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Investigating small intestine's permeability via TEER and tight junction expression. Can these parameters evaluate gut health in the pre-weaning piglet?

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ABSBSTRACT

The use of hiyper-prolific sows is a common practice in the swine industry. Improving larger sized litters enhance the presence of low birth weight piglets (LBW< 0.8 kg) which are more likely to die. The transition to weaning is a stressful event compromising the gut homeostasis, growth and survival rates, especially on LBW piglets. Currently, the early-weaning is a strategy performed consisting in the weaning time anticipation to 2-3 weeks of age. In addition with the high mortality and morbidity rates the swine production is hampered resulting in economic losses.

The effect of the birth weight (BW), weaning strategy (WS) and the small intestine area (SIA) were studied in newborn piglets on the pre-weaning phase by the expression of zonula occludens-1 (ZO-1). 72 pairs of *Hypor* sex-matched littermates catalogued as low birth weight (LBW< 1 kg, n=36) or normal birth weight (NWB, n=36) were randomly distributed to the rearing systems from 3 days to 3 weeks of age. The early-weaned (EW) piglets were artificially fed in brooders with a milk-replacer provided *ad libitum*. On conventional weaning (CW), piglets stayed with sow and intake colostrum. Pairs of LBW-NBW were slaughtered at 3, 5, 8 and 19 days old. Tissue samples from duodenum (5%) and ileum (75%) were harvested to quantify the ZO-1 expression. Any significant differences related to BW and WS were found although colostrum exerted a greater effect the ileum and in LBW piglets. Relevant differences were observed within time (p= 0.001) and ileum (p=0.0005), improving the expression in 5-19 (p<0.001) and 8-19 (p=0.007) days.

In parallel, *in vitro* experiments on IPEC-J2 cell monolayers tested the permeability (FITC-Dextran, FD4), the trans-epithelial electrical resistance (TEER) and the immunolocalization of ZO-1 after 1 mM H_2o_2, 4 mM DEM and ethoxyquin treatments. The effect of 1 mM H_2o_2 affect the permeability (p =$2.73\cdot10^{-5}$) while time and time: stressor (p< $2\cdot10^{-16}$) influenced TEER values. The 4 mM DEM incubation showed significant differences within time and stressor (p<$2\cdot10^{-16}$), ethoxyquin (p=0.00041) and DEM:ethoxyquin (p=$1.11\cdot10^{-5}$). In conclusion, the IPEC-J2 cell line is a reliable model to screen the antioxidant potential reducing the animal experimentation. Moreover, the permeability and TEER evaluate the damage of the intestinal barrier due to the dependent doses-effect between the ethoxyquin and oxidant.

RESUM

L'ús de truges *híper-prolífiques* és una pràctica comuna a la indústria porcina. La producció de ventrades més grans incrementa la incidència de garrins de baix pes (LBW<0.8 kg) propensos a morir. El deslletament és un procés d'estres que compromet l'homeòstasi intestinal i les taxes de creixement i supervivència, en especial als LBW. Actualment, el deslletament precoç és una estratègia que anticipar el temps de deslletament fins a 2-3 setmanes d'edat. En conjunt amb l'alta mortalitat i morbiditat, la producció porcina es dificulta comportant pèrdues econòmiques.

L'efecte del pes (BW), l'estratègia de cria (WS) i la regió intestinal (SIA) van ser estudiats en garrins nounats a la fase prèvia del deslletament mitjançant l'expressió de la proteïna zonula occludens-1 (ZO-1). 72 *Hypor* sexats i aparellats en la ventrada van ser catalogats com baix (LBW<1kg,n=36) i normal (NBW,n=36) pes. Posteriorment es van distribuir aleatòriament segons el mètode de cria des del 3 dia fins a la 3 setmanes d'edat. Els garrins deslletats prematurament (EW) van ser alimentats artificialment amb un substitutiu de llet proveït *ad libitum*. Al deslletament convencional (CW), els garrins romanen amb la truja essent alimentats amb calostre. Parelles de LBW-NBW van ser sacrificats als 3, 5, 8 i 19 dies d'edat. Mostres de teixit del duodè (5%) i l'ili (75%) van ser recollides per quantificar l'expressió de ZO-1. No hi han diferències significatives d'acord amb el BW i WS encara que el calostre exerceix un efecte òptim a l' ili i als LBW. Hi han diferències

rellevants amb el temps (p = 0.001)i a l'ili (p = 0.0005) augmentat l'expressió als dies 5-19 (p<0.001) i 8-19 (p=0.007).

En paral·lel, experiments *in* vitro van analitzar la permeabilitat (FITC-Dextrà,FD4), la resistència elèctrica transepitel·lial (TEER) i l'immunolocalització de ZO-1 en cultius de cèl·lules IPEC-J2 tractades amb 1 mM H_2O_2, 4 mM DEM i etoxiquina. L'acció d'1 mM H_2O_2 afecta la permeabilitat (p =$2.73 \cdot 10^{-5}$) mentre el temps i temps: estressor ($p < 2 \cdot 10^{-16}$) influencien la TEER. L'incubació amb 4 mM DEM mostra diferències significatives amb el temps i l'estressor ($p<2 \cdot 10^{-16}$), l'etoxiquina (p=0,00041) i DEM: etoxiquina (p=$1.11 \cdot 10^{-5}$). En conclusió, la línia cel·lular IPEC-J2 és un model fiable analitzant el potencial antioxidant reduint l'experimentació amb animals. A més, la permeabilitat i la TEER avaluen el dany de la barrera intestinal degut a la relació dosis-efecte dependent de l'etoxiquina i l'estressor.

RESUMEN

El uso de cerdas híper-prolíficas es una práctica común en la industria porcina. La producción de camadas más grandes incrementa la incidencia de lechones de bajo peso (LBW<0.8 kg) propensos a morir. El destete es un proceso de estrés que compromete la homeostasis intestinal y las tasas de crecimiento y supervivencia, en especial en LBW. Actualmente, el destete precoz es una estrategia que anticipa el tiempo de destete hasta 2-3 semanas de edad. Junto a la alta mortalidad y morbilidad, la producción porcina se dificulta comportando pérdidas económicas.

El efecto del peso (BW), la estrategia de cría (WS) y la región intestinal (SIA) fueron estudiados en lechones recién nacidos en la fase previa al destete mediante la expresión de la proteína zonula occludens-1 (ZO-1). 72 *Hypor* sexados y emparejados en la camada fueron catalogados como bajo (LBW<1kg,n=36) y normal (NBW,n=36) peso. Posteriormente se distribuyeron al azar según el método de cría desde el 3 día hasta las 3 semanas de edad. Los lechones destetados prematuramente (EW) fueron alimentados van artificialmente con un sustitutivo de leche provisto *ad libitum*. En el destete convencional (CW), los lechones permanecen con la cerda siendo alimentados con calostro. Parejas de NBW-LBW fueron sacrificadas en 3,5,8 y 19 de edad. Muestras de tejido del duodeno (5%) y del íleon (75%) fueron recogidas para cuantificar la expresión de ZO-1. No hay diferencias significativas de acuerdo con el BW y WS aunque el calostro ejerce un efecto óptimo en el íleon y en LBW. Hay diferencias relevantes en el tiempo (p=0.001) y en el íleon (p=0.0005) aumentado la expresión de los días 5-19 (p<0.001) y 8-19 (p=0.007).

En paralelo, los experimentos *in vitro* analizaron la permeabilidad (FITC-Dextrano,FD4), la resistencia eléctrica transepitelial (TEER) y la inmunolocalización de ZO-1 en cultivos de células IPEC-J2 tratadas con 1 mM H_2O_2, DEM 4 mM y Etoxiquina. La acción de 1 mM H_2O_2 afecta la permeabilidad ($p=2.73 \cdot 10^{-5}$) mientras el tiempo y tiempo:estresor ($p<2 \cdot 10^{-16}$) influencian la TEER. La incubación con 4 mM DEM muestra diferencias significativas en el tiempo y estresor ($p<2 \cdot 10^{-16}$), la etoxiquina (p=0.00041) y DEM:Etoxiquina ($p=1.11 \cdot 10^{-5}$). En conclusión, la línea celular IPEC-J2 es un modelo fiable que analiza el potencial antioxidante y reduce la experimentación con animales. Además, la permeabilidad y la TEER evaluan el daño de la barrera intestinal debido a la relación dosis-efecto dependiente de la etoxiquina y el estresor.

ABBREVIATIONS

BCA	*Bicinchoninic acid*
CP	*Control pig*
CW	*Conventional weaning*
DPX	Distyrene plasticizer xylene
EW	*Early-weaning*
FITC, FD-4	*Fluorescein isothiocyanate dextran 4 kDa*
FBS	*Fetal bovine serum*
GIT	*Gastrointestinal tract*
IEC	*Intestinal epithelial cell*
IUGR	*Intra-uterine growth restriction*
LBW	*Low birth weight*
NBW	*Normal birth weight*
PBS	*Phosphate-buffered saline*
ROS	*Reactive oxygen species*
RT	*Room temperature*
TEER	*Transepithelial electrical resistance*
TJ	*Tight junction*
TJC	*Tight junctional complex*
TJP	*Tight junction protein*
ZO-1	*Zona occludens protein-1*

1. INTRODUCTION

1. 1. THE WEANING STRATEGY

Hybrid sows with high litter sizes are increasingly used in pig farms to enhance piglet production (Andersen et al., 2014). Consequently, the incidence of the low birth weight piglets (LBW) has risen, resulting in a negative profitability as they are more likely to die (see Figure 1) (Milligan et al., 2002). In 2002, Lay *et al.* reported that newborn piglets weighing less than 0.8 kg (LBW) have a survival rate of 32 % in comparison with larger piglets (≥ 2 kg) that show a 97 % probability to survive the first week after birth. Overall, the individual birth weight is considered to be a key factor that has an impact on the optimal growth and survival ratios in a piglet (Marchant et al., 2000).

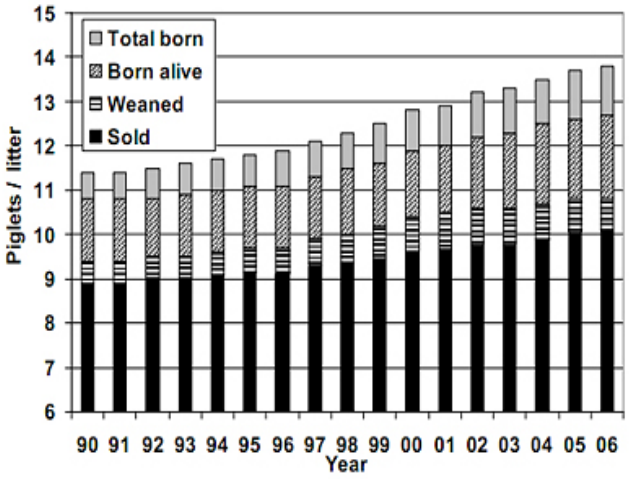

Figure 1. Higher number of piglets born per litter increased over the past years in parallel with the mortality percentage. Litter size evolution during 16 years (Boulot et al.2010. Management of high prolificacy in french herds: can we alleviate side effects on piglet survival?. Pig Industry).

