Universitat Autònoma de Barcelona

EXPLORING DIETARY STRATEGIES TO ENHANCE FEED INTAKE AND GROWTH OF PIGLETS AFTER WEANING BY A MULTIDISCIPLINARY APPROACH

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Certifiquen:

Que la memòria titulada **"Exploring dietary strategies to enhance feed intake and growth of piglets after weaning by a multidisciplinary approach"**, presentada per Laia Blavi Josa amb la finalitat d'optar al grau de Doctor en Veterinària, ha estat realitzada sota la seva direcció i, considerant-la acabada, autoritzen la seva presentació perquè sigui jutjada per la comissió corresponent.

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Ivan Pavlov

Resum

El deslletament és un període d'adaptació i estrès on els porcs poden patir el síndrome post-deslletament, caracteritzat per una baixa ingestió i creixement i per diarrees. És de gran interès per a la indústria porcina la recerca de solucions per incrementar el consum i el creixement després del deslletament. Per tant, l'objectiu d'aquesta tesi és investigar l'optimització de l'estatus mineral (Ca i Zn), l'aprenentatge matern, i la combinació d'extractes de stevia (SE) amb neohesperidina dihidrocalcona (NHDC) com a estratègies per millorar el consum i el creixement després del deslletament.

S'ha observat que baixos nivells de Zn poden conduir a la pèrdua de gana. En conseqüència, en el **Capítol 4**, es van realitzar quatre assajos per estudiar la deficiència de Zn després del deslletament. Hem observat que l'administració de nivells nutricionals de Zn condueixen a una disminució temporal dels nivells sèrics de Zn després del deslletament amb conseqüències sobre la mortalitat i el creixement a llarg termini. També, que la lactació va ser inadequada per satisfer els requeriments de Zn dels garrins, especialment en els garrins lleugers. I finalment, la suplementació diària de 30 mg de Zn durant l'última setmana de lactació podria atenuar la caiguda de Zn al deslletament.

Alts nivells de Ca poden reduir els rendiments productius. Per tant, en el **Capítol 5** es van avaluar els efectes de la inclusió de nivells alts i baixos de calci en la dieta sobre els rendiments productius, la microbiota i l'expressió gènica en quatre assajos diferents. Els nivells alts de Ca (0,95% i 1,55% de $CaCO_3$) van disminuir el PV, el GMD i van augmentar l'IC, aquest efecte va ser més evident amb nivells farmacològics de Zn (2.480 mg/kg de Zn). A més, nivells alts de Ca mostren una major expressió dels gens relacionats amb el procés inflamatori (IFNα, i INFγ) en el jejú i també una comunitat microbiana més heterogènia amb augment dels gèneres *Bacterioides* al còlon dels garrins deslletats.

Les dietes per a garrins es formulen amb alts nivells de Ca i amb nivells farmacològics de Zn, això pot originar formar complexos de fitat-Ca-Zn. En el **Capítol 6** es va realitzar un assaig per observar si la inclusió en dietes post-deslletament de nivells farmacològics de Zn i l'addició de fitasa afecten la digestibilitat aparent del Ca i

P i la digestibilitat estandarditzada del Ca. Hem observat que els nivells farmacològics de Zn disminueixen la digestibilitat i la retenció del Ca i P, però aquest efecte va ser parcialment atenuat per la inclusió de fitasa.

A causa de que els porcs tenen una capacitat sensorial molt desenvolupada, en el **Capítol 7** es va estudiar l'aprenentatge matern; basat en aprendre de les aromes afegides a la dieta materna. Hem detectat per primer cop en el líquid amniòtic els principals compostos volàtils de l'aroma. A més, la inclusió de l'aroma en les dietes de les truges va millorar el consum i el creixement dels garrins després del deslletament.

En el **Capítol 8**, es van realitzar dos experiments per estudiar la preferència de SE i la NHDC amb un test d'elecció doble. Hem observat que la combinació de 150 mg/kg de SE amb 2-4 mg/kg de NHDC augmenta la ingestió i la preferència en comparació amb les dietes que només inclouen SE.

En conclusió, és important l'equilibri entre el Zn i el Ca, pel fet que nivells alts o baixos d'aquests tindran conseqüències sobre el rendiment productiu, l'expressió gènica i la microbiota del garrí deslletat. L'aprenentatge matern i l'addició de SE i NHDC són dues estratègies vàlides per augmentar la ingestió després del deslletament.

Resumen

El destete es un periodo de adaptación y estrés donde los cerdos frecuentemente sufren el síndrome post-destete; caracterizado por una baja ingestión y crecimiento, y por diarreas. Es de gran interés para la industria porcina la búsqueda de soluciones para incrementar el consumo y el crecimiento después del destete. Por lo tanto, el objetivo de esta tesis es investigar la optimización del estatus mineral (Ca y Zn), el aprendizaje materno, y la combinación de extractos de stevia (SE) con neohesperidina dihidrocalcona (NHDC) como estrategias para mejorar el consumo y el crecimiento después del destete.

Bajos niveles de Zn pueden conducir a la pérdida de apetito. Por lo tanto, en el **Capítulo 4**, se realizaron cuatro ensayos para estudiar la deficiencia de Zn después del destete. Observamos que la administración de niveles nutricionales de Zn pueden conducir a una disminución temporal de los niveles séricos de Zn después del destete con consecuencias sobre la mortalidad y el crecimiento a largo plazo. También que la lactación fue inadecuada para satisfacer los requerimientos de Zn de los lechones, especialmente en los lechones ligeros. Finalmente, la suplementación diaria de 30 mg de Zn durante la última semana de lactación podría mitigar la caída de Zn al destete.

Altos niveles de Ca pueden reducir los rendimientos productivos. En consecuencia, en el **Capítulo 5** se evaluaron los efectos de la inclusión de niveles altos y bajos de calcio en la dieta sobre los rendimientos productivos, la microbiota y la expresión génica en cuatro ensayos diferentes. Los niveles altos de Ca (0,95% y 1,55% de $CaCO_3$) disminuyeron el PV, la GMD y aumentaron el IC. Este efecto fue más evidente con niveles farmacológicos de Zn (2.480 mg/kg de Zn). Además, niveles altos de Ca mostraron en el yeyuno una mayor expresión de los genes relacionados con el proceso inflamatorio (IFNα, e INFγ) y también una comunidad microbiana más heterogénea con aumento de los géneros *Bacterioides* en el colon de los lechones destetados.

Las dietas para lechones se formulan con altos niveles de Ca y Zn, y esto puede originar la formación de complejos de fitato-Ca-Zn. En el **Capítulo 6** se realizó un ensayo para observar si la inclusión en dietas post-destete de niveles farmacológicos de Zn y la adición de fitasa afectan a la digestibilidad aparente del Ca y P y a la

digestibilidad estandarizada del Ca. Observamos que los niveles farmacológicos de Zn disminuyeron la digestibilidad y la retención del Ca y P, pero este efecto fue parcialmente mitigado por la inclusión de fitasa.

Debido a que los cerdos tienen una capacidad sensorial muy desarrollada, en el **Capítulo 7** se estudió el aprendizaje materno; basado en el hecho de aprender de los aromas añadidos en la dieta materna. Hemos detectado por primera vez la presencia de compuestos volátiles del aroma en el líquido amniótico. Además, la inclusión del aroma en las dietas de las cerdas mejoró el consumo y el crecimiento de los lechones después del destete.

En el **Capítulo 8**, se realizaron dos experimentos para estudiar la preferencia de SE y la NHDC con un test de elección doble. Observamos que la combinación de 150 mg/kg de SE con 2-4 mg/kg de NHDC aumenta la ingestión y la preferencia en comparación con las dietas que incluyen sólo SE:

En conclusión, es importante el equilibrio entre el Zn y el Ca, debido a que niveles altos o bajos de estos tendrán consecuencias sobre el rendimiento productivo, la expresión génica y la microbiota del lechón destetado. El aprendizaje materno y la adición de SE y NHDC son dos estrategias válidas para aumentar la ingestión después del destete.

Summary

Weaning is a period of adaptation and stress where pigs frequently suffer the post-weaning "growth check", characterized by low feed intake, poor growth, and diarrhea. The search for multiple and combined solutions to increase feed consumption and growth performance after weaning is of great practical interest for the swine industry. The purpose of this thesis is, therefore, to investigate the use of optimizating mineral status (Ca and Zn), maternal learning, and stevia (SE) with neohesperidine dihydrochalcone (NHDC) combination as strategies to improve feed intake and growth performance after weaning.

Low levels of zinc may lead to anorexia and loss of appetite. Therefore, in **Chapter 4**, four trials were performed to study the post-weaning Zn deficiency, its origin, the consequences, and a possible solution. The results showed that the administration of nutritional Zn levels may lead to a temporary decrease in serum Zn levels after weaning, which was more pronounced in light pigs than heavy pigs, and with consequences on mortality and long-term growth performance. The lactation was inadequate to meet pigs' Zn requirements, especially in light pigs of the litter. Finally, we proved that the supplementation of 30 mg of Zn during the last week of lactation mitigated the fall of serum Zn at weaning.

High Ca levels may depress feed intake, weight gain, and feed efficiency. Therefore, in **Chapter 5** effects of low and high dietary calcium on growth performance, intestinal morphology, microbiota and gene expression were evaluated in four different trials. High Ca levels (0.95% and 1.55% limestone) reduced growth performance, decreased BW, ADG and increased feed conversion ratio, but the effect was more evident with in-feed pharmacological levels of Zn (2,000 to 3,000 mg/kg of Zn) than without. Also, high Ca levels showed higher expression of genes related to inflammatory process (e.g. IFNα, and INFγ) in the jejunum of weaned pigs and, a higher heterogeneous microbial community with increased of the *Bacterioides* genera in colon.

Weaned pigs' diets are formulated with high Ca levels and with pharmacological Zn levels that may form Ca-Zn-phytate complexes. In **Chapter 6**, a trial was conducted to observe if inclusion of Zn at pharmacological levels in diets and microbial phytase

affects the apparent total tract digestibility of Ca and P and standardized total tract digestibility of Ca. The results showed that pharmacological levels of Zn reduced Ca and P digestibility and retention, but this effect was partly mitigated by the inclusion of phytase in the diets.

As pigs have a highly developed sensorial capacity, in **Chapter 7**, maternal learning was studied; which it is based on learning from flavors added in the maternal diet. It was detected for the first time major volatile compounds in the amniotic fluid as a link between the intrauterine events and extra uterine responses to flavors. In addition, the inclusion of flavor in the sow diets improved piglet consumption and growth after weaning.

In **Chapter 8**, two double-choice feeding experiments were conducted to study the effect of stevia extracts (SE) and neohesperidine dihydrochalcone (NHDC) on feed preference in pigs. The results showed that the combination of 150 mg/kg SE with 2-4 mg/kg NHDC in starter diets increases feed intake and preference compared to diets including only SE:

Taken together, it is important the balance among nutrients, especially with Zn and Ca, due to high or low levels of them will have consequences on growth performance, gene expression and, microbiota. Maternal learning and the addition of SE and NHDC are two valid strategies to increase feed intake after weaning.

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Abbreviations

The present dissertation follows the standard *Journal of Animal Science* abbreviations.

ANOSIM	analysis of similarities
ATTD	apparent total tract digestibility
BOND	biomarkers of nutrition for development
CD	crypt depth
CEAAH	ethical committee on human and animal experimentation
EFSA	european food safety authority
FCR	feed conversion ration
FDR	false discovery rate
FEDNA	federación española para el desarrollo de la nutrición animal
FTU	phytase activity
GIT	gastrointestinal tract
IAEA	international atomic energy agency
IP	*myo*-inositol phosphate
IZiNCG	international zinc nutrition consultive group
MRE	metal response element
MTF	metal-binding transcription
ND	no data
NES	normalized enrichment score
NHDC	neohesperidine dihydrochalcone
NNS	non-nutritive sweetener
NPY	neuropeptide y
NRC	national research council
OCLN	occluding
OD	odds ratio
OTU	operational taxonomic unit
PC	principal components
PZCs	plasma zinc concentrations
SE	stevia extracts
SEM	standard error of mean
STTD	standardized total tract digestibility

SZCs	serum zinc concentrations
tP	total phosphorus
TRPV	transient receptor vanilloid
TTTD	true total tract digestibility
UNICEF	united nations children's fund
VH	villous height
WHO	world health organization
ZO1	zonula occludens 1

CHAPTER 1

General introduction

Commercial weaning is a sudden change in pig life during which pigs have to be adapted to eat a novel food, usually solid diet, after the separation from their mother. This occurs at d 21 to 28 of age, and pigs pass from a liquid diet of 20% DM to a compound diet with 85% DM (Varley and Wiseman, 2001). The nutritional, psychological and environmental changes produce stress response and anorexia on the first days after weaning (Pluske et al., 2007b). Anorexia produces gastrointestinal disturbances: alterations in small intestine architecture and enzyme activities, transiently-increased mucosal permeability, disturbed absorptive-secretory electrolyte balance and altered local inflammatory cytokine patterns (Lallès et al., 2007).

Besides, weaned pigs have low acid secretion, because of low levels of lactose substrate and consumption of large meals at infrequent intervals, resulting in elevated pH (Kidder and Manners, 1978); the addition of limestone and ZnO in weaned diets, with the highest acid-binding capacity, also raise the pH. At higher pH, the gastric conditions could allow pathogens to survive and to have a greater opportunity to colonise the digestive tract (Yen, 2001), as well as pH $\geq$ 5 in the small intestine precipitates the Ca-Zn-phytate complex, reducing the Calcium and Zinc absorption (Selle et al., 2009).

Another factor which influences feed consumption at weaning is neophobia. It is known as rejection or aversion to the new ingredients, taste like sour and bitter, non-familiar flavors or scents. It happens when an animal is exposed to novel food and is reinforced by the associated novel environment (Hursti and Sjödén, 1997). However, neophobia can be reduced through a learning process before or after birth involving contact with flavor cues and associations between those components and positive consequences that induces a food recognition and more consumption of the flavored diet (Mennella et al., 2001; Wells and Hepper, 2006; Oostindjer et al., 2010; Figueroa et al., 2013).

<u>Methods to increase feed consumption at weaning:</u>

a. To wean the pigs according to their size and ability to cope (including the possibility of weaning different pigs in a single litter at different times).
b. To familiarize pigs to the consumption of significant amounts of solid feed before they are weaned (creep-feeding). These diets should be highly digestible and include ingredients with high palatability (Solà-Oriol et al.,

2012). Creep-feed may also help to satisfy requirements of piglets to achieve a good growth potential and weaning weight, in addition to prepare the digestive system of the sucking pigs to cereal-based solid diets after weaning (Pluske et al., 2003).

c. To avoid environmental (especially temperature) challenges and competitive stress.
d. Good quality and fresh ingredients: weaned pig is very sensitive to dietary mold growth and rancidity, so addition of proper amount of mold inhibitors and antioxidants to the manufactured diet is very important to prevent reduction in palatability during storage.
e. **Optimum balance among nutrients in the diet**: provide adequate amounts of vitamins, trace minerals and limiting amino acids in the diet. Specifically, zinc deficiency triggers a profound loss of appetite (King, 2011; Solomons, 2013a) and high Ca levels depress feed intake (Lei et al., 1994).
f. Use of weaning diets that are highly digestible: the digestibility of diets has a positive relationship with the feed intake.
g. Palatable feedstuff: in the revision of Dong and Pluske (2007) showed that lactose-containing products, spray-dried plasma, spray-dried blood meal, and high quality fish meal are palatable ingredients for the newly-weaned pigs. In addition, the inclusion of whey or lactose in the starter diet ensures continuation of bacterial fermentation and some, though reduced, lactic acid production (Kidder and Manners, 1978). However, improving the palatability of feed has variable and mostly only minor effects (Appleby et al., 1991; Pajor et al., 2002).
h. In relation with the previous point, inclusion of **flavors and taste enhancers** (sensory additives) in the weaning diets may increase fed intake, but not always (Dong and Pluske, 2007). Pigs have an innate preference for sweet (Kennedy and Baldwin, 1972); and sweeteners, which impart a sweet taste, improve feed palatability and zootechnical performance (Yebra-Biurrun, 2005).
i. Promoting ways of a **maternal learning** based on learning from flavors added in the maternal diet. It has been suggested that flavor cues from the maternal diet in uterus can reach the fetus through the amniotic fluid and/or the placental blood stream. This early exposure of fetus to certain cues

generally may result in a preference for these flavors later in life and consequently can positively affect the acceptance of food with a similar flavor before and after weaning (Hepper, 1988; Mennella et al., 2001; Figueroa et al., 2013). This flavor preference can be strengthened when the flavor is also present in maternal milk (Galef and Henderson, 1972). It has been demonstrated that maternal learning improves feed intake, maladaptive behavior, gastro-intestinal problems and poor growth (Oostindjer et al., 2010).

Among the previous strategies, we will focus on those that involve: i.- and optimum balance among minerals (Zn, Ca, and P), ii.- an early learning of piglet to the new food, and iii.- the search of synergisms between sweeteners as feed palatability enhancers. Therefore, the following sections of this literature review will focus on: 1) Minerals: zinc and calcium and their interaction, 2) Learning strategies: maternal transfer and learning, and 3) Sensory feed additives: sweeteners, stevia and neohesperidin dihydrochalcone (**NHDC**) as likely strategies to smooth the weaning period and to improve feed intake and growth.

CHAPTER 2

Literature review

The following literature review highlights the importance of three different strategies in pig nutrition to encourage feed consumption after weaning. It starts with minerals, especially Zinc, Calcium, and also phytate as source of variability of Ca and Zn availability. Afterwards, it follows with learning strategies, focusing on maternal learning. And finally describes sensory feed additives, in particular, the sweeteners with Stevia and Neohesperidine Dihydrochalcone.

2.1. MINERALS

All animal tissues and all feeds contain inorganic or mineral elements in widely varying amounts and proportions (Underwood and Suttle, 1999). The term 'essential mineral element' is restricted to a mineral element that has been proven to have a metabolic role in the body. Before an element can be classed as essential it is generally considered necessary to prove that purified diets lacking the element cause deficiency symptoms in animals and that those symptoms can be eradicated or prevented by adding the element to the experimental diet (McDonald et al., 2002). In 1981, 22 mineral elements were believed to be 'essential' for the higher forms of animal life (Underwood and Suttle, 1999). These comprised seven major or macronutrient minerals – calcium, phosphorus, potassium, sodium, chlorine, magnesium and sulphur – and 15 trace or micronutrient mineral elements – iron, iodine, zinc, copper, manganese, cobalt, molybdenum, selenium, chromium, tin, vanadium, fluorine, silicon, nickel and arsenic (Table 2.1). Since then, aluminum, lead and rubidium have been shown to be beneficial in some circumstances. This dissertation focuses on Calcium and Zinc, and their interaction in diets fed to pigs.

Table 2.1. *Nutritionally important essential mineral elements and their approximate concentration in the animal (McDonald et al., 2002).*

Major elements	g/kg	Trace elements	mg/kg
Calcium	**15**	Iron	20-80
Phosphorus	10	**Zinc**	**10-50**
Potassium	2	Copper	1-5
Sodium	1.6	Molybdenum	1-4
Chlorine	1.1	Selenium	1-2
Sulphur	1.5	Iodine	0.3-0.6
Magnesium	0.4	Manganese	0.2-0.5
		Cobalt	0.02-0.1

2.1.1 ZINC

2.1.1.1 Functions

Zinc is an essential micronutrient for multiple aspects of metabolism. Since 1933 Zinc was shown to be an essential nutrient for rats and mice, in 1955 for pigs and 1963 for humans (Todd et al., 1933; Tucker and Salmon, 1955; Prasad et al., 1963). Although it is a dietary trace element, it is one of the most abundant elements within cells. Approximately 95% of the body zinc is within the cells (King, 2011). Zinc is a type II nutrient, like P or Mg, which means that is required for general metabolism, and physiologic signs of zinc deficiency are linked with diverse biochemical functions rather than with a specific function. Type I nutrients, e.g. Fe, Cu, vitamin C, folic acid, are required for one or more specific functions (Golden, 1989). Therefore, when dietary zinc is insufficient, a marked reduction on endogenous zinc loss occurs immediately to conserve the nutrient; this process is accompanied by a profound loss of appetite and a cessation in growth (King, 2011; Solomons, 2013b).

Zinc has three very basic functions, classified as catalytic, structural, and regulatory (Cousins, 2006). The information is presented in table 2.2.

Table 2.2. *Basic zinc functions, adapted from King (2011) and King et al. (2016).*

Enzyme catalyst (Cousins, 2006; Eide, 2006)	• Zinc is a catalyst for > 300 different enzymes • Zinc metalloenzymes decrease activity under conditions of zinc deficiency, but their protein structure does not change; adding zinc restores enzyme activity. • There is no direct link between zinc deficiency symptoms and the function of an individual enzyme or enzymes.

Structural component (Cousins, 2006)	• 2,500 transcription factors or 8% of the human genome require zinc for their structural integrity.
	• Zinc fingers
	  ○ Zn fingers motif in frog were discovered in 1985 and established a structural role for zinc
	  ○ Their function is stabilize the structure of DNA, RNA, proteins or other molecules (Keen et al., 2003)
	  ○ Contain 4 cysteines that allow zinc to be bound in a tetrahedral complex. Some zinc fingers have histidine

	substituted for cysteine. o Occur in proteins involved in signal transduction, cellular differentiation or proliferation, cellular adhesion, and transcription • There is a tight homeostatic control of zinc because of the abundance of zinc fingers. • Zinc is also involved in maintaining the structure of enzymes such as CuZn superoxide dismutase where copper is at the active site and zinc maintains enzymatic structure.
Regulation of gene expression (Cousins et al., 2006)	• Zinc regulates more than thousand gens through the metal response element (**MRE**) and a metal-binding transcription factor (**MTF**) in the promoter of the regulated gene. o The MTF acquires Zn in the cytosol or nucleus and then interacts with the MRE to stimulate transcription. o Depending on the cellular zinc status, MTF-1 can negatively or positively regulates numerous genes. o It is thought that dietary Zn can be transported into the cells and interact with MTF-1, facilitating translocation to the nucleus for MRE binding and stimulating transcription o It could be explained the effects of zinc deficiency on lipid peroxidation, immune function, apoptosis, and neuronal function by this mechanism of gene expression regulation • Zinc controls numerous cell-signaling pathways by modulating kinase and phosphorylase activities

Therefore, Zn has a big impact on growth because it stimulates connective tissue development and maintenance (Fukada et al., 2008), bone mineralization (Yamaguchi, 1998) and regulates the function of insulin-like growth factor 1. It is also essential also for both innate and adaptive immunity (Prasad, 2009) and is a good antioxidant agent (Eide, 2011). Zn is also involved in other functions like wound healing and blood clotting, taste

acuity and appetite control (Stefanidou et al., 2006; Murakami and Hirano, 2008; Prasad, 2009; Bhowmik et al., 2010; Plum et al., 2010).

2.1.1.2. Absorption and bioavailability

Zinc bioavailability and absorption are complex to understand and assess as there are several factors that have an impact on them:

- Dietary factors: presence of nutrients that interact with Zn (e.g. Ca, Fe) or anti-nutritional factors (e.g. phytate) in the diet. In the followings sections the minerals and phytate interactions will be discussed
- Zn source: the specific chemical properties of each Zn source (Zn from feedstuffs vs. Zn from supplements, different chemical properties between Zn compounds) and their level in the diet
- Physiological factors: such as the Zn status of the individuals, expression of several proteins (metallothioneins, Zn transporters) that regulate its absorption, gastric pH, or the fasting or level of DM intake.

Absorption of dietary zinc is best characterized as a saturation process and is primarily determined by two factors: the amount of zinc ingested and dietary phytate (Miller et al., 2007; Hambidge et al., 2010).

Zinc is released from food as free ions during digestion. Zinc absorption occurs in the small intestine, in the distal duodenum and proximal jejunum; with very little being absorbed from the stomach (Underwood, 1977). However, colon and cecum can contribute to Zn absorption when it is impaired in the small intestine (Hara et al., 2000).

There are two mechanisms for the intestinal transport of Zn from the lumen of the intestine (the basolateral membrane of the enterocyte; Hambidge et al., 2010) to the portal circulation (Menard and Cousins, 1983; Steel and Cousins, 1985):

1. Transcellular: regulated at the intestine level, saturable, carrier-mediated, minerals pass through apical and basolateral membrane of the cell
2. Paracellular: non-regulated, unsaturable, diffusional-mediated, minerals pass between cells.

Transcellular: the uptake of zinc into cells and its transport into and out of intracellular organelles requires transporter proteins that span these membranes to facilitate the movement of zinc. Zinc transporters come from two families, the ZIP (SLC39) and CDF/ZnT (SLC30) proteins (Eide, 2006; Figure 2.1). The ZIP family transport Zn and/or other metal ion substrates from the extracellular space or organellar lumen into the cytoplasm. The CDF/ZnT family transport Zn and/or other metal ions from the cytoplasm into the lumen of intracellular organelles or to the outside of the cell. Thus, CDF/ZnT proteins work in opposition to the ZIP transporters (Figure 2.1; Eide, 2006). There is an important Zn transporter for the acquisition of zinc from the diet, ZIP4 (Zrt/Irt-like protein 4; SLC39A4). The presence of ZIP4 into the apical surface of intestinal cells is sensitive to dietary Zn, increased with Zn deficiency and decreased during Zn sufficiency (Kim et al., 2004) and can also be influenced by the genotype (Siebert et al., 2013). Thus, genotype should be taken into consideration in animal breeding and experimental animals for the influence on Zn absorption. The ZnT5 (Zn transporter 5; SLC30A5) may function as both an influx and efflux Zn transporter in the intestinal cell (Valentine et al., 2007) and it seems to respond to lumen Zn concentration (Cragg et al., 2002). However, the expression of ZnT5 and also ZnT1 increase in the jejunum when high levels of Zn are fed, to increase the export of Zn from epithelial cells (Chai et al., 2014). The mechanism of the intracellular transport of Zn from the apical to the basolateral intracellular surface for transport to the portal circulation is not fully known at this time.

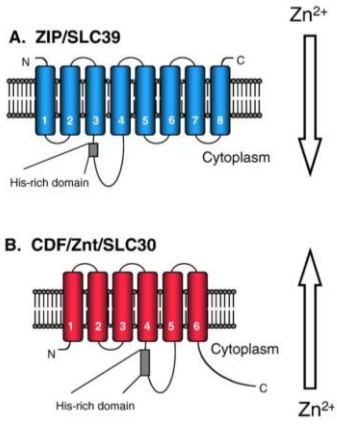

Figure 2.1. *The predicted membrane topologies of the ZIP/SLC39 and CDF/Znt/SLC30 families of metal ion transporters (Eide, 2006).*

2) Paracellular: as the dietary level of the Zn increases the total amount of Zn entering the body increases due to the existence of both carrier-mediated and non-regulated diffusion absorption. However, the efficiency of absorption falls. This fact has special relevance in piglets receiving therapeutic doses of ZnO.

Zinc metabolism has specific considerations that limit our ability to measure Zn absorption: high endogenous intestinal excretion, rapid turnover of Zn in plasma and constant urinary excretion over a wide range of dietary intake. The apparent absorption is calculated as the difference between dietary Zn intake and fecal Zn content and to measure the true absorption of Zn, endogenous Zn should be separated from unabsorbed dietary Zn and isotope techniques are necessary (Hotz and Brown, 2004). A new experimental model has been developed to determine Zn requirement in piglets and hence to assess bioavailability of dietary Zn sources (Brugger et al., 2014) based on Zn digestibility, Zn concentration in different tissues (liver, bone, blood) and gene expression (hepatic metallothionein [**MT**]).

2.1.1.3. Available biomarkers

There is a lack of a good indicator of zinc status, the signs of depletion are diverse and cannot be attributed to a defect in a specific function. A specific biomarker has not yet been identified and is unlikely that a specific biomarker of Zn exists (King, 2011). The Biomarkers of Nutrition for Development (**BOND**) Zinc Expert Panel recommended three zinc biomarkers for estimating zinc status in humans: dietary intakes, plasma zinc concentration (**PZCs**) or serum zinc concentration (**SZCs**), and height-for-age of growing infants and children.

2.1.1.3.1. Dietary Zn assessment

An assessment of dietary Zn intakes is the best method for estimating Zn exposure in humans (King et al., 2016). For individuals clinical application, a Zn assessment can be estimated from a diet history or food frequency questionnaire. For human populations, the degree of risk of deficiency and the need of intervention (Hotz and Brown, 2004) can be estimated from food balance-sheet data provided by the Food and Agriculture Organization. However, in livestock animals their feed is usually formulated following the guidelines of

each specie and age. In swine, values can be obtained from national committees or from the industry, such as for e.g. NRC or FEDNA (Table 2.3). However, the difficulty is to know exactly the quantity of feed that they eat, due to animals in commercial conditions are allotted in group. Another individually and more accurate Zn status assessment is needed.

Table 2.3. *Recommended dietary and daily allowances for Zn depending on body weight and productive stage of swine.*

	NRC, 2012		FEDNA, 2013
BW Range (kg)	mg/kg	mg/day	mg/kg
5-7	100	26.6	120 (100-130)
7-11	100	46.8	
11-25	80	72.4	110 (110-120)
25-50	60	90.2	
50-75	50	105.9	
75-100	50	125.3	80 (90-110)
100-135	50	139.4	
Gestation sows	100	210.0	100 (95-120)
Lactating sows	100	596.6	100 (95-120)
Sexually active boars	50	118.8	120 (95-140)

2.1.1.3.2. Plasma/serum zinc concentration (PZCs / SZCs)

Even though only 1% of the total body Zn is presented in circulating blood, plasma (or serum) Zn concentration responds to dietary manipulation in both depletion and supplementation studies (Hotz, 2007; Lowe et al., 2009). Consequently several expert committees, WHO, UNICEF, IAEA, and IZiNCG have endorsed PZCs or SZCs as the only biochemical indicator recommended of Zn status, especially for assessing the risk of Zn deficiency in populations (Benoist et al., 2007; Hess et al., 2007; Lowe et al., 2009).

Zn concentration are greater in serum than plasma, this difference appear to be partly dependent on the time between collection and separation (English and Hambidge,

1988). When both types of samples (plasma and serum) are retained for identical periods of time before separating the cells, the Zn concentration results for plasma and serum no longer differed (King et al., 2016). Anti-coagulants, such as heparin or EDTA, required for the separation of plasma are potential sources of zinc contamination (Pineau et al., 1993). Therefore, using serum also avoids the possibility of contamination by anti-coagulants. All Zn analyses of the present thesis are based on serum.

2.1.1.3.3. Effect of zinc supplementation on plasma/serum zinc concentration

Plasma zinc is a pool of zinc readily available for uptake by tissues when needed, and it is a component of the small, vulnerable zinc reserve (King, 2011). During a severe dietary Zn restriction (< 1 mg/day for 4−5 weeks) there is a marked drop (about 35%) of Zn in the rapidly exchangeable Zn pool, but little or no measurable effect is registered on the size of the slow Zn pool, which is maintained as the expense of the rapid pool (King et al., 2001). Therefore, a plasma/serum Zn concentration of less than about 0.70 mg/L suggests deficiency (Hotz and Brown, 2004; Maret and Sandstead, 2006; Suttle, 2010; Crook, 2011), 0.4-0.8 mg/L are associated with marginal status (Puls, 1988). In pigs, plasma Zn concentrations of 0.33 mg/L has been found in Zn-deficient pigs and, 0.85-1.2 mg/L in Zn sufficient pigs (Johanning et al., 1990).

Several reviews and meta-analyses have provided a sizeable body of information on the effects of zinc supplementation on PZCs/SZCs among children (Brown et al., 2002; Brown et al., 2009a; Brown et al., 2009b; Haider and Bhutta, 2009; Moran et al., 2012) and adults (Lowe et al., 2009; Lowe et al., 2012). For pigs, also the supplementation of pharmacological levels of zinc had an increase on PZCs/SZCs (Hahn and Baker, 1993; Hill et al., 2000; Walk et al., 2015).

2.1.1.3.4. Uses and limits of plasma/serum Zn concentration

The utility of plasma/serum Zn concentration as a biomarker of Zn nutrition can be assessed in several ways (King et al., 2016):

1) Measure the PZC/SZC response after controlled manipulations of Zn intake including both zinc-depletion/repletion studies and zinc supplementation trials.
2) Asses the relation between usual dietary zinc intake and PZC/SZC.

3) Compare PZCs/SZCs between individuals by using clinical signs that are generally recognized as functional outcomes of severe Zn deficiency.
4) Compare initial PZCs/SZCs between individuals who do or do not show a functional response to changes in their Zn intakes.

Plasma/serum Zn concentrations may have limitations (King et al., 2016):

1) Heterogeneity in all population groups
2) PZC/SZC does not respond to short-term exposure for fortified zinc foods; some response may occur for longer periods.
3) It is not known if functional changes occur without changes in plasma zinc
4) Biological factors, other than zinc intake, influence plasma zinc concentrations:
    a. Acute infection and inflammation reduce serum Zn concentration, due to the redistribution of Zn from the plasma to the liver carried out by MT (Moshage, 1997).
    b. Time of day, plasma/serum Zn concentration fluctuate as much as 20% during 24-h period, due to the effects of food ingestion (Hambidge et al., 1989).
    c. sex, age, pregnancy, oral contraceptive use, severe stress, position of subject during blood drawing, and length of time subject's arm is occluded with a tourniquet.

2.1.1.3.5. Other biomarkers

Other possible biomarkers have been tested to measure serum or plasma zinc and growth. The BOND zinc expert panel reviewed the data and divided them into 3 categories: potentially useful but needing more information, emerging biomarkers, and those that are not useful (Table 2.4).

2.1.1.4. Zinc deficiency and physiological implications

The demonstration of zinc deficiency as a cause of clinical disease in mammals was first shown in swine and was manifested by parakeratosis, a skin disorder of eczema and erythematous dermatitis (Tucker and Salmon, 1955). According to Chasapis et al. (2012) Zn is such a critical element in health that even a small deficiency is a disaster.

Multiple body functions are affected by zinc deficiency, including physical growth, immune competence, reproductive function, neurobehavioral development (King, 2011; Grider, 2013), and digestive function (Pallauf and Kirchgessner, 1976; Roth et al., 1992). Also lack of zinc leads to anorexia, loss of appetite, smell and taste failure, alopecia, and impaired wound healing (Chasapis et al., 2012). However, the deficiency is sometimes difficult to diagnose (Prasad and Oberleas, 1971) and the underlying biochemical defects responsible for most of them have not been found (Grider, 2013). There is no body store of available zinc, except maybe in the liver of infants (Pierpaolo et al., 1996) but not in dogs younger than 1 year (Paßlack et al., 2015). Therefore a regular, adequate dietary supply is required.

Table 2.4. *Potential, emerging, and not useful zinc biomarkers, adapted from (King et al., 2016).*

	Potential	Emerging	Not useful
Definition	Biomarkers that show promise, but data are insufficient to establish specific cut-offs indicating zinc inadequacy in populations	Biomarkers for which there is some theoretical basis for a relation to zinc intake or status, but testing is insufficient to confirm the relation	Biomarkers that do not relate consistently to zinc intake or status
Biomarkers	- Hair zinc (for long-term Zn status (Hotz and Brown, 2004)). - Urinary zinc - Neurobehavioral function	- Nail zinc - Zinc-dependent proteins - Oxidative stress and DNA integrity - Zinc kinetics - Taste acuity	- Zinc-dependent enzymes - Erythrocyte and leukocyte zinc - PMNCs, mononuclear cells, platelet Zn and plasma alkaline phosphatase

A variety of physiological mechanisms including neurotransmitter and endocrine secretions have been explored as potential mediators of zinc-induced anorexia, including norepinephrine, gama-aminobutyric acid, dopamine, dinorphin, and galanin, without consistent results (Teegarden and Gunther, 2008). The regulation of neuropeptide Y (**NPY**)

a hunger-stimulating peptide, may also play a role in zinc-mediated reduction in food intake although it has been controversial because hypothalamic NPY levels are elevated in both zinc deficiency and food restriction (Fukagawa, 2003); reduced food intake despite elevated NPY levels in zinc deficiency is described as NPY resistance (Baltaci and Mogulkoc, 2012).

Zinc deficiency causes can be divided into (Hambidge, 2000; Maret and Sandstead, 2006; Crook, 2011):

1. Primary causes (genetic): acrodermatitis enteropathica, a rare autosomal-recessive condition in human individuals caused by a mutation of the ZIP4 gene (Slc39a4), leading to low Zn absorption (Geiser et al., 2012)
2. Secondary causes: include poor intake and absorption due to the presence of phytate in the diet, malabsorption states (e.g. inflammatory bowel disease, coeliac disease) and, long-term parenteral nutrition. Zn deficiency can also result from increased utilization such as sepsis, and trauma and enhanced loss as in extensive burns and renal tubule disease. Zinc deficiency is also associated with vitamin A and D deficiency and also iron deficiency.

However, the predominant Zn malnutrition phenotype in men and animals is a subclinical deficiency associated with reduction of Zn status parameters without development of visible symptoms (Brugger and Windisch, 2016). Weaned pigs with subclinical Zn deficiency impair Zn status parameters in plasma (PZCs, plasma Zn-binding capacity, plasma alkaline phosphatase activity), femur and liver Zn, apparent digestibility of Zn (Brugger et al., 2014), and pancreatic exocrine enzymes (Brugger and Windisch, 2016).

In pigs, the lowest Zn plasma levels are reached 2-3 days post-weaning, but supplementing pharmacological levels of ZnO rapidly increase Zn plasma levels. This drop is independent of a 24h fasting period, suggesting a direct effect of weaning on the plasma values, likely by increasing endogenous Zn losses in the feces or after sequestration by internal organs (Davin, 2014). **Therefore, more research is needed to elucidate factors and solutions that influence Zn deficiency in weaned pigs.**

Hypothesis

Pigs have a deficit of Zn after weaning that could limit the functional and immunologic response of pigs. Therefore, supplementing with Zn during lactation may increase post-weaning serum Zn levels. This deficit is variable depending on different productive conditions

2.1.2. CALCIUM

2.1.2.1. Functions and physiological role

Calcium and Phosphorus are two important minerals in the body that are interrelated because the absorption and utilization of one mineral may influence the absorption and utilization of the other. Calcium is the 5th most abundant element in the body with 96 to 99% residing in the skeleton as hydroxyapatite (Weaver and Peacock, 2011); and phosphorus is the 6th most abundant element in the body with 60 to 80% of the P in the skeleton tissue, and only 20 to 40% of P is present in soft tissue or fluids (Crenshaw, 2001; Vitti et al., 2010). The greater variation in P in skeletal tissue compared with Ca is due to variation in the proportion of soft tissue to skeletal tissue during the growing phases of pigs (Crenshaw, 2001).

In the 1800s, the association of Ca and P to rickets was clear, but the relationship of the disease to vitamin D was not established until the 1920s. They have important functions in the body such as formation and maintenance of bones, transmission of nerve impulses, cofactors of enzymes, muscle contraction, synthesis of protein and phospholipids, components of nucleic acids (Crenshaw, 2001; Ewing and Charlton, 2007; Vitti et al., 2010). In addition, Ca has been recognized to have protective effects on the resistance to enteropathogenic infections by decreasing colonization and translocation of common intestinal Gram-negative pathogens in rats (Bovee-Oudenhoven et al., 1997; ten Bruggencate et al., 2011) and P participates in energy metabolism and storage (as ATP, GTP, creatine phosphate, arginine phosphate etc.), and is an important buffer in most body compartments (Kohlmeier, 2003).

Calcium and P are one of the most abundant mineral elements in the animal body. However, they are frequently found in insufficient quantities in common feedstuffs to meet requirements of livestock. Calcium is generally deficient in grains and abundant in most forages, whereas P is higher in seeds than roughages and seed by-products. Feeds containing milk and bone are high in both P and Ca (McDowell, 2003). Therefore, Ca deficiency is more a problem of animals fed mostly on concentrates, especially pigs and poultry, whereas, P deficiency is predominantly a condition of grazing ruminants, especially cattle (McDowell, 2003).

2.1.2.2. Absorption of Ca

Calcium needs to be in a soluble or ionic form to be absorbed. Calcium is mainly absorbed in the small intestine (Partridge, 1978; Liu et al., 2000), mostly in the duodenum, but the place where Ca is absorbed may vary among Ca sources and types of diets (Partridge, 1978; González-Vega et al., 2014a). Although some studies indicated absorption of Ca is also in the colon (Liu et al., 2000), results of recent studies have indicated that no absorption of Ca takes place in the large intestine (González-Vega et al., 2014a).

Calcium can be absorbed by (Bronner, 1987):

1. Saturable transcellular absorption (active transport): at lower Ca levels, using cellular routes. Transcellular Ca^{2+} absorption takes place against an electrochemical gradient and, therefore, requires energy. This active Ca^{2+} transport is under the control of hormones in a Ca^{2+}-dependent manner (van Abel et al., 2003).
2. Nonsaturable paracellular (diffusion) absorption: at high levels of dietary Ca, using paracellular routes.

The transcellular absorption: Ca is transported through Ca channels that are located in the apical side of the cell. These channels are called transient receptor potential vanilloid (**TRPV**), and designated as TRPV6 and TRPV5 for the intestines and the kidneys, respectively (Christakos, 2012). Once Ca is inside the cell, the Ca binding proteins (CaBP), also known as calbindin, diffuses the Ca across the cell. The CaBP9K is principally found in the intestines and CaBP28k in kidneys. Calcium exits the cell from the basolateral side

through a Na/Ca exchanger with the plasma membrane Ca-ATPase. The saturable mechanism is vitamin D (1,25 dihydroxycholecalciferol (1,25-(OH)2D3)) –dependent because 1,25-(OH)2D3 influences the expression of CaBP9k and TRPV6 (Christakos, 2012), and the expression of CaATPase (Figure 2.2).

Some studies have demonstrated that vitamin D up-regulated TRPV5, TRPV6, CaBP, and Ca-ATPase (van Abel et al., 2003; Kutuzova and DeLuca, 2004) and dietary Ca up-regulates only TRPV6 and CaBP (van Abel et al., 2003). It has been believed that 1,25-(OH)2D3 influences only the saturable mechanism, but results of recent studies indicated that 1,25-(OH)2D3 may also play a role in the nonsaturable paracellular mechanism (Kutuzova and DeLuca, 2004; Christakos, 2012).

