UNIVERSITÀ DEGLI STUDI DI MILANO

PhD school in Veterinary and Animal Science

Department of Health, Animal Science and Food Safety (VESPA)

(Class XXXIII)

NUTRITIONAL STRATEGIES FOR IMPROVING THE GUT HEALTH OF MONOGASTRIC ANIMALS

AGR/18 – Nutrizione e Alimentazione Animale

SILVIA GIORGI
Matr. R11902

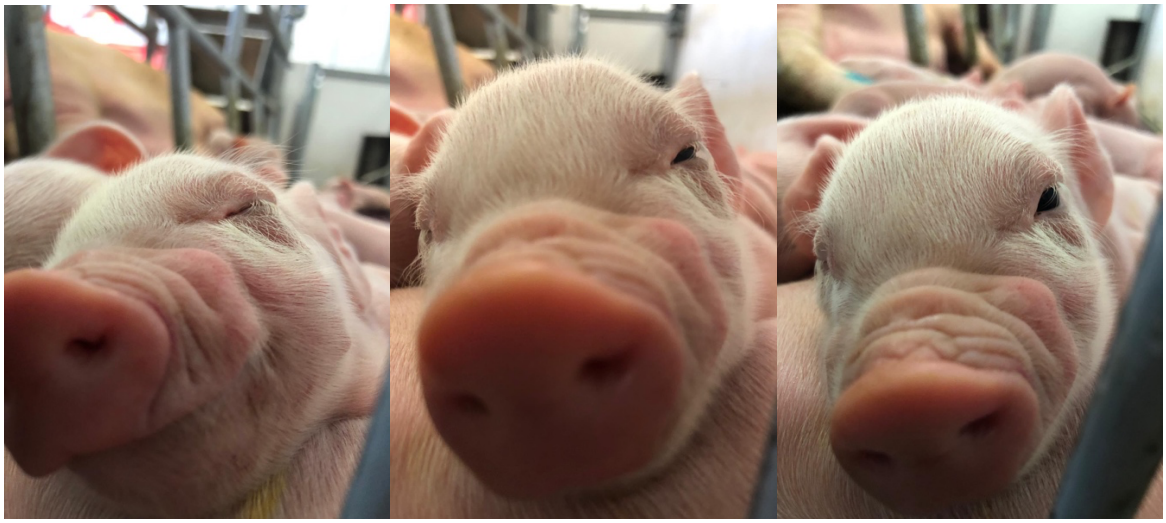
ADVISOR
Prof. Valentino Bontempo

PhD COURSE COORDINATOR
Prof. Valeria Grieco

Academic Year
2019/2020

Alle persone che guardano le stelle ed esprimono desideri.
Alle stelle che ascoltano e ai sogni che si avverano.
E a te nonno: la mia stella più bella.

TABLE OF CONTENTS



ABSTRACT

Optimal gastrointestinal functionality and health is essential for sustainable animal production, especially considering that nowadays, the challenge in livestock sector is to maintain a high productivity and food security in a sustainable way, reducing the use of antimicrobials. Nutritional strategies that aim to improve animals' performance preserving their intestinal health, are essential for achieving these goals. Therefore, in the present thesis, various nutritional interventions were evaluated, using short and medium chain fatty acids in particular, on the growth and intestinal well-being of pigs. The purpose of this thesis was to: 1) evaluate the effects of an innovative form of a medium chain fatty acid, lauric acid saponified with calcium, supplemented in post-weaning piglet diet on their growth and gut health and as a possible alternative to reduce the use of antibiotics; 2) asses the effectiveness of the combination of a short chain fatty acid (tributyrin) and a medium chain fatty acid (monolaurin) in post-weaning piglet diet on their growth performance and some gut health parameters; 3) evaluate the effects of lauric acid saponified with calcium administered to sows diet starting from the last 3 weeks of gestation, on sows productivity and on the growth performance and health of their offspring; 4) to assess the effect of fatty acids and different milk fractions on porcine IPEC-J2 cell line proliferation and viability. The results presented in this thesis highlight how short and medium chain fatty acids are effective candidates for improving animal health, reducing the use of antibiotics. This may contribute to the development of a more sustainable livestock production system based on the respect of animal health and the reduction of antimicrobials, as recommended by the "*One Health*" approach. However, further investigations are necessary to better understand the mechanisms of action, the dosage and the best forms of administration of short and medium chain fatty acids (alone or synergistically) integrated in pigs' diets, in particular during weaning, to further improve their growth performance and gut health.

RIASSUNTO

Una funzionalità ottimale e una buona salute gastrointestinale sono essenziali per una produzione animale sostenibile, soprattutto se si considera che, oggigiorno la sfida nel settore zootecnico è mantenere un'elevate produttività e sicurezza alimentare che sia sostenibile, riducendo al contempo l'uso degli antibiotici. Strategie nutrizionali che mirino a migliorare le performance degli animali preservandone la salute intestinale sono indispensabili per il raggiungimento di tali obiettivi. In questa tesi sono quindi state valutati diversi interventi nutrizionali, utilizzando in particolare acidi grassi a corta e media catena, sulla crescita e il benessere intestinale dei suini. Lo scopo di questa tesi è stato: 1) valutare gli effetti di una forma innovativa di un acido grasso a media catena, l'acido laurico saponificato con il calcio, integrato nella dieta post-svezzamento dei suinetti sulla loro crescita e salute intestinale e come possibile alternativa per la riduzione degli antibiotici; 2) valutare l'efficacia della combinazione di un acido grasso a corta catena (tributirrina) e un acido grasso a media catena (monolaurina) nella dieta post-svezzamento dei suinetti, sulle loro performance di crescita e su alcuni parametri di salute intestinale; 3) valutare gli effetti dell'acido laurico saponificato con il calcio, somministrato nella dieta delle scrofe a partire dalle ultime 3 settimane di gestazione, sulla produttività delle scrofe e sulle performance di accrescimento e sulla salute della prole; 4) valutare l'effetto degli acidi grassi a catena di diversa lunghezza e di diverse frazioni del latte bovino sulla proliferazione e vitalità della linea cellulare intestinale suina IPEC-J2. I dati presentati in questa tesi evidenziano come gli acidi grassi a corta e media catena siano dei candidati efficaci per migliorare la salute degli animali riducendo l'uso degli antibiotici. Ciò potrà contribuire allo sviluppo di un sistema di produzione zootecnica più sostenibile basato sul rispetto della salute degli animali e alla riduzione degli antimicrobici, come raccomandato dall'approccio "*One health*". Ulteriori indagini sono comunque necessarie per approfondire maggiormente la conoscenza sui meccanismi d'azione, sul dosaggio e le forme di somministrazione degli acidi grassi a corta e media catena (singoli o in sinergia) integrati nelle diete dei suini, in particolare nel periodo dello svezzamento, per migliorarne ulteriormente le performance di crescita e la salute intestinale.

CHAPTER 1 -

General Introduction

1.1 ANTIBIOTICS IN LIVESTOCK PRODUCTION

Our diets and food production systems have changed dramatically over the past decades and the way antibiotics are used every day is closely linked to these changes. Population growth and changing food preferences are largely driven by economic growth and urbanization. The consequence is an exponential increase in the demand for food of animal origin (Alexandratos and Bruinsma, 2012). The increase in production, particularly in swine, poultry and cattle farming, has been achieved largely through intensification: increasingly large, highly specialized farms, geographically disconnected from the land that produces their feed, containing high densities of animals genetically homogeneous and highly selected for production traits. As reported by Van Boeckel et al., (2015), intensive livestock farming has been and still is dependent on antibiotics for growth promotion, prevention and disease treatment. Therefore, these structural changes result in a considerable increase in the use of antibiotics. The authors also predicted that antibiotic use will double in fast-growing economies such as Brazil, Russia, India, China and South Africa by 2030.

Livestock production sits at the nexus of three global public goods: (1) health and nutrition; (2) climate and natural resource use; and (3) equity and growth. Each of these domains includes both positive and negative consequences on livestock sector growth (Food and Agriculture Organization, 2009). Antibiotic resistance epitomizes the issues around this nexus with dramatic impacts on health, equity and the environment. There is growing consensus that antibiotic use in livestock production is linked to antibiotic resistant human infections based on the evidence available from either whole-genome sequencing and phylogenetic or natural experiments involving the introduction or withdrawal of antibiotics from livestock production systems (Robinson et al., 2016).

Antibiotics were first studied in the late 1800s and it was in the early 1900s that penicillin was first discovered (Abraham and Chain, 1940). Since the application of penicillin in the 1940s, antibiotics have played an important role in the prevention, control, and treatment of infectious diseases both in humans and animals. In the animal production field, the benefits of using antimicrobials as growth promoters (AGPs) was firstly reported to promote growth in poultry in a study conducted by Moore et al., (1946) and subsequently in pigs (Jukes et al., 1950). Since then, antibiotics have been routinely used in animal production as AGPs and for disease prevention, reducing mortality and morbidity (Dibner and Richards, 2005). Typically, the

antibiotic is distributed to animals through the feed, as it is more efficient and less expensive to medicate entire groups than individual treatments.

The problem of antibiotic resistance has emerged because many of the antibiotics used in livestock are identical, or closely resemble to the drugs used in humans. Moreover, and unfortunately, continuous long-term exposure of gut commensals and pathogens to low-dose antibiotics in animals has been linked to the increased emergence of antibiotic-resistant pathogens (Lhermie et al., 2016).

1.2 THE ANTIBIOTIC RESISTANCE ISSUE AND THE "ONE HEALTH" APPROACH

Munita and colleagues (2016) deeply studied the mechanisms that regulate the development of bacteria antibiotic resistance. It is an inevitable and natural phenomenon for bacteria to develop resistance against antibiotics, but this is exacerbated by their inappropriate use. This, in turn, leads to treatment failure with detrimental effects on the health of both people and livestock, and on farmers' livelihoods. In fact, bacteria have a notable genetic plasticity that allows them to respond to a wide array of environmental threats, including the presence of antimicrobials. Bacterial cells derived from a susceptible population are able to develop gene mutations that negatively affect drug activity, causing cell survival in the presence of antimicrobial molecules (Munita et al., 2016).

According to O'Neill (2016), although the global burden of antibiotic resistance in livestock has not been estimated, 700.000 human deaths in 2010 have been linked to antimicrobial resistance (AMR) and this is projected to increase dramatically if no action is taken (O'Neill, 2016). The proportion attributable to bacterial infections is not distinguished but it is certainly considerable and is growing rapidly. The use of antibiotics as growth promoters has thus been prohibited in many countries, with Sweden being the first to ban antibiotics in 1986. Subsequently, Denmark banned their use in 1998 and was followed by the European Union, which introduced a total ban in 2006 (Castanon, 2007).

However, the prohibition on the subtherapeutic use of antibiotics in animal feed resulted in decreased animal production (Cheng et al., 2014) due to higher rates of infections in livestock and has also increased the risk of food-borne infections in consumers (Hao et al., 2014). As a

result, ten years later, prophylactic antibiotics are still used at high levels in many countries to sustain animal health and welfare (Aarestrup, 2012; Callens et al., 2012).

AMR is in rapid evolution and greatly affects the efficacy of antimicrobial treatment of patients infected with multi-drug resistant organisms. Therefore, AMR is currently considered to be one of the major public health threats for the near future. Suffice it to say that in 2016 a colistin-resistant, plasmid-mediated gene (MCR-1) was discovered in commensal *Escherichia coli* from pigs, pork meat products and humans in China (Rhouma et al., 2016). His discovery sparked global concern. In fact, colistin is considered an antibiotic of last resort that is one of the only antibiotics active in severe infections caused by multidrug-resistant pathogens such as *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Enterobacteriacee* (Catry et al., 2015).

Measures are needed worldwide to reduce the use of antimicrobials. In the last few decades, a new approach defined as "*One Health*" has been adopted. This concept recognizes that the health of people is connected to the health of animals and the environment. Physicians, veterinarians, ecologists, and many others have been working together to monitor public health threats and to learn about how diseases spreads. Thus, it is crucial to find a way to improve livestock health and welfare, to decrease antimicrobial use and to produce safe products in a sustainable way. Consequently, the research and development of innovative strategies is essential for improving animal health by reducing antimicrobial residues and antibiotic-resistant microorganisms and ensuring consumer health (Cheng et al., 2014).

1.3 ANTIBIOTIC USE IN PIG FARMING

Generally, antibiotics are used in pig farming in three main ways: 1) as growth promoters, 2) as prophylactic or metaphylactic treatment to prevent disease and 3) for therapeutic purposes to treat disease.

Traditionally, the use of the AGPs has been the most controversial because it has involved adding antibiotics to pig feeds that belong to the same chemical family as antibiotics that are valuable or critical in the treatment of human infections. Unfortunately, the AGPs treatment regimen created the ideal situation for the selection of antibiotic-resistant bacteria and the spread of antibiotic-resistance genes among enteric bacteria in the pig's intestinal tract. Feed companies used to prepare AGPs medicated feeds on instructions from farmers, and there has been often no veterinary supervision over their use. As mentioned above, for these reasons, the

use of AGPs was banned by the EU in 2006 (some had been removed from the market before then) and many other countries have also significantly restricted AGPs (Maron et al., 2013).

A wide range of antibiotics are used therapeutically in pigs. Pigs can be dosed individually orally or by injection, although the antibiotic is usually included in the feeding. The effectiveness of the latter can be questioned, as the farmer cannot guarantee that every pig receives the appropriate dose of antibiotic and, of course, sick animals often experience loss of appetite. The objective is that the medicated feed is only used when there is a threat of an outbreak of an infectious disease and is only used for a short period of time, perhaps 5–10 days. However, there is clearly the opportunity to use these medicated feeds repeatedly during one cycle of production or to use them for extended periods of time. However, in most countries, as in Italy, medicated feed for prophylactic/metaphylactic use require a veterinary prescription.

The fact that the purpose for antibiotic use is disease control means that an even wider range of antibiotics important in human medicine can be used in animal feeds. In a study by Callens et al., (2012) conducted in Belgium, where no guidelines for prudent use were implemented, it was reported that nearly half of the oral antibiotics administered were inadequate and that the antibiotics used included some important human antimicrobials such as colistin and amoxicillin. Furthermore, a systematic review concluded that oral use of antibiotics in animals increases the risk of antibiotic resistant *Escherichia coli* in treated pigs and by extension the risk of transfer of this resistance to humans (Burow et al., 2013).

Although the use of antibiotics in pig farming is currently a primary topic, limited information is available on the real amounts of antimicrobials used in pigs. A study conducted in Denmark reported an increase in the use of tetracyclines between 2002 and 2008, but a decrease in the use of macrolides, sulfonamides-tri-metoprim, cephalosporins and fluoroquinolones (Vieira et al., 2011). Interestingly, Denmark also imposed restrictions on pig farmers using more than double the average amounts of antimicrobials (Alban et al., 2012). Estimates from the United States indicate that annual use is higher for chlortetracycline (533.973 kg) and tylosin (165.803 kg) (Apley et al., 2012) while Canadian data suggests penicillin (35%), tetracyclines (11%) and ceftiofur (8%) were the most frequently used antibiotics, based on reports from veterinarians (Glass-Kaalastra et al., 2012). Jordan and colleagues (2009) reported that in Australia few of the antibiotics used to control *Escherichia coli* were significant in human medicine, although ceftiofur was used in nearly 25% of the herds sampled.

In general terms, in pig farming, antibiotics are commonly used in all the stages of pigs' life, but during lactation and post-weaning periods above all. Callens et al., (2015) reported that

more than 80% of antibiotics is applied to pigs at less than ten weeks of age and other similar studies reported that weaners received the major amount of antibiotics (Jensen et al., 2012; Fertner et al., 2015).

In nursery pigs, the removal of AGPs is causing several problems. In Sweden, mortality during the nursery period increased approximately 1.5 percentage units after the ban of AGPs, and total feed consumption from weaning to 25 kg increased by 2–3 kg. In addition, daily gain was reduced, resulting in animals requiring 5–6 days more to reach the target weight of 25–30 kg. Moreover, omitting the AGPs from diets fed to newly weaned pigs in particular, does cause several problems resulting in reduced performance and increased morbidity. Thus, a total elimination of antibiotics may not be the best solution. Raising antibiotic-free pigs leads to challenges in maintaining similar health, production, performance and profitability as compared to conventionally raised pigs. Specifically, antibiotic-free challenges include (but are not limited), to increased risk of:

- exposure to bacterial and viral pathogens within facilities;
- disease stress;
- body condition variability and days to market;
- mortality;
- reduced water intake and feed consumption;
- higher costs for alternative treatments against disease.

Moreover, pathogenic bacterial effects on lower gut integrity, associated with lack of antibiotic support, can lead to increased individual pig and farm population risks.

In general terms, the best way to protect pigs in an antibiotic-free environment seems to be proactive management practices, namely: judicious use of vaccines, rigorous cleaning and biosecurity measures, and the implementation of nutritional strategies designed to strengthen the animal's natural immunity, especially in the most delicate phase of pig life, as weaning.

1.4 “THE WEANING PROBLEM”

Weaning pigs from the sow is one of the most stressful events in pig’s life. In fact, weaning imposes tremendous stress on piglets and is usually accompanied by marked changes in gastrointestinal physiology, immunology and microbiology (Pluske et al., 1997; Campbell et al., 2013). Owing to these changes, the period following weaning is characterized by a high incidence of intestinal disturbances with diarrhea (PWD) and depression of growth

performance. Poor growth performance associated with weaning is a result of multi-factorial stressors, including environmental, nutritional and psychological stressors (Williams, 2003). In fact, at weaning, pigs have to deal with the abrupt interruption in the established social interaction with sow and littermates, and the stress of adapting to a new environment (Lalles et al., 2007). In addition, the piglet has to cope with the sudden withdrawal of sow milk and adapt to less digestible, plant-based dry diets containing complex protein and carbohydrate including various antinutritional factors (Lalles et al., 2007). Hence, piglets have a sharp reduction in feed intake immediately after weaning (Pluske et al., 1997). While approximately 50% of weaned pigs consume their first feed within 24-h post-weaning, in approximately 10% weaning anorexia persists for up to 48-h (Brooks et al., 2001). Antibiotics and minerals, especially zinc oxide (ZnO) and copper sulfate ($CuSO_4$), are often included in the diets of weaning pigs to control post-weaning PWD and optimize growth performance. However, in addition to the ban on antibiotics as growth promoters, a reduction in the use of $CuSO_4$ and ZnO is also foreseen due to environmental pollution caused by their marked presence in pig manure (Verstegen and Williams, 2002; Jondreville et al., 2003).

Technological improvements in housing, nutrition, health, and management have been used to minimize some of the adverse effects of weaning stress, but a greater understanding of the biological impact of stress is needed to improve strategies to overcome weaning stress (Campbell et al., 2013).

1.4.1 Feed intake and weaning

When the piglet is weaned, it must suddenly adapt from highly digestible and palatable liquid milk from its mother to a solid dry diet that is less digestible and palatable. As a consequence, feed intake is usually initially reduced after weaning. As reviewed by Le Dividich and Sève (2000), the extent and duration of reduced feed intake are variable. It is estimated that by the end of the first week post-weaning, metabolizable energy (ME) intake is about 60-70% of pre-weaning milk intake and that it takes approximately 2week post-weaning to achieve full recovery to the pre-weaning ME intake level. In a study conducted by Spreeuwenberg et al., (2001), was evaluated the relationship between low feed intake with different diet compositions (lactose/protein ratios) and small intestinal barrier function. They reported that during the first 4 days post-weaning, that diet composition was not as important a factor to maintain intestinal barrier function; but that continued low feed intake was more important to predispose the pig to intestinal barrier dysfunction. The direct consequence of a lower feed intake is a reduction

in growth performance. In general, pigs lose about 100–250 g body weight (BW) the first day after weaning, regardless of weaning age and recover this loss in BW by about 4 days post-weaning (LeDividich and Sève, 2000). Tokach et al., (1992) reported that weight gain in the first week after weaning has an impact on total days to market (at approximately 110 kg body weight). When pigs gained more than 227 g/day during the first week after weaning, the days to market were shortened by approximately 6-10 days compared to pigs gaining 0 g/day to 150 g/day the first week. Therefore, it is important that pigs eat and grow as soon as possible after weaning.

1.4.2 Intestinal structure and functional changes at weaning

Along with experiencing low feed intake, weaned pigs experience physiological changes in structure and function (enzyme activities and absorption or secretion) of the intestine. These physiological changes affect the small intestine absorbs capacity, which can likely influence feed efficiency (Pluske et al., 1997). Pluske et al., (1997) and Boudry et al., (2004) reported that weaning induces both acute and long-lasting structural and functional changes in the small intestine, including shortening of the villi (villous atrophy) and an increase in crypt depth (crypt elongation). The decrease in villous height continued until about 5 days after weaning, when the villi were approximately only half of the initial height. Crypt elongation was also evaluated with slower changes occurring over the first 11 days post-weaning. After weaning, pigs also experience reduced brush border digestive enzyme activities, as highlighted by Pluske et al., (1997). Accordingly, Lalles et al., (2004) reported reductions in lactase and amino-peptidase activity from 2 to 15 post-weaning days, while maltase was reduced for 2 days post-weaning, then increased from 8 to 15 post-weaning days. In addition, pancreatic secretions had a transient decrease to 15 days after weaning, before trypsin and amylase activity began to increase. Alkaline phosphatase, an enzyme that plays a role in detoxification of pathogenic bacterial lipopolysaccharide endotoxin and impacts intestinal inflammation (Lalles, 2010), is also reduced in early weaned pigs (Lackeyram et al., 2010). These alterations can impact the ability of the small intestine' s digestive, absorptive, and secretory capacity and ultimately the intestinal barrier function, which may contribute to PWD.

1.4.3 Intestinal inflammation associated with weaning

Beyond the compromised digestive and absorptive capacity, consequences associated with weaning also induce a negative effect on intestinal barrier function (Spreeuwenberg et al., 2001; Boudry et al., 2004; Moeser et al., 2007). The epithelial layer of the intestinal lumen represents the body' s first line of defense for protecting the pig from various harmful pathogens, toxins, or antigens that reside within the lumen of the small intestine. When the intestinal barrier is disrupted, the result is increased permeability that allows toxins, bacteria, or feed-associated antigens to cross the epithelium, resulting in inflammation, malabsorption, diarrhea, and reduced growth and production. In a study conducted by Nabuurs et al., (1994) was evaluated the effects of weaning and *Escherichia coli* infection on the absorption capacity of the small intestine of pigs. Pigs were either weaned at 30 to 32 days of age or unweaned by remaining with the sow, while *Escherichia coli* infection consisted of infecting segments of the small intestine using perfusion procedures. The results of the study allowed the authors to conclude that after weaning the net absorption of fluid and electrolytes is temporarily decreased which may contribute to PWD.

Studies conducted at North Carolina State University have investigated the effects of stress-induced intestinal damage associated with a weaning stress model. Moeser et al., (2007) evaluated intestinal dysfunction in 19 days old weaned pigs compared to unweaned pigs. Twenty–four hours after weaning, the pig's intestinal barrier function was evaluated for secretory activity by transepithelial resistance (TER) and intestinal permeability by paracellular mannitol flux in the jejunum and colon. In both the jejunum and colon, weaned pigs had greater secretory activity and intestinal permeability than unweaned pigs. This research was in agreement with the work of Boudry et al., (2004), who demonstrated a transient reduction in jejunal TER, but not in the colon. Moeser et al., (2007) also evaluated stress hormones and intestinal barrier function over 7 days post-weaning. They reported increased serum corticotrophin releasing factor (CRF) and cortisol in weaned pigs, indicating that weaning induces activation of the stress pathways which may be mediating the intestinal dysfunction. Finally, Smith et al., (2010) utilizing the weaning stress model evaluated the effects of early weaning stress on intestinal barrier function and intestinal health. Pigs from 5 different weaning ages (15, 18, 21, 23, and 28 days of age) were utilized. At 35 days of age, all pigs were evaluated for secretory activity and intestinal permeability in the jejunum. The results indicate that as weaning age was incrementally increased, improvements in intestinal barrier function were observed, as indicated by improved TER and lower permeability, as measured by mannitol and

inulin flux. To evaluate if the intestinal dysfunction was sustained, 15 or 28 days old weaned pigs were evaluated after 9 weeks of age. The results were similar to previous observations; earlier weaned pigs had a reduced TER and increased permeability. Thus, the research demonstrated that the stress resulting from weaning induces a breakdown in intestinal barrier function associated with increased permeability and mucosal inflammation and that weaning age can impact present and future mucosal barrier function of piglets (Moeser et al., 2007; Smith et al., 2010).