Another important factor is the transition from the suckling to the weaning phase, as lower growth and higher mortality rates have been reported (Pluske et al., 1997). During the pre-weaning (birth until 3 weeks of age) the development of the immune system and the gut's maturation are induced by the absorption of bioactive substances present in the mother's sow milk (colostrum) (Bourne, 1973; Skrzypek et al., 2005). In fact, the small intestine doubles its absorptive capability by increasing an 80 % in length and 30 % in diameter on the first 10 days after birth. The beginning of the weaning phase (3-4 weeks of age) is one of the most stressful events in the piglet lifetime as morphological, structural and physiological gut modifications are induced by the solid food feeding. Challenging factors as the removal from the mother and the the exposure to antigens in new food, force a rapid adaptation that must be coped to ensure the piglet's survival. Nowadays, the rearing early-weaning strategy is a practice commonly extended in the swine industry. Based on advancing the weaning age (2-3 weeks of age) some benefits as a faster growth, healthier piglets and an economic return rapidly are expected overcoming the death losses by enhacing the sow's productivity (more litters/year) (King, 2001). Otherwise, an abnormal food intake promoting the down-regulation of the enzyme development was reported (Van Heugten, 1997).

Due to the increased morbidity and mortality risk, an economical loss over 17 million in Flanders was estimated. Similarly in U.S, a loss between $130 and $330 million in 2010 resulted from the weaning challenges (Lay et al., 2002). The economical aspect impulses technological improvements in nutrition and breeding management in order to minimize the collateral adverse effects (Campbell et al., 2013). Currently, the pork industry is actively seeking for measures to decrease mortality and morbidity ratios during the pre-weaning

phase, especially focusing on LBW piglets. This project aimed to evaluate the effect of the birth weight (LBW vs NBW), weaning strategy (pre-weaning, early vs conventional) and oxidative stress on the roles developed by the tight junction proteins during the pre-weaning using *in vitro* and *in vivo* experiments.

1.2. SMALL INTESTINAL BARRIER IN PIGLETS

1.2.1. Structure and function:

The gastrointestinal tract provides one of the largest interfaces between the host and the hostile environment. The small intestine is divided into the duodenum, jejunum and ileum, each consisting of four different layers: mucosa, submucosa, muscularis externa and serosa (connective tissue). The mucosal layer is situated on the luminal side and is mostly populated by enterocytes (> 80 %) and secretory cells (enteroendocrine, globet cells). The intestinal epithelial cells (IECs) or enterocytes are columnar cells forming a monolayer settled into the *lamina propria*. Enterocytes have specialized structures on the apical side managing the absorption of nutrients towards the lumen. These prolongations known as microvilli are amplifying 30 times the absorptive capacity due to the brush border membrane built. Furthermore, the IECs display an enzymatic and immune activity participating in the homeostasis of the immune system (Peterson et al., 2014).

The epithelial cohesiveness is managed by cell to cell junctions, involving different groups of transmembrane proteins known as tight junctions. These proteins are assembled making up the tight junctional complex in neighboring enterocytes through intimate contacts ("kissing-points") between the extracellular membranes. Thus, a continuous sealed barrier is formed regulating the permeability and the transport of substances. This network of intercellular proteins are limiting the paracellular space to 10-15 Å restricting the passage of toxins, antibodies and harmful molecules (>3.5 kDa). Meanwhile, the electrolytes, nutrients and solutes can easily diffuse from the lumen to the circulatory system (Tripathi et al., 2009). The tight junctions participate in the cell polarity (see Figure 2) as separate the apical and basolateral domains which are respectively, facing the lumen and the extracellular matrix (Lu et al, 2013).

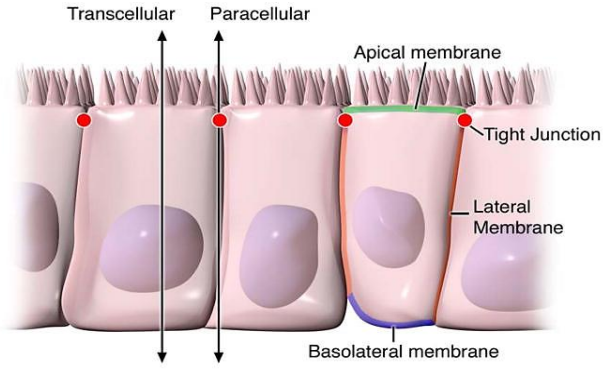

Figure 2. The fence function developed by tight junction proteins. Definition of the apical and basolateral domains and pathways of epithelial permeability (Groschwitz and Hogan, 2009. Intestinal barrier function: molecular regulation and disease pathogenesis. J Allergy Clin Immunol)

The reduction of the antigenic tolerance is a collateral effect from a damaged tissue that allows the exogenous proteins (e.g. non-self antigens) to go across the paracellular space. In this way, the interaction with the immune cells from the *lamina propria* is promoted and consequently the immune response is insulted with inflammatory processes and hyperpermeability (Lallès et al., 2004; Ulluwishewa et. al, 2011; Anderson et al., 2012). Thus, an injured integrity triggers a dysfunctional selectivity that make the immune response

inefficient (Ulluwishewa et. al, 2011). This might result in gut bacterial overgrowth, infectious diarrhea, Crohn's disease, necrotizing enterocolitis or death (Lambert, 2009; Anderson et al., 2012; Hu et al., 2013).

The TJ proteins are categorized according to: integral membrane proteins (e.g. occludins (6082 kDa), claudins (2027kDa)) and adhesion molecules (e.g. IgG-like family, ~35 kDa), peripheral membrane proteins (e.g. cytoplasmic zonula occludens (ZO) isoforms) and signaling proteins (e.g. PKC, MAPKS) (Groschwitz and Hogan, 2009).

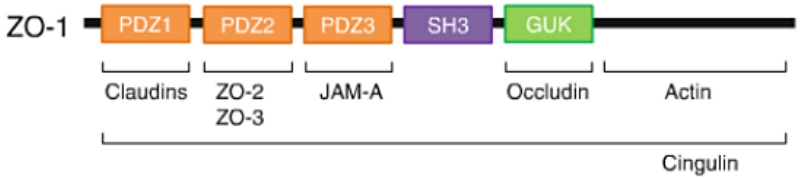

Figure 3. The structure of zonula occludens proteins and domains displaying the tight junctional complex assembly (Christensen. 2014. Effect of gut microbiota on intestinal integrity. National Food Institute)

To make up the tight junction complex (TJC), an interaction between TJ (occludins, junctional adhesion molecules and claudins) and the compounds from the cytoskeleton (actin/myosin filaments) is required for maintenance of the cell polarity. This role is mediated by clustering scaffolding proteins, as ZO-1 alternatively known as tight junction protein 1 (TJP-1). The role of ZO-1 is to organize the integral membrane proteins and mediate the interaction with actin filaments or other cytoplasmic proteins (e.g. cingulin) (Xu et al., 2013). The recognition of the integral proteins is carried out by the Post synaptic density proteins (PDZ) and Guanylate kinase (GUK) domains from ZO-1, being a high-regulated and dynamic network (see Figure 3) (Utepbergenov et al., 2006; Niessen, 2007). Therefore, ZO-1 is a key element in managing the cell-cell contacts and maintaining the tight junction complex between neighboring IECs.

The well-formed tight junctions ensure a suitable barrier developing the fence (cell polarity) and gate (diffusion) functions successfully (Anderson et al., 2012). A reliable procedure to monitor the organization of the tight junction network in epithelial layers is the transepithelial electrical resistance assay (TEER). The TEER measurements indicate alterations in the barrier integrity costing the disruption of the permeability. In the present study, the evaluation of the permeability and trans-epithelial electrical resistance (TEER) was performed *in vitro* on IPEC-J2 cell monolayers. This non-transformed and non-tumorigenic cell line was originally derived from the jejunum of unsuckled piglets maintains differentiated characteristics and the tool-like receptors, mucins and cytokines expression (Liu et al., 2010). Due to the physiological features maintained *in vitro*, the experimental results can be extrapolated towards *in vivo* situations (De Vos et al., 2012). This technique is sensitive to the flux of ions traversing the layer, although the route of transport (paracellular vs transcellular) cannot be determined. Besides that, permeability assays are required to ensure the analysis of the paracellular state. This parameter is estimated with the flux of FITC-Dextran 4 (FD-4) running into the paracellular space which depends on the tightness of the monolayer.

The permeability and the integrity of the barrier are terms closely linked although different concepts are concerned. Nevertheless, an impaired permeability reflects an impaired integrity of the small intestine barrier as a disrupted integrity arises by changes in the mucus layer, TJ connections and an imbalanced cell death-proliferation rate (Christensen, 2014). Other factors as the intracellular signaling and gene expression regulate the ZO-1 activity, altering the strength of the TJs as several studies demonstrated (Matter and Balda, 2003; Ebnet, 2008).

1.2.2. A dysfunctional small intestinal barrier

Some studies indicate that the integrity of the tight junction complex (TJC) in piglets is compromised by intrauterine growth restriction (IUGR), a factor affecting to the 20% of the piglets from the litter (Boudry et al., 2011; Roger, 2012). This factor increases the risk of death conditioning the fetus and newborn survival in piglets during the perinatal period. In previous studies a wall thickness and reduced length, weight, villus height and crypth depth was observed resulting in a decreased nutrient absorption (D'Inca et al., 2010). As a consequence, an altered intestinal growth was concluded in the study of Wang *et al.* related with alterations in the morphology and the endocrine homeostasis that contribute to the predominance of lower growth rates (Wang et al., 2005).

Other influent factor is the red-ox imbalance resulting in the ROS production by external stress stimuli or natural metabolic processes as cell respiration in mitochondria (Kirkinezos and Moraes, 2001). Nowadays, the attention for the antioxidants is increasing as they protect the organelles and the membranes against an excess of reactive oxygen species (ROS). This noxious compounds are highly unstable and reactive due to the unpaired valence (Majewska et al., 2011). The ROS molecules can interact with organic elements like proteins, lipids, nucleic acids and transcription factors inducing irreversible alterations (Brieger et al., 2012). Consequently, secondary effects as chronic inflammation or neurodegenerative diseases are induced by the intracellular oxidative stress see (Figure 4).

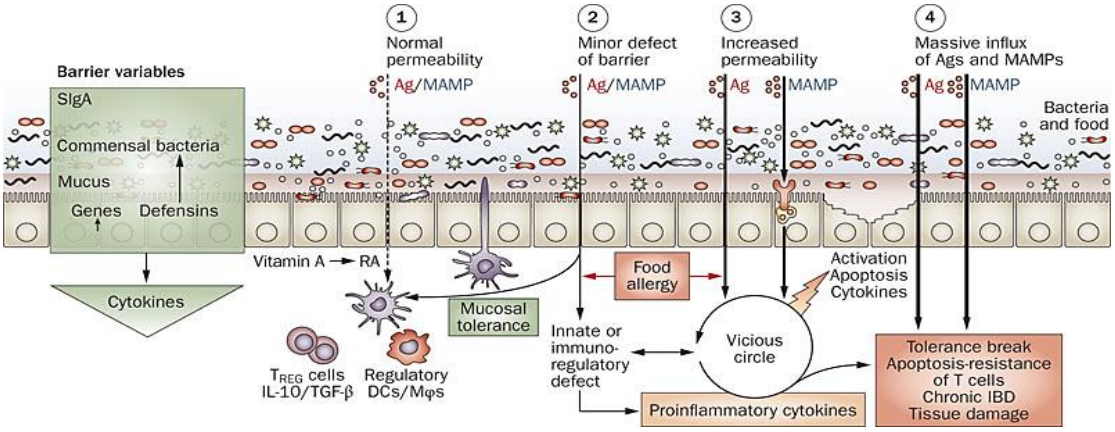

Figure 4. Impaired barrier permeability results in inflammatory and apoptotic processes. Consequences of the dysfunctional homeostasis in mucosal barrier (Brandtzaeg, P., Food allergy: separating the science from the mythology, 2010, Nature Reviews Gastroenterology and Hepatology, 7: 380-400).