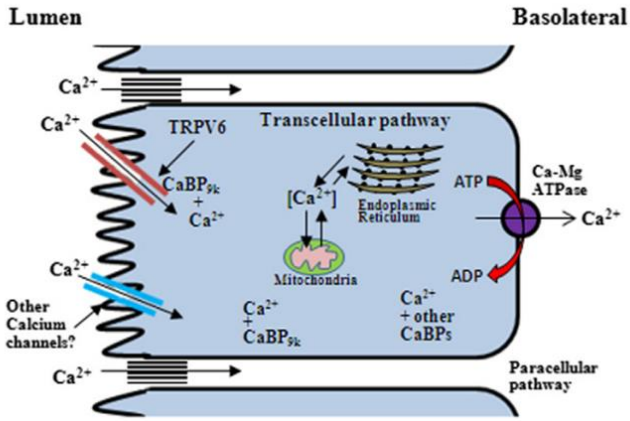

Figure 2.2. *Model of vitamin D mediated intestinal calcium absorption. The model of transcellular calcium transport consists of influx through an apical calcium channel (TRPV6), diffusion through the cytosol and active extrusion at the basolateral membrane by the plasma membrane ATPase (PMCA1b; Christakos, 2012).*

Increasing dietary Ca decreases the mRNA expression of TRPV6, but this does not result in less absorption of Ca, because dietary Ca increases and an increased proportion of Ca is absorbed via the paracellular route in the small intestine. The total percentage of Ca that is absorbed is almost constant regardless of dietary Ca concentration (Stein et al., 2011). High concentrations of dietary Ca will also reduce the mRNA expression in the kidneys of TRPV6, TRPV5, S100 calcium binding protein G, and calbidin 1(González-Vega et al., 2016), but because there is no paracellular reabsorption of Ca in the kidneys

excretion of Ca in the urine is increased as dietary Ca concentration increases. As a consequence, body Ca concentrations are primarily regulated in the kidneys (González-Vega et al., 2016).

2.1.2.3. Digestibility of Ca

The concentration and digestibility of Ca differ among Ca sources. Animal and inorganic sources have relatively high concentrations of Ca, whereas plant sources have relatively low concentrations of Ca. As a consequence, Ca originating from animal proteins or inorganic sources are used to supply Ca in swine and poultry diets (NRC, 2012).

The concentration of Ca in most feed ingredients has been reported; however, few digestibility values are available. Digestibility can be defined as apparent, standardized, or true digestibility (Stein et al., 2007), and can be measured by segments as ileal, colon, cecal, etc, or by the total tract.

Apparent digestibility values of a nutrient may vary with the concentration of the nutrient in the diet if there are endogenous losses of the nutrient. Therefore, apparent digestibility values are not expected to be always additive in mixed diets, whereas values for standardized or true digestibility are additive in mixed diets (Stein et al., 2005) because these values are corrected for endogenous losses. Thus, if basal or total endogenous losses are subtracted from the output in the digestibility calculations, values are not influenced by the nutrient concentration. It is, therefore, recommended to formulate diets using standardized or true digestibility values (NRC, 2012). Calcium has important endogenous losses; therefore digestibility values need to be calculated as standardized or true digestibility (Gonzalez-Vega et al., 2013).

2.1.2.4. Ca deficiency

Failure to provide adequate Ca, P, or vitamin D in the diet may result in abnormalities in bone structure. A deficiency of anyone or all three of these nutrients produces rickets in young, growing animals, and osteomalacia in older swine. During growth, Ca deficiency causes poor growth, lameness, stilted gait, a general tendency to go down or lose the use of the limbs (posterior paralysis), frequent cases of fractures, softness

of bones, bone deformation, beading of the ribs, enlargement and erosion of joints, and unthriftiness. Poor growth is primarily a sign of a P deficiency, while growth retardation results only when Ca is severely deficient over a long period while P is adequate (McDowell, 2003).

2.1.2.5. High Ca levels

High Ca levels depress weight gain, feed efficiency (Lei et al., 1994; Fan and Archbold, 2012; González-Vega and Stein, 2016), and feed intake (Lei et al., 1994). In addition, elevated levels of Ca reduce phytase efficacy (Lei et al., 1994; Selle et al., 2009) and the digestibility of P (Stein et al., 2011; González-Vega et al., 2014a). The interaction could be explained because excess dietary Ca tends to form insoluble complexes with phytate or phosphate in the small intestine (Cromwell, 1996), or compete for the active site of phytase (Wise, 1983; Pointillart et al., 1985; Qian et al., 1996) rendering them unavailable for hydrolysis in the stomach (Wise, 1983) and small intestine (Cromwell, 1996).

Among ingredients used in pig diets, limestone and ZnO show the highest acid-binding capacity values (Lawlor et al., 2005). Therefore, high dietary Ca or Zn may also favor an increase in digestive tract pH, which in turn decreases phytate solubility (Sandberg et al., 1993; Selle and Ravindran, 2008).

High Ca and P levels downregulate the expression of proinflammatory cytokines and increase digestive and absorptive functions (Metzler-Zebeli et al., 2012); but affect negatively the intestinal microbiota by decreasing gastric streptococci and lactate (Metzler-Zebeli et al., 2011) and, decreasing the crypt depth on the cecum (Metzler-Zebeli et al., 2012).

Hypothesis:

High Ca levels decrease feed intake, weight gain and feed efficiency; also high Ca and P levels may affect negatively intestinal microbiota. Therefore, low Ca diets (without limestone) early after weaning may increase pigs performance and promote changes in microbiota and intestinal mucosa.

2.1.3. MINERAL INTERACTIONS

Minerals may interact each other and with other nutrients and non-nutritive factors. This interaction, which may be synergistic or antagonistic, takes place in the feed itself, in the digestive tract and during tissue and cell metabolism (Georgievskii et al., 1982).

Synergism interaction: elements that mutually enhance their absorption in the digestive tract and jointly fulfil some metabolic function at the tissue or cell level.

Antagonisms interaction: elements that inhibit the absorption of each other in the digestive tract and produce opposite effects on any biochemical function in the organism. On the contrary from synergism, which is most often mutual, antagonism may be one- or two-sided. For example, Zn and Cu inhibit the absorption of each other in the intestine, whereas K inhibits the absorption of Zn and Mg, but not the other way round. Another examples of antagonistic interactions is the formation of the triple Ca-P-Zn salt in the presence of high Ca concentrations in the diet, explained in more detail in the next section, or Fe^{+2} competing with Zn^{+2} for the bond with plasma transferrin.

The antagonistic interaction of elements may be predicted from their position in the Periodic Table. Such interaction is the result of their physicochemical similitude, their tendency to form complexes and their degree of affinity to the corresponding reactive groups of biopolymers. This explains the antagonisms of elements such as Zn and Cd, Zn and Cu, Ca and Fe (Figure 2.3).

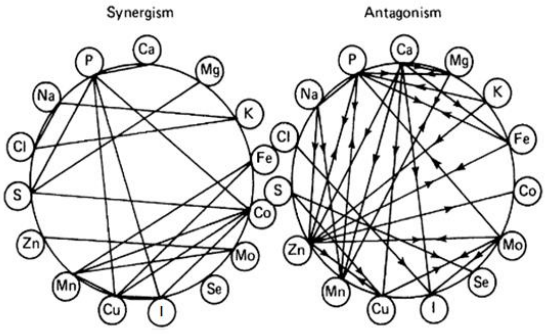

Figure 2.3. *Biochemical interrelation of 15 essential minerals; synergistic on the left, antagonistic on the right. The scheme also shows the mutual relations both in the digestive tract and during metabolism (Georgievskii et al., 1982).*

2.1.4. PHYTIC ACID

Availability of Ca, P and Zn may vary considerably according to their chemical combination or physical association with other compounds in feeds, such as phytate.

Phytate, which is a salt of phytic acid and is formed from six phosphate molecules combined with myo-inositol (myo-inositol 1,2,3,4,5,6-hexakis dihydrogen phosphate); is present in most plant seeds as Mg and K salt mixtures (Onyango et al., 2009). The concentration of phytate in plant seeds is variable, but mostly between 1 and 3% (Graf, 1983). Phytic acid has a molecular weight of 660 g/mol and has 12 negative charges that may chelate different cations such as Ca, Mg, K, Cu, Zn, Fe and also may bind protein, starch, and lipids (Onyango et al., 2009; Selle et al., 2009). The functions of phytic acid are to protect the seed from oxidative stress (Graf, 1983) and to retain some P, inositol, and other minerals in the seed for the germination process (Kies, 2005).

2.1.4.1. Phytate and Minerals

In grains and plant protein supplements, most of the P is bound to phytate, which is unavailable for absorption by pigs and poultry. The proportion of total P contained as phytate P may range from 59 to 70% in cereal seeds, 20 to 46% in legume seeds, and from 34 to 66% in oilseed meals (Eeckhout and De Paepe, 1994). Phytate contains 282 g P per kg (Selle et al., 2009). The majority of the phytate-bound P is, therefore, excreted in the feces and may contribute to increase P-pollution. Consequently, diets for pigs are most correctly formulated based on standardized total tract digestibility (**STTD**) of P (NRC, 2012). Inclusion of inorganic P in the diets is needed to compensate for the low STTD of the phytate-bound P. However, this solution is environmentally not sustainable and becoming progressively more expensive (Kies, 2005). Therefore, during the last decades research has been focused on identify solutions that may contribute to a reduction in the amount of P excreted in the feces and also to make more phytate bound P available to pigs and poultry.

Phytate is negatively charged and as phytate moves from low pH in the stomach to a greater pH in the small intestine, phytate becomes more negative (Santos, 2012). This enhances the reaction between phytate and cations or proteins (Figure 2.4) resulting in the

precipitation of these salts (Santos, 2012).Phytate has more affinity for Cu and Zn, but Ca is the most abundant cation in swine and poultry diets. Then, Ca is more likely than other cations to form such salts (Selle et al., 2009). One molecule of phytic acid can bind an average of 3 to 6 moles of Ca to form insoluble phytates at the pH of the intestine (McDowell, 2003). However, Cu and Zn are usually incorporate at high doses in weanling diets due to this minerals may act as growth promoters (Jay et al., 2010) and/or prevent the post-weaning diarrhea (Hahn and Baker, 1993; Carlson et al., 2006; Hu et al., 2012). Therefore, Cu and Zn also bind to phytate in the intestinal tract, and thereby, reduce the availability of these minerals for absorption (Kerovuo, 2000; Kornegay, 2001; Santos, 2012).

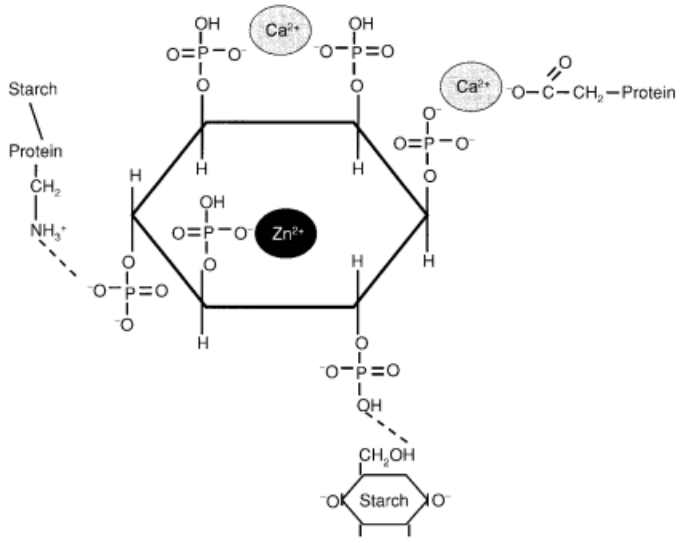

Figure 2.4. *Structure of phytate and possible bonds (Kornegay, 2001).*

Most of the plant ingredients have an average low content of Ca and Zn, and relatively high content of phytate (Table 2.5). Therefore, most of the Ca and Zn in diets for swine and poultry are supplemented as inorganic Ca and Zn (NRC, 2012). The addition of microbial phytase increase apparent total tract digestibility (**ATTD**; Poulsen et al., 2010; Rodríguez et al., 2013; Almaguer et al., 2014), STTD (González-Vega et al., 2014b; González-Vega et al., 2015b) and true total tract digestibility (**TTTD**) of Ca (González-Vega et al., 2013). In addition supplementing with microbial phytase increases the serum Zn levels (Revy et al., 2004; Walk et al., 2013). The reason that phytase increase ATTD,

STTD, TTTD of Ca and serum Zn is that the enzyme hydrolyzes the phytate, reducing the chance to bind Ca and Zn (Selle et al., 2009).

Phytate decrease the bioavailability of Zn (Ashida et al., 2000), whereas Ca *per se* has not a negative effect on zinc absorption (Lönnerdal et al., 1984; Dawson-Hughes et al., 1986). However, the calcium content of the diet may affect Zn absorption from phytate-containing meals (Lönnerdal, 2000). Adverse effects of calcium–phytate interactions on the absorption of manganese and zinc have been demonstrated (Biehl et al., 1995). The reason for this is that Ca has the propensity to form complexes with phytate and zinc. Multiple mineral complexes, such as Ca-Zn-phytate, are more stable than single mineral complexes, such as Ca-phytate or Zn-phytate (Maenz et al., 1999). When two cations are presented simultaneously, as Ca^{+2} and Zn^{+2}, they act together to increase the quantity of phytate precipitation (Simpson and Wise, 1990). The formation of Zn-Ca-phytate complexes in the small intestine may be a major mechanism by which phytate reduces dietary Zn availability (Fordyce et al., 1987).

Table 2.5. *Zn, Ca, P, phytic P and phytate content of common feed ingredients. Source: Schlemmer et al, 2009 and FEDNA 2010.*

Feed Ingredients	Zn (mg/kg)	Ca (g/kg)	P (g/kg)	Phytic P (g/kg)	Phytate content (g/100g)
Rice	21	0.4	1.0	0.6	0.06-1.08
Dehulled Oats	23	0.8	3.8	1.8	0.42-1.16
Barley	30	0.6	3.6	2.1	0.38-1.16
Corn	24	0.2	2.7	1.9	0.72-2.22
Wheat	50	0.5	3.0	2.0	1.14-3.91
Wheat bran	83	1.3	9.5	7.5	2.1-7.3
Rice bran	45	1.0	13.5	12.0	2.56-8.7
Peas	45	0.8	4.0	2.1	0.22-1.22
Soymeal 44	48	2.9	6.1	4.0	1.0-2.22
Soybean hulls	42	5.0	1.5	1.1	-

2.1.4.2. Phytase

As it can be observed, phytic acid is an antinutritive component in plant-derived food and feed, and therefore enzymatic hydrolysis of phytic acid is desirable (Kerovuo, 2000). Phytases catalyze the hydrolysis of phytate and are able to improve the nutritional quality of phytate-rich diets.

During the last decades it has become common the addition of microbial phytase in monogastric diets to make phytate-bound P available for absorption. Phytase can be derived from various sources including plants, animals and microorganisms (bacteria, yeast and fungi; Jain et al., 2016). Although some cereals such as wheat and rye contain phytase, the efficacy of this phytase is only 40% of the efficacy of microbial phytase (Zimmermann et al., 2002). Therefore, inclusion of microbial phytase is common in poultry and pig diets.

Phytase (myo-inositol hexakisphosphate phosphohydrolase) hydrolyze phosphomonoester bonds of phytate, which releases bound P and produces lower forms of *myo*-inositol phosphates (**IP**: IP5, IP4, IP3, IP2, IP1, and inositol; Wyss et al., 1999; Lassen et al., 2001). Phytases can be divided mainly into 3 categories by the position of the phosphomonoester group on the phytate molecule at which hydrolysis is initiated (Greiner and Konietzny, 2010). There are the 3-, 5-, or 6- positions:

1) 3-phytases: e.g. *Aspergillus niger* based phytase
2) 5-phytases: e.g. *Pisium sativum* based phytase
3) 6-phytases: e.g. *Escherichia coli* based phytase

Most diets fed to pigs and poultry contain 3-phytases or 6-phytases. In general, 3-phytases are from microbial origin, whereas 6-phytases are from plant origin. However, there are some exceptions, e.g. *E. coli* and *Periophora lycii* have 6-phytase activity (Greiner and Konietzny, 2010). The pH in the gastrointestinal tract plays an important role in the efficacy of the enzyme. Low pH in the stomach enhances the activity of microbial phytase (60%) and plant origin phytase (40%), but a greater pH in the small intestine and secretion of HCl, pepsin, and proteases may reduce the activity of the enzyme (Rapp et al., 2001). Therefore, the hydrolysis of phytate in the stomach plays an important role for the digestibility of P (Jongbloed et al., 1992).

Microbial phytases may originate from either fungi or bacteria, but both have similar effects on Ca and P digestibility (Guggenbuhl et al., 2007; Selle and Ravindran, 2008). Research on other sources of production of phytase such as phytases produced by genetically modified plants (e.g., wheat and corn) and production of phytase by pigs are still being conducted. It has been observed that phytase from transgenic corn increases energy and P digestibility (Li et al., 2013), and improve the growth performance (Wang et al., 2011) when fed to pigs.

The advantage of adding microbial phytase to the diets is the increase in digestibility of P (Akinmusire and Adeola, 2008; Almeida and Stein, 2010; Rodríguez et al., 2013), Ca (Brady et al., 2002; Gonzalez-Vega et al., 2013; Rodríguez et al., 2013; González-Vega et al., 2014b), and sometimes also protein and energy (Kies et al., 2001; Selle and Ravindran, 2008).

The efficacy of phytase is influenced by many factors such as stability under pH conditions, resistance of pepsin or proteases in the gastrointestinal tract (Greiner and Konietzny, 2010), feeding management (e.g., feeding level and feeding frequency; Mroz et al., 1994), age or physiological status of the animal (Kemme et al., 1997), and others.

Phytase activity (**FTU**) is generally defined as follows: one unit of phytase activity is the amount of enzyme that liberates 1μmol of inorganic phosphorus in 1 min from 5.1 mmol solution of sodium phytate at 37°C and pH 5.5 (Kornegay, 2001). The activity of phytase may be affected by metal ions, however, it is not clear the cause of this negative effect. Binding of metal ions to the enzyme, formation of metal ion-phytic acid complexes, or increased intestinal pH may be some of the possible reasons for the decrease in the efficacy of the enzyme (Liu et al., 1998; Kerovuo, 2000). As we explained earlier, one of the most abundant metal ions in diets fed to pigs is Ca and Zn during the first weeks post-weaning. The amount of Ca that is included in diets fed to pigs sometimes exceeds the requirement established by NRC (2012) because Ca supplements such as calcium carbonate are inexpensive; therefore, excess of Ca has not been a concern from an economic stand point. However, it has been reported that increased levels of dietary Ca may decrease phytase activity (Lei et al., 1994; Lantzsch et al., 1995; Brady et al., 2002; Selle et al., 2009) and reduce P digestibility (Stein et al., 2011). The digesta pH is crucial to the

solubility of Ca-phytate complexes (Selle et al., 2009). Increasing dietary Ca as limestone increases digesta pH along the length of the small intestine (McDonald and Solvyns, 1964). Phytase can only act on phytates in solution and the extent to which phytates are hydrolyzed depends largely on their solubility (Lantzsch et al., 1995).

The addition of pharmacological doses of Zn as ZnO to the pig's diets to prevent the post-weaning diarrhea also reduce P absorption (Meyer et al., 2002). Apparent digestibility of Ca seems not to be influenced by dietary Zn in the absence of phytase, but if phytase is added to the diet, a decrease in Ca digestibility is observed as dietary Zn increased from 0 to 3,500 mg/kg (Walk et al., 2015). Moreover, a lack of response to phytase on growth performance was also reported if pharmacological levels of ZnO are supplemented to the diets (Martínez et al., 2005). **Therefore, validate that high Zn levels in the diet affect the digestibility of Ca in pigs will improve the formulation of these minerals in pig industry.**

Hypothesis:

Therefore, high Zn levels may affect Ca absorption (it has never been studied on standardized digestibility), and the response may be different depending on phytase supplementation.

2.2 SENSORY FEED ADDITIVES

According to the definition of the European Commission, "*Feed additives are products used in animal nutrition for purposes of improving the quality of feed and the quality of food from animal origin, or to improve the animals' performance and health*". There are different categories of feed additives (European community, regulation number 1831/2003):

1) Technological additives: any substance added to feed for a technological purpose;
2) **Sensory additives**: any substance, the addition of which to feed improves or changes the organoleptic properties of the feed, or the visual characteristics of

the food derived from animals; For example flavors or sweeteners like Stevia and Neohesperidine dihydrochalcone.

3) Nutritional additives;
4) Zootechnical additives: any additive used to affect favorably the performance of animals in good health or used to affect favorably the environment;
5) Coccidiostats and histomonostats

In pig industry it is common the utilization of sensory feed additives based on essential oils, aromatic herbs, and/or spices to improve feed palatability and zootechnical performance. The sense of taste is responsible for detecting and responding to sweet, bitter, sour, salty, and umami (amino acid) stimuli. It is also capable of distinguishing between these various taste modalities to generate innate behavioral responses (Zhao et al., 2003). Pigs are innately averse to bitter-tasting compounds, but are attracted to sweet and umami stimuli (Kennedy and Baldwin, 1972;Tedo, 2009). Sweeteners are defined as feed additives that are used to impart a sweet taste (Yebra-Biurrun, 2005), which it is known that improve feed palatability and zootechnical performance in piglets. **Selection of new additives to encourage feed intake at weaning will contribute to prevent digestive problems related with weaning**.

2.2.1 FLAVORS AND LEARNING STRATEGIES: how to increase feed consumption

For mammals, the selection and ingestion of an appropriate diet is a complex challenge that needs the incorporation of information from a wide variety of sources. It has been observed that mammals are able to have an innate recognition and preference for high energy (sweet savors), proteins (umami savors) and even electrolytes (savory savors) feed (Pérez et al., 1995; Wald and Leshem, 2003). The rest of flavors are identified like a challenge. When the animal learn to discern if post-eating consequence of these feeds are positive or negative, then the animal acquire an associative learning that relates the sensory cues of food with the benefit (hedonic and/or postingestive) or disadvantage. Then, learning is considered important for developing feed preferences.

Learning of the feeding behaviors appears as an evolutionary mechanism that has been established to facilitate the search of food, making it more efficient and adaptive. Three types of learning have been described: trial and error (or direct experience with food

and its consequence) (Myers and Sclafani, 2006; Dwyer et al., 2009), social learning acquired with direct or indirect contact with others animals (Galef and Whiskin, 2004) and **maternal learning**, which is established during gestation or lactation (Mennella et al., 2001).

The dissertation will focus on maternal learning as learning strategy using feed additives to promote feed intake after weaning.

2.2.1.1 Maternal learning

It is known that pigs have a well-developed sense of smell (Morrow-Tesch and McGlone, 1990) and piglets can discriminate among auditory, olfactory, visual and tactile stimuli immediately after birth (Parfet and Gonyou, 1991). Furthermore it has been observed that pigs can learn olfactory cues for faster discrimination than visual discrimination tasks (Croney et al., 2003). Pigs have a well-developed olfactory system at birth, so neonatal pigs are able to recognize their mother's faecal, skin odours (Morrow-Tesch and McGlone, 1990), and amniotic fluid but this preference disappeared with age (Figueroa et al. 2013). It can also be observed in other mammals, birds and amphibians (Bolhuis et al., 2009). For example, rat pups recognize the flavor previously offered to the mother, indicating the preference is acquired prenatally (Hepper, 1987). Therefore, when a mammal is born they have a rudimentary idea of who are their parents/family. This leads to animals a smoothly transition from the prenatal environment to postnatal life, a maternal recognition, nipple localisation and initiation of suckling (in mammals; Hepper, 1987).

If mothers know what kind of food is good to eat and what kind of food is present to their environment, maternal learning would allow offspring to prefer to eat the same. Then, maternal learning starts before farrowing, when the sow eats some compounds of feed that pass to foetus.

It has been described that some volatile compounds may cross the placental barrier and enter the foetal blood stream, consequently diffuse out of the nasal blood capillaries and come into contact with foetal olfactory receptors. In addition, flavors come into the amniotic fluid; the foetus inhales or swallows it and then stimulates olfactory receptors or taste buds (Hepper, 1988). As a result this exposure to cues may lead to create preference

for this flavors later in life and also can positively modulate the acceptance of food with a similar flavor before and after weaning (Mennella et al., 2001). After birth, maternal learning could continue through milk, the hedonism and lactic postingestive effect with the pleasure of nursering may create an associative learning with cues in milk (Hepper and Wells, 2006). Thus, the flavor preference might be strengthened more when the flavor is also present in the maternal milk (Mennella et al., 2001; Oostindjer et al., 2009). However only milk exposure does not increase flavor preference in all species (Hepper and Wells, 2006). Although in rabbit pups the ingestion of mother's milk was sufficient to influence the dietary preference (Bilkó et al., 1994); in piglets postnatal exposure alone did not reduce weaning-associated problems (Oostindjer et al., 2010).

Hypothesis:

Flavor incorporation in late pregnancy diets could represent a familiar volatile cue for weanling piglets. The link between the intrauterine and extra uterine period can be established by the presence of volatile compounds in the amniotic fluid.

What kinds of flavors can cross the placental barrier? Table 2.6, shows some of the flavors that have been described to create postnatal effects. Most of them increases postnatal olfactory preference for these flavors or reduces aversion. Some of them only focussed on the short-term recall by neonates and the others observed that flavor preferences may be long-lasting and persist until weaning and beyond.

The first evidence about maternal learning in pigs was on 1976, where pigs were able to choose those diets that were given previously to their mothers during lactation, increasing feed intake and growth after weaning (Campbell, 1976). However, it has been long later when new studies on maternal learning in pigs have been published reporting different results; some of them that maternal learning has positive effects on feed intake, growth (Oostindjer et al., 2009, 2010), behavior (Oostindjer et al., 2009, 2010), and preference (Figueroa et al., 2013) and others did not observe those responses, but a reduction on stress at weaning was observed when the familiar flavor was present

(Oostindjer et al., 2011). Therefore, early experience with flavors (prenatal learning), plays a role in postnatal feeding behavior and development of dietary habits, increasing later acceptance and improving adaptation to weaning.

Focusing on postnatal learning, volatiles can be also transferred to the mammary glands via vascular routes from the digestive or respiratory system (Dougherty et al., 1962). It has been observed that pre-weaning exposure to feed flavor (during lactation) from the maternal diet can reduce food neophobia and promote the intake of an otherwise unfamiliar feed after weaning (Bolhuis et al., 2009). For example in Table 2.7, the studies used two- or three-way food choice test to assess the influence of the maternal diet on feed preferences of the offspring in a wide range of species.

In pig, Campbell (1976) demonstrated that flavor can be transferred by mother's milk and affect flavor preferences after weaning, but Oostindjer et al. (2010) was not able to demonstrate it. There are reinforcing conditions like contact with the smell of the mother's body, breath, feed particles clinging to her skin, fur or teeth or faecal droppings during lactation which may further strenghten their preference and acceptance of these flavors (Bolhuis et al., 2009). As a result, the postnatal learning can also facilitate the acceptance of a similarly flavored feed at weaning and thus increases the consumption after weaning. In other words, breast milk may bridge the experiences of flavors in utero to those in solid feeds.

As seen in this review many studies have studied maternal learning in different species. However, **there is little practical knowledge in the pig industry about the mechanisms underlying flavor learning and how perinatal flavor learning can increase acceptance of diets after weaning**.

Table 2.6. *Overview of experiments demonstrating that prenatal exposure to flavors from the maternal diets affects postnatal preferences. Species, type of flavor, postnatal effects and references are indicated, adapted from Bolhuis et al., (2009).*

Species	Flavor[1]	Postnatal effects[6]	References
Rats	Garlic	↑ olfactory preference d 12	Hepper, 1988
Rabbits	Juniper[2]	↑ intake juniper at weaning d 28	Bilkó et al., 1994
	Cumin	↑ olfactory preference d 0	Coureaud et al., 2002
Sheeps	Citral[2]	↑ olfactory preference d 0	Schaal et al., 1995
	Oregano[3]	↑ intake oregano-flavored feed at 3,4.5,6,7.5 mo	Simitzis et al., 2008
Dogs	Anise (trans-anethol)	↑olfactory preference d 0	Wells and Hepper, 2006
		↑ intake anise-flavored treats w 10, only when exposed through milk also	Hepper and Wells, 2006
Pig	Anise[2]	↑ intake the same flavor on diet, ↑ body weight first days post-weaning and ↓ diarrhea	Oostindjer et al., 2009
		Positive effects on behavior	Oostindjer et al., 2009
	Milky-cheese[2]	↑olfactory preference d14, 21 and 26	Figueroa et al., 2013
		↑olfactory preference d14, 21 and 26	Figueroa et al., 2013
Humans	Anise[2]	↑ olfactory preference d0 and d4	Schaal et al., 2000
	Garlic	↑ olfactory preference d0	Hepper, 1995
	Carrot[4]	↓ negative facial responses while eating carrot-flavored cereal at 6 month	Mennella et al., 2001
		↑ enjoyment perceived by mother while eating carrot-flavored cereal	Mennella et al., 2001

[1]Provided in the maternal diet throughout gestation unless indicated otherwise.
[2]Last two gestational weeks.
[3]Day 50-130 of gestation.
[4]Last three gestational weeks.
[5]Fluidarom® is a commercial flavor based on anethol, cinnamaldehyde and eugenol.
[6]Relative to (non-exposed) control groups. d = day; w = weeks; mo = months.

Table 2.7. *Overview of experiments demonstrating that exposure to flavors from the diet of the lactating mother affects post-weaning preferences. Species, type of flavor, post-weaning and references are indicated. Adapted from Bolhuis et al. (2009).*

Species	**Flavor**[1]	**Testing Age**[8]	**Post-weaning effects**[9]	**References**
Rats	Lab chow[2]	d 21 (w)	↑ intake lab chow maternal diet	Galef and Sherry, 1973
		d 19-23 (w)	↑ intake lab chow maternal diet	Galef and Henderson, 1972
	Lab chow[3]	d 23 (w)	↑ intake in 30-min test if weanling diet matched with maternal diet	Bornstein et al., 1975
	Garlic[4]	d 29-40	↑ intake of garlic-flavored water	Capretta and Rawls, 1974
	Onion	d 21, d23 (w) d25	↑ intake of onion-flavored diet	Wuensch, 1978
Mice	Fennel	d22 (w)	↑ intake of fennel	Mainardi et al., 1989
Rabbits	Juniper	d28 (w)	↑ intake of juniper	Bilkó et al., 1994
Goats	Rice straw[5]	6 mo	↓ latency to first ingestion ↑ intake of rice straw	Van Tien, 2002
Dogs	Anise[6]	10 w	↑ intake anise-flavored treats, only when exposed prenatally also	Hepper and Wells, 2006
Pigs	Firanor nº3	3 w	↑ intake firanor nº3 and ↑growth rate	Campbell, 1976
Humans	Carrot[7]	6 mo (w)	↓ negative facial responses while eating carrot-flavored cereal	Mennella et al., 2001
			↓ intake of carrot-flavored cereal shortly after exposure to carrot-flavored mother's milk	Mennella and Beauchamp, 1999

[1]Provided in the maternal diet throughout the lactation period unless indicated otherwise.

[2]Day 5-weaning.
[3]Day 2-weaning.
[4]Three days before parturition-weaning at day 22.
[5]Last three weeks before weaning at 3 months.
[6]First 20 days of lactation
[7]First two months of lactation, for four days per week.
[8](w) indicates that animals were tested at weaning or were not fully weaned at the time of testing. d = days; w = weeks; mo = months.
[9]Relative to (non-exposed) control groups.

2.2.2. SWEETENERS

Sweeteners can be grouped in various ways. In this dissertation will be grouped as nutritive and non-nutritive.

1) Nutritive sweeteners: also called sugars, sugar, caloric sweeteners, and added sugars. They contain carbohydrate and provide energy. It can be further classified as monosaccharide, like glucose, fructose, and galactose or as disaccharides like sucrose and maltose. Sugars occur naturally (intrinsic) in all fruit, vegetables, and dairy foods or are added (extrinsic) to foods. Sugar often refers to sucrose, which is derived from sugar cane or sugar beets (Fitch and Keim, 2012).
2) Non-nutritive sweeteners (**NNS**): also called artificial sweeteners, non-caloric sweeteners, or sugar substitutes which mimics the effect of sugar on taste (Chattopadhyay et al., 2011). There are a large variety of them and they are differentiated based on whether they are high-intensity, low-calorie, high-potency, and/or non-nutritive (Shankar et al., 2013). Non-nutritive sweeteners are known to be at least 30 to 13,000 times sweeter in taste compared with sucrose (Zygler et al., 2011). Non-nutritive sweeteners offer little to no energy when ingested, so they can replace the sweetness of sugar or energy-containing sweeteners but they do not have the same functional properties (Fitch and Keim, 2012). Some examples of NNS are saccharin, sucralose, aspartame, acesulfame-K, neotame, and stevia, an herb extract of intense sweetness (Mattes and Popkin, 2009).

2.2.2.1. Relative sweetness

Perceived sweetness depends and can be modified by a number of factors: the chemical and physical composition, the concentration of the sweetener, the temperature at which the product is consumed, the pH, other ingredients in the product, and the sensitivity of the taster. Sucrose is the usual standard (Nabors, 2012). The intensity of the sweetness of a given substance in relation to sucrose is determined on a weight basis. In Table 2.8 it can be consulted the approximate relative sweetness values for the most important sweeteners in humans and also the gustatory responses of pigs to sweeteners known to be sweet in humans.

2.2.2.2. Stevia Extract

Stevia extract (**SE**), a natural NNS, is a glycoside isolated from the plant *Stevia rebaudiana* Bertoni (Shankar et al., 2013; Figure 2.5). This plant produces diterpene glycosides (Figure 2.6) that are low calorie sweeteners, about 300 times sweeter than saccharose (Lemus-Mondaca et al., 2012). The proportion for the four major glycosides (on a dry weight basis) are 9.1% stevioside, 3.8% rebaudioside A, 0.6% rebaudioside D, and 0.3% dulcoside (Brandle et al., 1998; Chaturvedula et al., 2011).

The sweet steviol glycosides (E 960; EFSA, 2014) have functional and sensory properties superior to those of many other high-potency sweeteners (Brandle et al., 1998). In addition, the steviol glycosides have therapeutic benefits as anti-hyperglycemic, anti-hypertensive, anti-inflammatory, anti-tumor, anti-diarrheal, diuretic and immunomodulatory effects (Chan et al., 2001; Chatsudthipong and Muanprasat, 2009; Anton et al., 2010) and are thought to possess antioxidant, antimicrobial and antifungal activity (Lemus-Mondaca et al., 2012).

The sweetness properties of the glycosides differ from one another (Table 2.9). For example, rebaudiose A, which has an extra glucose unit relative to stevioside, is superior in terms of both sweetness and quality of taste. Some SE less purified also might contain high-saponin plants that could reduce the diet palatability (Carlson et al., 2012) and pure stevioside produces a significant bitter after taste (de Oliveira et al., 2007).

There is no industrial way to produce these glycosides and their production relies on the harvest from the herb, especially in China (Carocho et al., 2015). Steviol glycosides have been approved as a sweeter for humans in many countries, including the EU and USA and are used in beverages, dairy products, ice cream, frozen desserts, sugar-free confectionary, mints, dried sea-foods and sauces (Carocho et al., 2015). EFSA determined that the consumption of the steviol glycosides not pose a toxicological threat as a food additive (EFSA 2014). For animals, stevia extracts (**SE**) are authorized as additives in Animal Feed as flavoring agent and palatant enhancer (2.B category; European Community, Regulation EU No 1831/2003).

Table 2.8. *Relative sweetness in humans (Adapted from Nabors, 2012) and gustatory responses in pigs to sweeteners and the number of pigs tested (Adapted from Glaser et al., 2000).*

Sweetener	Approximate sweetness Humans	Number of pigs tested	Gustatory responses Pigs
Sucrose	**1**	42	22+, 20−
Lactitol	0.4	-	ND
Polyglycitol, maltitol syrups	0.4-0.9	-	ND
Isomaltulose	0.48	-	ND
Sorbitol	0.6	34	15+,19−
Erythritol	0.7	6	4+, 2−
Mannitol	0.7	8	5+, 3−
Maltitol	0.9	-	ND
Tagatose	0.9	-	ND
Xylitol	1.0	36	25+, 11−
High-fructose corn syrup, 55%	1.0	-	ND
High-fructose corn syrup, 90%	1.0+	-	ND
Crystalline fructose	1.2-1.7	-	ND
Glycyrrhizin	50-100	-	ND
Aspartame	180	9	9−

Sweetener	Approximate sweetness Humans	Number of pigs tested	Gustatory responses Pigs
Acesulfame K	200	12	6+, 6−
Saccharin	300-500	12	6+, 6−
Steviol glycosides	**300**	**-**	**ND**
Sucralose	600	11	9+, 2−
Monellin	1,500-2,000	4	4−
Neohesperidine dihydrochalcone	**1,800**	**4**	**4−**
Alitame	2,000	8	4+, 4−
Thaumatin	2,000-3,000	4	4−
Neotame	8,000	-	ND
Advantame	20,000	-	ND

+ indicates a preference over 80%; − indicates an indifferent or rejection.
ND: No Data

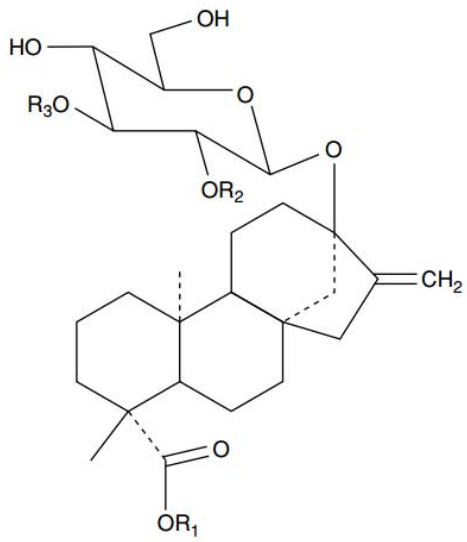

Figure 2.5. *Chemical structure of the sweet glycoside of Stevia rebaudiana Bertolini (Glória, 2003).*

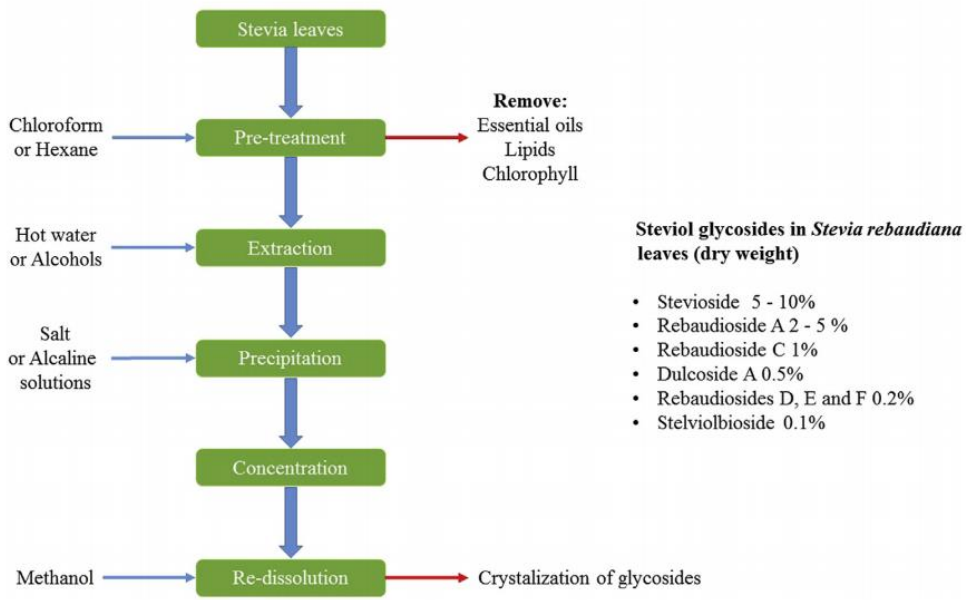

Figure 2.6. *Representation of the extraction method of Steviol glycosides from the leaves of Stevia rebaudiana (Bertoni), along with the relative percentage in dry weight of the different glycosides (Carocho et al., 2015).*

Table 2.9. *Molecular formulas, molecular weights, potencies and tastes of the steviol glycosides. Adapted from Prakash et al. (2014).*

Name	Formula	Molecular weight (g/mol)	Potency	Quality of taste
Steviol	$C_{20}H_{30}O_3$	318.45	ND	Very bitter
Steviolmonoside	$C_{26}H_{40}O_8$	480.58	ND	ND
Rebaudioside A	$C_{44}H_{70}O_{23}$	967.01	200	Less bitter
Rebaudioside B	$C_{38}H_{60}O_{18}$	804.88	150	Bitter
Rebaudioside C	$C_{44}H_{70}O_{22}$	951.01	30	Bitter
Rebaudioside D	$C_{50}H_{80}O_{28}$	1129.15	221	Like sucrose
Rebaudioside E	$C_{44}H_{70}O_{23}$	967.01	174	Like sucrose
Rebaudioside F	$C_{43}H_{68}O_{22}$	936.99	200	ND
Stevioside	$C_{38}H_{60}O_{18}$	804.88	210	Bitter
Steviolbioside	$C_{32}H_{50}O_{13}$	642.73	90	Unpleasant
Rubusoside	$C_{32}H_{50}O_{13}$	642.73	114	Very bitter
Dulcoside A	$C_{38}H_{60}O_{17}$	788.87	30	Very bitter

ND = No Data

There are some studies of the SE effects on feed intake, growth performance and feed preference in pigs. Clouard et al. (2012) observed that SE and high-saponing plants triggered positive feed responses during the preferences test but did not enhance middle-term food intake, pigs' appetite and weight gain during the starter phase. Other studies reported that stevioside slightly reduced feed intake (Geuns et al., 2003) and there was no advantage of stevia compared to sucrose on feed intake, weight gain and feed/gain ratio of pigs (Munro et al., 2000). Watanasit et al. (2003) observed that supplementation of 0.4% stevia in the diet of suckling pigs (from d 7 to d 21 of live) increased feed intake and weight gain compared to 0, 0.2 and 0.6% stevia supplementation. All together, these contradictory findings make it **difficult to conclusively assume that stevia per se enhances food palatability in pigs**.

2.2.2.3. Neohesperidine dihydrochalcone

Neohesperidine dihydrochalcone is a semisynthetic NNS and a flavor enhancer (Bassoli and Merlini, 2003). It is produced by alkaline hydrogenation of nehoesperidine, which it is a biflavonoid occurring naturally in bitter oranges (*Citrus aurantium*) (Bassoli and Merlini, 2003; Glória, 2003). Its molecular formula is $C_{28}H_{36}O_{15}$ and its molecular weight is 612.60 (Figure 2.7).

