis and adipocyte differentiation and modulate inflammation processes by providing protection against proinflammatory signaling pathways NF-κB (Mach et al., 2014). In addition, anti-inflammatory cytokines, such as TGF-β and IL-10, regulate the intestinal barrier by attenuating defects in tight junction permeability in intestinal morphology of early weaned piglets (Hu et al., 2013). Recently, Meng et al. (2018) also showed that resveratrol, a plant phenol supplemented during pregnancy and lactation, improves the antioxidant status of both sows and piglets and regulates placental antioxidant gene expression by the Keap1-Nrf2 and Sirt1 pathway in placenta. Graugnard et al. (2015) also described that maternal supplementation of a yeast mannan-rich fraction during pregnancy and lactation increased protein and Immunoglobulin G content in milk (at day 20 of lactation), with alterations in the intestinal gene expression in the progeny.

Some studies have described the presence of exosomes in porcine milk, a heterogeneous group of cell-derived membranous structures that are present in biological fluids (Zhang et al., 2018) and contain mRNA, microRNA, DNA, proteins, and lipids. They are transferred from maternal milk to neonates via the digestive tract, participating in the regulation of neonatal immune system (Gu et al., 2012), stimulating gastric and pancreatic digestion, as well as regulating intestinal cell proliferation and digestive tract development (Chen et al., 2017). Present results suggest an early activation of the immune mechanism in piglets, probably due to the transfer of different compounds into colostrum and milk. However, there is hardly any evidence regarding the effects of BPA supplementation of sow diets on the offspring, and further studies are required in order to know and understand their likely long-term effects on piglet performance and resilience against challenging diseases.

In conclusion, the results confirmed the prenatal and postnatal maternal transfer of dietary BPA supplemented to sows, with major effects on sow reproductive performance (litter size born alive), colostrum and milk composition, as well as bacteriostatic effects in milk, and interesting results on piglet oxidative status and gut health gene expression. The relevance of these results and likely changes in the early gut microbiota colonization in piglets and the growth performance of the animals after weaning should be also explored.

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Conflict of interest statement.

None declared.

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CHAPTER 5

Phytogenic compounds supplemented to gestating hyperprolific sows affects the gut health-related gene expression and histological responses in neonate piglets

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BRIEF RESEARCH REPORT
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Phytogenic Compounds Supplemented to Gestating Hyperprolific Sows Affects the Gut Health-Related Gene Expression and Histological Responses in Neonate Piglets

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This research aims to determine whether a specific blend of phytogenic compounds (BPC) supplemented in gestating hyperprolific sow diets can promote prenatal maternal effects in terms of piglet gut function and morphology. Twenty-eight (Landrace × Yorkshire) gilts and sows (parity 0 to 7) were randomly distributed by parity number and body weight into two dietary treatments: unsupplemented Control (CON) (n = 14) or CON diet supplemented with 1 g/kg feed of BPC during gestation (n = 14). The BPC supplementation during gestation of sows downregulated the neonate piglets' jejunal genes involved in oxidation (SOD2) and nutrient transport (SLC16A1/MCT1, SLC11A2/DMT1, and SLC39A/ZIP4), while IFNG and CLDN4 related to immune response and barrier function, respectively, were upregulated ($q < 0.10$). In addition, the jejunal villus height and the ratio of the villus height to crypt depth tended to increase ($p < 0.10$), while goblet cell volume density was higher ($p < 0.05$) in BPC compared to CON. In conclusion, dietary supplementation of BPC in gestating diets for hyperprolific sows influences neonatal histomorphology and expression of genes related to the intestinal function and health.

Keywords: hyperprolific sows, prenatal exposure, neonatal programming, phytogenic compounds, piglet gut health

INTRODUCTION

In modern pig breeds, hyperprolific dam-line sows are characterized by a high number of fetuses in utero compared to conventional sow lines. This has increased the litter size; however, it has also increased the percentage of piglets affected with varying degrees of intrauterine growth retardation (IUGR) and a low birth weight (LBW) (1). The IUGR characteristics may be derived from a reduced utero-placental blood flow (2), as well as increased oxidative damage, decreased mitochondrial function, impaired angiogenesis, and downregulated protein levels of glucose transporters in

placentae for LBW piglets (3). Indeed, limitations in available nutrients supplied by the sow may slow the growth of fetuses and their intestinal development (4), which are the major causes of morbidity and impaired growth performance in LBW piglets (5). Perinatal (fetal/neonatal) gut dysfunction in newborn mammals not only restricts their primary function of nutrient digestion and absorption but also could compromise their epithelial barrier function and the activation and maturation of the submucosal immune system (6). In fact, among the frequent problems suffered by IUGR piglets are intestinal growth and morphology as well as altered gene expression (7).

In previous research, we and other authors have shown that phytogenic compounds (PCs) in the sow's feed can be transferred to the offspring through amniotic fluid and milk (8, 9). This prenatal and postnatal exposure to PCs, through nutritional programming during the perinatal period, influences the oxidative status of sows and piglets (9) and also reduces weaning-associated health and welfare problems in piglets (8, 9). However, we were not able to identify the importance and relevance of early effects promoted by the prenatal dietary treatments to counteract signs of impaired gastrointestinal function in neonatal piglets. In the present study, we explored whether a blend of PCs (BPC) supplemented to the diets of gestating hyperprolific sows influences the small-intestine histomorphology and gene expression in neonate piglets. It was hypothesized that BPC supplementation would enhance the parameters related to gut health and function in neonate piglets.

MATERIALS AND METHODS

Experimental Design, Animals, and Housing

Twenty-eight DanBred hybrid line (Landrace × Yorkshire) gilts and sows (parity 0 to 7) were randomly distributed by parity number and body weight (BW) into two dietary treatment groups (n = 14 per treatment). After breeding, sows were fed an unsupplemented control diet (CON) or the CON diet supplemented with 1 kg/MT of a blend of PCs (Delacon Biotechnik GmbH, Engerwitzdorf, Austria) during the entire gestation (BPC). During the entire gestation, the sows were individually controlled, while pen was included as a random effect. Sows were assigned to individual cages (1.8 × 0.8 m) from weaning (d 0) until confirmed gestation (day 35). On day 35, sows were allotted by parity and BW into group pens of seven sows/pen (5.0 × 5.0 m) until day 110 of gestation. Thereafter, sows were moved to the farrowing unit, where they were placed in individual farrowing pens (2.0 × 2.6 m) mounted over a partially slatted floor with a heated floor pad for piglets. Water was provided ad libitum through a nipple waterer, and experimental diets were provided to sows in pelleted form. In addition, the sows' reproductive performance was monitored at farrowing.

Abbreviations: BPC, blend of phytogenic compounds; GIT, gastrointestinal tract; IUGR, intrauterine growth retardation; LWB, low birth weight; NBW, normal birth weight; PCs, phytogenic compounds; VH, Villus height.

TABLE 1 | Composition of supplemented EO in the BPC[a] .

Component	g/kg in premix
trans-Anethole	12.17
1.8-Cineole	9.73
Camphor	7.42
p-Cymene	2.56
D-Limonene	2.31
α-Terpineol	2.07
Borneol	1.83
α-Pinene	1.70
Linalool	1.46
β-Pinene	1.34

[a]Total EO content was about 45 g/MT of feed with the 1 kg/MT premix dosage.

Diets and Feeding

The Control diet offered in gestation was formulated to meet or exceed nutrient requirements for DanBred sows (10), with adaptations based on Spanish recommendations for gestating sows (11). The BCP experimental diet was the CON diet plus 1 g/kg of BPC supplement. The BPC contained 45 g/kg essential oils (EO) (EO composition described in Table 1). Based on individual body condition, sows were fed their corresponding diet as follows: 2.1 kg/d from weaning to service, an average of 2.9 kg/d from service to day 35 of gestation, and 2.5 kg/d from day 35 to day 110 of gestation (flat line). Each gestating pen was equipped with a mechanical free-access self-closing semi-cage without pneumatic actuators (Rotecna, Lleida, Spain) to keep animals individually monitored during feeding. From day 110 of gestation, sows were fed their corresponding treatment diet with ad libitum intake.

Sampling

Eight piglets per treatment (one piglet per liter, the animal representing the median BW within each litter) were selected and euthanized at farrowing (without sucking colostrum) to obtain jejunum tissue samples. Before euthanasia, piglets were anesthetized by intramuscular injection of 100 mg Telazol, 50 mg ketamine, and 50 mg xylazine per 1 ml/22 kg of BW. Thereafter, pigs were euthanized by jugular vein injection using sodium pentobarbital 0.5 ml/kg of BW. The jejunum tissue obtained for the gene expression analysis (~1.5 cm^2) was placed in 1 ml of RNAlater (Applied Biosystems, Foster City, CA, USA) and stored at room temperature (25°C) during the first 24 h after collection. Thereafter, the tissue samples were stored at −80°C until analysis. Additional jejunum tissue samples (~2 cm^2) were placed in individual tubes containing formaldehyde solution for histomorphology determination.

Jejunal Gene Expression Study by qPCR

Piglet jejunum gene expression of 56 genes related to intestinal health and functionality was quantified by RT-qPCR using an OpenArray Real-Time PCR Platform as specified by Reyes-Camacho et al. (9). Briefly, total RNA was extracted from 50 mg of jejunum tissue using the Ambion RiboPure Kit

(Life Technologies, Carlsbad, CA, USA) by following the manufacturer's protocol. RNA concentration was measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop products, Wilmington, DE, USA), and RNA quality was checked using Agilent 2100 Bioanalyzer equipment (Agilent Technologies, Santa Clara, CA, USA). Primers were designed by spanning exon-exon boundaries using the Primer Express 2.0 software (Applied Biosystems), and genomic DNA amplification and primer dimer formation were controlled. One replicate per sample was run in a TaqMan OpenArray gene expression custom plate format for gene expression with 56 assays of 48 samples per plate (OpenArray plate) in a QuantStudio 12K Flex Real-Time PCR System (Applied Biosystems, Foster City, CA). A total of 56 genes were previously selected based on the bibliography, including four reference genes. Details regarding genes and primers can be found in previously published work by González-Solé et al. (12).

Jejunal Histomorphology Measurement

For the histomorphology evaluation, jejunum samples were fixed for 24-48 h in neutral-buffered 10% formalin. After dehydration and embedding in paraffin wax, sections of ~3 µm were stained with hematoxylin and eosin. Villus height (VH), crypt depth (CD), number of intraepithelial lymphocyte (IEL), and number of goblet cells (GC) per 100 micrometers of villus height were measured blinded in 10 well-oriented villi and crypts per sample by the same person using a light microscope (BHS, Olympus, Barcelona, Spain).

Statistical Analyses

The individual newborn piglet was considered as the experimental unit. All data were analyzed considering the treatment as the main effect. The results are presented as means with their corresponding SEM. For morphology, data were analyzed by using the TTEST procedure of the statistical package SAS 9.4 (SAS Inst. Inc., Cary, NC, USA), and significant differences were declared at $p < 0.05$, while $0.05 < p < 0.10$ were considered significant tendencies.

Gene expression statistical analysis was performed as specified by González-Solé et al. (12). Briefly, data were collected and analyzed using the Thermo Fisher Cloud software 1.0 (Applied Biosystems) applying the applying the 2^{-11Ct} method for relative quantification (RQ). The normal distribution of the RQ values was checked with the Shapiro.test function of R 3.5.3 software, and log2 transformation was applied if required. One-way ANOVA was performed, and the Benjamini-Hochberg false discovery rate (FDR) was used for multiple-testing correction of p-values, defining the model:

$$Y_{ij} = \mu_i + treat_j + \varepsilon_{ij}$$

where Y_{ij} is each observation of the outcome variable, μ_i is the global mean, $treat_j$ is the main effect of the treatment, and ε_{ij} is the experimental error term. Significant gene expression differences between functional groups were accepted at $p < 0.05$, whereas significant gene expression differences between treatments were accepted at FDR (q-values) < 0.10.

TABLE 2 | Effects of BPC supplementation during gestation of hyperprolific sows on jejunal histomorphology of neonate piglets[a].

Jejunum morphology	Treatments		SEM[b]	p-value[c]
	CON	BPC		
Villus height (VH), µm	621	756	55.4	0.072
Crypt depth (CD), µm	65.4	66.2	2.12	0.746
VH:CD ratio, µm/µm	9.8	11.9	0.72	0.060
Goblet cell density/100 µm VH	0.76	1.02	0.11	0.033
Lymphocyte density/100 µm VH	0.95	0.91	0.13	0.788

[a]Values are expressed as means of eight newborn pigs per treatment (n = 8). Treatments: CON, control diet; BPC, control plus blend of phytogenic compounds.
[b]Standard error of the mean. Statistical significance was assumed at ($p < 0.05$) while statistical tendency was assumed at $p < 0.10$ using the T-test.

RESULTS

The Piglets' Jejunum Histomorphology

The jejunal histomorphology results (Table 2) showed that, compared to the CON group, BPC tended to increase the VH ($p = 0.072$) and VH:CD ratio ($p = 0.060$) in neonate piglets. The GC density improved in the BPC group ($p = 0.033$). However, no significant differences ($p > 0.05$) were observed in CD or IEL density.