2. OBJECTIVES

- The present study investigates within *in vivo* trials the relationship of birth weight (NBW, LBW) and the rearing system (CW, EW) factors with a functional barrier in neonatal individuals using the scaffolding ZO-1 protein as an indicator of the state and strength barrier. The evaluation was done during the pre-weaning phase in samples harvested from the duodenum and ileum region.

- The purpose of *in vitro* experiments was to figure out whether the permeability variation can be linked to changes in the TJ expression under pathological (direct vs indirect red-ox imbalance) or improved physiological (endogenous vs synthetic antioxidant treatments) conditions.

- Studying the permeability and the TEER parameters as a potential candidates of the small intestinal barrier evaluation. To achieve this purpose, *in vitro* experiments were carried out by serial TEER measurements, FD-4 permeability assays and immunocytochemical staining on IPEC-J2 cell monolayers.

➢ The global goal of *in* vitro experiments was to investigate whether the analysed parameters served as an indicators capable to alert against the dysfunctionality and physiological gut alterations.

➢ Using the IPEC-J2 cell line as a model to evaluate the effect of intracellular the red-ox imbalance measuring quantitative and qualitative the ROS production. Provide reliable *in vitro* models to test the oxidant and antioxidant potential as an alternative to the animal usage.

3. MATERIALS AND METHODS

This experiment was approved by the *Ethical Committee on Animal Experimentation of Ghent University* and the *University of Antwerp* (Belgium) for the use of animals in research and lead by *EC2011/195* regulation.

3.1. Experimental design and sample collection

*Hypor s*ex-matched littermates were selected within 24 hours after birth according to their weight. Newborn piglets were catalogued as LBW (72 pairs) when birth weight was between 750-950 g (0, 84 ± 0, 09 kg). The birth weight of NBW piglets (72 pairs) is above 1 kg (1, 37 ± 0, 18 kg).

The *in vivo* experiments performed were based in 72 piglets from two commercial farms weighed within 24 h after birth (1.37 ± 0.18 kg) identified as LBW or NBW within 1 SD unit of the mean within-litter birth weight. According with birth weight (LBW/NBW), pairs of neonatal piglets were randomly assigned to different rearing systems during the pre-weaning phase (3 days after birth till 3 weeks of age). The experimental conditions performed were; i.e. weaning at approximately 3 weeks of age (3w, 19.6 ± 0.50 d), weaning at approximately 4 weeks of age (4 w, 26.5 ± 0.50 d) and removal from the sow at 3 days of age and fed with a milk replacer until weaning time at 3 weeks of age (19.8 ± 0.38 d, 3d3w). Within LBW and NBW groups, early and conventional weaning treatments were tested with 6 piglets per condition and per time point (see table 2). The early weaned (EW) piglets were transferred to brooders from 3 until 21 days of age fed with a milk replacer provided by an automated milk dispensing system (Rescue Deck®) simulating *ad libitum* rearing (see Figure 5). Conversely, the piglets following the conventional weaning (controls pigs, CP) strategy were left with the sow until 3 weeks of age. On table 1 was shown the temperature range performed in both rearing strategies depending on the age.

Table 1. Optimal temperature conditions during the pre-weaning phase.

Age (days)	0-3	3-7	7-14	14-21	21-28
Temperature (°C)	29-35	25-29	24-28	22-26	21-25

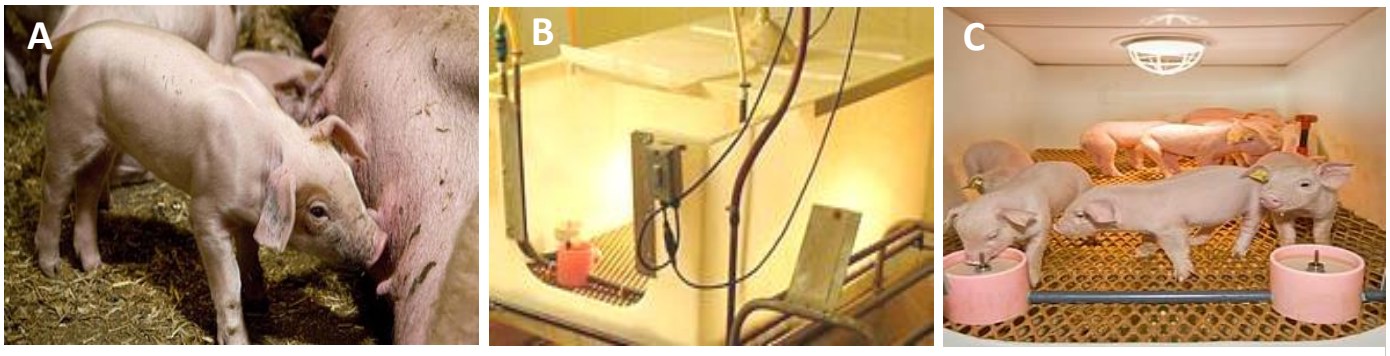

Figure 5. Rearing strategies during the pre-weaning phase. A) Under conventional weaning piglets are fed with colostrum. B) Rescue desks are used to feed piglets *ad* libitum with milk replacer. C) Early-weaning conditions during pre-weaning (fed from 3 days age until 3 weeks of age).

In total, 72 piglets were sacrificed. The slaughtering was done with anesthesia preparing a mixture of 40 mg/kg Ketamine and 2 mg/kg Xylazine in the same syringe. The solution was injected in the hamstring of the right leg and the euthanasia consisted in bled out piglets via the carotid arteries and jugular veins at 3,5, 8 and 19 days of age. *Per piglet,* tissue samples were taken at the duodenum (5%) and ileum (75%) region of the small. After rinsing the tissue samples with PBS, they were snap frozen in liquid nitrogen and stored at -80 °C. On table 2 was shown the amount of samples harvested per condition.

Table 2. Number of slaughtered piglets per condition during the pre-weaning period.

Age (days)	LBW		NBW	
	Early-weaning	Conventional-weaning	Early-weaning	Conventional-weaning
3	6		6	
5	6		6	
8	6	6	6	6
19	6	6	6	6

3.2. SAMPLE PREPARATION (ELISA)

To quantify the level of TJ proteins expression, approximately 0.4 g of crushed tissue from the collected duodenum and ileum samples frozen with liquid nitrogen (-196 ºC) was used at least. The cell disruption was done by the mechanical action performed with a mortar and pestle. They were previously cleaned with distilled water and ethanol and wrapped it with aluminum foil for storage o/n at -80°C. Afterwards, the crushed tissue was collected in frozen falcons tubes to be weighted (*Sartorious, BP 210s*). Finally, samples were stored at -80°C until sonication step.

Figure 6. Summary of main crushing steps followed.

Per 0.4 g of crushed tissue, an amount of in 1200 µLof sterile filtered PBS using an Acrodisc® Syringe Filters (Life Sciences) was dissolved before the sonication for 6 times 5 seconds per sample using an Ultrasonic Vibra-Cell (VCX 130, Newtown, USA). The samples were kept on ice for 30 min. and centrifuged at 19000 x G for 2 min. at 4 °C (Heraeus X3R with TX-750 swinging bucket rotor, Thermo Scientific, Rockford, USA). The total protein concentration contained on the supernatant was measured using the Bicinchoninic acid test (BCA). Samples were diluted 1:30 in filtered PBS for the analysis with Pierce TM BCA Protein Assay Kit (Thermo Scientific, Rockford, USA). Concentrations of the test samples were calculated using a standard curve of serial dilutions (0 – 2000 µg/mL) of bovine serum albumin. 25 µL/well of standard and test samples were transferred to a 96-well plate in triplicates. A working solution (WS) was made mixing reagent A and B in 1:50 proportion containing the bicinchoninic acid (BCA). A volume of 200 µL/ well was added to the plate before 30 min. incubation at 37 °C conditions following a 1:8 ratio. The BCA reagent reacted with the reduced cuprous cation (Cu^{1+}) from proteins forming a purpled-coloured product. Absorbance was measured at 550 nm using a Sunrise spectrophotometer (Freedom Evolyzer-z, Tecan Group Ltd., Männedorf, Switzerland). The rank of total proteins estimated in samples was 20-30 µg/µL. When the BCA test was finished, samples were diluted in filtered PBS to yield a final total concentration of 10 ng/µL. Finally, samples were aliquoted and stored at -80 °C.

3.3. SANDWICH ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

For analyzing tight junction proteins an ELISA kit from Cloud-Clone Corporation® (Houston, TX, USA) was used.

Standard curve was made from stock solution by addition of standard diluent (1:2) to the following final concentrations: 10, 5, 2.5, 1.25, 0.625, 0.313, 0.156 and 0 ng/mL for quantification of tight junction protein-1 (SEC262Hu, *Homo sapiens)*. A volume of 100 µL/well from samples (10 ng/µL) and standards was added in duplicates into the flat bottom of the pre-coat 96-wells plate. Then was covered with a sealer and incubated

during 2 hours at 37ºC. Remaining liquid on wells was removed by snapping the plate onto absorbent paper. Afterwards, 100 µL /well of detection reagent A was added and incubated for 1 h at 37 ºC. Remaining liquid was removed again and three washing steps were followed adding 350 µl/well of wash solution for 1 minute. A volume of 100 µl /well of detection reagent B (conjugated antibody linked to HRP enzyme) was added and incubated at 37 ºC during 30 min. and afterwards a washing step was repeated 5 times. By putting 90 µl/well of substrate solution, samples turned blue according to the total amount of proteins. Last incubation step was for 15 min, 37 ºC. Finally, 50 µl /well of stop solution were added. Immediately, a change in color was observed. Measurement of absorbance was performed with Infinite M200 Pro spectrophotometer and X-Fluor software at 450 nm and 25°C (Freedom Evolyzer-z, Tecan Group Ltd., Männedorf, Switzerland).

The quantification of total ZO-1 proteins in the collected samples resulted by interpolating the absorbance measurements into the standard curve assessed. Then, data were plotted in spreading graphics, indicating the average and SEM values within different individuals under the same experimental conditionss. The standard error measure (SEM, $\sigma/\sqrt{n}$) was used to described the precision criteria of the sampling and analysis methods.

3.4. IPEC-J2 CELL CULTURE

A porcine epithelial jejunocyte cell line (IPEC-J2 ACC 701, Leibniz-Institut DSMZ, Braunschweig, Germany) from an unsuckled neonatal piglet was grown in Dulbecco's modified Eagle (DMEM, Life Technologies, USA), 1.5 mM HEPES, 5% FBS, 1% Insulin/Transferrin/Selenium, 1% Penicillin/Streptomycin and 2.5 µg/mL fungizone medium. Afterwards, 1 x 10^5 cells/well were seeded in a 12-well plate with inserts (ThinCerts™, pore size 0.4 µm, 1.12 cm^2 surface, Greiner Bio-one, Frickenhausen, Germany) and cultured for a week by incubation at 37°C, 5% CO_2 with medium without phenol red (Dulbecco's modified Eagle, 1.5 mM HEPES, 5% FBS, 1% Insulin/Transferrin/Selenium, 1% Penicillin/Streptomycin and 2.5 µg/mL fungizone medium, Life Technologies, USA) renewed at the third day. Washing steps were performed after seeding and incubation with the stressor agent by the addition of 1 and 2 ml x 2 of DMEM/F-12 (1:1) (X1), Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12, Gibco, Life Technologies,USA medium without phenol red on the and the basolateral chambers respectively.