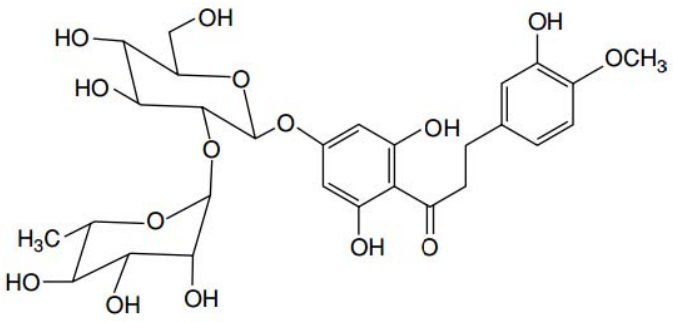

Figure 2.7. *Chemical structure of neohesperidine dihydrochalcone (Glória, 2003).*

The NHDC is 250 to 2000 sweeter than sucrose for humans (Baêr et al., 1990; Glória, 2003; Table 2.10) although pigs do not seem to detect as sweet (Glaser et al., 2000). It has a pleasant taste which it is characterized by a delay before reaching its maximum intensity. At high concentrations, NHDC exhibits long-lasting sweetness associated to licorice-like aftertaste (Borrego et al., 1995; Bassoli and Merlini, 2003). Neohesperidine dihydrochalcone has the property to decrease the perception of bitterness, saltiness, sharp, and spicy attributes (Glória, 2003). Therefore, it has been reported that when it is used at low concentrations in combination with other intense sweeteners, NHDC enhances the quality of the sweetness given to the food (Borrego et al., 1995).

The sweetness intensity of NHDC depends on its concentration, the pH, and the product to which it is combined; for example, as the concentration increases, the sweetness of NHDC decreases compared to sucrose (Glória, 2003). Neohesperidine dihydrochalcone shows synergism with saccharin, aspartame, cyclamate, sucralose, acesulfame-K, sugar alcohol (Glória, 2003). And, NHDC is not absorbed, but it is metabolized by the intestinal flora, yielding the same or similar breakdown products to its naturally occurring analogs (Bassoli and Merlini, 2003).

Table 2.10 *Properties of some intense sweetners. Adapted from (Glória, 2003).*

Sweetener (INS)[1]	Sweetness[2]	Sweetness characteristics	Synergism[3]
Alitame	2,000	Clean, no unpleasant aftertaste	Sac, Cyc, Aces
Sucralose (955)	400-800	Slow onset, clean sweet sugar-like, prolonged sweetness	Cyc, Aces, NHDC
NHDC (959)	**250-2,000**	**Delayed onset, lingering licorice-menthol-like aftertaste**	**Sac, Asp, Aces, Cyc, Sucralose, Sugar alcohols**
Glycyrrhizin (958)	50-100	Slow onset, long aftertaste, licorice flavor	Stev, Thau, Asp
Stevioside	100-300	Slow onset, menthol at high levels, bitter	Asp, Cyc, Aces, Glyc
Thaumatin-Talin (957)	2,000-3,000	Slow onset, persistent, licorice-like	Sac, Aces, Asp, Cyc, Stev, Glyc

[1]INS, international Numbering System
[2]In relation to sucrose
[3]sac: saccharin; cyc: cyclamate; aces: acesulfame-K; NHDC: neohesperidine dihydrochalcone; asp: aspartame; stev: stevioside; thau: thaumatin; glyc: glycyrrhizin

Hypothesis:

Stevia extracts, a natural NNS, produce bitter after taste but NHDC decreases the perception of bitterness in humans. Therefore, SE and their interaction with NHDC may enhance feed palatability and thus increase feed preference in pigs.

CHAPTER 3

Objectives and experimental design

Weaning is a sudden change in pigs life and represents a period of adaptation and stress in response to the separation from their mothers, mixing with other litters, reallocation to a different environment, and novel food (usually solid diet). Therefore, pigs frequently suffer post-weaning "growth check", characterized by low feed intake, poor growth, and diarrhea. The search for multiple and combined solutions to increase feed consumption and growth performance after weaning is of great practical interest for the swine industry.

This PhD dissertation is part of the project AGL2012-31924 focused on improving the mineral status of the pig (Chapter 3, 4 and 5); Chapter 5 was also possible with the cooperation of the Department of Animal Science, University of Illinois (US). Two different studies related to ameliorate post-weaning growth check were funded by Norel S.A. (maternal learning; Chapter 6) and by Ferrer Health Tech S.A. (synergisms between sweeteners; Chapter 7).

The hypotheses of the present PhD dissertation are:

1) Nutritional levels of Zn in the diet after weaning may have short-, medium-, and long-term consequences on growth performance and supplementing pigs with Zn during lactation may increase post-weaning serum Zn levels.

2) Low Ca diets, without adding limestone, might increase pigs' performance and promote changes in microbiota and intestinal mucosa.

3) Pharmacological Zn levels may affect Ca digestibility (it has never been studied on standardized digestibility), and the response may be different depending on phytase supplementation.

4) Flavor supplementation in late gestation may represent a familiar volatile cue for weanling pigs increasing feed intake and growth after weaning.

5) Stevia extracts combined with NHDC may enhance feed palatability and, therefore, may increase feed preference in pigs.

The purpose of this thesis is, therefore, to **investigate the use of optimization mineral status (Ca and Zn), maternal learning, and stevia with NHDC as strategies to improve feed intake and growth performance after weaning**. The specific objectives are:

1) To observe the short-, medium and, large-term consequences of nutritional levels of Zn on growth performance and mortality after weaning. The evolution of serum Zn levels from birth to post-weaning period and its consequence on diarrhea incidence. And to measure serum Zn levels after Zn supplementation during lactation (**Chapter 4**).

2) To test the productive performance response early after weaning of pigs fed on diets containing low or high Ca levels; and to evaluate if high Ca levels may modify the jejunum mucosal expression of genes, the colon microbiota and the intestinal morphology of the jejunum (**Chapter 5**).

3) To test that pharmacological level of Zn affects STTD of Ca, and that microbial phytase increases the ATTD and STTD of Ca and the ATTD of P regardless of the concentration of Zn in the diet (**Chapter 6**).

4) To determine the presence or absence of volatile compounds in amniotic fluid and milk after flavor supplementation in maternal diets; and to study the productive performance and feed preferences of pigs at weaning with different combinations of flavor inclusion in sow, creep-feed and/or weanling diets (**Chapter 7**).

5) To investigate how pigs respond to *Stevia rebaudiana* Bertoni and its combination with NHDC by a two-choice test (**Chapter 8**).

To asses these five objectives, 13 different trials were performed. Results were included in Chapters 4 to 8.

In **Chapter 4**, four trials were performed. In Trial 1 a total of 400 pigs were distributed into two dietary treatments (125 or 2,480 mg/kg of Zn) after weaning to observe the short-, medium- and long-term consequences on growth performance and mortality. In trial 2 to compare serum Zn levels by using diarrhea sign, a total of 110 pigs were distributed at weaning by their serum Zn level (low < 0.71 mg/L or high > 0.9 mg/L) and the presence of diarrhea was recorded daily until d 35 post-weaning. In Trial 3, to measure serum Zn levels evolution during lactation, a serial blood samples were taken from 8 pigs during lactation and 60 pigs after weaning. Finally Trial 4 was aimed to measure the serum Zn levels after the supplementation of Zn citrate during last week

of lactation; a total of 96 pigs were distributed into four treatments, which were the daily administration of 0, 6, 18 or 30 mg of Zn during lactation.

In **Chapter 5**, four trials were performed. The first three experiments were conducted to observe the effects of dietary Ca on growth performance. In Trial 1, a total of 240 pigs were distributed into three different dietary treatments: 0.35, 0.65 and 0.95% of Ca. In trial 2 and 3 a total of 320 pigs were distributed into two treatments 0.35 and 0.95% of Ca with 125 mg/kg of Zn or 2,675 mg/kg of Zn, respectively. Trial 4 was conducted to observe the effects of dietary Ca on intestinal morphology, microbiota and gene expression. It was used a total of 18 pigs distributed into two dietary treatments 0.45 and 0.95% of Ca.

In **Chapter 6**, a trial was conducted to observe the effects of ZnO and microbial phytase on Ca digestibility. A total of 56 barrows were distributed into 6 dietary treatments following a 2 × 3 factorial arrangement where the main factors were Zn (0 or 2,400 mg/kg of Zn) and phytase inclusion (0, 1,000, or 3,000 FTU).

In **Chapter 7 (Blavi et al., 2016)**, two trials were designed with the aim to observe the effect of flavor incorporation in late pregnancy on feed intake, growth performance and feed preference of weaned pigs. In trial 1 a total of 80 sows and in Trial 2 a total of 24 were exposed to a flavored or control diet from d 73 to farrowing.

Finally, in **Chapter 8 (Blavi et al., 2016)**, two double-choice feeding trials were conducted to study the effect of SE and NHDC on feed preference in pigs. In Trial 1, the doses of SE were 0, 100, 200, 300, 400 and 500 mg/kg were compared to sucrose (4%) in feed; in Trial 2, the dose of SE (150 mg/kg) plus 0, 2, 3, 4 or 5 mg/kg NHDC were compared to sucrose (4%) in feed.

CHAPTER 4

Exploring Zinc deficiency after weaning using serum Zn levels: the consequences and possible solutions in pigs.

Exploring Zinc deficiency after weaning using serum Zn levels: the consequences and possible solutions in pigs.

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4.1. ABSTRACT

Four trials were performed to give an answer to different questions: 1.- how piglets' serum Zn levels change after weaning? what are the short-, medium-, and long-term consequences of nutritional or therapeutic doses of Zn oxide?; 2.- are serum Zn levels in piglets soon after weaning a predisposing factor to diarrhea?; 3.- are suckling piglets able to maintain high serum Zn levels throughout lactation and do these levels vary between high and low BW piglets?; 4.- is it possible to increase serum Zn levels at weaning by supplementing Zn during lactation. In **Trial** 1 a total of 400 pigs were allotted to 2 treatments: the Nutritional diet (125 mg/kg Zn, 16.5 % CP and 0.35% Ca) or the Therapeutic diet (2,480 mg/kg Zn, 20.2% CP and 0.8% Ca). Pigs were individually weighed at weaning, d 14, d 35 post-weaning, and every three weeks until slaughter (d 142). A blood sample was taken from 15 piglets from each treatment at d 0 (weaning), d 7, d 35 and d 49 post-weaning. There were no differences in BW during all periods, except at d 14 pigs that were fed the Nutritional diet had greater BWs than pigs fed the Therapeutic diet ($P < 0.05$). However, at the end of the fattening phase the pigs fed Nutritional diet with lower BWs tended to have lower BWs than pigs fed Therapeutic diet ($P = 0.06$). No differences in mortality rate ($P > 0.10$) were observed in general, however, lower BW pigs had greater mortality than heavy pigs. Pigs fed the Nutritional diet had lower ($P < 0.001$) serum Zn levels (0.7 mg/L) at d 7 post-weaning than piglets fed the Therapeutic diet (1.2 mg/L), but no differences were observed at older ages. **Trial 2** included 240 piglets at weaning with an average initial BW of 6.94 ± 1.87 kg. A blood sample was obtained at weaning to analyze the serum Zn, and they were distributed into 24 pens (10 pigs/pen) by weight block. Diarrhea incidence was recorded daily from d 0 to d 35 post-weaning. From the 240 pigs, group of 110 pigs with uniform BW (6.5 ± 1.9 kg) was selected and separated into two groups based on serum Zn levels at weaning with 55 piglets with low serum Zn (**LZn;** < 0.71 mg/L) and 55 piglets with high serum Zn (**HZn**; > 0.9 mg/L). Piglets with LZn were 2.49 times as likely to have diarrhea as piglets with HZn ($P < 0.02$). In **Trial 3**, blod samples were taken from piglets during lactation. Eight piglets (one piglet per litter) had blood drawn at d 0 (farrowing), 7, 14, 21 and 28 (weaning) of age, and 60 piglets, categorized as either heavy (8.63 kg) or as light (5.50 kg), had blood drawn at d 28. Serum Zn levels at birth were 1.2 mg/L and decreased ($P < 0.01$) to 0.67 mg/L at d 28. Heavy pigs showed greater ($P < 0.01$) serum Zn levels (0.98 mg/L) than light BW pigs (0.79 mg/L). In

Trial 4, a total of 96 suckling piglets were allotted to 4 treatments that consisted of the daily administration of 0, 6, 18 or 30 mg of Zn as Zn Citrate in capsul-forme during the last 7 d of lactation. Piglets were individually weighed and a blood samples were obtained on d 14, d 21 (weaning), and d 7 after weaning. Serum Zn levels linearly increased by day and as Zn citrate supplementation increased (interaction, $P < 0.001$). However, only light pigs supplemented with 18 and 30 mg/L of Zn had an increase in serum Zn levels during lactation. In conclusion, low Ca (0.35%) and low CP (16.5 %) diets without ZnO supplementation fed after weaning promoted a decrease in serum Zn levels early after weaning, but serum Zn levels recovered at the end of the nursery period without evidence of decreased growth performance or increased mortality. Low Zn status (< 0.7 mg/L) at weaning may be a predisposing factor of diarrhea. A decrease in serum Zn levels occurs during lactation, and is more severe in low BW piglets. However, supplementation of Zn during lactation can mitigate this decrease.

4.2. INTRODUCTION

Zinc (Zn) is an essential micronutrient for all living organisms with different roles. It can be a structural component of proteins, an enzymatic co-factor, and a transcriptional regulator of cellular and biochemical processes (Solomons, 2013). A regular, adequate dietary supply is required because there is no functional reserve or body store of available Zn. Consequently, humans and pigs are at risk of Zn deficiency when dietary inadequacies such as low zinc intakes or poor Zn absorption occur (King et al., 2016). Breastfed infants can be also at elevated risk of deficiency when Zn intake solely from human milk is inadequate to meet their growth requirements (Brown et al., 2009). Zinc milk concentration declines during lactation in several mammals, including humans (Donangelo and King, 2012), rats (Keen et al., 1981) and dogs (Lönnerdal et al., 1981a), but not in sows (Hill et al., 1983).

Zinc deficiency affects multiple body functions, including growth, reproductive function, neurobehavioral development (King, 2011), digestive function (Roth et al., 1992), and both innate and acquired immunity (Raiten et al., 2015) which is often followed by higher incidences of infectious disease. Also, lack of zinc leads to anorexia and loss of appetite (Chasapis et al., 2012). In infants, Zn deficiency is a predisposing factor for diarrhea and is implicated in >50% of diarrhea deaths in developing countries (King et al. 2016) but it has been shown that this can be reduced by supplementation of

20 mg of Zn for 10 to 15 days (King et al., 2016; Krebs, 2013). The young pig may also suffer a decrease on serum Zn levels and increase in diarrhea incidence after weaning when fed diets with nutritional Zn levels (Davin et al., 2013). However, this may be prevented by the use of therapeutic doses (2,000 to 3,000 mg/kg) of ZnO (Poulsen, 1995; Hill et al., 2001; Heo et al., 2013; Kim et al., 2015). But, environmental concerns about the accumulation of Zn in soils (Berenguer et al., 2008) and its likely connection with antimicrobial resistance (Bednorz et al., 2013) forces most to formulate with Zn at nutritional levels.

Therefore, the general hypothesis of this study was that piglets after weaning may show variable periods of Zn deficiency that may compromise their growth later in life, and that it may be ameliorated through nutritional interventions. The purpose of this study was to test the following hypothesis: 1) pigs supplemented with therapeutic doses of ZnO for 35 d after weaning will prevent the occurrence of temporary serum Zn deficiency in piglets and will promote greater performance in the short-, medium-, and long-term, when compared with piglets fed diets not supplemented with ZnO, 2) low serum Zn levels may occur in some piglets at weaning, and that this will be a predisposing factor for post-weaning diarrhea; and 3) supplementing Zn during lactation will increase serum Zn concentration at weaning.

4.3. MATERIALS AND METHODS

All experimental procedures were approved by the Ethical Committee on Human and Animal Experimentation of the UAB (CEAAH 1406).

Four trials were performed on a commercial farm with a breeding stock of 400 sows (Landrace x Large White) and a weaning program at d 28; except for Trial 4 which was carried out in a different commercial farrowing facility with a breeding stock of 850 sows (Landrace x Large White) and with piglets weaned at d 21. A schematic representation of all experimental designs and the timing of the different treatments and procedures are presented in Figure 4.1.

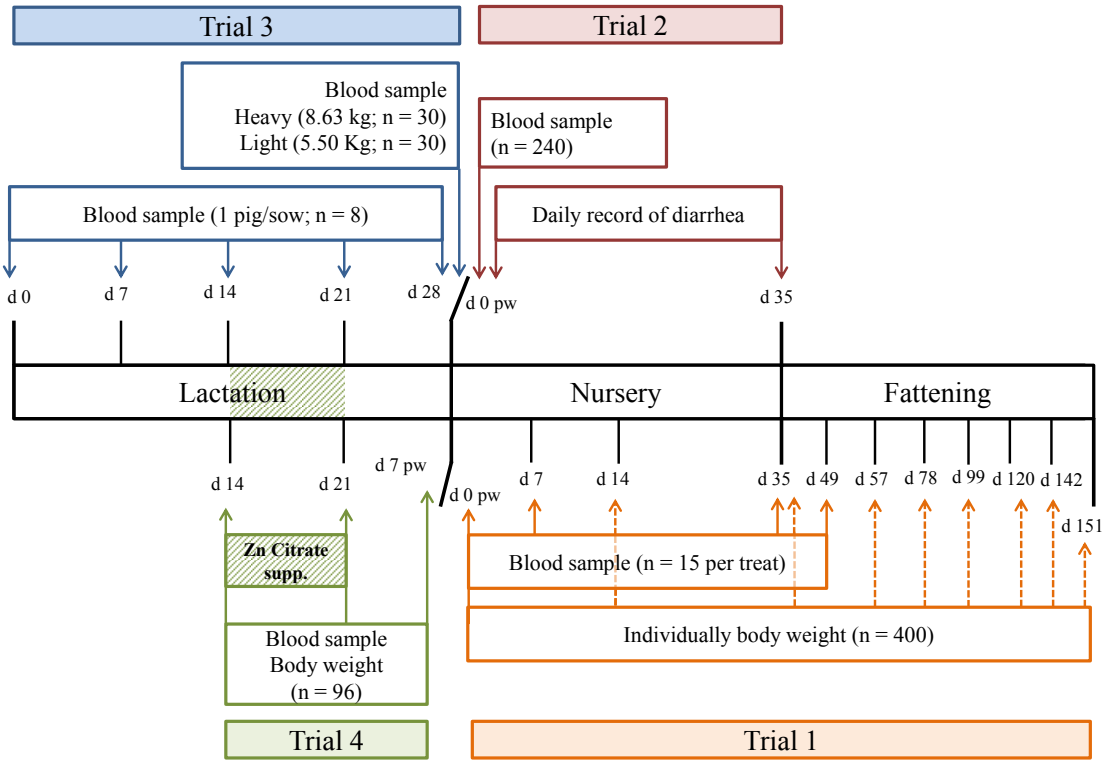

Figure 4.1. *The experimental design and the timing of the different treatments and procedures of Trial 1, 2 3 and 4. pw = post-weaning; supp. = supplementation.*

4.3.1. Trial 1: *short-, medium- and long-term responses to therapeutic doses of ZnO after weaning.*

Four-hundred pigs [Pietrain x (Landrace x Large White)] were selected at weaning (28 ± 1d of age; BW= 7.2 ± 1.80kg) and moved to the weanling unit, with two rooms of 24 pens each. The rooms were equipped with central heating, automatic forced ventilation, and a completely slatted floor. Each pen (3.2 m^2 in floor area) had *ad libitum* access to feed and water. Pigs were distributed into 40 pens (10 animals/pen), by initial body weight, into two blocks (BW category; heavy = 8.4 ± 0.85 kg and light = 5.8 ± 1.07 kg), and allotted to two dietary treatments (Therapeutic diet with 2,480 mg/kg of Zn or Nutritional diet with 125 mg/kg of Zn, and low levels of CP and Ca; Table 4.1; 20 pens/treatment).

Table 4.1. *Ingredients and nutrient composition (% as-fed basis, unless otherwise indicated) of the pre-starter, starter and, fattening diets.*

	Pre-Starter		Starter	
Ingredients	Nutritional	Therapeutic	Nutritional	Therapeutic
Maize	56.9	26	55.8	35
Wheat	12	17.1	10	18
Barley	13	11.565	15	18.7
Rice	-	1	-	-
Extruded soybeans	-	17.869	-	10.9
Soybean meal 44%CP	3.6	4	9.7	5.9
Spray dried porcine plasma	3.8	1.5	-	-
Sweet milk whey	2.7	6.5	-	-
Whey powder 50% fat	-	2.5	-	2.5
Carob meal	-	0.25	-	-
Dried egg powder	-	0.1	-	-
Soybean oil	-	1	0.17	-
Coconut oil	-	0.5	-	-
Fish meal	5.3	2.5	6	5
Calcium carbonate	-	0.803	0.41	0.7
Mono calcium phosphate	0.51	1.01	0.78	0.87
Salt	0.3	0.266	0.34	0.45
Sodium bicarbonate	-	0.152	-	-
Dextrose	-	0.8	-	-
Sucrose	-	0.25	-	-
L-Lysine HCL	0.57	0.428	0.55	0.52
DL-Methionine	0.22	0.19	0.21	0.18
L-Threonine	0.26	0.178	0.26	0.22
L-Tryptophan	0.11	0.038	0.1	0.05
L-Valine	0.19	-	0.19	-
L-Isoleucine	0.14	-	0.09	-
Vit-Min premix	0.4[a]	3.5[b]	0.4[a]	1[b]
Dry Matter	88.2	90.79	87.9	89.06
Ash	3.7	6.59	4.2	5.14
NE, kcal/Kg	2,476	2,629	2,450	2,475
Ether Extract	3.1	8.34	3.4	5.93
Crude Protein	16.5	20.23	16.5	17.98
d-Lys	1.02	1.32	1.03	1.20
Ca	0.48	0.85	0.63	0.76
Zinc, mg/Kg	150	2580	210	2740

[a]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 mg of Se (organic); 0.1 mg of Se (sodium).

[b]Supplied the following per kg of diet: 7,500 UI of vitamin A; 750 UI of vitamin D3; 450 mg of vitamin E; 15 mg of vitamin B1; 40 mg of vitamin B2; 38 mg of vitamin B6; 0.38 mg of vitamin B12; 38 mg of vitamin K3; 150 mg of niacin; 110 mg of calcium pantothenate; 2.25 g of choline (clorur); 22 mg of folic acid; 1.5 mg of biotin; 750 mg of Zn (oxide); 225 mg of Mn (oxide); 750 mg of Fe (sulfate); 1.4 g of Cu (sulfate); 2.2 mg of Se (sodium selenite); 1.5 mg of I (potassium iodide); 1.2810^{10} of bacillus; 2.9 g of lactic acid; 4.1 g of orto phosphoric acid; 23 g of flavors.

To formulate the Nutritional diet we decided to decrease CP and Lys from 20.2% and 1.37% to 16.5 %, and 1.20% to avoid dysbiosis and to formulate on an ideal protein basis. The changes assumed that an increased level of protein would cause protein fermentation (Bertschinger et al., 1979), potentially causing the proliferation of pathogenic bacteria (Ball and Aherne, 1987; Prohászka and Baron, 2010). We also reduced Ca levels for 14 d after weaning, due to the observation in previous trials that this improved in growth performance (Blavi et al., 2016). At d 35 post-weaning, pigs were moved to the growing-finishing facility, and sorted according to treatment, by sex, and approximate BW. Density of the pens during the growing-fattening phase was 10 pigs per pen. Each pen (7.5 m^2 in floor area) was equipped with a nipple drinker and a concrete hopper with 2 feeder spaces. Pigs had *ad libitum* access to feed and water. From d 35 post-weaning, pigs received the same diet until d 63 (2,480 kcal/kg NE, 18.0% CP, 1.20% digestible Lys, 0.65% Ca and, 125 mg/kg Zn). During the growing (d 63 to d 127) and finishing period (d 127 to slaughter) pigs were fed two other diets, containing 2,375 kcal/kg NE, 16.0% CP, 0.99% digestible Lys, 0.60% Ca and, 125 mg/kg Zn and 2,400 kcal/kg NE, 16% CP and 0.95% digestible Lys, 0.65% Ca and, 125 mg/kg of Zn, respectively.

Fifteen piglets were randomly selected from each treatment and blood samples were obtained at d 0 (weaning), 7, 35, and 49. Blood samples were obtained via puncture of the jugular vein and collected into 10mL vacutainer® tubes (BD Vacutainer®, Z, BD-Plymouth, UK) free of detectable Zn. Serum was obtained via centrifugation (2,000 x *g*, 10 min, 15˚C) of blood samples and was immediately frozen

at -20˚C. Serum samples were diluted in a 0.05% p/v EDTA and 0.5% v/v NH3 solution to analyze Cu, Fe and Zn content using an inductively coupled plasma optical spectrophotomer (ICP-OES model Optima 4300DV, PerkinElmer Inc., Waltham, MA, USA).

All piglets were individually weighed at weaning (d 0) and, 14, 35, 57, 78, 99, 120, 142, and 151 d post-weaning. Mortality rate was calculated at d 14, 35, 57, 142, and for the entire period.

4.3.2. Trial 2: *is serum Zn level a predisposing factor to diarrhea?*

240 pigs [Pietrain x (Landrace x Large White)] were used and blood samples were taken individually at weaning via puncture of the jugular vein (the material and the analysis were conducted as explained for Trial 1). Pigs were moved to the weanling room and distributed into 24 pens (10 pigs/pen) by weight. The diet fed from weaning to d 14 contained 2,455 kcal/kg NE, 19.0% CP, 1.28% digestible Lys, 0.53% Ca and, 125 mg/kg Zn, and from d 14 to d 35 contained 2,460 kcal/kg NE, 19.0% CP, 1.20% digestible Lys, 0.7% Ca and, 125 mg/kg. All diets were without ZnO supplementation. Any presence of diarrhea described as watery feces was recorded daily during the pre-starter and starter phase (0 to 35 d post-weaning). Afterwards, a total of 110 piglets were selected and allotted to two experimental groups according to their serum Zn and BW at weaning in order to observe the link between serum Zn levels and diarrhea. The low serum Zinc group (LZn; < 0.71 mg/L Zn) had an average BW of 6.5 ± 1.9 kg, the high serum Zinc group (HZn; > 0.9 mg/L Zn) had an average BW of 6.5 ± 1.2 kg. The comparison between the two different levels of Zn serum at weaning and the effect on diarrhea incidence were analyzed via logistic regression used to calculate the Odds Ratios (**OD**).

4.3.3. Trial 3: *is maternal lactation able to maintain serum Zn levels of piglets until weaning and how do these levels change between high and low BW piglets?*

A total of 8 sows (Landrace x Large White) and their litters were randomly selected. Parity of the sow was not considered as Davin (2014) did not observe differences in trace mineral content of colostrum and milk among sows of different parities. Creep-feed was offered in commercial pan feeders with a hopper to ensure ad libitum access to feed from d 12 onward. Blood samples were taken from the same

piglet per sow (one piglet/sow) at d 0 (farrowing), 7, 14, 21, and 28 (weaning). Blood samples were obtained via puncture of the jugular vein using a 20G syringe and collected into 2mL Aquisel® tubes (Aquisel®, Z, Barcelona, Spain). To obtain the serum we followed the same protocol as Trial 1. In addition, a total of 60 piglets [Pietrain x (Landrace x Large White)] were selected (26 ± 2 d old) and allotted into two blocks by BW (heavy = 8.6 ± 0.17 kg and light 5.5 ± 0.17 kg) and from each piglet a blood sample was taken at weaning by puncture of the jugular vein (the material and the procedure are explained in section 4.3.1. Trial 1).

4.3.4. Trial 4: *is it possible to increase serum Zn levels at weaning by Zn supplementation during lactation?*

A total of 96 piglets [Pietrain x (Landrace x Large White)] at 14d of age were selected from 24 litters (2 piglets/litter categorized as heavy 4.9 ± 0.32 kg BW, and 2 piglets/litter as light 2.9 ± 0.31 kg BW). Piglets did not have access to the creep-feed. From d 14 to 21, piglets received a daily administration of an opaque, red, hard gelatin capsule (size 4, Acofarma, Terrassa, Spain). One piglet from each BW category in the same sow received the capsule containing 0 mg of Zn (n= 48), while the other piglet received the capsule containing 6, 18 or 30 mg of Zn as Zn citrate (8 replicates per treatment and BW category). The source of Zn selected was zinc citrate, because Zn in milk is loosely bound to citrate (Lönnerdal et al., 1980). The dose of 6 mg Zn/d was chosen to be similar to the amount of Zn provided in one kg of sow milk. The 18 and 30 mg/d doses of Zn were three and five times the increment of the initial dose, respectively. Individual weights and blood samples were obtained on d 14 and 21 of lactation and on d 7 post-weaning. Blood samples were taken via puncture of the jugular vein (the material and the procedures are explained in section 4.3.1. Trial 1).

4.3.5. Statistical Analysis

All results were analyzed by using the SAS® statistical package (version 9.3, SAS Institute; Cary, USA). Determination of odds ratios in Trial 1 were calculated using the FREQ procedure and with 95% confidence intervals. Zinc, Fe and Cu levels were analyzed with an ANOVA using the GLM procedure for Trials 2 and 4, and using the MIXED procedure for Trial 3. For these analyses, the statistical unit was the pig. Body weight in trials 2 and 4 was analyzed with an ANOVA using the GLM procedure

and the statistical unit was the pig. The mortality rate in trial 4 was analyzed with the FREQ procedure and the statistical unit was the pen.

All results are presented as Least Square Means by taking into account a Tukey adjustments, and the alpha level used for the determination of significance for all of the analyses was 0.05.

4.4. RESULTS AND DISCUSSION

4.4.1. How do the serum Zn levels change after weaning? Which are the short-, medium- and long-term consequences of nutritional or therapeutic doses of Zn?

In trial 1, it was observed that heavy piglets (8.6 kg) presented a greater ($P < 0.01$) level of serum Zn (1.1 vs 0.8 mg/L of Zn) compared to light piglets (5.5 kg) at weaning (Table 4.2). Pigs fed the Nutritional diet had lower serum Zn concentrations (0.67 mg/L) compared with pigs fed the Therapeutic diet (1.2 mg/L; $P < 0.001$; Table 6) at d 7 post-weaning. At d 35 post-weaning, light pigs fed the Nutritional diet had lower serum Zn levels compared with light pigs fed the Therapeutic diet (0.9 vs. 1.4 mg/L of Zn), but heavy pigs fed the Therapeutic and Nutritional diet had the same serum Zn levels (1.2 mg/L; interaction; $P = 0.05$). After obtaining blood samples, pigs were moved to the growing facilities where all pigs were fed the same diet without pharmaceutical ZnO levels. The serum Zn levels at d 49 was similar for all pigs ($P = 0.17$).

Normal serum Zn concentrations are reported to be within the range of 0.7–1.5 mg/L and serum zinc concentrations associated with marginal status are within the range of 0.4–0.8 mg/L (Puls, 1990). Therefore, our results show that light pigs at weaning showed a marginal Zn status, which may be explained by the fact that heavier piglets usually suck from anterior teats, which are the most productive (Rosillon-Warnier and Paquay, 1984). In addition, piglets fed the Nutritional diet were marginally zinc-deficient one week after weaning. These result are in agreement with Davin et al. (2013), who observed that Zn concentration in plasma was lower in animals fed an adequate Zn diet as compared to unweaned littermates or weaned littermates fed therapeutic doses of ZnO. However, Carlson et al. (2007) did not observe a decrease in plasma Zn levels from d 1 to d 15 after weaning in pigs fed 150 mg/kg of Zn in the diet.

Table 4.2. *Zn, Cu and Fe concentration (mg/L) during post-weaning period (0 to 49d) between animals fed the Nutritional (120 mg/kg Zn) diet and animals fed the Therapeutic diet (2,480 mg/kg Zn); n = 15 per treatment.*

	Nutritional		Therapeutic		Pooled	P-value		
	Heavy	Light	Heavy	Light	SEM	Treatment	Block	Treatment*Block
Zn, mg/L								
d 0	1.053	0.846	1.144	0.76	0.1	0.977	**0.002**	0.324
d 7	0.735	0.608	1.35	1.05	0.14	**0.0002**	0.092	0.484
d 35	1.237^{ab}	0.91^{b}	1.201^{ab}	1.421^{a}	0.17	0.079	0.683	**0.046**
d 49	1.205	1.188	1.028	1.067	0.19	0.167	0.913	0.794
Cu, mg/L								
d 0	2.11	2.15	2.15	2.49	0.11	0.074	0.076	0.165
d 7	1.99	1.95	1.750	1.97	0.14	0.369	0.456	0.270
d 35	2.07^{a}	1.80^{b}	1.60^{b}	1.64^{b}	0.08	0.0001	0.108	0.034
d 49	2.48	2.72	1.82	2.00	0.22	0.001	0.275	0.878
Fe, mg/L								
d 0	10.38	5.63	5.24	5.93	1.98	0.188	0.267	0.142
d 7	5.02	3.24	2.57	3.82	0.90	0.238	0.723	0.063
d 35	1.38	2.09	2.33	2.11	0.53	0.231	0.535	0.245
d 49	3.28	3.76	4.68	3.21	0.50	0.389	0.324	0.060

$^{a-b}$Values within a column without a common superscript are different ($P < 0.05$).

Piglet weaning is associated with an early and transient increase of inflammatory cytokines in the gut (Pié et al., 2004) which may produce a transient depression in the serum Zn concentration with a redistribution of Zn among various tissues such as liver, bone marrow, and thymus (Cousins and Leinart, 1988; Liuzzi et al., 2005). This may explain the low serum Zn levels in pigs fed the Nutritional diet at d 7 and the return to normal values at d 35

No differences in serum Cu levels at d 0 and 7 were observed ($P > 0.1$). On d 35 and 49, pigs fed the Nutritional diet presented greater serum Cu levels compared with pigs fed the Therapeutic diet (1.93 vs 1.62 mg/L on d 35, respectively, and 2.6 vs 1.9 mg/L on d 49, respectively; $P < 0.01$). It has been observed that plasma Cu concentrations decrease when high ZnO levels are added to the diet (> 1,000 mg/kg; Hill et al., 2001) because Zinc and Cu have an antagonistic interactions on each other's absorption (NRC, 2012). Serum Fe levels were not affected during the entire period (Table 4.2).

All piglets were individually weighed from weaning until slaughter. No differences were observed between treatments, except at d 14 post-weaning, when pigs fed the Nutritional diet (125 mg/kg Zn) had greater BW than pigs fed 2,500 mg/kg Zn (9.55 vs. 9.14 kg, respectively; $P < 0.05$; Table 4.3). However, at the end of the productive life (d 142) light pigs fed the Nutritional diet during the nursery period tended to have less weight than light pigs fed Therapeutic diet ($P = 0.06$).

Table 4.3. *Body weight changes from weaning until slaughter (d 142) between animals fed Nutritional Zn levels (125 mg/kg) or Therapeutic Zn levels (2,480 mg/kg).*

	Nutritional		Therapeutic		Pooled	P-value		
	Heavy	Light	Heavy	Light	SEM	Treat	Block	Treat*Block
BW 0, kg	8.40	6.07	8.31	6.00	0.11	0.539	<0.0001	0.940
BW 14, kg	10.99	8.12	10.52	7.76	0.15	**0.036**	<0.0001	0.763
BW 35, kg	21.05	16.21	20.26	16.67	0.31	0.694	<0.0001	0.131
BW 57, kg	34.67	27.74	34.43	28.77	0.55	0.554	<0.0001	0.345
BW 78, kg	51.71	43.60	51.02	44.87	0.66	0.739	<0.0001	0.261
BW 99, kg	70.18	60.72	68.16	61.63	0.77	0.581	<0.0001	0.148
BW 120, kg	89.06	78.30	87.83	80.73	0.83	0.581	<0.0001	0.094
BW 142, kg	104.21	93.32	103.90	97.50	0.90	0.105	<0.0001	**0.060**

Mortality rate was higher in light pigs (5.81 kg at weaning) than in heavy pigs (8.4 kg at weaning; Figure 4.2). Although, no differences were observed between all pigs fed the Nutritional and those fed the Therapeutic diet, mortality rate tended to be higher in pigs fed the Nutritional diet compared with pigs fed the Therapeutic diet in two critical days (d 14 postweaning, and d 57, which one week after moving the pigs to the growing facilities; 6.95 vs. 3.23% and 1.99 vs. 0.57%, respectively; $P < 0.10$). Moreover, at d 14 and 57 the mortality rate was higher in light pigs than in heavy pigs ($P < 0.05$). However, at the end of the nursery period (d 35) light pigs fed the Therapeutic diet had a higher mortality rate compared with light pigs fed the Nutritional diet (1.18 vs. 2.96%, $P <0.10$; Figure 4.2).

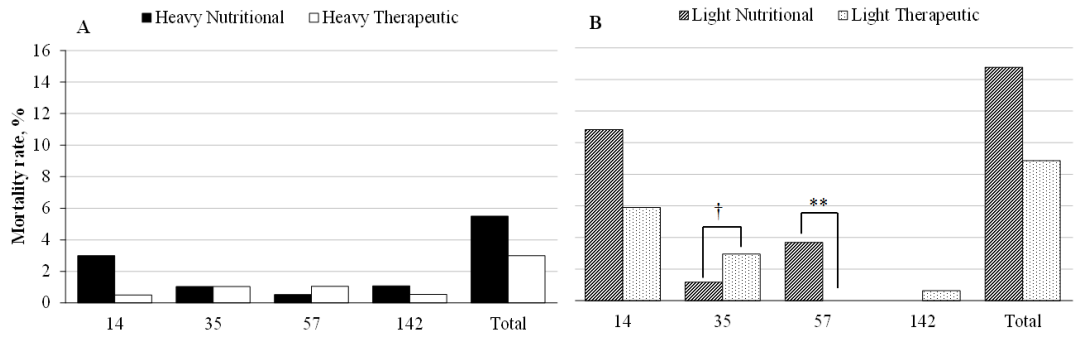

Figure 4.2*. Mortality rate from d 14 post-weaning until slaughter (d 142) and for the total period of heavy pigs (8.4 kg at weaning; A) and light pigs (5.81 kg at weaning; B) fed the Nutritional (125 mg/kg of Zn) or the Therapeutic (2,480 mg/kg of Zn) diets. †: P < 0.1, **: P < 0.05*

Poulsen (1995) and Hill et al. (2001) observed that as dietary ZnO level increases, growth performance increases, but there are other studies that did not observe this (Schell and Kornegay, 1996; Martínez et al., 2005; Broom et al., 2006). Unlike Poulsen (1995) and Hill et al. (2001), which formulated a basal diet with 60 or 300 mg/kg of Zn and then supplemented ZnO, in the present study, two different dietary strategies were compared. Based on the results of this experiment, reducing CP and Ca levels without supplementing ZnO after weaning does not have short or medium term consequences on growth performance, but may have long-term consequences, especially in light pigs considering that those pigs fed the Nutritional diet that had lower serum Zn concentration for 35 d but not in heavy pigs that only had lower serum Zn levels for 7 d.

Intra-uterine growth retarded (**IUGR**) piglet are defined as those whose birth weight are below the mean birth weight of the total population. It has recently been observed that in IUGR piglets, the metabolism of trace minerals such as Fe, Cu, and Zn is reduced (Zhang et al., 2015) and is most likely due to reduced feed intake. Low feed intake and anorexia (Chasapis et al., 2012), weight loss, poor food efficiency, and growth impairment are all signals of Zn deficiency (Grider, 2013). The mechanism by which Zn deficit alters the appetite may include changes in endocrine secretions and neurotransmitters concentrations at the hypothalamus, such as a decrease in galanin, or by a resistance to NPY, both hunger-stimulating peptides (Teegarden and Gunther, 2008; Baltaci and Mogulkoc, 2012). Light pigs fed nutritional Zn levels, had serum Zn levels below 0.7 mg/L, which is considered deficient [Hotz and Brown, 2004; Maret and Sandstead, 2006] for 35 d. Thus, light pigs with Zn deficient serum levels and reduced Zn metabolism may have been experienced loss of appetite, lower vitality, and may have been more at risk to develop diarrhea. This was observed by the higher mortality and impaired growth at the end of the 142 d period.

One of the major causes of productivity loss and mortality after weaning is related to post-weaning diarrhea (PWD), specifically enterotoxigenic *Escherichia coli*, which has been estimated to cause 50% of the mortality in pigs (Gyles, 1994). Therapeutic doses of ZnO could reduce post-weaning diarrhea (Poulsen, 1995; Hu et al., 2012; Heo et al., 2013; Kim et al., 2015; Stensland et al., 2015) and thus reduce the post-weaning mortality related to diarrhea.. Light pigs were shown to be more vulnerable to diarrhea than heavy pigs, especially at two critical periods, after weaning and after moving to the growing facilities. As well, light pigs had long-term consequences on growth performance if the starter diet contained only nutritional levels of Zn.

4.4.2. Are serum Zn levels of piglets at weaning a predisposing factor to diarrhea?

The odds ratio between the two piglets groups with different levels of Zn serum at weaning and its likelihood to develop diarrhea was 0.401 (0.184 – 0.874; Table 4.4). Therefore, pigs with low Zn serum levels (< 0.71 mg/L Zn) at weaning had 2.49 times the likelihood to have diarrhea when compared to pigs with high Zn serum levels (> 0.9 mg/L Zn) at weaning (P < 0.02; Table 4). Serum Zn levels below 0.7 mg/L are considered deficient in pigs, according to Hotz and Brown (2004) and Maret and

Sandstead (2006). This is first time in pigs that it has been demonstrated that low Zn levels may be a predisposing factor for diarrhea independent of pigs' weight. Also, in infants, Zn deficiency is a predisposing factor for diarrhea and is implicated in >50% of diarrhea-related deaths (King et al. 2016).

Table 4.5. *Number of animals with or without diarrhea according to the levels of Zn at weaning (n = 55) and their body weight.*

	LZn (<0.7)	HZn (>0.9)
Diarrhea	38	26
No diarrhea	17	29
BW 0, kg	6.5	6.5

OR = 0.401 (0.184 - 0.874)

Zinc is considered to be crucial for immune response. It has been described that Zinc deficiency impairs both innate function (via compromised epithelial barrier, macrophage, and neutrophil function) and acquired immunity (via reduction in the number of CD4 T cells, NF-kB, and IL-2 gene expression; Raiten et al., 2015). Consequently, the improvement of cellular immune statue in infants may be one of the mechanisms by which Zn supplementation reduces diarrhea morbidity (Salgueiro et al., 2000). Another possibility is that diarrhea negatively affects intestinal health by increasing intestinal losses of endogenous zinc, thus increasing zinc requirements, and therefore contributing to a zinc deficiency perpetuating a vicious cycle (Hambidge, 2000; Hotz and Brown, 2004).

4.4.3. Is maternal lactation able to maintain serum Zn levels of piglets at weaning, and how do these levels change between high and low BW piglets?