The Piglets' Jejunum Gene Expression

The results for the jejunum gene expression analysis of neonate piglets are shown in Table 3. Although only six genes showed statistical differences between treatments ($p < 0.05$, $q < 0.10$), the other six genes that showed statistical differences ($p < 0.05$) between functional groups are presented. Compared to the CON, a downregulation ($q < 0.10$) was observed in the BPC treatment for the SOD2 gene from the oxidation group and the SLC16A1/MCT1, SLC11A2/DMT1, and SLC39A/ZIP4 genes from the nutrient transport group, while from the immune response and barrier function groups an upregulation ($q < 0.10$) was observed for IFNG and CLDN4, respectively. Furthermore, there were no significant differences between treatments ($p < 0.05$, $q > 0.10$) for SLC15A1/PEPT1, ALPI, IDO1, DAO1, CCK, and OCLN from nutrient transport, immune response, enzyme/hormone, and barrier function group, respectively.

DISCUSSION

Although the study did not aim to research the sow's reproductive performance, our findings indicate that BPC improved both the total number of piglets born (19.9 vs. 17.8) and the number born alive (17.2 vs. 14.7), while the neonate piglet BW tended to decrease (1.17 vs. 1.33 kg) (Supplementary Table 1) in agreement with previous studies with a larger sample size in commercial conditions (9). LBW in piglets has been associated with a certain degree of IUGR (1), with impaired intestinal structure, and with a transcriptomic profile and bacterial colonization in neonatal IUGR piglets (13). In previous studies by our group, it has been observed that at birth, the jejunum expression of 10 genes, involved in the immune, digestive,

TABLE 3 | Effects of BPC supplementation during gestation of hyperprolific sows on related jejunal health-function mRNA relative expression of neonate piglets[a] .

Function	Genes[b]	Treatments		SEM[d]	p-value[e]	q-value[f] (FDR)
		CON	BPC[c]			
Oxidation	SOD2	1.00	0.71	0.051	0.009	0.023
Nutrient transport	SLC15A1/PEPT1	1.00	0.71	0.074	0.048	0.256
	SLC16A1/MCT1	1.00	0.55	0.083	0.002	0.040
	SLC11A2/DMT1	1.00	0.58	0.070	0.004	0.018
	SLC39A4/ZIP4	1.00	0.42	0.141	0.012	0.087
Immune response	IFNG	1.00	6.18	1.393	0.003	0.043
	ALPI	1.00	0.58	0.090	0.011	0.117
	IDO1	1.00	3.43	0.634	0.031	0.155
Enzyme/hormone	DAO1	1.00	0.67	0.068	0.008	0.106
	CCK	1.00	0.66	0.082	0.032	0.238
Barrier function	CLDN4	1.00	3.15	0.601	0.010	0.087
	OCLN	1.00	0.82	0.046	0.040	0.250

[a]Data are means of eight newborn pigs per treatment (n = 8).
[b]Genes: SOD2, superoxide dismutase-2; SLC15A1/PEPT1, solute carrier family 15 (oligopeptide transporter) member 1; SLC16A1/MCT1, solute carrier family 16 (monocarboxylate transporter 1) member 1; SLC11A2/DMT1, solute carrier family 11 (proton-coupled divalent metal ion transporter) member 2; SLC39A4/ZIP4, solute carrier family 39 (zinc transporter) member 4; IFNG, interferon gamma; ALPI, intestinal alkaline phosphatase; IDO1, indoleamine 2,3 dioxygenase; DAO1, diamine oxidase; CCK, cholecystokinin; CLDN4, claudin-4; OCLN, occludin. Treatments: CON, control diet; BPC, control plus blend of phytogenic compounds.
[c]Gene expression means for BPC are calculated in relation to the mean from CON group. [d]
Standard error of the mean.
[e]p-Values come from the ANOVA test.
[f] Significant gene expression differences between treatments were accepted at FDR (q-values) < 0.10.

and stress responses, is clearly different according to the piglet BW category between half-sibling piglets (light vs. average littermates) (unpublished data).

In this study we explored the effects of prenatal BPC exposure on histomorphology, and gene expression related to the health and functioning of the newborn piglet jejunum from hyperprolific dam-line sows. The jejunum plays an important role in nutrient uptake as well as immune system programming and metabolic programming in the pigs' early life (14). To our knowledge, there are no studies reporting the effects of PCs on the intestinal gene expression and morphology of neonate pigs. Positive findings were observed in histomorphology in neonate piglets of the BPC group, which showed improvements in the VH, the ratio of VH:CD, and the goblet cell density compared to newborn piglets from the CON group. The effects promoted by BPC appear to attenuate the early effects derived from IUGR, which is known to affect the intestinal cell proliferation-apoptosis balance in neonate piglets (5). They also affect the intestinal growth and morphology in association with the altered gene expression of growth-related proteins (7) and modify the volume density and function of epithelial cell types such as goblet cells, which are important constituents of the innate defense system (15). An increased VH and ratio VH:CD may suggest an improvement in the digestive and absorptive function of the intestine as a result of an increased absorptive surface, an expression of brush border enzymes, and nutrient transport systems (16).

In highly prolific sows, uterine capacity or insufficiency can affect fetal growth when competition by littermates for limited uterine space and nutrients becomes increasingly critical (17). Evidence in human studies has shown that placental uptake and transport of nutrients such as thiamin, folic acid, and glucose by BeWo cells are modulated by PCs, such as polyphenols (18). The solute carrier (SLC) genes are a large family of protein transporters in mammals, and their gene expression is notably affected if there is a deprivation of nutrients such as amino acids (AAs), which implies an effect on the capacity of the SLC to regulate intracellular nutrient concentrations and, in addition, detect alterations in extracellular nutrient levels (19). For instance, the gene expression of the SLC38 family was upregulated in hypothalamic cells N25/2 of mice after AA starvation (20). Glucose deprivation increases MCT1 protein expression and their interaction in oxidative tumor cells (21). Moreover, the hypoxia induced an increase in MCT1 plasma membrane expression in glioma cells, both in in vitro and in vivo models (22). The gene expression analysis of the current study showed that prenatal exposure of the fetus to BPC led to downregulation in the jejunum of nutrient transport-related genes SLC16A1/MCT1, SLC11A2/DMT1, and SLC39A/ZIP4 in newborn piglets. This may indicate that compared to BPC, potential deprivation of nutrients, especially for Fe^{2+} (23), short-chain fatty acids (24), and zinc (25), respectively, induced an adaptative upregulation of the abovementioned SLC genes in newborn piglets from the CON group.

When inflammation occurs in the gastrointestinal tract (GIT), this can result in a decrease in digestive efficiency and reduced absorption of nutrients (26). In this study, the prenatal exposure of the fetus to BPC induced an upregulation of the immune response IFNG and barrier function CLDN4 genes. IFNG is critical in increasing and mediating intestinal immunity because of its role in recognizing and eliminating pathogens. IFNG can have several functions; it can exhibit its immunomodulatory

effects by controlling inflammatory response, enhancing antigen processing and presentation, increasing leukocyte trafficking, inducing an antiviral state, boosting the antimicrobial functions, and affecting cellular proliferation and apoptosis (27). On the other hand, expression of Claudin proteins such as CLDN4 within the small intestine of newborn piglets plays a vital role in controlling barrier function and mucosal homeostasis (epithelial tight junctions), particularly on the apical aspect of lateral surfaces of intestinal epithelial cells where they help regulate ion and macromolecule movement across the intestinal epithelium (28). It has been described that transcription levels of IFNG and CLDN4 showed a lower expression in LBW than normal birth weight (NBW) piglets (29). Thus, results shown in the BPC group suggest that upregulation of IFNG and CLDN4, together with the development in jejunal histomorphology, could help to improve the gut health of LBW piglets.

In addition, the induction of mitochondrial oxidative stress during periods of nutrient deprivation in animals has been associated with decreased metabolic requirements, higher mitochondrial membrane potential, and increased superoxide production at the level of the complex III of the electron transport chain (30). Since SOD2 activity is regulated in response to mitochondrial superoxide production, the upregulation of mitochondrial SOD activity results in an overproduction of hydrogen peroxide as a product of the disproportionation reaction of superoxide anion catalyzed by SOD (31). In the present study, the SOD2 gene was downregulated in the jejunum of BPC newborn piglets, which may indicate that compared with CON, undue intrauterine oxidative stress was avoided in newborn piglets from the BPC group. In swine, it has been described that oxidative damage to DNA (8-hydroxy-20-deoxyguanosine, 8-OHdG) and proteins (carbonyls) measured in plasma increased after the stress period such as weaning, and this coincides with a rise in enzymatic antioxidant activity including SOD. Furthermore, oxidative damage to macromolecules by mitochondrial dysfunction and cellular oxidative stress is more important in LBW than in NBW piglets, as measured concentrations of 8-OHdG and carbonyls are significantly higher (32). Thus, it suggests that upregulation of the oxidation SOD2 gene shown in CON may indicate a defense mechanism against mitochondrial dysfunction and cellular oxidative stress conditions.

It has also been reported that several dietary plants and their bioactive components have the potential to modulate human transcriptome profiles of various biological pathways involved in oxidative, immune response, glucose and fatty acid metabolism, and inflammatory and cell signaling pathways (33). In previous studies, we have observed that perinatal exposure to a blend of PCs containing eucalyptol, p-cymene, linalool, anethole, and thymol influenced the overexpression of the barrier function (MUC2), and immune response (PPARGC-α, TNF-α, TGF-β1, IDO1, and IL-10) genes in suckling piglets (9). In this study, we have observed that these effects may be anticipated by administering BPC in the sow gestation diet. In terms of fetal programming, maternal nutrition status during fetal development may result in a permanent imprint by changing the epigenetic state of the fetal genome and its gene expression (34). Although the action modes of several PCs are not yet fully clarified, the main action modes of the major compounds used in BPC, such as trans-anethole (35), 1.8-cineole (36), and Camphor (37), exhibit important biological properties. These include anti-inflammatory, antioxidant, and antimicrobial properties that could have influenced the changes on the intestinal histomorphology and gene expression of neonate piglets in this study. Therefore, the prenatal exposure of the fetus to PCs may indicate a positive means to attenuate the intestinal dysfunction in LBW piglets, especially in terms of structure and functionality.

In conclusion, BPC supplemented to hyperprolific sows during gestation influenced the prenatal programming of some intestinal biological functions. This was evidenced by the improved histomorphology and gene expression related to the nutrient absorption, immune response, intestinal integrity, and oxidative stress in the jejunum of neonate piglets.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by CEEAH-Univesritat Autònoma de Barcelona.

AUTHOR CONTRIBUTIONS

DR-C performed the animal trial, the statistical analyses, and writing—original draft preparation. JP and DS-O were the principal investigator and contributed to conceptualization and experimental design. EP and JV conducted the conceptualization and experimental design. TA supervised the interpretation of the study results and reviewed the draft. LC-M and JF contributed to the design and set-up of the gene expression studies. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.639719/full#supplementary-material

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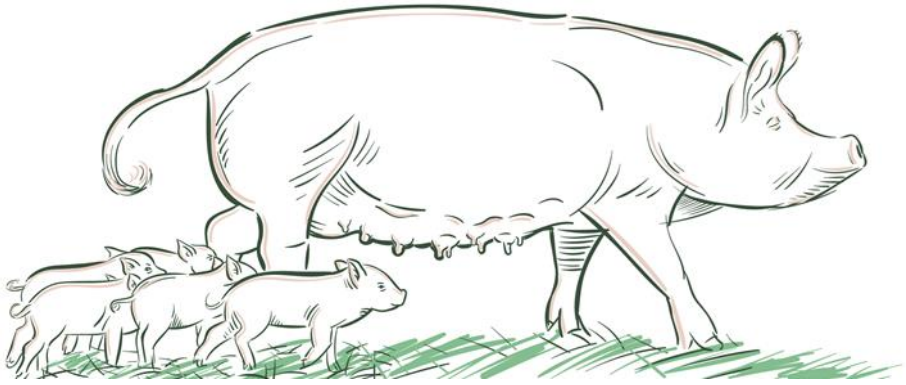
Conflict of Interest: EV, TA, and JV are employees of DELACON Biotechnik GmbH. (Engerwitzdorf, Austria) which is a global supplier of phytogenic compounds.

The remaining 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.

Chapter 5



CHAPTER 6

Prenatal exposure to innately preferred D-limonene and *trans*-anethole does not overcome innate aversion to eucalyptol, *affecting* growth performance of weanling piglets

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Article

Prenatal exposure to innately preferred D-limonene and *trans*-anethole does not overcome innate aversion to eucalyptol, *affecting* growth performance of weanling piglets

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Simple Summary: Weanling piglets appear to be poorly adapted and motivated to ingest solid feed due to the innate reluctance of young animals to ingest an unfamiliar feed or flavor, i.e., feed neophobia, which commonly results in a period of underfeeding. This, and other common wean stress factors, lead to gastrointestinal disorders and impaired growth performance. Increasing the preference or familiarity for a certain type of food or for specific flavors may improve voluntary feed intake in weanling piglets. Botanical compounds (BCs) are described as functional feed additives and include sensorial properties that are able to influence feed intake and growth in pigs by dietary supplementation or sensory maternal learning. In this study, the effects of BCs such as D-limonene, *trans*-anethole, and eucalyptol on innate feed preference and growth performance of weanling piglets were evaluated by means of a double-choice feeding test and pre- and postnatal exposure to these compounds.