3.5. ANTIOXIDANT PRE-TREATMENT AND INDUCTION OF OXIDATIVE STRESS

Effects on GIT permeability were studied by adding stressor agents 1 mM H_2O_2 and 4 mM DEM treatments by 1 h incubation step at 37 °C, 5 % CO_2. Also treatments with a pre-treatment with 2 Mm Trolox ((±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid, water-soluble vitamin E analogue, Sigma-Aldrich®, St. Louis, MO) and 25, 12.5, 6.25, and 3.125 µM (1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline, Sigma-Aldrich®, St. Louis, MO) were performed in combination with the stressor agents under the same incubation conditions.

3.6. TRANSEPITHELIAL ELECTRICAL RESISTANCE

TEER was measured *in vitro* with a volt-ohm meter under sterile conditions using a Millipore MilliCell-ERS® set-up with a range of 20 kΩ (mode R, Merck Millipore, Darmstadt, Germany) to evaluate the resistance before and at 1, 24, 41 and 49 h after 1 h incubation with oxidative stress agents (1 mM H_2O_2 and 4 mM DEM). Then, incubation of the 12-well plate was performed at 37°C and 5 % CO_2 until the measurement time within the medium renewal and washing steps mentioned (see section 3.5). TEER values are expressed as kΩ x cm^2,

the surface area represent the area of the insert where the cells grow. TEER values exceeding 1 kΩ x cm^2 resistance indicate that the monolayer is confluent.

3.7. PERMEABILITY ASSAY

1 x 10^5 IPEC-J2 cells were seeded per insert (ThinCerts™, pore size 0.4 µm, 1.12 cm^2 surface, Greiner Bio-one, Frickenhausen, Germany) in a 12-well plate and grown to confluence at 37°C, 5% CO_2 for one week. Achieved the confluent point, a pre-treatment overnight with different concentrations of ethoxyquin (25, 12.5, 6.25, and 3.125 µM) were carried out. Washing steps with 1 ml and 2 ml of filtered DPBS on upper and lower chamber respectively were performed twice. Then 1 h incubation at 37°C, 5% CO_2 with oxidant agents (1 mM H_2O_2 and 4 mM DEM) and overnight pre-treatment with 2mM Trolox into the apical Boyden chamber was followed. After, the washing steps were repeated again in the same way. To assess the paracellular permeability the flux of FITC-dextran 4 kDa (FD-4, Sigma-Aldrich®, St. Louis, MO) was investigated by adding 1 µg/mL FD-4 into 500 µL/well of colorless medium (DMEM/F-12) in the upper-well. Subsequently, the FD-4 was left to permeate overnight and 100 µ/welll from the lower chamber was transferred to a black with a transparent, flat bottom 96-well plate. Finally, the fluorescence ($\lambda_{excitation}$ = 488 nm, $\lambda_{emission}$ = 520 m) from samples and the standard curve with known concentrations (25, 12, 6. 3, 1.5 and 0.75 FD-4 µg/ml) was measured.

The permeated flux was estimated by interpolating the mean of the fluorescence intensity into the standard calculated curve. The total permeated FD4 (µg/ml) from samples were converted into a picomoles according to the molecular weight (1 Da = 1.66053886 x 10^{-24} g).

3.8. IMMUNOSTAINING OF TIGHT JUNCTION IN IPEC-J2 CELLS

The effect of 1 mM H_2O_2 and 4 mM DEM on the expression of ZO-1 was studied *in vitro* in IPEC-J2 cells.

Glass coverslips were pre-coated with polylysine for the IPEC-J2 cells adhesion. Then were distributed into a 12-well plate and were cultured for 1 week. Conditions of treatments evaluated the damage resulted from oxidative stress (1 mM H_2O_2, 4 mM DEM). All the wells were washed twice with PBS after achieving 100% of confluence. Cells were fixated using paraformaldehyde (4%, 30 min, room temperature). Another 3 washes x 5 min with 0.2 % Tween-20 dissolved in PBS were needed. For permeabilization, 0.2% Tween-20 and 0.2 % Triton X-100 in PBS was added and incubated for 1 h at room temperature (RT) with a shaker. Afterwards the fixation solution excess was removed with 2 washing steps. To reduce the non-specific binding from the goat second antibody a blocking solution in PBS with 1 % skim milk powder, 10 % normal goat serum (NGS), 2.25 % 0.3 M glycine and 0.2 % Tween-20 was incubated 1 h at RT. The primary polyclonal rabbit antibody anti-ZO-1 (Santa Cruz Biotechnology, Santa Cruz, CA) was diluted 1:50 in PBS with 0.2% Triton X-100, 0.2% Tween-20 and 10% NGS and incubated overnight at 4°C on shaker. Wells were washed three times for 5 min in PBS with 0.2 % Tween-20. Incubation for 1 h at RT with a secondary biotinylated goat anti-rabbit antibody (Dako, Glostrup, Denmark) diluted 1:200 in PBS with 0.2% Triton X-100, 0.2% Tween-20 and 10% NGS solution was added followed by 3 washing steps. Streptavidin-HRP was diluted 1:200 in PBS with 0.2 % Triton X-100, 0.2 % Tween-20 and 10% NGS solution and incubated for 1 h at RT. Afterwards, two washing steps for 5 min were performed with 0.2% Tween-20 in PBS followed by 5 min in distilled water. Finally, staining process with 3, 3'-Diaminobenzidine (1-5 min). Carazzi's hematoxylin staining was performed to stain the nuclei. A washing step with tap water was performed for 5 min. The monolayers were transferred into an alcohol series with increasing percentage (first isopropanol: 50, 70, 90, 100% and then xilol). The coverslips were covered with Distyrene Plasticizer Xylene (DPX). Microscopic evaluation was performed using a BX 61 microscope (Olympus, Belgium)

equipped with a DP 50 camera (Olympus). The surface area of the wound was measured using the analySIS-Pro software (Olympus).

3.9 STATISTICAL ANALYSIS

Data were analyzed using the Rcmdr (R-3.1.2) software with a $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***) significance code. Mixed models were used to study the influence of birth weight (BW), weaning strategy (WS) and if applicable the slaughtering time (T) on the ZO-1 *in vivo* concentration. Differences exerted by the categories mentioned above were tested performing pairwise interactions based on independent observations. The relationship among both small intestinal areas (SIA) was considered as paired-data within the same individual and analyzed by separate with the simplified model. The fixed effects were BW, WS, and SIA in contrast with random effects accounted from time, piglets and litter variability. The categories BW, WS and SIA encompassed two levels each one (LBW, NBW, CW, EW and Duodenum,Ileum respectively) while time depends on the rearing system (CP: d3, d8, d19; EW: d3, d5, d8, d19). To assess the differences among means, one-way and multifactorial ANOVA test with Tukey post-hoc comparison were used to evaluate the 12 treatments performed.

4. RESULTS

4.1. IN VIVO EXPERIMENTS

4.1.1. Significant differences in ZO-1 concentration related to time and ileum with a huge recovery and improved expression by colostrum intake.

Samples were collected at 3, 8, 5 and 19 days (d3, d8, d5, d19). In the control group (CW) only three measurement times were done in comparison with the early-weaned piglets. The concentration of ZO-1 in samples was extrapolated from standard curves which showed high coefficients of determination ($R^2 > 0.991$), meaning reliable estimations within the concentration range.

The relationship among the BW, WS, SIA, T categories and the ZO-1 concentration was analyzed with a multi-factorial ANOVA test. The small intestine area ($p = 0.0263$*) and time ($p = 0.001$**) were statistically significant. Nevertheless, any relevant variability resulted from birth weight and weaning strategy factors. According to the SIA, a 1-way ANOVA was done within time and proximal/distal region by separate. Any significant differences were found in the duodenum. In contrast, the slaughtering time ($p = 0.00046$***) induced a relevant effect on ZO-1 concentration from the ileum.

The evolution of ZO-1 in LBW piglets over-time was showed in Figure 7. The interpretation was based on the experimental results as any significant differences were found. Due to similar ZO-1 concentrations assessed at d3 (0.422 ± 0.007 ng/mL ZO-1), the intake of sow's milk since birth had an equal effect in all the piglets. The TJ proteins were reduced at d5 as a consequence of the new type of milk (0.3075 ± 0.005 ng/mL ZO-1). At 8 days of age, all the treatments stabilize the concentration of ZO-1 despite the marked reduction (0,247 ± 0.055 ng/mL ZO-1) assessed in duodenum from CW piglets. Between d8 and d19, a recovery of the initial ZO-1 expression was generally observed. Moreover, the ZO-1 expression placed on the distal area from CW piglets was almost doubled respect d8 (0.542 ± 0.098 ng/mL ZO-1). Conversely, the proximal area showed a decrease regardless the WS performed (0.329 ± 0.005 ng/mL ZO-1). Although any significant differences were found statistically, the general trend observed in Figure 7 is the higher sensitivity of the proximal area based on the impaired ZO-1 expression. On the other hand, both weaning strategies exerted greater benefits in the ileum.

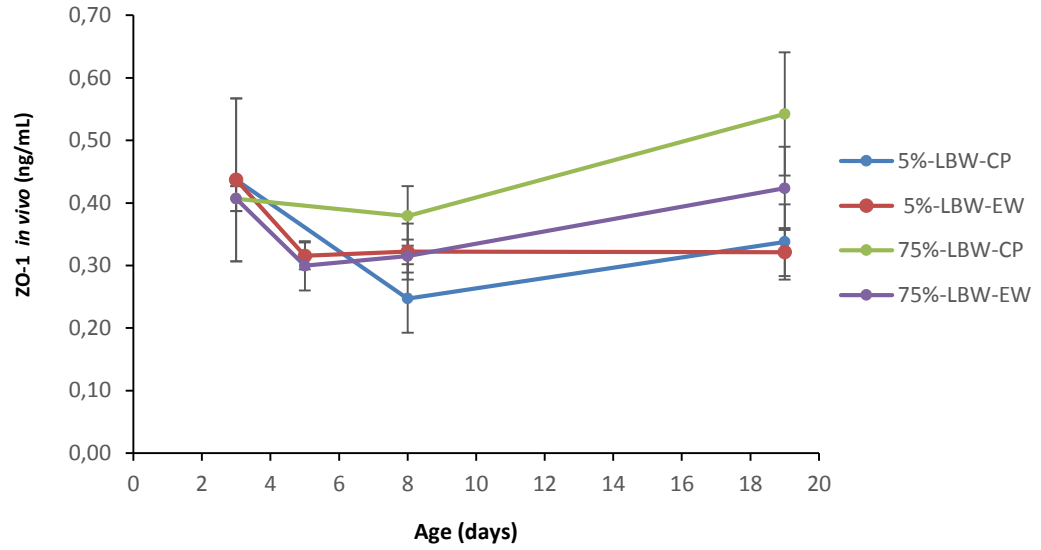

Figure 7. ZO-1 expression was improved in ileum during conventional weaning while was reduced in the proximal region in both WS performed. Quantification *in vivo* of ZO-1 in duodenum (5%) and ileum (75%) tissue samples from LBW piglets fed with formula-milk (EW) and sow's milk (CP) from 3 days aged during pre-weaning phase. Data were represented as means ± SEM (n =

The presence of ZO-1 was studied in NBW piglets (see Figure 8). The ZO-1 concentration in ileum was recovered and progressively increase until 19 days of age (0.504 ± 0.039 ng/mL) on artificially feed piglets. On the other hand, the expression on the same small intestine area was established by the influence of the sow's milk (0.429 ± 0.045 ng/mL ZO-1) after the decrease at d5. Despite of, under both rearing strategies was noticed a parallel evolution since the 8th day of age in the distal region. The opposite effect was showed in the proximal area as ZO-1 concentration was improved gradually (0.263 ± 0.03 to 0.416 ± 0.07 ng/mL) on CW piglets whereas the expression was stabilized in EW piglets.