In Trial 3, the serum Zn levels of pigs decreased as lactation continued. Pigs started at 1.18 mg/L of serum Zn at d 0 (birth) and ended at 0.67 mg/L of serum Zn at d 28 (weaning; $P < 0.01$; Table 4.5) and light piglets (5.5 kg) had lower serum Zn levels at weaning than heavy piglets (8.6 kg), 0.79 vs. 0.98 mg/L of Zn ($P < 0.01$; Table 4.6) as in Trial 1.

Table 4.5. *Changes in serum Zn, Cu and Fe levels during lactation (0 from 28 days), n = 8 animals.*

	0	7	14	21	28	SEM	P-value
Zn	1.18[a]	1.01[ab]	0.92[b]	0.79[bc]	0.67[c]	0.087	<0.0001
Cu	0.37[b]	1.31[a]	1.35[a]	1.58[a]	1.49[a]	0.121	<0.0001
Fe	1.78	1.70	2.10	1.63	3.05	0.533	0.147

[a-c]Values within a column without a common superscript are different ($P < 0.05$).

Table 4.6. *Zn status at day 28 of life (weaning) between animals categorized as heavy BW (8.6 kg) and as light BW (5.5 kg); (P < 0.01, n = 30 per block of weight).*

	Heavy	Light	Pooled SEM	P-value
Zn, mg/L	0.98	0.79	0.038	0.001
Cu, mg/L	1.99	2.17	0.059	0.030
Fe, mg/L	5.15	3.92	0.834	0.267
BW, kg	8.63	5.50	0.171	0.001

In contrast with the present study, Mahan and Shields (1998) observed that body Zn increased rapidly from birth to weaning (10.93 to 15.28 mg/kg). Unlike Mahan and Shields' (1998) study, which collected total body components (internal tissue, whole blood, and hair) at birth and at weaning from different pigs to determine body Zn, in the present study, only the circulating pool of Zn in blood samples were analyzed from the same piglet at birth, at d 7, 14, 21 and at weaning.

Mobilization of Zn body stores into milk by sows is tightly regulated (McCormick et al., 2014). Plasma Zn is transferred to the mammary gland, and byactive transport is secreted into milk to meet the high Zn requirements of piglets (Matte et al., 2014; Davin et al., 2015). However, although the serum Zn levels of pigs may be high during the first days of life, as lactation progresses the serum Zn levels decrease. The reduction of Zn concentration in pigs' serum may be explained because of a decline in milk Zn concentration as lactation progresses (Krebs et al., 1995; Donangelo and King, 2012). It has been reported that, in humans, there is a pronounced decrease in Zn concentration of about 75% over lactation (Lönnerdal et al., 1981b; Donangelo and

King, 2012) and that is independent of maternal Zn intake. Reductions in milk Zn concentrations have also been observed in others mammals and by as much as 60 % in rats (Keen et al., 1981) and 9.4% in dogs (Lönnerdal et al., 1981a). However, in sows, there is not a decrease of the Zn concentration in milk (Hill et al., 1983), but a decrease in milk production during long lactations (Hansen et al., 2012).

Zinc requirements during lactation are greater than during pregnancy in humans, especially in the first weeks postpartum (Krebs, 1998), which results in a redistribution of Zn to the mammary gland. Zinc is mobilized from involuting tissues (uterus) and maternal blood, bone (Tamura and Goldenberg, 1996; Krebs, 1998; Donangelo and King, 2012), and by increasing efficiency of Zn absorption in the intestine (Donangelo and King, 2012). In sows, Van Riet et al. (2015) observed that at the end of lactation, plasma Zn concentrations of sows tended to increase returning to the initial values at early gestation, likely as a result of increased absorption or decreased nutrient requirements (Donangelo and King, 2012).

Serum Cu levels increased during lactation as serum Zn levels decreased, starting with 0.4 mg/L of Cu at d 0 and ending at 1.5 mg/L of Cu ($P < 0.01$). However, this result is in contrast with the study by van Riet et al. (2015), where plasma Cu concentration decreased linearly during lactation. There were no significant differences in the serum Fe levels during lactation ($P = 0.15$), and this was likely due to the high variation between piglets.

Therefore, these results may indicate that during the latter 2 weeks sows' milk is inadequate to meet the piglets' Zn requirements, especially in the case of light pigs which have a greater risk of developing Zn deficiency at the end of lactation.

4.4.4. Is possible to increase serum Zn levels at weaning by Zn supplementation during lactation?

Pigs supplemented with 18 and 30 mg of Zn had more serum Zn levels at weaning and 7 d after than pigs not supplemented with Zn ($P < 0.001$, Figure 4.3).

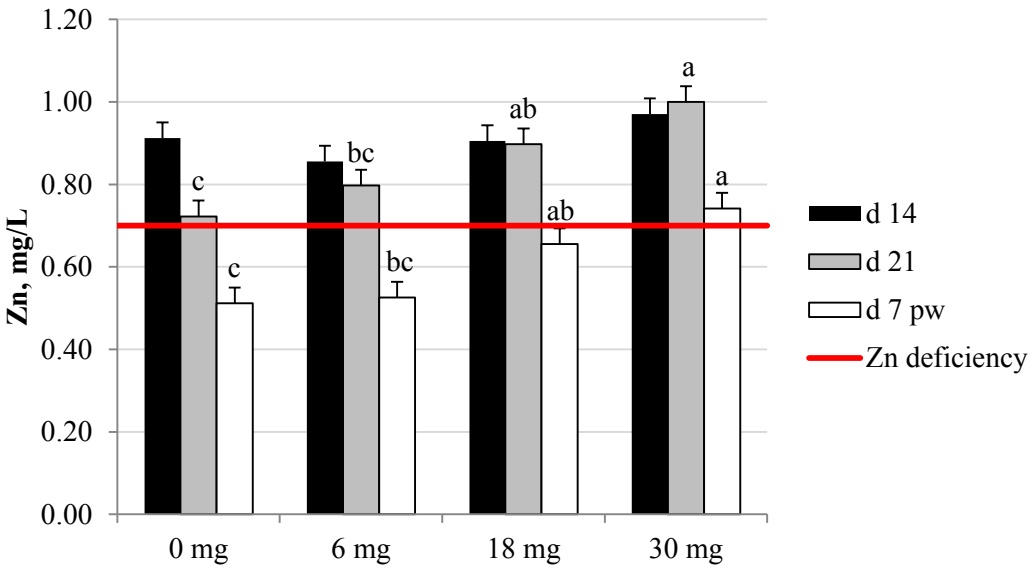

Figure 4.3. *Effect of daily Zn supplementation (0, 6, 18 or 30 mg) from d 14 to d 21 on serum Zn concentration at d 14, 21 (weaning) and d 7 post-weaning (pw). The red line establishes the Zn deficiency (< 0.7 mg/L of serum Zn).* $^{a\text{-}c}$ *Values on top of a column without a common superscript are different (interaction between treatment and day, P < 0.01).*

Only pigs supplemented with 30 mg of Zn had serum Zn levels over a deficient level (0.7 mg/L) one week after weaning. Light pigs supplemented with 18 and 30 mg/kg of Zn at weaning had an increase in serum (12 and 21 %, respectively) between d 14 and d 21 compared with light pigs without Zn supplementation ($P < 0.01$). This may explain the previous result. But, heavy pigs supplemented with Zn (6, 18, and 30 mg) did not differ from heavy pigs without supplementation (Figure 4.4). The difference in serum Zn level at weaning between heavy and light pigs was repeated in Trial 4. Pigs categorized as light showed lower serum Zn levels compared with heavy pigs (0.86 vs. 0.95 mg/kg of Zn, respectively; $P < 0.001$). This reinforces the idea that light pigs have higher risk to develop Zn deficiency at weaning and, thus its consequences, like anorexia, loss of appetite, impaired growth, diarrhea, etc.

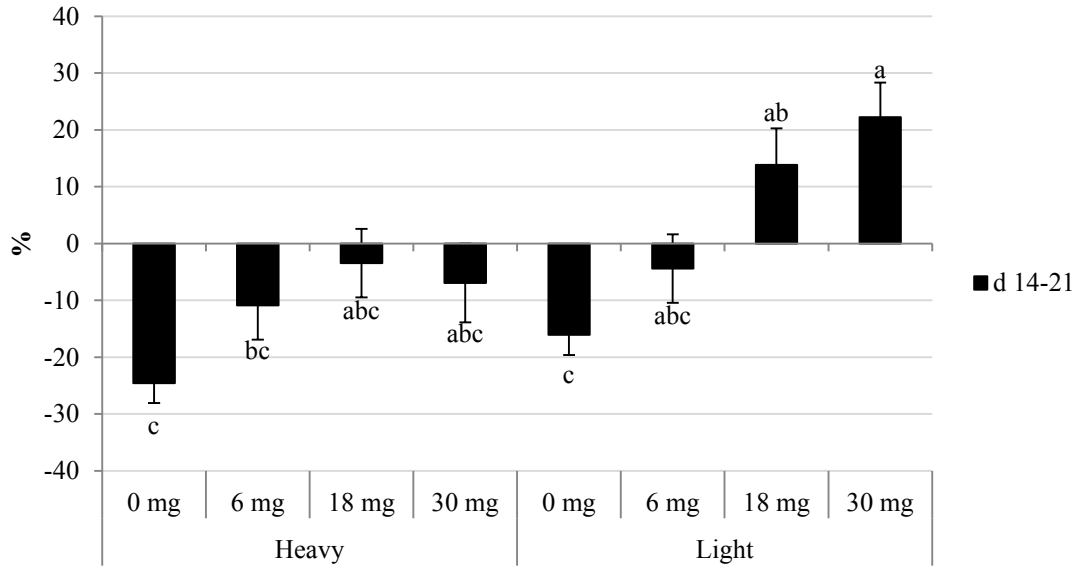

Figure 4.4. *Relative change on the serum Zn concentration between d 14 and d 21 of age in heavy (4.9 kg BW at d 14) and light pigs (2.9 kg BW at d 14) supplemented with 0, 6, 18 or 30 mg Zn/d during the last week of lactation (d 14 to d 21).*[a -c]*Values on top of the column without a common superscript are different (interaction between treatment and block of weight, P < 0.01).*

These results are in agreement with Caine et al. (2009) and Metzler-Zebeli et al. (2010), where the nutriment-intubation of 40 mg Zn from Zn methionine increased Zn serum and liver tissue concentration of suckling pigs at weaning, but with a limited improvement to intestinal morphology of weaned pigs (Metzler-Zebeli et al., 2010).

The estimation of the total amount of Zn present for a weaned pig is 15.3 mg/kg fat-free empty body weight (Mahan and Shields, 1998), therefore, the supplementation of the light pigs with 18 and 30 mg of Zn was the equivalent of 370 and 617 % the total Zn in the body based on metabolic weight as stated by Fageström (1977), and for the high pigs was 250 and 417 %. Light pigs received much more Zn compared with heavy pigs, and this may explain why light pigs were the only ones that presented an increase in serum Zn levels during lactation. However, the amount of dietary Zn administered was not representative of the increase in serum Zn levels. Several studies in animals and humans have shown that the whole-body content of Zn remains relatively constant over a wide range of intake. For example, it was observed that weanling rats maintain the whole body zinc constant as dietary Zn increased from 10 to 100 µg Zn/g of diet, because the efficiency of Zn absorption decreased and endogenous excretion increased

(Kirchgessner, 1993). In humans, studies have described that zinc losses and absorption are adjusted to match intakes over a 10-fold range (Johnson et al., 1993). As a general rule, Zn is relatively non-toxic when it is consumed in the diet (Grider, 2013), and pigs are possibly the livestock specie with the highest tolerance for Zn. The National Research Council set the maximum tolerable level of Zn for pigs at 1,000 mg/kg diet (NRC, 2005) and the NRC proposes that Zn accumulates in tissues such as the liver, kidney and bone in order to protect other organs from failure induced by Zn accumulation.

Our results therefore indicate that the administration of nutritional Zn levels may lead to a temporary decrease in serum Zn levels after weaning, and that this is pronounced in lighter piglets and has additional consequences on mortality and long-term growth performance. The initial stages of life are very important and lactation is inadequate to meet piglets' Zn requirements. This is especially true for the lighter piglets of the litter. Finally, it was confirmed that is possible to revert the fall of Zn serum concentrations in lighter piglets by supplementation of Zn during lactation.

CHAPTER 5

High dietary calcium levels in low phosphorus diets affects growth performance, intestinal microbiota, and the inflammatory gene expression response in the intestinal mucosa of weaning pigs

High dietary calcium levels in low phosphorus diets affects growth performance, intestinal microbiota, and the inflammatory gene expression response in the intestinal mucosa of weaning pigs

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5.1. ABSTRACT

Effects of low and high dietary calcium on growth performance, intestinal morphology, microbiota and gene expression were evaluated in four different trials. In Trial 1, a total of 240 piglets (26 ± 2 d old, average BW = 7.7 ± 1.04 kg) were distributed into 3 dietary treatments: low (0.35%; **LCa**), medium (0.65%; **MCa**), and high (0.95%; **HCa**) Ca. Feed intake and individual BW were registered during the pre-starter phase (d 0 to 14 post-weaning). Pigs fed HCa presented lower BW, ADG and higher FCR than pigs fed the LCa and MCa ($P < 0.05$). Two other performance trials (160 pigs each; 26 ± 2 d old, average BW = 7.5 ± 0.88 and 7.7 ± 1.03 kg in Trial 2 and Trial 3, respectively) were conducted to study the effects of low (0.35%) or high (0.95%) Ca levels in diets including ZnO at a nutritional (125 mg/kg, Trial 2) or therapeutical (2,480 mg/kg ZnO, Trial 3) level. Feed intake and individual BW were registered from d 0 to 35 post-weaning. Pigs fed the low Ca diet tended ($P < 0.1$; Trial 2) to have higher BW compared to pigs fed the high Ca diet in diets with 125 mg/kg of Zn, and showed a higher BW ($P < 0.05$; Trial 3) with diets containing 2,480 mg/kg of Zn. In Trial 4, a total of 18 pigs (28 ± 0 d old, average BW = 7.2 ± 0.24 kg) were allocated individually in cages and assigned to 2 dietary treatments: low (0.45%) and high (0.95%) Ca levels. Piglets were fed for 14 d and then euthanized to obtain jejunum tissue for gene expression and histology and colon digesta for 16sRNA microbiota analyses. No significant differences in small intestinal morphology were observed. However, pigs fed high levels of Ca presented a higher heterogeneous microbial community with increased of the *Bacterioides* genera in colon, while the transcriptomic profile of pigs fed LCa diets indicate a lower activation of the inflammatory pathways than pigs fed HCa diet.

5.2. INTRODUCTION

Plant ingredients contain low Ca levels and 1/3 may be bound to P phytate (Selle et al., 2009). Therefore, Ca has to be supplemented with animal or mineral sources, such as limestone or calcium phosphates. Increasing calcium carbonate in Ca-deficient diets results in a concomitant increase in P and Ca bone retention in growing pigs (Létourneau-Montminy et al., 2015). However, growth performance and feed intake may decrease linearly with increasing dietary Ca (González-Vega et al., 2016; Rousseau et al., 2016), and the magnitude of the effect is exacerbated when low dietary P levels

are provided (Rousseau et al., 2016). There are different mechanisms that have been reported to explain the negative effects of Ca in performance. Elevated levels of Ca may reduce phytase efficacy (Lei et al., 1994; Selle et al., 2009) and P digestibility (Stein et al., 2011). Excess dietary Ca tends to form insoluble complexes with phytate or phosphate in the small intestine (Cromwell, 1996), or compete for the active site of phytase (Wise, 1983; Pointillart et al., 1985; Qian et al., 1996) rendering them unavailable for hydrolysis in the stomach (Wise, 1983) and small intestine (Cromwell, 1996). Moreover, Maenz et al. (1999) described that multiple mineral complexes such as Ca-Zn-phytate are thought to be more stable than single mineral complexes.

Limestone and ZnO also show a high acid-binding capacity (Lawlor et al., 2005). Therefore, high dietary Ca or Zn may also favor an increase in digestive tract pH, which in turn decreases phytate solubility (Sandberg et al., 1993; Selle and Ravindran, 2008) and affect protein digestibility (Selle and Ravindran, 2008) and microbiota (Metzler-Zebeli et al., 2011).

Therefore, the hypothesis of this study was that using low Ca diets (zero limestone) early after weaning increases piglets performance, either in diets containing or not therapeutic doses of ZnO, associated with changes in the microbiota and intestinal mucosa.

5.3. MATERIAL AND METHODS

The Ethical Committee on Animal Experimentation at the UAB, reviewed and approved the protocols for the experiments. Pigs used in the study were the offspring of Pietrain boars and Landrace x Large White females.

5.3.1. Animals, Experimental Design, and Diets

5.3.1.1. Trial 1

The first trial was performed to study the effects of three different dietary Ca levels (low, medium and high; Table 5.1 and 5.2) in piglet performance the first 14 d after weaning.

Table 5.1. *Ingredients composition of Trial 1, 2, 3 and 4 diets, as-fed basis*[1].

	Trial 1[2]			Trial 2 and 3[3,4]			Trial 4	
	Pre-starter			Pre-starter		Starter	Pre-starter	
Ingredients, %	LCa 0.35%	MCa 0.65%	HCa 0.95%	LCa 0.35%	HCa 0.95%		LCa 0.35%	HCa 0.95%
Maize	36.31	35.02	33.73	36.31	33.73	48.08	47.88	47.2
Barley	13	13	13	13	13	7.28	13.19	13
Wheat	17	17	17	17	17	17	1.2	1.18
Soybean meal 44%CP	10	10	10	10	10	8	7.1	7
Extruded Soybean meal	-	-	-	-	-	8.67	11.16	11
Fish meal LT	6	6.08	6.17	6	6.17	2.5	2.54	2.5
Sweet Milk Whey	10	10	10	10	10	-	12.17	12
Spray Dried Porcine Plasma 80%CP	3.86	3.92	3.98	3.86	3.98	-	2.9	2.86
HP300	-	-	-	-	-	5.01	-	-
Soybean oil	2.14	2.52	2.9	2.14	2.9	-	-	-
Calcium carbonate	0	0.78	1.55	0	1.55	-	0	1.42
Mono-calcium phosphate	0.09	0.08	0.07	0.09	0.07	0.6	0.87	0.86
Lard	-	-	-	-	-	0.05	-	-
Salt	0.3	0.3	0.3	0.3	0.29	0.36	-	-
ZnO[3]	-	-	-	0.00 - 0.30	0.00 - 0.30	0.00 - 0.30	-	-
L-Lysine-HCL	0.3	0.3	0.29	0.3	0.3	0.48	0.43	0.42
DL-Metionine	0.21	0.21	0.22	0.21	0.22	0.22	0.21	0.21
L-Threonine	0.2	0.2	0.2	0.2	0.2	0.2	0.17	0.17
L-Tryptophan	0.09	0.09	0.09	0.09	0.09	0.05	0.08	0.08
L-Valine	0.1	0.1	0.1	0.1	0.1	-	0.1	0.1
Vit-Min complex	0.4[5]	0.4[5]	0.4[5]	0.4[6]	0.4[6]	1.5[6]	-	-

[1]1,000 Phytase units (FTU) were added to all diets on top. Phytase was ronozyme® (Ronozyme® NP, DSM, Kaiseraugust, Switzerland).

[2]Antibiotics in feed: Zn Oxide: 2,480 mg/kg; Amoxicilin: 300 mg/kg; Colistin sulphate: 120mg/kg.

[3]Antibiotics in feed: amoxicilin, 300 mg/kg; colistin sulphate, 120 mg/kg.

[4]The ZnO inclusion was 0 mg/kg in Trial 2 and 3,000 mg/kg in Trial 3.

[5]Premix Supplied: Magnesium (0.12, 0.12 and 0.12%), Sodium (0.41, 0.41 and 0.42%), Clorur (0.65, 0.65 and 0.65%), Potassium (0.73, 0.72 and 0.72%), Copper (5.95, 6.01 and 6.06%), Iron (63.26, 67.56 and 71.87%), Vitamin E (14.4214.17 and 13.91%), Biotin (0.15, 0.15 and 0.15%), Coline (1233.1, 1230.3 and 1227.6%), Sulphur (0.2, 0.2 and 0.2%), Zinc (33.4, 33.1 and 33.0%) and Manganese (13.5, 13.4 and 13.3%) for LCa, MCa, HCa respectively.

[6]Pre-starter and starter diets Premix Supplied (g/kg): 7000 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 glycine), 0.05 mg of Co (sulphate), 40 mg of Zn (chelate of amino acids), 12.5 mg of Mn (oxide), 7.5 mg of Mn (chelate of glycine), 0.35 mg of I, 0.5 mg of Se (organic) and 0.1 mg of Se (sodium).

Table 5.2. *Analyzed composition of Trial 1, 2, 3 and, 4 diets, as-fed basis (unless otherwise indicated).*

	Trial 1			Trial 2			Trial 3			Trial 4	
	Pre-starter			Pre-starter		Starter	Pre-starter		Starter	Pre-starter	
	LCa	MCa	HCa	LCa	HCa		LCa	HCa		LCa	HCa
Item	0.35%	0.65%	0.95%	0.35%	0.95%		0.35%	0.95%		0.35%	0.95%
DM, %	89.88	90.01	90.14	92.28	92.32	90.61	92.41	92.39	90.27	90.78	90.73
NE, kcal/kg[1]	2520	2520	2520	2520	2520	2460	2520	2520	2460	2460	2460
Ash, %	4.18	4.93	5.69	4.85	6.39	4.94	5.14	6.56	5.02	5.06	6.47
CP, %	19.42	19.31	19.28	19.32	19.12	18.52	19.15	19.03	18.34	18.11	17.87
EE, %	4.86	5.2	5.54	3.55	4.01	3.68	3.42	3.89	3.62	4.48	4.33
NDF + ADF, %	8.35	8.25	8.15	10.8	10.74	12.15	10.79	10.69	12.04	9.43	9.96
Ca, %	0.64	0.92	1.16	0.55	1.11	0.51	0.49	1.13	0.51	0.65	1.4
P, %	0.58	0.6	0.58	0.59	0.58	0.54	0.6	0.56	0.55	0.62	0.63
Zn, mg/kg	-	-	-	220	231	170	2624	2686	2793	-	-
Phytase, FTU[2]/kg	1903	1891	1028	2426	2374	2008	2327	2559	1710	-	-

[1]Nutrient composition of Net Energy was estimated.

[2]FTU = phytase units.

A total of two hundred and forty piglets (26 ± 2 d old, average BW = 7.7 ± 1.04 kg) were distributed at weaning into 24 pens (10 pigs/pen) by initial body weight into two blocks (heavy = 8.6 ± 0.46 kg, and light = 6.7 ± 0.37 kg) and randomly allotted to 3 diets with 4 replicate pens per diet and block resulting in a total of 8 replicate pens per diet for the 2 blocks.

Pigs were settled to the weanling unit with a room of 24 pens. The room was equipped with central heating, automatic forced ventilation and a completely slatted floor. Pigs had *ad-libitum* access to feed and drinking water. All diets were formulated to contain 0.35% (**LCa**), 0.65% (**MCa**) or 0.95% (**HCa**) total Ca with variable amounts of limestone, and 0.33% STTD P until d 14 post-weaning by including monocalcium phosphate and 1,000 FTU (Ronozyme® NP, DSM, Kaiseraugust, Switzerland). Vitamins and all minerals were included to meet or exceed the requirements for 11 to 25 kg pigs (NRC, 2012). A mineral matrix (g/kg NPP; Ca or Na) of the phytase was not considered for diet formulation.

Body weight and feed intake were monitored on d 0, 7 and 14 post-weaning. Piglets were weighted individually. ADFI, ADG and FCR were calculated by pen.

5.3.1.2. Trial 2 and 3

Two trials were conducted to determine the effects of differing dietary Ca content on piglet performance, either in diets containing or not therapeutic Zn supplementation. Each trial consisted of one hundred and sixty (26 ± 2 d old) pigs (average BW = 7.5 ± 0.88 kg in trial 2; and 7.7 ± 1.03 kg BW in trial 3). Pigs were randomly allotted to a randomized complete block design with 2 blocks of 80 pigs (heavy = 8.6 ± 0.38 kg, and light = 6.7 ± 0.44 in trial 2; and heavy = 8.3 ± 0.88 kg, and light = 6.7 ± 0.37 in trial 3) distributed by BW into 16 pens (10 pigs/pen). The weanling room was equipped with central heating, automatic forced ventilation and a completely slatted floor. Pigs had *ad-libitum* access to feed and drinking water. Dietary treatments consisted in low (0.35%, without limestone; LCa) or high (0.95%; HCa) Ca levels diets containing a nutritional level of Zn (125 mg Zn/kg; Trial 2) or a therapeutical level of ZnO (2,675 mg Zn/kg, 3,000 mg/kg ZnO; Trial 3). The ingredient and nutrient composition are presented in Table 5.1 and 5.2, respectively. Pigs were fed the diets during the first 14 d after weaning. From d 14 until d 35 post-weaning, the level of Ca was fixed at 0.77% for all diets. The source of inorganic Ca used in the 95% Ca diet was calcium carbonate. Both

diets included low monocalcium phosphate to contain 0.33% STTD P, and were supplemented with 1,000 FTU (Ronozyme® NP, DSM, Kaiseraugust, Switzerland). Vitamins and all minerals were included to meet or exceed the requirements for 11 to 25 kg pigs (NRC, 2012). A mineral matrix (g/kg NPP; Ca or Na) of the phytase was not considered for diet formulation.

Body weight and feed intake were monitored on d 0, 14 and 35 post-weaning. Piglets were weighted individually. ADFI, ADG and FCR were calculated by pen.

5.3.1.3. Trial 4

Eighteen growing barrows (28 ± 0 days old) with an average initial BW of (7.2 ± 0.24 kg) were randomly allotted to 2 diets (0.45% or 0.95% Ca; Table 5.1 and 5.2) with 9 replicate pigs per diet to evaluate the effects of dietary Ca on small intestinal morphology, gut microbial ecology. To study the intestinal gene expression only 12 pigs were selected based on his final BW. Pigs were individually settled into metabolic cages with 0.36 useful m^2, and were fed dietary treatments for 14 days. They had *ad-libitum* access to feed and drinking water.

Sample collection

On d 14, pigs were euthanized and sequentially sampled during the morning (between 08:00 and 12:00 h). Piglets received an intravenous lethal injection of sodium pentobarbital (200 mg/kg BW; Dolethal, Vetoquinol, S.A., Madrid, Spain). Once dead, animals were bled, the abdomen was immediately opened and the whole gastrointestinal tract was removed and carefully dissected from the mesentery. For candidate gene expression, the mid-jejunum (30 cm in the middle of the jejunum) was selected as important intestinal site for digestion. Jejunum pieces of 3 to 4 cm were cut, and digesta were removed. Jejunum tissues were thoroughly washed in ice-cold PBS, and were placed into 2 ml RNA-free vials and immediately frozen in liquid nitrogen and stored at -80˚C. For microbiota analyses, digesta from the proximal-colon (considered to be 1 m from the ileocecal junction) were homogenized and aseptically collected. The digesta samples were placed in ice until transfer to -80˚C. For the histology analyses, 3-cm sections from the middle jejunum were removed, opened longitudinally, and fixed by immersion in 10% (vol/vol) buffered formalin.

Gene expression profiling

Total mRNA was isolated from 50 mg of intestinal tissue (mid jejunum) according to the Takara Fast Pure kit (Takara Bio, Japan) protocol. The purity and concentration of total RNA were checked using the Nanodrop ND 1000 (Thermo Fisher Scientific Inc., Waltham, MA, USA), while RNA integrity was assessed by Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA).

Total mRNA was hybridized on Affymetrix Porcine Gene 1.1 ST array strips. Hybridized arrays were scanned on a GeneAtlas imaging station (Affymetrix, Santa Clara, CA, USA). Performance quality tests of the arrays including the labelling, hybridization, scanning and background signals by a Robust Multichip Analysis were performed on the CEL files using Affymetrix Expression Console.

The Affymetrix Transcripts ID's were associated to 13,494 Human gene names, based on Sus scrofa Ensembl (release 83, www.ensembl.org). On processed gene expression values, an exploratory functional analysis was applied with Gene Set Enrichment Analysis software, using the Hallmark.v5.1 and the C5.v5.1 catalogs of gene sets (based on Gene Ontology) from Molecular Signatures Database v3.1 (http://www.broadinstitute.org/gsea/msigdb/Index.jsp).

Microbiota analyses

Bacterial DNA was extracted from ~250 mg of colonic digesta by using the commercial QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA, USA), according to the manufacturing instructions and optimization recommendations. The quality and quantity of extracted DNA was assessed using a NanoDrop (ND-1000 spectrophotometer, Nano-Drop Techonologies, Wilmington, DE, USA).

For the 16S rRNA gene high-throughput sequencing, amplicon libraries were prepared using Nextera XT Index Kits 16S v3 – v4 Amplicon-Seq Kit (Illumina). Metagenomic DNA (5 ng/µl) was used as template for the first PCR (PCR-I) amplification (95 C for 3 min, 25 cycles at 95°C for 30 s, 55°C for 30 s, 72°C for 30 s plus 72°C for 5 min). PCR product was purified with AMPure XP beads (Beckman Coulter). A second PCR (PCR-II) used five µl of resuspended PCR product to add the Illumina sequencing adapters and dual-index barcodes (95°C for 3 min, 8 cycles at 95°C for 30 s, 55°C for 30 s, 72°C at 30 s plus 72°C for 5 min). The final product was

cleaned up using the AMPure XP Beads and the library was analyzed using the Bioanalyzer DNA 1000 (Agilent Technologies). Five µl of diluted DNA from each library were mixed for pooling libraries and denatured with NaOH, diluted with hybridization buffer and heat denatured. Five % PhiX served as an internal control. For sequencing on the MiSeq Illumina platform instrument a v3 – v4 Miseq sequencing kit (2x250 bp paired-end reads; Illumina) was used.

For the bioinformatics analysis, sequence reads generated by 16S rRNA were processed following QIIME (Caporaso et al., 2010) v1.9.1 pipeline with default settings. Firstly, paired-end reads were merged and subsequently quality filtering was performed at a quality score of Q20. Subsampled pick open-reference operational taxonomic unit (**OTU**) strategy (Rideout et al., 2014) was followed at 10% of subsampling. Reads were clustered to OTU using uclust (Edgar, 2010) with 97% sequence similarity. Representative sequences were assigned to taxonomy against bacterial 16S GreenGenes v13.8 reference database (DeSantis et al., 2006) at a 90% confidence threshold and sequence alignment and phylogenetic tree building were obtained through uclust and FastTree. Thereafter, chimeric sequences were removed with ChimeraSlayer (Haas et al., 2011), and singletons and OTUs below 0.005% as recommended by Bokulich et al. (2013) were removed.

Jejunum mucosa morphology measurements

Tissue samples were dehydrated and embedded in paraffin wax, sectioned at 4 µm, and stained with hematoxylin and eosin. Morphological measurements of villous height (**VH**), crypt depth (**CD**) and ratio VH:CD were performed with a light microscope (BHS; Olympus, Spain). Mitosis activity and the number of intraepithelial lymphocytes were measured in 10 well-oriented crypts using a linear ocular micrometer, according to published parameters (Nofrarias et al., 2005).

5.3.2. Statistical Analysis

5.3.2.1. Performance parameters

Performance results were analyzed by using the SAS® statistical package (version 9.4, SAS Institute; Cary, USA).

In Trial 1, 2, and 3, piglet performance was analyzed with an ANOVA by using the GLM procedure, and the statistical unit was the pen.

5.3.2.2. Transcriptomic profile

Normalized enrichment score (**NES**) was calculated for each gene set, and statistical significance was considered when false discovery rate (**FDR**) % < 25 and *P*-values of NES < 0.05. Enrichment score *P*-values were estimated using a gene set-based permutation test procedure and the FDR *P*-value were calculated for the comparison between LCa and HCa. Transcript ID's can be considered significant when FDR < 0.05.

5.3.2.3. Microbiota

For the biostatistics analysis, R v3.3.0 was used. Firstly, OTU table was imported to R with phyloseq package (McMurdie and Holmes, 2013). Diversity analysis was performed using vegan package (Oksanen et al., 2016) at OTU level. Richness and alpha diversity (Shannon, Simpson and Simpson inverse indexes) were calculated with raw counts while beta diversity (Bray-curtis distance) with relative abundances. To compare treatment effects a rarefaction analysis of richness and an ANOVA analysis were performed for alpha diversity. An analysis of similarities (**ANOSIM**) and a PERMANOVA based on Bray-curtis distance and a non-metric multidimensional scaling were performed for beta diversity. Finally, normalization of raw counts and differential abundance analysis were performed using metagenomeSeq package (Paulson et al., 2013). For this aim, taxa were aggregated at Phylum and Genus level.

5.4. RESULTS

The analyzed P concentrations in diets were in agreement with the expected values (Table 5.2), but the Zn concentrations were slightly greater than expected (220 and 231 vs 125 mg/kg). These differences were probably due to differences among theoretical and actual composition of feed ingredients. This situation might be common in commercial practice because Zn is not routinely analyzed in feed ingredients and its addition to diets is based in theoretical values. Analyzed Ca contents and phytase activities were also higher than expected (0.49-0.64% vs a theoretical 0.35%, 0.92 vs a theoretical 0.65%, and 1.11-1.4 vs a theoretical 0.95% of Ca, and phytase activity was between 1710 to 2559 FTU). However, we registered the data and confirmed that differences among levels of Ca were maintained to evaluate their effects on performance and physiological parameters.

Table 5.3. *Productive performance (BW, ADFI, ADG and feed conversion ratio [FCR]) of pigs fed diets with 0.35% (LCa), 0.65% (MCa) or 0.95% (HCa) of Ca after weaning (n = 8 per treatment; 0 to 7, 7 to 14 and 0 to 14 days post-weaning; Trial 1).*

	Heavy			Light			SEM	*P*-value		
	LCa (0.35%)	MCa (0.65%)	HCa (0.95%)	LCa (0.35%)	MCa (0.65%)	HCa (0.95%)		Ca	block	Ca*block
BW, kg										
d 0	8.63	8.65	8.63	6.73	6.73	6.74	0.01	0.620	<.0001	0.231
d 7	9.37^{a}	9.29^{a}	8.97^{a}	7.49^{b}	7.37^{b}	7.45^{b}	0.08	0.041	<.0001	0.048
d 14	11.97^{a}	11.64^{ab}	10.97^{b}	9.53^{c}	9.54^{c}	9.36^{c}	0.11	0.000	<.0001	0.006
ADG, g/d										
d 0-7	105.8	90.7	48.7	108.1	91.8	100.6	11.8	0.044	0.073	0.074
d 7-14	370.9^{a}	336.6^{ab}	285.2^{c}	291.9^{c}	309.9^{bc}	272.6^{c}	10.7	0.000	0.000	0.015
d 0-14	238.4^{a}	213.7^{ab}	166.9^{c}	200.0^{bc}	200.9^{bc}	186.6^{bc}	8.1	0.000	0.129	0.008
ADFI, g/d										
d 0-7	172.0	157.2	132.3	153.0	139.7	167.1	11.4	0.411	0.951	0.058
d 7-14	457.5^{a}	423.5^{ab}	403.0^{ab}	371.5^{b}	407.4^{ab}	378.2^{b}	13.3	0.134	0.001	0.034
d 0-14	365.3	346.73	313.1	319.4	299.3	316.2	12.8	0.113	0.010	0.108
FCR										
d 0-7	1.63^{a}	1.84^{a}	3.19^{b}	1.46^{a}	1.54^{a}	1.69^{a}	0.28	0.012	0.011	0.048
d 7-14	1.24	1.26	1.42	1.28	1.32	1.39	0.05	0.024	0.641	0.655
d 0-14	1.53^{ab}	1.63^{ab}	1.88^{c}	1.60^{ab}	1.49^{a}	1.70^{bc}	0.04	<.0001	0.033	0.022

[a-c] Values within a column without a common superscript are different ($P < 0.05$).

5.4.1. Growth Performance

In Trial 1, pigs fed high Ca diet (HCa) had lowest BW at d 7 and 14 (LCa; $P < 0.05$; Table 5.3). Heavy pigs fed the HCa diet presented lower BW at d 14 than those fed the LCa diet while light pigs were not affected by the dietary Ca level (interaction, $P < 0.01$). Heavy pigs fed HCa diet also had lower ADG compared to heavy pigs fed MCa and LCa from d 7 to d 14 and from d 0 to d 14 but, no differences were observed for light pigs (interaction, $P < 0.05$). Moreover, for the entire period (d 0 to d 14) pigs categorized as light showed similar ADG than heavy pigs (block $P = 0.13$). For the entire period no different ADFI was observed due to the dietary Ca level or between light and heavy pigs during the first week post-weaning (block, $P = 0.95$). However, higher ADFI was observed for heavy pigs fed the LCa diet than light pigs fed the LCa diet from d 7 to d 14 post-weaning (interaction, $P < 0.05$). Pigs fed HCa diets showed higher FCR compared than those fed LCa diet ($P < 0.05$). From d 0 to d 7, heavy pigs fed HCa diet also showed higher FCR compared with light pigs fed LCa (interaction, $P < 0.05$).

In Trial 2, BW of piglets was not affected by the dietary treatments ($P > 0.05$; Table 5.4). However, lower BW tended to be observed for those piglets fed the high Ca diet than those fed the low Ca diet after 14 and 35 d post-weaning ($P = 0.07$ and $P = 0.08$, respectively). Pigs fed HCa diet tended to show lower ADG than those fed LCa for the pre-starter period (d 0 to d 14 post-weaning) and for the entire nursery period (0 to 35 d post-weaning). Similar growth rate was observed for light pigs than heavy ones (block effect, $P > 0.1$). For the pre-starter period (0 to 14d post-weaning) pigs fed HCa showed lower feed intake than pigs fed LCa diet ($P < 0.05$). However, the effect diluted during the starter period (14 to 35 d post-weaning) and was not carried over up to the end of the nursery period (35d post weaning). But, heavy pigs fed HCa diet sowed higher feed intake than those fed the LCa from d 14 to d 35 and for the entire period but, light pigs fed HCa diet did not show the same effect (interaction, $P < 0.05$). Finally, Ca level did not affect feed conversion ratio but, similar response pattern the previously observed for ADFI was achieved either for heavy and light pigs in both, from d 14 to d 35 and from d 0 to d 35 (interaction, $P <0.05$).

Table 5.4. *Productive performance (BW, ADFI, ADG and feed conversion ratio[FCR]) of pigs fed diets with 0.35% (LCa), or 0.95% (HCa) of Ca after weaning and 0 mg/kg of ZnO (n = 8 per treatment; 0 to 14, 14 to 35 and 0 to 35 days post-weaning; Trial 2).*

	Heavy		Light		SEM	*P*-value		
	LCa	HCa	LCa	HCa		Ca	block	Ca*block
	0.35%	0.95%	0.35%	0.95%				
BW, kg								
d 0	8.29	8.28	6.79	6.79	0.01	0.945	<.0001	0.780
d 14	11.96	11.57	10.65	9.69	0.34	0.071	0.001	0.423
d 35	23.13	22.5	21.27	19.76	0.57	0.083	0.002	0.451
ADG, g/d								
d 0-14	262.6	234.6	275.72	206.8	24.2	0.069	0.767	0.414
d 14-35	524.4	520.7	505.9	473.5	18.2	0.339	0.095	0.445
d 0-35	419.7	406.3	413.8	366.8	15.9	0.082	0.180	0.312
ADFI, g/d								
d 0-14	325.9	299.7	328.4	257.8	22.0	0.048	0.387	0.333
d 14-35	674.8	790.2	789.0	604.2	64.7	0.601	0.589	0.039
d 0-35	535.2	594.0	604.8	465.6	39.7	0.331	0.472	0.028
FCR								
d 0-14	1.24	1.31	1.21	1.25	0.05	0.391	0.415	0.806
d 14-35	1.29	1.52	1.56	1.28	0.11	0.794	0.879	0.041
d 0-35	1.28	1.46	1.46	1.27	0.08	0.938	0.963	0.039

In trial 3, the use of low Ca diet also showed higher BW at the end of the pre-starter period (d 14 post-weaning; $P < 0.05$; Table 5.5). Pigs fed the LCa diet had also showed higher ADG than those pigs fed the high Ca diet (from d 0 to d 14; $P < 0.05$) while no different feed intake was observed. Therefore, according to ADG and ADFI, pigs fed the low Ca diet tended to show lower FCR than pigs fed the high Ca diet ($P = 0.06$). However, no different ADG and ADFI were observed for the starter period (14 to 35 d post-weaning) and for the entire nursery period (0 to 35d post-weaning). Lower FCR was observed for heavy pigs fed HCa diet than those fed LCa diet but, contrarily light pigs fed HCa diet did not show an improved FCR compared to those fed LCa (interaction, $P = 0.05$ in FCR d 14 to d 35 and $P = 0.06$ in FCR d 0 to d35).

Table 5.5. *Productive performance (BW, ADFI, ADG and feed conversion ratio[FCR]) of pigs fed diets with 0.35% (LCa), or 0.95% (HCa) of Ca after weaning and 3,000 mg/kg of ZnO (n = 8 per treatment; 0 to 14, 14 to 35 and 0 to 35 days post-weaning; Trial 3).*

	Heavy		Light		SEM	*P*-value		
	LCa	HCa	LCa	Hca		Ca	block	Ca*block
Item	0.35%	0.95%	0.35%	0.95%				
BW, kg								
d 0	8.63	8.63	6.75	6.75	0.01	0.713	<.0001	0.981
d 14	11.35	11.19	9.53	8.88	0.16	0.028	<.0001	0.158
d 35	21.37	21.61	19.02	17.6	0.52	0.276	<.0001	0.133
ADG, g/d								
d 0-14	194.90	183.23	198.83	152.27	11.54	0.027	0.264	0.156
d 14-35	476.79	496.02	451.85	415.11	19.58	0.663	0.019	0.179
d 0-35	364.04	370.90	350.65	309.97	14.74	0.274	0.027	0.133
ADFI, g/d								
d 0-14	269.45	278.95	252.96	275.27	19.773	0.437	0.620	0.752
d 14-35	695.37	615.45	560.23	627.80	54.800	0.912	0.285	0.203
d 0-35	525.00	480.85	437.32	486.79	38.184	0.946	0.306	0.244
FCR								
d 0-14	1.383	1.525	1.275	1.883	0.181	0.061	0.503	0.224
d 14-35	1.455	1.248	1.238	1.523	0.110	0.739	0.804	0.051
d 0-35	1.440	1.300	1.245	1.587	0.114	0.390	0.691	0.055

5.4.2. Gene Expression

The hierarchical clustering of pigs for all the transcribed Affymetrix identities are represented in Figure 5.1. The transcriptomic profile clearly discriminates between the two dietary levels of Ca (HCa-green; LCa-orange), only one HCa subject, clustered with LCa pigs.