Other immunological responses that occur during the weaning process are alterations in pro-inflammatory cytokines. Pro-inflammatory cytokines have an influence on intestinal integrity and epithelial function as it relates to permeability and transport of nutrients (McKay and Baird, 1999). Pié et al., (2004) evaluated gene expression of pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α during the weaning process. The research demonstrated that weaning is associated with an up regulation of pro-inflammatory cytokines. Pié et al., (2004) also reported increased TNF-α expression initially by day 1 in the proximal and mid intestine followed by the distal small intestine and proximal colon from day 2 to 8. Thus, indicating that weaning is associated with an early up-regulation of genes of pro-inflammatory cytokines that may contribute to functional disorders, resulting in reduced subsequent performance and play a role in PWD. Regulation of metabolism may also be altered in response to weaning when inflammation associated with increased expression of pro-inflammatory cytokines occurs.

Pro-inflammatory cytokines regulate both immune function and growth or metabolic processes (Johnson, 1997; Spurlock; 1997). Thus, weaning stress impacts both structural alterations and active immune responses. As reviewed by Burrin et al., (2003), the intestine is a major site for amino acid oxidation, net synthesis, and utilization of amino acids for protein synthesis. Alterations in amino acid metabolism occur after weaning (Montagne et al., 2007), which may impact protein synthesis and subsequent tissue deposition. Williams et al., (1997) demonstrated that when the immune system is activated, growth, feed intake, feed efficiency, and lean tissue deposition is reduced. Therefore, the reduction or mitigation of post-weaning stress and subsequent effects in the structural changes of the intestine and activation of the inflammatory immune response, is critical for improving swine performance from weaning to market.

1.5 GIT PHYSIOLOGICAL AND METABOLIC CHANGES AROUND WEANING

The GIT of a pig is a very complex environment. In particular, the GIT of young pigs around the time of weaning undergoes rapid changes in size, protein turnover rates, microbiota mass and composition, and quick and marked alterations in digestive, absorptive, barrier and immune functions (Pluske et al., 1997). Burrin and Stoll (2003) divided these changes into the *acute phase*, observed within the first 5-7 days after weaning, and the *adaptive phase*, which occurs after this (Fig. 2). The authors distinguished between the acute and adaptive phases based primarily on the changes in feed intake and the subsequent impacts that enteral (luminal) nutrition has on the GIT, considering it takes 7-14 days for weaned pigs to learn how to eat and resume a level of dry matter intake (at least) that is comparable to that during the pre-weaning period (Burrin and Stoll, 2003).

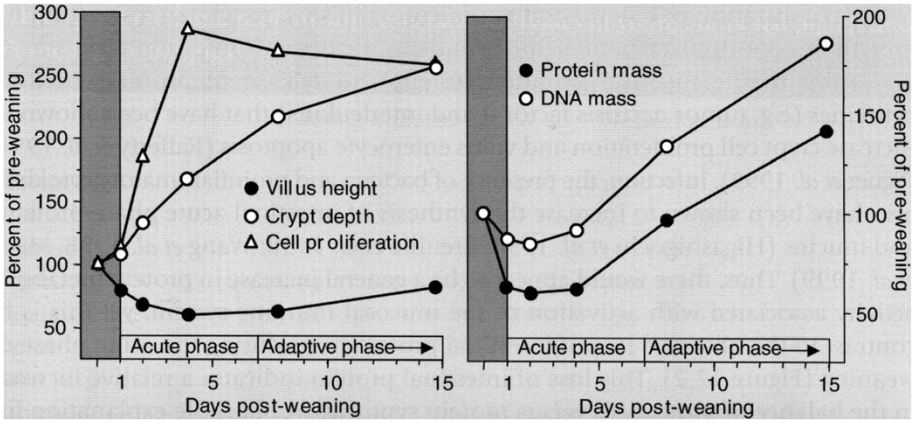


Fig. 1 – Acute and adaptive phases in development of weaned pigs, after Burrin and Stroll, 2003.

If the GIT is deficient in macronutrients, micronutrients and energy, then its health, development and any subsequent recovery in the adaptive phase will be impaired. As seen, the GIT plays a key role in achieving growth performance. For this reason, it is important to underline the effect of weaning on the different part of the GIT as was excellently done, in the review by Heo et al., in 2013.

1.5.1 Stomach

As studied by Barrow et al., (1977) in the past, and more recently by Zhang and Xu (2003), the functions of the stomach include feed mixing, partial digestion of feed and serving as a barrier against the external environment. Considering that the optimal pH for dietary protein digestion in the stomach is 3.0 (Proha´szka and Baron, 1980), to achieve the digestive function, the stomach is endowed with acid (hydrochloric acid (HCl)) secreting cells that help to keep its pH low (Yen, 2000) because a lower pH is required for conversion of the gastric zymogens into active enzymes (Khan et al., 1999). Neonatal pigs have a rather high gastric pH (5.0 to 6.0) facilitated by the strong buffering capacity of colostrum and milk. This might appear contradictory to the above, but there is a reason. A more tolerant gastric pH allows passage of ingested environmental bacteria (not all of them are pathogens, after all) from the stomach to the small and large intestine for the establishment of normal gastrointestinal microbiota. This is considered essential and even beneficial for the animal's long-term health, as in this way, a healthy and diverse microbiota can develop in the gut. Usually, the predominant beneficial bacteria in the stomach are lacto- and bifido-bacteria, whereas in the intestine there is a mix of bacteria. Nevertheless, after the first few hours of suckling, gastric pH drops to about 4 to remain there until weaning, and in most cases, during the first three to four weeks post-weaning. Callegari and colleagues (2015) found that at 63 days of life (40 days post-weaning) the pH of the stomach of piglets that had received a basal diet without any supplement, was on average 4.39. In fact, weaning pigs have a limited ability to secrete HCl, which may result in higher gastric pH (Efird et al., 1982). It is only after about 3 weeks post-weaning that gastric pH drops gradually until it reaches mature levels (2 to 3) (Roth, 2000; Callegari et al., 2015). Exposure to low pH values (i.e. 3.0–4.0) is bactericidal for many pathogenic bacteria, including *Escherichia coli* (Yen, 2000). Hence, in addition to its influence on nutrient digestion, maintenance of a low gastric pH value is essential for a healthy gut because this can help to reduce the passage of pathogenic bacteria into the small intestine.

Weaning also reduces gastric motility. This was demonstrated by Snoeck et al., (2004) reporting a reduction in the stomach emptying rate in pigs on 3 and 14 days after weaning, compared with suckling pigs. Given the high gastric pH that is usually observed post-weaning, gastric stasis may contribute to the development of PWD in piglets by allowing the proliferation of pathogens. In addition, it has been reported that a stress gene, corticotrophin-releasing factor receptor 2, whose activation has been implicated in the inhibition of gastric motility (Martinez et al., 2004), is up-regulated in the jejunum of weaned pigs (Moeser et al., 2007). Although

corticotrophin-releasing factor receptor 2 is yet to be identified in the stomach of the weaned pig, the finding of Moeser et al. (2007) suggested that changes in gastric emptying rate could be modulated by intestinal feedback (Boudry et al., 2004a; Lalles et al., 2007). Other factors that can be involved in gastric emptying rate are feed intake and composition of the diet (Lalles et al., 2007). For example, switching pigs abruptly from a milk-based diet to a wheat-based diet at 5 weeks after weaning resulted in a transient increase in the gastric emptying rate (Boudry et al., 2004a).

1.5.2 Small intestine

The small intestine performs many physiological functions, the most important of which is certainly the digestion of nutrients as well as the secretion and absorption of liquids and electrolytes. Similarly, as has already been mentioned, significant changes in the structure and function of the small intestine are known to occur in the immediate post-weaning period (Pluske et al., 1997; Hopwood and Hampson, 2003; Heo et al., 2013).

The epithelial lining of the small intestine has finger-like projections known as villi, which help to increase its surface area for digestion and absorption processes (Zhang and Xu, 2003). In addition, the mucosal surface of the small intestine has tubular glands that open into the intestinal lumen at the base of the villi, known as crypts. Crypts contain epithelial stem cells required for repopulation of epithelial cells (Zhang and Xu, 2003; Llyod and Gabe, 2008). For optimal function of the small intestine, longer villi are desirable. However, there is a period of transient villous atrophy and crypt hyperplasia after weaning, and post-weaning anorexia has been suggested to be the main aetiological factor in these changes, as energy intake after weaning is positively related to the small intestinal architecture (Pluske et al., 1997). In an old work by McCracken et al., (1999), is reported that weaning anorexia is correlated with crypts hypertrophy and local inflammatory responses: reduced feed intake but not diet composition compromised epithelial architecture in jejunum of pigs fed a diet based on soybean meal compared with those fed a milk replacer. In general, it is rather difficult to compare data on intestinal morphology from different experiments because of differences in the age, breed, diets and experimental conditions and also because there are no known standards for villus heigh and crypt depth measurements. Collectively, it is evident that maintaining energy intake and reducing weaning stress are important factors for maintaining the integrity of the small intestinal structure immediately after weaning (Pluske et al., 1997; Moeser et al., 2007).

The brush-border surface of enterocytes performs digestive actions in the small intestine. Enterocytes account for approximately 90% and 95% of the epithelial cells in the crypt and villus, respectively, and they are responsible for releasing digestive enzymes. These enzymes are mainly mucosa-based and can be easily distinguished from pancreatic enzymes that act mainly on the luminal content of the intestine (Adeola and King, 2006). Activities of the brush-border enzymes in weaned pigs have been used as indicators of maturation and digestive capacity of the small intestine since 1985 (Henning, 1985). A reduction in lactase activity is usually observed after weaning, and this is partly related to ontogenic decline in brush-border lactase activity (Kelly et al., 1991a; Motohashi et al., 1997). However, there is a lack of consistency in the literature as to the effect of weaning on the activities of other brush-border disaccharidases. For example, weaning caused an increase in the activity of sucrase, maltase and glycoamylase during the first week after weaning, as reported in the study of Kelly et al., (1991b). On the contrary, Hedemann and Jensen (2004) highlighted a decrease in sucrase and maltase activities. Discrepancies between studies are probably a result of multiple variations such as experimental design, experimental diets, age of the animals, analytical and statistical methodologies and days post-weaning at which measurements were taken.

Starvation owing to anorexia and the presence of immature enterocytes owing to villous atrophy could play a role in the decline in brush-border peptidase activities around weaning (Hedemann et al., 2003). In studies where transient reductions in brush border enzyme activities have been reported, enzyme activities reached minimum levels between 3- and 5-days post-weaning and increased gradually thereafter. The increase in brush-border enzyme activities after the first 5 days post-weaning is probably due to an increase in substrate availability as daily feed intake increases (Pluske et al., 1997).

Secretion of fluids and electrolytes from crypt cells and nutrients absorption from the intestinal lumen are part of the primary functions of the small intestine (Pa´cha, 2000; Xu, 2003). Small intestinal secretion is a natural physiological phenomenon and is essential for nutrient digestion and absorption. However, a net secretory condition occurs when fluids and electrolytes influx into the gut lumen exceeds its efflux into the blood, and this may serve as a predisposing factor for PWD (Pa´cha, 2000; Wapnir and Teichberg, 2002).

Changes in the absorptive and secretory function of the small intestine after weaning are segment dependent. For example, Boudry et al., (2004a) reported an increase in Na+-dependent glucose absorption in the jejunum of weaned pigs, but the opposite occurred in the ileum. Likewise, basal short-circuit current, which is a measure of ion transport, was increased in the jejunum. The authors, however, called for caution in the interpretation of increased jejunal

absorptive capacity as this was accompanied by villous atrophy and decreased enzymatic activities in the jejunum. Hence, the increased jejunal absorptive capacity might have little or no biological significance. The low ileal absorptive capacity shortly after weaning, could contribute to high incidences of osmotic diarrhea in piglets by increasing the amount of nutrients in the hindgut.

1.5.3 Large intestine

The three components of the large intestine are the caecum, colon and rectum. Physiological functions of the large intestine of the pigs include fluids and electrolyte absorption, and provision of a physical barrier against microbial invasion (Williams et al., 2001; Zhang and Xu, 2003). Hence, alteration in these functions may play a role in physio-pathogenesis of post-weaning intestinal problems.

The mucosal surface of the large intestine is lined with crypts, but unlike the small intestine, it lacks villi. Weaning decreased the crypt density and increased the mitotic index in the caecum of piglets (Castillo et al., 2007). Weaning also caused a transient reduction in the absorption capacity of the colon as indicated by basal short-circuit current at 2 days after weaning (Boudry et al., 2004b). It has been documented that excessive fluid loss in the small intestine will only result in PWD when the absorption capacity of the large intestine is exceeded. Based on the available data, it appears that a combination of alteration in the structure and absorptive function of the large intestine could contribute to the increased incidence of PWD in piglets (Hopwood and Hampson, 2003). Nabuurs (1998) reported that simulated halving of the absorption capacity of the large intestine increased the adverse effects of the activity of ETEC in the small intestine of piglets. Future research should investigate changes in absorption capacity of the large intestine in piglets after weaning.

1.6 GUT HEALTH

As shown above, the optimal gastrointestinal function is of prime importance for sustainable animal production, especially at weaning. The efficiency of the GIT and its health are important factors in determining animal performance (Celi et al., 2017). Over the last few decades, the adoption of genetic selection for high growth and reproductive traits, the implementation of advanced husbandry techniques (vaccination, hygiene, housing, transport, management, etc.), better understanding in digestive physiology and dietary requirements of farmed animals, has

led to significant gains in productive performance. In this regard, the crucial question that animal scientists have asked themselves was: “Has the performance of farm animals reached their genetic / physiological limits?”.

It is within this context that the concept of “gut health” has begun to attract significant interest within the animal science community (Kogut and Arsenault, 2016). However, while gut health is an increasingly important topic in animal nutrition, a clear scientific definition is still lacking, although it has been used repeatedly in animal health (Kogut and Arsenault, 2016). A clear definition of gastrointestinal health and functionality and how it can be measured is required to monitor animal health and to evaluate the effects of any nutritional intervention on animal performance. While in human medicine, gut health is often associated with the “absence of clinical diseases”, this definition cannot be applied to farm animals as it is well known that animal performance can be impaired without any clinical signs of diseases.

Several publications have summarized timely information regarding the definition of “gut health” (Bischoff, 2011; Pluske, 2013; Kogut and Arsenault, 2016; Adewole et al., 2016; Celi et al., 2017; Moeser et al., 2017; Jayareman and Nyachoti, 2017). Kogut and Arsenault (2016) defined a healthy gut as the “absence/prevention/avoidance of disease so that the animal is able to perform its physiological functions in order to withstand exogenous and endogenous stressors”. In a recent publication of Pluske et al., 2018, whilst not disagreeing with Bischoff definition, stated that gut health should be more general and can be described as a generalized condition of homeostasis in the GIT. In accordance with the World Health Organization (WHO) definition of “health” from 1948 (cited by Bischoff, 2011), which proposed a positive definition instead of “the absence of diseases”. In this regard, Bischoff (2011) also commented that “gut health” is “a state of physical and mental well-being in the absence of GI (gastrointestinal) complaints that require the consultation of a doctor, in the absence of indications of risks for bowel disease and in the absence of confirmed bowel disease”. This clearly refers to human health. However, Bischoff (2011) also argued that although the WHO has defined health as being more than just the absence of disease, the prevention or avoidance of GIT disease forms an integral part of our understanding of the general issue. Bischoff (2011) then further defined 5 major criteria that could form the basis of an overarching definition of gut health: 1) effective digestion and absorption of food, 2) absence of GIT illness, 3) normal and stable intestinal microbiota, 4) effective immune status, and 5) general status of well-being. Similarly, Celi et al., (2017) remarked that the key components of GIT functionality are diet, effective structure and function of the gastrointestinal barrier, host interaction with the gastrointestinal microbiota, effective digestion and absorption of feed, and effective immune status.

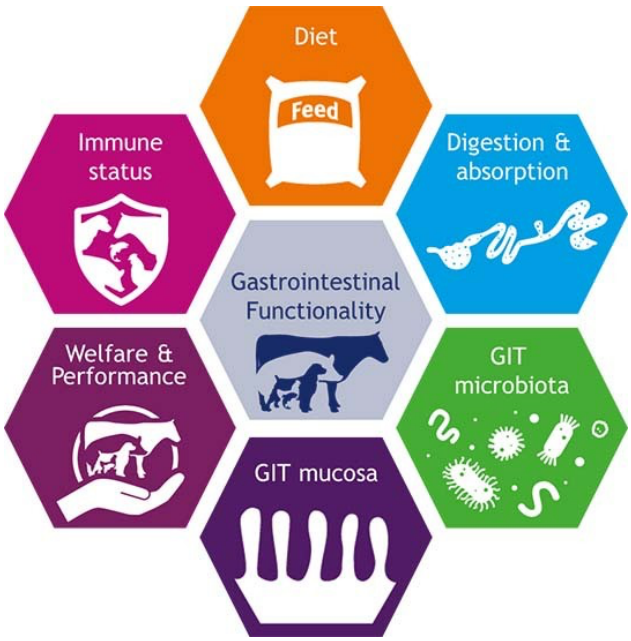


Fig. 2 - Main components contributing to animals' gastrointestinal functionality (adapted from Celi et al., 2017).

However, and although correct, the functions of the GIT extend beyond the processes associated with feed intake, digestion, and the subsequent active or passive absorption and barrier function. In fact, the GIT also plays an important role in the regulation of epithelial and immune systems of vital importance for normal biological functioning and homeostasis in both the GIT and the body. The association between the enteric nervous system (ENS) and the higher centers, via the parasympathetic nervous system and/or endocrine system, also plays a major role in animal well-being, health, and structure and function of the GIT (Moeser et al., 2017).

Regarding pigs' gut health, these definitions typically associate gut health with pathogens that cause, either clinically or sub clinically illness, mortality and (or) morbidity to pigs, and subsequent economic losses. However, and in agreement with the definition of Bischoff (2011), Pluske et al., (2018) proposed that gut health in pigs can be compromised even in the absence of any over disease(s) in the GIT. The low feed intake after weaning means a lack of nutrition, and stressors and challenges associated with weaning also cause changes to the structure and function of the GIT (Pluske et al., 1997; Kim et al., 2012, Celi et al., 2017; Moeser et al., 2017). As already seen, the immediate post-weaning period in pigs not only causes marked structural and functional changes to the small intestine (Pluske et al., 1996a, b), but also contributes to an intestinal inflammatory status that in turn compromises villus-crypt architecture (Pié et al.,

2004), GIT barrier function (Camilleri et al., 2012; Kim et al., 2012; Moeser et al., 2017; Wijtten et al., 2011), and disruption of the microbiota (Fouhse et al., 2016; Gresse et al., 2017; Schachtschneider et al., 2013).

1.6.1 Microbiota of the gastrointestinal tract: implications for gut health

The general features of the GIT microbiota are well known. It is also evident that a myriad of factors influences the diversity and activity of the GIT microbiota, including: colonization and associated succession of microbial populations, the age of the pig and the environment it inhabits, antimicrobial agents, dietary composition, feed additives, feed processing, feeding methods, disease load, weaning, season, environment, stress and genetics. Furthermore, the intestinal microbiota represents a compromise between helpful barrier functionality, synthesis of beneficial nutrients and proteins and improved energy harvest from dietary components with low inherent potential, and the deleterious effects of inflammation and sub-clinical (and clinical) pathologies (Celi et al., 2017). As expected, there is a varied assemblage of bacteria that diverges in population density and diversity in different compartments of the GIT and at different stages in the life of a pig (Holman et al., 2017; Zhao et al., 2015). Additionally, the microbiota is intimately involved in cross talk between the enteric bacteria and the host, with the chemistry and distribution of bacterial binding sites on gut mucosal surfaces playing key roles in determining host and tissue susceptibility and in triggering host responses, especially in young animals (Kelly and King, 2001; Montagne et al., 2003; Celi et al., 2017).

Some of the discussion with regard to the microbiota and gut health focusses simplistically to ‘good’ versus ‘bad’ bacteria and their impact on GIT structure and function. However, Hillman (2004) proposed that emphasis in relation to the composition and diversity of the GIT microbiota, should be placed on an optimal microbiota being present in the GIT, rather than a normal microbiota, because commensal and pathogenic co-exist normally, even in the absence of overt disease, for much of a pig's productive life. Hence, the presence or absence of a pathogenic organism may not necessarily predict that disease will occur unless numbers proliferate to such an extent to overwhelm the general microbial population in the GIT, or more specifically a specific region of the GIT (Hopwood et al., 2005). This will vary according to production sites (e.g., indoor or outdoor sites; Schmidt et al., 2011), genetics, diets and so on. In this respect, Hillman (2001) showed that lactobacilli having antipathogenic activity against pathogenic F4 *Escherichia coli,* were unevenly distributed across 19 Scottish pig farms, likely explaining variations seen in the efficacy of probiotics and other additives.

The addition of an antimicrobial to the GIT where there is a population of bacteria already possessing a high indigenous antimicrobial activity, is likely to be less effective than adding it where there is a population that shows little or no pre-existing antimicrobial activity (Hillman, 2001). The consistency of activity (e.g., across farms, across diets, across seasons) rather than the degree of activity of selected feed additives that modulate the GIT bacteria, that in turn may impact on gut health, is currently poorly understood with respect to the large number of antibiotic replacements/ alternatives on the market at present. Arguably, in this regard, a focus for gut health should be on supporting the animal to regulate shifts in the intestinal microbiota such that rapid population swings are avoided, and equilibrium can be maintained.

1.6.2 Gut microbiota disruptions and post-weaning diarrhoea

Amongst the physiological and GIT factors impacted by the weaning transition, microbiota disruption in the GIT is likely a key influence, usually leading to PWD.

Most of the studies conducted during the weaning transition have reported a decrease in bacteria of the *Lactobacillus* spp. group and a loss of microbial diversity. On the other hand, Clostridium spp., Prevotella spp. or facultative anaerobes such as *Proteobacteriaceae*, including *E. coli*, were positively impacted (Gresse et al., 2017).

The piglets' change from sows' milk to a solid diet of different composition and form undeniably plays a major role in the predisposition to diarrhea after weaning, both of microbial and dietary origin. Furthermore, in-feed and (or) in-water antibiotics also cause differences in the GIT microbiota at weaning due to their wide spectrum of activity and thus their potential ability to kill or prevent the growth of both pathogenic and beneficial microbes (Gresse et al., 2017). The diversity of the microbiota may be even more decreased (Looft et al., 2012) with the extended use of AGPs, which can increase opportunities for pathogenic microorganisms to colonize and trigger diseases (Fouhse et al., 2016). In this regard, antimicrobial resistance is also key to any discussions pertaining to the use of antibiotics. Consequently, weaning under commercial conditions has been associated with a disrupted state of the microbiota, or dysbiosis (Lalles et al., 2007a). However, the precise underlying characteristics allowing the prediction of such a state are not completely clear.