Abstract: In the present research, two studies were performed to determine the effects of specific botanical compounds (BCs) on the innate feed preference and feed intake of piglets, as follows: Exp. 1 studied the innate feed preferences of post-weaning piglets using a double-choice feeding test. A total of 828 weaned piglets were distributed into 36 pens (23 pigs/pen) and assigned to three dietary pair choice feeding options (n = 12): unsupplemented prestarter diets (reference) versus reference plus D-limonene, *trans*-anethole, or eucalyptol. Piglets showed a preference for diets with D-limonene (53.8%) and *trans*-anethole (54.5%), and an aversion to eucalyptol (41.6%) ($p < 0.05$). Exp. 2 studied whether the prenatal and perinatal exposure to D-limonene, *trans*-anethole, and eucalyptol influences the feed intake and growth of newly-weaned piglets. Twenty-eight gestating and lactating sows were distributed into two dietary treatments (n = 14): unsupplemented Control diets or Control plus a blend of BCs (BBC; containing D-limonene, *trans*-anethole, and eucalyptol). D-limonene, *trans*-anethole, and eucalyptol were transferred into the placental fluid, and D-limonene and *trans*-anethole into the milk ($p < 0.05$). Furthermore, weanling piglets (n = 200; Control) and (n = 203; BBC) received the same treatment as their mothers in prestarter diets. The early response after weaning showed that piglets' post-weaning BW gain was higher in the Control ($p < 0.05$) group than in those exposed to BBC. In conclusion, prenatal exposure to preferred D-limonene and *trans*-anethole, or familiarity to eucalyptol did not help to overcome the innate aversion to eucalyptol and its negative effect on weanling piglets' BW.

Keywords: botanical compounds, weanling piglets, innate feed preference, sensory maternal learning, growth performance, maternal transfer, hyperprolific sows

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1. Introduction

Farm pigs are fed nutritionally balanced diets with no choice, a practice that implies that voluntary feed intake is mostly based on nutritional needs rather than sensory profiles. However, there are critical phases in pig management, such as the post-weaning period [1], when palatability may have a great influence on feed intake. A major challenge for raising pigs is the transition from highly-digestible (liquid) milk to the less-digestible and more complex solid feed, and neophobia is an important aspect of post-weaning feed refusal [2]. Common consequences of this behavior are anorexia and undernutrition [3] until piglets are completely familiarized with their new feeding and environmental conditions. These effects on feed intake, coupled with other wean stress factors, i.e., physiological, environmental, and social, contribute to gastrointestinal and immune system dysfunctions leading to impaired health and growth [4]. Botanical compounds (BCs), such as those used in the present study, D-limonene [5], *trans*-anethole [6], and eucalyptol [7], have been reported to act as sensory feed additives that are able to influence feed preference and intake in pigs [8] [9].

In addition, it has been described that young animals, including pigs, can learn about flavors based on the flavors in the maternal diet that are part of the amniotic fluid (prenatal) or milk (postnatal), which may reduce neophobia and modulate preference for similarly flavored food types at weaning [10]. For instance, prenatal exposure to anise [10], perinatal exposure (amniotic fluid and milk) to limonene, menthol, carvone [11], and anethol, cinnamaldehyde, and eugenol [12], all increased feed intake and growth of piglets after weaning. The literature has also described the influence of BCs on the mammalian chemosensory taste system by activating the transient receptor potential channels and taste receptor cells (referred to as taste and nutrient chemosensing) [13]. It has been suggested that in mammals, the sweet, umami, and salty tastes are innately preferred, whereas bitter and many sour tastes are innately rejected [14].

In the present study it was hypothesized that different BCs may promote innate feed preference responses, either preferred or aversive, in early weaning piglets; while prenatal exposure to these compounds will help to reduce neophobia and enhance the early feed intake and growth of piglets after weaning. Thus, two studies were conducted under commercial conditions designed to assess the effects of specific dietary BCs on innate feed preference and sensory maternal learning as follows: Exp. 1 used a double-choice feeding test (DCHT) to investigate the effect of prestarter diets supplemented with D-limonene, *trans*-anethole, or eucalyptol versus unsupplemented control diets on feed preference at one week of post-weaning (d 8 to d 14). Meanwhile, Exp. 2 aimed to determine the transfer (presence or absence) of D-limonene, *trans*-anethole, and eucalyptol from feed into the placental fluid and milk of hyperprolific sows supplemented with a blend of BCs (BBC) including the above-mentioned compounds. Exp. 2 also studied the influence of these compounds on the growth performance (BW) of newly-weaned piglets (d 1 to d 7).

2. Materials and Methods

All animal experimentation procedures were approved by the Ethics Committee of the Universitat Autònoma de Barcelona in compliance with the European Union guidelines 2010/63/EU for the care and use of animals in research (code CEEAH2788M2).

2.1. Exp. 1 (Double-choice feeding test)

2.1.1. Experimental design, animals, and housing

A total of 828 male and female (50:50) weanling pigs ([Large White × Landrace] × Piétrain) were selected for use in Exp 1. Piglets were weaned at d 21 of age and housed in the weaning unit on the same commercial farm (Farm 1). During the first post-weaning week (d 1 to 7) piglets were adapted to the new environmental conditions by being offered an unflavored commercial prestarter diet. At post-weaning day 7, weaned pigs with an initial BW of 6.11 ± 0.83 kg were blocked by sex and distributed according to BW into 36

pens (18 male pens and 18 female pens; 23 pigs/pen). Thereafter, pens were randomly assigned to three different dietary treatments (n = 12 replicate pens per treatment). Male and female pens were assigned equally to treatments. Each pen (3.20 m2) was equipped with two commercial pan feeders (Maxi hopper, Rotecna, Spain) and a nipple bowl drinker to provide ad libitum access to feed and water. The floor was completely slatted, and the temperature and ventilation rates were controlled using central and forced ventilation with an automatic cooling system.

2.1.2. Feeding programme and dietary treatments

The double-choice feeding test (DCHT) was performed during the second week after weaning (d 8 to 14). Before offering the new diets, all remaining feed was removed from the feeder used during the prestarter period. Two commercial pan feeders with hoppers were then placed in each pen to provide the reference diet and the assigned experimental diet. To avoid biases, both pan hopper feeders were hand-filled to ensure totally free access to both diets. In addition, to prevent side-effect bias in feeding behaviors, the position of the feeders inside the pen (right or left) was switched once at day 4. Experimental diets in the DCHT were the unsupplemented (basal) reference diet or the reference diet plus a botanical compound (BC) (D-limonene (7.50 g/kg), *trans*-anethole (12.17 g/kg), or 1,8-cineole (Eucalyptol) (9.73 g/kg) of feed, respectively with 2 kg/t premix dosage (Delacon Biotechnik GmbH, Engerwitzdorf, Austria) added as essential oils with wheat bran and wheat semolina as the carrier. For each dietary treatment, the BCs were pre-mixed with 5 kg of basal diet before being included in the mixer during the feed preparation process. All diets were formulated to meet the nutrient requirements for growth of newly-weaned piglets [15]. The composition of the prestarter basal diet used in the DCHT is presented in Table 1.

2.1.3. Experimental Procedures, Data and Sample Collection

Piglet BW was recorded at day 8 and day 14 of post-weaning. The feed intake for the reference and the experimental diets was measured in the second post-weaning week (day 8 to 14). In the present study, this was considered to be the innate response period of animals to the different sensory features of the feed (short-term preference) according to the methodology and reference periods described by Solà-Oriol et al. [16] and Roura et al. [17]. The feed intake values of each diet were expressed as described in Villagómez-Estrada et al. [18]. Briefly, feed intake per replicate pen was standardized by dividing feed intake by the average pig BW and by the number of pigs per replicate pen. Consequently, preference for the supplemented test diet relative to the reference diet was calculated as the percentage contribution of the test diet to total feed intake according to the following equation described by Solà-Oriol et al. [16]:

%PREFERENCE = Test diet intake / (Test diet intake) + (Reference diet intake) x 100

Therefore, preference values can range between 100 and 0%. A value of 50% indicates indifference with respect to the reference diet, whereas values significantly higher or lower than 50% indicate a significant preference or aversion, respectively.

2.1.4. Statistical analysis

In this study, different procedures with the SAS 9.4 (SAS Inst. Inc., Cary, US) statistical package were used to analyze all of the data.

Standardized feed intake, percentage preference values, and piglet performance were analyzed with ANOVA using the MIXED procedure. The model included the fixed effects of treatment and the random effects of sex. The pen was considered the experimental unit (23 pigs/pen). Data were examined for outliers using the ROBUSTREG procedure. The percentage preference values for each experimental diet were transformed using the logit transformation log according to the following equation: Ln(Pref/(1-Pref)) and, thereafter compared to the neutral value of 50% using a Student's TTEST procedure. All the results are presented as least square (LS) means with their corresponding SEM considering a

Tukey adjustment. Significant difference was determined at a probability $p < 0.05$ and tendencies were considered when *p*-values were between >0.05 and <0.10.

Table 1. Ingredients and nutrient composition of diets used in Experiment 1 and 2.

	Experiment 1	Experiment 2		
Item	**Piglets pre-starter**	**Sows′ gestation**	**Sows′ lactation**	**Piglets pre-starter**
Ingredients, %				
White broken rice	60.00	-	-	-
Soybean meal, 47 % CP	28.55	2.50	13.50	5.00
Wheat	3.86	9.00	25.55	10.00
Soy oil	3.00	-	-	-
Barley	-	35.00	10.00	15.00
Maize	-	22.70	27.01	44.00
Wheat middling´s	-	15.00	7.00	-
Sweet milk whey	-	-	-	10.26
Sunflower meal	-	5.65	4.50	-
Sugar beet pulp	-	3.10	2.50	-
Maize flour	-	-	-	1.01
Soybean protein concentrate	-	-	-	5.00
Rapeseed meal	-	2.50	4.50	-
Calcium carbonate	-	0.99	1.25	-
Lard	-	1.05	1.00	-
Plasma	-	-	-	1.50
Extruded soybean	-	-	-	5.13
Dicalcium phosphate	2.27	0.99	1.25	0.87
Salt	0.57	0.40	0.50	0.25
L-Lysine HCl	0.56	0.31	0.63	0.81
DL-Methionine	0.29	-	-	0.23
L-Threonine	0.28	0.10	0.18	0.21
L-Valine	0.15	-	-	0.09
L-Tryptophan	0.08	-	-	0.03
Mycofix plus 3.E	-	0.10	0.10	-
Vit-Min Premix	0.40[1]	0.50[2]	0.50[2]	0.60[3]
Calculated nutrient composition				
Net energy, kcal/kg	2591	2261	2455	2438
Crude protein, %	19.5	13.0	16.7	17.0
Calcium, %	0.70	0.85	0.91	0.30
Total phosphorus, %	0.72	0.56	0.57	0.40
Digestible phosphorus, %	0.38	0.35	0.37	0.30
SID Lysine, %	1.46	0.60	1.00	1.11

[1] Supplied the following per kg of diet: 7,000 IU of vitamin A (acetate); 500 IU of vitamin D3 (cholecalciferol); 250 IU of vitamin D (25-hydroxicholecalciferol); 45 mg of vitamin E; 1 mg of

vitamin K3; 1.5 mg of vitamin B1; 3.5 mg of vitamin B2; 1.75 mg of vitamin B6; 0.03 mg of vitamin B12; 8.5 mg of D-pantothenic acid; 22.5 mg of niacin; 0.1 mg of biotin; 0.75 mg of folacin; 20 mg of Fe (chelate of amino acids); 2.5 mg of Cu (sulphate); 7.5 mg of Cu (chelate of glycine); 0.05 mg of Co (sulphate); 40 mg of Zn (oxide); 12.5 mg Zn (chelate of amino acids); 12.5 mg of Mn (oxide); 7.5 of Mn (chelate of glycine); 0.35 mg of I, 0.5 of Se (organic); 0.1 mg of Se (sodium). [2] Supplied the following per kg of diet: vitamin A (retinyl acetate), 10,000 IU; vitamin D3 (cholecalciferol), 2,000 IU; vitamin E (acetate de tot-rac-3-tocopheryl), 45 mg; vitamin K3 (menadione nicotinamide bisulphite), 3 mg; vitamin B1 (thiamine mononitrate), 3 mg; vitamin B2 (riboflavin), 9 mg; vitamin B6 (pyridoxine hydrochloride), 4.5 mg; vitamin B12 (cyanocobalamin), 0.04 mg; nicotinamide, 51 mg; pantothenic acid (calcium D-pantothenate),16.5 mg; biotin (D-(+)-biotin), 0.15 mg; folic acid, 1.8 mg; choline chloride, 350 mg; iron (as iron sulphate monohydrate), 54 mg; zinc (as zinc oxide), 66 mg; man-ganese (as manganese oxide), 90 mg; iodine (as calcium iodine anhydrous), 1.2 mg; selenium (as sodium selenate), 0.18 mg; copper (as copper sulphate pentahydrate), 12 mg; ethoxyquin, 4 mg; D,L-malic acid, 60 mg; fumaric acid, 75 mg; sepiolite, 907 mg; vermiculite 2001 mg; colloidal silica 45 mg. [3] Supplied the following per kg of diet: vitamin A (retinyl acetate), 10,000 IU; vitamin D3 (cholecalciferol), 2,000 IU; vitamin E (allrac α-tocopheryl-acetate) 100 ppm; choline chloride, 187 ppm; iron (as iron sulphate monohydrate),100 ppm; iodine (potassium iodide), 100 ppm; copper (as copper sulphate pentahydrate),149 ppm; manganese (as manganese oxide), 58 ppm; zinc (as zinc oxide), 120 ppm; selenium (as sodium selenate), 0.30 ppm; selenomethionine (produced by Sac-charomyces cerevisiae), 0.1 ppm; butyl-hydroxytoluene (BHT), 63 ppm; citric acid, 8 ppm.