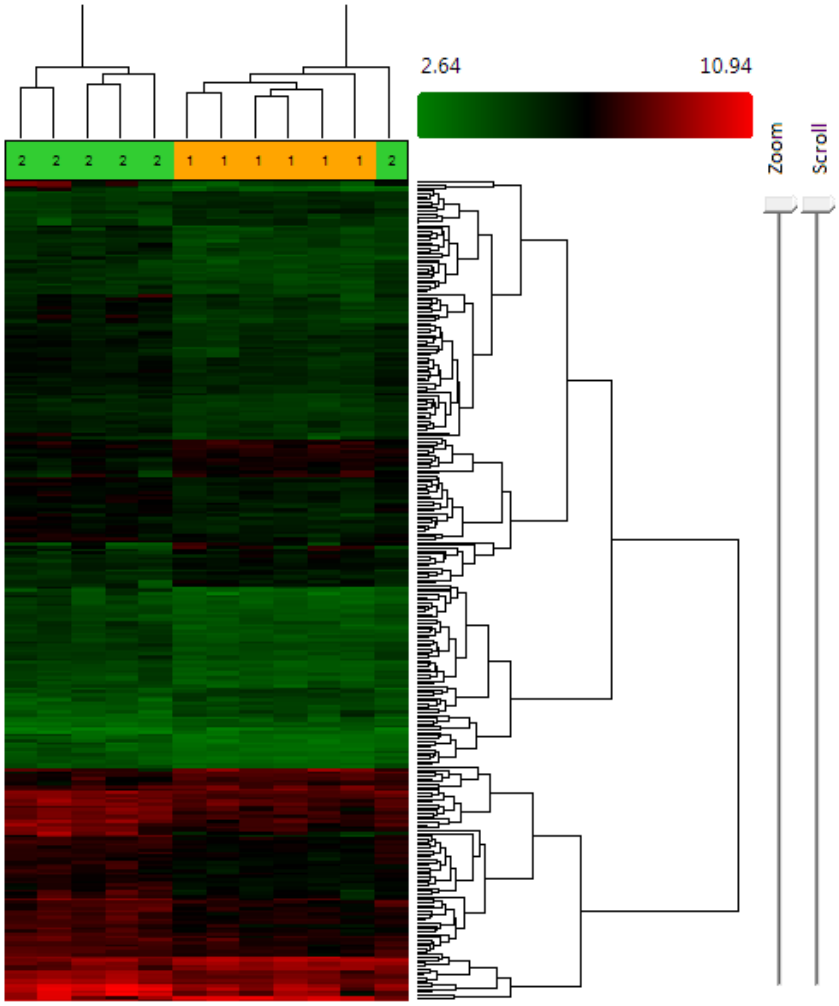

Figure 5.1. *Hierarchical clustering for all the transcribed Affymetrix identities in jejunum mucosa of weaned pigs fed low Ca diet (LCa; 0.45% of Ca; orange) or high Ca diet (HCa; 0.95% of Ca; green).*

To discriminate specific effects of the dietary treatments, a first exploratory run for the enrichment of large gene sets (Hallmark) has been done. In this procedure the comparative enrichment of significant gene is tested in 50 very large and classic sets of genes. Information of each set can be obtained at http://software.broadinsitute.org/gsea/msigdb/genesets.jsp. The comparison between HCa vs. LCa showed that 19 among 50 gene sets were significantly enriched for HCa (Table 5.6). In HCa group, gene sets related to IFNα and IFNγ ranked the top. Moreover, several gene sets grouped to be involved in cell cycle regulation, DNA and RNA transcription (E2F_TARGETS, MYC_TARGETS_V1 and V2), and DNA replication (G2M_CHECKPOINT) ranked the top. Other gene sets related to response

to luminal signals via T cells and macrophages (IL6; JAK; STAT3; TNFα; NF-*k*B; complement) were also enriched.

Table 5.6. *Gene sets significantly enriched in jejunum mucosa of weaned pigs fed high Ca diet (HCa; 0.95% of Ca) vs. low Ca diet (LCa; 0.45%) among those tested in hallmark database.*

Gene sets enriched HCa vs. LCa	Size	NES	FDR q-val
INTERFERON_ALPHA_RESPONSE	79	2.82	0
E2F_TARGETS	152	2.76	0
INTERFERON_GAMMA_RESPONSE	156	2.71	0
G2M_CHECKPOINT	151	2.58	0
MYC_TARGETS_V1	154	2.49	0
UNFOLDED_PROTEIN_RESPONSE	95	2.18	0
MYC_TARGETS_V2	51	2.1	0
IL6_JAK_STAT3_SIGNALING	74	1.95	0
MTORC1_SIGNALING	167	1.88	0
INFLAMMATORY_RESPONSE	166	1.82	0.001
MITOTIC_SPINDLE	159	1.61	0.006
IL2_STAT5_SIGNALING	172	1.6	0.006
TNFA_SIGNALING_VIA_NFKB	168	1.56	0.009
COMPLEMENT	160	1.52	0.014
KRAS_SIGNALING_UP	164	1.47	0.023
PROTEIN_SECRETION	72	1.46	0.022
APOPTOSIS	144	1.43	0.03
PI3K_AKT_MTOR_SIGNALING	88	1.36	0.052
DNA_REPAIR	99	1.36	0.053

The comparison between LCa vs. HCa showed that 9 among 50 gene sets were significantly enriched for LCa (Table 5.7). In the LCa group the statistical significance, as well as the number of sets was clearly less intense than observed for HCa. Gene sets related to Myogenesis and Xenobiotic metabolism ranked the top.

Table 5.7. *Gene sets significantly enriched in jejunum mucosa of weaned pigs low Ca diet (LCa; 0.45%) vs. high Ca diet (HCa; 0.95% of Ca) among those tested in hallmark database.*

Gene sets enriched LCa vs. HCa	SIZE	NES	FDR q-val
MYOGENESIS	160	2.1	0.002
XENOBIOTIC_METABOLISM	160	1.78	0.009
ESTROGEN_RESPONSE_EARLY	172	1.46	0.122
HYPOXIA	168	1.37	0.188
OXIDATIVE_PHOSPHORYLATION	162	1.35	0.165
P53_PATHWAY	164	1.32	0.173
GLYCOLYSIS	166	1.26	0.232
WNT_BETA_CATENIN_SIGNALING	33	1.25	0.225
REACTIVE_OXIGEN_SPECIES_PATHWAY	41	1.25	0.209

In order to deeply study the difference between the two diets, a deeper run for gene enrichment was tested, by a more detailed gene sets (C5 database). This database consists of 1,454 gene sets, but in this analysis, were excluded the gene sets of extreme size (keeping them when min = 15, max = 50 genes), as suggested by the system guidelines. This resulted in filtering out 546 gene sets.

The comparison between HCa vs. LCa showed that 274 gene sets were significantly enriched at FDR < 25% (Supplementary Table S5.1). All the gene sets ranking in the first 30 were related to CELL_CYCLE_PHASE.

The comparison between LCa vs. HCa showed that 91 gene sets significantly enriched at FDR < 25% (Supplementary Table S5.2). In general, as seen for the previous reduced database, the statistical significance was lower than for the test of HCa vs. LCa. The first gene sets ranked the top was ACTIN, moreover, several gene sets related with ion, mineral and amino acid trans membrane transport ranked the top (i.e. ION_CHANNEL_ACTIVITY, POTASSIUM_ION_TRANSPORT, AMINO_ACID_TRANSPORT, AMINO_ACID_TRANSMEMBRANE_TRANSPORTER_ACTIVITY, TRANSMEMBRANE_TRANSPORTER_ACTIVITY).

5.4.3. Microbiota Profile in Colon Content

A total of 849 OTUs were found among all samples. The rarefaction curves reported a uniform distribution of the abundance of OTUs among the different samples (log_{10}[reads] between 4.5 – 4.8) and reached the plateau phase meaning a saturated richness (data not shown).

The alpha diversity based on Simpson (0.96 ± 0.32 and 0.97 ± 0.32 for LCa and HCa, respectively) and Simpson inverse (29.5 ± 9.82 and 37.9 ± 12.6 for LCa and HCa, respectively) did not show significant differences between groups except a trend in Shannon index (4.34 ± 1.45 and 4.56 ± 1.52 for LCa and HCa, respectively; $P = 0.07$) with higher values for HCa group.

The beta diversity based on Bray-curtis dissimilarity distance (Figure 5.2) unveiled two potential clusters differentiated by the two dietary treatments. This difference was further supported with ANOSIM ($P = 0.01$) and PERMANOVA ($P = 0.02$) tests where HCa group presented a higher heterogeneous community compared to a more homogeneous community in LCa group.

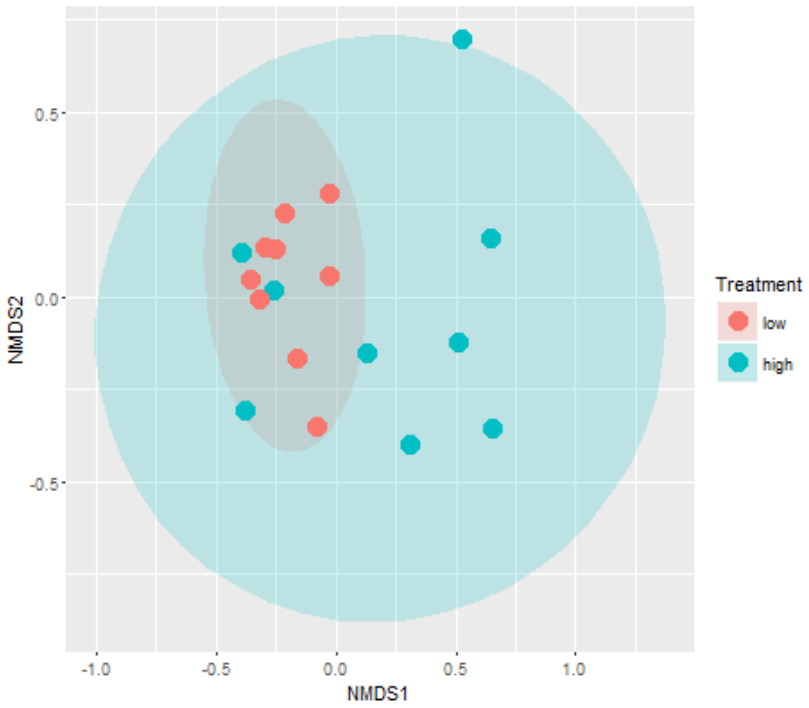

Figure 5.2. *Non-metric multidimensional scaling of OTU relative abundances. Distance are calculated based on Bray-curtis distance.*

Regarding the taxonomic composition, 15 different phyla were detected (Table 5.8) which were by decreasing abundance as follows: Firmicutes, Bacteroidetes, Spirochaetes, Proteobacteria, Planctomycetes, Cyanobacteria, Fibrobacteres, Chlamydiae, TM7, Tenericutes, Actinobacteria, Euryarchaeota, Verrumicrobia, WPS-2 and Synergistetes. Firmicutes and Bacteroidetes were the major phyla among all samples representing 47.9 and 46.0%, respectively. Only one phylum, Euryarchaeota, within Archaea domain separated from Bacteria, was found to be significantly higher in HCa compared to LCa group ($P < 0.05$). At a genus level, a total of 54 different genera were detected (Table 5.9). In this case, unknown genera were the major members representing 34.2% among all samples and only 16 genera, included unknown, rendered the 95%. Ten genera differed between groups ($P < 0.05$): *Bacteroides*, *Parabacteroides*, *Anaerostipes*, *Dorea*, *Methanibrevibacter*, *Anaeroplasma* and rc4-4 were enriched in HCa group; and *Helicobacter* and *Butyrivibrio* were enriched in LCa group. It is notable that these genera were within the 5% (*Bacteroides* and *Parabacteroides*) and 1% less abundant genera (*Anaerostipes*, *Dorea*, *Methanobrevibacter*, *Anaeroplasma*, rc4-4, *Helicobacter* and *Butyrivibrio*).

Table 5.8. *List of the mean percentage (standard deviation) of all phyla detected in Low Calcium and High Calcium treatments. Phyla are ordered in decreasing amounts.*

	LCa		HCa	
Firmicutes	46.4	(7.35)	49.5	(4.05)
Bacteroidetes	48.7	(7.33)	43.2	(5.71)
Spirochaetes	1.67	(1.136)	2.89	(2.749)
Proteobacteria	1.81	(0.671)	2.51	(2.654)
Planctomycetes	0.196	(0.258)	0.604	(0.859)
Cyanobacteria	0.464	(0.475)	0.204	(0.251)
Fibrobacteres	0.308	(0.305)	0.252	(0.207)
Chlamydiae	0.098	(0.133)	0.277	(0.329)
TM7	0.126	(0.119)	0.148	(0.237)
Tenericutes	0.111	(0.101)	0.150	(0.148)
Actinobacteria	0.073	(0.037)	0.068	(0.031)
Euryarchaeota	0.011	(0.009)	0.080	(0.091)
Verrucomicrobia	0.003	(0.009)	0.035	(0.083)
WPS-2	0.026	(0.065)	0.018	(0.040)
Synergistetes	0.008	(0.005)	0.006	(0.006)

Table 5.9. *List of the mean percentage (standard deviation) of all genera detected in LCa and HCa treatments. Genera are ordered in decreasing amounts.*

	LCa		HCa	
Unknown	28.2	(6.56)	40.2	(10.70)
Prevotella	37.1	(8.79)	25.5	(7.39)
Phascolarctobacterium	5.58	(2.030)	5.59	(2.189)
Roseburia	5.46	(2.444)	3.12	(3.976)
[*Prevotella*]	3.25	(1.828)	3.42	(1.842)
Oscillospira	2.58	(0.838)	2.56	(0.693)
Treponema	1.58	(1.100)	2.61	(2.761)
Anaerovibrio	2.10	(1.191)	1.49	(0.954)
CF231	1.67	(0.535)	1.64	(1.114)
Streptococcus	1.94	(1.675)	1.79	(1.037)
Megasphaera	1.01	(1.225)	1.66	(2.433)
Lachnospira	1.01	(0.763)	0.982	(0.844)
Faecalibacterium	1.27	(1.415)	0.674	(0.304)
Ruminococcus	0.797	(0.680)	0.940	(0.401)
Clostridium	1.000	(0.634)	0.836	(0.385)
p-75-a5	0.782	(0.461)	0.819	(0.478)
Lactobacillus	0.733	(1.152)	0.767	(0.791)
Coprococcus	1.006	(1.376)	0.635	(0.521)
Campylobacter	0.174	(0.201)	0.890	(2.596)
Psychrobacter	0.422	(0.239)	0.391	(0.185)
Fibrobacter	0.310	(0.306)	0.253	(0.207)
Selenomonas	0.159	(0.474)	0.310	(0.464)
Desulfovibrio	0.197	(0.121)	0.273	(0.091)
YRC22	0.131	(0.143)	0.246	(0.583)
Chlamydia	0.099	(0.134)	0.277	(0.329)
*Bacteroides**	0.020	(0.026)	0.315	(0.409)
Sphaerochaeta	0.099	(0.055)	0.284	(0.244)
Bulleidia	0.231	(0.210)	0.100	(0.077)
*Parabacteroides**	0.070	(0.069)	0.223	(0.165)
[*Eubacterium*]	0.132	(0.070)	0.058	(0.049)
*Anaerostipes**	0.004	(0.004)	0.163	(0.235)
Moraxella	0.092	(0.062)	0.070	(0.045)
Lactococcus	0.085	(0.057)	0.072	(0.042)
Blautia	0.078	(0.043)	0.068	(0.029)
Enhydrobacter	0.081	(0.044)	0.067	(0.034)
*Dorea**	0.025	(0.019)	0.102	(0.089)
Succinivibrio	0.089	(0.139)	0.041	(0.066)
Mitsuokella	0.016	(0.033)	0.066	(0.155)
*Methanobrevibacter**	0.011	(0.009)	0.080	(0.091)

Turicibacter	0.023	(0.029)	0.062	(0.062)
Sutterella	0.044	(0.032)	0.049	(0.046)
[*Ruminococcus*]	0.065	(0.072)	0.022	(0.021)
*Anaeroplasma**	0.002	(0.007)	0.071	(0.103)
Anaerovorax	0.024	(0.051)	0.043	(0.048)
Actinobacillus	0.037	(0.033)	0.035	(0.023)
L7A_E11	0.026	(0.030)	0.032	(0.042)
RFN20	0.037	(0.047)	0.009	(0.008)
rc4-4*	0.004	(0.004)	0.027	(0.023)
*Helicobacter**	0.038	(0.056)	0.003	(0.004)
Flexispira	0.023	(0.028)	0.004	(0.005)
*Butyrivibrio**	0.021	(0.041)	0.000	(0.000)
Lachnobacterium	0.009	(0.025)	0.007	(0.019)
Peptococcus	0.006	(0.007)	0.010	(0.012)
Anaerobiospirillum	0.012	(0.029)	0.002	(0.004)
Pyramidobacter	0.008	(0.005)	0.006	(0.006)

*: Significant differential abundances of the genus between groups.

5.4.4. Jejunum Mucosa Morphology

There were no differences between treatments in villous height, crypt depth, ratio VH:CD, the number of intraepithelial lymphocytes and mitosis activity ($P > 0.1$; data not show).

5.5. DISCUSSION

In the present study, we investigated whether different levels of dietary Ca in combination with therapeutics levels of Zn modifies the productive performance, gastrointestinal microbiota and gene expression in the gastrointestinal tract of weaned pigs.

Productive performance

Calcium and P are considered macrominerals because they are required at levels greater than 100 mg/kg in the diets (Ewing and Charlton, 2007) to satisfy whole body accretion. Calcium requirements for piglets are proposed to be 0.85 - 0.80% for 5 to 11 kg BW piglets (NRC, 2012). In addition, adequate dietary Ca level and Ca:P ratio are essential for proper bone accretion in young pigs but excess or deficiency in one of the minerals causes impaired utilization of the other (González-Vega and Stein, 2014).

Increasing concentration of Ca in diets reduced BW, ADG and increased FCR in weaned pigs (Trial 1) which is in agreement with results observed by Lei et al. (1994), although they found differences in feed intake and we only detected them when ZnO was not supplemented (Trial 2). Also a decrease in ADG and G:F ratio as the concentration of Ca increased were observed in 11 to 25 kg pigs (González-Vega et al., 2016), and in 30 kg pigs (Larsen and Sandström, 1993).

In studies using constant concentration of dietary Ca and increasing concentrations of STTD P pig growth performance is clearly increased (Ekpe et al., 2002; Zhai and Adeola, 2013). Gutzwiller et al. (2014) observed that wide Ca:P ratio reduced feed intake, growth rate and impaired feed conversion ratio but increased the mineral concentration of tibia. The Ca:P ratio is strongly important because and excess or deficiency of one of both minerals may seriously affect the utilization of the other (Crenshaw, 2001). In our trials, the Ca:total P ratio (Ca:**tP**) analyzed vary from 1.1 to 2.1:1 in Trial 1, 0.9 to 1.9:1 in Trial 2, 0.8 to 2.0:1 in Trial 3, and 1.1 to 2.2:1 in Trial 4. Therefore, high Ca:tP ratios may reduce growth performance. It has been observed that reducing Ca:tP from 1.5 to 1.0:1 in low P diets supplemented with microbial phytase in growing pigs resulted in higher ADG, G:F, BW at slaughter (Liu et al., 1998), and increased apparent absorption of P in the small intestine (Liu et al., 2000). However, the negative effects of the wide dietary Ca:P ratio on growth performance could not be verified in studies of Lantzsch et al. (1995) and of Létourneau-Montminy et al. (2010) because feed intake of the pigs was restricted. González-Vega et al. (2016) stated that to maximize growth performance in pigs, the ratio of STTD Ca to STTD P likely is more important than the absolute concentration of both minerals, and the quantity of Ca in diet to maximize retention of Ca and maximize bone ash is different from the quantity needed to maximize growth performance.

The negative effect of Ca in growth performance could be explained by different mechanisms; pigs fed with high dietary Ca level (limestone) may exert a buffering effect, increasing the pH in the gastrointestinal tract and therefore promoting the formation of insoluble Ca-Phytate complexes (Wise, 1983; Sandberg et al., 1993; Selle et al., 2009), decrease of phytate solubility (Sandberg et al., 1993; Selle and Ravindran, 2008) and microbial phytase activity (Sandberg et al., 1993; Lei et al., 1994; Lantzsch et al., 1995; Selle et al., 2009). A higher Ca concentration may also increase the formation of Ca- mineral P complexes in the gastrointestinal tract, which reduces digestibility of P

(Lei et al., 1994; Lantzsch et al., 1995; Stein et al., 2011; González-Vega and Stein, 2014).

It is noteworthy that the effects observed with Ca on the performance of piglets were significant with diets containing a therapeutical level of ZnO (Trial 1 and 3), but a tendency was observed when Zn was incorporated at a nutritional level (Trial 2). Zinc oxide is administered in early weaning diets at therapeutic doses (>2.500 mg/kg) to prevent post-weaning diarrhea (Poulsen, 1995) and to promote growth performance. However, our results may indicate an interaction between high levels of Ca and high levels of Zn. It has been described that Ca-Zn-phytate complexes may precipitate likely affecting the growth performance by reducing P absorption. When two cations are presented simultaneously, as Ca^{+2} and Zn^{+2}, they act together to increase the quantity of phytate precipitation (Simpson and Wise, 1990). Also, multiple mineral complexes, such as Ca-Zn-phytate, are more stable than single mineral complexes, such as Ca-phytate or Zn-phytate (Maenz et al., 1999). However, we hypothesized that other changes on the physiology of the gastrointestinal tract could be related with differences on performance. These changes were explored in Trial 4.

Gene expression

To investigate the mechanism underlying the effects of high dietary Ca we evaluated the transcriptome of the pig jejunum by RNA microarray analysis. The present results demonstrated that single changes on dietary Ca concentration modified the jejunal expression of genes related to inflammatory process in weaned pigs. Compared with the LCa group, pigs fed high level of dietary Ca (1.4% of Ca; 1.55% of limestone) showed greater expression of genes related to the cell cycle regulation, DNA and RNA transcription and inflammatory response in the jejunum. The results are in contrast with Metzler-Zebeli et al. (2012) who observed that high Ca (1.2%) and P (0.8%) levels supplemented by limestone and dicalcium phosphate downregulate the expression of pro-inflammatory cytokines in duodenum and may increase digestive and absorptive functions; and also stated that those effects were specific to intestinal segment. In rats, it has also been observed that high calcium phosphate levels downregulate pro-inflammatory cytokine expression in colon (Schepens et al., 2009). Recently, it has been observed that high Ca and P levels (1.4 and 1.2%, respectively) downregulate the expression of genes that encode for tight junction proteins (occluding

(***OCLN)*** and zonula occludens 1 (***ZO1***) and increase the expression of TRL2 gene in the jejunum of weaned pigs (Metzler-Zebeli et al., 2015). The stability of intestinal barrier is part ensured by the thigh junctions (Ukena et al., 2007), and a proper intestinal barrier function is of great importance for uptake of nutrients and to prevent pathogens and toxins entering the organism. In addition, Ca absorption occurs by two mechanisms, nonsaturable paracellular absorption and saturable transcellular absorption (Bronner, 1987). At high dietary Ca levels the non-saturable mechanism is more active (Bronner, 2003). Using the passive transport pathway, Ca is passively moved from the lumen of the small intestine through the tight junctions between the enterocytes (Gropper et al., 2009). Therefore high dietary Ca may influence the expression of genes involved in nutrient absorption such as *OCLN* and *ZO1*.

Weaning is the most critical period in swine industry, because pigs have to face serial challenges, such as new diet and environment, when their immune and digestive system is still immature. The early weaning provokes alteration of gut integrity and appears to be one of the major aetiologic factors in gut-associated disorders (Hampson, 1994). It is characterised by shortened villous length (Pluske et al., 1997a), disturbed absorptive-secretory electrolyte and fluid balances, increased mucosal permeability (Boudry et al., 2004), decreased enzymatic activities (Pluske et al., 1997a), stimulation of pro-inflammatory cytokine gene expression (McCracken et al., 1999; Pié et al., 2004), activation of heat shock proteins in the mucosa (David et al., 2002), lowered levels of mucins (Lopez-Pedrosa et al., 1998) and decreased globet cell density (Brown et al., 1988; Nuñez et al., 1996). Therefore, in the present study it has been observed that high levels of Ca enriched several gene sets related with inflammatory process (e.g. IFNα, and INFγ) and this suggest that high Ca levels at weaning may aggravate intestinal integrity loss with consequences on the growth performance. In support to these assumption, the obtained results confirm the key role of Ca in the regulation of the mucosa homeostasis, by affecting apoptosis and mitotic function as reported by (Pinton et al., 2008). Moreover, the enrichment of the E2F_TARGETS pathway in the HCa group, that include genes involved in DNA replication, cell proliferation and apoptosis confirms the mucosal integrity impairment in according with (Pediconi et al., 2003). On the other hand, the transcriptomic profile in the LCa group reflects a positive pressure on the development of the jejunum, as signal of recovering from the post-weaning

impairment, firstly enriching the myogenic potential of the intestine, which is positively related with the epithelial differentiation (Barbieri and Sestili, 2012).

Microbiology profile in colon content

Different studies have demonstrated changes in intestinal microbial communities related to Ca supplementation (Trautvetter et al., 2012; Metzler-Zebeli et al., 2013; Chaplin et al., 2016) although very few has been performed with pigs. Mann et al. (2014) by pyrosequencing of 16S rRNA showed structural changes in the mucosa-associated microbiota of weaned piglets related to the dietary supplementation of Ca and P, especially at stomach. However, they did not study luminal populations. In our study, we showed a clear effect of Ca supplementation on the dynamics of colonic luminal microbiota with distinct beta diversities, despite alpha diversity was not modified. This suggests that the inclusion of $CaCO_3$ in the diets could promote a differential adaptation of the microbiota between animals resulting in less homogenous ecosystems.

Defining what kind of changes promotes Ca in the intestinal microbiota is not an easy task as probably it depends on the interaction with other different variables. The effect of dietary Ca on gastrointestinal microbial communities has been demonstrated to differ depending the gastrointestinal site (Mann et al., 2014) and the type of diet the animals is fed (Metzler-Zebeli et al., 2010). One of the most consistently reported effects of Ca supplementation has been some kind of prebiotic effect on lactic acid bacteria inhabiting the hindgut. Supplementary Ca phosphate in diets increased resistance to *Salmonella* by strengthening the endogenous ileal lactobacilli (Bovee-Oudenhoven et al., 1997) and decreasing fecal enterobacteria in mice (Ten Bruggencate et al., 2004). Mann et al. (2014) also described a significantly increase in *Lactobacillus* at the gastric *Pars* non-glandularis in weaned piglets. However, in our study we were not able to identify changes in these bacteria in the colon. It is possible those effects are restricted to foregut or that they depend on the basal diet and the source of Ca the animal receive. In this regard other authors describe how high Ca levels reduce the proliferation of ileal *Enterococcus* spp., *Enterococcus faecicum*, and the *Clostridium leptum* cluster and also tended to decrease *Lactobacillus reuteri* and *Lactobacillus mucosae* at distal ileum in growing pigs (Metzler-Zebeli et al., 2010).

In our study high levels of Ca increased the abundance of Euryarchaeota phylum, within Archaea domain, increasing *Methanobrevibacter* which is the predominant genus among methanogens found in pig feces (Mao et al., 2011). The other 14 phylum detected were not significantly affected by the diet although it was possible to detect changes in particular microbial genera. The scarce effects of Ca supplementation in main phyla has been also described by other authors suggesting the robustness of the colonic microbial ecosystem in their main structure that is not easily modified by the diet (Metzler-Zebeli et al., 2013). Nonetheless, it is notable the significantly enrichment found in some particular microbial genera, and particularly in one of the most relevant in pig colon as is *Bacteroides*, that was significantly increased by the HCa diet (log2-fold change higher than 4). This is in agreement with Metzler-Zebeli et al. (2013) who also observed an increase in the *Bacteroides-Prevotella-Porphyromonas* group in ileal digesta of weaned pigs although they barely detected differences in the lower gut. Another study in mice (Chaplin et al. 2016) also found that the administration of high levels of Ca in high-fat diets increased *Bacteroides*/*Prevotella* in cecum and also *Bifidobacterium* and *Lactobacillus* spp.

Different mechanisms by which dietary Calcium may exert its effects in the animal and its microbiota has been proposed in the literature. Ca would induce beneficial effects on the host through a protective cytotoxic effect (Ten Bruggencate et al., 2004; Schepens et al, 2009; Gomes et al., 2015) via formation of an amorphous calcium-phosphate complex in non-acidic pH values, i.e. ileal and colonic pH, which would precipitate bile acids and fatty acids (Trautvetter et al., 2012) protecting the intestinal permeability (Schepens et al., 2009; Ten Bruggencate et al., 2011) and thus reducing inflammation (Schepens et al., 2009) or bind to pathogens such *Salmonella* (Ten Bruggencate et al., 2011). However, in our study the performance of the animals impaired by Ca supplementation, and also the gene expression analysis did not support this hypothesis. As discussed in the next section, our results showed a promotion of intestinal inflammation such increasing expression of cytokines and complement system. Partly in accordance, the study of Metzler-Zebeli et al. (2015) observed how high dietary Ca down-regulated *OCLN* and *ZO1* (thigh junction proteins) expression genes in jejunum related to mucosal barrier function, as compared to adequate Ca diets. It could be hypothesized that high Ca diets may reduce the mucosal barrier integrity against possible pathogens and toxins entering the organisms.

Other mechanism proposed for Ca has been its buffering potential when supplemented as Ca phosphate or Ca carbonate. This buffering capacity might protect acid-sensitive bacteria (Metzler-Zebeli et al., 2013; Mann et al., 2014) such as *Bacteroides* which is suppressed at lower pH than 5.5 (Chung et al., 2016) and showed to be increased in our study. Here it is also interesting to remark that *Bacteroides,* which co-occurs with *Parabacteroides,* has been precisely assigned to human enterotype 1 (Arumugam et al., 2011 Wu et al., 2011) associated with Western diets, high in animal protein and saturated fats, and related to a higher incidence of diseases (Wu et al., 2011). In this study we could hypothesize that a higher concentration of Ca could had increase the amount of undigested fat arriving to the hindgut through the formation of Ca-soaps (Govers et al., 1993; Ten Bruggencate et al., 2011; Trautvetter et al., 2012) and modifying microbiota. In pigs this genus is naturally present in higher amounts during suckling period (Mach et al., 2015; Bian et al., 2016) and has been shown to correlate negatively with body weight (Mach et al., 2015) and protein concentration but positively with lactose (Bian et al. 2016).

In conclusion, the present results demonstrated that high dietary Ca level (0.95% of Ca; 1.55% of limestone) decreases growth performance (reduces BW and ADG, and increases FCR) but no feed intake in weaned pigs. Also, pigs fed high Ca levels during 14 d after weaning express genes related to the inflammatory response, and a higher heterogeneous microbial community with increased of the Bacterioides genera in colon, which may be related with the detrimental growth performance. However, the present results did not find differences in the jejunum morphology between Ca levels. These results, suggest that is better no include limestone in diets during the first two weeks post-weaning.

Supplementary Table S5.1. *Detailed gene sets significantly enriched among those tested in hallmark database in jejunum mucosa of weaned pigs fed high dietary level of Calcium - 0.95% (HCa) vs. low dietary level of Calcium - 0.45% (LCa).*

Gene sets enriched HCa vs. LCa	Nominal *P*-val	FDR *q*-val
CELL_CYCLE_PHASE	0.000	0.000
CELL_CYCLE_PROCESS	0.000	0.000
M_PHASE	0.000	0.000
MITOTIC_CELL_CYCLE	0.000	0.000
RNA_PROCESSING	0.000	0.000
M_PHASE_OF_MITOTIC_CELL_CYCLE	0.000	0.000
MITOSIS	0.000	0.000
CHROMOSOME	0.000	0.000
NUCLEAR_PORE	0.000	0.000
NUCLEAR_MEMBRANE_PART	0.000	0.000
NUCLEAR_MEMBRANE	0.000	0.000
CENTROSOME	0.000	6.89E-05
NUCLEAR_PART	0.000	7.47E-05
CHROMOSOMAL_PART	0.000	1.26E-04
NUCLEAR_ENVELOPE	0.000	2.62E-04
DNA_RECOMBINATION	0.000	2.78E-04
PORE_COMPLEX	0.000	2.97E-04
RIBONUCLEOPROTEIN_COMPLEX_BIOGENESIS_AND_ASSEMBLY	0.000	3.11E-04
NUCLEOLUS	0.000	3.27E-04
DNA_REPLICATION	0.000	3.45E-04
MICROTUBULE_ORGANIZING_CENTER	0.000	3.81E-04
NUCLEOCYTOPLASMIC_TRANSPORT	0.000	4.84E-04
RNA_SPLICING	0.000	5.42E-04
TRNA_METABOLIC_PROCESS	0.000	7.80E-04
NUCLEOPLASM	0.000	9.27E-04
NUCLEAR_LUMEN	0.000	9.28E-04
NUCLEAR_TRANSPORT	0.000	0.001
CELL_CYCLE_GO_0007049	0.000	0.001
DNA_DEPENDENT_DNA_REPLICATION	0.000	0.002
REGULATION_OF_MITOSIS	0.000	0.002
MRNA_METABOLIC_PROCESS	0.000	0.002
ORGANELLE_LUMEN	0.000	0.002
SPINDLE	0.000	0.002
MEMBRANE_ENCLOSED_LUMEN	0.000	0.002
RIBOSOME_BIOGENESIS_AND_ASSEMBLY	0.002	0.003
NUCLEAR_EXPORT	0.003	0.003
MRNA_PROCESSING_GO_0006397	0.000	0.003
LIGASE_ACTIVITY	0.000	0.003
MICROTUBULE_CYTOSKELETON	0.000	0.004

INTERPHASE	0.000	0.004
DNA_DIRECTED_RNA_POLYMERASEII_HOLOENZYME	0.000	0.004
DNA_DAMAGE_RESPONSESIGNAL_TRANSDUCTION	0.000	0.004
RNA_BINDING	0.000	0.004
PROTEASOME_COMPLEX	0.002	0.005
CHROMATIN	0.003	0.005
RNA_HELICASE_ACTIVITY	0.002	0.006
MACROMOLECULE_LOCALIZATION	0.000	0.006
HELICASE_ACTIVITY	0.000	0.008
DNA_INTEGRITY_CHECKPOINT	0.000	0.008
CHROMATIN_BINDING	0.000	0.008
DNA_METABOLIC_PROCESS	0.000	0.008
RIBONUCLEASE_ACTIVITY	0.000	0.008
PROTEIN_TRANSPORT	0.005	0.008
CHROMOSOME_ORGANIZATION_AND_BIOGENESIS	0.000	0.008
CHROMOSOME_SEGREGATION	0.003	0.009
CELL_CYCLE_CHECKPOINT_GO_0000075	0.000	0.009
NUCLEOPLASM_PART	0.000	0.009
ESTABLISHMENT_OF_PROTEIN_LOCALIZATION	0.000	0.010
DNA_DAMAGE_CHECKPOINT	0.003	0.010
MITOTIC_CELL_CYCLE_CHECKPOINT	0.003	0.011
CHROMOSOMEPERICENTRIC_REGION	0.001	0.011
RESPONSE_TO_VIRUS	0.001	0.011
PROTEIN_TARGETING	0.000	0.013
INTRACELLULAR_NON_MEMBRANE_ BOUND_ORGANELLE	0.000	0.014
CHEMOKINE_ACTIVITY	0.000	0.014
RESPONSE_TO_DNA_DAMAGE_STIMULUS	0.000	0.015
ENDONUCLEASE_ACTIVITY	0.003	0.015
INTRACELLULAR_TRANSPORT	0.000	0.015
NON_MEMBRANE_BOUND_ORGANELLE	0.000	0.016
PROTEIN_RNA_COMPLEX_ASSEMBLY	0.001	0.016
PROTEIN_LOCALIZATION	0.002	0.017
ADAPTIVE_IMMUNE_RESPONSE	0.006	0.017
CHEMOKINE_RECEPTOR_BINDING	0.000	0.017
CYTOKINE_ACTIVITY	0.001	0.018
ENDOMEMBRANE_SYSTEM	0.000	0.019
INTRACELLULAR_PROTEIN_TRANSPORT	0.000	0.019
KINETOCHORE	0.007	0.020
ADAPTIVE_IMMUNE_RESPONSE_GO_0002460	0.007	0.021
INTERPHASE_OF_MITOTIC_CELL_CYCLE	0.004	0.024
RIBONUCLEOPROTEIN_COMPLEX	0.001	0.024
NUCLEAR_CHROMOSOME	0.004	0.025
REGULATION_OF_DNA_METABOLIC_PROCESS	0.001	0.025
REGULATION_OF_CELL_CYCLE	0.004	0.026
PROTEIN_IMPORT_INTO_NUCLEUS	0.000	0.026

POSITIVE_REGULATION_OF_I_KAPPAB_KINASE_ NF_KAPPAB_CASCADE	0.000	0.027
CELLULAR_LOCALIZATION	0.003	0.027
ESTABLISHMENT_OF_CELLULAR_LOCALIZATION	0.000	0.027
CONDENSED_CHROMOSOME	0.000	0.027
IMMUNE_RESPONSE	0.000	0.027
LOCOMOTORY_BEHAVIOR	0.001	0.028
TRANSCRIPTION_INITIATION_FROM_RNA_ POLYMERASE_II_PROMOTER	0.003	0.028
ACETYLTRANSFERASE_ACTIVITY	0.008	0.029
DEOXYRIBONUCLEASE_ACTIVITY	0.006	0.029
LIGASE_ACTIVITY_FORMING_CARBON_ NITROGEN_BONDS	0.004	0.029
RESPONSE_TO_BIOTIC_STIMULUS	0.004	0.029
PROTEIN_N_TERMINUS_BINDING	0.001	0.029
NUCLEOBASENUCLEOSIDENUCLEOTIDE_AND_ NUCLEIC_ACID_TRANSPORT	0.006	0.029
POSITIVE_REGULATION_OF_MULTICELLULAR_ ORGANISMAL_PROCESS	0.001	0.029
NUCLEASE_ACTIVITY	0.012	0.030
REGULATION_OF_I_KAPPAB_KINASE_ NF_KAPPAB_CASCADE	0.003	0.030
TRANSCRIPTION_INITIATION	0.007	0.031
PROTEIN_FOLDING	0.008	0.031
TRANSLATION_INITIATION_FACTOR_ACTIVITY	0.000	0.034
NUCLEAR_IMPORT	0.003	0.034
DNA_REPAIR	0.019	0.034
DOUBLE_STRAND_BREAK_REPAIR	0.013	0.037
I_KAPPAB_KINASE_NF_KAPPAB_CASCADE	0.003	0.037
NUCLEAR_BODY	0.014	0.037
MEIOTIC_RECOMBINATION	0.016	0.038
PEPTIDYL_AMINO_ACID_MODIFICATION	0.004	0.040
PHOSPHOINOSITIDE_METABOLIC_PROCESS	0.016	0.043
TRANSLATIONAL_INITIATION	0.015	0.045
PROTEIN_DNA_COMPLEX_ASSEMBLY	0.022	0.046
PROTEIN_IMPORT	0.009	0.047
IMMUNE_SYSTEM_PROCESS	0.000	0.048
RESPONSE_TO_ENDOGENOUS_STIMULUS	0.000	0.048
GOLGI_VESICLE_TRANSPORT	0.014	0.050
SINGLE_STRANDED_DNA_BINDING	0.021	0.050
POSITIVE_REGULATION_OF_IMMUNE_RESPONSE	0.012	0.050
SMALL_CONJUGATING_PROTEIN_LIGASE_ACTIVITY	0.007	0.050
RNA_SPLICINGVIA_TRANSESTERIFICATION_REACTIO NS	0.021	0.054
PROTEIN_KINASE_CASCADE	0.001	0.055
SPLICEOSOME	0.014	0.056
DRUG_BINDING	0.018	0.056

ESTABLISHMENT_AND_OR_MAINTENANCE_ OF_CHROMATIN_ARCHITECTURE	0.011	0.056
TRANSLATION_FACTOR_ACTIVITY_ NUCLEIC_ACID_BINDING	0.026	0.058
POSITIVE_REGULATION_OF_SIGNAL_ TRANSDUCTION	0.000	0.058
HYDROLASE_ACTIVITY_ACTING_ON_ ESTER_BONDS	0.020	0.059
EXONUCLEASE_ACTIVITY	0.003	0.059
TRANSFERASE_ACTIVITY_TRANSFERRING_ ALKYL_OR_ARYLOTHER_THAN_METHYLGROUPS	0.026	0.059
CHROMATIN_ASSEMBLY_OR_DISASSEMBLY	0.026	0.059
CYTOSKELETAL_PART	0.001	0.061
PROTEIN_AMINO_ACID_LIPIDATION	0.028	0.062
LIPID_RAFT	0.026	0.062
TRANSLATION_REGULATOR_ACTIVITY	0.039	0.062
INTERLEUKIN_RECEPTOR_ACTIVITY	0.027	0.062
RESPONSE_TO_OTHER_ORGANISM	0.024	0.063
G1_S_TRANSITION_OF_MITOTIC_CELL_CYCLE	0.014	0.063
UBIQUITIN_PROTEIN_LIGASE_ACTIVITY	0.014	0.063
MICROTUBULE_ORGANIZING_CENTER_PART	0.022	0.066
UBIQUITIN_LIGASE_COMPLEX	0.002	0.066
PROTEOLYSIS	0.033	0.066
GENERAL_RNA_POLYMERASE_II_ TRANSCRIPTION_FACTOR_ACTIVITY	0.016	0.068
PHOSPHOINOSITIDE_BIOSYNTHETIC_PROCESS	0.040	0.071
CHROMATIN_REMODELING	0.026	0.072
PHOSPHOPROTEIN_PHOSPHATASE_ACTIVITY	0.026	0.073
PROTEIN_HETERODIMERIZATION_ACTIVITY	0.008	0.073
IMMUNE_EFFECTOR_PROCESS	0.008	0.073
POSITIVE_REGULATION_OF_IMMUNE_ SYSTEM_PROCESS	0.011	0.074
CYSTEINE_TYPE_PEPTIDASE_ACTIVITY	0.021	0.075
SMALL_PROTEIN_CONJUGATING_ ENZYME_ACTIVITY	0.034	0.076
LIPOPROTEIN_METABOLIC_PROCESS	0.036	0.077
GTP_BINDING	0.000	0.077
CELL_PROLIFERATION_GO_0008283	0.026	0.077
LIPOPROTEIN_BIOSYNTHETIC_PROCESS	0.050	0.077
MICROTUBULE_ASSOCIATED_COMPLEX	0.000	0.079
JAK_STAT_CASCADE	0.031	0.079
REGULATION_OF_PROGRAMMED_CELL_DEATH	0.029	0.079
N_ACYLTRANSFERASE_ACTIVITY	0.033	0.082
REGULATION_OF_APOPTOSIS	0.025	0.083
ACID_AMINO_ACID_LIGASE_ACTIVITY	0.000	0.083
ATP_DEPENDENT_HELICASE_ACTIVITY	0.044	0.085
DOUBLE_STRANDED_DNA_BINDING	0.021	0.086