As alluded to previously, a feature of the GIT after weaning is an inflammatory response. Zeng et al., (2017) remarked that perturbations of the microbiota are commonly observed in diseases involving inflammation in the GIT, with the inflamed microenvironment being particularly conducive to overgrowth of *Enterobacteriaceae*, which acquire fitness benefits

while other families of symbiotic bacteria succumb to environmental changes inflicted by inflammation. GIT inflammatory host-response mechanisms produce reactive species such as nitric oxide that, when released into the GIT lumen, is rapidly transformed into nitrate. This nitrate-rich environment confers growth advantages on some strains of *E. coli*, which possess nitrate reductase genes that are absent in species of *Clostridia* or *Bacteroidia* (Gresse et al., 2017). A recent study conducted by Wei et al., (2017), reported an increased concentration of reactive oxygen species in the intestine coupled with an expansion of the *E. coli* population 7 days after weaning. Consequently, there is much interest in the use of assessing antioxidant status (Buchet et al., 2017) and compounds (Zhu et al., 2012) mitigate this aspect of gut health.

1.6.3 Disbiosis

As underlined above, the structural and functional changes occurred at weaning in pigs' gut, are correlated with alterations of the intestinal microbiota, which can predispose to the onset of enteric pathologies (Gresse et al., 2017).

In pigs, the intestine is colonized at birth. *E. coli* and Streptococcus spp create an anaerobic environment that is colonized by bacteria, such as *Bacteroides*, *Lactobacillus*, B*ifidobactrium* and *Clostridium*, selected by the milk diet. At the time of weaning, with the transition to a diet based on cereals and often with a high protein content (not easily digestible in relation to the age of the animals), there is a net decrease in *Lactobacillus* and a loss of microbial diversity, while *Clostridium* spp., *Prevotella* spp., or facultative anaerobes, such as *Proteobacteriaceae,* increase.

The loss of microbial diversity and the decrease in *Lactobacillus*, bacteria among the most important for maintaining the balance of the intestinal microbiota, increase the risk of onset of dysbiosis and infectious diseases. Gresse et al., (2017) hypothesized that the series of events leading to dysbiosis, although not fully understood, start from the lesser diversity of the intestinal microbiota, which makes the glycans, which make up the intestinal mucus, more available for pathogenic microorganisms. In fact, the degradation of mucus by commensal bacteria (such as *Bacteroides*) releases sugars such as fucose, galactose or mannose, used by potentially pathogenic species as *E. coli* or *Salmonella* spp. Intestinal inflammation, in turn, favors the development of *Enterobacteriaceae*. The nitric oxide produced by the inflamed tissue is transformed into nitrate in the intestinal lumen, favoring strains of *E. coli* compared to *Clostridium* or *Bacteroides* as the latter do not possess the nitrate reductase enzyme. Finally, the concentration of oxygen in the inflamed tissues (linked to the increased blood flow), can

favor facultative anaerobes, such as *Enterobacteriaceae* at the expense of strict anaerobes, leading to a further loss of bacterial diversity, and triggering a vicious circle that potentially causes enteric infection (Figure 3).

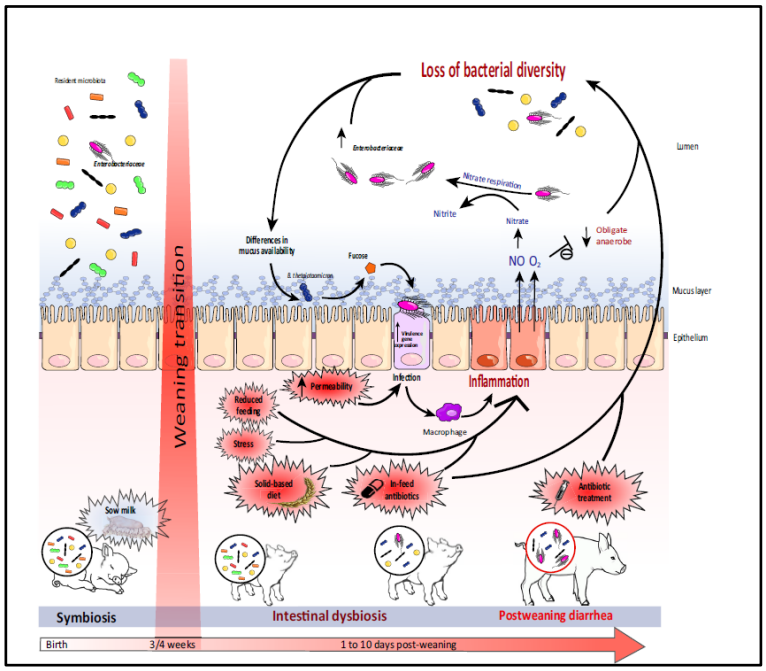


Fig. 3 - Diagram of events related to the onset of dysbiosis in post-weaning pigs (from Gresse et al., 2017)

1.6.3.1 Streptococcosis

Streptococcosis is a disease that causes significant economic losses in the pig industry, due to limited but persistent mortality (especially at weaning), as well as from the formation of waste and increased health costs (Sala and Zavattini, 2018). The causative agent is *Streptococcus suis*, a gram-positive bacteria. Among the 35 serotypes identified on the basis of capsular polysaccharides (CPSs), serotypes 2 and 9 are the most frequently isolated from pigs affected by the disease; serotype 2 is also a zoonotic agent (Segura et al., 2017). The natural habitat of this pathogen in pigs, is the upper respiratory tract (tonsils and nasal cavities), the genitals, and the intestine. Adult pigs are often healthy carriers of *Streptococcus suis*. In fact, the incidence of the disease affects about 5% of carriers and, in the absence of treatment, mortality reaches 20%. In most cases, the disease affects animals between 4 and 10 weeks of age, particularly after 10-15 post-weaning days. The concomitant presence of immunosuppressive viruses (PRRS and PCV2) can further complicate the situation.

The most frequent clinical manifestation is that related to meningitis, with the appearance of symptoms such as lethargy, hyperthermia, incoordination, atypical postures that result in decubitus, opisthotonos, pedaling, convulsions and nystagmus (Figure 4 and 5). Other symptoms are anorexia and lameness due to arthritis (Sala and Zavattini, 2018; Gottschalk, 2012).

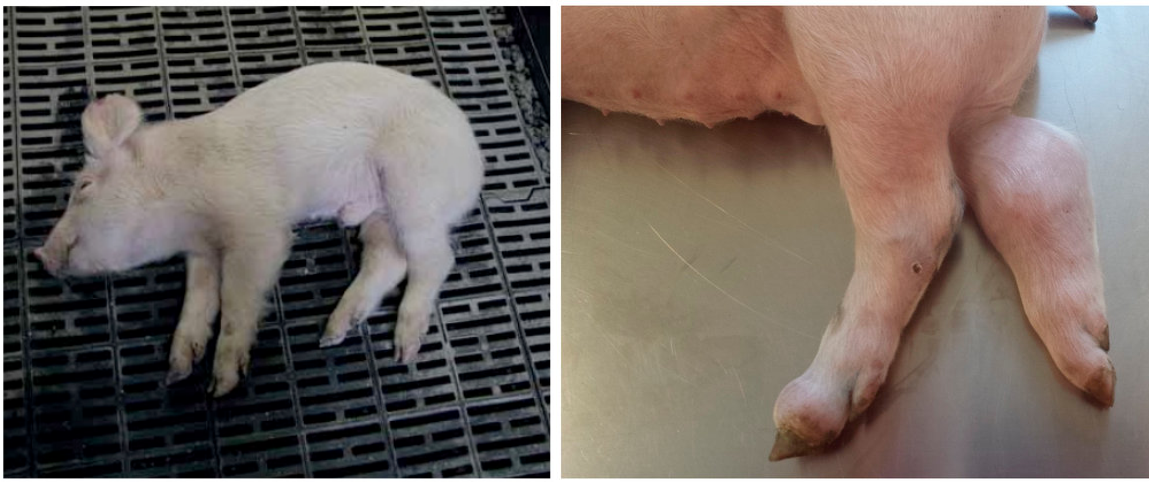


Fig. 4 and 5 - Acute symptoms of Streptococcus suis infection during meningitis and arthritis (from Sala and Zavattini, 2018).

The main route of transmission of *Streptococcus suis* occurs through the respiratory tract (oro-nasal). During farrowing, *Streptococcus suis* is transferred from sows' vaginal secretions or saliva to the piglet's nasal cavity and colonizes the tonsils soon after birth. In post-weaning, transmission occurs by direct contact (nose-nose) or via saliva and blood (Segura et al., 2016). Practices of cutting teeth or tail, castration or tattooing are also possible routes of infection.

It is generally accepted that the main route of penetration into the body is represented by the tonsils. In recent years, however, various evidences have shown that the GIT cannot be excluded as a secondary site of infection. Su et al., (2008) studied changes in the composition of the gut microbiota, with an emphasis on *Lactobacillus* spp. and *Streptococcus suis*, in the stomach, jejunum and ileus of piglets after weaning (21 days after farrowing). At 24 days, *S. Suis* predominated in digestive samples at the stomach, jejunum and ileus levels, with levels up to 10^7/g but in the stomach was not detectable before weaning. At the same time, the proportion of *Lactobacillus* was significantly decreased (see Table 1).

Tab. 1 - Quantitative real-time PCR analysis of total bacteria load, Lactobacilli, Lattobacillus sobrius and Streptococcus suis in stomach, jejunum and ileum content samples at 21 and 24 piglets post-weaning days (adapted Su et al., 2008).

Pathogens	**Stomach**		**Jejunum**		**Ileum**	
	21d	24d	21d	24d	21d	24d
Total bacterial load	5,40	7,95*	8,99	8,23	9,72	8,29*
Lactobacillus	4,55	6,04*	8,11	5,60*	9,29	6,80*
Lactobacillus/bacteria (%)	14,13	1,23	13,18	0,23	37,15	3,24
L. sobrius	4,14	5,64*	7,12	5,05*	8,65	5,94*
Lactobacillus sobrius/bacteria (%)	5,50	0,49	1,35	0,07	8,51	0,45
S. suis	< 4	7,36*	6,34	7,04	7,24	7,60
S. suis/bacteria (%)	< 3,98	25,70	0,22	6,49	0,33	20,42

The results suggested that, in the immediate post-weaning, the defensive barrier of the stomach may not offer sufficient challenges to the propagation of *Streptococcus suis*, favoring an increase in concentration in the intestinal contents. Piglets with high intestinal concentrations of *Streptococcus suis* can be an important source of transmission within the herd and weaning groups, and strategies aimed at decreasing its concentration result in lower environmental pressure and lower probability of disease spread.

In some in vitro studies, specific interactions between porcine intestinal epithelial cells and *Streptococcus suis* have been described, showing that this pathogen is able to translocate from the intestine and reach the bloodstream, colonize different tissues, and cause disease (Segura et al., 2016). Ferrando and Schultsz (2016) proposed a probable mechanism of *S. suis* infection via the intestinal mucosa. According to the authors, the translocation of the bacteria could take place in the small intestine, due to their abundance in post-weaning period. Colonization of the mucosa is mediated by adhesins (ApuA and SadP) which interact with cell receptors. In addition, ApuAs, by degrading α-glucans, allow bacteria to proliferate. Translocation through the epithelium can occur via the paracellular and / or transcellular route, favored by the production of enzymes that damage the intestinal barrier such as Suilisina (Sly). Once it reaches the subepithelial connective tissues, *S. suis* can enter the lymphatic and blood systems and reach the target organs as shown in Figure 6.

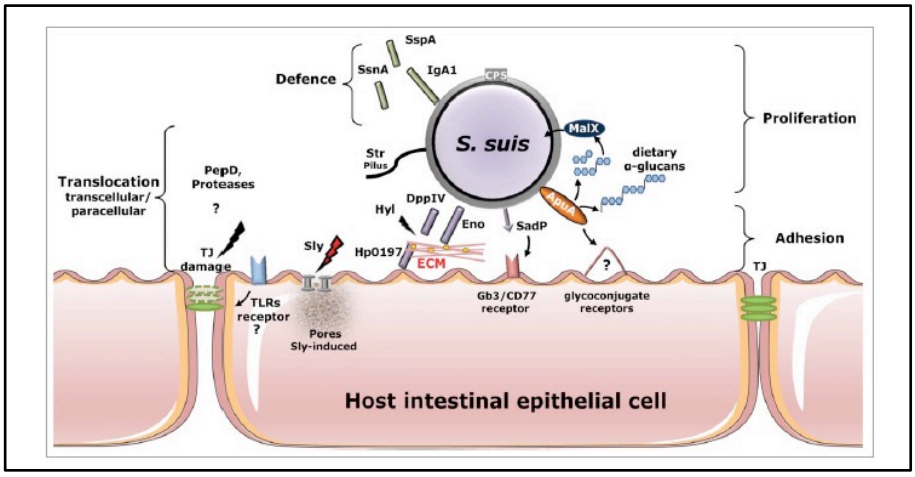


Fig. 6 – S. suis virulence factors and their role during adhesion and penetration of the intestinal epithelium (from Ferrando and Schultsz, 2016).

The control and the prevention actions against *S. suis* have been, and still are, based mainly on the use of antibiotics. The antibiotics of first choice are β-lactams (e.g. Amoxicillin). However, this strategy has not actually changed the farm epidemiology of infections, instead determining a gradual selection of resistances, visible above all in the progressive increase in therapeutic dosages and administration times, which can last for several weeks after weaning (Sala and Zavattini, 2018).

1.6.4 Barrier function and mucosal immune aspects of the GIT

An integral issue when discussing gut health, interconnected to the effects of the microbiota and the host, is that of epithelial barrier function and the mucosal immune system (Pluske et al., 2018).

The mucosal immune system is continuously challenged by external (e.g., diet, aerosols) and internal (e.g., the microbiota) factors, hence numerous cell types such as dendritic cells, lymphocytes (adaptive immune system), macrophages and cytokines (innate immune system) have evolved to play important functions in the regulation of the communication between the GIT microbiota and its mucosal immune system. As described by Moeser et al., (2017), the intestinal epithelial cells act as immune sentinel cells by recognizing pathogenic signal molecules and secreting interleukins (IL) and growth factors (i.e., IL-17A, IL-33, IL-23 and transforming growth factor-b), which have important immunomodulatory properties. The resident immune cells and related gut-associated lymphoid tissue constitutes the largest immune organ in the body. Given the massive antigenic luminal environment and continual

exposure to luminal products, the GIT immune system is tightly regulated via a number of molecular mechanisms, to prevent excessive activation and inflammation in response to these factors.

Conversely, the GIT immune system must also rapidly and strongly respond to any violation in barrier function or in the event of a pathogenic/antigenic challenge, to mobilize innate and adaptive immune responses, which is critical in preventing the systemic spread of infection and inflammation (Moeser et al., 2017; Pluske et al., 2018).

As previously mentioned, the epithelial barrier function is impaired in the immediate post-weaning period, where weaning results in a more permeable small intestine (Spreeuwenberg et al., 2001; Moeser et al., 2007; Pohl et al., 2017). Inflammation of the intestine is associated with increased permeability which can lead to the translocation of toxins, allergens, viruses or bacteria. If and when bacteria cross this first line of defense and reach the lamina propria, their metabolites or mediators released by the epithelial cells can provoke an inflammatory response, and in this case the measurement of pro-inflammatory cytokines provides some information on the degree of inflammation localization, as highlighted by Johnson (1997). Thus, weaning itself, and essentially the period of anorexia that occurs immediately after weaning, causes an inflammatory response (McCracken et al., 1999; Pie et al., 2004) that initiates perturbations to intestinal health. In this case, simply encouraging pigs to eat more feed after weaning helps improve these responses (Pluske et al., 1997).

Moeser et al., (2017) provided a comprehensive review of the interactions and associations between weaning stress and GIT barrier development and function, with a discussion of the implications for lifelong gut health in pigs. In particular, the authors found that the concept of early origin of susceptibility to GIT disease in pigs is supported by paradigms in humans in which early adverse events (e.g., psychological trauma, inflammation, infections) are risk factors for GIT inflammatory and functional diseases later in life. Greater understanding and appreciation of how early childhood stressors and challenges, such as weaning, epithelial and immune are needed to discover new goals and (or) management interventions to promote optimal GIT development and long-term gut health.

1.6.5 Correlation between intestinal inflammation and antioxidant status at weaning

The stress associated with weaning represent a powerful pro-inflammatory stimulus, with very significant effects on the structure of the intestinal barrier.

Following weaning at 21 days, Hu et al., (2013) measured a production of pro-inflammatory cytokines, TNF-α, IL-6 and IFN-γ. These cytokines had a negative effect on the mRNA expression of tight junction proteins, Occludin, Claudin and on ZO-1, which in turn determines a negative impact on the permeability of the intestinal barrier, demonstrated by the decrease in transepithelial electrical resistance (TER) and an increase in the paracellular flow of dextran. At the same time, morphological changes were also detected in the intestinal villi (shortening) and in the crypts (in depth). After 14 days from weaning the intestinal morphology had normalized, unlike the alterations of the barrier, indicating the need for longer times for its functional restoration.

In nature, weaning occurs between 10 and 12 weeks of age, when there is an almost complete maturation of the epithelium, nervous and immune systems of the intestine. Stressogenic and inflammatory stimuli during critical stages of maturation, such as those following early weaning, result in an imbalance that precludes developments, but also the long-term function of the intestinal system. In such situations, in fact, there is an early and persistent activation of the hypothalamus-pituitary-adrenal axis, which is followed by a high activation of mast cells, which can remain evident throughout the life of the animal. Mast cells produce proteases and cytokines (TNF-α) which act directly on the expression of barrier proteins, resulting in increasing permeability, as well as enterocyte apoptosis (Hausmann, 2010). The same authors pointed out that weaning at the age of less than 28 days is more likely to have a negative impact on the development of the barrier, with obvious consequences on functionality, which lasts for the entire life of the animal (Figure 7).

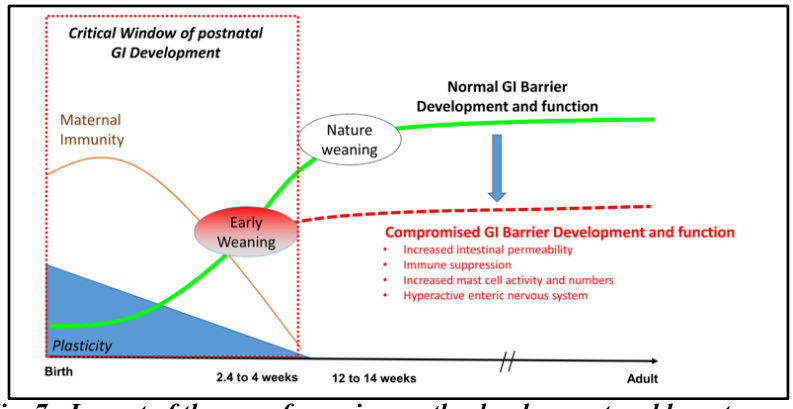


Fig. 7 - Impact of the age of weaning on the development and long-term functionality of the intestinal barrier (from Moeser et al., 2017)

Stressful events, such as those related to early weaning, through the activation of the hypothalamus-pituitary-adrenal axis, can also cause oxidative stress (Costantini et al., 2011).

Oxidative stress is an imbalance between the production of molecules with a strong oxidizing action (ROS), such as superoxide anion and hydrogen peroxide, and the ability of tissues to eliminate them through antioxidant mechanisms, by enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx). Cao et al. (2018), observed, during the first 7 days after weaning (21 days of pigs' life), a tissue, decrease of enzymes involved with antioxidant systems such as GPx and SOD, with an increase in Malondialdehyde (MDA, produced by lipid peroxidation). At the mucosal level, ROS, acting as powerful oxidants on protein and lipid macromolecules, can cause alterations to tight junctions and cause cell apoptosis, with impairment of the intestinal barrier function.

Oxidative stress and the inflammatory process are closely related. The cells of the immune system activated by inflammatory processes, neutrophils, monocytes, lymphocytes, in fact, produce ROS, which in turn are able to directly activate the production of inflammatory cytokines; the process is mediated by NF-kb (Biswas, 2016; Figure 6).

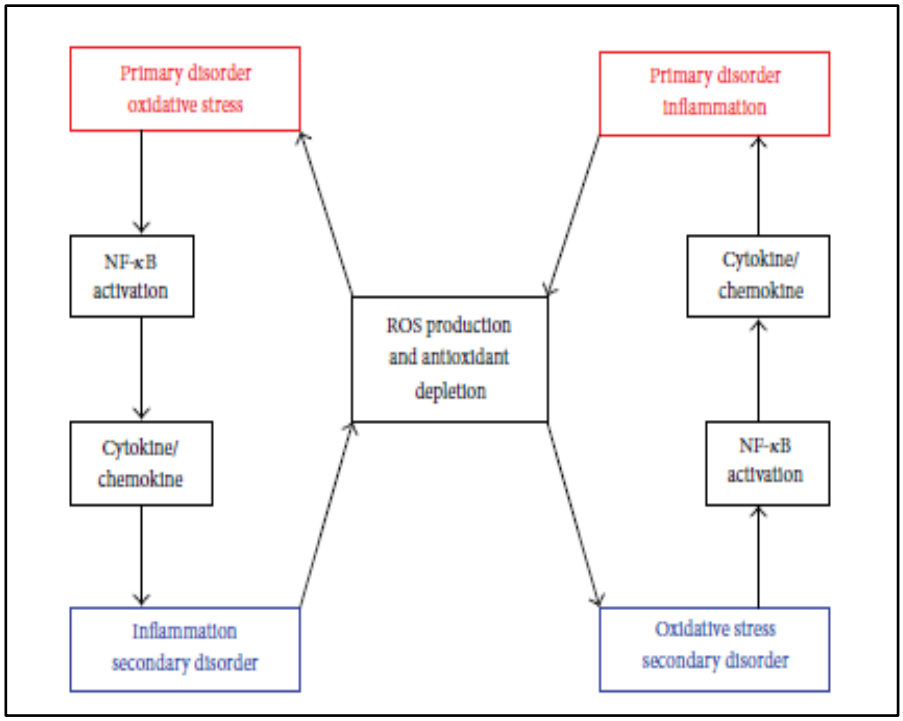


Fig. 8 - In the event that oxidative stress is the primary alteration, the inflammation develops secondarily and in turn stimulates oxidative stress. If inflammation is the primary alteration, it induces oxidative stress which, in turn, increases oxidative stress. The activation of NF-kb plays a central role (from Biswas, 2016).

1.6.6 Impacts of pre-weaning nutrition and of new management approach on gut health after weaning

In the literature, numerous studies and reviews have investigated the importance of nutritional management and GIT control in influencing gut health, even in the absence of overt enteric disease. In particular, in a study by Jayaraman and Nyachoti (2017), the authors pointed out that farming practices related to food and nutrition, animal welfare, biosecurity and disease prevention are important determinants of intestinal health and piglets' growth performance. Subsequently, the adoption of high husbandry practices is fundamental in implementation of strategies aimed at raising pigs in environments with reduced use of antibiotics, or without the use of AGP and (or) minerals such as Zn and Cu.

Among the managerial strategies useful for optimal pre- and post-weaning management, there is the supplementary feeding of lactating piglets to improve their gut health after weaning. The so-called "crawl" feeding is an established practice which consists of offering a solid diet (a "crawling" feed) to the piglets while they are still suckling from the sow. Traditionally, this feed was usually presented in a sectioned area (the "creep") of the farrowing cage to prevent the mother from accessing the feed. However, the term is now used simply for the feed offered to suckling piglets (English, 1981).