2.2. Exp. 2 (Sensory maternal learning)

2.2.1. Experimental design, animals, and housing

According to findings of innate feed preference for D-limonene, *trans*-anethole, and innate aversion for eucalyptol in Exp. 1, Exp. 2 was performed to study whether the sensory maternal learning could mitigate (familiarize) the innate aversion to eucalyptol of weanling pigs. Thus, Exp. 2 was performed as described below.

From farm 2, a total of 28 gilts and sows (up to parity 7) from a hyperprolific DanBred hybrid line (Landrace x Yorkshire) and their piglets (n = 409) were distributed by parity number and BW into two dietary treatment groups (n = 14 dams per treatment). After breeding, sows were fed unsupplemented control diets during gestation and lactation (Control) or Control diets supplemented with 1 kg/t of a blend of botanical compounds (Delacon Biotechnik GmbH, Engerwitzdorf, Austria) throughout the whole gestation and lactation period (BBC). The BBC contained 45 g/kg essential oil (EO) of feed with a 1 kg/t premix dosage (EO composition described in Table 2). After weaning, piglets from sows that had received the same diet were mixed together in a single group (n = 200; Control) and (n = 203; BBC), respectively, and moved to the nursery unit on the same farm in order to evaluate individual BW at weaning and growth performance during the prestarter phase (day 7) of post-weaning. Piglets were weaned at 23.82 ± 2 days of age with a mean BW of 5.35 ± 1.05 kg. Room temperature and ventilation rate were automatically controlled at approximately 24 ºC using thermostatically controlled heaters and exhaust fans.

2.2.2 Feeding programme and dietary treatments

Control diets for each experimental period were formulated to meet or exceed nutrient requirements for DanBred sows [19], with adaptations based on Spanish recommendations for gestating and lactating sows, and for prestarter piglets [20] (Table 1). Experimental diets were Control plus 1 kg/t of BBC. Sows were fed an average of 2.65 ± 0.05 kg

feed per day of the gestation diet of their corresponding treatments from service to 110 d of gestation, based on individual body condition. From 110 d of gestation and during lactation, sows were fed *ad libitum*. In addition, piglets received the same experimental treatments in the creep feed (as mash from lactation day 7 to weaning) and prestarter diets. The Control creep feed and prestarter diets were unsupplemented, whereas the experimental diets were Control supplemented with 1 kg/t of BBC. At weaning, all piglets from the same experimental treatment were allotted to a single large pen (1 pen 32.00 m^2/treatment) with (n = 200; Control) and (n = 203; BBC) piglets in each pen, respectively, and received corresponding pelleted prestarter experimental treatments until day 7 of post-weaning. Each pen was equipped with a fully slatted floor, three *ad libitum* pan hoppers (Swing Feeder R3 Wet WTF, Rotecna, Spain) in the middle of the pen and nipple drinkers on the wall to provide ad libitum access to feed and water.

Table 2. Botanical composition of supplemented EO in the BBC[1].

Botanical component	g/kg in premix
trans-anethole	12.17
1,8-cineole	9.73
Camphor	7.42
p-cymene	2.56
D-limonene	2.31
α-terpineol	2.07
Borneol	1.83
α-pinene	1.70
Linalool	1.46
β-pinene	1.34

[1] Total EO content was about 45 g/t of feed with the 1 kg/t premix dosage.

2.2.3. Experimental Procedures, Data and Sample Collection

Placental fluid samples (60 mL per sow) were collected at farrowing as described by Blavi et al. [12], whereas milk samples (30 mL per sow) were collected at day 20 of lactation, both from the same subset of sows to determine maternal transfer of compounds (n = 12 per treatment). Samples were not filtered and were immediately chilled on-farm and stored at −20 °C until analysis. In addition, the individual piglet BW was recorded at weaning and day 7 post-weaning.

Maternal transfer of volatile BCs through placental fluid and milk was determined by solid-phase microextraction, gas chromatography-mass spectrometry based on the volatile BC profile characterized in the tested BBC as follows: Twelve milliliters of amniotic fluid or milk were placed into 20-mL sample vials containing 2 g NaCl (Supelco Inc., Bellefonte, PA), and 40 μL of eugenol at 50 ppm was added as internal pattern. A HP6890 Series II gas chromatograph (Agilent Technologies, Salt Lake City, UT) equipped with an electronic impact HP5973 detector (Agilent Technologies) was used for 30 min to extract volatiles and analyze the analytic content in the headspace. In addition, a Comb pal autosampler (CTC Analytics AG, Zwingen, Switzerland) was used to perform solid-phase microextraction. Injection was made in a splitless mode for 1 min at 265°C. A TRB-WAX gas chromatographic column with the dimensions 60 m/mm length, 0.25-mm i.d., and 0.25-μm film thickness (Supelco Inc.) was used. Column flow (He) was 1.5 mL/min. Injector temperature was maintained at 100°C for 10 min and raised to 265°C, at 12°C/min, for 17 min. The data were processed using data analysis (Agilent Technologies). The minimum detection limits were 16 ppb.

In addition, the control samples (without BBC supplementation) were used for the systematic analysis of samples, with 4.8 ppm of anethole to verify the instrumental sensitivity response to the tested BBC. Results for relative peak abundance were estimated

based on the ratio (abundance/retention time) for each compound and expressed as a percentage of the proportional increase in concentrations in relation to the assigned reference values (basal value = 100%) in unsupplemented Control sows.

2.2.4. Statistical analysis

Each piglet was considered an experimental unit for BW evaluation. After transformation, placental and milk transfer values, as well as piglet BW were analyzed using the TTEST procedure of the SAS statistical package 9.4 (SAS Inst. Inc., Cary, US). All data were analyzed considering the treatment as the main effect, and the results are presented as least square (LS) means with their corresponding SEM. Finally, mean significant differences were declared at $p < 0.05$, while $0.05 \leq p < 0.10$ were considered significant trends.

3. Results

3.1. Exp. 1 (Double-choice feeding test)

3.1.1. Innate feed preference

The piglets' innate preference for the experimental diets is shown in Table 3. The results indicate a greater preference among piglets for diets supplemented with D-limonene (53.8% *vs* 46.2%; $p = 0.021$) and *trans*-anethole (54.5% *vs* 45.5%; $p = 0.049$), while piglets exposed to the diet supplemented with eucalyptol preferred the unsupplemented reference diet, indicating an aversion to the supplemented diet (41.6% *vs* 58.4%; $p = 0.002$).

Table 3. Feed preference percentage of weaned piglets (d 8 to d 14) for tested BCs versus unsupplemented reference diets by using a double-choice feeding test.

Item	Treatment groups		
	D-limonene	**trans-anethole**	**Eucalyptol**
Feed preference[1, 2], %	53.8 ± 3.77	54.5 ± 5.93	41.6 ± 5.74
p-value[3]	0.021	0.049	0.002

[1] Data are means of ($n = 12$) pens with 23 pigs per replicate pen. [2]Preference index significantly ($p < 0.05$) >50% indicate preference, while <50% indicate aversion using T-test. [3]*p*-value were calculated by using the logit transformation log.

3.1.2. Piglet's growth performance

Although Exp. 1 was not designed to study the piglets' performance due to the simultaneous exposure to the standard and supplemented diet, interestingly, piglets within the group exposed to D-limonene and *trans*-anethole showed a higher ($p < 0.05$) average daily gain (ADG) and total average daily feed intake (ADFI) when compared with piglets exposed to eucalyptol. In addition, the feed conversion ratio (FCR) was lower ($p < 0.001$) in piglets from the D-limonene and *trans*-anethole group than in the eucalyptol group (Table 4). Furthermore, a mortality rate (%) of 0.00 was recorded for D-limonene and *trans*-anethole, while 0.72 in the eucalyptol group ($n = 2$ dead piglets).

Table 4. Effects of BCs on piglet's post-weaning performance (d 8 to d 14) within test groups of a double-choice feeding test.

Item	Treatment groups			SEM[1]	*p*-value
	D-limonene	***trans*-anethole**	**Eucalyptol**		
Initial BW, kg	5.86	6.14	6.32	0.300	0.558
Final BW, kg	7.44	7.86	7.31	0.369	0.529
ADG, g	225.4[a]	255.1[a]	143.0[b]	0.017	0.002
ADFI[2], g	252.9[a]	264.3[a]	213.7[b]	0.014	0.040
FCR	1.14[b]	1.05[b]	1.50[a]	0.043	<0.001

[1] Data are means of ($n = 12$) pens per treatment with 23 pigs per replicate pen. [2]Values for ADFI consider combined feed intake of reference and supplemented diet. [a,b]Means within a row with different superscripts indicate significant differences ($p < 0.05$).

3.2. Exp. 2 (Sensory maternal learning)

3.2.1. Placental and milk maternal transfer

Dietary BBC supplementation during gestation increased ($p < 0.05$) the relative concentrations of D-limonene, *trans*-anethole, and eucalyptol in sows' placental fluid (Figure 1). During lactation, supplementation of BBC increased ($p < 0.05$) the concentrations of D-limonene and *trans*-anethole in sows' milk, whereas eucalyptol was not detected in milk (considering a detection limit of 16 ppb) (Figure 2).

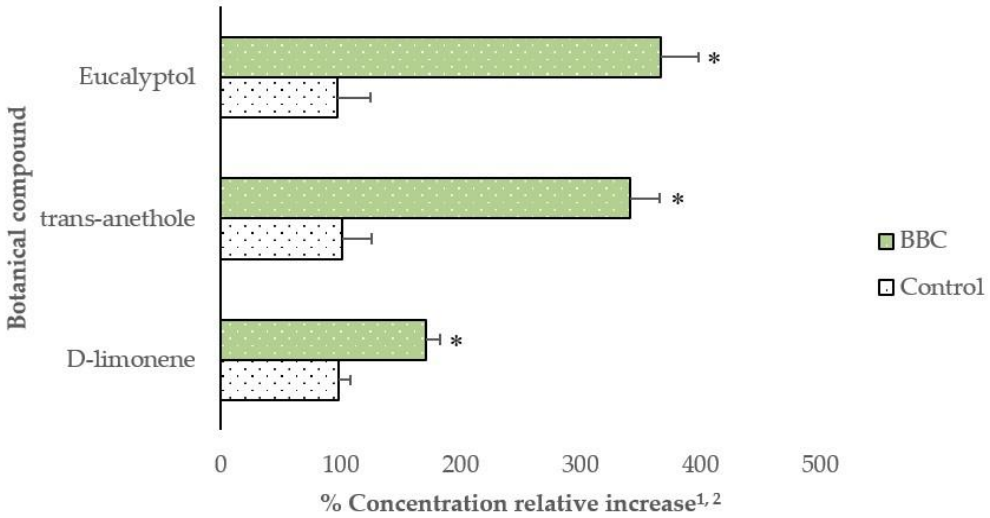

Figure 1. Relative concentration of botanical compounds in placental fluid of hyperprolific sows supplemented with dietary BBC. [1]Data are means of 12 sows per treatment ($n = 12$). [2]Difference in abundance/retention time of the BBC relative to assigned reference values (100%) in Control sows. Treatments: Control: unsupplemented diet; BBC: Control plus blend of botanical compounds. (*) Indicate significant differences ($p < 0.05$) using T-test.

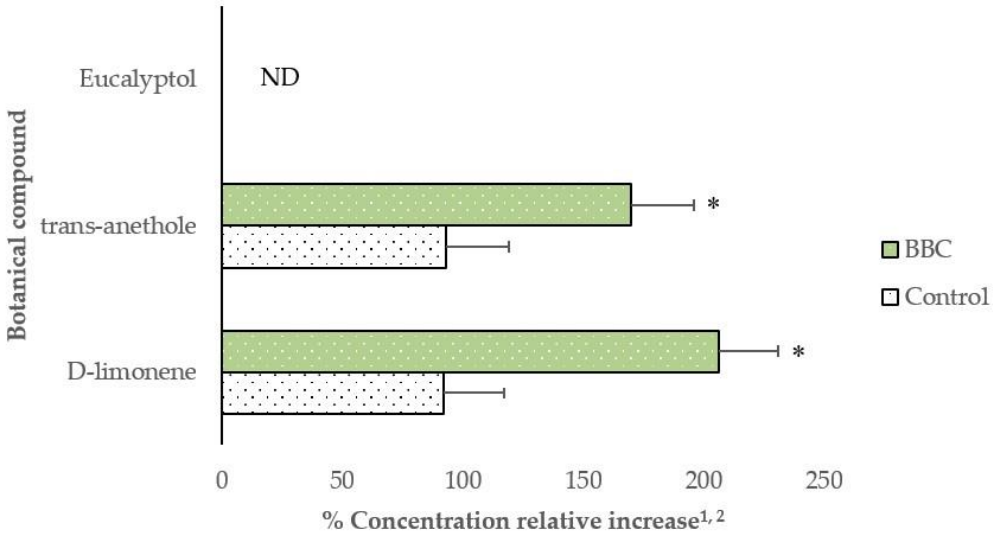

Figure 2. Relative concentration of botanical compounds in milk of hyperprolific sows supplemented with dietary BBC. [1]Data are means of 12 sows per treatment ($n = 12$). [2]Difference in abundance/retention time of the BBC relative to assigned reference values (100%) in Control sows. Treatments: Control: unsupplemented diet; BBC: Control plus blend of botanical compounds. ND: not detected. (*) Indicate significant differences ($p < 0.05$) using T-test.