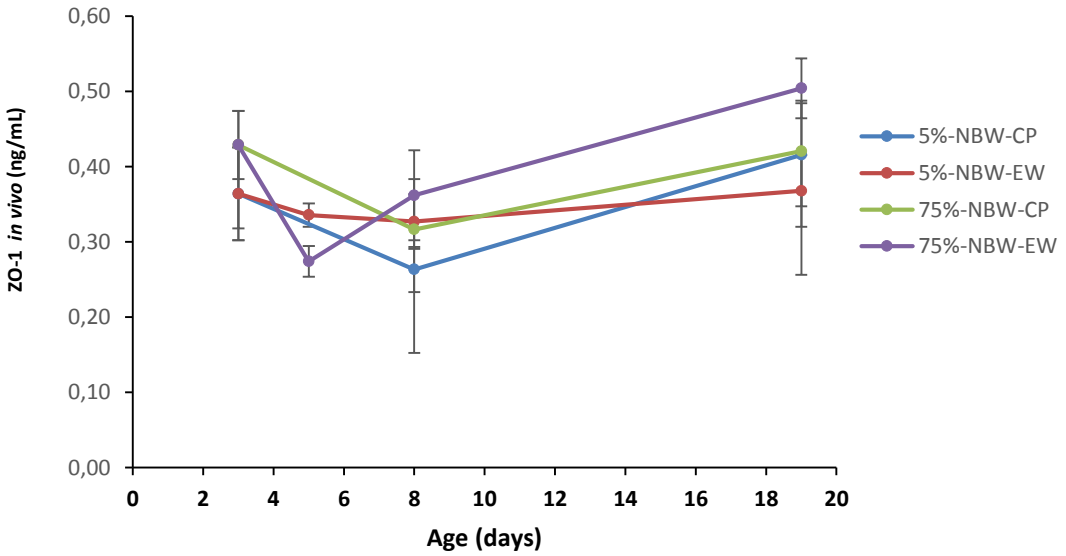

Figure 8. Evolution of ZO-1 expression showed more stability over-time in weaned piglets with the sow although the hugest recovery observed was in ileum from artificially reared piglets. Quantification *in vivo* of ZO-1 in duodenum (5 %) and ileum (75 %) tissue samples from NBW piglets fed with formula-milk (EW) and sow's milk (CP) from 3 days aged during pre-weaning phase. Data were represented as means ± SEM (n = 6).

In order to evaluate the effect of the rearing strategies an analysis within EW piglets was done (see Figure 9). The ZO-1 concentrations assessed at 3 days of age showed values close to 0.424±0.0153 ng/ mL ZO-1. Any treatment mitigated the ZO-1 decrease at d5, although the duodenum of NBW piglets showed less impairment (0,335 ± 0.0155 ng/mL ZO-1). Despite of, the recovery of the expression was achieved equally overcoming the first week of age in all the treatments although was higher in ileum on NBW individuals (0.362 ± 0.006 ng/ml ZO-1). Between 8 and 19 days of age, the distal region displayed a greater improvement of ZO-1 being higher in NBW piglets (0.504 ± 0.04 ng/mL ZO-1). The samples taken from duodenum of the oldest piglets displayed a recovered ZO-1 concentration in NBW piglets (0.367± 0.047 ng/mL ZO-1) in contrast with the reduced expression on LBW group (0.3215 ± 0.04 ng/mL ZO-1).

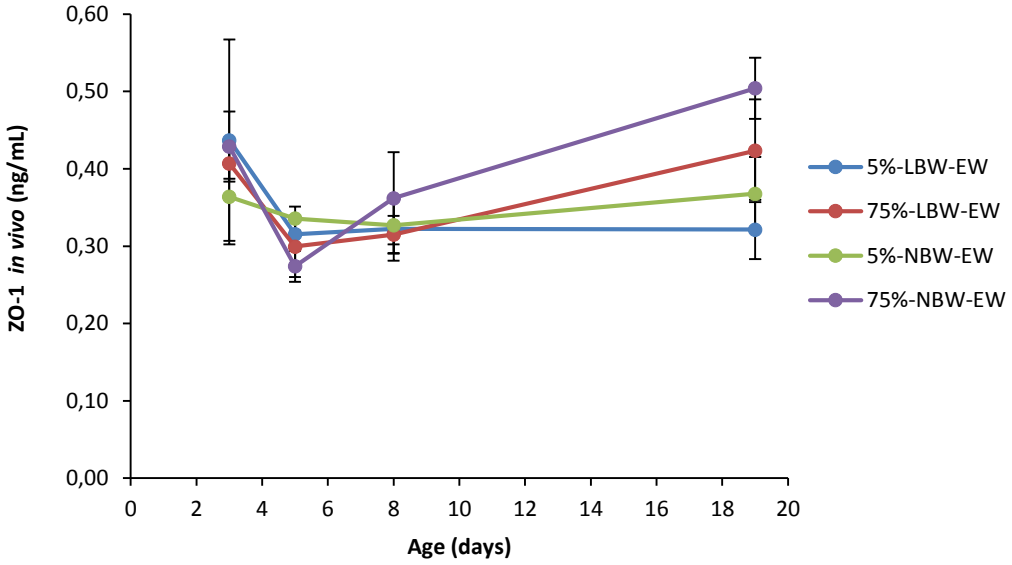

Figure 9. Lower ZO-1 concentration on LBW early-weaned piglets and higher integrity recovery on ileum region with normal weight preference. Quantification *in vivo* of ZO-1 in duodenum (5%) and ileum (75%) tissue samples from LBW and NBW piglets fed with formula-milk from 3 days aged during pre-weaning phase. Data were represented as means ± SEM (n = 6).

In Figure 10, a representation of the ZO-1 concentration from weaned piglets with the sow was done. The values from the ZO-1 quantification performed on the youngest piglets were considered. In general, was observed less impaired concentration in comparison when the EW strategy was performed. Due to the colostrum feeding performed between birth and 3 days of age, the measured values of ZO-1 assessed in CW piglets at d3 were considered equal in the EW graphics. Thus, the initial concentration resulted from CW was the same as figure 9 showed (0.424±0.0153 ng/ mL ZO-1). The whole amount of proteins dropped below the initial values at 8 days of age, especially the proximal region of LBW piglets (0.247±0.055 ng/ mL ZO-1). However, the ileum of LBW piglets displayed the most well-balanced evolution between d3 and d8. The highest ZO-1 concentration at d19 was found in the ileum of LBW piglets (0.542±0.098 ng/mL ZO-1). In reference to the duodenum and the initial values, the expression of ZO-1 was raised in the oldest NBW piglets (0.415±0.030 ng/mL ZO-1) whereas a lower quantification resulted from the LBW group (0.338±0.060 ng/mL ZO-1).

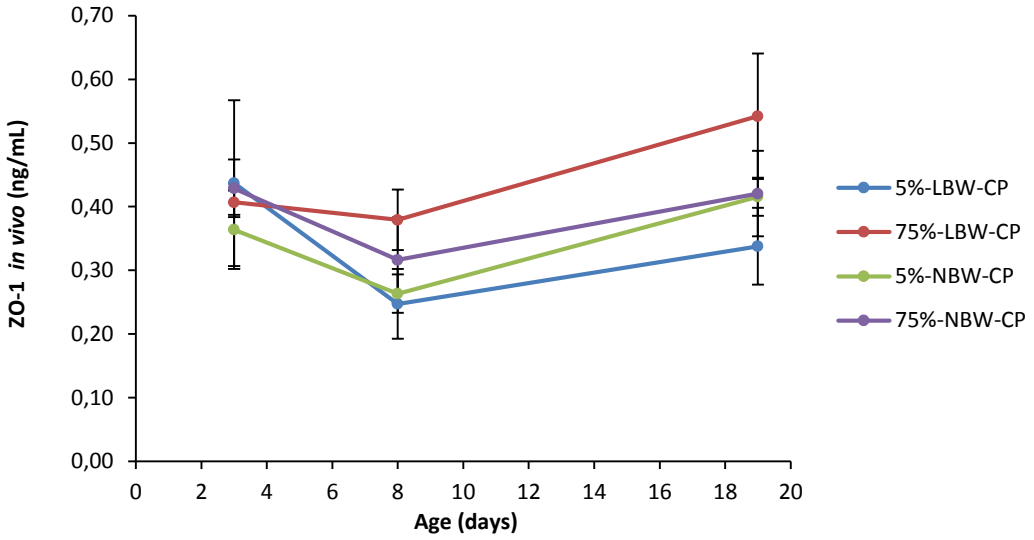

Figure 10. Higher ZO-1 expression on LBW than in NBW piglets in the distal region while NBW piglets showed a greater recovery after the first week of age. Quantification *in vivo* of ZO-1 in duodenum (5 %) and ileum (75 %) tissue samples from LBW and NBW piglets fed with sow-milk from 3 days aged during pre-weaning phase. Data were represented as means ± SEM (n = 6).

The effect of the small intestine area (SIA) was studied during the pre-weaning phase. Firstly, was evaluated the evolution of the TJ proteins in the duodenum (see Figure 11). An average of 0.400 ± 0.042 ng/ mL ZO-1 was estimated at d3 as the brief differences among the birth weight were no significant. After the first week of age, the ZO-1 proteins were mainly decreased reaching equal levels within the same WS regardless the type of piglet (0.2472 ± 0.055, 0.263 ± 0.030; 0.322 ± 0.045, 0.327 ± 0.037 ng/ mL ZO-1). Between the 8 and 19 days of age, the total amount of ZO-1 was reduced in LBW piglets independently of the WS while the normal weight and sow's milk increased the concentration in the proximal small intestine (0.416 ± 0.068 ng/ mL ZO-1). The NBW piglets resulted in a stable ZO-1 expression over-time even on early-weaned piglets (0.349 ± 0.020 ng/ mL ZO-1).

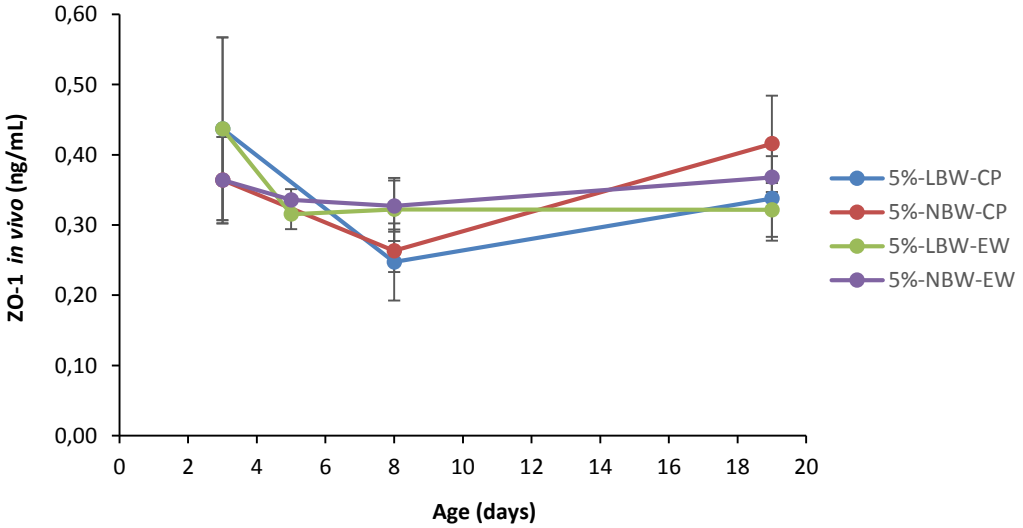

Figure 11. The CW increased the ZO-1 concentration in NBW piglets while in LBW piglets was reduced regardless the weaning strategy. Quantification *in vivo* of ZO-1 in duodenum (5 %) and ileum (75 %) tissue samples from LBW and NBW piglets fed with sow-milk from 3 days aged during pre-weaning phase. Data were represented as means ± SEM (n = 6).