Providing dry (creep) diets to piglets in lactation presents opportunities for improving weaning weights and post-weaning pig performance, ostensibly through the stimulation of digestive enzymes associated with carbohydrate and protein digestion, and (or) tolerance to antigens present in the diets fed after weaning. It has been proposed that creep feeding becomes more important and beneficial as weaning age increases. In fact, as piglets grow, their demand for nutrients similarly grows and with increasing age this demand outstrips the capacity of the sow to supply them, as the sow's milk yield peaks at around 3weeks and then slowly declines (Pluske et al., 1995).

English et al., (1980) suggested that a piglet should consume, on average, 600 g of creep feed before weaning to become prepared for the process and mitigate the post-weaning growth check. At current weaning ages, this level of individual intake is unattainable. Not surprisingly, therefore, this area of feeding and management has attracted research and development given its potential impacts on post-weaning GIT structure and function, and hence performance. In many studies, in which piglets were categorized into eaters and non-eaters of creep feed, have provided some new insights on the value of creep feeding after weaning (Bruininx et al., 2002; Pluske et al., 2007; Sulabo et al., 2010). These studies have generally shown that only a certain

proportion of pigs (about 45% to 65%) within the litter consume creep feed. Moreover, eaters (e.g., piglets in the litter that positively consumed creep feed) have better initial post-weaning exploratory behaviors, feed intake and growth performance than non-eaters (e.g., piglets that did not consume creep feed). Increasing the proportion of individual pigs consuming creep feed within litters may elicit positive effects on nursery performance. Therefore, it is important to identify factors that may create more eaters of creep feed in whole litters.

An alternative, or complementary, approach to improving adaptation time to weaning, with possible impacts on gut health, is to mimic the gradual weaning process that occurs in pigs in a natural environment. This may address piglet needs in such a way that it brings about long term benefits with respect to performance and welfare. In nature, a sow will isolate herself before farrowing and return to the group to allow her litter to mix with the other piglets at approximately 10 days after birth (Jensen, 1986). There is no particular point where the sow begins to wean her piglets as it is a gradual process that occurs over 13 to 19 weeks, with the sow predominately controlling the initiation and termination of suckling bouts (Jensen, 1988; Jensen and Recen, 1989). As the benefits of suckling begin to decrease for the piglets (e.g., too much energy expended for not enough reward), piglets solid feed intake from exploration of the environment begins to increase (Bøe, 1991). Interestingly, there is no drop in daily weight gain during weaning under natural conditions (Bøe, 1991).

A system of lactation housing, where a sow can leave her piglets at will by stepping over a barrier, provides an option for the sow to gradually wean her piglets in a commercial setting. In such systems, sows will generally choose to spend less time with their litter at the end of lactation, resulting in an increase in creep feed consumption by the piglets compared with conventionally housed piglets (Pajor et al., 2002; Weary et al., 2002). However, this effect is often coupled with a decrease in growth rate, suggesting that the increase in creep feed consumption is not enough to compensate for the loss of milk consumption as a result of the sow spending less time with the piglets (Pajor et al., 2002; Weary et al., 2002). However, data pertaining to indices of gut health, were not reported. It is also interesting to note that the group lactation systems, where there was freedom for sows to spend less time with the piglets and opportunity for piglets to socialize with non-littermates before weaning, seem to have longer-lasting improvements in performance with higher weight gains in pigs exposed to group lactation reported up to 5 weeks after weaning, compared with conventionally housed pigs (Kutzer et al., 2009; van Nieuwamerongen et al., 2015 and 2017).

Similar in concept to controlled sow housing, intermittent suckling (IS) involves the daily separation of sows and piglets for a specified period of time during the latter part of lactation.

Studies conducted on this practice have highlighted benefits for the welfare of piglets as evidenced by improvements in post-weaning performance (Berkeveld et al., 2009; Turpin et al., 2017a, b) and behavior (de Ruyter et al., 2017). However, there is concern that repeated and forced episodes of sow-piglet separation is detrimental to the welfare of piglets and sows with possible implications for gut health after weaning. Physiological and behavioral studies that further examined this aspect reported only a transient increase in cortisol in piglets (Turpin et al., 2016a) and activity (Berkeveld et al., 2007a) on the former 1 to 2 days of separation, after which the levels stabilize at baseline or at the same levels as their conventionally weaned counterparts. Turpin (2017) did not report any adverse effects of this increase on aspects of intestinal health (mast cell degradation, CRF-1 receptor density) after weaning, unlike other reports related to weaning stress (Moeser et al., 2017).

Therefore, it is evident that the application of managerial strategies in the management of pre-weaning phase have a positive effect on the subsequent growth of piglets. However, in addition to these, in the last years, the scientific community has focused on the search for substances of various kinds which, added to the diet, have positive effects on both growth and intestinal health of animals.

1.7 NUTRITIONAL STRATEGIES FOR A HEALTY GASTROINESTINAL TRACT

There is a wide array of products, such as feed additives, and feeding/management strategies available that influence, or purport to influence, different aspects of gut health (Adewole et al., 2016; Cheng et al., 2014; de Lange et al., 2010; Heo et al., 2013; Jayaraman and Nyachoti, 2017; Pluske, 2013; Bontempo et al., 2016).

In general, the large number of feed and (or) water additives available to swine farmers to use as alternatives or replacements to AGPs that have been evaluated, are generally aimed at: 1) enhancing the pigs' immune responses (e.g., immunoglobulin, u-3 fatty acids, yeast derived b-glucans), 2) reducing pathogen load in the pig's GIT (e.g., organic and inorganic acids, high levels of zinc oxide, essential oils, herbs and spices, some types of prebiotics, bacteriophages, anti-microbial peptides), 3) stimulating establishment of beneficial GIT microbiota (e.g., probiotics and some types of prebiotics), and (or) 4) stimulating digestive process (e.g., butyric acid, gluconic acid, lactic acid, glutamine, threonine, cysteine, and nucleotides) (de Lange et al., 2010).

According to Adewole et al., (2016), feed additives have been used by several researchers and in research models for the purpose of improving growth performance and also to prevent diseases, but the effectiveness of these additives depends mainly on the amount added to the diet. Along the same line of reasoning, Celi et al., (2017) reiterated that the reason for this inconsistency may be that the effectiveness of each additive depends on diet (e.g. composition, diet processing and feeding methods), colonization and associated succession of microbial populations, stress and genetics. Therefore, it is not possible to recommend a specific additive that has positive effects on all diets, but it is likely that if no AGPs is used, at least some additives are beneficial in diets fed to piglets (Liu et al. 2018).

Pluske et al., (2018) reported that additives are predominantly characterized not only by their different modes of action, but also by the variation in responses obtained when added to pig diets. This variation is presumably a consequence, in part, of many different management conditions pigs are subjected to, which in turn influence factors such as microbiota composition and intestinal mucosal immunity. Thus, there is a need for additional research in order to validate the findings, but in summary, inconsistent results obtained with feed additives may be due to differences in the pig age, health status, environmental conditions or management. Therefore, research on new alternatives that are efficient, safe and that can truly replace AGPs still is and will be a long process (Cheng et al. 2014).

1.8 FATTY ACIDS AS FEED ADDITIVES: Who are they?

Among the alternatives proposed and which appear to be excellent candidates for replacing, or at least reducing AGPs, and for having positive effects on the intestinal health of pigs, are short and medium chain fatty acids.

Acids are molecules able to donate protons and are characterized by a dissociation constant and acidifier power that influence its effect. Acids may be administered as feed additives in various ways, including as a pure form, as a blend of organic and/ or inorganic acids, or in association with phytoextracts or enzymes that can enhance the beneficial impacts. The group of feed additives categorized as acids includes inorganic acids, organic acids (OA), fatty acids (FA) and their salts. The members of the OA group may be classified by their saturation level (saturated or unsaturated) and/ or carbon chain length (short, medium, or long chain) (Rossi et al., 2010). The members of the OA group may be used alone or in blends. Each acid presents

chemical properties that influence its beneficial effects, and the dose response is not always constant (Ferronato and Prandini, 2020).

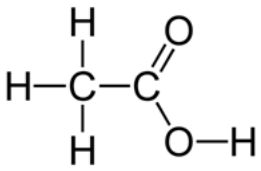
FAs are a category of OA characterized by aliphatic chains with 4–28 carbons, where the carboxylic group is hydrophilic, while the carbon chain is hydrophobic. These could be chemically classified according their carbon chain length into short chain fatty acids (SCFAs; 1–5 carbon atoms), medium chain fatty acids (MCFAs; 6–12 carbon atoms), or long chain fatty acids (LCFAs; 13–21 carbon atoms), and according to saturation level, into saturated or unsaturated fatty acids. In Table 2 are presented details on the most relevant studies about SCFAs and MCFAs.

Tab. 2 - Studies about the most common fatty acids used in pig farming adapted from Ferronato and Prandini, 2020.

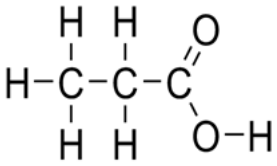
Compound	Animal category	Dose	Response	Reference
SHORT CHAIN FATTY ACIDS	Weaning pigs	1mM, coated form	Reduction of hilA expression, Salmonella invasion, and fecal shedding	Boyen et al., 2008
	Weaning pigs	0.20% free sodium butyrate or 0.06% protected sodium butyrate	Increased villus height, growth, and surviving rate of E. coli challenged pigs	Bosi et al., 2009
	Weaning pigs	0.17% sodium n-butyrate	Increased Lactobacillus count, ileal microvilli length, and depth of cecal crypts. Reduction of coliform bacteria in ileum, feed and costs	Galfi and Bokori,1990
	Weaning pigs	0.08% sodium n-butyrate	Increased ADG, daily feed intake (DFI), feed efficiency (FE), and live weight (LW)	Piva et al., 2002
	Weaning pigs	0.1% tributyrin	Alleviation of acetic acid-induced intestinal injury. Increased caspase-3 levels, claudin-1 protein, and epidermal growth factor receptor mRNA expression	Hou et al., 2014
MEDIUM CHAIN FATTY ACIDS	Newborn pigs	(1) 0.50% or 1.0% or 2.0% medium chain fatty acids (MCFA) blend 1:1:1 (C6:0 + C8:0 + C10); (2) 1.0% of 4 MCFAs blend (50% C6:0 + 20% lactic acid + 10% or 20% or 30% monolaurin)	(1) Increased ADG, ADFI, and feed efficiency; (2) increased ADFI and ADG with 1.0%	Thomas et al., 2020
	Newborn pigs	0.02% MCFA– long chain fatty acids (LCFA) blend (0.9% C6:0 +29% C8:0 + 19% C10:0 + 8% C14:0 + 5% C16:0 + 6% C18:1n -7 + 8% C18:2n – 6 + 1% C18:3n -3)	Higher live weight/body weight ratio; possible toxic effect	Casellas et al., 2005
	Weaning pigs	0.2% MCFA blend	Increased ADG (MCFA), gain to feed ratio (G/F), glucose level in blood, nutrient digestibility of dry matter (DM), N, and energy (MCFA + 0.01% probiotic)	Mohana Devi et al., 2014
	Weaning pigs	8% MCFA blend (60% C8:0 + 40% C10:0)	Increased triglyceride concentrations. Reduction of feed intake and superior G/F ratio for 3 weeks, and serum urea concentration	Cera et al., 1989
	Weaning pigs	0.003% (salts form; 48% C12:0 + 18.6% C14:0 + 9.9% C16:0 + 6.8% C18:1 + 6.2% C8:0 + 5.8% C10:0+ 3.6% C18:0 + 1.3% C18:2)	Reduction of Salmonella count in cecum, Enterobacteriaceae and total coliform in ileum/colon, and intraepithelial lymphocyte counts	Lopez-Colom et al., 2019
	Weaning pigs	0.2% OAs blend (C12:0 + butyrates, MCFAs + sorbic acid + phenolic compounds)	Increased Lactobacillus and Faecalibacterium, growth performance, and serum immunoglobulin (Ig; IgG, IgA). Reduced diarrhea rate	Han et al., 2018
	Weaning pigs	0.2% or 0.4% OA and MCFA (17% fumaric acid + 13% citric acid + 10% malic acid + 1.2% MCFA C10:0/C8:0)	Increased body weight (BW), ADG, ADFI, and G/F. Reduction of diarrhea incidence	Lei et al., 2017
	Weaning pigs	0.2% or 0.4% organic acids (OA) and MCFA (17% fumaric acid +13% citric acid + 10% malic acid + 1.2% MCFA C10:0/C8:0)	Decreased pH digesta, MCFA induced minor changes in microbiota composition instead of OA. Interaction of OA and MCFA to reduce E. coli virulence and prevent post-weaning diarrhea	Zentek et al., 2013
	Sows	Medium chain triglyceride (MCT) (92% C8:0 + 2% C10:0 + 6% C12:0) 9:1 by weight	Improved survival rate to day 21, muscle glycogen, and serum albumin of the smallest pigs	Jean and Chiang, 1999
	Sows	10% supplemental fat as MCT source	Increased pig ADG and average pig weaning weight	Gatlin et al., 2002

1.8.1 SHORT CHAIN FATTY ACIDS

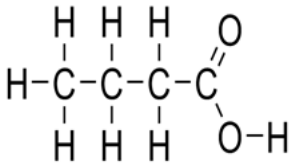
Fatty acids with a chain of less than six carbon atoms are called short-chain fatty acids (SCFA), which include acetate (C:2), propionate (C:3) and butyrate (C:4).



Acetic acid (acetate)



Propionic acid (propionate)



Butyric acid (butyrate)

SCFAs are bacterial metabolites produces as a result of the fermentation of dietary partially and non-digestible polysaccharides, performed by specific anaerobic bacteria (den Besten et al., 2013). The beneficial effect of SCFAs is closely correlated with increasing proliferation and decreasing apoptosis of enterocytes. They are the major fuels of enterocytes and provide 60–70 % of the energy requirement for colonocytes in particular (Jacobi and Odle, 2012). Both weaning and growing pigs have a great capacity to absorb and metabolize SCFAs from the hindgut (Liu, 2015). Moreover, a reduced capacity of the intestinal mucosa to oxidize butyrate has been implicated in the pathogenesis of ulcerative colitis (Scheppach et al., 1992). Thus, SCFAs are essential for maintaining the normal metabolism of colon mucosa, regulating colonocyte growth and proliferation (Rossi et al., 2010).

However, the beneficial effect of SCFAs is not restricted to the colon, and SCFAs also stimulates cell proliferation and growth of the small intestine. This effect on distal mucosa is likely mediated by a systemic mediatory mechanism (Sakata and Inagaki, 2001).

In general terms, SCFAs have been shown to play an important role in improving intestinal health and limiting intestinal inflammation in pigs. Among them, butyric acid has numerous positive effects on the health and growth of piglets.

1.8.1.2 Butyric acid (C:4)

Butyric acid is one of the main SCFAs that arise as a result of bacterial fermentation at the gastrointestinal level, particularly in the colon. Although, compared to acetic and propionic acid, butyric acid is the least abundant, in recent years, it has been extensively studied (both in human

medicine and in veterinary medicine) for the various positive actions it carries out in the intestine.

Butyric acid is synthesized, by the microbiota of the large intestine, starting from undigested carbohydrates, mainly soluble and fermentable, such as oligosaccharides, inulin, glucans, but also from starch that escapes the digestion in the small intestine, which is the most powerful butyrrogenic substrate. The bacterial groups that have been identified as major producers of butyric acid belong to the order of *Clostridiales* and are those of Cluster IV and XIVa, including *Faecalibacterium prausnitzii*, *Eubacterium rectale*, *Roseburia* spp. Butyric acid is absorbed both in the small and large intestine, by diffusion (non-dissociated forms of the acid) and through various active transport and ion exchange mechanisms (e.g., transporters of mono-carboxylates). It represents the preferred energy source for enterocytes and is essential for maintaining the normal metabolism of the intestinal mucosa (Guilloteau et al. 2010).

Butyric acid has a pKa of about 4.8. Therefore, in acidic environments, such as gastric ones, the undissociated form prevails, while at more neutral levels of the intestine, it is found mainly in dissociated form. As an acid, it is irritating and characterized by a very pungent odor. Considering the behavior in relation to the external pH, the undissociated forms, due to their lipophilic nature, tend to be absorbed at the level of the initial part of the GIT. In this regard, forms have been studied to allow its release in the lower tracts, such as esterification with glycerin or microencapsulation (Bedford et al., 2017), also improving the organoleptic characteristics. Generally, non-esterified forms are commonly salified to obtain butyrate (e.g., Sodium Butyrate).

Mallo et al., (2012), compared the use of encapsulated butyrate or monobutyrin in 21-day-old pigs, noting a higher concentration of butyric acid in the colon, especially in the encapsulated form. In human medicine, several studies have highlighted the positive effects of butyrate in the treatment of colon diseases, often on an inflammatory basis, such as ulcerative colitis or Crohn's disease. The anti-inflammatory effects are mediated by mechanisms of regulation of the expression of pro-inflammatory cytokines, through the inhibition of the biochemical pathway of the factor NF-kb, which determines the production of IFN-γ, TNF-α, IL-6, IL-8, and at the same time the induction of regulatory cytokines such as IL-10 or TGF-B. Positive findings on oxidative stress have also been identified, with increased glutathione production (Bedford et al., 2017).

At the level of the healthy intestinal mucosa, especially in the colon, it reduces apoptosis and promotes the secretory activity of goblet cells; in a very interesting way, however, it stimulates apoptosis in cancer cells (Guilloteau et al., 2010). Also, in the zootechnical field,

interest in butyric acid has grown, as one of the nutritional interventions to support intestinal health, especially in the post-weaning phase, identifying in many cases positive effects on growth performance and on health status. In recent years, several in vitro and in vivo studies have investigated the effects of butyrate in pigs, demonstrating its action as a regulator of mucosal inflammatory processes and maintenance of the integrity of the intestinal barrier. Fang et al., (2014) reported that dietary supplementation of sodium butyrate significantly decreased diarrhea incidence of weaned piglets and thus reduced the adverse effects of weaning stress and maintained the integrity of intestinal mucosa.

In the same way, tributyrin (an ester of butyric acid) improves intestinal morphology, improves feeding, growth and efficiency during 3-4 weeks after weaning and mitigates adverse changes in the intestinal structure and function associated with weaning stress. It also improves morphology and intestinal enzymatic activity (Hou et al., 2006; Hou et al., 2014). Moreover, dietary supplementation with tributyrin alleviated intestinal injury by inhibiting apoptosis, promoting tight-junction formation and activating epidermal growth factor receptor signaling in piglet colitis (Hou et al., 2006; Hou et al., 2014; Liu, 2015).

Yan et al., (2017), challenged IPEC-J2 cell cultures with *E. coli* LPS showed that butyrate preserved the integrity, and decreased the permeability, of the intestinal barrier, increasing and restoring the expression of the proteins Claudin 3 and 4, the main components of tight junctions. This effect was obtained through the activation of specific biochemical pathways (AKT-mediated protein synthesis pathway). Ma et al., (2012), again using IPEC-J2 cell cultures, subjected the cells to physical damage (scratching). The treated cells showed a faster damage repair process, mediated by increased expression of the barrier proteins Occludin and ZO1. Furthermore, the authors highlighted a higher production of SOD, GPx and GSH and a lower concentration of MDA, suggesting positive effects on the oxidative stress of the treated tissue.

Feng et al., (2018), administered 2 g/kg of sodium butyrate to 21-day weaned pigs over a three-week period. Growth performance results did not differ, but treated pigs had a lower incidence of diarrhea. In addition, at the level of the intestinal mucosa, the authors showed lower permeability and higher production of Occludin, ZO1 and Claudin in the colon, also in this case by regulating the activation of biochemical pathways of synthesis.

Wang et al., (2018), for 14 days after weaning (28 days of pigs' life), provided 450 mg/kg of protected sodium butyrate. The treated animals had a higher weight gain and, at the jejunal mucosa level, the height of the villi and the villi: crypts ratio (V: C) increased. The treatment also resulted in a lower activation of the Mast-cells. Mast-cells play a central role in mediating intestinal inflammation; following their activation, preformed mediators are released. At the

tissue level (jejunum), the authors showed a decrease in these mediators and cytokines, such as histamine, TNFα and IL-6.

The pro-inflammatory cytokines have a negative effect on the integrity of the intestinal barrier by increasing its permeability and inducing apoptosis. Positive effects on the permeability of the intestinal barrier, with greater production of the Occludin protein (jejunum and colon) and on inflammation (lower secretion of TNFα) were highlighted by Huang et al., (2015) with the use of 1 g/kg protected butyrate, together with Kytasamycin and Colistin. Moreover, the treated pigs showed a lower concentration of MDA and a higher concentration of GPx, with the latter due to the use of butyrate, in comparison with the group that was fed only antibiotics and the control group. This supports the evidence of Ma et al., (2012) described above.

Over the years there have been numerous studies on integration with butyrate and the impact on livestock performance both in post-weaning and even before weaning in pigs. Already in 1990, Galfi et al., (1990) found that 0.17% sodium butyrate improves the dietary intake, conversion index and weight gain of piglets from about 7 to 100 kg, accompanied by a greater length of the ileum villus. In 2002, Piva et al., (800 mg/kg of Sodium Butyrate) recorded an increase in feed intake and weight gain only in the first 14 days after weaning (total period: 56 days), assuming a positive effect especially when switching from a liquid to a solid diet. Lu et al., (2008), with a dosage of 1 g/kg of sodium butyrate in 21-day-old piglets, obtained, after 30 day trial, better dietary feed intake, conversion index and weight gain. At the same time, at the mucosa level of the small intestine, the authors showed greater length of the villus and the Villus: Crypts ratio; in addition, at the serum level, the concentration of TNFα and IL-6 decreased. According to the authors (Piva et al., 2008), the best morphological characteristics may depend both on the reduction of the direct negative action of cytokines on the mucosa, and on the indirect effect on the appetite of animals; in fact, high concentrations of cytokines depress feed intake.

Low feed intake causes negative effects on the intestine, with atrophy of the villus. Chiofalo et al., (2014), supplementing the diet of pigs weaned at 21 days with 440 mg/kg of protected sodium butyrate or 1.47 g/kg of unprotected salt for 45 days, recorded a higher final weight of the pigs treated, with improvements in the feed conversion ratio in the final phase of the study, and a higher length of small intestine villus, especially in the group fed with butyrate protected form. Other works have investigated the use of sodium butyrate in the undercutting period. Kotunia et al., (2004), administered through a milk-replacer, found a higher length of the villi in the jejunum and ileum. Le Gall et al., (2009) provided the product before weaning and / or

after weaning. The data emerging from the study suggest that pre-weaning use provides the most positive responses, such as an improvement in weight gain, better feed intake and fecal digestibility of the feed in the phase after weaning.

On the other hand, some other studies have not shown improvements following the use of butyrate in post-weaning. Fang et al., (2014), with 1% of encapsulated product, did not detect effects on feed intake, weight gain and feed conversion ratio, even though the pigs treated, compared to the controls, had a lower incidence of diarrhea. Also, in a study by Biagi et al., (2007) no differences were observed in terms of growth performance, intestinal morphology or intestinal microbiota in 32-day weaned piglets, which were given different amounts of sodium butyrate (1,000, 2,000, or 3,000 mg / kg) up to 6 weeks.