3.2.2. Piglet's growth performance

Regarding piglet growth performance in Exp. 2 (Table 5), there were no differences between treatments in terms of weaning piglet BW ($p > 0.05$). At post-weaning d 7 the individual piglet BW was higher ($p = 0.037$) in the Control, and the ADG decreased ($p < 0.001$) in weaning piglets from the BBC group. Moreover, it was recorded a mortality rate (%) of 1.00 in Control, while 1.93 in the BBC group (Control $n = 2$ and BBC $n = 4$ dead piglets respectively).

Table 5. Effects of supplemented BBC in prestarter diets on newly-weaned piglet´s growth performance (d 1 to d 7).

Item	Treatments		SEM[1]	*p*-value
	Control	BBC		
Individual piglet BW, kg				
Weaning	5.34	5.41	0.101	0.588
Post-weaning d 7	5.43	5.14	0.098	0.037
ADG weaning to post-weaning d 7	12.5	-38.9	8.932	<0.001

[1] Data are means of ($n = 200$) and ($n = 203$) piglets for Control and BBC, respectively. Treatments: Control: unsupplemented diet; BBC: Control plus blend of botanical compounds. Statistical significance was assumed at ($p < 0.05$) using T-test.

4. Discussion

4.1. Innate preference or aversive responses of weaning piglets to BCs

According to the results, weanling piglets showed significant preferences for diets supplemented with D-limonene or *trans*-anethole and an aversion to diets supplemented with eucalyptol. In addition, piglets exposed to preferred flavors of D-limonene or *trans*-anethole showed an improved growth performance (BW) when compared with exposure to the avoided eucalyptol flavor. This is rather interesting since by using the DCHT all piglets were offered an alternative option to the unsupplemented standard prestarter diet. Thus, the previous results indicate that under the conditions of this study, compared to eucalyptol, chemosensory features of the taste, such as D-limonene and *trans*-anethole, have the potential to improve the palatability and feed preferences. This would be efficient for decreasing the feed neophobia and maintaining voluntary feed intake during a feed transition, as well as growth performance (BW) in weaned piglets.

The sensorial perceptions of a feed (i.e., palatability) are mainly defined by a combination of three chemical sensations: smell (aroma), taste (flavor), and somatosensory (e.g., texture). These are important determinants for feed acceptance and later feeding behaviors [21]. Several studies have reported that certain BCs used as flavors and sensory additives have the ability to modify feed palatability and, thus, modulate feed intake and preferences in piglets [8], [9], [22]. However, the results are contradictory and depend on factors including additives and diet composition, inclusion rate and periods of exposure, as well as age and sex. For instance, Clouard and Val-Laillet [8] described that at postweaning d 16 (i.e., the day of feed transition) a diet supplemented with *Citrus sinensis* immediately increased palatability and acceptance of the unfamiliar starter diet, while supplementation with *Stevia rebaudiana* (stevia) and an extract of high-saponin plants increased palatability only after a few days of exposure, suggesting a long-term familiarization processes. However, no effects on feed intake and growth performance were observed. In addition, Clouard et al. [9] found that during one-way and/or two-way choice tests, one-week weaned piglets consumed the meals supplemented with extracts of *Citrus sinensis* 0.12 ml/kg, *Cinnamomum camphora L. (*camphor*), Cinnamomum aromaticum Nees (*cinna-

mon), and *Illicium verum* (star anise) less than the standard diet. Thus, these authors suggest that these functional ingredients in food, at these concentrations, did not improve food palatability and did not increase food intake.

Moreover, Michiels et al. [22] used a two-way choice test 10 days post-weaning, and described that, compared to the control standard diet, feed supplemented with 125, 500, 1250, and 2000 mg/kg thymol was avoided by weanling piglets. However, when feed contained 2000 mg/kg thymol plus flavor A (intense artificial sweeteners) it was preferred, but not for the feed with 2000 mg/kg thymol plus flavor B (containing flavor A plus a caramel aroma). In addition, camphor is a known TRPA1 inhibitor and hence a candidate for reducing thymol's TRPA1 activation potential [23]. However, Michiels et al. [22] concluded that exposure to camphor (50 and 200 mg/kg did not mitigate feed avoidance caused for thymol, thus, thymol's bitter taste sensations might be largely responsible for feeding aversions. In this regard, the BC eucalyptol is a known TRPM8 agonist, whose activation induces a noxious cooling/burning sensation [24]. Meanwhile, TRPM5 activation by sweet tastants by an indirect mechanism causes an increase in intracellular Ca^{2+} levels due to the phytochemicals activating the sweet-taste receptor [13]. In addition, TRPM5 is specifically expressed in oral and extra-oral taste receptor cells (TRCs) such as sweet heterodimer T1R2+T1R3, which in turn is co-expressed with glucose transporter (GLUT2) and Na+ -dependent glucose/galactose co-transporter (SGLT1) [25], which is responsible for stimulating the secretion of glucagon-like peptide (GLP)-1 and -2 [26].

Eucalyptol can be described as having a fresh camphor-like smell and a spicy cooling taste [7] compared to the sweet citrus-like flavor and aroma of D-limonene [27] or the sweet anise-like flavor of *trans*-anethole [6]. Although it was not an aim of this study to study the effects of BCs on the chemosensory system, the results suggest that compared to eucalyptol, growth performance improvements in weanling pigs that preferred D-limonene and *trans*-anethole in the DCHT, may be related to a stimulus of voluntary feed intake due to higher appetence, nutrient absorption, and peptide secretion in the gastrointestinal tract. Studies in mammals, for instance in marsupials such as the brushtail possum (*Trichosurus vulpécula*), described that food intake is constrained in diets containing 1,8-cineole (eucalyptol) [28]. However, to our knowledge, there are no studies describing this effect on pigs.

4.2. Effects of prenatal exposure to BBC on weaning piglet's growth performance

Oostindjer et al. [29] mentioned that pre- and postnatal exposure to flavors through the amniotic fluid and mother's milk derived from the maternal diet has been shown to modulate food preferences and neophobia of young animals of several species. Regarding the possible biological explanations for the previous findings, it is important to highlight the role of the sensory maternal learning on the porcine nutritional programming and/or sensory conditioning during the pre- and postnatal period. Early exposure to volatile flavor components detected by the olfactory and taste systems beginning *in utero* and continuing during early milk feedings is considered to have a strong influence on flavor preference or aversion during weaning. Thus, pre- and postnatal experiences with food flavors transmitted from the mother´s diet provide the earliest opportunity to present specific flavors to the offspring in order to influence food acceptance and preferences [30]. In fact, sensory programming effects or familiarity impact by transfer of BCs from the sow's diet to the amniotic fluid and/or milk have been shown particularly for BCs such as anise [29], limonene, menthol and carvone [11], anethol, cinnamaldehyde, and eugenol [12], which influenced the feeding behaviors and growth performance of weanling piglets.

In this study, it was hypothesized that prenatal exposure of piglets to innately avoided BCs such as eucalyptol could have a favorable impact on familiarity and later acceptance.

However, the present results do not support this hypothesis. Considering the results on an innate preference for D-limonene and *trans*-anethole in Exp 1, and an innate aversion for eucalyptol, Exp. 2 aimed to study whether a sensory maternal learning by pre- and postnatal exposure to these BCs could help to mitigate the aversion and familiarize the weanling pigs to eucalyptol. According to the observations in Exp. 2, all BCs tested in the DCHT (D-limonene, *trans*-anethole, and eucalyptol) added as supplements to sow feed were transferred to the placental fluid. In contrast, only D-limonene and *trans*-anethole were detected in the milk. A possible metabolization effect for metabolites of eucalyptol that are transferred to the blood, or even metabolic activity in the milk itself [31], may be the reason for the non-transfer of eucalyptol through sows' milk. Furthermore, these results demonstrate a potential difference in exposure of piglets before and after farrowing to selected BCs from maternal diets. Weanling piglets receiving the same BBC (containing preferred D-limonene and *trans*-anethole) in the pre-starter diet as the BBC supplemented to their mothers' diet during gestation showed lower BW gain during the first week post-weaning compared to the Control group.

In mammals, including humans, taste begins to emerge earlier than other senses such as sight and sound. During the last trimester of prenatal development, the taste buds are already capable of detecting information and transmitting it to the central nervous system [32]. In fact, the fetus can detect pleasant and unpleasant tastes and flavors. For instance, fetal swallowing frequency increases in response to the introduction of sweet solutions to the amniotic fluid and decreases in response to the introduction of bitter solutions [30]. However, at post-weaning (first week), even though eucalyptol was transferred only prenatally from sow to offspring, no positive familiarization effect was observed. Apparently, the aversive effect of eucalyptol seems to override any positive effects of preference on feed intake of D-limonene and *trans*-anethole when they were mixed in the BBC. The above observations suggest a mixture effect with the BBC where the positive response (pleasant sensations) of D-limonene and *trans*-anethole on the chemosensory system of weanling piglets could be suppressed due to the negative response (noxious sensations) to eucalyptol.

There is a well-known mixture suppression effect in gustation. Unpleasant odors and tastants are known to prevail over pleasant ones when they are mixed together [24]. For instance, Michiels et al. [22] described that in weaned piglets camphor did not avoid aversions caused by thymol's bitter taste. In addition, mixture-specific effects of phytochemicals, including eucalyptol and menthol, have been described for the modulation of trigeminal chemosensory systems, with an intensification of noxious sensations such as burning, cooling, and tingling, even in the absence of an olfactory percept [24]. This suggests that potential mixture effects on trigeminal stimuli are similar to the mixture effects observed for smell and taste. Therefore, the BW results for the newly-weaned piglets in Exp. 2 suggest that the pleasant flavors of D-limonene and *trans*-anethole that led to their preference in the DCHT did not mask the innate aversion to eucalyptol, which could be prenatally conditioned.

It must be concluded that one-week weanling piglets showed preferences for pre-starter diets supplemented with BC such as D-limonene or *trans*-anethole, while eucalyptol supplementation led to a reduced feed acceptance. However, when the above-mentioned BCs are mixed, the apparent positive effects (pleasant odor and/or taste) of D-limonene and *trans*-anethole are not sufficient to overcome the aversion caused by eucalyptol, resulting in impaired growth performance (BW) of newly-weaned piglets. Moreover, sensory maternal learning (prenatal exposure) to eucalyptol is not sufficient to compensate for this effect. Behavior towards these types of substances may be innate and cannot be trained easily through prenatal exposure.

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CHAPTER 7

General discussion

In the present PhD thesis, it was investigated the effects of dietary phytogenic feed additives (PFAs) supplementation as early feeding strategies to ameliorate the physiological, health, and productive challenges that face both hyperprolific sows and their offspring in modern swine system. Thus, the increased litter size has led to oxidative stress and intrauterine growth retardation, resulting in lower and more variable birth weights and performance of piglets, including an increased offspring pre- and postnatal mortality (Matheson et al., 2018). Both, birth weight of the individual pig and within-litter BW weight variability are of considerable economic interest for swine production, as postnatal growth performance, carcass quality and efficiency of the whole production cycle are compromised (López-Vergé et al., 2018a). Therefore, due to potential functional properties of PFAs (Durmic and Blache, 2012), in this thesis it was explored if PFAs supplementation in the sows and piglets feed may promote the piglet health and well-being, and growth and production efficiency in the swine system.

We proposed the following hypotheses that will be used to guide our General Discussion:

1) Dietary specific blends of phytogenic compounds (BPC) supplemented to hyperprolific sows may influence on oxidative status and performance of sows and offspring. The effect maybe higher if supplementation starts from the early gestation.
2) The BPC in the feed of gestating and lactating sows may be transferred to the offspring through placental fluid and milk.
3) The pre- and postnatal exposure (by maternal transfer) of the offspring to BPC may affect the gut health-related functions and histomorphology.
4) Both colostrum and milk composition might be modified by dietary supplementation of sows with BPC.
5) Prestarter diets supplemented with phytogenic compounds may improve feed palatability and, therefore, innate feed preference in weanling piglets.
6) Sensory maternal learning (by maternal transfer) to BPC might influence feed intake and growth of newly-weaned piglets.

Dietary specific blends of phytogenic compounds (BPC) supplemented to hyperprolific sows may influence on oxidative status and performance of sows and offspring. The effect maybe higher if supplementation starts from the early gestation.

It has been suggested that pregnancy promote a state of oxidative stress. Elevated oxidative stress is reported to be associated with pregnancy complications in hyperprolific sows (Berchieri-Ronchi et al., 2011). Previous studies showed that oxidative stress can cause vascular dysfunction in the placenta, suggesting that oxidative stress in the placenta may be involved in the development of IUGR piglets through modulating placental vessel development, oxidative damage, mitochondrial dysfunction, and impaired angiogenesis (Hu et al., 2020). In fact, porcine placentae with high vascular density can help to improve placental efficiency, and, consequently increase maternal-fetal nutrients, respiratory gases, and waste exchanges, thus promoting fetal growth and survival (Song et al., 2018). In the present thesis (Chapter 4) it has been observed that dietary supplementation of a specific blend of phytogenic compounds (BPC1) during gestation of hyperprolific sows increased the sow plasma levels of catalase (CAT), total antioxidant capacity (TAC), and nitric oxide (NO) on day 35 of gestation. Meanwhile, at the end of the gestation period (day 110), BPC1 increased plasma levels of superoxide dismutase (SOD) and glutathione peroxidase (GSH-PX). These findings suggest a strengthening of the antioxidant status of gestating sows, which may play a crucial role in overcoming pathophysiological of gestating hyperprolific sows including angiogenesis, and, thus, placental efficiency.