The statistical analysis revealed a significant influence of the SIA in the ZO-1 concentration assessed (P = 0.0263*). Moreover, the pairwise contrasts revealed the significance of the time (p = 0.001**). Significant differences were found in the distal section (p = 0.00046***). The concentrations measured at 3 days of age, revealed similar concentrations (0.418 ± 0.012 ng/ mL ZO-1). At 5d, the ZO-1 levels were halved in early-weaned piglets independently of the birth weight (0.287 ± 0.013 ng/ mL ZO-1). The transition of the first week of age resulted in a restored expression below the values initially measured. Between 8 and 19 days old, relevant differences within the quantified ZO-1 were found as all the treatments reached a higher concentration. The LBW piglets conventionally weaned performed the highest expression improvement (0.379 ± 0.047 to 0.542 ± 0.067 ng/ mL ZO-1, p = 0.0067**). Underneath, NBW piglets artificially fed also suffered an important enhancement in the total ZO-1 concentration (0.274± 0.020 to 0.504 ± 0.039 ng/ mL ZO-1, p < 0.001***) between 5-19 days of age. Also during this period, a significant increased expression was noticed on LBW (0.30± 0.04 to 0.423 ± 0.066 ng/ mL ZO-1, p < 0.001***) early-weaned piglets. In reference to the other several times, an equal ZO-1 concentrations were found at d3, d19 and d3, d5, d8 as any significant differences were stablished.

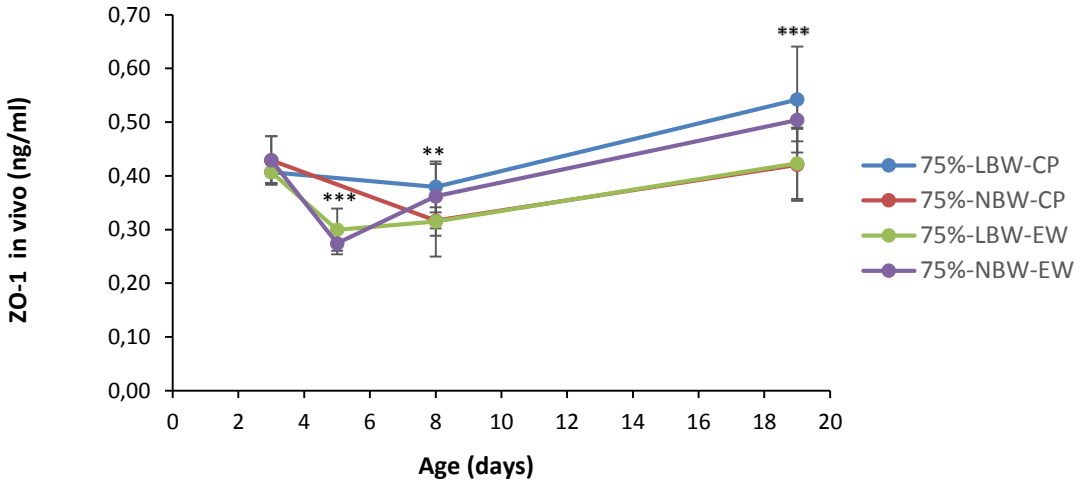

Figure 12. Differences in the ZO-1 quantification from distal samples were significant over-time. Quantification *in vivo* of ZO-1 in duodenum (5 %) and ileum (75 %) tissue samples from LBW and NBW piglets fed with sow-milk from 3 days aged during pre-weaning phase. Data were represented as means ± SEM (n = 6).

4.2. IN VITRO EXPERIMENTS

4.2.1. Disruption of the cell-cell contacts managed by ZO-1 proteins in IPEC-J2 cell monolayer treated with 1 mM H_2O_2 and 4 mM DEM.

The nuclei from the IPEC-J2 cells were observed as purple spots because the hematoxylin staining was bound to the nucleic acids. In parallel, the visualization of ZO-1 expression was immune-located through the brown staining mainly in cell-cell contacts and inside the cell body.

Untreated IPEC-J2 cell monolayers were used as a control of a functional small intestine's barrier (physiological conditions). In figure 13, the arrows and the circle emphasized the expression of ZO-1 in the jejunocytes pointing a well-formed TJ network assuming a balanced redox status. The intercellular contacts highly defined and the similarly sized nuclei positioned in the center of the cell body, indicate a normalized expression of ZO-1 under the physiological conditions. Some intracellular vesicles were observed, indicating apoptotic processes related with the mucosal turnover. The multiple spots on the nucleus belong to mitotic

cells during the DNA replication phase, showing a higher cytoplasmic expression of ZO-1 by the enlarged interactions with the actin filaments improving the vesicles' migration and cytokinesis.

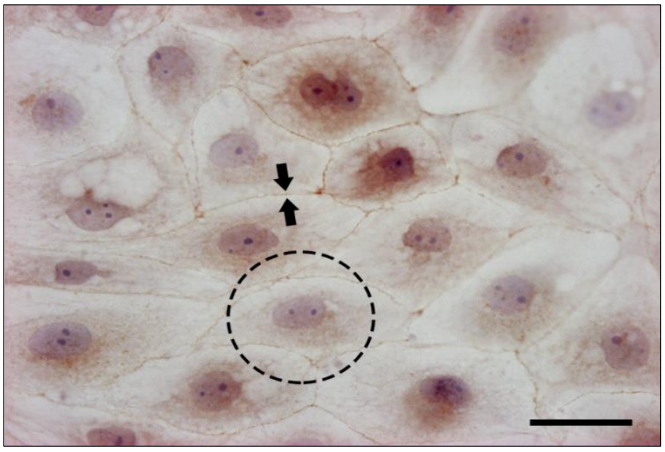

Figure 13. Culturing IPEC-J2 cells in complete medium served as a control of ZO-1 expression. Expression of ZO-1in IPEC-J2 cells incubated with medium DMEM/F-12 without phenol red at 37 °C, 5% CO_2. Scale bar: 30 µm.

The treatment with 1 mM H_2O_2 (figure 14), showed a larger amount of vesicles when the cells are directly stressed (see square). The number of apoptotic cells increased when the cells were treated with 1 mM H2O2 compared to the untreated control. In comparison, the continuous epithelial monolayer was disrupted as the cell-cell contacts were vaguely defined. Thus, the paracellular space managed by the intercellular ZO-1 expression was augmented and consequently impaired the cohesiveness and the sealed the epithelial barrier.

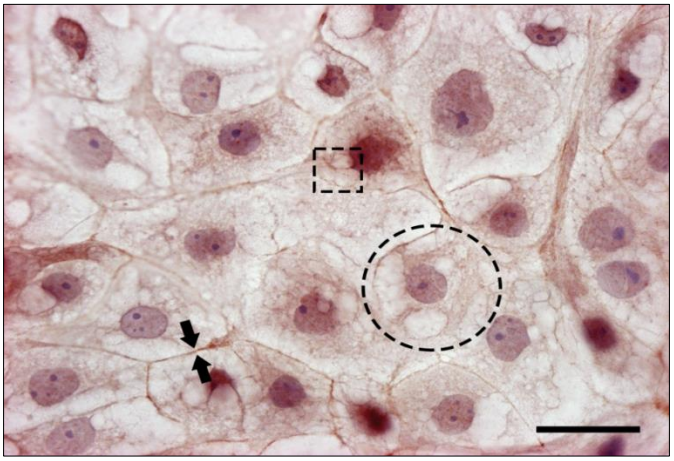

Figure 14. IECs intracellular impaired expression of ZO-1 as a result of ROS. Expression of ZO-1in IPEC-J2 cells incubated with 1 mM $H_2O_2$1 h at 37°C, 5% CO_2. Scale bar: 30 µm.

After DEM treatment a damaged ZO-1 expression was appreciated due to the impairment of the glutathione antioxidant system (GSH) (see Figure 15). The cytoplasmic ZO-1 levels were brief. A shift in the pattern expression was reported by the relocation of the structural protein between neighboring jejunocytes. It was supposed to be a consequence of the cellular compounds delivered during the programmed cell death processes. Respect the paracellular space, not differences could be appreciated between the intracellular and the extracellular areas as the expression of ZO-1 was not surrounding the IECs. The mitosis was considerably reduced and exceeded by the apoptotic process observed in the left-hand side of the picture. Different nuclei

joining together were found and hypothesized to result from the external membrane completely disrupted as the cytokinesis process doesn't involve the TJP's disorganization around. Moreover, the huge damage displayed by the stressor agent impaired the adherence capacity of the cells as the expression of ZO-1 was overlapped in different membranes.

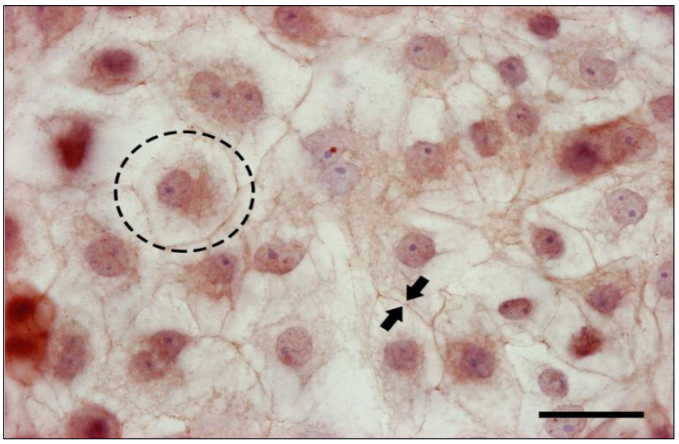

Figure 15. Reduced ZO-1 expression lead the intercellular network disruption. Expression of ZO-1in IPEC-J2 cells incubated with 4 Mm DEM 1 h at 37°C, 5% CO_2. Scale bar 30 µm.

4.2.2. Overnight 12.5 µM ethoxyquin pre-incubation rescued the TEER values assessed on IPEC-J2 monolayers after oxidative stress treatments, especially after 1 mM H_2O_2.

Development over time of the transepithelial electrical resistance after 1 h, 37 °C, 5% incubation with different oxidative stress treatments was performed.

On the first trial the intracellular stress was induced by 1 mM H_2O_2, 4mM DEM agents and both in combination with 2 mM Trolox antioxidant. Among all of the treatments, the IPEC-J2 cell monolayers cultured in DMEM/F-12, 5% FBS, phenol red free medium and 2 mM Trolox control showed the recovery of the impaired resistance values assessed after 1 h (see Figure 16). The pre-incubation with 2 mM Trolox before oxidative treatments, mitigated the adverse effects restoring the tightness of the epithelial monolayers after 17 h (0.56 ± 0.002 kΩ x cm^2).