STRUCTURE_SPECIFIC_DNA_BINDING	0.036	0.086
REGULATION_OF_IMMUNE_SYSTEM_PROCESS	0.020	0.087
MOTOR_ACTIVITY	0.051	0.088
REGULATION_OF_IMMUNE_RESPONSE	0.037	0.091
PURINE_RIBONUCLEOTIDE_BINDING	0.050	0.093
MRNA_BINDING	0.004	0.093
GUANYL_NUCLEOTIDE_BINDING	0.033	0.095
DNA_HELICASE_ACTIVITY	0.048	0.097
PROTEIN_CATABOLIC_PROCESS	0.025	0.099
CELLULAR_DEFENSE_RESPONSE	0.041	0.099
ATP_BINDING	0.039	0.099
MEIOTIC_CELL_CYCLE	0.052	0.099
POSITIVE_REGULATION_OF_RESPONSE_TO_STIMULUS	0.002	0.099
VIRAL_INFECTIOUS_CYCLE	0.002	0.100
NUCLEOTIDYLTRANSFERASE_ACTIVITY	0.052	0.100
MEIOSIS_I	0.055	0.100
PURINE_NUCLEOTIDE_BINDING	0.037	0.100
VIRAL_REPRODUCTION	0.043	0.100
TRANSCRIPTION_FACTOR_COMPLEX	0.023	0.101
G_PROTEIN_COUPLED_RECEPTOR_BINDING	0.049	0.103
PROTEIN_AMINO_ACID_DEPHOSPHORYLATION	0.038	0.104
MICROTUBULE_CYTOSKELETON_ORGANIZATION_AND_BIOGENESIS	0.052	0.104
CELLULAR_PROTEIN_CATABOLIC_PROCESS	0.033	0.105
PROTEIN_SERINE_THREONINE_KINASE_ACTIVITY	0.040	0.110
CHROMATIN_MODIFICATION	0.009	0.110
ADENYL_RIBONUCLEOTIDE_BINDING	0.012	0.114
LEUKOCYTE_ACTIVATION	0.042	0.117
B_CELL_ACTIVATION	0.055	0.117
DEFENSE_RESPONSE	0.004	0.118
ENVELOPE	0.016	0.119
VIRAL_REPRODUCTIVE_PROCESS	0.057	0.120
LYMPHOCYTE_ACTIVATION	0.029	0.120
CYTOKINE_BINDING	0.056	0.121
REGULATION_OF_T_CELL_ACTIVATION	0.021	0.123
PHOSPHORIC_MONOESTER_HYDROLASE_ACTIVITY	0.027	0.123
MICROTUBULE_BASED_PROCESS	0.056	0.123
DNA_DEPENDENT_ATPASE_ACTIVITY	0.056	0.124
PHOSPHORIC_ESTER_HYDROLASE_ACTIVITY	0.023	0.124
DEPHOSPHORYLATION	0.044	0.124
HORMONE_RECEPTOR_BINDING	0.074	0.125
NUCLEAR_HORMONE_RECEPTOR_BINDING	0.010	0.127
PROTEIN_MODIFICATION_PROCESS	0.001	0.127
ORGANELLE_ENVELOPE	0.081	0.128
REGULATION_OF_LYMPHOCYTE_ACTIVATION	0.008	0.129
ORGANELLE_MEMBRANE	0.077	0.129

BIOPOLYMER_CATABOLIC_PROCESS	0.050	0.133
PROTEIN_TYROSINE_PHOSPHATASE_ACTIVITY	0.039	0.134
RESPONSE_TO_STRESS	0.001	0.136
ADENYL_NUCLEOTIDE_BINDING	0.018	0.141
T_CELL_ACTIVATION	0.064	0.145
TRANSFERASE_ACTIVITY_TRANSFERRING_ PHOSPHORUS_CONTAINING_GROUPS	0.002	0.147
PHOSPHORYLATION	0.009	0.147
PROTEIN_AMINO_ACID_PHOSPHORYLATION	0.093	0.147
REGULATION_OF_DNA_REPLICATION	0.010	0.148
REGULATION_OF_TRANSLATIONAL_INITIATION	0.076	0.149
ORGANELLE_ORGANIZATION_AND_BIOGENESIS	0.010	0.150
AMINO_SUGAR_METABOLIC_PROCESS	0.093	0.150
NUCLEOTIDE_BINDING	0.003	0.151
POST_TRANSLATIONAL_PROTEIN_MODIFICATION	0.005	0.157
APOPTOSIS_GO	0.002	0.158
RNA_CATABOLIC_PROCESS	0.108	0.160
REGULATION_OF_CYTOKINE_PRODUCTION	0.042	0.160
ATPASE_ACTIVITY	0.103	0.160
PROGRAMMED_CELL_DEATH	0.005	0.160
SOLUBLE_FRACTION	0.036	0.161
GOLGI_APPARATUS_PART	0.053	0.162
UNFOLDED_PROTEIN_BINDING	0.095	0.163
PROTEIN_KINASE_ACTIVITY	0.017	0.169
DNA_PACKAGING	0.098	0.170
POSITIVE_REGULATION_OF_DEVELOPMENTAL_ PROCESS	0.019	0.172
MULTI_ORGANISM_PROCESS	0.033	0.177
SECRETION_BY_CELL	0.062	0.181
REGULATION_OF_CELL_PROLIFERATION	0.014	0.193
TRANSFERASE_ACTIVITY_TRANSFERRING_ GROUPS_OTHER_THAN_AMINO_ACYL_GROUPS	0.097	0.199
NUCLEAR_ENVELOPE_ENDOPLASMIC_ RETICULUM_NETWORK	0.064	0.201
NUCLEAR_CHROMOSOME_PART	0.106	0.202
POSITIVE_REGULATION_OF_T_CELL_ACTIVATION	0.131	0.208
REGULATION_OF_CYCLIN_DEPENDENT_ PROTEIN_KINASE_ACTIVITY	0.129	0.210
NEGATIVE_REGULATION_OF_DNA_ METABOLIC_PROCESS	0.105	0.211
GOLGI_ASSOCIATED_VESICLE	0.018	0.212
KINASE_REGULATOR_ACTIVITY	0.112	0.213
MICROTUBULE_BINDING	0.110	0.213
REGULATION_OF_DEVELOPMENTAL_PROCESS	0.003	0.213
DNA_BINDING	0.117	0.214
CYTOSKELETON	0.032	0.218
PROTEIN_AMINO_ACID_N_LINKED_GLYCOSYLATION	0.068	0.219

INTEGRAL_TO_ORGANELLE_MEMBRANE	0.118	0.219
NEGATIVE_REGULATION_OF_APOPTOSIS	0.059	0.220
POSITIVE_REGULATION_OF_CELL_PROLIFERATION	0.092	0.220
NEGATIVE_REGULATION_OF_PROGRAMMED_ CELL_DEATH	0.045	0.229
NEGATIVE_REGULATION_OF_MULTICELLULAR_ ORGANISMAL_PROCESS	0.032	0.232
PHOSPHOTRANSFERASE_ACTIVITY_ALCOHOL_ GROUP_AS_ACCEPTOR	0.021	0.232
METHYLTRANSFERASE_ACTIVITY	0.105	0.232
TRANS_GOLGI_NETWORK	0.139	0.232
PROTEIN_C_TERMINUS_BINDING	0.151	0.233
POSITIVE_REGULATION_OF_CYTOKINE_ BIOSYNTHETIC_PROCESS	0.133	0.233
POSITIVE_REGULATION_OF_LYMPHOCYTE_ ACTIVATION	0.130	0.233
POSITIVE_REGULATION_OF_TRANSCRIPTION_ FROM_RNA_POLYMERASE_II_PROMOTER	0.137	0.233
REGULATION_OF_TRANSCRIPTION_FROM_RNA_ POLYMERASE_II_PROMOTER	0.095	0.233
RESPONSE_TO_UV	0.143	0.233
GLYCEROPHOSPHOLIPID_BIOSYNTHETIC_PROCESS	0.146	0.234
CELLULAR_MACROMOLECULE_CATABOLIC_PROCES S	0.116	0.234
PEPTIDASE_ACTIVITY	0.132	0.234
KINASE_ACTIVITY	0.122	0.234
INTRINSIC_TO_ORGANELLE_MEMBRANE	0.026	0.235
POSITIVE_REGULATION_OF_TRANSLATION	0.064	0.235
GOLGI_MEMBRANE	0.096	0.236
INTERLEUKIN_BINDING	0.174	0.238
CELL_ACTIVATION	0.052	0.242
MACROMOLECULAR_COMPLEX_ASSEMBLY	0.108	0.243
REGULATION_OF_KINASE_ACTIVITY	0.068	0.247

Supplementary Table S5.2. *Detailed gene sets significantly enriched among those tested in hallmark database in jejunum mucosa of weaned pigs fed low dietary level of Calcium - 0.45% (LCa) vs. high dietary level of Calcium - 0.95% (HCa).*

Gene sets enriched LCa vs. HCa	Nominal *P*-value	FDR q-val
COLLAGEN	0.000	0.125
RHO_PROTEIN_SIGNAL_TRANSDUCTION	0.012	0.146
ION_CHANNEL_ACTIVITY	0.000	0.150
POTASSIUM_ION_TRANSPORT	0.003	0.151
AMINO_ACID_TRANSPORT	0.025	0.152
AMINO_ACID_TRANSMEMBRANE_TRANSPORTER_ ACTIVITY	0.008	0.154
REGULATION_OF_SMALL_GTPASE_MEDIATED_ SIGNAL_TRANSDUCTION	0.030	0.155
ORGANIC_ACID_TRANSMEMBRANE_TRANSPORTER_ ACTIVITY	0.023	0.155
NEURITE_DEVELOPMENT	0.019	0.155
TRANSMEMBRANE_TRANSPORTER_ACTIVITY	0.000	0.155
AMINE_TRANSMEMBRANE_TRANSPORTER_ACTIVITY	0.000	0.155
HORMONE_ACTIVITY	0.035	0.156
HEART_DEVELOPMENT	0.018	0.157
ANATOMICAL_STRUCTURE_MORPHOGENESIS	0.000	0.158
CATION_TRANSMEMBRANE_TRANSPORTER_ACTIVITY	0.000	0.158
METAL_ION_TRANSPORT	0.000	0.159
INTERCELLULAR_JUNCTION	0.007	0.159
L_AMINO_ACID_TRANSMEMBRANE_ TRANSPORTER_ACTIVITY	0.018	0.160
NEGATIVE_REGULATION_OF_DNA_BINDING	0.031	0.160
SODIUM_ION_TRANSPORT	0.013	0.160
CELL_JUNCTION	0.000	0.161
ION_TRANSMEMBRANE_TRANSPORTER_ACTIVITY	0.000	0.161
AMINE_TRANSPORT	0.003	0.161
CELLULAR_MORPHOGENESIS_DURING_DIFFERENTIATION	0.023	0.162
CATION_CHANNEL_ACTIVITY	0.004	0.163
GATED_CHANNEL_ACTIVITY	0.000	0.166
DETECTION_OF_EXTERNAL_STIMULUS	0.025	0.166
SYSTEM_PROCESS	0.000	0.167
SUBSTRATE_SPECIFIC_CHANNEL_ACTIVITY	0.000	0.168
ACTIVE_TRANSMEMBRANE_TRANSPORTER_ACTIVITY	0.000	0.168
CARBOXYLIC_ACID_TRANSMEMBRANE_ TRANSPORTER_ACTIVITY	0.020	0.170
DETECTION_OF_STIMULUS	0.010	0.171
REGULATION_OF_CELL_MIGRATION	0.009	0.171
VOLTAGE_GATED_CATION_CHANNEL_ACTIVITY	0.011	0.172
ACTIN_BINDING	0.004	0.174

GLUTAMATE_RECEPTOR_ACTIVITY	0.022	0.174
GENERATION_OF_A_SIGNAL_INVOLVED_IN_ CELL_CELL_SIGNALING	0.024	0.174
HYDROGEN_ION_TRANSMEMBRANE_ TRANSPORTER_ACTIVITY	0.011	0.175
ACTIN_FILAMENT_ORGANIZATION	0.003	0.176
AXONOGENESIS	0.009	0.176
VOLTAGE_GATED_POTASSIUM_CHANNEL_ACTIVITY	0.030	0.177
INORGANIC_ANION_TRANSPORT	0.007	0.177
TRANSMISSION_OF_NERVE_IMPULSE	0.006	0.179
VOLTAGE_GATED_CHANNEL_ACTIVITY	0.003	0.179
GLUTAMATE_SIGNALING_PATHWAY	0.061	0.179
NEGATIVE_REGULATION_OF_BINDING	0.023	0.180
SYNAPTIC_TRANSMISSION	0.000	0.182
MONOVALENT_INORGANIC_CATION_TRANSPORT	0.004	0.182
G_PROTEIN_SIGNALING_COUPLED_TO_CAMP_NUCLEOTIDE_ SECOND_MESSENGER	0.010	0.182
POTASSIUM_CHANNEL_ACTIVITY	0.018	0.183
LIPID_TRANSPORT	0.003	0.196
ORGANIC_ACID_TRANSPORT	0.033	0.197
CATION_TRANSPORT	0.000	0.198
RAS_PROTEIN_SIGNAL_TRANSDUCTION	0.011	0.199
CAMP_MEDIATED_SIGNALING	0.018	0.200
OXIDOREDUCTASE_ACTIVITY	0.000	0.200
EXTRACELLULAR_MATRIX_STRUCTURAL_ CONSTITUENT	0.059	0.200
ENZYME_LINKED_RECEPTOR_PROTEIN_ SIGNALING_PATHWAY	0.005	0.201
ANION_TRANSPORT	0.056	0.201
PROTEIN_OLIGOMERIZATION	0.056	0.201
NEURON_DIFFERENTIATION	0.030	0.202
SUBSTRATE_SPECIFIC_TRANSMEMBRANE_ TRANSPORTER_ACTIVITY	0.000	0.202
ENDOSOME	0.023	0.203
ENERGY_DERIVATION_BY_OXIDATION_OF_ ORGANIC_COMPOUNDS	0.034	0.204
HEPARIN_BINDING	0.064	0.205
CELLULAR_CARBOHYDRATE_CATABOLIC_PROCESS	0.057	0.206
CELL_PROJECTION	0.012	0.206
MONOVALENT_INORGANIC_CATION_TRANSMEMBRANE_ TRANSPORTER_ACTIVITY	0.063	0.207
PATTERN_BINDING	0.024	0.207
REGULATION_OF_MUSCLE_CONTRACTION	0.060	0.208
CARBOXYLIC_ACID_TRANSPORT	0.027	0.209
NERVOUS_SYSTEM_DEVELOPMENT	0.000	0.209
AXON_GUIDANCE	0.064	0.209
NEUROLOGICAL_SYSTEM_PROCESS	0.000	0.209

OXIDOREDUCTASE_ACTIVITY_ACTING_ON_THE_CH_CH_ GROUP_OF_DONORS	0.063	0.210
APICAL_PART_OF_CELL	0.061	0.211
GENERATION_OF_PRECURSOR_METABOLITES_ AND_ENERGY	0.000	0.212
METAL_ION_TRANSMEMBRANE_TRANSPORTER_ ACTIVITY	0.005	0.215
CARBOHYDRATE_METABOLIC_PROCESS	0.006	0.216
NEURON_DEVELOPMENT	0.038	0.224
SUBSTRATE_SPECIFIC_TRANSPORTER_ACTIVITY	0.000	0.225
CARBOHYDRATE_CATABOLIC_PROCESS	0.079	0.226
GENERATION_OF_NEURONS	0.029	0.227
ACTIN_FILAMENT_BINDING	0.081	0.228
NEUROTRANSMITTER_BINDING	0.062	0.229
ELECTRON_CARRIER_ACTIVITY	0.048	0.240
EXTRACELLULAR_MATRIX	0.018	0.242
DIGESTION	0.063	0.243
ION_TRANSPORT	0.000	0.243
ACTIN_CYTOSKELETON_ORGANIZATION_ AND_BIOGENESIS	0.025	0.243
ACTIN_FILAMENT_BASED_PROCESS	0.046	0.245

CHAPTER 6

Effects of zinc oxide and microbial phytase on digestibility of calcium and phosphorus in maize-based diets fed to growing pigs

Chapter 6

Effects of zinc oxide and microbial phytase on digestibility of calcium and phosphorus in maize-based diets fed to growing pigs

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6.1. ABSTRACT

An experiment was conducted to test the hypothesis that inclusion of Zn at a pharmacological level in diets fed to pigs affects apparent total tract digestibility (ATTD) of Ca and P and standardized total tract digestibility (STTD) of Ca. The second hypothesis was that inclusion of microbial phytase increases the ATTD of Ca and P and the STTD of Ca regardless of the concentration of Zn in the diet. Fifty six growing barrows (average BW: 15.4 ± 1.9 kg) were allotted to a randomized complete block design with 7 dietary treatments and 8 pigs per treatment. A maize-based basal diet was formulated with either 0 or 2,400 mg/kg Zn from ZnO and 0, 1,000, or 3,000 FTU per kg. A Ca-free diet was used to determine basal endogenous losses of Ca. Experimental diets were fed for 13 d and feces were collected from the feed provided from d 6 to 11 using the marker-to-marker approach; urine was also collected from d 6 to 11. Retention of Ca, ATTD of Ca, and STTD of Ca increased ($P < 0.01$) as the concentration of phytase in the diet increased, and were less ($P < 0.01$) if ZnO was used than if no ZnO was added to the diet. Retention of P and the ATTD of P increased ($P < 0.0001$) as the concentration of phytase increased in the diet, but the increase was greater if ZnO was not added than if ZnO was added to the diet (interaction, $P < 0.05$). In conclusion, pharmacological levels of Zn reduced Ca and P digestibility and retention, but this effect was partly mitigated by the inclusion of phytase in the diets. Inclusion of microbial phytase increased the ATTD and STTD of Ca in diets and also the ATTD of P.

6.2. INTRODUCTION

Use of values for STTD of Ca may result in improved diet formulations for pigs compared with use of values for total Ca because STTD of Ca takes the basal endogenous loss of Ca into account (González-Vega et al., 2015a; 2015b; Merriman and Stein, 2016). Values for STTD of Ca are believed to be additive in mixed diets, which is not always the case for values for ATTD. Values for ATTD or STTD of Ca may be increased by microbial phytase (Selle et al., 2009; Almeida and Stein, 2013, González-Vega et al., 2013), which is likely a result of hydrolysis of phytate esters and a subsequent reduction of the ability of phytate to chelate Ca (Selle et al., 2009; González-Vega et al., 2013).

It is common industry practice to use diets with pharmacological concentrations of zinc (i.e., up to approximately 3,000 mg/kg) during the post-weaning period to prevent post-weaning diarrhea in pigs (Poulsen, 1995; Hill et al., 2000). However, Zn competes with Ca for absorption through channel proteins on the brush border membrane in the pig small intestine (Bertolo et al., 2001a), and it is, therefore, possible that elevated levels of dietary Zn interferes with absorption of Ca. In addition, Ca and Zn may bind to phytate, which may also affect absorption of Ca. However, possible interactions between Zn and phytase on the STTD of Ca have not been reported. Therefore, the objectives of this experiment were to test the hypothesis that 1) pharmacological levels of Zn affects ATTD of Ca and P and STTD of Ca, and 2) microbial phytase increases the ATTD and STTD of Ca and the ATTD of P regardless of the concentration of Zn in the diet.

6.3. MATERIALS AND METHODS

The Institutional Animal Care and Use Committee at the University of Illinois, Urbana, IL, reviewed and approved the protocol for the experiment. Pigs used in the experiment were the offspring of L 359 boars and C–46 females (PIC, Hendersonville, TN).

6.3.1. Animals, Experimental Design, and Diets

Fifty six growing barrows with an average initial BW of 15.4 ± 1.9 kg were randomly allotted to a randomized complete block design with 2 blocks of 28 pigs. Within each block, pigs were randomly allotted to 7 diets with 4 replicate pigs per diet resulting in a total of 8 replicate pigs per diet for the 2 blocks. A diet based on maize, potato protein isolate, cornstarch, and soybean oil was formulated with either 0 or 2,400 mg/kg of added Zn and 0, 1,000, or 3,000 FTU per kg (Quantum Blue, AB Vista Feed Ingredients, Marlborough, UK; Table 6.1). The 2,400 mg/kg of Zn was provided by addition of 3,000 mg/kf of ZnO to the diets.

Table 6.1. *Ingredient composition of experimental diets, as-fed basis.*

Ingredient, %		No added ZnO			3,000 mg/kg added ZnO		
	Ca-free	0 FTU[1]	1,000 FTU	3,000 FTU	0 FTU	1,000 FTU	3,000 FTU
Corn	75.80	70.00	69.00	69.00	70.00	69.00	69.00
Cornstarch	2.22	2.08	2.27	2.27	1.78	1.97	1.97
Potato protein isolate	12.00	12.00	12.00	12.00	12.00	12.00	12.00
Soybean oil	3.00	3.00	3.00	3.00	3.00	3.00	3.00
Calcium carbonate	-	1.75	1.75	1.75	1.75	1.75	1.75
Monosodium phosphate	0.95	0.95	0.76	0.76	0.95	0.76	0.76
L-Lys HCL	0.13	0.61	0.61	0.61	0.61	0.61	0.61
DL-Met	0.08	0.11	0.11	0.11	0.11	0.11	0.11
L-Thr	0.08	0.09	0.09	0.09	0.09	0.09	0.09
L-Trp	0.05	0.05	0.05	0.05	0.05	0.05	0.05
L-His	0.09	0.10	0.10	0.10	0.10	0.10	0.10
Lactose	5.00	8.66	8.66	8.66	8.66	8.66	8.66
Phytase premix[2]	-	-	1.00	1.00	-	1.00	1.00
Sodium chloride	0.40	0.40	0.40	0.40	0.40	0.40	0.40
Vitamin mineral premix[3]	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Zinc oxide	-	-	-	-	0.30	0.30	0.30
Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00

[1]FTU = phytase units.

[2]The phytase premix was prepared by mixing 980 g ground corn and 20 g Quantum Blue 5000 G (AB Vista Feed Ingredients, Marlborough, UK) or 940 g ground corn and 60 g Quantum Blue 5G to provide 1,000 or 3,000 FTU of phytase per kilogram complete diet.

[3]The vitamin-micromineral premix provided the following quantities of vitamins and micro minerals per kilogram of complete diet: vitamin A as retinyl acetate, 11,136 IU; vitamin D3 as cholecalciferol, 2,208 IU; vitamin E as DL-alpha tocopheryl acetate, 66 IU; vitamin K as menadione dimethylprimidinol bisulfite, 1.42 mg; thiamin as thiamine mononitrate, 0.24 mg; riboflavin, 6.59 mg; pyridoxine as pyridoxine hydrochloride, 0.24 mg; vitamin B12, 0.03 mg; D-pantothenic acid as D-calcium pantothenate, 23.5 mg; niacin, 44.1 mg; folic acid, 1.59 mg; biotin, 0.44 mg; Cu, 20 mg as copper sulfate; Fe, 126 mg as iron sulfate; I, 1.26 mg as ethylenediamine dihydriodide; Mn, 60.2 mg as manganous sulfate; Se, 0. , 0.125 mg as sodium selenite and 0.125 mg as selenium yeast; and Zn, 124.9 mg as zinc sulfate.

Each diet was mixed in one 125 kg batch and all diets were fed in mash form. A 500 g sample of each diet was collected at the time of mixing and used for diet analysis. All diets were formulated to contain 0.70% total Ca and 0.33% standardized total tract digestible P, and the Ca to standardized total tract digestible P ratio was 2.10:1. A Ca-free diet that was used to measure basal endogenous losses of Ca, was also included in the experiment. Vitamins and all minerals were included in all diets except the Ca-free diet to meet or exceed the requirements for 11 to 25 kg pigs (NRC, 2012). Microminerals were included in all diets from the vitamin-mineral premix, which supplied 125 mg/kg of Zn from zinc sulfate. Therefore, the 3 diets without ZnO were calculated to contain 125 mg/kg of added Zn and the 3 diets containing 3,000 mg of ZnO were calculated to contain a total of 2,525 mg/kg of added Zn. The Zn provided by maize and potato protein isolate was not included in the calculated concentrations of Zn in the diets.

The Experimental Animal Allotment Program (Kim and Lindemann, 2007) was used to randomly allot the 28 pigs in each block to the 7 diets. Pigs were housed individually in stainless steel metabolism crates that were equipped with a slatted metal floor, a stainless steel feeder, a nipple drinker, and a screen floor that allowed for total fecal collection. A urine tray was installed below the screen floor, which allowed for total collection of urine.

6.3.2. Feeding and Sample Collection

Pigs were fed experimental diets for 13 d and the quantity of feed provided per pig daily was calculated as 3 times the daily maintenance energy requirement (i.e., 197 kcal ME/kg $BW^{0.60}$; NRC, 2012) and divided into 2 equal meals that were provided at 0800 and 1700 h. Pigs had free access to water throughout the experiment. The initial 5 d were considered an adaptation period to the diets. A color marker (indigo carmine) was added to the morning meal on d 6 and a second marker (ferric oxide) was added to the morning meal on d 11 according to the marker-to-marker approach (Adeola, 2001). Fecal collections were initiated when the first marker appeared in the feces and ceased when the second marker appeared. Fecal samples were stored at -20°C immediately after collection. Urine collection was initiated on d 6 in the morning and ceased on d 11 in the morning, and 20% of the collected urine was stored at -20°C. Orts that were

collected during the collection period were dried in a forced-air oven at 65ºC, and the weight was subtracted from feed allotments to calculate feed consumption.

6.3.3. Sample Analysis

Fecal samples were dried in a forced-air oven at 65˚C, ground in a Wiley mill (Model 4; Thomas Scientific, Swedesboro, NJ) using a 1-mm screen, and subsamples were collected for analysis after all the ground materials had been mixed. Diets and fecal samples were analyzed for DM by oven drying at 135˚C for 2h (Method 930.15; AOAC, 2007). Diets, fecal, and urine samples were analyzed for Ca and P by inductively coupled plasma-optical emission spectroscopy (ICP-OES; Method 985.01A, B, and D; AOAC, 2007) after wet ash sample preparation (Method 975.03 B[b]; AOAC, 2007). Diets were analyzed for GE using an isoperibol bomb calorimeter (Model 6300; Parr Instruments, Moline, IL) and benzoic acid was the internal standard. Diets were also analyzed for N using the combustion procedure (Method 990.03; AOAC, 2007) on an Elementar Rapid N-cube protein/nitrogen apparatus (Elementar Americas, Inc., Mt. Laurel, NJ) with aspartic acid used as the internal standard, and CP was subsequently calculated as N × 6.25. Diets were also analyzed for ash (Method 942.05; AOAC, 2007), and for ADF and NDF using Ankom Technology method 12 and 13, respectively (Ankom^{2000} Fiber Analyzer, Ankom Technology, Macedon, NY). Alfalfa meal was used as the internal standard for the ADF and NDF analyses. Diets were also analyzed for phytase activity (Method 2000.12, AOAC, 2007) and for Zn by inductively coupled plasma-optical emission spectroscopy (ICP-OES; Method 985.01A, B, and D; AOAC, 2007) after wet ash sample preparation (Method 975.03 B[b]; AOAC, 2007).

6.3.4. Calculations and Statistical Analysis

Values for ATTD of Ca and P were calculated for the 6 Ca-containing diets (NRC, 2012). The basal endogenous losses of Ca were determined from pigs fed the Ca-free diet according to González-Vega et al. (2015a). To obtain the STTD of Ca, ATTD values were corrected for basal endogenous losses according to González-Vega et al. (2015a). Retention of Ca was calculated as previously outlined (Almeida and Stein, 2010) using the following equation:

$$Car = \{[Cai - (Caf + Cau)]/Cai\} \times 100,$$

where Car is Ca retention (%), Cai is the intake of Ca (g), Caf is the fecal output of Ca, and Cau is the urinary output of Ca (g) over the collection period. Retention of P was also calculated using this equation.

Data were analyzed as a 2 × 3 factorial using the Proc MIXED procedure of SAS (SAS Inst. Inc., Cary, NC). The model included the fixed effects of Zn, phytase, and the interaction between Zn and phytase and the random effects of block and replicate within block. The LSMEANS procedure was used to calculate mean values, and means were separated using the PDIFF option if significant differences were observed. The pig was the experimental unit and an alpha level of 0.05 was used to assess significance among means, whereas differences were considered tendencies if the P-value was between 0.05 and 0.10.

6.4. RESULTS

Pigs readily consumed their assigned diets and remained healthy throughout the experiment. Values for analyzed concentrations of Zn in diets without ZnO were between 57.8 and 138.0 mg/kg and diets with added ZnO all analyzed between 2,525 and 2,670 mg/kg of Zn (Table 6.2).

There were no differences in feed intake among dietary treatments, but daily Ca intake increased ($P < 0.05$) as the concentration of phytase increased (Table 6.3). Fecal Ca output increased ($P < 0.05$) if ZnO was added to the diets, and the output of Ca in feces tended to decrease ($P = 0.058$) as the concentration of phytase increased regardless of concentration of ZnO in the diets. Urine Ca output was not affected by addition of ZnO to the diet. However, the output of Ca in urine was reduced if phytase was added to diets without ZnO, but that was not the case if phytase was added to the diets with ZnO (interaction, $P < 0.05$).

Total Ca excretion was reduced ($P < 0.0001$) as the concentration of phytase increased and was greater if ZnO was used ($P = 0.001$) than if no ZnO was added to the diet. An interaction between phytase and ZnO was observed for Ca excretion because pigs fed diets without ZnO and 3,000 FTU of phytase had the least excretion of Ca, but that was not the case if ZnO was added to the diet ($P < 0.05$). The ATTD and STTD of Ca increased ($P = 0.001$) as the concentration of phytase increased and were ($P < 0.01$) if ZnO was used than if no ZnO was added to the diet. However, for Ca retention, an

interaction ($P < 0.05$) between phytase and ZnO was observed because pigs fed the diet without ZnO and 3,000 FTU of phytase had greater retention of Ca than pigs fed the diet without ZnO and 1,000 FTU of phytase, but if ZnO was added to the diet, no difference between the diets with 1,000 and 3,000 FTU of phytase was observed for Ca retention.

Intake of P was not affected by inclusion of phytase or ZnO in the diets (Table 6.4). Excretion of P was reduced as diet phytase concentration increased, but the reduction was less in diets that contained ZnO than in diets that contained no ZnO (interaction, $P < 0.01$). Urine P output was not affected by addition of phytase or ZnO to the diets. Total P excretion decreased ($P < 0.0001$) as the concentration of phytase increased in the diet, but the excretion was greater if ZnO was added than if ZnO was not added to the diet (interaction, $P < 0.05$). Likewise, the percentage of P retention and ATTD of P increased ($P < 0.0001$) as the concentration of phytase increased in the diet, but the increase was greater if ZnO was not added than if ZnO was added to the diet (interaction; $P < 0.05$).

Table 6.2. *Analyzed composition of experimental diets, as-fed basis.*

Item		No added ZnO			3,000 mg/kg added ZnO		
	Ca-free	0 FTU[1]	1,000 FTU	3,000 FTU	0 FTU	1,000 FTU	3,000 FTU
DM, %	87.20	87.69	87.89	88.07	87.97	88.11	88.07
Ash, %	2.18	3.86	3.84	3.57	3.67	4.12	4.23
GE, kcal/g	4147	4059	4005	4038	3981	4020	4037
CP, %	14.39	16.30	16.20	16.40	15.66	15.44	16.53
ADF, %	2.59	2.33	2.51	2.29	2.54	2.27	2.32
NDF, %	8.66	8.09	8.21	7.96	8.45	7.83	7.84
Ca, %	0.03	0.63	0.77	0.69	0.65	0.70	0.66
P, %	0.39	0.43	0.40	0.40	0.39	0.41	0.36
Zn, mg/kg	110	58	93	138	2640	2520	2670.00
Phytase, FTU/kg	<70	<70	1,100	3,700	<70	1,300	3,200

[1]FTU = phytase units.

Table 6.3. *Calcium balance and apparent total tract digestibility (ATTD) and standardized total tract digestibility (STTD) of Ca for pigs fed diets containing different levels of microbial phytase (0, 1,000, or 3,000 FTU1 phytase) without or with ZnO addition*[2].

Item	No added ZnO			3,000 mg/kg added ZnO			SEM	P-value		
	0 FTU	1,000 FTU	3,000 FTU	0 FTU	1,000 FTU	3,000 FTU		Phytase	Zn	Phytase × Zn
Feed intake, g/d	724	725	806	750	781	774	140	0.723	0.914	0.966
Ca intake,[4] g/d	4.65	5.34	5.52	4.85	5.45	5.14	0.25	0.023	0.907	0.426
Fecal Ca output, g/d	1.51	1.40	1.12	1.69	1.62	1.47	0.13	0.058	0.021	0.804
Urine Ca output, mg/d	618^{ab}	887^{a}	463^{b}	856^{a}	613^{ab}	517^{ab}	90	0.037	0.552	0.0103
Ca excretion, % of intake	44^{ab}	41^{b}	28^{c}	52^{a}	41^{b}	41^{b}	2.4	<0.0001	0.001	0.024
ATTD of Ca, %	68.1	74.5	79.7	65.4	69.7	72.0	2.3	0.001	0.008	0.555
STTD of Ca,[3] %	70.0	76.1	81.3	67.2	71.3	73.7	2.3	0.001	0.008	0.578
Ca retention, % of intake	55^{bc}	58^{b}	71^{a}	47^{c}	58^{b}	58^{b}	2.4	<0.0001	0.001	0.024

[a-d]Values within a column without a common superscript are different (P <0.05).

[1]FTU = phytase units.

[2]Data are means of 8 observations per treatment, except for the 3,000 ZnO and 0 phytase diet that had only 7 observations.

[3]Values for standardized total tract digestibility were calculated by correcting apparent total tract digestibility values for basal endogenous losses. Basal endogenous losses were determined from pigs fed the Ca-free diet as 0.430 ± 0.18 g/kg of DMI.

6.5. DISCUSSION

Zinc is an essential micronutrient for all living organisms with different roles as a structural component of proteins, an enzymatic co-factor, and transcriptional regulator in cellular and biochemical processes (Solomons, 2013b). Requirements in pigs expressed as mg/kg diet is reduced as pig BW increases (NRC, 2012). For pigs between 11 and 25 kg, the dietary requirement is 80 mg/kg (NRC, 2012), but pharmacological concentrations of Zn (2,000 to 3,000 mg/kg) may enhance growth performance and reduce the prevalence of diarrhea (Hahn and Baker, 1993; Case and Carlson, 1996; Hill et al., 2000; Carlson et al., 2006; Hu et al., 2012).

The bioavailability of several minerals including Zn is affected by phytate (*myo*-inositol hexaphosphate; Lopez et al., 2002). Phytate is the main storage form for P in plants (Selle et al., 2009), but is also considered an antinutritional factor for humans and animals as it has the capacity to chelate nutritionally important cations such as Cu^{+2}, Zn^{+2}, Mg^{+2}, Mn^{+2}, Fe^{+2}, and Ca^{+2} (Persson et al., 1998; Maenz et al., 1999; Selle et al., 2009). Phytase (*myo*-inositol hexaphosphate phosphohydrolase) hydrolyzes the phosphor-ester bond between phytate and P, which will release phytate-P and may enhance macro- and micro mineral availabilities by releasing the cations that are bound to phytate and possibly also increase the utilization of energy and amino acids (Selle and Ravindran, 2008).

The observation that microbial phytase supplementation increased the ATTD of Ca and P is in agreement with results from previous experiments (Igbasan et al., 2001; Guggenbuhl et al., 2007; Almeida and Stein, 2010; Poulsen et al., 2010; González-Vega et al., 2013; 2015a; 2015b). Correction of ATTD values of a nutrient for basal endogenous losses results in calculation of values for STTD (NRC, 2012). The basal endogenous loss of Ca obtained from pigs fed the Ca-free diet was 0.43 g/kg DMI, which is in agreement with the value (0.40 g/kg DMI) reported by González-Vega et al. (2015b) who also used a diet based on maize and potato protein isolate.

Table 6.4. *Phosphorus balance and apparent total tract digestibility (ATTD) of P for pigs fed diets containing different levels of microbial phytase (0, 1,000, or 3,000 FTU1 phytase) without or with ZnO addition*[2].

Item	No added ZnO			3,000 mg/kg added ZnO			SEM	P-value		
	0 FTU	1,000 FTU	3,000 FTU	0 FTU	1,000 FTU	3,000 FTU		Phytase	Zn	Phytase × Zn
P intake, g/d	3.15	2.88	3.23	2.89	3.18	2.80	0.16	0.996	0.286	0.042
Fecal P output, g/d	1.17^{a}	0.85^{b}	0.62^{c}	1.23^{a}	1.21^{a}	0.96^{b}	0.05	<0.0001	<0.0001	0.008
Urine P output, mg/d	62	56	57	49	58	49	9	0.962	0.497	0.619
P excretion, % of intake	40.5^{ab}	31.9^{c}	21.1^{d}	46.1^{a}	35.1^{bc}	34.3^{bc}	1.8	<0.0001	<0.0001	0.015
ATTD of P, %	61.5^{cd}	70.0^{b}	80.6^{a}	55.6^{d}	63.2^{c}	67.5^{bc}	1.5	<0.0001	<0.0001	0.040
P retetention, % of intake	59.5^{cd}	68.2^{b}	78.9^{a}	53.9^{d}	64.9^{bc}	65.7^{bc}	1.8	<0.0001	<0.0001	0.015

[a-d]Values within a column without a common superscript are different (P <0.05).

[1]FTU = phytase units.

[2]Data are means of 8 observations per treatment, except for the 3,000 ZnO and 0 phytase diet that had only 7 observations.

The increase in STTD of Ca that was observed as phytase was added to the diets also is in agreement with previous data (González-Vega et al., 2015a; 2015b). These results confirm that phytate may chelate dietary Ca, but if phytate is hydrolyzed by phytase, the chelated Ca will be released and absorbed, which will increase retention of both Ca and P (Sauer et al., 2003; Poulsen et al., 2010). However, González-Vega et al. (2013) did not observe an increase in P-retention as phytase was added to the diet as was the case in this experiment, but the diets used by González-Vega et al. (2013) were very low in Ca, which may have prevented P from being retained because for P to be retained in bone tissue, both Ca and P need to be available (Crenshaw, 2001; Stein et al., 2006).

The reduction in ATTD and STTD of Ca that was observed as ZnO was added to the diet is in agreement with data indicating that pharmacological levels of ZnO reduced Ca and P absorption in 7.3 kg pigs fed diets containing 0.78% total P and 0.95% Ca (Meyer et al., 2002). However, Walk et al. (2015) reported that ATTD of Ca was not influenced by dietary Zn in the absence of phytase, but if phytase was added to the diet, a linear reduction in Ca digestibility was observed as dietary Zn increased from 0 to 3,500 mg/kg. In the present experiment, a reduction in retention of Ca was also observed in pigs fed diets containing ZnO compared with pigs fed diets without ZnO, confirming that pharmacological levels of Zn has a negative effect on absorption and retention of Ca, and that the positive effect of phytase is reduced if ZnO is added to the diets. Recently, it was reported that addition of pharmacological concentrations of ZnO and 1,000 FTU of phytase to diets fed to weanling pigs reduced growth performance during the nursery phase compared with pigs fed diets containing phytase, but no added ZnO (Blavi et al., 2016). A lack of response to phytase on growth performance of 7.2 kg pigs was also reported if pharmacological concentrations of ZnO were included in diets (Martínez et al., 2005).

High dietary Ca accentuates the negative effect of phytate on Zn bioavailability in broiler chickens (Bafundo et al., 1984), rats (Forbes et al., 1984), and fish (Gatlin and Phillips, 1989) although the mechanism by which Ca reduces Zn availability is different from the effects of phytate. High dietary Ca also reduces blood and bone concentrations of Zn in post-weaning pigs (Hsu et al., 1975), but does not affect the apparent absorption of Zn (Whiting and Bezeau, 1958). (Whiting and Bezeau, 1958). It is, therefore, possible that if addition of microbial phytase to diets increases the

digestibility of Ca as was demonstrated in this experiment and in previous experiments (Gonzalez-Vega et al., 2015a; 2015b) the additional absorbed Ca may have a negative effect on the Zn status of pigs. However, Ca may improve Zn absorption from phytate containing foods in humans (Lönnerdal et al., 1984; Petterson et al., 1994).

The approximate pH of the intestine where absorption of metal ions takes place coincides with the pH at which these complexes precipitate (Champagne, 1988). The order of mineral potency as inhibitors of phytate hydrolysis at a neutral pH is Zn^{+2} >> Fe^{+2} > Mn^{+2} > Fe^{+3} > Ca^{+2} > Mg^{+2} (Maenz et al., 1999). Multiple mineral-phytate complexes such as Ca-Zn-phytate are more stable than single mineral complexes such as Ca-phytate or Zn-phytate (Maenz et al., 1999) and if 2 cations, such as Ca^{+2} and Zn^{+2}, are presented simultaneously, they act together to increase phytate precipitation (Simpson and Wise, 1990). The formation of Zn-Ca-phytate complexes in the small intestine may be a major mechanism by which phytate reduces dietary Zn availability (Fordyce et al., 1987). Therefore, it may be hypothesized that high dietary Zn increases the negative effect of phytate on Ca digestibility, which may be the reason for the reduction in STTD of Ca that was observed as ZnO was added to the diets.

Another possible explanation for the negative effects of pharmacological levels of Zn on STTD of Ca is that Ca^{+2} and Zn^{+2} compete for a common transport pathway on the brush border membrane, and this transporter has greater affinity for Zn^{+2} than for Ca^{+2} (Bertolo et al., 2001b). High dietary ZnO may, therefore, produce more ionic Zn^{+2} ready for absorption in the stomach and proximal parts of the small intestine, and as a consequence, transport capacity for Ca^{+2} is reduced, which results in a reduced absorption and digestibility of Ca. However, additional research is needed to confirm this hypothesis. The observation that there was no interaction between addition of ZnO and phytase on ATTD and STTD of Ca indicates that the increased digestibility of Ca that is caused by phytase is independent of the concentration of Zn in the diet.