Butyric acid, like other SCFAs, has an antimicrobial activity, mainly against Gram-negative bacteria and linked above all to its undissociated (fat-soluble form), able to penetrate inside cell membranes, lowering the bacteria intracellular pH and causing a toxic accumulation of anions. In fact, the antimicrobial mechanism of action is influenced by the shape and size of the fatty acid chain as well as by the composition: the presence of a -OH group is decisive for the antibacterial properties, while the presence of a methylated group confers little or no antibacterial properties (Desbois and Smith, 2010; Abdelli et al, 2020). Other studies suggested that FAs act as a non-ionic surfactant, which became incorporated into the bacterial cell membrane. Taking into consideration some studies mentioned in the previous paragraph, Galfi et al., (1990), had shown a reduction in the proportion of *Coliforms* in the ileum and a relative increase in *Lactobacilli*. On the other hand, Chiofalo et al., (2014) an increase in lactic bacteria in the intestinal mucosa in pigs treated with protected butyrate, while Lu et al., (2008), a lower concentration of *Clostridium* and *E. coli* in the chyme of the small intestine, without absolute changes of *Lattobacillus* and *Bifidobacterium*. Roca et al., (2014), with the inclusion of 0.3% sodium butyrate or Avilamycin or a mixture of plant extracts, in diets for 21-day weaned piglets (3 weeks of treatment), concluded that, among the diets, especially the butyrate was able to increase the biodiversity of the microbiota in the distal portions of the intestine.

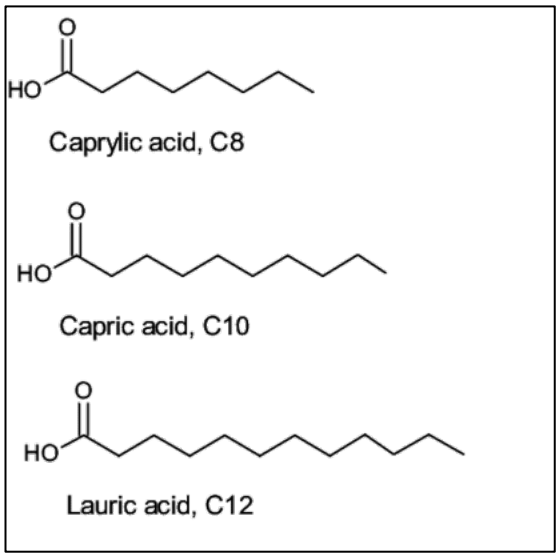
The direct antimicrobial effect against pathogens was the subject of a study that involved a challenge with *Salmonella Typhimurium* (24-day piglets, dosage 2.1 g/kg of protected product). Treatment resulted in less intestinal colonization and fecal excretion of Salmonella (Barba-Vidal et al., 2017). In a recent work, Xiong et al., (2016) proposed a new possible and interesting mechanism of butyrate antibacterial action (0.2% in the diet) against *E. coli* O157: H7, with which 24-day-old piglets were challenged. Compared to the controls, the treated did not show clinical symptoms of the infection and, at the same time, had less fecal excretion of

the microorganism. The authors showed that butyrate increased the synthesis of Host Defense Peptides (HDP), which, in turn, stimulated the activity of macrophages, increasing their antibacterial activity and consequently reducing inflammation, as evidenced by lower serum levels of TNFα and IL-6 in the treated. HDPs are peptides that exist in all living organisms and are divided into the family of Difensins or Cathelicidins. HDPs possess a broad-spectrum direct antimicrobial effect against Gram-negative, Gram-positive, viruses and fungi, exhibiting an action aimed at the integrity of the membranes. Furthermore, these peptides play an important role in modulating the immune response (innate and acquired) and protecting intestinal mucosal barrier (Robinson et al., 2018).

1.8.2 MEDIUM CHAIN FATTY ACIDS

MCFAs are saturated 6- to 12-carbon acids, including caproic acid (C6:0), caprylic acid (C8:0), capric acid (C10:0), and lauric acid (C12:0). These compounds are widely distributed in nature. As triglycerides, they are present in mammalian milk (in the sow the level is low), and in plant byproducts such as coconut and palm oil (Jensen, 2002). Furthermore, in the skin, they are secreted by the sebaceous glands and their presence has a controlling effect on the microbial population.

MCFAs administration seems to provide a positive effect through nutritional, metabolic, antimicrobial, and immune-stimulating effects. MCFAs undergo simpler degradation to FAs and glycerol with the help of pancreas lipase, after which they diffuse into portal blood, where they associate with albumin for direct transport to the liver and are used for energy production via mitochondrial ß-oxidation. MCFAs enters the mitochondria independently of the carnitine transport system and undergo preferential oxidation (Baltic et al., 2017). The contribution of MCFAs as an energy source for the distal GIT epithelium is minor compared with that of butyrate, and only a small portion of the systemic pool of MCFAs is stored in adipose tissues (Zentek et al., 2011). MCFAs may also be generated by hydrolysis of medium chain triglycerides (MCTs). Therefore, in general, MCFAs



represent a rapidly available source of energy for the intestine, making them particularly interesting for the nutrition of young animals (Hanczakowska et al., 2017).

For these reasons, MCTs and MCFAs are often used for feed supplementation, but a high dosage may decrease feed palatability and/ or create a false sense of satiety due to their rapid oxidation in the liver. Inconsistent results have been obtained in newborn and lactating piglets in this regard, with the results suggesting that MCFAs may have toxic effects at high concentrations (Peffer et al., 2005). Regarding supplementation during the weaning phase, Thomas et al., (2020) and Mohana Devi et al., (2014) reported improvements of daily gain, feed intake and feed conversion ratio under MCFA supplementation. There is no evidence that dietary administration of MCFAs can affect the reproductive performance of pigs, yield maternal or fetal toxicity, or exhibit teratogenic effects at doses up to 12,500 mg/ kg (Traul et al., 2000).

MCFAs supplementation in sows during gestation and lactation reportedly had positive effects on the sows' body condition score but did not alter their milk composition; among the offspring, there were improvements in maturity at birth, body glycogen stores, and the survival rate (Gatlin et al., 2002). MCFAs can also help to support the integrity of the intestine in young piglets. For example, Dierick et al. (2003; 2004) reported that feeding of MCFAs to weaning pigs influenced the intestinal morphology, resulting in a significant increase in the length of the villous in the small intestine combined with a lower crypt depth and a lower number of intraepithelial lymphocytes. More recently, Hanczakowska et al., (2011), by administering both caprylic acid and capric acid, separately or together (0.2%) to piglets, from 7 to 84 day of life, showed a significant weight increase and a better digestibility of proteins and fiber. At the mucosal level, an increase in the height of the ileum villi was observed, which was significant with the administration of capric acid.

MCFAs or MCTs have been suggested to improve gut health under inflammatory conditions. Activation of the immune system and the inflammatory response is a fundamental process in the body's defense. However, these processes need to be finely regulated so that they are not excessive. In fact, inflammation and tissue damage are correlated and can trigger a reciprocal stimulation mechanism. Recently, Xu et al., (2018), highlighted a possible role of MCFAs as modulators of the inflammatory and immune response. The study was conducted to evaluate whether the administration of MCTs (4% of a mixture of caprylic and capric acid) had an impact in alleviating the inflammatory response of the intestinal mucosa, and its associated damage and necroptosis, following exposure of pigs with *E. coli* LPS (intraperitoneal injection). The results showed that the treatment improved intestinal morphology, enzymatic digestive activity

and the barrier function of the epithelium: in fact, an increase in intestinal villi, an increase in the activities of Saccharase and Maltase was observed at the jejunal level, as well as a higher expression of the Claudin-1 protein, essential for maintaining the barrier function. Furthermore, following the treatment, a reduced expression of Toll-Like receptor 4 (TLR4) mRNA and of the Nucleotide-binding Oligomerisation Domain (NOD) protein genes was highlighted in jejunum and ileum. TLRs and NODs play a central role in the detection of pathogens and in the activation of innate inflammatory and immune responses. Consequently, at the plasma level, the concentration of TNF-α, a pro-inflammatory cytokine, was decreased as well as the expression of the heat-shock protein 70 (HSP70) in the jejunum. HSP70 is an anti-inflammatory protein that is produced as a result of inflammation. The treatment also inhibited the necroptosis of enterocytes in the jejunum and ileum, which may have been caused by a direct effect or by inhibition of the inflammatory pathways mediated by TLR or NOD. According to the authors, these results indicate that MCFAs can modulate inflammatory processes in the intestinal mucosa, and improve the integrity of the barrier, the morphology of the villi and digestive functions (Xu et al., 2018).

The strong antibacterial activity of MCFAs is a long-known feature and makes MCFAs attractive as possible alternatives to antibiotics. Already in the early 1970s, in vitro studies conducted by Kabara et al., (1972) and Conley et al., (1973), had highlighted the antibacterial activity of MCFAs and, even more, of their monoglycerides, showing how this was higher in the cases of lauric acid and monolaurin, with a spectrum of action particularly effective against Gram-positive bacteria. In an in vitro study of Bergsson et al., (2001) was found that monocaprin, especially at low concentrations, was more active than capric acid against Gram-positive bacteria. In particular, through fluorescence and two-color electron microscopy, the authors highlighted the disintegration of the cell membrane but leaving the cell wall intact.

Batovska et al., (2009), examined the antibacterial activity of different MCFAs and their monoglycerides against some Gram-positive bacteria. From the results obtained, it emerged that, in general, the monoglycerides had higher efficacy, and monolaurin, in particular, was the most active compound. According to the authors, a possible explanation lies in the hydrophilic portion (glycerol), which acts as a carrier of the FA across the membrane (Batovska et al., 2009). In another study by Shilling et al., (2013), lauric acid caused damage to cell membranes and the cytoplasmic structure, and inhibition of growth of *Clostridium* bacteria. A study by Schlievert et al., (2012), pointed out that monolaurin is 200 times more effective against *Staphylococcus aureus* and *Streptococcus pyogenes* than both lauric acid and other MCFAs. The same authors then tested the in vitro sensitivity of numerous microorganisms towards

monolaurin (Table 3). The table shows that, in general, Gram-positives bacteria are sensitive. In particular, it is very interesting that *Streptococcus suis*, the causative agent of porcine Streptococcosis, is very sensitive, with an inhibitory concentration of 50 µg / ml. On the other hand, among the resistant microorganisms there are *Enterobacteriaceae*, including *E. coli*.

Bacterium	Gram or Other Stain	Oxygen Tolerance	Strains Tested	Average Bactericidal Concentration (µg/ml)
Staphylococcus aureus	Positive	Aerobe	54	300
Streptococcus pyogenes	Positive	Aerotolerant Anaerobe	4	30
Streptococcus agalactiae	Positive	Aerotolerant Anaerobe	3	30
Group C Streptococcus	Positive	Aerotolerant Anaerobe	1	30
Group F Streptococcus	Positive	Aerotolerant Anaerobe	1	20
Group G Streptococcus	Positive	Aerotolerant Anaerobe	1	50
Streptococcus suis	Positive	Aerotolerant Anaerobe	1	50
Streptococcus sanguinis	Positive	Aerotolerant Anaerobe	1	50
Streptococcus pneumoniae Serotype III	Positive	Aerotolerant Anaerobe	2	10
Enterococcus faecalis	Positive	Aerotolerant Anaerobe	1	100
Listeria monocytogenes	Positive	Aerobe	1	50
Bacillus anthracis Sterne	Positive	Aerobe	1	50
Bacillus cereus	Positive	Aerobe	1	50
Peptostreptococcus species	Positive	Anaerobe	1	1
Clostridium perfringens	Positive	Anaerobe	1	1
Neisseria gonorrhoeae	Negative	Aerobe	1	20
Haemophilus influenzae Non-typable	Negative	Aerobe	2	50
Gardnerella vaginalis	Negative	Aerobe	2	10
Campylobacter jejuni	Negative	Aerobe	1	1
Bordetella bronchiseptica	Negative	Aerobe	1	1
Pseudomonas aeruginosa	Negative	Aerobe	1	Not Susceptible
Burkholderia cenocepacia	Negative	Aerobe	1	500
Pasteurella multocida	Negative	Aerobe	1	500
Prevotella melaninogenica	Negative	Anaerobe	1	50
Bacteroides fragilis	Negative	Anaerobe	2	50
Fusobacterium species	Negative	Anaerobe	1	50
Escherichia coli	Negative	Aerobe	2	Not Susceptible
Salmonella minnesota	Negative	Aerobe	1	Not Susceptible
Enterobacter aerogenes	Negative	Aerobe	1	Not Susceptible
Proteus vulgaris	Negative	Aerobe	1	Not Susceptible
Shigella sonnei	Negative	Aerobe	1	Not Susceptible
Klebsiella pneumoniae	Negative	Aerobe	1	Not Susceptible
Mycobacterium phlei	Acid Fast	Aerobe	1	100
Mycobacterium tuberculosis	Acid Fast	Aerobe	1	100
Mycoplasma hominis	Cell Wall deficient	Aerobe	1	1

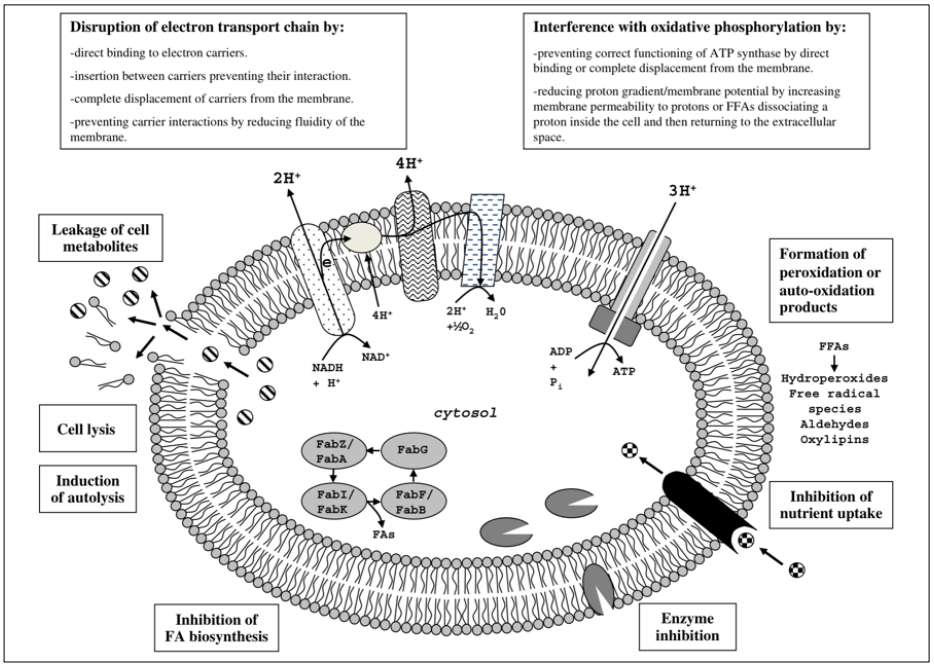
Tab. 3 - Sensitivity of various microorganisms to Monolaurin (from Schlievert et al., 2012)

Regarding the sensitivity of Gram-negative (*E. coli* in particular), an in vitro work by Kim et al., (2013) highlighted a bactericidal synergistic effect between MCFAs (caprylic, capric and lauric acids) and organic acids (acetic, lactic, malic and citric acids) against *E. coli* O157: H7. Indeed, bacteria treated with the individual substances showed cell membrane damage, but were

still able to form colonies. Otherwise, the combined treatment shows membrane disintegration with cell death. The authors believe that initially organic acids, or MCFAs, cause damage to the bacterial cell membrane and that this subsequently accelerates the entry of the same substances in the cell, causing irreversible toxic effects (Kim et al., 2013).

To date, the mechanisms of action of the antimicrobial activity of MCFAs are not fully understood. However, the main target seems to be the cell membrane (Schlievert et al., 2012). The membrane-lithic behavior of FAs and monoglycerides derives from their amphipathic properties, which allow these molecules to adhere to the bacterial cell membrane, leading to the destabilization of the membrane and the formation of pores on it (Yoon et al., 2018). In particular, the destabilizing activity of the membrane causes an increase in cell permeability and lysis, leading to inhibition of bacterial cell growth (bacteriostatic action) or to cell death (bactericidal action). At higher concentrations, FAs can solubilize the membrane to such an extent that various membrane proteins or larger sections of the lipid bilayer are released (Desbois and Smith, 2010; Yoon et al., 2018). Among the vital processes involving bacterial cell membranes, two of the most important are the electron transport chain and oxidative phosphorylation, which are essential for the production of energy in bacterial cells. The two processes are interconnected, and MCFAs have the potential to interrupt the electron transport chain process by binding to electron carriers or by altering the integrity of the membrane and interfering with oxidative phosphorylation by decreasing the membrane potential and the proton gradient. Furthermore, FAs can directly inhibit membrane enzymes such as glucosyltransferase, presumably due to similar molecular structures of fatty acids with known inhibitors of small molecules (Zhou et al., 2018).

Fig. 9 - Summary of the antibacterial action mechanisms of MCFAs (from Desbois et al., 2010).



Furthermore, in conditions of relatively acid pH, the absorption of the undissociated MCFAs into the bacterial cell determines a cytotoxic effect: in the cytoplasm, in fact, with a pH close to neutrality, the acid dissociates, decreasing its pH and inactivating various enzymatic activities (Zentek et al., 2011).

Considering their antibacterial effects, MCFAs are typically supplemented into the diet in blends comprising other MCFAs or OA, in order to harness a synergic effect and widen the antibacterial spectrum. López-Colom et al., (2019) confirmed that MCFA salts from coconut oil (C12:0, C8:0, and C10:0) had antipathogenic effects, such as reducing *Salmonella* in cecum, reducing *Enterobacteriaceae* and Coliform bacteria in the ileum and colon and increasing the antimicrobial effects of lauric, caprylic, and capric acids beyond the effects seen with long-chain fatty acids (LCFAs) (López-Colom et al., 2019). Marounek et al., (2004) reported that MCFAs supplementation had a coccidiostatic effect, delaying shedding and shortening the patent periods for cryptosporidial oocysts of *Cryptosporidium parvum* and *Isospora suis*. MCFAs are also supplemented in blends along with others OA. According to Zentek et al., (2013) the combination of OA (fumaric acid and lactic acid) and MCFA (C8:0; C10:0) could modify the microbiota and prevent post-weaning diarrhea. Han et al., (2018) confirmed that MCFAs blends increased growth performance, decreased the diarrhea rate, and enhanced serum immunity. The diarrhea incidence in weanling pigs challenged with *E. coli* was reduced by supplementation with an OA-MCFA (C8:0, C10:0) blend (Lei et al, 2017).

1.8.2.1 Lauric acid (C:12)

In a recent study, Kour et al., (2020) reviewed the use of MCFAs in veterinary practice with a particular focus on lauric acid. Lauric acid is one of the MCFAs with 12 carbon atom chain. Like many other FAs, lauric acid has long shelf life and is non-toxic in nature. It is also having a more favorable effect on total HDL cholesterol than any other FAs (Mensink et al., 2003). Moreover, antiprotozoal, antifungal, antibacterial, and antiviral, and other activities of lauric acid reflected unique features that put it on the top of the food additives (Galib, 2018). Furthermore, the inability of bacteria to develop resistance against monolaurin was considered vital in attracting more attention to this compound.

In another review by Dayrit (2015), the author highlighted the properties of lauric acid and their significance in coconut oil. The author also investigated how lauric acid is metabolized in the body and also investigated the antibacterial mechanisms (Dayrit, 2015). The mechanism by which lauric is consumed by the body is - more amount of it gets converted into monolaurin (glyceryl laurate). After the release of lauric acid from the triglyceride, it is either transported directly to the liver via the portal vein or reformed into new triglycerides which enter the lymphatic system.

The fate of lauric acid in the blood stream has been particularly studied in rats. Using isotopically labeled lauric acid and palmitic acid, Goransson (1965) observed that lauric acid disappeared more rapidly than palmitic acid from the blood and was also oxidized more rapidly. He also showed that small amounts of lauric acid are found in the liver as triglycerides and that it is not incorporated into phospholipids.

In the liver FAs are metabolized for energy in the mitochondria. Depending on its chain length, a FA can cross the mitochondrial membrane either by passive diffusion or by carnitine-assisted transport. Gharlid and co-workers (1996) showed that lauric acid is rapidly transported across the membrane bilayer by non-ionic passive diffusion. Measurements using dynamic NMR spectroscopy showed that MCFAs are able to diffuse more rapidly than LCFAs across the membrane through a “flip-flop” mechanism; a lengthening of the carbon chain by two carbons slows down the rate of diffusion by about 100 times (Hamilton, 1998). Consistent with this, lauric acid has been shown to diffuse freely across the mitochondrial membrane, while longer chain fatty acids require carnitine (Bremer, 1983). Thus, lauric acid can be rapidly transported into the mitochondria via physical diffusion or with assistance from carnitine.

Lauric acid is rapidly metabolized in the liver in a number of ways. β-Oxidation accounts for the major pathway for fatty acid metabolism producing acetyl-CoA for the citric acid cycle.

In liver mitochondria, acetyl-CoA can also be converted to acetoacetic acid and then to beta-hydroxybutyric acid and acetone; these compounds are collectively called ketone bodies. Although the liver synthesizes ketone bodies, it has little β -ketoacyl-CoA transferase and is therefore not able to utilize ketone bodies. The ketone bodies are transported to other tissues such as the brain, muscle and heart which have the enzymes to convert ketone bodies to acetyl-CoA to serve as energy source.

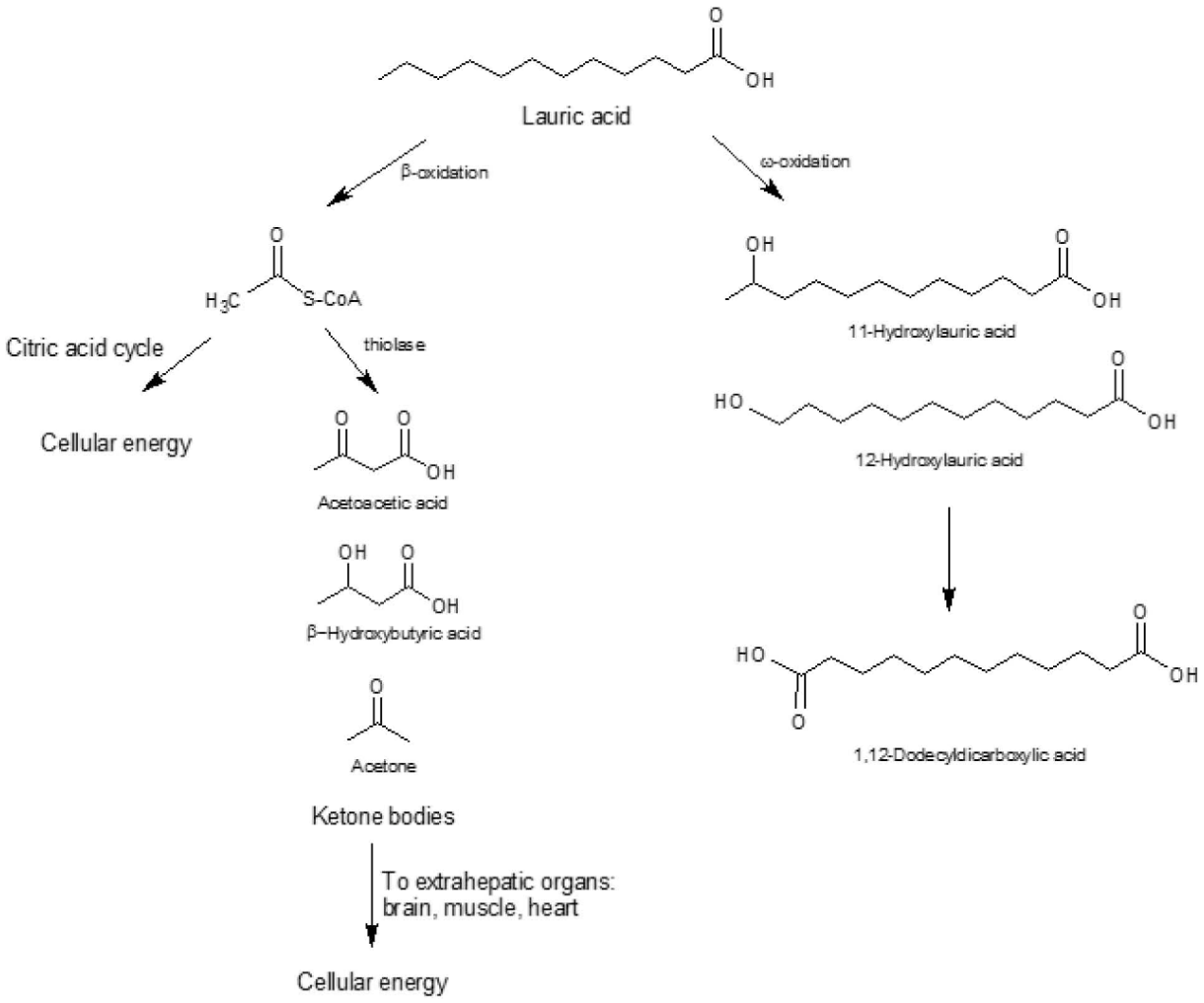


Fig. 10 – Representation of the metabolism of lauric acid in the liver (from Dayrit, 2015).