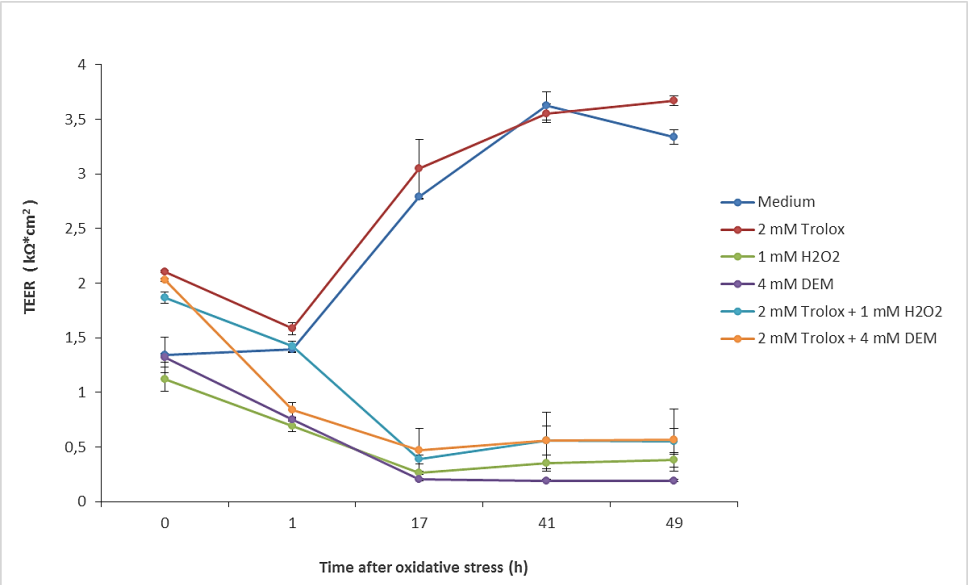

Figure 16. Incubation 1 h with 2 Mm Trolox shown a resistance recovery in a barrier made of IPEC-J2 cells. Evolution of TEER at several time points in medium, 2 mM Trolox, 1 mM H_2O_2 and 4 mM DEM controls. Data were represented as means ± SEM (n ≥ 2) and normalized by multiplying with the insert surface.

In figure 17 were observed the most hazardous effects induced by 4 mM DEM (p = $2.2 \cdot 10^{-16}$ ***), involving a ten-fold decreased in TEER values (1.32 ± 0.042 kΩ x cm^2, $p < 0.001$ ***) in comparison with 1 and 17 h after oxidative stress (0.202 ± 0.011 kΩ x cm^2). Statistically significant differences with regard to time (p = $2.2 \cdot 10^{-16}$ ***), time: DEM (p = $2.2 \cdot 10^{-16}$ ***), ethoxyquin (p = 0.000415 ***), DEM: ethoxyquin (p = $1.104 \cdot 10^{-5}$ ***) and time: ethoxyquin (p = 0.0332*) were determined. The triple interaction within DEM: time: ethoxyquin was not significant.

In line with the statistical analysis, the time after oxidative stress was an influent factor. Thus, the comparison of the measured TEER values between 49-1 h, 41-1 h and 49-0 h revealed significant differences ($p < 0.001$***) in all the treatments tested. The epithelial resistance assessed before the incubation with 4 mM DEM and 41 h after showed relevant differences (p = 0.00349**). Between 0 and 41 h after inducing stress, only the oxidative treatments after 1 and 17 h presented significant values of TEER ($p=0.0332$*).

The previous IPEC-J2 cell monolayer incubation with a wide concentration range of ethoxyquin (3.124 to 25 µM) also induced the variability of TEER (p = 0.000415***) .The first hour after incubation with the stressor cost the half-resistance of the initial value assessed before the treatment. On the other hand, the ethoxyquin controls showed a high improvement of the TEER. Thus, the largest resistance 4.02 ± 0.11 kΩ x cm^2 observed was due to the effect of 3.125 µM ethoxyquin at 41 h. The strength of the barrier was accentuated over-time by lower concentrations of the antioxidant 1-41 h after treatments. Nevertheless, at the last incubation time 12 and 25 µM conditions showed a larger antioxidant potential.

In combination with DEM, the ethoxyquin was able to restore the resistance featured before the stressor incubation (p = $1.104 \cdot 10^{-5}$***). Also the IPEC-J2 monolayers treated with 4 mM DEM and 3.125 µM ethoxyquin showed a briefly improvement in tightness. In similar conditions, within the range 6.25-25 µM any significant differences were appreciated after 1 h of oxidative stress. Almost all the treatments performed, exceeded the typical values from a confluent monolayer recovering the tightness progressively.

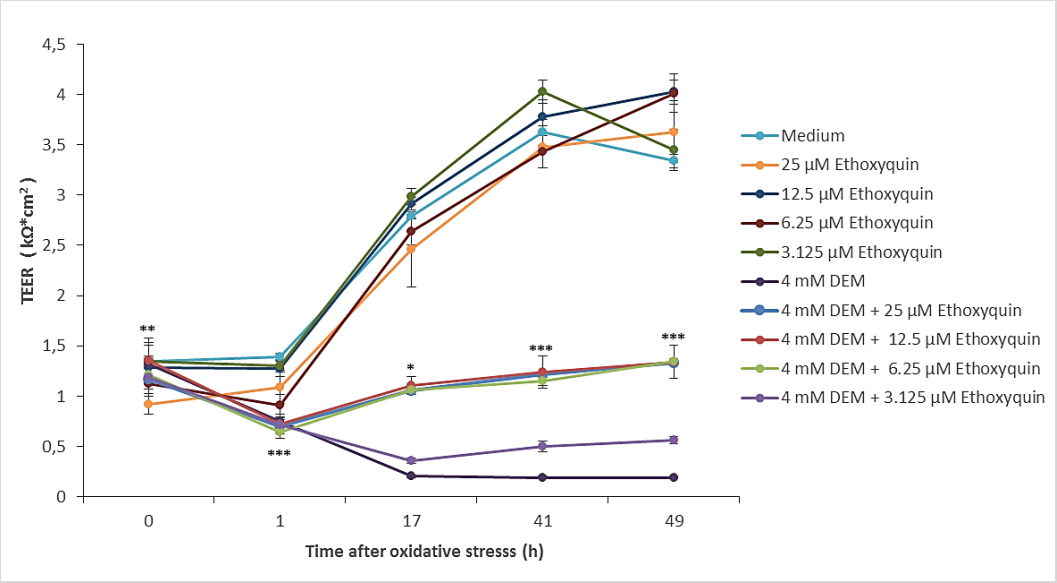

Figure 17. Ethoxyquin was able to repair oxidative stress induced as a beneficial potential by itself was shown. Evolution of TEER at several time points in medium, 4 mM DEM and ethoxyquin (25, 12.5, 6.25 and 3.125 µM) treatments performed. Data were represented as means ± SEM (n ≥ 2) and normalized by multiplying with the insert surface.

The effect of 1 mM H_2O_2 (p = $2.2 \cdot 10^{-16}$ ***) and 25-3.125 µM concentrations of ethoxyquin was determined by measuring over-time the TEER values in IPEC-J2 cell monolayers (see Figure 18). The values resulted from 3.125 and 6.25 µM treatments combined with 1 mM H_2O_2 evolved in parallel at several times, without being

able to carry out significant differences among the ethoxyquin concentrations. The highest TEER values assessed in ethoxyquin treatments were found 41 h after the incubation with 12.5 and 3.125 µM (3.5-4 kΩ x cm^2). In comparion with 1 mM H_2O_2 an improvement in the tight junction expression was noticed due to the overnight pre-treatments nevertheless was only observed after 17 h of incubation. In this way, 12.5 µM of ethoxyquin was the most suitable concentration restoring the strength of the epithelial monolayer (1.18 ± 0.2 kΩ x cm^2) indicating confluent cultures.

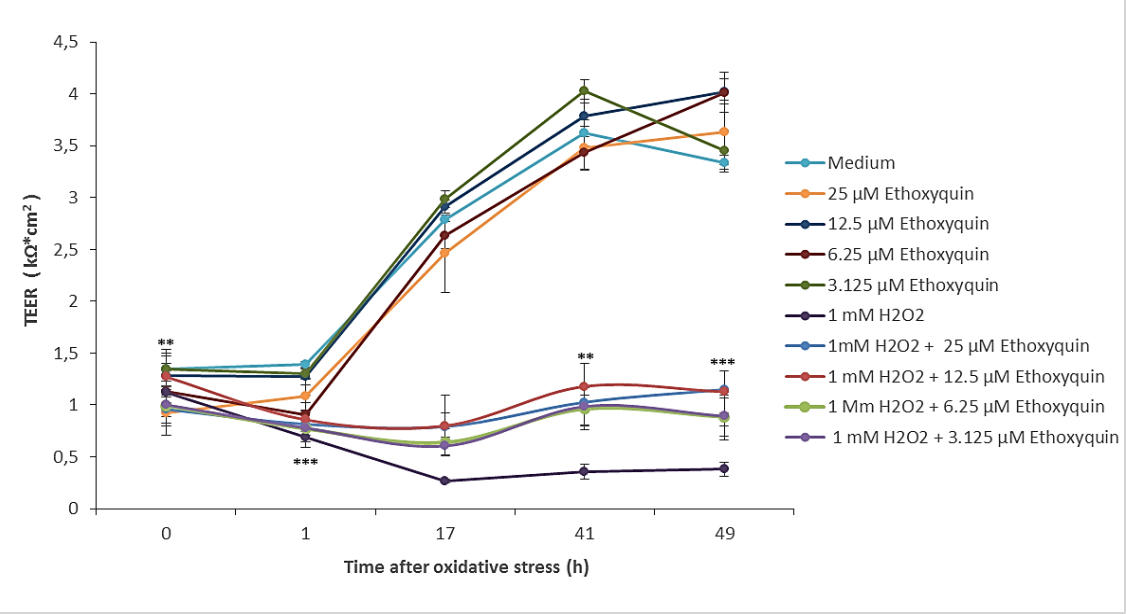

Figure 18. 1 mM H_2O_2 decreased the TEER in IPEC-J2 cell monolayers but was rescued by ethoxyquin pre-treatment over time. Evolution of TEER at several time points in medium, 1 mM H_2O_2 and ethoxyquin (25, 12.5, 6.25 and 3.125 µM) treatments performed. Data were represented as means ± SEM (n ≥ 2) and normalized by multiplying with the insert surface.

4.2.3. Overnight pre-treatment of IPEC-J2 cells with ethoxyquin concentrations higher than 3.125 µM recovered the functional permeability after 4 mM DEM but not after 1 mM H_2O_2 treatment.

The permeability values collected were based in the flux of FD-4 from the apical to the lower chamber after o/n inserts incubation with oxidant and antioxidant agents (see Figure 19).