The reduction in P retention that was observed as ZnO was added to the diets is in agreement with data indicating that pharmacological concentrations of ZnO decreases plasma P, regardless of phytase supplementation (Walk et al., 2013). Digestibility of P, in pigs fed 4.5 g/kg digestible P, was also reduced as supplemental Zn was added at pharmacological levels to the diet, and this effect was greater in pigs fed diets without phytase than if phytase was included in the diet (Walk et al., 2015). However, there

were no effects of ZnO addition on P digestibility by pigs fed 5.5 g/kg digestible P (Walk et al., 2015) indicating that the negative effects of ZnO on ATTD of P may be overcome by addition of excess P in the diet. The antagonistic relationship between Zn and P has also been demonstrated in rats; and rats fed low Zn (18 mg/kg) with 1.20% Ca and 1.20% P had reduced weight gain compared with rats fed diets containing 42 mg/kg of Zn. However, if both Ca and P in the diet were reduced to 0.30% no benefit of increasing dietary Zn from 18 to 42 mg/kg was observed (Cabell and Earle, 1965). Thus, several mechanisms may be involved in reducing Zn availability and effects of phytase are likely influenced by dietary concentrations of Ca and P.

In conclusion, results of this experiment indicate that pharmacological levels of Zn in diets for pigs may reduce Ca and P digestibility, but addition of microbial phytase to these diets may partly ameliorate this effect. As a consequence, if pigs need pharmacological levels of Zn, dietary concentrations of Ca and P in diets for pigs that are around 15 kg may need to be increased by 4% and 9.5%, respectively, or diets need to be supplemented with microbial phytase to prevent reduced absorption of Ca and P. Results of the experiment also indicate that interactions between Zn, Ca, P, and phytate may take place in the intestinal tract of pigs, which is the likely reason that supplemental Zn from ZnO reduced Ca and P digestibility and retention. The reason for the reduced absorption of Ca in diets containing pharmacological levels of Zn may be that Zn and Ca compete for the same calcium channels to be absorbed into the enterocytes. Inclusion of microbial phytase increased the ATTD and STTD of Ca and also the ATTD of P, confirming that dietary phytate interferes with Ca and P digestibility.

CHAPTER 7

Anethol, cinnamaldehyde and eugenol inclusion in feed affects post-weaning performance and feeding behavior of piglets

Chapter 7

Anethol, cinnamaldehyde and eugenol inclusion in feed affects post-weaning performance and feeding behavior of piglets

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7.1. ABSTRACT

The early exposure of the fetus to certain volatiles may result in a further preference for these compounds later in life and could positively affect the acceptance of feed containing a similar flavor and the zootechnical responses. The study consisted of 2 trials to determine if including Fluidarom 1003® (a commercially flavored feed-additive containing > 25% of anethol and cinnamaldehyde and > 10% of eugenol; Norel S.A.; Madrid, Spain) in sow and post-weaning piglet diets: 1) provokes the presence or absence of three major volatile compounds (anethol, cinnamaldehyde and eugenol) in amniotic fluid and milk, affecting piglet performance (BW, ADG, ADFI and FCR) after weaning, and 2) modifies creep-feed consumption and feed preference in a two-choice test. The major compounds: anethol, cinnamaldehyde and eugenol were detected in amniotic fluid, however, only traces were observed in milk. The inclusion of flavor in the sow diets improved piglet consumption and growth after weaning ($P = 0.001$). Furthermore, the positive reward associated with the flavor included in the sow diet was stronger when piglets were offered a non-flavored creep-feed ($P < 0.05$). Therefore, early exposure of pigs' fetuses to maternal dietary clues at the end of gestation might allow for conditioning pigs after weaning.

7.2. INTRODUCTION

Anethol, cinnamaldehyde, and eugenol are plant extracts widely used as sensory additives in human and animal feed (Lee et al., 2011; Komala et al., 2012; Kim et al., 2013). They are mostly used as feed flavors but they may also show benefits such as improved animal performance, better nutrient digestibility and faster gut maturation (Burt, 2004; Manzanilla et al. 2004; Lillehoj and Lee, 2012).

Weaning is stressful for piglets, having to face social stress by mixing with other pigs and adapting to eat a novel food, usually solid diet which may generate neophobia. A learning process by flavors added in maternal diets before or after birth, known as maternal learning (Mennella et al., 2001; Wells and Hepper, 2006; Oostindjer et al., 2010; Figueroa et al., 2013) could smooth the weaning changes and optimize the production efficiency of pigs. In maternal-learning process, volatile flavors coming from maternal diets may reach the fetus via the amniotic fluid and/or the placental bloodstream (Hepper, 1988 and Figueroa et al., 2016). During the last trimester of

gestation, fetuses of several mammalian species appear to be able to detect and retain chemosensory information (Schaal and Orgeur, 1992). After birth, the flavor contact may continue through the milk (Mennella and Beauchamp, 1991). In pigs, it has been observed that pre-natal exposure to flavors via maternal diet influences feed preferences of pigs in two-choice test (Figueroa et al., 2013) and enhances their feed intake and growth performance (Oostindjer et al., 2009, 2010, 2011).

Therefore, the objective of these experiments was to test the hypothesis that: 1) a flavor incorporation in late pregnancy diets could represent a link between the intrauterine and extra uterine period, and a familiar volatile cue for weanling piglets; 2) plant extracts incorporated in sow, creep-feed and/or weanling diets increases performance of piglets.

7.3. MATERIALS AND METHODS

All experimental procedures were approved by the Ethical Committee on Human and Animal Experimentation of the UAB (CEAAH 1406).

7.3.1. Flavors

The flavors used in the experimental trials were commercially flavored feed additives from Norel S.A., Madrid, Spain. Fluidarom 1003® (375 mg/kg) was used in feed containing > 25% of anethol and cinnamaldehyde and > 10% of eugenol (onwards as F treatment) and the remaining percentage is a complex mixture of different compounds. Lacto-Vanilla (500 mg/kg in feed), based on > 25% of vanillin, > 10% of butyric acid and > 10% of diacetyl, was used in the preference test as negative control.

7.3.2. Diets

The composition for the gestation, lactation, creep-feed and weanling (pre-starter and starter) diets were the same for both trials and are presented in Table 7.1. Diets were formulated to meet or exceed the nutrient requirements for each period (NRC, 2012) and were presented in mash form. Creep-feed was offered in commercial pan-feeders with a hopper in order to ensure *ad-libitum* access to feed from d 12 onwards (Trial 1 and 2). Pre-starter diets were offered *ad-libitum* for fourteen consecutive days, and starter diets were offered from days 14 to 35 post-weaning (Trial 1).

Table 7.1. *Ingredients and nutrient composition (% as-fed basis, unless otherwise indicated) of the gestation, lactation, creep-feed, pre-starter and starter diets.*

Ingredient	Gestation	Lactation	Creep-Feed	Pre-Starter(*)	Starter(**)
Barley	27.3	22.0	22.0	15.0	17.1
Wheat	20.0	20.0	17.5	15.0	18.0
Wheat bran	16.0	5.0	-	-	-
Maize	10.0	25.0	15.1	28.0	35.0
Sweet beet pulp	7.5	-	-	-	-
Gluten feed	5.0	2.5	-	-	-
Canola meal	4.5	2.5	-	-	-
Soybean meal 44% CP	3.0	17.5	-	5.0	6.0
Soybean meal concentrate	-	-	2.5	-	-
Soybean hulls	2.4	-	-	-	-
Full-fat soybean meal	-	-	16.5	-	-
Extruded Soybeans				9.44	11.44
Sweet Milk Whey	-	-	14.1	10	-
Fishmeal	-	-	-	2.5	5.0
Animal plasma 80% CP	-	-	5.0	5.0	-
Soybean oil	-	-	3.8	1.85	-
Lard	1.33	2.29	-	-	-
Whey powder (50% Fat)	-	-	-	5.0	2.5
Calcium carbonate	1.53	1.37	0.94	1.13	0.25
Mono-calcium phosphate	0.52	0.79	0.85	0.84	2.84
Salt	0.30	0.30	0.27	0.08	0.45
Sodium bicarbonate	0.20	0.30	-	-	-
L-Lysine-HCl	0.11	0.13	0.48	0.47	0.63
DL-Methionine	-	-	0.24	-	-
L-Threonine	-	-	0.20	-	-
L-Tryptophan	-	-	0.50	-	-
Vit-Min complex	0.40[a]	0.40[a]	0.40[b]	0.40[c]	0.40[c]

[a] Sow gestation and lactation diets Premix Supplied (g/kg): 12,500 IU of vitamin A, 2,000 IU of vitamin D3, 20 mg of vitamin E, 2 mg of vitamin K3, 4 mg of vitamin B1, 5 mg of vitamin B2, 25 mg of vitamin B3, 2.6 mg of vitamin B6, 0.02 mg of vitamin B12, 12 mg of calcium pantothenate, 25 mg of Nicotinic acid, 0.100 mg of biotin, 300 mg of Choline-Cl, 100 mg of Fe, 10 mg of Cu, 0.5 mg of Co, 100 mg of Zn, 80 mg of Mn, 0.5 mg of I and 0.22 mg of Se.

[b] Creep-Feed diet Premix Supplied (g/kg): 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 glycine), 0.05 mg of Co (sulphate), 40 mg of Zn (chelate of amino acids), 12.5 mg of Mn (oxide), 7.5 mg of Mn (chelate of glycine), 0.35 mg of I, 0.5 mg of Se (organic) and 0.1 mg of Se (sodium).

[c] Pre-starter and starter diets Premix Supplied (g/kg): 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 glycine), 0.05 mg of Co (sulphate), 40 mg of Zn (chelate of amino acids), 12.5 mg of Mn (oxide), 7.5 mg of Mn (chelate of glycine), 0.35 mg of I, 0.5 mg of Se (organic) and 0.1 mg of Se (sodium).

(*) Antibiotics in feed: Zn Oxide: 3,000 mg/kg; Amoxicilin: 300 mg/kg; Colistin sulphate: 120mg/kg.

(**) Antibiotics in feed: Zn Oxide: 1,500 mg/kg; Amoxicilin: 250 mg/kg; Colistin sulphate: 120mg/kg.

7.3.3. Experimental Procedures

The following procedures were the same in Trials 1 and 2. Litter size was standardized at 12 piglets per sow immediately after birth by cross-fostering within the same experimental treatment. All piglets were individually identified by using plastic ear tags and were weighed at weaning (d 28 of lactation).

At weaning, piglets were moved to the weanling unit, with two rooms of 24 pens each. The rooms were equipped with central heating, automatic forced ventilation and a completely slatted floor. Each pen (3.2 m^2 in floor area) had *ad-libitum* access to feed and drinking water.

7.3.3.1. Trial 1

The first trial was designed to determine if a flavor included in the sow diet (F) can provide a transfer of volatile compounds to amniotic fluid and to milk and consequently modify the piglet feed intake and performance after weaning. A total of 80 sows (Landrace x Large White) were selected and distributed into two experimental

groups according to parity number, body condition score, and body weight at d 72 of gestation (late gestation). Sows were either exposed to a flavored (diet F, 375 mg/kg, n = 40) or control (C, n = 40) feed from d 73 to farrowing (last trimester of gestation) and for 28 days of lactation. The following sow's productive parameters per litter were registered: number of piglets (born, alive and stillbirth), weight of piglets born alive, number of weaned piglets, and pre-weaning mortality. During lactation, piglets from C sows were offered a non-flavored creep-feed (CC), while piglets from F sows were offered a flavored creep-feed (FF treatment) from day 12 of life onwards (Figure 7.1).

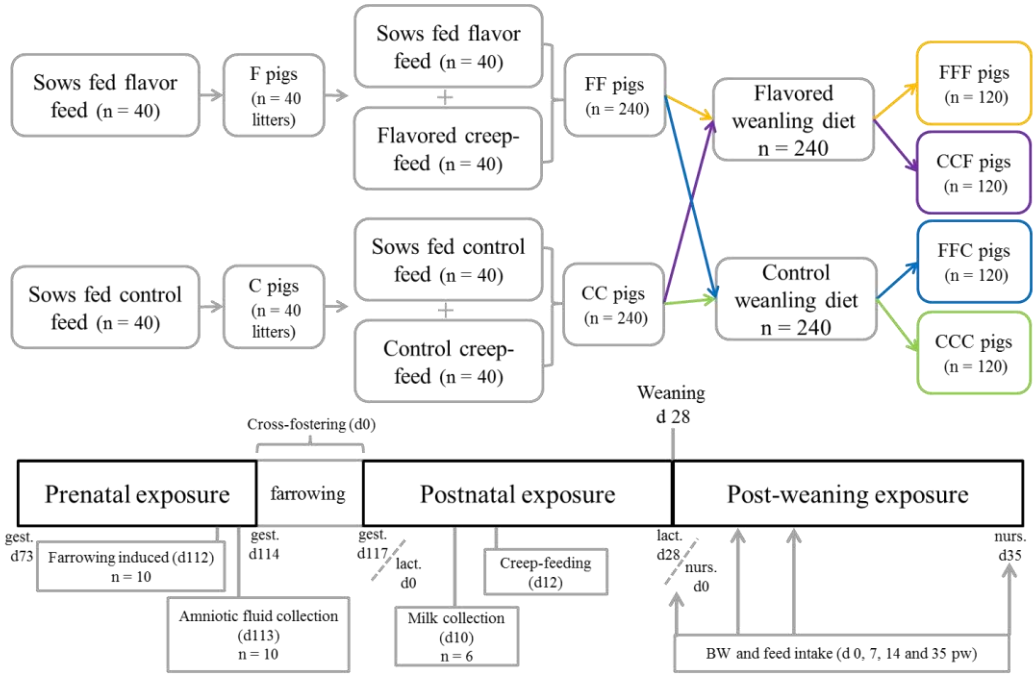

Figure 7.1. *The experimental design and the timing of the different treatments and procedures of Trial 1. F pigs = pigs from sows that received flavored feed during gestation and lactation; C pigs = pigs from sows that received non-flavored feed during gestation and lactation; FF pigs = pigs from F sows received flavored creep-feed; CC pigs = pigs from C sows received non-flavored creep-feed; FFF pigs = pigs from F sows received flavored creep-feed and flavored weanling diet; CCF pigs = pigs from C sows received non-flavored creep-feed and flavored weanling diet; FFC pigs = pigs from F sows received flavored creep-feed and non-flavored weanling diet; CCC pigs = pigs from C sows received non-flavored creep-feed and non-flavored weanling diet. gest. = gestation; lact. = lactation; nurs. = nursering; pw = post-weaning*

The presence of volatile compounds in the amniotic fluid was determined in 10 sows (four sows from the C and six from the F treatment). Farrowing was induced, two

days before the expected date of farrowing, by 2 cm^3 of D-cloprostenol ($PGF_{2\alpha}$) followed 24h later by 2 cm^3 of oxytocin. After approximately 1 hour, farrowing began and the amniotic fluid was collected. There were no problems with the sows and their litters, and all were included in the trial. On d 10 of lactation, a total of six samples of milk were collected from four F sows and from two C sows. All of the samples were kept frozen until lab analysis.

A total of 480 weanling piglets [Pietrain x (Landrace x Large White)] were selected at weaning (26 ± 2 days old; average BW = 7.4 ± 1.28 kg). Piglets were distributed into 48 pens (10 pigs/pen), by initial body weight, into three blocks (body weight category; heavy = 8.67 ± 0.52 kg; medium = 7.66 ± 0.22 kg and light = 5.76 ± 0.29 kg) and allotted into four experimental treatments following a 2 x 2 factorial arrangement where the main factors were the flavor inclusion in the sow diets plus creep-feed (CC vs FF treatments) and in weanling diets (C vs F diets, 375 mg/kg in pre-starter and starter feed) until d 35 post-weaning, corresponding to four pens per treatment and block.

Body weight and feed intake were monitored on d 0, 7, 14 and 35 post-weaning. Feed intake was calculated by the difference between the initial and final weight of feeders and accepting possible feed losses. Piglets were weighed by pen. ADFI, ADG and FCR were calculated by pen.

An analysis for the detection of the different volatile compounds present in the samples of sow milk and in amniotic fluid was conducted by solid-phase micro-extraction, gas chromatography and mass spectrometry (SPME-GC-MS). Twelve mL of amniotic fluid or milk were placed into 20mL sample vials (Supelco Inc; Bellefonte, PA, USA) and 2 g NaCl was added. The contents of the vials were mixed by gently swirling the vials by hand and heating at 70ºC for 10 minutes. A HP6890 Series II gas chromatograph (Agilent Technologies; Salt Lake City, UT, USA) equipped with an electronic impact HP5973 detector (Agilent Technologies) containing 50/30 μm DVB-CAR-PDMS fiber (Supelco Inc.; Bellefonte, PA, USA), was used for 30 minutes to extract volatiles and analyze the analytic content in the headspace. A Combipal auto-sampler (CTC Analytics AG, Zwingen, Switzerland) was used to perform SPME. Injection was made in a splitless mode, one min at 265ºC. The gas chromatographic column used was a TRB-WAX one with the dimensions of 60 m/mm length, 0.25 mm

i.d., and 0.25 μm film thickness (Supelco; Bellefonte, PA, USA). Column flow (He) was 1.5 mL/min. Injector temperature was maintained at 100ºC for 10 min and raised to 265ºC, at 12ºC/min, for 17 min. The data were processed using data Analysis (Agilent Technologies; Salt Lake City, UT, USA).

Specific emphasis was done in the detection of the three main volatile compounds supplemented to the experimental diets (anethol, eugenol and cinnamaldehyde). The minimum detection limits were 10 μg/L of anethol, 30 μg/L of eugenol, and 30 μg/L of cinnamaldehyde. The standards were Anethol (99% Sigma-Aldrich; Madrid, Spain), Cinnamaldehyde (≥95% Sigma-Aldrich; Madrid, Spain), and Eugenol (99% Sigma-Aldrich; Madrid, Spain).

7.3.3.2. Trial 2

The second trial was designed to determine if a flavored diet fed to sows and piglets may modify the piglets feeding behavior, expressed either as changes in the creep-feed intake or changes on the preference of piglets towards a flavored diet after weaning. A total of 24 sows (Landrace x Large White) were used. Three groups of sows were arranged according to parity, expected farrowing date and body condition score at d 72 of gestation, and allotted to three experimental treatments. The treatments only differed in flavor inclusion: FFF had flavor in gestation-lactation, creep-feed and weaning; FCF in gestation-lactation and weaning; and CCC no flavor inclusion (Figure 7.2). Sows were exposed to a flavored feed from d 73 to farrowing and for 28 days of lactation. From d 12 until weaning (d 28), all litters were offered free access to creep-feed, either flavored or not flavored. Creep-feed consumption was estimated by weighing the disappearance of creep-feed from the pan-feeder at d 17, 23 and 28.

The animals had *ad-libitum* access to feed and drinking water, except for 1h before the preference test, during which piglets had no access to the commercial feeders.

On d 2 after weaning, a preference test was conducted in the housing pens by a two-choice test between two diets supplemented either with Fluidarom (375 mg/kg) or Lacto-Vanilla (500 mg/kg) with four animals from each pen for 30 minutes (adapted from Solà-Oriol et al., 2011). A total of 24 piglets per treatment were used (n = 6 replicates per treatment).

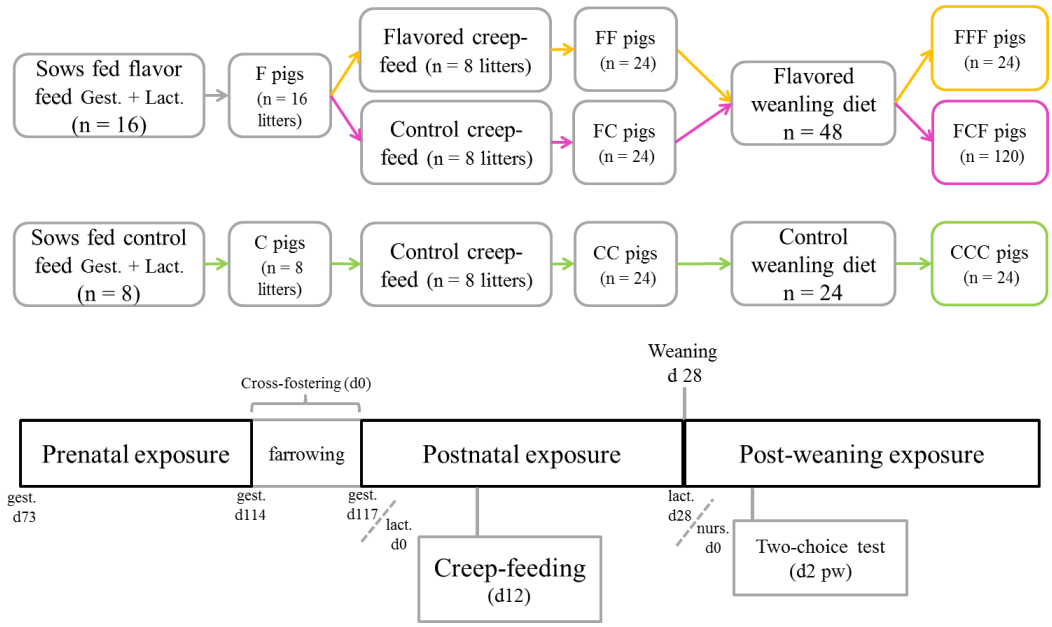

Figure 7.2. *The experimental design and the timing of the different treatments, procedures, and the behavioral test of Trial 2. F pigs = pigs from sows that received flavored feed during gestation and lactation; C pigs = pigs from sows that received non-flavored feed during gestation and lactation; FF pigs = pigs from F sows received flavored creep-feed; FC pigs = pigs from F sows received non-flavored creep-feed; CC pigs = pigs from C sows received non-flavored creep-feed; FFF pigs = pigs from F sows received flavored creep-feed and flavored weanling diet; FCF pigs = pigs from F sows received non-flavored creep-feed and flavored weanling diet; CCC pigs = pigs from C sows received non-flavored creep-feed and non-flavored weanling diet. gest. = gestation; lact. = lactation; nurs. = nursering; pw = post-weaning*

7.3.4. Statistical Analysis

All results were analyzed by using the SAS® statistical package (version 9.2, SAS Institute; Cary, USA). A contrast analysis was used to determine if there were differences between volatile compounds in amniotic fluid of C sows and F sows. The relationship among volatile compounds in feed and in amniotic fluid was studied with principal components (**PC**) analysis, by using PRINCOMP procedure.

7.3.4.1. Performance parameters

In Trial 1, piglet performance was analyzed with an ANOVA following a 2 x 2 factorial arrangement where the two main factors were flavor supplementation on sows' diet (gestation, lactation and creep-feed) and on pre- and starter diets by using the GLM procedure. Pen was the statistical unit for all production measurements.

In Trial 2, the creep-feed intake was analyzed with an ANOVA by using the GLM procedure, and the statistical unit was the litter.

7.3.4.2. Preference test

Preference values were calculated by dividing the feed intake by body weight and the number of piglets in the test. Preference values were analyzed with an ANOVA using the MIXED procedure. The statistical unit was each pen of four piglets. Additionally, the preference values for the flavor were compared to the neutral value of 50% by using a Student's T-test procedure.

All of the results are presented as Least Square Means by taking into account a Tukey adjustment, and the alpha level used for the determination of significance for all of the analyses was 0.05.

7.4. RESULTS

7.4.1. Trial 1

Amongst other compounds, anethol, cinnamaldehyde and eugenol were detected in amniotic fluid within the most influent compounds differing F vs. C sows (Figure 7.3) but, only traces were observed in milk. There was an increase ($P < 0.01$) in anethol abundance of sows fed the flavor diet compared with sows fed the control diet (1601 vs. 7579 mass number/charge number (m/z)). There was also an increase ($P < 0.001$) for sows fed the flavor diet compared with the control diet for cinnamaldehyde (1346 vs. 2376 m/z) and eugenol (1209 vs. 12606 m/z). The PC analysis of the volatile compounds showed clear differentiation between C Sows and F Sows and also the presence of anethol, cinnamaldehyd and eugenol was only in the amniotic fluid of sows fed the flavor diet (Figure 7.4). The first 4 PC had eigenvalues above 1 (141.2, 24.9, 17.9 and 5.6), and these explained 70.6, 12.4, 8.9 and 2.8% of total variability, respectively. Therefore the two first PC already explained a 83% of the total variability.

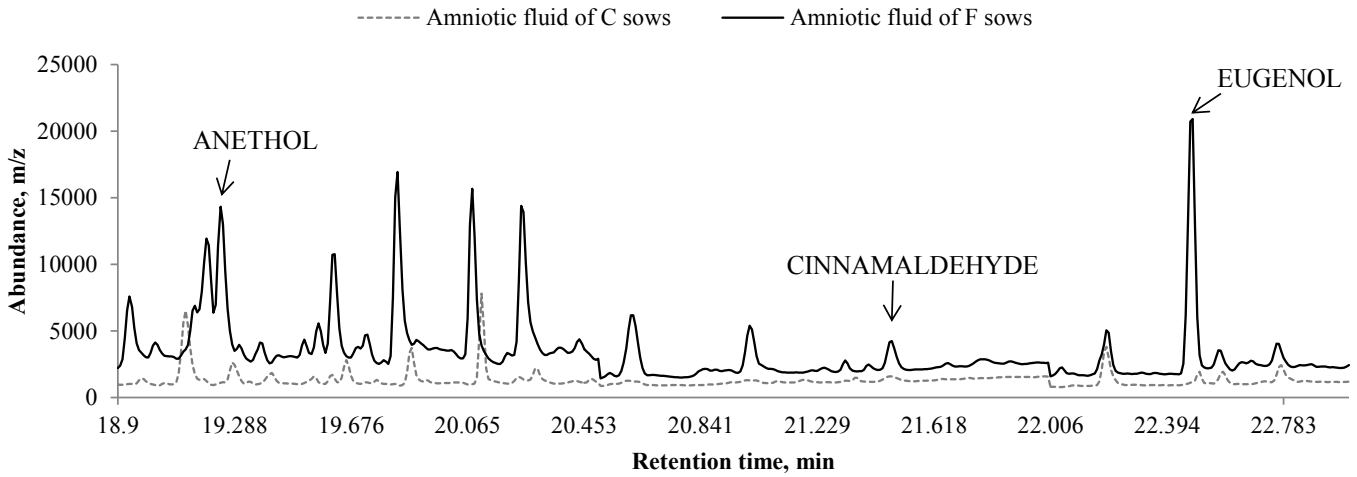

Figure 7.3. *Presence of anethol, cinnamaldehyde and eugenol in amniotic fluid. The solid line represents the mean of amniotic fluid samples of the Flavored (F) sows and the dotted line represents the mean of amniotic fluid samples of the Control (C) sows (Trial 1).*

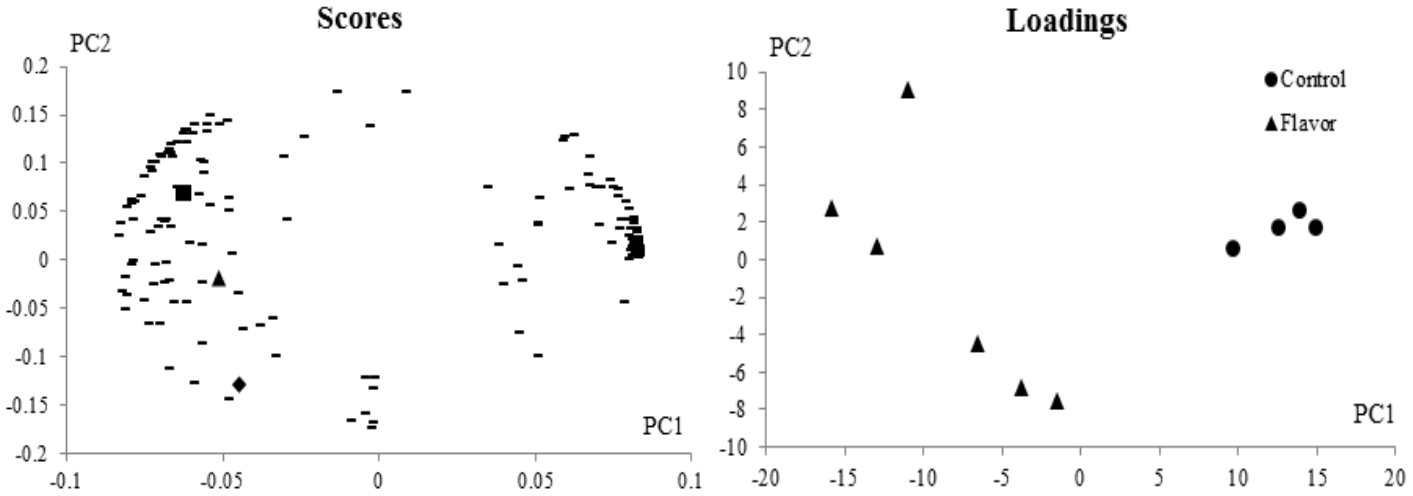

Figure 7.4 *Principal component (PC) analysis of the volatile compound in the amniotic fluid of sows fed a control diet (Control) or the flavored diet with 375mg/kg of Fluidarom (Flavor): undefined compounds (—), cinnamaldehyd (■), eugenol (▲), and anethol (♦).Principal component 1 explained 70.6% of the total variation, and PC2 explained 12.4% of the variation. Scores: orientation of the volatile-compounds relative to the PC. Loading: orientation of sow amniotic fluid relative to PC.*

No differences were observed on sow's productive performance ($P > 0.1$; results not shown). Table 7.2 shows the productive performance for Trial 1. The supplementation with flavor to the sows' diets at the end of the gestation, lactation and in creep-feeding increased the feed intake of their piglets in the pre-starter (0-14d) and in the starter period (14-35d) ($P < 0.01$). Moreover, these pigs gained more weight during the first 14d post-weaning; consequently they had a higher BW at d 14 and 35 post-weaning ($P < 0.05$). Supplementing the pre-starter and starter diets with the flavor increased ($P = 0.01$) feed intake and tended to increase BW gain early after weaning but, did not affect the performance from d 14 to d 35. However, there were no differences in the interaction between sow and weaning diets ($P > 0.1$).

Table 7.2 *Productive performance of piglets (BW, ADFI, ADG and feed conversion ratio [FCR]) obtained from sows fed a Control or Flavored diet during late-gestation and lactation and a Control or Flavored diet after weaning (n = 12 per treatment; 0 to 14, 14 to 35 and 0 to 35 days post-weaning; Trial 1).*

	Sow diet (Gestation –Lactation)					*P*-values		
	Control		Flavored					
	Weanling diet		*Weanling diet*		Pooled	Sow diet	Weaning diet	Sd x
Item	Control CCC	Flavored CCF	Control FFC	Flavored FFF	SEM	(Sd)	(Wd)	Wd
BW d 0, g	7415	7423	7426	7407	27.6	0.939	0.837	0.633
BW d 7, g	**8066**[b]	8319[ab]	**8453**[a]	**8393**[a]	75.5	**0.004**	0.208	**0.045**
BW d 14, g	**9892**	10219	**10427**	**10453**	108.1	**0.001**	0.111	0.722
BW d 35, g	**18933**	19774	**20100**	**20036**	348.5	**0.048**	0.273	0.203
ADFI d 0-14, g/d	238.0	259.5	**286.6**	**297.0**	8.72	**0.000**	0.076	0.525
ADG d 0-14, g/d	174.4	199.8	**212.0**	**217.5**	8.04	**0.002**	0.063	0.224
FCR d 0-14	1.399	1.299	1.364	1.367	0.034	0.326	0.162	0.136
ADFI d 14-35, g/d	649.6	**721.5**	**722.5**	**741.5**	16.65	**0.008**	**0.010**	0.121
ADG d 14-35, g/d	360.9	393.3	407.0	402.5	28.28	0.335	0.625	0.519
FCR d 14-35	1.864	1.917	1.852	1.912	0.124	0.944	0.651	0.976
ADFI d 0-35, g/d	484.9	**536.7**	**548.1**	**563.6**	12.34	**0.001**	**0.010**	0.151
ADG d 0-35, g/d	286.3	315.9	328.9	328.5	16.73	0.107	0.390	0.375
FCR d 0-35	1.678	1.67	1.656	1.694	0.069	0.983	0.833	0.742

[a-b]Means within a row with different superscripts are different ($P < 0.05$).

CCC pigs = pigs from C sows received non-flavored creep-feed and non-flavored weanling diet.

CCF pigs = pigs from C sows received non-flavored creep-feed and flavored weanling diet.

FFC pigs = pigs from F sows received flavored creep-feed and non-flavored weanling diet.

FFF pigs = pigs from F sows received flavored creep-feed and flavored weanling diet.

7.4.2. Trial 2

Total creep-feed intake from d 10 until weaning (d 28) is presented in Table 7.3. Treatments with unflavored creep-feed (FCF and CCC) promoted a greater feed intake than flavored creep-feed diets early in the period d 12 to 16 and d 17 to 22; and during the entire period (days 12 to 28) ($P = 0.034$).

Table 7.3. *Daily intake of creep-feed (g·piglets-1·d-1) among the treatments during the suckling period (d 12 to 28, n = 8 per treatment; Trial 2).*

	Treatment (G&L+CF+W)			Pooled	*P*-values
ADFI	CCC	FCF	FFF	SEM	Treatment
12-16	8.87[a]	8.94[a]	1.80[b]	1.33	0.003
17-22	11.32[a]	14.75[a]	5.58[b]	1.61	0.004
23-28	24.14	18.31	9.37	5.98	0.195
12-28	14.46[a]	13.77[a]	5.22[b]	2.53	0.034

[a-b]Means within a row with different superscripts are different ($P < 0.05$).

CCC pigs = pigs from C sows received non-flavored creep-feed and non-flavored weanling diet.

FCF pigs = pigs from F sows received non-flavored creep-feed and flavored weanling diet.

FFF pigs = pigs from F sows received flavored creep-feed and flavored weanling diet.

The preference for Fluidarom (375 mg/kg of feed, positive control) over the lacto-vanilla (500 mg/kg of feed, negative control) was directly compared in piglets two days after weaning. The percentage of preference is reported in Figure 7.5. Piglets from the control group CCC showed higher preference for Lacto-Vanilla; while no preferences for any flavor were observed for piglets corresponding to FFF ($P > 0.1$). However, a preference ($P = 0.014$) for the Fluidarom 1003® diet was observed in FCF piglets.

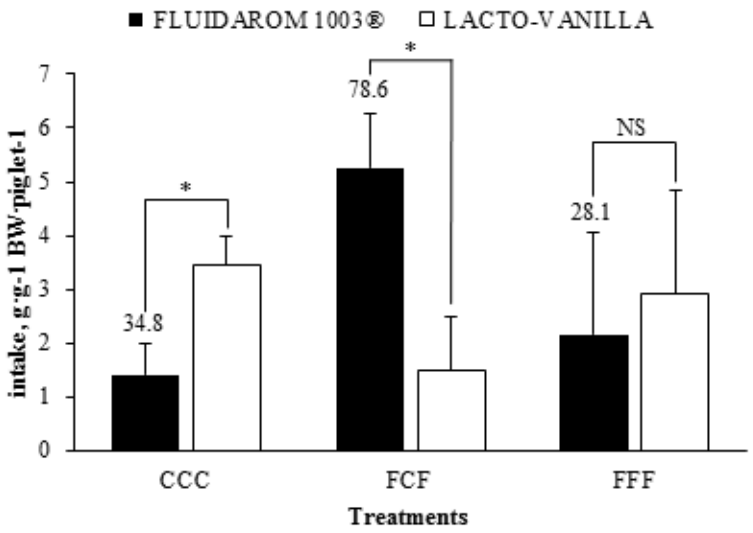

Figure 7.5. *Mean (+SEM) feed intake of the three treatments after 30 min; feed intake was corrected by BW and number of piglets. The number at the top of the bars indicates the average percent preference for Fluidarom (Norel, S.A., Madrid, Spain). Asterisks indicate that Fluidarom is significantly different than Lacto-Vanilla (Norel, S.A., Madrid, Spain) feed intake and preference, statistical comparison against the preference neutral value (50%; indifference; n = 6, Trial 2). NS P > 0.1; * P <0.05.*

7.5. DISCUSSION

The results showed that a mixture of flavors included in the sow diet improved growth performance of piglets after weaning. Greater feed intake and daily BW gain were observed during the pre-starter and starter phases (0 to 35 days post-weaning) mainly due to maternal exposure to the flavor. Flavor incorporation in weaning diets increased feed intake early after weaning but did not modify BW gain. In contrast to our hypothesis, no interactions were observed between the flavor inclusion in sow and piglets diets. However, the results of Trial 2 suggest that flavor inclusion in the sow diet modified feed preference after weaning. Wang et al. (2014) observed that adding a fruit-milk-anise flavor (with 9.4% anethol and 33.2% of eugenol) to the lactating sow diet, creep-feed, and into the weaning diet during the first 8 days, increased the ADFI and ADG of piglets when compared to the control.

Anethol, eugenol and cinnamaldehyde are phytogenic feed compounds extracted from garlic, clove and cinnamon, respectively (Mattson et al., 2011; Kim et al., 2013). Even though they are considered sensory additives and no demonstration of efficacy is

required by EFSA, the aim of using phytogenic additives in animal feeding is to improve zootechnical performance. Thus, botanically derived products have shown also benefits such as stimulation of digestive secretions and gut maturation, an enhanced immune response, microbiota modulation, and/or anti-inflamatory and antioxidant properties (Windisch et al., 2008). The supplementation with capsicum oleoresin, garlic or turmeric oleoresin to pigs reduced diarrhea and inflammation caused by *Escherichia coli* infection by decreasing gene expression associated with antigen presentation, and also reduced the adverse effects of porcine reproductive and respiratory syndrome by improving the immune responses of pigs (Liu et al., 2013a,b, 2014). The use of cinnamaldehyde with thymol improved feed intake, growth and immune status in pigs (Li et al., 2012a,b). In the present study it has also been observed an increase of feed intake after weaning with the presence of the flavor. Phenolic compounds, such as eugenol and thymol, have stronger bactericidal activities against foodborne pathogens (Burt, 2004). Farhath et al. (2013) also detected that eugenol enhanced the humoral antibody response, with effects that could depend on the specific dosages. It is generally accepted that a combination of more than one type of plant extract may elicit a stronger antimicrobial response than if only one type is used (Piccaglia et al., 1993). For example, cinnamaldehyde possess antioxidant, antimicrobial, and larvicidal activities (Lee et al., 2011), but cinnamaldehyde along with other plant extracts, such as carvacrol and capsicum, show synergistic enhancement of gut innate immunity against intestinal parasitic and bacterial infections. Although the mechanisms for these synergistic effects are unknown, they may involve morphological modification of gastrointestinal mucosal cells or altered expression of metabolism-related genes (Lillehoj and Lee, 2012). In the present study, the beneficial effects of adding the flavor in sows diets may indicate direct effects on the digestive tract, microbiota or the immunity response that deserve to be further studied.

The results of trial 2 showed that prenatal flavor in the diet affected feed preference of piglets after weaning. The basis of this effect could be related to the transference of some volatile compounds to the womb and/or the milk, producing a conditioning process that may occur inside the womb or an effect of familiarity due to repeated exposure. Greater feed intake after weaning could also be related to a stress-reducing effect by piglet exposure to a familiar flavor acquired before weaning (Oostindjer et al., 2011). This could explain the beneficial effects in piglets exposed

prenatally to flavor but not in the post-weaning (Trial 1), when considering that piglets shared the same environment and cross-contamination of volatiles within the room could have been occurred. This effect has generally been accepted in other mammals such as humans (Varendi et al., 1998) and rats (Smotherman, 1982).

In fact, the detection of anethol, cinnamaldehyde and eugenol as the most influent compounds amongst other in the amniotic fluid of sows fed the flavored diet (Figure 7.3 and 7.4), confirms that prenatal exposure may be a powerful pathway for maternal flavor-conditioning of newborn piglets, and may be enough to establish a link between prenatal life and the post-weaning period. On the other hand, only traces of anethol, cinnamaldehyde and eugenol were detected in milk indicating a lower transference of these compounds by this pathway. Oostindjer et al. (2010) observed that pre-natal exposure, but not postnatal exposure alone, to aniseed flavor through the maternal diet increased feed intake and higher body weight after weaning in prenatally exposed animals. Mennella et al. (1995) evaluated the flavor in amniotic fluid by a sensory panel and observed that some volatile compounds, like garlic consumed by pregnant women significantly alter the odor of their amniotic fluid. However, Hausner et al. (2008) observed that volatiles from the maternal diet are transferred selectively and in relatively low amounts to breast milk.

Piglets, during lactation, consumed more non-flavored than flavored creep-feed. The results show that the inclusion of an exogenous flavor in the creep-feed may reduce feed intake during lactation, as compared to those piglets fed a non-flavored diet. Figueroa et al. (2013) also observed that suckling piglets did not prefer a creep-feed supplemented with an anise or garlic flavor when compared to an unflavored diet, even if it had previously been given to the sows. It seems that during the lactating period piglets preferred a simple, unflavored diet (cereal base, without added flavors). Moreover, Sulabo et al. (2010) reported that flavor supplementation to creep-feed diets did not improve daily creep-feed intake, ADG or feed efficiency. The high content of dairy by-products in the creep-feed, like milk whey, lactose and even skimmed milk, may be one of the main reasons to explain why piglets preferred a plain creep-feed over a flavored, modified diet.

Prenatal olfactory learning has been demonstrated in rats (allyl-sulphide) (Hepper, 1988), humans (aniseed) (Schaal et al., 2000), (carrot) (Mennella et al., 2001), dogs

(anise) (Wells and Hepper, 2006), sheep (oregano) (Simitzis et al., 2008), rabbits (juniper) (Bilkó et al., 1994) and pigs (aniseed and milky-cheese flavors [counterbalanced]) (Figueroa et al., 2013), where flavors in the maternal diet lead to a preference for these flavors post-natally. Feed preference in Trial 2 follows the same direction, as piglets perinatally exposed to the flavor and without the flavored creep-feed had a preference for the flavor studied. The results also suggest that flavor inclusion in the creep-feed may exert a negative effect on the newborn piglet, showing a greater variability and inconsistent post-weaning feeding behavior in the feed choice study. So, regarding creep-feed results, it can be concluded that: 1) by giving the piglets an unflavored creep feed, their intake is higher than by giving them a flavored one (not related to maternal flavor exposure) and 2) flavored creep feed may erase the effects of a prenatal maternal learning. Consequently, and based on these results, it could be suggested that in order to prepare piglets with a strategy to promote dietary flavor familiarity with their mothers, it is recommended not to include the flavor in the creep-feed.