Numerous reports have been published on the antimicrobial properties of lauric acid and monolaurin both in vitro and in vivo. Among the saturated FAs, lauric acid and monolaurin have been shown to be very active against Gram-positive bacteria and a number of viruses and fungi. The antimicrobial activity of lauric acid, monolaurin, and other ester derivatives can be classified under three main mechanisms: 1) destruction of the cell membrane of Gram-positive bacteria and lipid-coated viruses by physicochemical processes, 2) interference with cellular

processes, such as signal transduction and transcription, and 3) stabilization of cell membranes. The availability of these multiple mechanisms may be one of the reasons why bacteria have been unable to evolve resistance against the action of these compounds. Early studies on the antimicrobial activity of FAs identified lauric acid as the most active among the saturated fatty acids; a systematic study of the in vitro anti-microbial activities of fatty acids, monoglycerides and diglycerides showed that lauric acid was the most active fatty acid against gram-positive bacteria and 1-monolaurin as more active than lauric acid (Kabara, 1972; 1979).

Aside from its antimicrobial activity, monolaurin was also shown to be effective in blocking or delaying the production of exotoxins by pathogenic Gram-positive bacteria (Schlievert et al., 1992). The mechanism by which monolaurin inhibits the synthesis of staphylococcal toxins and other exoproteins was shown to be at the level of transcription. Further, it was shown that monolaurin can block the induction of β -lactamase by interfering with signal transduction (Projan et al., 1994). Monolaurin inhibited the expression of virulence factors in *Staphylococcus aureus* and the induction of vancomycin resistance in *Enterococcus faecalis*. It was suggested that monolaurin acted by blocking signal transduction (Ruzin and Novick, 2000).

Various studies have also hypothesized that FAs may damage outer or cytoplasmic membrane, hinder macromolecular synthesis or denature proteins and DNA and the effect of monolaurin may be similar (Skrivanova et al., 2006). Furthermore, lauric acid has also been shown to have positive effects on the health and growth of pigs (Pluske et al., 2018). It most likely exerts its effects on the voluntary intake of feed through reduced motility (peristalsis) thus increasing transit time through the gastrointestinal tract and (or) through increased production and (or) secretion of hormones (e.g., CCK, PYY) known to have adverse effects against feed intake (Little et al., 2005; Black et al., 2009).

1.9 FORM OF SUPPLEMENTATION OF FAs IN PIG'S DIET

As has been amply explained above, FAs have numerous positive effects on pig health and growth. However, giving them in the feed is not easy and the choice of the supplementation form is crucial. In fact, when administered in the free form, they are rapidly absorbed by the cells of the intestinal mucosa, thereby reducing or even nullifying their effects. The digestion of free FAs starts in the stomach and only a little amount of them reaches the small intestine (Liu, 2015). Thus, a longer retention time in the gut may afford more extended effects on piglets' gut health. Moreover, the pure form of FAs has an unpleasant odor and taste (Oprean et al., 2011).

1.9.1 Esterification

To overcome this problem, FAs are usually used in an esterified form with glycerol. In this way, it is possible to delay the absorption of FAs to prolong their effectiveness along the gastrointestinal tract. Esterification is a chemical reaction in which an ester and water are obtained from an alcohol and an acid. Usually, esters are obtained from the reaction between glycerol and one or more fatty acids, or mono-di-triglycerides of FAs (Otera, 2006).

Numerous studies have reported that it is possible to administer esterified FAs in piglet diet. In this way, it is possible to solve the problem related to the unpleasant odor and taste typical of free fatty acids, and their absorption in the stomach and in the initial part of the intestine is prevented, so as to prolong their effectiveness throughout the GIT (Dierick et al., 2002; 2003).

However, this process has some disadvantages: the product obtained is a substance similar to a paste, whose consistency does not allow its insertion in feed or in drinking water. It is necessary to add an inorganic base (usually silica) which modifies its consistency, obtaining a more granular and simpler product to be feed mixed. However, adding the inorganic base leads to a reduction in the amount of fatty acid in the final product (even 35-40%) because the base takes up a lot of space within the molecule. This entails an increase in the dosage, which can be disadvantageous also in economic terms, not only for the health of piglets.

To overcome these problems, another possible and innovative solution can be saponification.

1.9.2 Saponification

Saponification is a process that consists in saponified FAs, adding calcium hydroxide or other elements, such as magnesium or potassium. It is a hydrolysis between FAs (usually triglycerides) and alkali-metal hydroxides, to obtain the salts of fatty acids, also called soaps. It is the reverse process of esterification (Schumann and Siekmann, 2005).

The reaction takes place at about 200°C and 3-4 atmospheres of pressure. The soaps obtained are milled in coarse flakes of white-yellowish color, which varies according to the starting fatty acid (Cevolani, 2016). The salts of FAs are non-corrosive compounds, easily included in meal feed for animals as a homogeneous mixture (Cevolani, 2016) due to their powder form.

Moreover, their use allows to solve the problems related to the unpleasant odor and taste of free FAs and to delay their absorption along the GIT. Saponification is also an effective method for bringing a high concentration of FAs into place; in the finished product, only about 7-10% is occupied by the element (i.e. Calcium). When it reaches the site of action, the soap breaks down and the fatty acid can perform its functions.

An example of the saponification of a fatty acid is the saponification of lauric acid (C:12) with calcium. The production process of the product is as follows:

- MCFAs (in this case lauric acid) are loaded into a heated mixer, at a temperature for which they are already in liquid form;
- An aqueous dispersion (suspension) of calcium hydroxide is prepared separately;
- Then, the suspension is added slowly under stirring. The reaction is exothermic and causes partial evaporation of the water.
- When all the suspension has been added, the mixture is heated to terminate the reaction and to almost completely eliminate the water, obtaining the soap. Heating is maintained until the softening point is reached; at this point zinc oxide (ZnO) is added as a powder (40 thousand ppm).
- Subsequently, heating is interrupted but the mixing continued until the mixture is completely uniform. At this point, cooling phase started in which the product will start to solidify and then break and crumble;
- At the end of the cooling process a very heterogeneous solid particle compound will be obtained. Through the mill grinding, the desired granulometry is obtained.

The final composition of the product obtained comprises 10% calcium, ZnO (30 ml/kg) and 75% of lauric acid.

1.10 CELL CULTURE IN PIG RESEARCH

Nowadays, alternatives are being developed not only to reduce the use of antibiotics and improve the gut health in animal production, but also to reduce the use of animals in scientific studies. Intestinal epithelial cell lines (IEC) provide an approach to the mechanisms of signaling pathways related to the interaction between the epithelial cell and bacteria or viruses. Compared to the animal model, cell line studies are less expensive, are not associated with ethical concerns, and provide a simple, highly controlled model for studying isolated factors, such as the effect of innovative nutritional strategies, on the IEC response.

Basically, there are two types of cell culture: 1) primary cell cultures and 2) continuous cell lines. Primary cells are directly isolated from a subject that have limited lifespan, whereas continuous cell lines, also referred as established or immortalized, have acquired the ability to proliferate with infinite lifespan. There are several means to achieve the immortality of a cell line. Tumor cells and undifferentiated cells are inherently immortal, otherwise normal cells can be immortalized (confer tumor ability) adding foreign genes through a virus. Continuous cell lines from what the scientific community designates as cell lines, are one of the most used and useful models to study the effect of a specific factor (feed additives for instance) on a specific cell type a the most basic level.

IEC's are involved in multiple functions of the digestive system, such as the absorption of nutrients and water, exchange of electrolytes and hormone production. Because of its permanent exhibition to the external environment, IECs are of key importance for maintaining the integrity of the intestinal epithelium and reducing the risk of invasion by external agents, forming a physical barrier. They are also crucial for the maintenance of intestinal homeostasis.

Epithelial intestinal cells help on the physical barrier function due to two physical structures of the epithelium (microvillus and tight junctions) that separates the external environment of the organism, preventing the invasion of microorganisms. IECs also secrete a large variety of antimicrobial peptides, cytokines and chemokines that are activated in a pathological or infectious situation enhancing the inflammatory response. During an infection, when a pathogen breaches the intestinal mucosa, it is recognized by toll-like receptors (TLRs). TLRs are expressed at the intestinal surface playing a key role in the innate immune system, since they activate immune cell responses. Between the IEC there are goblet cells that secrete mucus. This mucus contains mucins that form a permeable barrier that protects and repairs the intestinal

epithelium of any damage. Mucins can also interact with bacteria and have the ability to immobilize and eliminate them through the movements of the gut.

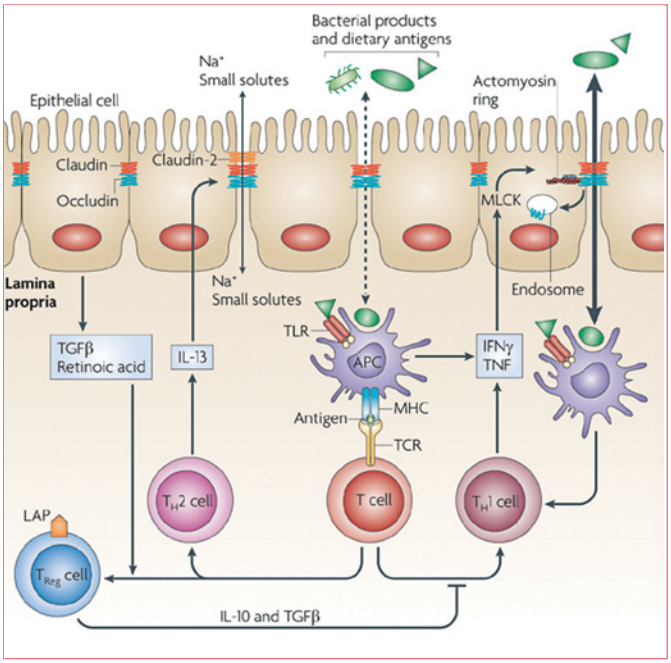


Fig. 11 – Barrier function of IECs (from Turner, 2009)

Most research has been conducted on human intestinal cell lines that are derived from a cancerous colon (e.g., Caco-2) or duodenum and also in small intestinal rat cell lines (e.g., IEC-6 and IEC-18) which are not cancerous. However, the structure and function of the GIT of rodents appear to differ from that of other domestic mammals like swine. Nowadays, there are three different porcine intestinal epithelial cell lines:

- IPI-2I cells isolated from ileal tissue of an adult boar and transformed with a plasmid that immortalizes the cells;
- IPEC-1 cells isolated from a mixture of ileal and jejunal tissues of a day-old piglet. It is a non-transformed cell line;
- IPEC-J2 cells isolated from jejunal tissue of a day-old piglet and is non-transformed.

1.10.1 IPEC-J2 PORCINE CELL LINE

IPEC-J2 has become more used since the characterization done by Shierack et al., (2006) but they were originally isolated from the jejunal epithelium of a neonatal unsuckled piglet in 1989 by Helen Berschneider at the University of North Carolina (Berschneider 1989). Given the correct culture conditions, these cells will divide and grow for an infinite number of passages in vitro. To date, they have been cultured continuously for up to 98 passages.

The strength of the IPEC-J2 cell line as an in vitro model originates from its morphological and functional similarities with intestinal epithelial cells in vivo. IPEC-J2 cells have microvilli on their apical side and tight junctions sealing neighboring cells together (Schierack et al., 2006). To date, no brush border enzyme activity has been investigated in IPEC-J2 cells. IPEC-J2 cells form polarized monolayers when cultured on 0.4 μm pore-size filters, with or without a collagen basis. High transepithelial electrical resistance (TEER) values and low active transport rates are obtained when culturing the IPEC-J2 cells in fetal bovine serum (Geens and Niewold 2011). These atypically high TEER values can be beneficial to use when investigating compounds having a negative effect on the monolayer permeability or tight junction structures (Vergauwen et al., 2015). Porcine serum resulted in significantly lower TEER values and higher active transport rates comparable to the in vivo situation (Zakrzewski et al., 2013). When IPEC-J2 cells are grown in 10 % PS they will become taller and smaller in diameter, increasing the tight junction ultrastructure (Zakrzewski et al., 2013).

The protein expression of claudin-1, -3, -4, -5, -7, -8, -12, tricellulin, occludin, E-cadherin and zonula occludens-1 (ZO-1) by IPEC-J2 cells has been confirmed by immunoblotting (Zakrzewski et al., 2013. On the other hand, IPEC-J2 cells do not express claudin-2 and -15 resulting in lower cation selectivity, augmenting the ion permeability of tight junctions compared to the porcine jejunum (Zakrzewski et al., 2013; Schierack et al., 2006).

IPEC-J2 cells express and produce cytokines, defensins, toll-like receptors and mucins. The presence of glycocalyx-bound mucins like Muc1 and Muc3 has been confirmed in IPEC-J2 cells, respectively by RT-PCR and ELISA. The expression of the Muc2, the major gel-forming mucin in the human small intestine, was not detectable by RT-PCR (Schierack et al., 2006). Thus, the mucus production by IPEC-J2 cells is not comparable to the in vivo situation. The mucus layer is only a very thin layer that is superimposed on the IPEC-J2 cells when cultured with fetal bovine serum. However, PAS staining showed an increase in mucus production when IPEC-J2 cells were cultured with porcine serum (Zakrzewski et al., 2013).

IPEC-J2 cells express proteins such as MHC I and secrete cytokines like GM-CSF and TNF-α that can establish communication between enterocytes and the immune system. Furthermore, mRNA expression of TLR-1, -2, -3, -4, -5, -6, -8, -9, -10 and IL-1α, -1β, -6, -7, -8, -12A, -12B, -18 has been confirmed in IPEC-J2 cells. TLR-2, -3, -5, -9 and IL-6, -8 proteins were also detected in these cells (Brosnahan and Brown, 2012; Arce et al., 2010; Burkey et al., 2009). IPEC-J2 cells express the F4 fimbrial receptor but not the F18 fimbrial receptor as this has been correlated to older pigs (3–23 weeks). These features make IPEC-J2 cells an interesting in vitro model to investigate the pathogenesis of zoonotic enteric infections that also affect humans.

1.11 REFERENCES

Aarestrup F. 2012. Get pigs off antibiotics. Nature. 486: 465–466.

Abdelli N, Pérez JF, Vilarrasa E, Luna IC, Melo-Duran D, D'Angelo M, Solà-Oriol D. 2020. Targeted-release organic acids and essential oils improve performance and digestive function in broilers under a necrotic enteritis challenge. Anim 10, 259 doi:10.3390/ani10020259.

Abraham EP, Chain E. 1940. An enzyme from bacteria able to destroy penicillin. Reviews of Infectious Diseases 10: 677–678.

Adeola O, King DE. 2006. Developmental changes in morphometry of the small intestine and jejunal sucrase activity during the first nine weeks of postnatal growth in pigs. J Anim Sci 84: 112–118.

Adewole DI, Kim IH, Nyachoti CM. 2016. Gut health of pigs: challenge models and response criteria with a critical analysis of the effectiveness of selected feed additives – a review. Asian-Australas J Anim Sci 29: 909-924.

Alban L, Dahl J, Andreasen M, Petersen JV, Sandberg M. 2013. Possible impact of the 'yellow card' antimicrobial scheme on meat inspection lesions in Danish finisher pigs. Prev Vet Med 108: 334-341.

Alexandratos N and Bruinsma J 2012. World agriculture towards 2030/2050. The 2012 revision. Global Perspective Studies Team, ESA Working Paper No. 12-03, Food and Agriculture Organization of the United Nations (FAO), Rome, Italy.

Apley MD, Bush EJ, Morrison RB, Singer RS, Snelson H. 2012. Use estimates of in-feed antimicrobials in swine production in the United States. Foodborne Pathog Dis 9: 272-279.

Arce C, Ramirez-Boo M, Lucena C, Garrido JJ. 2010. Innate immune activation of swine intestinal epithelial cell lines (IPEC-J2 and IPI-2I) in response to LPS from Salmonella typhimurium. Comp Immunol Microbiol Infect Dis 33:161–174.

Baltic B. 2017. Importance of medium chain fatty acids in animal nutrition. IOP Conf. Ser.: Earth Enviromental Science, 85, Pp.12048.

Barba-Vidal E, Roll VFB, Castillejos L, Guerra-Ordaz AA, Manteca X, Mallo JJ, Martín-Orúe SM. 2017.vResponse to a Salmonella Typhimurium challenge in piglets supplemented with protected sodium butyrate or Bacillus licheniformis: effects on performance, intestinal health and behavior. Transl Anim Sci 1:186–200.

Barrow PA, Fuller R, Newport MJ. 1977. Changes in microflora and physiology of anterior intestinal tract of pigs weaned at 2 days, with special reference to pathogenesis of diarrhea. Infection and Immunity 18: 586–595.

Bedford A, Gong J. 2018. Implications of butyrate and its derivatives for gut health and animal production. Anim Nutr 4:151-159.

Berkeveld M, Langendijk P, Bolhuis JE, Koets AP, Verheijden JH, Taverne MA. 2007. Intermittent suckling during an extended lactation period: effects on piglet behavior. J Anim Sci 85: 3415-3424.

Berkeveld MP, Langendijk P, Soede NM, Kemp B, Taverne MA, Verheijden JH, et al. 2009. Improving adaptation to weaning: effect of intermittent suckling regimens on piglet feed intake, growth, and gut characteristics. J Anim Sci 87: 3156-3166.

Berschneider H. 1989. Development of normal cultured small intestinal epithelial cell lines which transport Na and Cl. Gastroenterology 96: A41.

Biagi G, Piva A, Moschini M, Vezzali E, Roth FX. 2007. Performance, intestinal microflora, and wall morphology of weanling pigs fed sodium butyrate. J Anim Sci 85:1184–91.

Bischoff SC. 2011. "Gut health": a new objective in medicine? BMC Med 9:24.

Biswas SK. 2016. Does the Interdependence between Oxidative Stress and Inflammation Explain the Antioxidant Paradox? Oxidative Medicine and Cellular Longevity, Volume 2016, Article ID 5698931.

Black JL, Williams BA, Gidley MJ. 2009. Metabolic regulation of feed intake. In Voluntary feed intake in pigs (eds. Torrallardona, D., Roura), pp. 189- 214. Wageningen Academic Publishers, The Netherlands.

Bøe K. 1991. The process of weaning in pigs: when the sow decides Appl Anim Behav Sci 30: 47-59.

Bosi P, Messori S, Nisi I, Russo D, Casini L, Coloretti F, Schwarzer K, Trevisi P. 2009. Effect of different butyrate supplementations on growth and health of weaning pigs challenged or not with E. coli K88. Ital J Anim Sci 8: 268–270.

Boudry G, Peron V, Le Huerou-Luron I, Lalles JP, Sève B. 2004. Weaning induces both transient and long-lasting modifications of absorptive, secretory, and barrier properties of piglet intestine. J Nutr 134:2256–2262.

Boyen F, Haesebrouck F, Vanparys A, Volf J, Mahu M, Van Immerseel F, Rychlik I, Dewulf J, Ducatelle R, Pasmans F. 2008. Coated fatty acids alter virulence properties of Salmonella Typhimurium and decrease intestinal colonization of pigs. Vet Microbiol 132: 319–327.

Bremer J. 1983. Carnitine-metabolism and functions. Physiol Rev 63:1420–1466.

Brooks PH, Moran CA, Beal JD, Demeckova V, Campbell A. 2001. Liquid feeding for the young piglets. In: MA Varley, JR Wiseman (eds), The Weaner Pig: Nutrition and Management. CAB International, Wallinford, Oxon, pp. 153.

Brosnahan AJ, Brown DR. 2012. Porcine IPEC-J2 intestinal epithelial cells in microbiological investigations. Vet Microbiol 156: 229–237.

Burkey TE, Skjolaas KA, Dritz SS, Minton JE. 2009. Expression of porcine Toll-like receptor 2, 4 and 9 gene transcripts in the presence of lipopolysaccharide and Salmonella enterica serovars Typhimurium and Choleraesuis. Vet Immunol Immunopathol 130: 96–101.

Burkey TE, Skjolaas KA, Minton JE. 2009. Board-Invited Review: porcine mucosal immunity of the gastrointestinal tract. J Anim Sci 87: 1493-1501.

Burow E, Simoneit C, Tenhagen BA, Kasbohrer A. 2014. Oral antimicrobials increase antimicrobial resistance in porcine E. coli — a systematic review. Prev Vet Med 113: 364-375.

Burrin D, Stoll B. 2003. Enhancing intestinal function to improve growth and efficiency. 9th International Symposium on Digestive Physiology of Pigs; 121–137.

Burrin D, Stoll B. 2003. Intestinal nutrient requirements in weanling pigs. In: Weaning the pig: concepts and consequences. Edited by Pluske JR, Vergesten MWA, Le Dividich J. The Netherlands: Wageningen Academic Publisher; p. 301-335.

Callegari MA, Novais AK, Olivieira ER, Dias CP, Schmoller DL, Pereira M Jr, Dario JGN, Alves JB, de Silva CA. 2015. Microencapsulated acids associated with essential oils and acid salts for piglets in the nursery phase. Ciencias Agrarias 4: 2193-2208.

Callens B, Persoons D, Maes D, Laanen M, Postma M, Boyen F, Haesebrouck F, Butaye P, Catry B, Dewulf J. 2012. Prophylactic and metaphylactic antimicrobial use in Belgian fattening pig herds. Prev Vet Med 106: 53-62.

Camilleri M, Madsen K, Spiller R, van Meerveld BG, Verne GN. 2012. Intestinal barrier function in health and gastrointestinal disease. Neurogastol and Motil 24: 503-512.

Campbell JM, Crenshaw JD, Polo J. 2013. The biological stress of early weaned piglets. J Anim Scie Biotechnol 4: 19.

Cao ST, Wang CC, Wu H, Zhang QH, Jiao LF, Hu CH. 2018. Weaning disrupts intestinal antioxidant status, impairs intestinal barrier and mitochondrial function, and triggers mitophagy in piglets. J Anim Sci 96:1073–1083.

Casellas J, Casas X, Piedrafita J, Manteca X. 2005. Effect of medium- and long-chain triglyceride supplementation on small newborn-pig survival. Prev Vet Med 67: 213–221.

Castanon J. 2007. History of the use of antibiotic as growth promoters in European poultry feeds. Poult Science 86: 2466–2471.

Catry B, Cavaleri M, Baptiste K, Grave K, Grein K, Holm A. Use of colistin-containing products within the European Union and European Economic Area (EU/EEA): Development of resistance in animals and possible impact on human and animal health. 2015. Intern J Antimic Agent 46: 297-306.