The control used were IPEC-J2 cells monolayer cultured with medium with an average of 231.817 ± 112.44 picomoles of FD-4. In comparison, the most adverse effect was observed by the 4 mM DEM action (1458.050 ± 239.811 picomoles of FD-4, $p = 2.342 \cdot 10^{-7}$ ***). A high flux of FD-4 was measured also after 1 mM H_2O_2 (854.26 ± 128.02 picomoles, $p = 2.728 \cdot 10^{-7}$ ***), 2 mM Trolox with 4 mM DEM (858.522 ± 350.124 picomoles) and 1 mM H_2O_2 with 25 µM ethoxyquin (877.885 ± 142.76 picomoles) treatments. Any relevant differences were found in ethoxyquin or in combinations with the stressor agent related to 1 mM H_2O_2 experiments. The solely incubation with the antioxidant proved the reduction of the permeability with all the concentrations incubated ($p = 2.283 \cdot 10^{-6}$ ***). A recovery of the barrier's permeability was noticed due to the combination of ethoxyquin with the stressor agents, showing the lower concentrations of the antioxidant a higher potential recovering the impaired selectivity after 1 mM H_2O_2 incubation. Also, the experiments with 4 mM DEM and > 3.125 µM of ethoxyquin ($p = 4.769 \cdot 10^{-6}$ ***) restored the physiological permeability according to treatments with medium (240.521 ± 10.13 picomoles of FD-4) after the incubation with 4 mM

DEM was noticed, except 3.125 µM of ethoxyquin (703.09 ± 35.08 picomoles). The solely action of 2 mM Trolox triggered the reinforcement of the TJ proteins in comparison with the medium (96.457 ±1 0.125 picomoles). The effect of 2 mM Trolox within stress treatments, showed an improvement in the barrier's functionality as the permeated concentration was reduced approximately a 40% (575.54 ± 115.1, 858.5 ± 350.12 picomoles of FD-4) in comparison with the rates from oxidative stress controls (854.26± 138.02, 1458.1 ± 240 picomoles of FD-4).

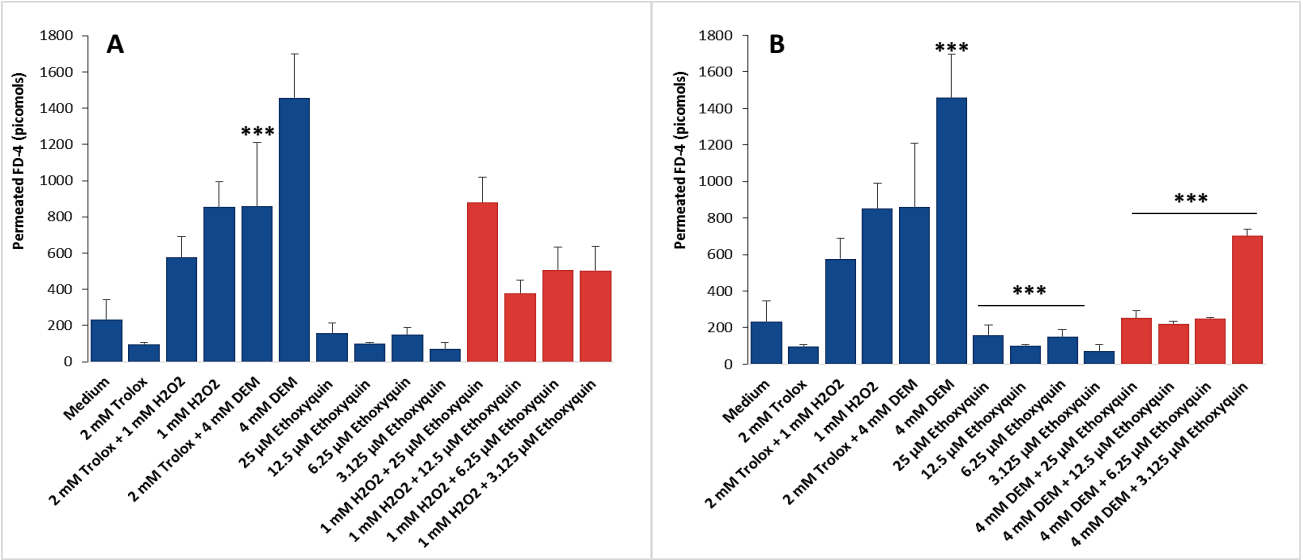

Figure 19. Pre-treatment with 2 mM Trolox and ≤ 25 µM Ethoxyquin incubation improved the permeability of IPEC-J2 cell monolayers treated with H_2O_2 whereas effects of DEM incubation were mitigated with ≤ 3.1225 µM Ethoxyquin. The paracellular flux to the basolateral chamber after o/n incubation with ethoxyquin and A) 1 mM H_2O_2 B) 4 mM DEM stressor agents was monitored by

5. DISCUSSION

5.1. IN VIVO EXPERIMENTS

As any significant differences were found, the birth weight and weaning strategy are not influencing the expression of ZO-1 either the barrier's function. According with the bibliography, any relation between the birth weight and a damaged intestinal barrier have been reported (Huygelen et al., 2014). Conversely to the experimental results, the huge potential of colostrum have been previously reported after the uptake on the first 24 h improving the survival rates during the transition to weaning (Cabrera et al., 2012). The sow's milk exert a beneficial effect raising the ZO-1 expression, more pronounced in LBW piglets especially in the ileum (was doubled in the oldest piglets). According to the theoretical frame, an intensive absorption of the immunoglobulins takes place in the distal area between 4 and 12 h after birth (King'ori, 2012).Similar consequences are observed in NBW piglets although a trend towards maintaining the expression is displayed. The colostrum intake promotes the gut development induced by the bioactive substances contained mostly on the first 36 hours (Bourne, 1973; Skrzypek et al., 2005). It contains growth factors (IGF-1, EGF and TGF-β) that accelerate the structural and functional maturation of the GIT. Thus, on the first 24 h after brith, a quick growth and maturation of the neonatal gut is achieved (King'ori, 2012). Furthermore, the small intestine's weight is increased more than 70% and the length is raised in a 20% until three days after birth (Xu et al., 1992). This post-natal growth is promoted by the incrustation of the immunoglobulins from colostrum in the mucosa. This fact, explains the intense remodeling of the small intestine and the high initial ZO-1 concentrations.

Furthermore, the neonatal epithelium is weaker allowing the transport of macromolecules (open gut barrier) and the absorption by pinocytosis until the second day of age (Barszcz and Skomiał, 2011). The absorption of the immunoglobulins is time-dependent due to the gut closure process. It takes place gradually along the small intestine meaning that the enterocytes form the proximal region are "closed" before the ones placed at the distal part. Initially, the uptake of macromolecules is a non-selective process that declines between 6-36 h after birth (Leary and Lecce, 1975, Sangild, 2003). This fact explains the dropped ZO-1 concentration measured at 5d on early-weaned piglets, emphasized by the low content of immunoglobulins in milk-replacers.

In contrast, the early-weaning strategy results in a delayed structural and functional maturation of the small intestine. The removal from the sow is stressful and challenging, inducing lower fed intakes propitiated by the new source of energy ant the exposure to new dietary antigens (Feed for health, 2011). Despite of, the expression of ZO-1 is in general well-maintained over-time involving a minor disruption of the barrier's homeostasis. Moreover, feeding neonatal piglets with formula-milk have showed less symptoms pointing out an impaired GIT (Huygelen et al., 2012). Nevertheless, the half of the pre-weaning mortality within 3 days after birth is associated with low colostrum intakes as plays a crucial role by providing an energy source, ensuring the immune transfer and promoting the intestinal maturation (Devillers et al., 2011). In this way, the supplementation with milk-replacer in conventional-weaned piglets is recommended during the suckling period trying to promote the nutrient intake and the daily weight gain (Croes, 2014).

5.2. IN VITRO EXPERIMENTS

The oxidative stress, as the immunostaining results reported in IPEC-J2 cultures.The direct and indirect stress induced by 1 mM H_2O_2 and 4 mM DEM agents cost the apoptosis in IPEC-J2 cells and disrupts the expression of ZO-1 proteins after 1 h incubation. The direct red-ox imbalance affects mainly the intracellular area while the indirectly ROS species produced also impair the intercellular contacts. Consequently, the cohesiveness of the sealed epithelial barrier is impaired resulting in a weaker barrier with a compromised homeostasis and function.

Particularly, the 4 mM DEM treatments show the most harmful effects as the cell polarity and the ZO-1 distribution is distrupted on the cell body and between neighboring IECs. The DEM agent alters the glutathione/glutathione disulfide system (GSH/GSSG) that participates in the intracellular red-ox homeostasis. Alterations in the GSH/GSSG cause the depletion of the glutathione generating oxidative stress in an indirect way (Franco and Cidlowsk, 2009). The TEER and FD-4 results are consistent with the effects observed in the controls from the stressor agents, as low TEER values and high permeated FD-4 are observed. Thus, an inverse the TEER and the permeability parameters are inversely correlated and experimentally proved. As the TEER assay evaluates the tightness of the epithelial barrier, low TEER values reflect a high paracellular flux of FD-4 resulted from the TJ opening and the diminished resistance played by IECs (Wijtten et al., 2011; Hu et al., 2013).

Nevertheless, the pre-treatments with ethoxyquin (except 3.125 µM) decrease the permeability and fortify the electrical resistance mitigating the effects associated with the 4 mM DEM incubation. Thus, the incubation with 12.5 µM of ethoxyquin restores the barrier's integrity coping the effect of the oxidative stress produced by both oxidants.

Normal values are assessed by TEER experiments in accordance to the range 1.2 - 6.5 kΩ x cm^2 reported by Zakrzewski *et al*. In general, the results are consistent with the FD-4 assays ensuring the comparison. Thus, the positive effect of the ethoxyquin controls is proved as well as low concentrations of the antioxidants resulted in high electrical resistances and low permeated picomoles of FD-4. Higher

concentrations of ethoxyquin in combination with 4 mM DEM, improve the TEER values and reduce the permeability with the 3.125 µM exception as the oxidant potential is higher than the restorative action of the ethoxyquin when the concentrations are equal.

An increase of confluence in the IPEC-J2 cultures is usually associated with larger electrical resistances and low permeability rates. This condition is observed in the Trolox and ethoxyquin controls. A high cellular density propitiates the establishment of intercellular unions, enhancing the ZO-1 expression. Consequently, the intercellular space is narrowed, encouraging the selectivity and hampering the passage of the voltage and the FD-4 molecules. The permeability test is based in a compound that easily diffuse across the paracellular space due to its molecular weight. In contrast with the TEER experiments, the conductivity of ions is compromised by the total cellular resistances performed by the paracellular and the transcellular pathways not being able the separation of the resistances (Günzel et al., 2012). In comparison with the experimental results from TEER, only the treatment with 25 µM ethoxyquin with 4 mM DEM show mixed results.

6. CONCLUSIONS

- The use of high prolific sows improving larger sized litters, tend to increase the incidence of the LBW piglets raising the mortality rates when the most suitable weaning strategy is not implemented (conventional weaning).
- The birth weight and the weaning strategy are not significant factors influencing the ZO-1 expression in neonatal piglets during the pre-weaning phase.
- The colostrum intake exert huge benefits improving the tightness of the barrier and the gut maturation in newborn piglets.
- The permeability, TEER and the tight junction expression are variables correlated with the status of the IPEC-J2 cell monolayers.
- The trans-epithelial electrical resistance and permeability are inversely correlated according to the experimental results performed in IPEC-J2 cell monolayers.
- The IPEC-J2 cell line is a suitable model to monitor *in vitro* the effect developed by stressor agents and evaluate the antioxidant potential.
- The of IPEC-J2 cultures 1 h incubated with 4 mm DEM and 1 mM H2O2 diminish the TEER parameter and increases the FD-4 permeation.
- The small intestine's barrier functionality is improved by dose-dependent concentrations of ethoxyquin.
- Overnight pre-treatments with ethoxyquin (especially with 12.5 µM) restore the permeability and the TEER over time and mitigates the effects of direct and indirect oxidative stress by the improvement of ZO-1 expression
- The permeability and the TEER parameters are suitable indicators to monitor the gut health *in vitro* reflecting changes in the tight junction expression related to adverse conditions.
- The experimental results from the TEER are more reliable than the permeability assays in the investigation of the cohesiveness from epithelial barriers managed by the tight junction proteins.

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