Even though many studies have examined perinatal flavor-learning in many species over the last four decades, there is still little information regarding the mechanisms affecting flavor-learning and how perinatal flavor-learning may improve feed acceptance in piglets (Oostindjer et al., 2010). Our results indicate that conditioning may be stronger during late gestation rather than during lactation. As was previously reported in dogs (Wells and Hepper, 2006) and in pigs (Oostindjer et al., 2010), postnatal exposure through milk alone did not show a preference for a new flavor. This result could be explained because piglets are less sensitive to postnatal modifications, as suggested by Oostindjer et al. (2009).

Inclusion of this flavor in the sow diet improved piglet's post-weaning performance, regardless of post-weaning diet. Suggesting that there are other physiological mechanisms involved that deserve further study. However, post-weaned piglets preferred the flavored feed over a different one. Young animals can learn about flavors from the maternal diet that appear in the amniotic fluid; in addition, these preferences acquired before birth are long-lasting. In conclusion, adding certain flavors during late-gestation and lactation could be used as a strategy to enhance voluntary feed intake and performance during weaning. A good strategy appears to be the incorporation of a flavor in sows (gestation and lactation) and post-weaning piglet diets, but not in creep-feed diets.

CHAPTER 8

The effects of including increasing doses of stevia and neohesperidine dihydrochalcone on feed preference in young piglets

Chapter 8

The effects of including increasing doses of stevia and neohesperidine dihydrochalcone on feed preference in young piglets

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8.1. ABSTRACT

Two double-choice feeding experiments were conducted to study the effect of stevia extracts and neohesperidine dihydrochalcone on feed preference in piglets. Pigs (14 to 35 d post-weaning) were offered a series of double choices between a common reference diet (**R**, containing 4% sucrose) and experimental diets (containing 4% maltodextrin plus different doses of SE and SE+NHDC). In Exp. 1, the doses of SE were 0, 100, 200, 300, 400 and 500 mg/kg (T1 to T6); in Exp. 2, the dose of SE was 150 mg/kg plus 0, 2, 3, 4 or 5 mg/kg NHDC (T1 to T5). The two diets (R and T1 to T6) were offered to pens of 3 piglets following a choice test protocol (n = 12 for each comparison). Feed intake and preference (relative intake of a given feed when offered as a double choice with a reference feed) for each diet were calculated. In Exp. 1, piglets in the comparisons of 0 and 100 mg/kg SE showed no preference or avoidance when contrasted with the R diet. However, feed with higher levels of SE (200, 300 and 500 mg/kg SE) were avoided (preferred less than 50%). In Exp.2, diets with 150 mg/kg SE plus 0 or 5 mg/kg NHDC were not preferred or avoided relative to the R diet, whereas diets with either 2, 3 or 4 mg/kg NHDC were preferred ($P < 0.05$) when contrasted with the R diet, following a quadratic response ($P = 0.08$). In conclusion, the combination of 150 mg/kg SE with 2-4 mg/kg NHDC in starter diets increases feed intake and preference (relative to 4% of sucrose) as compared to diets including only SE.

8.2. INTRODUCTION

It is well known that pigs have an innate preference for sweet (Kennedy and Baldwin, 1972) or umami taste (Tedo, 2009), and they reject bitter, sour taste or new and unfamiliars flavors (Blair and Fitzsimons, 1970). Sweeteners are defined as feed additives that are used to impart a sweet taste, which it is known that improve feed palatability and zootechnical performance in piglets (Yebra-Biurrun, 2005). Selection of new additives to encourage feed intake at weaning will contribute to prevent digestive problems related with weaning (Solà-Oriol, et al., 2009). Stevia extract is a natural sweetener extract derived from the plant *Stevia rebaudiana* Bertoni containing diterpene glycosides. The major glycosides are stevioside, rebaudioside A, rebaudioside D, and dulcoside, that are low calorie sweeteners, about 300 times sweeter than saccharose in humans (Lemus-Mondaca et al., 2012). However, some additives with stevia extract

less purified might contain high-saponin plants that could reduce the diet palatability (Clouard and Val-Laillet, 2014). Neohesperidine dihydrochalcone is a non-caloric sweetener that is 300 to 500 times sweeter than sucrose for humans (Baêr et al., 1990) although pigs do not seem to detect as sweet (Glaser et al., 2000). Feed preference between two diets can be measured with a two-choice test, in which pigs are offered both compounds simultaneously and the proportional intake of each feed is measured (Solà-Oriol, 2008).

The aim of the present study was to investigate how piglets respond to *Stevia rebaudiana* Bertoni extracts and the combination of Stevia extracts and NHDC when offered in a series of double choices with a reference feed. We hypothesized that exposure to Stevia Extracts and SE and NHDC will modify feed preference when contrasted with a reference diet containing sucrose (4%).

8.3. MATERIAL AND METHODS

Experimental procedures were approved by Ethical Committee on Human and Animal Experimentation of the UAB (CEAAH 1406).

A total of 396 piglets ([Large White x Landrace] x Pietrain) were used during the starter phase (14 to 35 d post-weaning), 216 piglets for Exp 1 (to study the preference for different levels of stevia extract) and 180 piglets for Exp. 2 (to study the preference for SE plus different doses of NHDC). After weaning, animals were offered *ad-libitum* a commercial pre-starter diet for the first 14 d and the experimental starter diets for the next 21 d post-weaning. At d 14, piglets were randomly selected in the range between 14 to 16 kg, according to a homogenous BW and were distributed into 12 pens (3 piglets/pen) in order to start the experimental phase. Each experimental phase was as follows: from d 3 to d 7; at the start (d 3) and at the end (d 7) of each phase all of the animals were weighed in order to calculate the feed intake expressed as g/kg BW. Piglets were adapted to the experimental conditions from d 1 to d 2, by offering them the commercial starter diet in two different mini hopper pan feeders (Rotecna, Agramunt, Lleida). From d 3 to d 7 the two experimental diets: the reference diet (**R**) and the experimental treatments T1 to T6 (Exp. 1) or T1 to T5 (Exp. 2) were offered *ad-libitum* to each pen following a two-choice test. On d 5, feeder position inside the pen, left or right, was changed in order to avoid side-effects. Feed disappearance from each feeder was measured on d 7. At the end of the experimental

phase those animals were removed and changed for other naïve animals to repeat the same protocol. The procedure described above was repeated for 6 times for Exp. 1 and Exp. 2. Each experimental phase included all treatments to be tested within an experiment (n = 2 per treatment and phase and 6 phases per experiment; n = 12 per treatment).

8.3.1. Diets

A single basal premix diet (96% of the complete diet) was prepared in order to produce the reference diet (R; basal diet + 4% of sucrose) and the treatments diets (basal diet + 4% of Maltodextrin + SE +/or NHDC). The basal diet was formulated to contain 69% of cereal (maize, barley and wheat), 9% of soybean meal, 18% of extruded soybeans, amino acids, vitamins and minerals to meet or exceed the animals' requirements (NRC, 2012). Maltodextrin, a polysaccharide composed of chains of glucose molecules with a dextrose equivalent of less than 20 (Wang and Wang, 2000), was used to equilibrate the energy and carbohydrate content of treatment diets. For Exp. 1 a total of 6 different treatments were prepared according to the increasing levels of stevia extract in feed: basal diet + 4% of maltodextrin + x mg/kg SE; x = 0 (T1), 100 (T2), 200 (T3), 300 (T4), 400 (T5) and 500 (T6). For Exp. 2 also a total of 5 different treatments were prepared according to the increasing levels of NHDC in combination with 150 mg/kg of stevia extract: basal diet + 4% Maltodextrin + 150 mg/kg SE + x mg/kg NHDC; x = 0 (T1), 2 (T2), 3 (T3), 4 (T4) and 5 (T5).

8.3.2. Experimental Products

The experimental products were PureCircle® (Stevia extract) composed of > 75% Total Steviol Glycosides (> 40% Stevioside and >15% Rebaudioside A), Oak Brook, Illinois, US. Neohesperidine dihydrochalcone, Interquim S.A., Spain. Sucrose, AB Azucarera Iberia S.L, Spain. And C*Pharm™ Dry (Maltodextrin) Cargill, Minneapolis, Minnesota, US.

8.3.3. Statistical Analysis

Standardized Feed Intake and % of preference (relative intake of a given experimental feed when offered as double choice with the reference diet) were analyzed with ANOVA using the MIXED procedure of the statistical package SAS® (version 9.3,

SAS Institute; Cary, USA), a linear and quadratic response was also analyzed by using the IML and GLM procedures of the statistical package SAS®. The statistical unit was the pen of three piglets. Additionally, the preference values for the treatments were compared to the neutral value of 50% by using a Student's t-test procedure of the statistical package SAS®. Feed Intake and preference results are presented as Least Square Means taking into account Tukey adjust. All the results used the alpha level (0.05) for the determination of significance.

8.4. RESULTS

8.4.1. Exp. 1 (increasing SE doses)

The intake and preference (%) for the different levels of Stevia extract when contrasted with the reference diet are presented in Figure 8.1. Piglets in the control comparative T1 (0 mg/kg SE) and T2 (100 mg/kg SE) showed no preference or avoidance when contrasted with the R diet. On the other hand, feed with (200, 300 and 500 mg/kg of SE, respectively) were avoided (preferred less than 50%, $P < 0.01$) relative to the R diet. The evolution of dose-response of SE was linear ($P < 0.01$) and quadratic ($P < 0.01$).

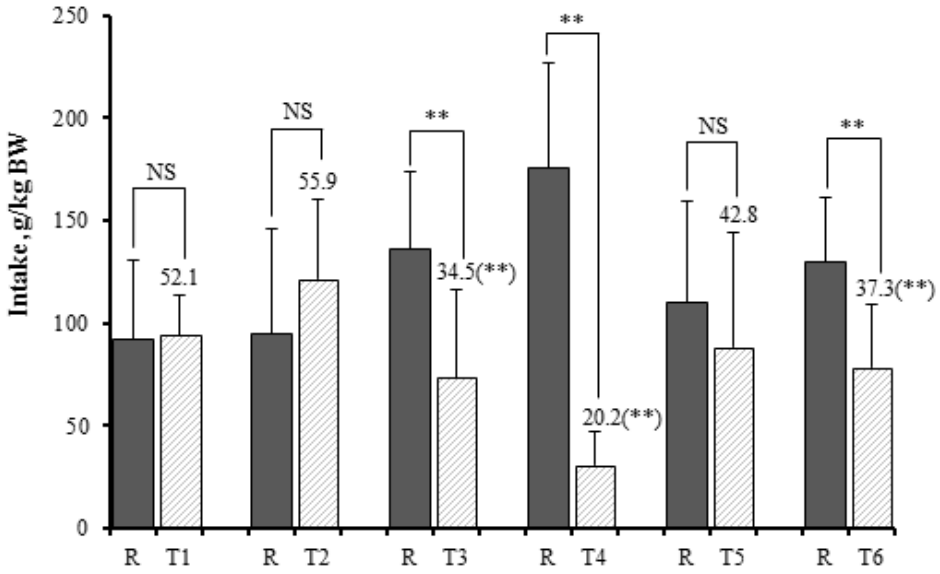

Figure 8.1 *Average feed consumption for a 5 d period and feed preference (number on top of the bars) for the different dietary treatments (T1 to T6) in Exp.1 as the % of contribution to the total feed intake, statistical comparison against the preference neutral value (50%). NS: P > 0.1; **:P < 0.01, R: 4% sucrose. T diets included 4%*

maltodextrin plus 0 mg/kg SE (T1); 100 mg/kg SE (T2); 200 mg/kg SE (T3); 300 mg/kg SE (T4); 400 mg/kg SE (T5); or 500 mg/kg SE (T6). Asterisks in parenthesis indicates different preference values against the preference neutral value (50%). Asterisks on brackets indicates different feed intake between T and R diets.

8.4.2. Exp. 2 (SE + increasing NHDC doses)

According to the SE response in Exp 1, an inclusion level of 150 mg/kg SE was selected to combine with increasing doses of NHDC. The intake and preference for SE and NHDC compared to the Reference diet are presented in Figure 8.2. Diets with 150 mg/kg SE + 0 or 5 mg/kg NHDC were not preferred or avoided relative to the R diet. In the other hand, diets with 150 mg/kg SE + 2, 3 and 4 mg/kg NHDC were preferred (P <0.05) relative to the R diet. The evolution of dose-response of SE+NHDC was quadratic (P = 0.08).

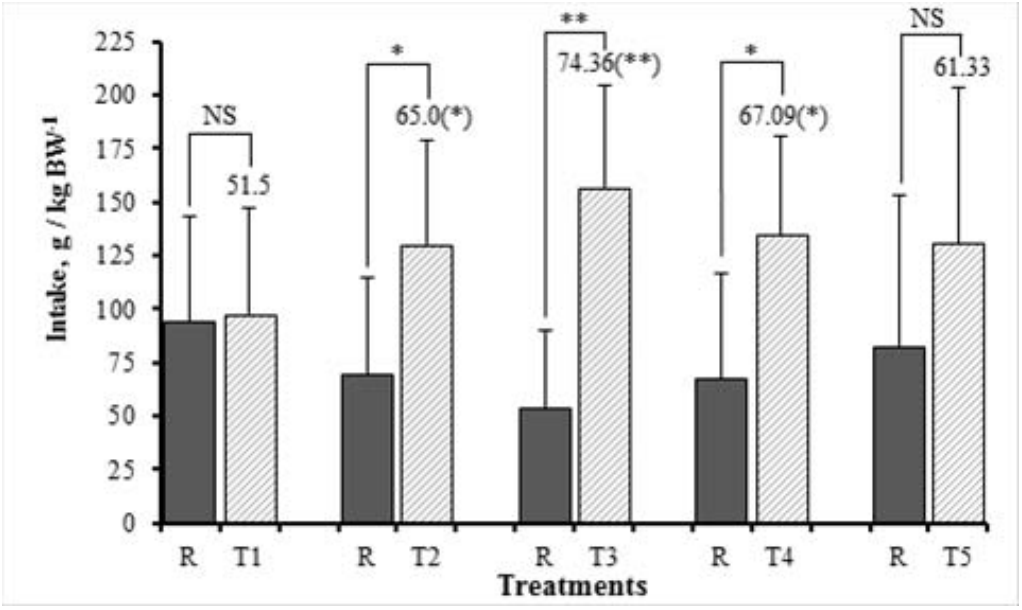

Figure 8.2 *Average feed consumption for a 5 day period and feed preference for the different dietary treatments (T1 to T5) in Exp. 2 as the % of contribution to the total feed intake (number on top of the bars), statistical comparison against the preference neutral value (50%). NS: P > 0.1; *:P < 0.05; **:P < 0.01, R: 4% sucrose. T diets included 4% maltodextrin plus 150 mg/kg SE plus 0 mg/kg NHDC (T1); 2 mg/kg NHDC (T2); 3 mg/kg NHDC (T3); 4 mg/kg NHDC (T4); 5 mg/kg NHDC (T5). Statistics symbols of preference values describe significance of comparison against the*

preference neutral value (50%). Statistical symbols on brackets describe feed intake differences between T and R diets.

8.5. DISCUSSION

According to our data, an inclusion of SE may decrease palatability. Moreover, the higher the SE inclusions (200, 300 and 500 mg/kg) the less preferred were the SE diets.

Pigs have 3-4 times more taste buds than humans (Chamorro et al., 1993), indicating that pigs have a superior sensorial capacity and could detect odd-flavors associated to SE at lower concentrations. In addition, pigs appear to taste stevioside as sweet, but not with strong responses (Danilova et al., 1999). The SE tested contained a minimum of 75% steviol glycosides (stevioside and Rebaudioside A). In humans, the different steviol glycosides have different taste properties. Stevioside and Rebaudioside A are 110-270 times and 150-320 times sweeter than sucrose, respectively; but both produce a bitter after taste at high concentrations (Bassoli and Merlini, 2003). It is known that steviol glycosides activate two bitter receptors (hTAS2R4 and hTAS2r14), and thus elicit a bitter taste (Allen et al., 2013). Moreover, Clouard and Val-Laillet (2014) found that their feed additive with 90% steviol glycosides also contained extracts of high-saponin plants, which reduce diet palatability in pigs due to an off-after-taste.

In Exp. 2, the combination of 150 mg/kg SE with increasing doses of NHDC (2, 3 and 4 mg/kg) enhanced feed preference (relative intake to the R diet) as compared to the diet including 0 mg/kg of NHDC. It is common in food industry to combine different sweeteners to provide synergistic effects as most non-caloric sweeteners present an unpleasant off-after-taste, e.g. saccharin, NHDC, acesulfame-k, aspartame, cyclamate. For example, rats and mice prefer saccharin to rebaudiose A, but the combination of erythriol and rebaudiose A enhanced the attractiveness of the mixture and stimulate intake (Sclafani et al., 2010).

In the present study, the use of NHDC may have stimulated the sweet potency of SE while covering up the bitter after-taste. In the European Union the use of NHDC at low concentrations (up to 3 mg/kg) may be used as flavoring substance (Regulation EU No. 872/2012). It has been observed that NHDC has the ability to decrease the perception of bitterness, saltiness, sharp and spicy attributes (Bassoli and Merlini,

2003). In our results, a quadratic response was observed, which could indicate a possible saturation level of the sweet perception at 5 mg/kg of NHDC.

It is concluded that the combination of 150 mg/kg SE with 2-4 mg/kg NHDC in starter diets increases feed intake and preference (relative to 4% of sucrose) as compared to diets including only SE.

CHAPTER 9

General discussion

Weaning is regarded as one of the most critical periods in pig production, which is often accompanied by a severe growth check, characterized by low feed intake and poor growth, and diarrhea. It is well established that this process is multifactorial, and that post-weaning anorexia and undernutrition are major aetiological factors (Pluske et al., 1997; Lallès et al., 2004). In addition, pigs have a limited digestion capacity due to its low acid secretion. For suckling pigs, its principal source of acidity is the bacterial fermentation of lactose from sows' milk to lactic acid (Cranwell et al., 1976; Kidder and Manners, 1978). After weaning, the problem gets worse because of the low levels of lactose subtract and consumption of large meals at infrequent intervals followed by an increase of stomach pH over 5.0 that may remain high for several days (Kidder and Manners, 1978). For this purpose, in this thesis we have investigated different strategies to ameliorate the post-weaning growth check in pigs. In general, we have observed that Zn supplementation during the suckling period, low Ca levels at weaning, maternal learning and the combination of Stevia with NHDC as feed taste enhancers have positive effects on feed intake or growth performance. Thus the aim of this general discussion is to integrate all the results reported in this thesis to highlight the most interesting findings.

Feed intake can be depressed due to alterations of nutrients in the diet, e.g. Zinc deficiency. In infants, poor appetite and impaired taste acuity are associated with decreased concentration of Zinc (Teegarden and Gunther, 2008). It has been described that pigs suffer a deficit of Zn after weaning (Davin et al., 2013) that could limit the functional and immunologic response of pigs. In the present thesis it has also been observed a decrease of serum Zn levels after weaning, but only in pigs fed nutritional levels of Zn in the diet and especially, in light piglets which had lower serum Zn values longer than heavy piglets. The low Zn concentration in light pigs may exert a negative impact on long-term growth; and may also increase mortality rate. One of the major causes of morbidity and mortality after weaning is related to post-weaning diarrhea, specifically enterotoxigenic *Escherichia coli* corresponding to 50% of the mortality in pigs (Gyles, 1994). In our results, we showed for the first time in pigs that the low serum Zn levels at weaning are a predisposing factor of diarrhea, which it is in agreement with the direct link between Zn deficiency and diarrhea described in infants (King et al. 2016).

It has to be highlight that light pigs showed lower serum Zn levels than heavy pigs at weaning. This result was confirmed in 3 trials reported in chapter 4. One possible explanation is that heavy pigs usually suck from anterior teats, which are the most productive (Rosillon-Warnier and Paquay, 1984); and thus they may consume more milk than light pigs. But, we also observed that serum Zn concentration in piglets decreases as lactation progresses, suggesting that the capacity of sow's milk production is not enough to reach pigs' Zn requirements during lactation. It could be suggested that milk Zn concentration might decrease during lactation like it has been reported in woman (Lönnerdal et al., 1981b; Donangelo and King, 2012), rats (Keen et al., 1981), dogs (Lönnerdal et al., 1981a) or, dairy animals (de Maria and Angelucci, 1978), but, not sows during the first 3 weeks of lactation (Hill et al, 1983).

As Zinc is required for multiple functions, zinc deficiency affects physical growth, immune competence, reproductive function, neurobehavioral development (King, 2011; Grider, 2013), and digestive function (Pallauf and Kirchgessner, 1976; Roth et al., 1992). Also limiting level of serum zinc may lead to anorexia, loss of appetite, smell and taste failure, and impaired wound healing (Chasapis et al., 2012). Therefore, light pigs in the litter may be at a higher risk to evolve towards Zn deficiency and thus may imply having poor growth, loss of appetite and have more risk to develop diarrhea, and thus to enter in a vicious circle of growth retardation and low feed intake. Recently, Zhang et al. (2015) found that metabolism of Zn in intra-uterine growth retardation pigs was inferior, which most likely due to simultaneously reduced feed intake. As consequence, we have proposed in this thesis to explore if light piglets may respond to a special Zn reinforce before weaning. To improve the Zn levels at weaning, we designed an experiment to supplement pigs with Zn Citrate during the last week of lactation. Although all pigs had a decrease of serum Zn levels after weaning, pigs supplemented with 30 mg of Zn did not present deficient levels of Zn, established as < 0.7 mg/L of Zn (Hotz and Brown, 2004; Maret and Sandstead; 2006). Moreover, the greater response in serum Zn levels was on light piglets. It is noteworthy however, that supplementation of light pigs with 18 and 30 mg of Zn was equivalent to 370 and 617% total Zn in the metabolic BW, as compared to lower equivalent metabolic BW values for heavy piglets (250 and 416%, respectively). Then, the same dose was higher for light than for heavy pigs.

Another mineral that can compromise feed intake is Calcium. It has been reported that high Ca levels depress feed intake (Lei et al., 1994), weight gain and, feed efficiency (Lei et al., 1994; Fan and Archbold, 2012; González-Vega, et al., 2016). As well, in this dissertation it has been observed that increasing concentration of Ca in diets reduced growth performance (decrease in BW, ADG and increased FCR), but the effect was more evident with in-feed pharmacological levels of Zn than without. High Ca levels also reduced feed intake but only when the diet was not supplemented with therapeutic levels of Zn. The negative effect of Ca in growth performance could be explained by different mechanisms. Pigs fed with high dietary Ca level (limestone) may increase the pH in the gastrointestinal tract and the increased pH favors formation of insoluble Ca-Phytate complexes (Selle et al., 2009), decrease phytate solubility (Selle and Ravindran, 2008), or reduce microbial phytase activity (Selle et al., 2009). It has been described that Ca-Zn-phytate complexes may precipitate likely affecting the growth performance by reducing P absorption. When two cations are presented simultaneously at high levels, as Ca^{+2} and Zn^{+2}, they act together to increase the quantity of phytate precipitation (Simpson and Wise, 1990). Also, multiple mineral complexes, such as Ca-Zn-phytate, are more stable than single mineral complexes, such as Ca-phytate or Zn-phytate (Maenz et al., 1999).

Therefore, an experiment was carried out to observe that high Zn levels may affect Ca and P absorption. It has been previously described that pharmacological levels of ZnO reduced the Ca and P absorption (Meyer et al., 2002). However it has never been studied on a standardized digestibility basis. Using standardized digestibility values rather than apparent digestibility values to formulate diets may be more accurate for nutrients that have endogenous losses in the intestinal tract (ie. Ca and P), because these values are additive in mixed diets (Stein et al., 2005). In this dissertation it has been observed a reduction in ATTD, STTD and retention of Ca and, ATTD and retention of P as ZnO was added to the diet, confirming that Zn has a negative effect on absorption and retention of Ca and P. Moreover, the response of supplementing the diets with phytase (1,000 and 3,000 FTU) was also tested. Phytate is the main storage for P in plants (Selle et al., 2009), and also an antinutritional factor that has the capacity to chelate bivalent cations such as Zn^{+2} and Ca^{+2} (Persson et al., 1998; Maenz et al., 1999; Selle et al., 2009). Phytase hydrolyzes the bond between phytate and P and may enhance macro- and trace mineral availabilities (Selle and Ravindran, 2008). Another

factor to take into consideration about using phytase is that may improve the efficacy of ZnO and allow for a reduction in starter diets, due to an increased Zn availability (Walk et al., 2013). We observed that the positive effect of phytase on ATTD and STTD of Ca was reduced if ZnO was added to the diets; Martínez et al. (2005) also reported a lack of response to phytase but on growth performance if pharmacological levels of ZnO are supplemented to the diets. It may be hypothesized that high dietary Zn may increase pH in the gastrointestinal tract (**GIT**), reduce phytate solubility, and reduce phytase efficacy to increase the ATTD and retention of Ca and P.

However, it could be hypothesized that not all post-weaning GIT disorders result from an alteration in nutrition (e.g. feed type, feed composition), but also from changes in enteric microbiota (e.g. density, location, pathogenicity) and mucosa of the GIT (e.g. type of receptors, mucin, inflammation activity, cytokine production), the so-called gut Health (Pluske et al., 2007a). Therefore, we evaluated the gene expression, microbiota and the morphology of different sections of the GIT in diets with high and low Ca levels. In the present dissertation, increasing Ca levels showed higher expression of genes related to inflammatory processes (e.g. IFNα, and INFγ) in the jejunum of weaned pigs. At weaning there is an acute response with increased gene expression of inflammatory cytokines (IL-β, IL-6. TNF-α; Pié et al., 2004). These genes appears to be upregulated with high Ca diets, likely explaining why pigs fed diets with high Ca levels showed lower BW gain than pigs fed low Ca diets after weaning. In contrast, Metzler-Zebeli et al. (2012) observed that high Ca and P levels, supplemented as limestone and dicalcium phosphate, downregulate the expression of proinflammatory cytokines and increase digestive and absorptive functions but affect negatively the intestinal microbiota by decreasing gastric streptococci and lactate (Metzler-Zebeli et al., 2011) and, decrease the crypt depth on the cecum (Metzler-Zebeli et al., 2012). Changes in diet composition may promote an alteration the composition of the microbiota and its metabolic activities. We also observed an alteration of colon microbiota but not in jejunum morphology. Although few differences were observed on microbiota, the high Ca diet showed a higher heterogeneous community and an increased on the *Bacteroides* genera (within the 5% less abundant genera) compared with a more homogeneous community in pigs fed LCa diet. An increase of *Bacteroides* genera in humans has been associated with Western diets, high in animal protein and saturated fats, and related to a higher incidence of diseases (Wu et al., 2011).In pigs, this genus is naturally present in

higher amounts during suckling period (Mach et al., 2015; Bian et al., 2016) and has been shown to correlate negatively with body weight (Mach et al., 2015). This result may possible add a new mechanism by which pigs fed HCa diets presented less growth than pigs fed LCa diets.

In previous paragraphs we have discussed the balance and interactions among nutrients, such as Zinc and Calcium. Some of these nutrients, such as for example Zn are known to have a major role on the development and functionality of olfactory receptors (Wang et al., 2003) and low Zn levels are link to taste disturbances (Giudice, 2006). We speculated that light piglets in the litter may be at a higher risk to evolve towards Zn deficiency and thus may imply having poor growth, loss of appetite and have more risk to develop diarrhea, and thus to enter in a vicious circle of growth retardation and low feed intake. Pigs have a highly developed sensorial capacity with a greater number of functional olfactory receptor genes (Groenen etal., 2012) and taste buds (Chamorro et al., 1993) compared with other mammal species. Thus, piglets respond positively to palatable feedstuff at weaning which could be a successful strategy to improve first feed contact (or reduce lack time to solid feed intake) and avoid further anorexia. A relatively high feed intake following the period of underfeeding may exceed the digestive or absorptive capacity and cause an overload of undigested nutrients triggering undesirable microbial activity (Makkink et al., 1994). Therefore, it is important for piglets to eat some feed early after weaning. In the present study we also explored the effect of offering diets with flavors and taste enhancers to increase palatability and stimulate, facilitate and promote feed intake after weaning. Maternal learning was also studied as a strategy to take profit of the link between the mothers' diet and the weaning diet to allow piglets recognize the new weanling diet as safe to eat.

Therefore, in Chapter 7 we used the maternal learning as strategy to increase feed intake after weaning which is based on learning from volatile compounds added to the maternal diet. It has been suggested that those compounds from the maternal diet in uterus can reach the fetus through the amniotic fluid and/or the placental blood stream. This early exposure of fetus to certain cues generally may result in a preference for these flavors later in life and consequently can positively affect the acceptance of food with a similar flavor before and after weaning (Hepper, 1988; Mennella et al., 2001; Figueroa et al., 2013). Prenatal olfactory learning has been demonstrated in several mammals such as rats (Hepper, 1988), dogs (Wells and Hepper, 2006), humans

(Mennella et al., 2001), pigs (Figueroa et al., 2013) etc. where flavors in the maternal diet lead to a postnatal preference for these flavors. However, we detected for the first time the volatile compounds in the amniotic fluid as a link between the intrauterine events and extra uterine responses to flavors. In addition, the inclusion of flavor in the sow diets improved piglet consumption and growth after weaning and the positive reward associated with the flavor included in the sow diet was stronger when pigs were offered a non-flavored creep-feed. The flavor used was a commercial flavor with anethol, eugenol and cinnamaldehyde as principal components, which also are phytogenic feed compounds extracted from garlic, clove and cinnamon, respectively (Mattson et al., 2011; Kim et al., 2013). Then, it is possible that the beneficial effects of adding the flavor in sow's diet may indicate direct effects on the digestive tract, microbiota, or the immunity response due to its phytogenic activity. However the detection of the principal components in the amniotic fluid and the preference showed by piglets towards the flavor included in the sow feed in a choice test after weaning, suggest that maternal learning may also contribute to the beneficial results of piglets after weaning.

Taste enhancers may be also useful to enhance feed palatability. Ii is widely described that pigs have an innate preference for sweet (Kennedy and Baldwin, 1972), therefore, the use of sweeteners may improve feed palatability and zootechnical performance (Yebra-Biurrun, 2005). Stevia extracts is a natural non-nutritive sweetener, the steviol glycosides from SE are 300 times sweetener than sucrose, but produce bitter after taste due to activation two bitter receptors (Allen et al., 2013) that pigs can detect (de Oliveira et al., 2007). In this dissertation, pigs preferred sucrose than SE, thus the supplementation of SE alone may decrease feed palatability (Chapter 8). In this thesis we tested the combination of SE and NHDC in pigs. NHDC is a semisynthetic non-nutritive sweetener and a flavor enhancer (Bassoli and Merlini, 2003) that decreases the perception of bitterness in humans. Pigs preferred the combination of SE and NHDC over sucrose which is highly preferred for pigs; thus suggesting that combination of SE and NHDC enhanced feed palatability. The use of NHDC may have stimulated the sweet potency of SE while covering up the bitter aftertaste due to NHDC has the ability to decrease the perception of bitterness, saltiness, and sharp and spicy attributes (Bassoli and Merlini, 2003).

CHAPTER 10

Conclusions

From the results presented in this dissertation, three blocks with its conclusions can be drawn:

Zinc deficiency: consequences and supplementation before weaning:

1) The administration of nutritional Zn levels lead to a temporary decrease in serum Zn levels after weaning. The decrease was more evident in light piglets, which also showed higher mortality and lower long-term growth performance.
2) Lactation was inadequate to meet piglets' Zn requirements, and a decay in the piglets serum Zn levels was observed, especially in the light piglets of the litter. A lower level of serum Zn at weaning was associated with an increased risk of suffering diarrhea after weaning.
3) Daily supplementation with 30 mg of Zn per piglet during the last week of lactation mitigated the fall of Zn during lactation.

High Calcium levels: negative impacts and the mineral interaction with phytate:

4) High dietary Ca level (0.95% of Ca; 1.55% of limestone) decreased growth performance (BW, ADG and feed efficiency) but no feed intake in weaned pigs when pharmacological levels of ZnO were supplemented. High dietary Ca levels upregulated the expression of genes related to the inflammatory response (IFNα, and INFγ) in the jejunum, higher heterogeneous microbial community and an increased the abundance of *Bacteroides* in colon.
5) Pharmacological levels of Zn (2,525 mg/kg of Zn) in diet reduced Ca and P digestibility, but the addition of 1,000 FTU microbial phytase ameliorates this negative impact. Interaction between Zn, Ca, P and phytate take place in the intestinal tract of pigs with negative consequences after weaning.
6) The inclusion of 1,000 FTU microbial phytase increased the ATTD and STTD of Ca and also the ATTD of P, confirming that dietary phytate interferes with Ca and P digestibility.

The utilization of different strategies to increase feed intake after weaning, by flavors and sweeteners:

7) Anethol, cinnamaldehyde and eugenol (main compounds of Fluidarom 1003®) were detected in amniotic fluid, but only traces were observed in milk, as a link to prenatal learning.

8) Inclusion of the flavor in the sow diet improved piglet's post-weaning performance, regardless of post-weaning diet. Post-weaning piglets preferred the flavored feed over a different one. Young pigs can learn about flavors from the maternal diet that appear in the amniotic fluid, and these preferences acquired before weaning are long-lasting.
9) Combination of 150 mg/kg SE with 2-4mg/kg NHDC in starter diets increased feed intake and preference (relative to 4% of sucrose) compared to diets including only SE.

CHAPTER 11

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ANNEX 1

Curriculum vitae of the author

Personal information

Surname, Name:	Blavi Josa, Laia	**Nationality:**	Spanish
E-mail:	laiablavi@gmail.com	**Date of birth:**	28/01/1988

Education

2014-present **Ph.D. Student in Animal Science**
Universitat Autònoma de Barcelona

2012-2013 **M.Sc. in Research in Animal Nutrition**
The Mediterranean Agronomic Institute of Zaragoza
Universitat Autònoma de Barcelona

2011-2012 **Postgraduate Specialization in Animal Nutrition**
The Mediterranean Agronomic Institute of Zaragoza

2006-2011 **B.Sc. in Veterinary Medicine**
Universitat Autònoma de Barcelona

Post-graduate courses

2016 **Training course on statistical techniques with R**
Servei d'Estadística Aplicada,UAB

2015 **English course (B2.2)**
Servei de Llengües, UAB

2015 **Generic Emergency Training**
Universitat Autònoma de Barcelona

2014 **Training course for researchers on animal experimentation and other scientific purposes**
Universitat Autònoma de Barcelona

2014 **English course (B2.1)**
Servei de Llengües, UAB

2013 **Biological Agents: Risk and Preventive Measures**
Universitat Autònoma de Barcelona

Professional experience

2012-present **Member of the Animal Nutrition and Welfare Service (SNiBA)**
Universitat Autònoma de Barcelona (Bellaterra)

- Collaboration in several research projects (experimental design, farm controls, laboratory analyses, statistical analyses and writing technical reports)

2016 **Member of the Stein Monogastric Laboratory** (6 month)
University of Illinois at Urbana-Champaign

- Collaboration in different research projects (experimental design, farm controls, laboratory analyses, statistical analyses and writing technical reports)

2012 **M.Sc. practice** (1 month)
Vall Companys (Lleida)

- Nutritionist practice

2011 **Student practice**
Animal Nutrition and Welfare Service (SNIBA)

2010 **Degree practice** (3 month)
Vall Companys (Lleida)

- Veterinary practice

2009 **Veterinary assistant** (3 month)
Clínica de la Conca (Montblanc)

Fellowships

2014-present **Pre-doctoral research grant (FI-DGR 2014)**
Agència de Gestió d'Ajuts Universitàris i de Recerca. Generalitat de Catalunya

2012-2013 **Scholarship for the tuition of M.Sc. in Animal Nutrition**
The Mediterranean Agronomic Institute of Zaragoza

Scientific publications

L. Blavi, D. Solà-Oriol, J.F. Pérez, and H.H. Stein. Effects of zinc oxide and microbial phytase on digestibility of calcium and phosphorus in maize-based diets fed to growing pigs. *Journal of Animal Science* (accepted).

L. Blavi, D. Solà-Oriol, J.J Mallo, J.F. Pérez. 2016. Anethol, cinnamaldehyde and eugenol inclusion in feed affects post-weaning performance and feeding behavior of piglets. *Journal of Animal Science, 94*

L. Blavi, D. Solà-Oriol, F.J. Crespo, M. del Mar Serra, and J. F. Pérez. 2016. The effects of including increasing doses of stevia and neohesperidine dihydrochalcone on feed preference in young piglets. *Journal of Animal Science*, *94:138-141.*

Divulgative publications

L. Blavi, D. Solà-Oriol y J.F. Pérez. 2015. Reforzar la alimentación del lechón antes y después del destete. *Nutrinews.*

L. Blavi y D. Solà-Oriol. 2015. ¿Estamos preparados para una prohibición del Zn a dosis terapéuticas en el post-destete? *Los expertos opinan, de la pàgina web 3tres3.*

L. Blavi y D. Solà-Oriol. 2013. ¿Es útil el creep feeding para afrontar un destete con éxito?. *Alimentación en primeras edades, Albéitar nº169.*

Conference proceedings

L. Blavi, D. Solà-Oriol, S.M. Martín-Orúe, and J.F. Pérez. 2016. Effect of different levels of zinc and calcium on growth performance in weanling pigs. *ADSA-ASAS Joint Annual Meeting*, Salt Lake City, USA. Type of presentation: Poster.

L. Blavi, D. Solà-Oriol, S.M. Martín-Orúe, and J.F. Pérez. 2016. Effect of different levels of zinc and phytase on growth performance in weanling pigs. *ADSA-ASAS Joint Annual Meeting*, Salt Lake City, USA. Type of presentation: Poster.

L. Blavi, D. Solà-Oriol and, J.F. Pérez. 2016. Could Zinc Citrate supplementation during lactation increase the serum Zn levels at weaning? *MidWest Meeting, ASAS Midwestern Section and ADSA Midwest Branch*, Des Moines, USA. Type of presentation: Poster.

L. Blavi, D. Solà-Oriol and, J.F. Pérez. 2016. Low calcium levels improve growth in piglets after weaning. MidWest Meeting, *ASAS Midwestern Section and ADSA Midwest Branch*, Des Moines, USA. Type of presentation: Poster.

E. Barba-Vidal, V.F. Buttow, E.G. Manzanilla[1], **L. Blavi**, C. Torrente, J.F. Pérez, S.M. Martín-Orúe. 2016. Blood parameters as piglet health biomarkers in an experimental infection with *Salmonella* spp. *MidWest Meeting, ASAS Midwestern Section and ADSA Midwest Branch*, Des Moines, USA. Type of presentation: Poster.

Ll. Fabà, D. Solà-Oriol, M. Caballero, S. López-Vergé, **L. Blavi**, and J. Gasa. 2016. Decisions based on body condition score (bcs) for feeding programs during gestation affect differently the production and state of reserves of gilts and first parity sows. *MidWest Meeting, ASAS Midwestern Section and ADSA Midwest Branch, Des Moines*, USA. Type of presentation: Oral.

S. López-Vergé, **L. Blavi**, D. Solà-Oriol, J.F. Pérez and J. Gasa. 2016. Alternative method to accurately predict the sows' body weight in their first month of gestation. *MidWest Meeting, ASAS Midwestern Section and ADSA Midwest Branch*, Des Moines, USA. Type of presentation: Poster.

L. Blavi, D. Solà-Oriol, F.J. Crespo, M.M Serra and J.F. Pérez. 2015. Effect of milky derived flavor inclusion in creep-feed diets on suckling piglet performance and litter homogenity. *ADSA-ASAS Joint Annual Meeting*, Orlando, USA. Type of presentation: Oral.

L. Blavi, S. López-Vergé, D. Solà-Oriol and J.F. Pérez. 2015. Comparing zinc status, growth and mortality in piglets fed with or without therapeutic doses of Zn oxide. *ADSA-ASAS Joint Annual Meeting*, Orlando, USA. Type of presentation: Poster.

S. López-Vergé, **L. Blavi**, D. Solà-Oriol, J.F. Pérez and J. Gasa. 2015. Could a reduction of crude protein content avoid the use of ZnO and Antibiotics in pigs diets without affecting their subsequent performance?. *ADSA-ASAS Joint Annual Meeting*, Orlando, USA. Type of presentation: Poster.

S. López-Vergé, **L. Blavi**, D. Solà-Oriol, and J. Gasa. 2015. Is the lactation period the main variable responsible for reducing the efficiency of the swine production? *ADSA-ASAS Joint Annual Meeting*, Orlando, USA. Type of presentation: Poster.

D. Solà-Oriol, P. Romero, D. Temple, **L. Blavi**, and J. Gasa. 2015. Feeder space may affect pig's performance in the early growing-finishing period. *ADSA-ASAS Joint Annual Meeting*, Orlando, USA. Type of presentation: Poster.

D. Solà-Oriol, **L. Blavi**, and R. Sala. 2015. Hammer status in hammer mill affects feed particle size and piglet performance after weaning. *ADSA-ASAS Joint Annual Meeting*, Orlando, USA. Type of presentation: Poster.

L. Blavi, D. Solà-Oriol and J.F. Pérez. 2015. Effect of supplementary feeding strategies during the suckling period to improve weanling performance. *13th Digestive Physiology of Pigs (DPP)*, Kliczków, Poland. Type of presentation: Oral.

L. Blavi, D. Solà-Oriol, F.J. Crespo, M.M Serra and J.F. Pérez. 2015. Neohesperidine dihydrochalcone increases stevia preference in young piglets. *13th Digestive Physiology of Pigs (DPP)*, Kliczków, Poland. Type of presentation: Poster.

L. Blavi, D. Solà-Oriol y J.F. Pérez. 2015. Oportunidades en el manejo de la alimentación de los lechones durante la lactación y tras el destete. *XVII Jornadas de Porcino de la UAB y la AVPC*, Bellaterra, Spain. Type of presentation: Oral.

L. Blavi, D. Solà-Oriol, J.J Mallo, L. Mesas, A. Ortiz, J.F. Pérez. 2013. Pre- and postnatal flavor exposure through maternal diet modifies feed preference and

productive performance in post-weaned pigs. *XIV Australasian Pig Science Association (APSA), Melbourne, Australia*. Type of presentation: Oral.

L. Blavi R. Franco, J.J Mallo, L. Mesas, A. Ortiz, D. Solà-Oriol J.F. Pérez. 2013. Efecto de la inclusion de una aroma commercial en la dieta de las cerdas y de sus lechones. *XV Jornadas sobre producción Animal, AIDA*. Zaragoza, Spain. Type of presentation: Oral.

Awards

2014 **Extraordinary M.Sc. award in Animal Nutrition**
The Mediterranean Agronomic Institute of Zaragoza
Universidad de Zaragoza

Skills and competences

Language		**Software**	
Catalan	● ● ● ● ●	Microsoft Office	● ● ● ● ●
Spanish	● ● ● ● ●	SAS	● ● ● ○ ○
English	● ● ● ● ○	R	● ● ○ ○ ○