Among the functional properties of phytochemicals contained in the BPC1, we may rise the antioxidant, anti-inflammatory, antimicrobial, and immunological effects of eucalyptol (Bhowal and Gopal, 2016), p-cymene (De Oliveira et al., 2015), linalool (Kamatou and Viljoen, 2013), anethole (Marinov and Valcheva-kuzmanova, 2015), and thymol (Nagoor Meeran et al., 2017). In addition, several preclinical studies reported the efficiency of phytochemicals in modulating angiogenesis, a process involved in the neovascularization (Rajasekar et al., 2019). Effective placental angiogenesis is crucially dependent on proper interactions between different types of cells (endothelial cells, pericytes, stromal cells), basement membrane and extracellular matrix which is mediated through the pro-angiogenic factors like vascular endothelial growth factor (VEGF), basic fibroblast growth factor (FGF-2), angiopoietins (Ang), interleukin (IL)-8 (Chung et al., 2010). These pathways lead to the downstream activation of intracellular proteins which induce proliferation, sprouting and tube formation of endothelial cells; as well as

vasodilation by increased NO production (known as major vasodilator and angiogenesis factor) stimulated by enhancement of nitric oxide synthase gene (Pereira et al., 2015). Regarding the latter, in this study it was observed that NO production values at day 35 of gestation was significantly increased by 30.8% with BPC1 (Chapter 4) and 25.7% with BPC2 (unpublished data) when compared with Control sows. Thus, it may suggest better placental angiogenesis in supplemented gestating sows.

The first month of gestation in pigs is critical for the successful establishment of pregnancy, but also to determine the potential litter size through the number of embryos that survive (Langendijk, 2015). As consequence, we have proposed in this thesis to explore if reproductive performance of sows may respond to a dietary supplementation of specific BPCs. In a recent study the supplementation of herbal antioxidants in gestating sows increased the number of live-born piglets by a mean of 1.5 (Parraguez et al., 2021). In our results we observed that the number of piglets born and born alive were significantly increased from 17.0 to 19.1 and from 14.5 to 17.5 with BPC1 (Chapter 4), while from 17.8 to 19.9 and from 14.7 to 17.2 with BPC2 (Chapter 5) (Table 7.1).

Table 7.1. *Reproductive performance of hyperprolific sows supplemented with BPC1 or BPC2 and its effect on the variability of piglet born alive weights.*

Variables	Control[1]	BPC1[2]	Control[1]	BPC2[3]
Number of sows, n	19	20	14	14
Parity, n	2.84	2.75	3.42	3.28
Total born, n	17.0^{y}	19.1^{x}	17.8^{y}	19.9^{x}
Born alive, n	14.5^{b}	17.5^{a}	14.7^{b}	17.2^{a}
Average BW of born alive, kg	1.41^{a}	1.16^{b}	1.33^{x}	1.17^{y}
Within-litter BW distribution of born alive, %				
Q1 < 1.0, kg	22	29	20	31
Q2 > 1.0 to 1.25, kg	24	26	20	23
Q3 > 1.25 to 1.5, kg	25	25	27	23
Q4 > 1.5, kg	29	20	33	23
Coefficient of variation, %	20.1	23.8	19.6	22.9

[1]Unsupplemented gestating sows; [2]Gestating sows supplemented with BPC1 (Exp 1). [3]Gestating sows supplemented with BPC2 (Exp 2). [a,b]Means within a row with different superscripts indicate significant differences ($p < 0.05$). [x,y]Means within a row with different superscripts indicate significant trends ($p < 0.10$).

However, it has been also reported that in pigs, litter size and offspring weight act antagonistically (Matheson et al., 2018). For instance, Moreira et al. (2020) by a systematic review and meta-analysis described that enhancement of litter size by an average of 4.4 live-born piglets reduced the average weight of live-born piglets by 12.4%

ranging 1.37 kg. In this thesis, an average increase of 3.0 and 2.5 live-born piglets decreased the average birthweight of live-born piglets by 17.7% with BPC1 and 12.0% with BPC2 respectively. These authors also reported that weight variability was affected in high prolificacy sows. For instance, the coefficients of variation (CV) of live-born piglets were increased by 4.5%. In this study, the CV of live-born piglets were increased by 3.7% and 3.3% with BPC1 and BPC2. Moreover, previous studies have categorized as LBW piglets those with <1.0 kg (Milligan et al., 2002). However, similar to other studies (Douglas et al., 2014), our study defined LBW piglets as <1.25 kg, corresponding to quartiles 1 (Q1) and 2 (Q2) from a total of 4 quartiles. In that regard, the number of LBW piglets were increased by 16.3% and 25.9% with BPC1 and BPC2. Therefore, according to the average BW of live-born piglets either BPC1 or BPC2, the live-born piglets from these sows were consider as LBW when compared to its respective control group (see Table 7.1).

The pre- and postnatal exposure (by maternal transfer) of the offspring to BPC may affect the gut health-related functions and histomorphology.

It has been described that compared with normal birthweight piglets (NBW), those affected by varying degrees of IUGR have smaller organs and dysfunction in their GIT system (Wang et al., 2008). In fact, differences in postnatal survival, health, growth, and performance between LBW and NBW pigs have been extensively reported (G. Wu et al., 2006). An impaired development of the gut including structure atrophy and alterations in transcription or epigenetic may be the major mechanisms responsible to these multiple defects during the early postnatal life of LBW piglets (Wang et al., 2010). Recently, Ayuso et al. (2021) identified the lower expression of genes involved in nutrient digestion (ANPEP and SI), barrier function (OCLN and CLDN4), and intestinal development markets (ALPI and OLFM), together with shorter villi and shallower crypts in LBW than NBW. In this thesis, it has been hypothesized that maternal supplementation of BPCs can change the jejunum transcriptional profile of the neonate and lactating piglets. It follows from the description of fetal programming that nutritional stimuli during fetal development may leave a permanent imprint by changing the epigenetic state of the fetal genome and gene expression (Wu et al., 2004).

The results of Chapter 5 showed that supplementation of BPC2 into the diet of gestating sows affected the gut (jejunum) health-related gene expression and histological responses in neonate piglets (Table 7.2). Compared to control (NBW), the

SLC16A1/MCT1, *SLC11A2/DMT1*, and *SLC39A/ZIP4* genes from nutrient transport were downregulated in neonates pigs from BPC2, which imply an effect in the SLCs capacity to regulate the intracellular nutrients concentrations and, in addition, detect alterations in the levels of extracellular levels (Beltrán Piña et al., 2019). For instance, an study determined that gene expression of SLCs were increase after deprivation of nutrients such as amino acids (Hellsten et al., 2017). Therefore, in this study, downregulation of above-mentioned SLCs (SLC16A1/MCT1, SLC11A2/DMT1, and SLC39A/ZIP4) genes may suggest that despite neonate piglets from BPC2 were categorized as LBW, it was prevented disproportionate hypoxia (Miranda-Gonçalves et al., 2016), and potential deprivation of nutrients (Beltrán Piña et al., 2019) including amino acids (Villodre Tudela et al., 2015) and glucose (De Saedeleer et al., 2014); iron (Fe), manganese and Copper (Hansen et al., 2009); and zinc (Takagishi et al., 2017), respectively.

Furthermore, nutrient deprivation of nutrients in animals leads to increased mitochondrial superoxide ($O_2^{-\cdot}$) production, and, consequently, metabolic oxidative stress (Salin et al., 2018). Since SOD2 activity is regulated in response to mitochondrial superoxide production (Zelko et al., 2002). Thus, in this study, compared to Control (NBW) neonate piglets, downregulation of *SOD2* gene from oxidation may suggests that undue intrauterine oxidative stress by nutrient deprivation of nutrients in LBW piglets from BPC2 it was avoided (Zelko et al., 2002). In addition, jejunal transcription levels of *IFNG* and *CLDN4* from immune responses and barrier function respectively, were higher in BPC2 neonate piglets than Control. In disagreement with other studies (Ayuso et al., 2021), that reported lower expression of these genes in LBW than NBW piglets, suggesting that maturation of the intestinal barrier is impaired in LBW piglets. Moreover, our recent study in neonate piglets found that prenatal exposure to BPC2 caused an increase in the villus height, villus height: crypt depth ratio, and goblet cells density. Thus, these results may encompasses an improvement of the 'health' of the GIT ('gut health') including nutrient digestion and absorption (Pluske et al., 2018), as well as the evolution of innate/adaptive immunity in neonate piglets of BPC2, where the later provides protection (together with the passive acquisition of antibodies) until immune system becomes competent (Butler et al., 2013).

Table 7.2. *Effects of BPC2 or BPC1 supplementation during gestation and/or lactation of hyperprolific sows on jejunal related health-function mRNA relative expression of LBW piglets.*[1]

		Lactation d 20 (BPC1)		Birth (BPC2)
Function	Gene	G+L[2]	L[2]	G[3]
Barrier function	CLDN4	↓	↓	↑
	OCLN	-	-	ns
	MUC2	↓	↑	-
Enzyme/hormone	DAO1	-	-	ns
	CCK	-	-	ns
	ANPEP	↓	↓	-
Immune response	IDO1	↑	↑	ns
	IFNG	-	-	↑
	ALPI	-	-	ns
	PPARGC1-α	ns	↑	-
	TNF-α	↑	↑	-
	TGF-β1	↑	↑	-
	IL-10	↑	↑	-
Nutrient transport	SLC15A1/PEPT1	-	-	ns
	SLC16A1/MCT1	-	-	↓
	SLC11A2/DMT1	-	-	↓
	SLC39A4/ZIP4	-	-	↓
Oxidation	SOD2	-	-	↓

[1]Data are means of eight newborn pigs per treatment (n = 8). [2]Suckling piglets (day 20) from supplemented sows with BPC1 (Exp 1) during gestation and lactation (G+L) or only lactation (L). [3]Neonates piglets from supplemented sows with BPC2 (Exp 2) during gestation (G). The symbol (↓) denotes downregulation, while symbol (↑) upregulation compared to unsupplemented Control (NBW). The (ns) denotes gene expression (p < 0.05) but no significant according to FDR (q < 0.10).

The basis of these effects could be related to the prenatal exposure (by maternal transfer through placental fluid) of the offspring to biological properties of some volatile compounds containing in BPC2 such as anti-inflammatory, antioxidant, antibacterial, and immunomodulatory activities of D-limonene (Baschieri et al., 2017), *trans*-anethole (Marinov and Valcheva-kuzmanova, 2015), borneol (Sokolova et al., 2017), p-cymene (De Oliveira et al., 2015), eucalyptol (Bhowal and Gopal, 2016), and camphor (Chaturvedi et al., 2018). For instance, eucalyptol (1,8-cineole) a terpenoid oxide isolated from *Eucalyptus species*, suppresses lipopolysaccharide-induced proinflammatory cytokine production through the action of NF-κB, TNF-α, IL-1β, and IL-6 and the extracellular signal-regulated kinase (ERK) pathway, and reduces oxidative stress through the regulation of signaling pathways and radical scavenging (Seol and Kim, 2016). In agreement with our results, other studies described that dietary supplementation of plant compounds such as grape seed and grape marc meal extract (GSGME) rich in

polyphenols are able to downregulated the mRNA abundances of various nutrients transports including glucose transporter (SLC2A2, SLC2A5 and SLC15A1) in piglets jejunum (Fiesel et al., 2014). Moreover, Gessner et al. (2013) also reported that dietary supplementation of GSGME increased the duodenal ratio of villus height:crypt depth, while decreased activities of the oxidative stress-responsive transcription factors NF-κB by translocation into the nucleus of Nrf2 to inhibit inflammation in the gut frequently occurring in pigs. Overall, our results regarding the jejunal gene expression and histological responses in neonate piglets prenatally exposed to BPC2 (Chapter 5), offers a window of opportunity for the early life programming of the gut health, especially in LBW piglets.

Both colostrum and milk composition might be modified by dietary supplementation of sows with BPC.

Immediately after parturition, maternal nutrition continues to regulate growth and development of piglets through mammary secretions (colostrum and milk), the sole nutritional sources for immunity and growth of piglets (Hurley, 2015). Li et al. (2018) described that colostrum and milk macronutrient components do not significantly increase with enhanced reproductive performance in sows. In fact, current composition of sow's colostrum is similar to those of 30 years ago (16% protein, 3% lactose and 5% fat), while only minor changes in the composition of the sow's milk (5% protein, 5% lactose and 7.5% fat). In present study, supplementation with BPC1 or BPC2 significantly improved the colostrum (protein) and milk (fat) compositions (Table 7.3). In the present work it was observed that compared with the composition of sow's colostrum and milk in Control, the colostrum protein concentrations were improved by 24.4% and 21.6% with BPC1 and BPC2. Meanwhile, milk fat contents were increased by 19.3% in supplemented sows with BPC1 during lactation, and 20.9% and 20.7% with BPC1 and BPC2 supplementation during gestation and lactation. In fact, these improvements of colostrum/milk compositions in supplemented sow´s were higher than current standard colostrum/milk concentrations described in literature.