Celi P, Cowieson AJ, Fru-Nji F, Steinert RE, Kluenter AM, Verlhac V. 2017. Gastrointestinal functionality in animal nutrition and health: new opportunities for sustainable animal production. Anim Feed Sci Technol 234: 88-100.

Cera KR, Mahan DC, Reinhart GA. 1898. Postweaning Swine Performance and Serum Profile Responses to Supplemental Medium-Chain Free Fatty Acids and Tallow. J Anim Sci 67, 2048.

Cevolani D. 2016. Prontuario degli alimenti per il suino. Terza edizione, Edagricole.

Cheng G, Hao H, Xie S, Wang X, Dai M, Huang L, Yuan Z. 2014. Antibiotic alternatives: the substitution of antibiotics in animal husbandry? Front Microbiol 5: 69–83.

Costantini D, Marasco V, Møller AP. 2011. A meta-analysis of glucocorticoids as modulators of oxidative stress in vertebrates. J Comp Physiol B 181:447–456.

de Ruyter EM, van Wettere WH, Lines DS, Plush KJ 2017. Gradually reducing sow contact in lactation is beneficial for piglet welfare around weaning. Appl Anim Behav Sci 193: 43-50

den Besten G, van Eunen K, Groen AK, Venema K, Reijngoud DJ, Bakker BM. 2013. The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. J Lipid Res 54: 2325–2340.

Desbois AP, Smith VJ. 2010 Antibacterial free fatty acids: Activities, mechanisms of action and biotechnological potential. Appl Microbiol Biotechnol 85: 1629–1642.

Dibner, J.D. Richards JD. 2005. Antibiotic growth promoters in agriculture: history and mode of action. Poult Sci 84: 634-643.

Dierick N, Michiels J, Van Nevel C. 2004. Effect of medium chain acid and benzoic acid, as alternatives for antibiotics, on growth and some gut parameters in piglets. Commun Agric Appl Biol Sci 69: 178-190.

Dierick NA, Decuypere JA, Degeyter I. 2003. The combined use of whole Cuphea seeds containing medium chain fatty acids and an exogenous lipase in piglet nutrition. Archiv für Tierernähr 57: 49-63.

Dierick NA, Decuypere JA, Molly K, Van Beek E, Vanderbeke E. 2002. The combined use of triacylglycerols (TAGs) containing medium chain fatty acids (MCFAs) and exogenous

lipolytic enzymes as an alternative for nutritional antibiotics in piglet nutrition. II. In vivo release of MCFAs in gastric cannulated and slaughtered piglets by endogenous and exogenous lipases; effects on the luminal gut flora and growth performance. LivesT Prod Scie 76: 1–16.

Efird RC, Armstrong WD, Herman DL. 1982. The development of digestive capacity in young pigs: effects of age and weaning system. J Anim Sci 55: 1380–1387.

English P, Robb CM, Bias MFM. 1980. Evaluation of creep feeding using a highly-digestible diet for litters weaned at four weeks of age. Anim Prod 30: 496.

English P. 1981. Establishing the early weaned pig. Pig Vet Soc Proc 7: 29-37.

Fang CL, Sun H, Wu J, Niu HH, Feng J. 2014. Effects of sodium butyrate on growth performance, haematological and immunological characteristics of weanling piglets. J AniM Physiol Anim Nutr 98: 680–5.

Feng W, Wu Y, Chen G, Fu S, Li B, Huang B, Wang D, Wang W, Liu J. 2018. Sodium Butyrate Attenuates Diarrhea in Weaned Piglets and Promotes Tight Junction Protein Expression in Colon in a GPR109A-Dependent Manner. Cell Physiol Biochem 47:1617-1629.

Ferrando ML, Schultsz C. 2016. A hypothetical model of host-pathogen interaction of Streptococcussuis in the gastro-intestinal tract. Gut Microbes 7: 154-162.

Ferronato G, Prandini A. 2020. Dietary supplementation of inorganic, organic and fatty acids in pig: a review. Anim 10: 17-40.

Fertner M, Boklund A, Dupont N, Enøe C, Stege H, Toft N. 2015. Weaner production with low antimicrobial usage: A descriptive study. Acta Veterinaria Scandinavica 57: 1-8.

Food and Agriculture Organization 2009. The state of food and agriculture: livestock in the balance. Food and Agriculture Organization of the United Nations, Rome, Italy.

Fouhse JM, Zijlstra RT, Willing BP. 2016. The role of gut microbiota in the health and disease of pigs. Anim Front 6: 30-36.

Gálfi P, Bokori J. 1990. Feeding trial in pigs with a diet containing sodium n-butyrate. Acta Vet Hung 38: 3–17.

Garlid KD, Orosz DE, Modriansky M, Vassanelli S, Jezek P. 1996. On the mechanism of fatty acid-induced proton transport by mitochondrial uncoupling protein. J Biol Chem 5:2615–2620.

Gatlin LA, Odle J, Soede J, Hansent JA. 2002. Dietary medium- or long-chain triglycerides improve body condition of lean-genotype sows and increase suckling pig growth. J Anim Sci 80, 38–44.

Geens MM, Niewold TA. 2011. Optimizing culture conditions of a porcine epithelial cell line IPEC-J2 through a histological and physiological characterization. Cytotechnol 63 :415–423.

Genova JL, Brandao Melo AD, Rupolo PE, Carvalho ST, Costa LB, De Oliveira Carvalho P. 2020. A summary of feed additives, intestinal health and intestinal alcaline phosphatase in piglet nutrition. Czech J Anim Scie 65: 281-294.

Glass-Kaastra SK, Pearl DL, Reid-Smith RJ, McEwen B, McEwen SA, Amezcua R, Friendship RM. 2013. Describing antimicrobial use and reported treatment efficacy in Ontario swine using the Ontario swine veterinary-based Surveillance program. BMC Vet Res 9: 238.

Goransson G. 1965. The metabolism of fatty acids in the rat VIII. Lauric acid and myristic acid. Acta Physiol Scand 64:383–386

Gottschalk M. 2012. Streptococcosis". In Diseases of Swine (Straw BE, Zimmerman JJ, D'Allaire S and Taylor DJ, eds), pp. 841–855. Blackwell Publishing, Ames, IA, USA.

Gresse R, Chaucheyras-Durand F, Fleury MA, van de Wiele T, Forano E, Blanquet-Diot S. 2017. Gut microbiota dysbiosis in postweaning piglets: understanding the keys to health. Trends Microbiol 25: 851-873.

Hamilton JA. 1998. Fatty acid transport: difficult or easy? J Lipid Res 39:467–481.

Han YS, Tang CH, Zhao QY, Zhan TF, Zhang K, Han YM, Zhang JM. 2018. Effects of dietary supplementation with combinations of organic and medium chain fatty acids as replacements for chlortetracycline on growth performance, serum immunity, and fecal microbiota of weaned piglets. Livest Sci 216: 210–218.

Hanczakowska E, Szewczyk A, Okon. 2011. Effects of dietary caprylic and capric acids on piglet performance and mucosal epithelium structure of the ileum. J Anim Feed Sci 20: 556-656.

Hanczakowska E. 2017. The use of medium-chain fatty acids in piglet feeding –a review. Ann Anim Sci 4: 967–977.

Hao H, Cheng G, Iqbal Z, Ai X, Hussain HI, Huang L, Dai M, Wang Y. 2014. Benefits and risks of antimicrobial use in food-producing animals. Front Microbiol 5: 288.

Hausmann M. 2010. How Bacteria-Induced Apoptosis of Intestinal Epithelial Cells Contributes to Mucosal Inflammation. International Journal of Inflammation, Volume 2010, Article ID 574568, doi:10.4061/2010/574568.

Hedemann MS, Højsgaard S, Jensen BB. 2003. Small intestinal morphology and activity of intestinal peptidases in piglets around weaning. J Anim Physiol Anim Nutr 87:32–41.

Hedemann MS, Jensen BB. 2004. Variations in enzyme activity in stomach and pancreatic tissue and digesta in piglets around weaning. Arch Anim Nutr 58: 47–59.

Henning SJ. 1985. Ontogeny of enzymes in the small intestine. Ann Rev Physiol 47: 231–245.

Hillman K. 2001. Bacteriological aspects of the use of antibiotics and their alternatives in the feed of non-ruminant animals. PC Garnsworthy, J Wiseman (Eds.). Recent advances in animal nutrition, Nottingham University Press, Loughborough, UK, pp. 107-134.

Hillman K. 2004. An analysis of gut microbes. Pig Int 34: 27-29.

Holman DB, Brunelle BW, Trachsel J, Allen HK. 2017. Meta-analysis to define a core microbiota in the swine gut. mSystems, 2 (2017), pp. e00004-17.

Hopwood DE, Hampson DJ. 2003. Interactionsbetween the intestinal microflora, diet and diarrhoea, and their influences on piglet health in the immediate post-weaning period. In: JR Pluske, J Le Dividich, MWA Verstegen (eds). Weaning the Pig: Concepts and Consequences. Wageningen Academic Publishers, Wageningen, Netherlands, pp. 199–218.

Hopwood DE, Pluske JR, Hampson DJ. 2005. Dietary manipulation of infectious bowel disease. R Mosenthin, J Zentek, E Zebrowska (Eds.). Biology of nutrition in growing animals, vol. 2005, Elsevier Limited, Amsterdam, The Netherlands, pp. 365-385.

Hou Y, Wang L, Yi D, Ding B, Chen X, Wang Q, Zhu H, Liu Y, Yin Y, Gong J. 2014. Dietary supplementation with tributyrin alleviates intestinal injury in piglets challenged with intrarectal administration of acetic acid. Br J Nutr 111: 1748–1758.

Hou YQ, Liu YL, Hu J, Shen WH. 2006. Effects of lactitol and tributyrin on growth performance, small intestinal morphology and enzyme activity in weaned pigs. Asian-Australas J Anim Sci 19: 1470–7.

Hu CH, Xiao K, Luan ZS, Song J. 2013. Early weaning increases intestinal permeability, alters expression of cytokine and tight junction proteins, and activates mitogen-activated protein kinases in pigs. J Anim Sci 91:1094–1101.

Huang WC, Tsai TH, Chuang LT, Li YY, Zouboulis CC and Tsai PJ. 2014. Anti-bacterial and anti-inflammatory properties of capric acid against Propionibacterium acnes: A comparative study with lauric acid. J Dermatol Sci 73: 232-240.

Huang Y, Li YL, Huang Q, Cui ZW, Yu DY, Rajput IR, Hu CH and Li WH. 2012. Effect of orally administrated Enterococcus faecium EF1 on intestinal cytokines and chemokines production of suckling piglets. Pak Vet J 32:81-84.

Jacobi SK, Odle J. 2012. Nutritional factors influencing intestinal health of the neonate. Adv Nutr 3: 687–96.

Jayaraman B, Nyachoti CM. 2017. Husbandry practices and gut health outcomes in weaned piglets: a review. Anim Nutr 3: 205-211.

Jean KB, Chiang SH. 1999. Increased survival of neonatal pigs by supplementing medium-chain triglycerides in late-gestating sow diets. Anim Feed Sci Technol 76: 241–250.

Jensen P, Recén B. 1989. When to wean—observations from free-ranging domestic pigs Appl Anim Behav Sci 23: 49-60.

Jensen P. 1986. Observations on the maternal behaviour of free-ranging domestic pigs. Appl Anim Behav Sci 16: 131-142.

Jensen P. 1988. Maternal behaviour and mother—young interactions during lactation in free-ranging domestic pigs. Appl Anim Behav Sci 20: 297-308.

Jensen VF, Emborg HD, Aarestrup FM. 2012. Indications and patterns of therapeutic use of antimicrobial agents in the Danish pig production from 2002 to 2008. J Vet Pharmacol Therap 35: 33-46.

Johnson RW. 1997. Inhibition of growth by pro-inflammatory cytokines: An integrated view. J Anim Sci 75:1244–1255.

Johnson RW. 1997. Inhibition of growth by pro-inflammatory cytokines: an integrated view. J Anim Sci 75:1244-1255.

Jondreville C, Revy PS, Dourmad JY. 2003. Dietary means to better control the environment impact of copper and zinc by pigs from weaning to slaughter. Livest Prod Sci 84: 147-156.

Jordan D, Chin JJ, Fahy VA, Barton MD, Smith MG, Trott DJ. 2009. Antimicrobial use in the Australian pig industry: results of a national survey. Aust Vet J 87: 222-229.

Jukes TH, Stokstad ELR, Taylor RR, Combs TJ, Edwards HM, Meadows GB. 1950. Growth promoting effects of aureomycin on pigs. Arch Biochem Bioph 26: 324–330.

Kabara JJ, Swieczkowski DM, Conley AJ, Truant JP. 1972. Fatty acids and derivatives as antimicrobial agents. Antimicrob Agents Chemother 2:23–28

Kabara JJ. 1979. Toxicological, bactericidal and fungicidal fatty acids and some derivatives. J Am Oil Chem Soc 56:760A–767A

Kelly D, King TP, Mcfadyen M, Travis AJ. 1991a. Effect of lactation on the decline of brush-border lactase activity in neonatal pigs. Gut 32: 386–392.

Kelly D, Smyth JA, McCracken KJ. 1991b. Digestive development of the early-weaned pig. II, Effect of level of food intake on digestive enzyme activity during the immediate post-weaning period. Brit J Nutr 65: 181–188.

Kemme PA, Jongbloed AW, Mroz Z, Kogut J, Beynen AC. 1999. Digestibility of nutrients in growing-finishing pigs is affected by Aspergillus niger phytase, phytate and lactic acid levels. Apparent ileal digestibility of amino acids. Livest Prod Sci 58: 107–117.

Khan AR, Khazanovich-Bernstein N, Bergmann EM, James MNG. 1999. Structural aspects of activation pathways of aspartic protease zymogens and viral 3C protease precursors. Proceedings of The National Academy of Sciences of The United States of America 96: 10968– 10975.

Kim JC, Hansen CF, Mullan BP, Pluske JR. 2012. Nutrition and pathology of weaner pigs: nutritional strategies to support barrier function in the gastrointestinal tract. Anim Feed Sci Technol 173: 3-16.

Kogut MH, Arsenault RJ. 2016. Editorial gut health: the new paradigm in food animal production. Front Vet Sci 3:71.

Kour P, Navjot SR, Singh H. 2020. Use of medium chain fatty acids in veterinary practice – a review. Inter J Med Vet Sci 1: 45-51.

Kutzer T, Bünger B, Kjaer JB, Schrader L. 2009. Effects of early contact between non-littermate piglets and of the complexity of farrowing conditions on social behaviour and weight gain. Appl Anim Behav Sci 121: 16-24.

Lackeyram D, Yang C, Archbold T, Swanson KC, Fan MZ. 2010. Early weaning reduces small intestinal alkaline phosphatase expression in pigs. J Nutr 140:461–468.

Lalles J, Boudry G, Favier C, LeFloc N, Luron I, Montagne L, Oswald IP, Pié S, Piel C, Sève B. 2004. Gut function and dysfunction in young pigs: physiology. Anim Res 53:301–316.

Lalles J. 2010. Intestinal alkaline phosphatase: multiple biological roles in maintenance of intestinal homeostasis and modulation by diet. Nutr Rev 68:323–332.

Lalles JP, Bosi P, Smidt H, Stokes CR. 2007. Weaning – A challenge to gut physiologists. Livest Sci 108: 82–93.

Lauridsen C. 2017. Digestion, absorption and metabolism of lipids, Chap. 22. In: Casimir CA, editor. Food lipids: chemistry, nutrition and biotechnology. Boca Raton (FL): Taylor & Francis Group; p. 591-602.

Le Gall M, Gallois M, Seve B, Louveau I, Holst JJ, Oswald IP, Lalles JP, Guilloteau P. 2009. Comparative effect of orally administered sodium butyrate before or after weaning on growth and several indices of gastrointestinal biology of piglets. Brit J Nutr 102: 1285–1296.

LeDividich J, Sève B. 2000. Effects of underfeeding during the weaning period on growth metabolism, and hormonal adjustments in the piglet. Dom Anim Endocrinol 19:63–74.

Lehninger AL, Nleson DL, Cox MM, Cox MM, Cox UMM, Fund SW. 2005. Principles of Biochemistry. Freeman WH.

Lei XJ, Park JW, Baek DH, Kim JK, Kim IH. 2017. Feeding the blend of organic acids and medium chain fatty acids reduces the diarrhea in piglets orally challenged with enterotoxigenic Escherichia coli K88. Anim Feed Sci Technol 224: 46–51.

Lhermie G, Grohn YT, Raboisson D. 2016. Addressing antimicrobial resistance: an overview of priority actions to prevent suboptimal antimicrobial use in food-animal production. Front Microbiol 7: 2114.

Little TJ, Russo A, Meyer JH, Horowitz M, Smyth DR, Bellon M, Wishart JM, Jones KJ, Feinle-Bisset C. 2005. Gatroenterol 133: 1124-1131.

Liu Y, Espinosa CD, Abelilla JJ, Casas GA, Lagos LV, Lee SA, Kwon WB, Mathai JK, Liu YY, Wang Y, Walsh TR, Yi LX, Zhang R, Spencer J. 2016. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: A microbiological and molecular biological study. Lancet Infect Dis 16: 161-168.

Liu Y. 2015. Fatty acids, inflammation and intestinal health in pigs. J Anim Sci Biotechnol 6: 41. DOI 10.1186/s40104-015-0040-1.

Llyod DAJ, Gabe SM. 2008. Intestinal morphology, intestinal regeneration and the promise of tissue engineering. In: AN Langnas, O Goulet, EMM Quigley, KA Tappenden (eds), Intestinal Failure: Diagnosis, Management and Transplantation. Wiley-Blackwell, Maiden, MA, USA, pp. 13.

Looft T, Johnson TA, Allen HK, Bayles DO, Alt DP, Stedtfeld RD, et al. 2012. In-feed antibiotic effects on the swine intestinal microbiome. Proc Nat Acad Sci 109: 1691-1696.

López-Colom P, Castillejos L, Rod Ríguez-Sorrento A, Puyalto M, Mallo JJ, Martín-Orúe SM. 2019. Efficacy of medium-chain fatty acid salts distilled from coconut oil against two enteric pathogen challenges in weanling piglets. J Anim Sci Biotechnol 10, 89.

Lu JJ, Zou XT, Wang YM. 2008. Effects of sodium butyrate on the growth performance, intestinal microflora and morphology of weanling pigs. J Anim Feed Sci 17 :568–578.

Luecke RW, McMillen WN, Thorp F Jr. 1950. Effect of vitamin B12, animal protein factor and streptomycin on the growth of young pigs. Arch Biochem, 26: 326-327.

Ma X, Fan PX, Li LS, Qiao SY, Zhang GL, Li DF. 2012. Butyrate promotes the recovering of intestinal wound healing through its positive effect on the tight junctions. J Anim Sci 90:266–268.

Mallo JJ, Balfagón A, Gracia MI, Honrubia P, Puyalto M. 2012. Evaluation of different protections of butyric acid aiming for release in the last part of the gastrointestinal tract of piglets. J Anim Sci 90:227–229.

Maron DF, Smith TJ, Nachman K. 2013. Restrictions on antimicrobial use in food animal production: an international regulatory and economic survey. Global Health 9: 48.

Marounek M, Skˇrivanová E, Skˇrivanová V. 2004. A note on the effect of caprylic acid and triacylglycerols of

Martinez V, Wang L, Million M, Rivier J, Tache´ Y. 2004. Urocortins and the regulation of gastrointestinal motor function and visceral pain. Peptides 25: 1733– 1744.

McCracken BA, Spurlock ME, Roos MA, Zuchermann FA, Gaskins HR. 1999. Weaning anorexia may contribute to local inflammation in the piglet small intestine. J Nutr 129:613–619.

McKay DM, Baird AW. 1999. Cytokine regulation of epithelial permeability and ion transport. Gut 44:282–289.

Merle R, Mollenhauer Y, Hajek P. 2013. Monitoring of antibiotic consumption in pigs on agricultural farms. Berliner und Munchener Tierarztliche Wochenschrift 126:326–32.

Moeser AJ, Pohl CS, Rajput M. 2017. Weaning stress and gastrointestinal barrier development: implications for lifelong gut health in pigs. Anim Nutr 3: 313-321.

Moeser AJ, Vander Klok C, Ryan KA, Wooten JG, Little JG, Cook VL, Blisklager AT. 2007. Stress signaling pathways activated by weaning mediate intestinal dysfunction in the pig. Am J Physiol Gastrointest Liver Physiol 292: G173–G181.

Mohana Devi S, Kim IH. 2014. Effect of medium chain fatty acids (MCFA) and probiotic (Enterococcus faecium) supplementation on the growth performance, digestibility and blood profiles in weanling pigs. Vet Med 59, 527–535.

Montagne L, Boudry G, Favier C, Le Huerou-Luron I, Lalles JP, Seve B. 2007. Main intestinal markers associated with the changes in gut architecture and function in piglets after weaning. Br J Nutr 97:45–57.

Moore PR, Evenson A, Luckey TD, McCoy E, Elvehjem CA, E, Hart EB. 1946. Use of sulfasuxidine, streptothricin, and streptomycin in nutritional studies with the chick. J Biol Chem 165: 437-441.

Mosenthin R, Sauer WC, Ahrens F, de Lange CFM, Bornholdt U. 1992. Effect of dietary supplements of propionic acid, siliceous earth or a combination of these on the energy, protein and amino acid digestibility and concentrations of microbial metabolites in the digestive tract of growing pigs. Anim Feed Sci Technol 37: 245–255.

Motohashi Y, Fukushima A, Kondo T, Sakuma K. 1997. Lactase decline in weaning rats is regulated at the transcriptional level and not caused by termination of milk ingestion. J Nutr 127: 1737– 1743.

Munita JM, Arias CA. 2016. Mechanisms of Antibiotic Resistance. Microbiology Spectrum 4:10.1128.

Nabuurs MJA, Hoogendoorn A, Van Zijderveld FG. 1994. Effects of weaning and enterotoxigenic Escherichia coli on net absorption in the small intestine of pigs. Res Vet Sci 56:379–385.

Nabuurs MJA. 1998. Weaning Piglets as a Model for Studying Pathophysiology of Diarrhea, Conference Proceedings, Gastro-Intestinal Disorders in Juveniles, Lelystad, Netherlands, 16–17 September, pp. S42–S45.

Navarro DMDL, Jaworski NW. 2018. Non-antibiotic feed additives in diets for pigs: A review. Anim Nutr 4: 113–125.

O'Neill J 2016. Tackling drug-resistant infections globally: final report and recommendations. The review on antimicrobial resistance. Retrieved on 19 May 2016 from http://amr-review.org/sites/default/files/160525_Final%20paper_with%20cover.pdf

Oprean L, Iancu R, Stand R, Trască. 2011. Comparison between types of feeding on goat milk composition. J Anim Sci Biotechnol 44: 76-79.

Otera J. 2006. Esterification: Methods, Reactions, and Applications. Wiley-VCH.

Pa´cha J. 2000. Development of intestinal transport function in mammals. Physiol Rev 80: 1633–1667.

Pajor EA, Weary DM, Caceres C, Fraser D, Kramer DL. 2002. Alternative housing for sows and litters: Part 3. Effects of piglet diet quality and sow-controlled housing on performance and behaviour. Appl Anim Behav Sci 76: 267-277.

Peffer PL, Lin X, Odle J. 2005. Hepatic-oxidation and carnitine palmitoyltransferase I in neonatal pigs after dietary treatments of cloridric acid, isoproterenol, and medium-chain triglycerides. Am J Physiol-Regul Integr Comp Physiol 288: 1518–1524.

Pié S, Lallès JP, Blazy F, Laffitte J, Seve B, Oswald IP. 2004. Weaning is associated with an upregulation of expression of inflammatory cytokines in the intestine of piglets. J Nutr 134: 641-647.