Regarding possible explanations to observed changes in colostrum/milk composition, in this study, we focused on the effects of BPCs on 1) the oxidative and 2) immune status and 3) nutrition of the sows because play an important role in regulating colostrogenesis and lactogenesis processes (Quesnel and Farmer, 2019). In that regard, colostrum/milk protein and lipid synthesis in porcine mammary gland are facilitated by

transport proteins. For instance, amino acids from blood are taken up by the porcine mammary gland epithelial cells through cationic (CAT-1 and CAT-2B), neutral (ASCT1, LAT2 and SNAT2), and anionic (EAAC1 and EAAC3) amino acid transporters. Meanwhile, fatty acids the enter porcine mammary gland, facilitated by transport proteins (mainly CD36). Subsequently, amino acids and fatty acids are synthesized into colostrum/milk proteins and lipids (Li et al., 2018). It has been suggested that the mammary gland has a transport system similar to other organs like intestine, kidney, and placenta. (Li et al., 2018). Thus, deficient vascularity of mammary gland may lead to oxidative stress and impaired angiogenesis (Forsythe et al., 1996). Recently, Holanda et al. (2019) described that supplementation of sows with L-arginine (a nitric oxide precursor) from 109 day of gestation to weaning (day 21) increased the transcription expression of GSH-PX (an important intercellular antioxidant enzyme), as well as activates proliferative mechanisms which improved mammary tissues' angiogenesis, and, thus, vascularity of mammary gland of lactating sows. Therefore, the improved antioxidant capacity of sows in the *peripartum* and lactation period may help to increase mammary gland vascularity of lactating sows, and, thus, synthesis and nutrient composition of colostrum and milk.

Table 7.3. *Effects of dietary BPC2 and BPC1 supplementation during lactation or gestation and lactation on colostrum and milk composition of hyperprolific sows.*

	BPC1[2]			BPC2[3]	
Variables	Control[1]	L	G+L	Control[1]	G+L
Analyzed nutrient content, %					
Colostrum[4]					
Protein	15.71	15.74	20.80*	15.43	19.68*
Fat	5.57	5.55	6.10	5.53	5.68
Lactose	3.05	3.13	2.48	3.00	3.10
Milk[5]					
Protein	6.85	6.61	6.16	6.81	6.42
Fat	7.21	8.94*	9.12*	7.03	8.87*
Lactose	4.89	4.57	4.75	4.88	5.38

[1]Unsupplemented sows. [2]Supplemented sows with BPC1 during lactation (L) or during gestation and lactation (G+L). [3]Supplemented sows with BPC2 during gestation and lactation. The (*) indicates higher significant values ($p < 0.05$). [4]Values within 12 h of farrowing. [5]Values at day 20 of lactation.

As above-mentioned, the results of Chapter 4 showed that plasma levels of GSH-PX and SOD were increased in gestating (day 110) sows from BPC1, which may suggest that like L-arginine effects, this improvement of antioxidant status of sows around *peripartum* was able to increase vascularity of mammary gland, and, consequently,

increase amino acid and fatty acids transport for synthesis of protein in colostrum and fat in milk respectively. In fact, we observed that supplementation of BPC1 during gestation and lactation of sows significantly increased the levels of plasma antioxidative enzymes (CAT and SOD) in suckling piglets (d 20), while (SOD and TAC) were increased in suckling piglets from sows supplemented with BPC1 only during lactation. In this sense, we suggest that these piglet's antioxidant capacities could be acquired through sow's antioxidant status, but also by the antioxidant properties of the BPC1 transferred maternally into milk. In addition, at post-weaning (day 7), these piglet's antioxidant capacities were maintained at higher levels of CAT, SOD, and GSH-PX than those levels observed in the Control group.

In addition, the synthesis of mammary secretions components takes place during late gestation, e.g., the colostrogenesis, when the transfer of immunoglobulins (proteins) from sow plasma to lacteal secretions slowly begins approximately 10 days before farrowing and is maximal at farrowing (Quesnel and Farmer, 2019). It has been suggested that all IgG and nearly 80% of IgM are directly transferred from blood to colostrum instead of their component's amino acids (Bourne and Curtis, 1973), a process mediated by the neonatal Fc receptor (FcRn) (Chen et al., 2018). Several substances including Eos and pre- probiotics have been showed immunomodulating effects on the Ig levels of swine colostrum (Farmer and Quesnel, 2009). Thus, we suggest that increased protein content in colostrum of supplemented sows with either BPC1 or BPC2 could be also related with an improvement of the sow's immune status. In fact, it has been described that porcine colostrum/milk contains substances with non-specific antimicrobial and/or immunomodulating activities, including lactoferrin and the derived peptide lactoferricin which protect against neonatal infections, as well as lysozyme, sIgA, oligosaccharides and milk mucins, such as MUC-1, which have bactericidal activity and may also protect against infection by inhibiting pathogen binding to epithelial cells (Salmon et al., 2009). In this sense, in this study (Chapter 4), we also observed that milk from sows supplemented with BPC1 during gestation and lactation showed bactericide activity against *Bacillus subtilis* and *Staphylococcus aureus.* Thus, antibacterial activity may be important for protection of both the mammary gland itself and the neonatal gut (Salmon et al., 2009).

Sow colostrum/milk also contain epidermal growth factor like amniotic fluid, insulin-like growth factor and TGF-β that may provide the neonatal intestine with

regulatory signals under both normal and pathophysiological conditions (Donovan et al., 1994), and immunomodulatory agents, including prolactin, and nucleotides enhancing the activity of NK cells, macrophages, T helper cells and cytokines. These cytokines include pro-inflammatory (TNF-α, IL-1β and IL-6) and anti-inflammatory factors (TGF-β and IL-10), but also Th-1 type response-promoting agents (IFN-γ and IL-12), and Th2-type response- promoting agents (IL-6, IL-4 and IL-10) and TGF-b1(Th-3) (Nguyen et al., 2007). In this sense, as is shown in Table 7.2, supplementation of sows with BPC1 during gestation and/or lactation (Chapter 4), influenced on jejunal related health-function gene expression in suckling piglets (d 20). For instance, the mRNA downregulation of *CLDN4* gene from barrier function, while upregulation of digestive enzyme *IDO1,* as well as *TNF-α, TGF-β1,* and *IL-10* genes from immune response were observed when compared with Control group. Thus, it could be suggested that mucosal barrier function, as well as intestinal maturation and immune response in piglets were modulated by the ingestion of maternal milk containing BPC1.

On the other hand, the effects of sow nutrition on macrochemical composition of mammary secretions, encompassing feed intake, energy and protein intake and source (Farmer and Quesnel, 2009). In fact, the fat content of colostrum/milk is considered as the most labile of the components (Hurley, 2015). As above-mentioned, in this study, we observed that supplementation of BPC1 or BPC2 significantly increased the milk fat concentrations. These results may also be related to an improvement of the standardized average daily feed intake (SADFI; from farrowing to lactation d 20) in supplemented lactating sows. In that regard, we observed that SADFI were significantly increased by 19.0% (7.37 kg) in supplemented sows with BPC2 during gestation and lactation, while tended to increase by 10.4% (6.63 kg) and 8.5% (6.49 kg) with supplementation of BPC1 during gestation and lactation or only lactation when compared with a mean SADFI of 5.94 kg in control groups (Unpublished data). Quesnel and Farmer, (2019) suggested that the fatty acid content of mammary secretions greatly depends on the amount of lipids provided in the sow diet, whereas the fatty acid profile is largely influenced by the type of lipid being fed to the sow. In this study, as a reference we also determinate the fatty acids profile in sow´s colostrum and milk by analyzing a single pool sample for each treatment group of both colostrum and milk, we observed (not statistically) that compared with values in Control groups and those reviewed in the bibliography (Li et al., 2018), the profiles of major fatty acids in sow colostrum and milk including C14:0, C16:0, C16:1

(*n*-7), C18:0, C18:1(*n*-9) and C18:2 (*n*-6) were higher in supplemented sows either BPC1 or BPC2 during gestation and/or lactation, which also coincide with the higher milk fat contents observed previously in supplemented sows (Table 7.4) (unpublished data).

Table 7.4. *Effects of dietary BPC1 or BPC2 supplementation during lactation and/or lactation on major fatty acid profiles in sow's colostrum and milk (mg/g).*[1]

	Colostrum 0 d			Milk 20 d			
Fatty acids	Control[2]	BPC1[3]	BPC2	Control[2]	BPC1[3] (L)	BPC1 (G+L)	BPC2 (G+L)
C14: 0	1.41	1.60	2.21	3.30	3.61	4.00	3.82
C16: 0	23.7	25.4	27.6	31.6	34.4	37.2	35.3
C16: 1	3.77	4.75	3.88	8.92	9.12	10.0	9.86
C18: 0	5.03	6.18	6.47	4.32	4.31	4.05	4.01
C18: 1	35.4	38.6	37.0	28.7	32.0	31.4	35.6
C18: 2(*n*-6)	18.2	19.2	19.6	11.8	12.8	12.2	12.7

[1]Values correspond to a single pool sample per treatment. [2]Unsupplemented sows. [3]Supplemented sows with BPC1 (Exp 1) or BPC2 (Exp 2) during gestation and lactation (G+L) or only lactation (L).

Lactation function by the sow is a maternal contribution to progressing postnatal growth and development of the piglets. Piglets are born deficient in energy, and intake of energy from colostrum and milk after the onset of lactation is of paramount importance for the newborn piglets, this may be an important factor in particular for LBW piglets (Quesnel et al., 2012). In this study, it has to be highlight that even though live-born piglets from either BPC1 or BPC2 (consider as LBW) showed significant lower average body weight than NBW in Control groups, this different disappear during the lactation time. In fact, piglets from BPC2 tended to show higher standardized weight gain (from birth to lactation day 20) than Control. In addition, there were no difference between treatments for the pre-weaning mortality rate (%) and days of lactation length (Table 7.5).

In this sense, it has been suggested that sow lacteal secretions are the major determinant of a suckling piglet's growth and survival, and the weight of piglets at weaning has a major impact on their post-weaning gain (Merlot et al., 2019). Unfortunately, the nutrients demand of lactating sows increases about 3.5 or more one week to ten days after farrowing (Theil et al., 2014). This metabolic effort is not fully compensated for by a parallel increase in feed intake, and sows have to lose their own body reserves to compensate the system. It is clear that feed intake is a key issue to optimize lactating sows nutrition (Solà-Oriol and Gasa, 2017). Our results regarding sow's BW and back-fat thickness parameters during lactation were not affected, thus, we suggest that the increased ADFI in supplemented lactating sows either BPC1 or BPC2,

helps to maintain its body condition, but also to compensate the growth performance and their weight at weaning in LBW piglets. In agreement with other studies, which described that supplementation of lactating sows' diet with PFAs such as 0.5% of powder star anise improved the ADFI and milk yield of sows, but also the weaning weight of piglets (Wang et al., 2015). These authors suggest that increase of ADFI and IGF-1 in milk and prolactin in serum of lactating sows may be related with these improvements.

Table 7.5. *Effects of dietary BPC1 or BPC2 supplementation during gestation and/or lactation on litter performance and individual piglet postweaning BW.*

	BPC1[1]			BPC2[1]	
	Control[2]	L	G+L	Control[2]	G+L
Piglets' average BW within-litter, kg					
Birth	1.41[a]	1.24[ab]	1.16[b]	1.33[x]	1.17[y]
Lactation day 20	4.94	5.04	4.70	4.80	5.11
Weaning	5.29	5.45	5.33	5.34	5.41
Weight gain from birth to lactation day 20, kg	3.53	3.80	3.54	3.47[y]	3.94[x]
Lactation length, d	23.4	22.9	23.9	23.4	24.2
Pre-weaning mortality rate,%	2.19	1.27	3.68	2.26	2.37
Individual piglet BW, kg					
Post-weaning day 7	5.41	5.58	5.51	5.43[a]	5.14[b]

[1]Piglet from supplemented sows with BPC1 (Exp 1) or BPC2 (Exp 2) during lactation (L) or during gestation and lactation (G+L). [2]Control, piglets from unsupplemented sows. [a,b]Means within a row with different superscripts indicate significant differences ($p < 0.05$). [x,y]Means within a row with different superscripts indicate significant trends ($p < 0.10$).

Prestarter diets supplemented with phytogenic compounds may improve feed palatability and, therefore, innate feed preference in weanling piglets.

The post-weaning period in pigs is often accompanied by a severe growth check and diarrhea. For the last decade, antimicrobial compounds have been used to promote piglet growth at weaning through the prevention of subclinical and clinical disease. However, according to EFSA (2017), several countries have restricted or banned the use of in-feed antibiotics (Ma et al., 2021). It is well established that poor growth performance associated with weaning is a result of multi-factorial stressors, however, post-weaning anorexia and undernutrition usually associated with neophobia to unfamiliar feed or flavor (Campbell et al., 2013). Overall, manipulating feed palatability e.g., by using sensory additives including phytochemicals offers the opportunity to influence feed intake, and, therefore, feed preferences and growth in weaning piglets (Michiels et al., 2012) (Caroline Clouard et al., 2012). Thus, in this thesis (Chapter 6), by using a double-

choice feeding test (DCHT) we also investigated the effect of offering diets with PFAs to increases feed palatability and promote feed intake after weaning. Our results indicated that weaned piglets showed significant innate preference for diets supplemented with D-limonene (53.8%) or *trans*-anethole (54.5%), while aversion for diets with eucalyptol (41.6%). In addition, piglets exposed to preferred flavors of D-limonene or *trans*-anethole showed an improved growth performance (BW) when compared with exposure to the avoided eucalyptol flavor. Therefore, in agreement with other studies using botanical compounds such as *Citrus sinensis* or *Stevia rebaudiana* (Clouard and Val-Laillet, 2014), we suggest that D-limonene or *trans*-anethole have the potential to improve the palatability and feed preferences by decreasing neophobia in weanling pigs.

Sensory maternal learning (by maternal transfer) to BPC might influence feed intake and growth of newly-weaned piglets.

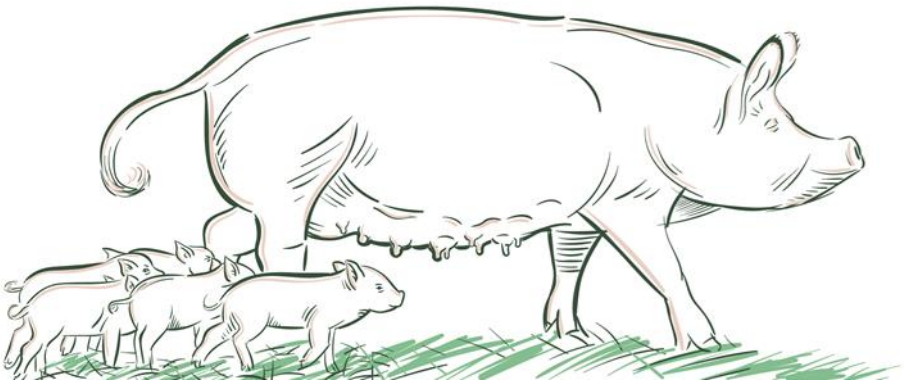
In swine, sensory maternal learning by pre- and postnatal flavor exposure has been study as strategy to reduce piglets neophobia for similarly flavor types from the sow diets and, therefore, influence feed preferences around weaning (Figueroa et al., 2013) (Oostindjer et al., 2010). In this sense, studies in pigs have been showed the maternal transfer into amniotic fluid and milk for phytochemicals such as anethol, cinnamaldehyde, and eugenol (Blavi et al., 2016a), or limonene, menthol, and carvone (Val-laillet et al., 2018) with improved piglet consumption and growth after weaning. In this thesis (Chapter 6), it was also hypothesized that pre- and/or postnatal flavor exposure through placental fluid and milk to BPCs supplemented to sows could help to reduce neophobia and stimulate voluntary feed intake, and therefore growth performance of piglets after weaning. In fact, the tymol, anethole, linalool, and eucalyptol detected as the most influent compounds in the amniotic fluid, while tymol, anethole, and p-cymene in the milk of sows fed the flavored diets containing BPC1 (Exp 1) improved the piglets post-weaning BW by (33.3%) when compared to Control group at day 7. Meanwhile, weanling piglets exposed to D-limonene, anethole, borneol, p-cymene and eucalyptol detected as the most influent compounds in the amniotic fluid, while D-limonene, anethole, and p-cymene in the milk of sows fed the flavored diets containing BPC2 (Exp 2), showed lower BW gain (-68%) at post-weaning day 7 than Control group (Table 7.5).

These findings confirm the pre- and/or postnatal exposure of newborn and suckling piglets to BPCs and may be enough to establish a link between prenatal life and the postweaning period. However, the results indicated that compared to weanling pigs in

BPC1, the sensorial maternal learning to BPC2 it was not effective to improved piglet's growth after weaning. This is rather interesting since we observed that while D-limonene and *trans*-anethole were transfer into placental fluid and milk, eucalyptol was only transferred into amniotic fluid. There is considerable interaction between ingested phytochemicals and tissues, enzymes and other compounds within the animal. The interaction during absorption, deposition and metabolism, and excretion are highly dependent on the physico-chemical attributes of the compounds involved and their susceptibility to transformation. Physico-chemical factors that are extremely influential and that could have determined the transfer of BPC to placental liquid and/or milk after ingestion are: molecular size and architecture; pH of the environment; hydrophilicity; lipophilicity; charge and polarity; ability to form micelles; solubility (Acamovic and Brooker, 2005).

For instance, a possible metabolization effect for metabolites of eucalyptol that are transferred to the blood, or even metabolic activity in the milk itself (Kirsch et al., 2013), may be the reason for the non-transfer of eucalyptol through sows' milk. Therefore, we suggest that perinatal exposure of piglets to pleasant sensations such as the sweet citrus-like flavor and aroma of D-limonene (Rodríguez et al., 2017) or the sweet anise-like flavor of trans-anethole (Marinov and Valcheva-kuzmanova, 2015) were not sufficient to overcome the unpleasant sensations such as fresh camphor-like smell and a spicy cooling taste of eucalyptol (Bhowal and Gopal, 2016), or other major compounds containing in the BPC2 such as camphor. For instance, Michiels et al. (2012) described that exposure to camphor did not improve feed preference in weaning piglets for a diet contained also thymol. In fact, according to the feed preferences observed in the DCHT we suggest that innate aversion to eucalyptol could be even prenatally conditioned.

Overall, the present PhD thesis provides evidence regarding the potential and mode of actions of dietary PFAs supplementation to implement effective early feeding strategies in the pig production. The objective will be to improve the performance of hyperprolific sow-lines in a scenario of restriction on the use of in-feed antibiotics and therapeutic ZnO, thus promoting the development of pig production in a safe, efficient, and sustainable context.



CHAPTER 8

Conclusions

Based on results presented in this PhD study, it can be concluded that:

1) Pre- and postnatal dietary supplementation of hyperprolific sows with BPCs lead to an improved antioxidant status of sows and their offspring. These improvements were associated with an increased number of piglets born alive with a lower mean birthweight (LBW).
2) Maternal transfer of BPCs through placental liquid and milk influenced the gut health-related functions and histomorphology in LBW piglets. This was evidenced by the improved villus height: crypt depth ratio and by changes on the gene expression related to the nutrient transport and digestion, immune response, intestinal integrity, and oxidative functions in the piglet's jejunum.
3) Colostrum protein and milk fat contents were enhanced in sows supplemented with BPCs. These changes were related with improvements of the sow's antioxidant and immune status, but also to enhanced feed intake of lactating sows.
4) Although the higher number of piglets per litter in BPC groups decreased the mean birthweight, these differences on BW disappeared during the lactation time. The changes observed during the lactation could be associated with the improvements observed on the piglet's gut-health and antioxidant status, but also with the changes observed on the colostrum and milk composition.
5) The supplementation of prestarter diets with D-limonene or *trans*-anethole improved innate feed preferences of weaned piglets, while eucalyptol induced feed aversion and lower performance. In addition, maternal transfer of D-limonene and *trans*-anethole to amniotic fluid and milk, while eucalyptol into amniotic fluid were not sufficient to overcome innate aversion to eucalyptol, which compromised weanling piglet's growth performance.

CHAPTER 9

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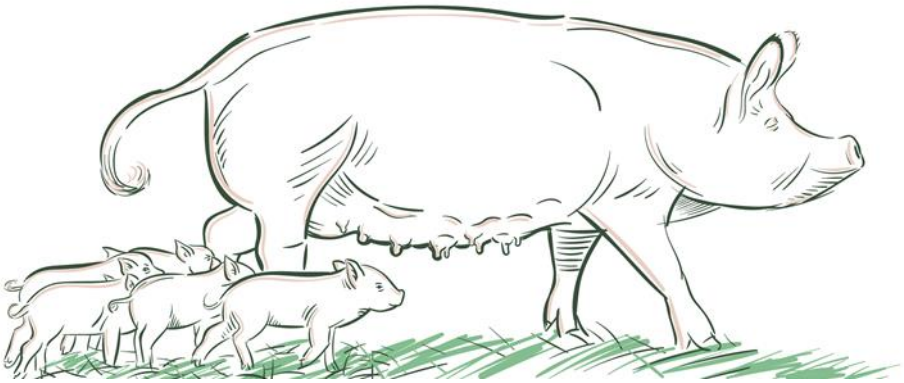

ANNEX 1

Curriculum vitae of the author

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Education

2020-present	**Master's Degree Student in Business Administration and Management focused on the Animal Health Industry**
	ESIC Business&Marketing School, Spain
2017-present	**Ph.D. Student in Animal Production**
	Universitat Autònoma de Barcelona, Spain
2016-2017	**M.Sc. in Research in Animal Nutrition**
	The Mediterranean Agronomic Institute of Zaragoza, Spain
	Universitat Autònoma de Barcelona, Spain
2015-2016	**Postgraduate Specialization in Animal Nutrition**
	The Mediterranean Agronomic Institute of Zaragoza, Spain
2006-2011	**B.Sc. in Agricultural Sciences**
	Universidad de Guadalajara, Mexico

Scientific publications

Prenatal exposure to innately preferred D-limonene and *trans*-anethole does not overcome innate aversion to eucalyptol, *affecting* growth performance of weanling piglets. *Animals, (11) July 2021.*

Phytogenic compounds supplemented to gestating hyperprolific sows affects the gut health-related gene expression and histological responses in neonate piglets. *Frontiers in Veterinary Science, (8) June 2021.*

Phytogenic actives supplemented in hyperprolific sows: effects on maternal transfer of phytogenic compounds, colostrum and milk features, performance and antioxidant status of sows and their offspring, and piglet intestinal gene expression. *Journal of Animal Science, (98) January 2020.*

Use of Spirulina maxima algae as a prebiotic additive in chicken for fattening and its nutraceuticals effects on the intestinal integrity and antimicrobial effect *vs Salmonella spp. ECORFAN-Ecuador Journal, (3) December 2016.*

Divulgative publications

D. Reyes-Camacho et al. 2020. Effects of maternal transfer of phytogenic compounds on piglet performance and gut health. *Pig333.com.*

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Conference proceedings

D. Reyes-Camacho, J.F. Pérez, T. Aumiller, J.D. van der klis, and D. Solà-Oriol. 2020. Maternal transfer of phytogenic compounds supplemented during gestation and/or lactation of hyperprolific sows: effects on reproductive performance and colostrum-milk feature. *MidWest Meeting, ASAS and ADSA Midwest Branch.* Omaha, Nebraska, USA. Type of presentation: Oral.

D. Reyes-Camacho. 2019. Dietary supplementation of phytogenic compounds in hyperprolific sows: synergistic effects on sows and their offspring. *XXVIII Premios FEDNA a investigadores jóvenes en XXXV Curso de Especialización FEDNA.* Madrid, Spain. Type of presentation: Oral.

D. Reyes-Camacho, M.A. Calvo, J.F. Pérez, y D. Solà-Oriol. 2019. Aditivos fitogénicos en cerdas hiperprolíficas: efectos sobre sus parámetros productivos, la composición del calostro y leche, y la transferencia materna de compuestos. *XVIII Jornadas sobre Producción Animal, AIDA.* Zaragoza, Spain. Type of presentation: Oral.

D. Reyes-Camacho, C. Villodre, J.F. Pérez, y D. Solà-Oriol. 2017. Evaluación de preferencias innatas en lechones post-destete frente a diferentes compuestos fitogénicos suplementados en dietas de iniciación. *XVII Jornadas sobre Producción Animal, AIDA.* Zaragoza, Spain. Type of presentation: Oral.

Awards

2019 **XXVIII FEDNA Awards for Young Researchers**

XXXV Curso de Especialización FEDNA. Madrid, Spain.

2011 **Extraordinary Agricultural Sciences Degree Practices**

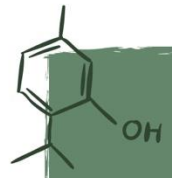
Centro Universitario de Ciencias Biológicas y Agropecuarias.

Guadalajara, Jalisco, México.

Gloria a Dios por las bendiciones, gracia y favor recibidos
para la realización y culminación de este anhelo.

Un poco de ciencia aleja de Dios, pero mucha ciencia devuelve a Él

- Louis Pasteur -



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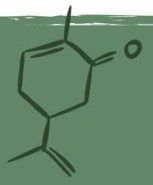

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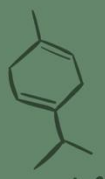
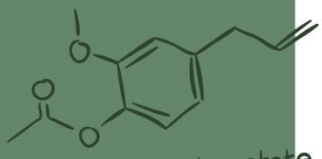
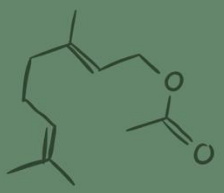


terpinene

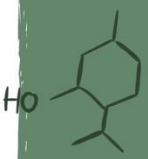


eugenyl acetate



geranyl acetate

limonene



menthol

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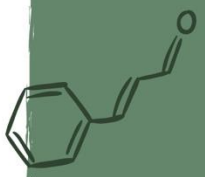

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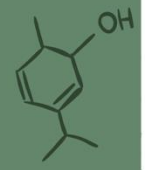

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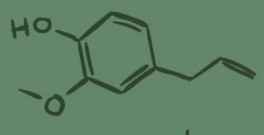

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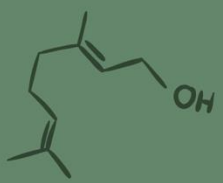

geraniol

p-cymene

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