Piva A, Morlacchini M, Casadei G, Gatta PP, Biagi G, Prandini A. 2002. Sodium butyrate improves growth performance of weaned piglets during the first period after weaning. Ital J Anim Sci 1: 35–41.

Pluske J, Turpin D, Abraham S, Collins A, Dunshea F. 2018. 2C-125: Lauric acid, a potentially new feed additive for the Australian pork industry. Report prepared for the Co-operative Research Centre for High Integrity Australian Pork.

Pluske JR, Hampson DJ, Williams IH. 1997. Factors influencing the structure and function of the small intestine in the weaned pig: a review. Livest Prod Sci 51:215-236.

Pluske JR, Kim JC, Hansen CF, Mullan BP, Payne HG, Hampson DJ, et al. 2007. Piglet growth before and after weaning in relation to a qualitative estimate of solid (creep) feed intake during lactation: a pilot study. Arch Anim Nutr 61: 469-480.

Pluske JR, Payne HG, Williams IH, Mullan BP. 2005. Early feeding for lifetime performance of pigs. PB Cronje, N Richards (Eds.), Recent advances in animal nutrition in Australia, volume 15. Animal science, University of New England, Armidale, NSW (2005), pp. 171-181.

Pluske JR, Turpin DL, Kim JC. 2018. Gastrointestinal tract (gut) health in the young pig. Anim Nutr 4: 187-196.

Pluske JR, Williams IH, Aherne FX. 1996a. Maintenance of villous height and crypt depth in piglets by providing continuous nutrition after weaning. Anim Sci 62:131-144.

Pluske JR, Williams IH, Aherne FX. 1996b. Villous height and crypt depth in piglets in in response to increases in the intake of cows'milk after weaning. Anim Sci 62: 145-158.

Pluske JR. 2013. Feed and feed additives-related aspects of gut health and development in weanling pigs. J Anim Sci Biotechnol 4:1.

Pohl CS, Medland JE, Mackey E, Edwards LL, Bagley KD, DeWilde MP, et al. 2017. Early weaning stress induces chronic functional diarrhea, intestinal barrier defects, and increased mast cell activity in a porcine model of early life adversity. Neuro Gastroenterol Motil. 10.1111/NMO.13118.

Proha´szka L, Baron F. 1980. The predisposing role of high dietary protein supplies in enteropathogenic E. coli infections of weaned pigs. Zentralblatt fu¨ r Veterina¨rmedizin Reihe B 27: 222–232.

Projan SJ, Brown-Skrobot S, Schlievert PM, Vandenesch F, Novick RP. 1994. Glycerol monolaurate inhibits the production of β-lactamase, toxic shock syndrome toxin-1, and other staphylococcal exoproteins by interfering with signal transduction. J Bact 176:4204–4209

Rhouma M, Beaudry F, Letellier A, 2016. Resistance to colistin: what is the fate for this antibiotic in pig production? Int J Antimic Ag 2: 119-126,

Robinson K, Ma X, Liu Y, Qiao S, Hou Y, Zhang G. 2018. Dietary modulation of endogenous host defense peptide synthesis as an alternative approach to in-feed antibiotics. Anim Nutr 4: 160-169.

Robinson TP, Bu DP, Carrique-Mas J, Fevre EM, Gilbert M, Grace D, Hay SI, Jiwakanon J, Kakkar M, Kariuki S, Laxminarayan R, Lubroth J, Magnusson U, Thi Ngo P, Van Boeckel TP, Woolhouse MEJ. 2016. Opinion paper: Antibiotic resistance: mitigation opportunities in livestock sector development. Anim 11: 1-3.

Roca M, Nofrarías M, Majó N, Pérez de Rozas A, Segalés J, Castillo M, MaríaMartín-Orúe S, Espinal A, Pujols J, Badiola I. 2014. Changes in Bacterial Population of Gastrointestinal Tract of Weaned Pigs Fed with Different Additives. BioMed Research International, Article ID 269402, 13 pages.

Rossi R, Pastorelli G, Cannata S, Corino C. 2010. Recent advances in the use of fatty acids as supplements in pig diets: A review. Anim Feed Sci Technol 162: 1–11.

Roth F. 2000. Acidos organicos en nutrition porcina: eficacia y modo de acción. In: Curso de especialización, 11, 2000, Barcelona. Fundacion Espanola para El Desarrollo de la nutrition animal – FEDNA 2000, pp. 169-181.

Ruzin A, Novick RP. 2000 Equivalence of lauric acid and glycerol monolaurate as inhibitors of signal transduction in Staphylococcus aureus. J Bact 182:2668–2671

Sakata T, Inagaki A. 2001. Organic acid production in the large intestine: implication for epithelial cell proliferation and cell death. In: Piva A, Bach Knudsen KE, Lindberg JE, editors. The Gut environment of pigs. Nottingham: Nottingham University Press.

Sala V, Zavattini S. 2018. Il condizionamento manageriale nella diffusione della Streptococcosi nell'allevamento suinicolo" – SUMMA animali da reddito n.9 novembre 2018.

Sandhya S, Talukdar J, Bhaishya D. 2016. Chemical and Biological Properties of Lauric Acid: A Review. Inter J Adv Res 7: 1123-1128.

Schachtschneider KM, Yeoman CJ, Isaacson RE, White BA, Schook LB, Pieters M. 2013. Modulation of systemic immune responses through commensal gastrointestinal microbiota. PLoS One 8(1): e53969.

Scheppach W, Sommer H, Kirchner T, Paganelli GM, Bartram P, Christl S. 1992. Effect of butyrate enemas on the colonic mucosa in distal ulcerative colitis. Gastroenterol 103:51–6.

Schierack P, Nordhoff M, Pollmann M, Weyrauch KD, Amasheh S, Lodemann U, Jores J, Tachu B, Kleta S, Blikslager A, Tedin K, Wieler LH. 2006. Characterization of a porcine intestinal epithelial cell line for in vitro studies of microbial pathogenesis in swine. Histochem Cell Biol 125: 293–305.

Schlievert PM, Deringer JR, Kim MH, Projan SJ, Novick RP. 1992. Effect of glycerol monolaurate on bacterial growth and toxin production. Antimicrob Agents Chemother 36:626–631

Segura M, Calzas C, Grenier D, Gottschalk M. 2016. Initial steps of the pathogenesis of the infection caused by Streptococcus suis: fighting against nonspecific defenses. FEBS Letters 590: 3772–3799.

Segura M, Fittipaldi N, Calzas C, Gottschalk M. 2017. Critical Streptococcus suis Virulence Factors: Are They All Really Critical? Trends Microbiol, July 2017, Vol. 25, No. 7.

Smith F, Clark JE, Overman BL, Tozel CC, Huang JH, Rivier JEF, Blisklager AT, Moeser AJ. 2010. Early weaning stress impairs development of mucosal barrier function in the porcine intestine. Am J Physiol Gastrointest Liver Physiol 298:G352–G363.

Snoeck V, Huyghebaert N, Cox E, Vermeire A, Saunders J, Remon JP, Verschooten F, Goddeeris BM. 2004. Gastrointestinal transit time of non-disintegrating radio-opaque pellets in suckling and recently weaned piglets. J ControL Rel 94: 143– 153.

Spreeuwenberg MAM, Verdonk JMAJ, Gaskins HR, Verstegen MWA. 2001. Small intestine epithelial barrier function is compromised in pigs with low feed intake at weaning. J Nutr 131:1520–1527.

Spurlock ME. 1997. Regulation of metabolism and growth during immune challenge: An overview of cytokine function. J Anim Sci 75:1773–1783.

Sulabo RC, Tokach MD, Dritz SS, DeRouchey JM, Nelssen JL. 2010. Effects of varying creep feeding duration on the proportion of pigs consuming creep feed and neonatal pig performance. J Anim Sci 88: 3154-3162

Thomas LL, Woodworth JC, Tokach MD, Dritz SS, DeRouchey JM, Goodband RD, Williams HE, Hartman AR, Mellick DJ, McKilligan DM. 2020. Evaluation of different blends of medium-chain fatty acids, lactic acid, and monolaurin on nursery pig growth performance. Transl Anim Sci 4: 548–557.

Tokach MD, Goodband RD, Nelssen JL, Kats LJ. 1992. Influence of weaning weight and growth during the first week postweaning on subsequent pig performance. Proc of Kansas State University Swine Day; 19–21.

Traul KA, Driedger A, Ingle DL, Nakhasi D. 2000. Review of the toxicologic properties of medium-chain triglycerides. Food Chem Toxicol 38: 79–98.

Turner J. Intestinal mucosal barrier function in health and disease. 2009. Nat Rev Immunol 9: 799–809.

Turpin DL, Langendijk P, Chen TY, Lines D, Pluske JR. 2016. Intermittent suckling causes a transient increase in cortisol that does not appear to compromise selected measures of piglet welfare and stress. Anim 6: 24.

Turpin DL, Langendijk P, Sharp C, Pluske JR. 2017a. Intermittent suckling with or without comingling of non-littermate piglets before weaning improves piglet performance in the immediate post-weaning period when compared with conventional weaning. J Anim Sci Biotechnol 8: 14.

Turpin DL, Langendijk P, Sharp C, Pluske JR. 2017b. Improving welfare and production in the peri-weaning period: effects of co-mingling and intermittent suckling on the stress response, performance, behaviour and, gastrointestinal tract carbohydrate absorption in young pigs. Livest Sci 203: 82-91

Turpin DL. 2017. The Gradual weaning of piglets: the influence of intermittent suckling and comingling on performance, the stress response, behaviour, and function and morphology of the gastrointestinal tract. PhD Thesis. Murdoch University, Murdoch WA, Australia.

Valencia Z, Chavez ER. 2002. Phytase and acetic acid supplementation in the diet of early weaned piglets: effect on performance and apparent nutrient digestibility. Nutr Res 22: 623–632.

Van Boeckel TP, Brower C, Gilbert M, Grenfell BT, Levin SA, Robinson TP, Teillant A and Laxminarayan R 2015. Global trends in antimicrobial use in food animals. Proceedings of the National Academy of Sciences 112: 5649–5654.

Vergauwen H, Tambuyzer B, Jennes K, Degroote J, Wang W, De Smet S, Michiels J, Van Ginneken C. 2015. Trolox and ascorbic acid reduce direct and indirect oxidative stress in the IPEC-J2 cells, an in vitro model for the porcine gastrointestinal tract. PLoS One 10(3):e0120485.

Verstegen MWA, Williams BA. 2002. Alternatives to the use of antibiotics as growth promoters for monogastric animals. Anim Biotechnol 13: 113–127.

Vieira AR, Pires SM, Houe H, Emborg HD. 2011. Trends in slaughter pig production and antimicrobial consumption in Danish slaughter pig herds, 2002–2008. Epidemiol Infect 139:1601-1609.

Wang CC, Wu H, Lin FH, Gong R, Xie F, Peng Y, Feng J, Hu CH. 2018. Sodium butyrate enhances intestinal integrity, inhibits mast cell activation, inflammatory mediator production and JNK signaling pathway in weaned pigs. Innate Immunity 24: 40–46.

Wapnir RA. Teichberg S. 2002. Regulation mechanisms of intestinal secretion: implications in nutrient absorption. J Nutr Biochem 13: 190–199.

Weary DM, Pajor EA, Bonenfant M, Fraser D, Kramer DL. 2002. Alternative housing for sows and litters: Part 4. Effects of sow-controlled housing combined with a communal piglet area on pre-and post-weaning behaviour and performance. Appl Anim Behav Sci 76: 279-290.

White F, Wenham G, Sharman GA, Jones AS, Rattray EA, McDonald I. 1969. Stomach function in relation to a scour syndrome in the piglet. Brit J Nutr 23: 847–858.

Wie HK, Xue HX, Zhou ZX, Peng J. 2017. A carvacrol-thymol blend decreased intestinal oxidative stress and influenced selected microbes without changing the messenger RNA levels of tight junction proteins in jejunal mucosa of weaning piglets. Anim 11: 193-201.

Wierup M. 1999. The Swedish Experience of Limiting Antimicrobial Use. Proceedings of Agriculture's Role in Managing Antimicrobial Resistance Conference. Oct 24–26, Toronto, Canada. Toronto, Canada.

Wijitten PJA, van der Meulen J, Vergesten MWA. 2011. Intestinal barrier function and absorption in pigs after weaning: a review. Br J Nutr 105: 967-981.

Williams IH. 2003. Growth of the weaned pig. In: JR Pluske, JV Le Dividich, MWA Verstegen (eds), Weaning the Pig: Concepts and Consequences, Wageningen Academic Publishers, Wageningen, Netherlands, pp. 17–36.

Williams NH, Stahly TS, Zimmerman DR. 1997. Effect of level of chronic immune system activation on the growth and dietary lysine needs of pigs fed from 6 to 112 kg. J Anim Sci 75:2481–2496.

Xiong H, Guo B, Gan Z, Song D, Lu Z, Yi H, Wu Y, Wang Y, Du H. 2016. Butyrate upregulates endogenous host defense peptides to enhance disease resistance in piglets via histone deacetylase inhibition. Scientific Reports 6: 27070. doi:10.1038/srep27070.

Xu RJ. 2003. Gastrointestinal secretory function. In: RJ Xu, PD Cranwell (eds). The Neonatal Pig: Feeding strategies without using in-feed antibiotics J. M. Heo et al. 236 Journal of Animal Physiology and Animal Nutrition. [a] 2012 Blackwell Verlag GmbH Gastrointestinal Physiology and Nutrition. Nottingham University Press, Thrumpton, Nottinghamn, pp. 117.

Xu X, Chen S, Wang H, Tu Z, Wang S, Wang X, Zhu H, Wang C, Zhu J, Liu Y. 2018. Medium-chain TAG improve intestinal integrity by suppressing toll-like receptor 4, nucleotide-binding oligomerisation domain proteins and necroptosis signaling in weanling piglets challenged with lipopolysaccharide. Brit J Nutr 119: 1019–1028.

Yan H, Ajuwon KM. 2017. Butyrate modifies intestinal barrier function in IPEC-J2 cells through a selective upregulation of TJ proteins and activation of the Akt signaling pathway. PloS ONE 12(6): e0179586.

Yen J. 2000. Anatomy of the digestive system and nutritional physiology. In: A. J. Lewis, L. L. Southern (eds), Swine Nutrition. CRC Press, Florida, USA, pp. 32.

Yoon B, Jackman J, Valle-González E, Cho NJ. 2018. Antibacterial free fatty acids and monoglycerides: biological activities, experimental testing, and therapeutic applications. Int J Molecul Sci 19: 1114.

Zakrzewski SS, Richter JF, Krug SM, et al. 2013. Improved cell line IPEC-J2, characterized as a model for porcine jejunal epithelium. PLoS One 8(11):e79643.

Zakrzewski SS, Richter JF, Krug SM, Jebautzke B, Lee IF, Rieger J, Sachtleben M, Bondzio A, Schulzke JD, Fromm M, Gunzel D. 2013. Improved cell line IPEC-J2, characterized as a model for porcine jejunal epithelium. PLoS One 8(11):e79643.

Zeng MY, Inohara N, Nuñez G. 2017. Mechanisms of inflammation-driven bacterial dysbiosis in the gut. Mucosal Immunol 10: 18-26.

Zentek J, Buchheit-Renko S, Ferrara F, VahjenW, Van Kessel AG, Pieper R. 2011. Nutritional and physiological role of medium-chain triglycerides and medium-chain fatty acids in piglets. Anim Health Res Rev 12: 83–93.

Zentek J, Ferrara F, Pieper R, Tedin L, Meyer W, Vahjen W. 2013. Effects of dietary combinations of organic acids and medium chain fatty acids on the gastrointestinal microbial ecology and bacterial metabolites in the digestive tract of weaning piglets. J Anim Sci 91: 3200–3210.

Zentek J, Ferrara F, Pieper R, Tedin L, Meyer W, Vahjen W. 2013. Effects of dietary combinations of organic acids and medium chain fatty acids on the gastrointestinal microbial ecology and bacterial metabolites in the digestive tract of weaning piglets. J Anim Sci 91: 3200–3210.

Zhang Y, Xu RJ. 2003. Anatomy and histology of the gastrointestinal tract. In: R. J. Xu, P. D. Cranwell (eds), The Neonatal Pig: Gastrointestinal Physiology and Nutrition. Nottingham University Press, Thrumpton, Nottingham, UK, pp. 1.

Zhao W, Wang Y, Liu S, Huang J, Zhai Z, He C, et al. 2015. The dynamic distribution of porcine microbiota across different ages and gastrointestinal tract segments PLoS One, 10 (2) Article e0117441.

Zhou X, Stevens MJ, Neuenschwander S, Schwarm A, Kreuzer M, Bratus-Neuenschwander A, Zeitz JO. 2018. The transcriptome response of the ruminal methanogen Methanobrevibacter ruminantium strain M1 to the inhibitor lauric acid. BMC Res. Notes 11: 135.

Zhu LH, Zhao KL, Chen XL, Xu JX. 2012. Impact of weaning and an antioxidant blend on intestinal barrier function and antioxidant status in pigs. J Anim Sci, 90: 2581-2589.

CHAPTER 2 -

Objectives

2.1 OBJECTIVES

Global food production is driving demand for increased livestock growth performance and animal product safety, resulting from an ever-growing human population. Innovative nutritional strategies have a key role in an optimized animal production system. The ultimate aim of these strategies is to maximize productivity, while minimizing the use of antibiotics as wanted from the "One health" approach. Therefore, the aim of this work was to evaluate the effects of different short and medium chain fatty acids on growth performance and gut health of pigs. To achieve this object four different trial were designed. In the first one, the aim was to study the consequences of dietary lauric acid saponified with calcium supplementation in weaning piglets as a potential antibiotic replacer acting on pig's growth and some gut health parameters. The second trial moved to the assessment of the effects of one short chain (tributyrin) and one medium chain (monolaurin) fatty acid supplemented together in pigs' diet to improve their growth performance and gut health. Then, with the third trial, considering the results obtained in the first trial, the focus was put on the effects of lauric acid saponified with calcium supplementation starting from the last 3 weeks of gestation in sows' diet on productivity, growth performance and health of the sows and their offspring. Finally, the fourth trial aim was to assess the effect of fatty acids and different milk fractions on porcine IPEC-J2 cell line proliferation and viability.

CHAPTER 3 –

Lauric acid saponified with calcium: a possible solution to reduce the use of antibiotics in post-weaning piglets

Adapted from:

Giorgi S., Comi M., Ghiringhelli M., Cheli F., Rebucci R. and Bontempo V. Lauric acid saponified with calcium: a possible solution to reduce the use of antibiotics in post-weaning piglets.

Manuscript submitted to Italian Journal of Animal Science

3.1 ABSTRACT

Decreasing the use of antibiotics in pig husbandry is one of the largest challenges for pig production. The scientific community has investigated numerous alternative substances to antibiotics, including medium chain fatty acids, due to their antimicrobial and protective effects on the gut health of piglets. The present study investigates the effect of lauric acid saponified with calcium on the growth performance and gut health parameters in post-weaning piglets. A total of 192 24-day-old piglets were assigned to one of three dietary treatments: CTR (basal diet alone), ANT (amoxicillin, 400 mg/kg), or C12-Ca (lauric acid saponified with calcium, 1 g/kg) for 28 days. C12-Ca did not affect performance, except for feed efficiency (FE), which increased ($P<0.05$) in the C12-Ca and ANT groups from 15 to 28 days. On days 0 to 28, FE was higher ($P<0.001$) in the C12-Ca group compared to the CTR group. C12-Ca and CTR supplementation decreased the antibiotic treatments against diarrhoea ($P<0.05$). A greater concentration of lactic acid was found in the C12-Ca group in the small intestine ($P<0.001$). Acetic acid concentration decreased under C12-Ca treatment in the caecum ($P<0.001$). No differences in the microbial populations of IL-10, IL-6, IgA, and IgG were found. In the duodenum and ileum, C12-Ca administration provided a higher total antioxidant capacity and lower malondialdehyde level ($P<0.001$). C12-Ca improved jejunal crypt depth and villus height: crypt depth ratio and ileal villus height and width ($P<0.001$). Our findings suggest that C12-Ca administration modulates the gut antioxidant status and ameliorates some parameters of the jejunum and ileum morphology of post-weaning piglets.

Keywords: Lauric acid, saponification, piglets, gut health

3.2. INTRODUCTION

It is widely reported that weaning stress entails changes in gut structure and function and may cause developmental delays in piglets (Pluske et al. 1997; Yin et al. 2014). Antibiotics have long been used as growth promoters and therapeutic medicine to address these issues. However, the scientific community has recognized that this practice poses a serious threat to both humans and livestock due to increases in drug-resistant pathogens (Czaplewski et al. 2016). Thus, interest in alternative substances, such as medium-chain fatty acids (MCFAs), has increased due to their antimicrobial and protective effects on the intestinal microarchitecture of piglets (Hanczakowska 2017).

MCFAs are a family of 6–12 carbon atoms in length and straight-chain saturated free fatty acids, including lauric acid (C12:0). MCFAs are naturally present in coconut oil (Dayrit 2015), cow's milk, and human breast milk (Jensen 2002). Previous studies have shown that MCFAs improve the growth performance of piglets and prevent the negative effects associated with weaning (Zentek et al. 2011). Furthermore, in vitro studies have shown that MCFAs have antibacterial activity, particularly lauric acid, which shows great efficacy against Gram-positive bacteria (Lieberman et al. 2006; Dayrit 2015).

The primary effect of the antimicrobial mechanism of MCFAs is to damage the bacterial cell membrane. The membranolytic behaviour of MCFAs is derived from their amphipathic properties, which allow these molecules to adhere to the cell membrane, causing destabilization and pore formation (Desbois and Smith 2010). Destabilization of the membrane causes an increase in cellular permeability, leading to the inhibition of bacterial cell growth or directly to cell death. MCFAs can also alter the vital processes of bacterial cells, including the electron transport chain and oxidative phosphorylation, which are essential for energy production (Yoon et al. 2018).

In this context, lauric acid could represent a valid alternative for the prevention of streptococcal infections in post-weaned piglets. However, when administered in a free form, MCFAs are rapidly absorbed by the cells of the intestinal mucosa, thereby reducing or nullifying their effects (Li et al. 2015). A longer retention time in the gut may afford more extended effects on piglets' gut health. To overcome this problem, MCFAs are used in an esterified form with glycerol. In this way, it is possible to delay the absorption of MCFAs to prolong their effectiveness along the gastrointestinal tract.

However, esterification has some disadvantages. The obtained product is a gel-like substance, the consistency of which makes it difficult to add to feeds. Furthermore, since the

antibacterial effects of fatty acids (FAs) also depend on the dosage and quantity of the active principle present in the intestinal lumen, esterification may limit the use of FAs. The esterification process requires the addition of an inorganic base (usually silica) that modifies the consistency of the mixture, resulting in a granular product that is easier to mix. However, the addition of inorganic bases leads to as much as a 40% reduction in the number of FAs in the final product, reducing their effects.

One innovative solution might be saponification, a process that involves saponifying the FAs of a triglyceride by adding calcium hydroxide or other elements to obtain the salts of the FAs (soaps), which can be easily included in meal feed for animals due to its form as a powder (Schumann and Siekmann 2005). Saponification could also be an effective method to introduce higher concentrations of MCFAs into the final product. In particular, lauric acid saponified with calcium is composed of 75% lauric acid and 10% calcium. Since there is no published information on the effects of the dietary supplementation of C12-Ca soaps in post-weaning piglets, we designed the experiment presented here to test the effectiveness of these soaps. Therefore, the aim of this study was to evaluate the effect of lauric acid saponified with calcium on the gut health and growth performance of post-weaning piglets and assess its possible efficacy as a dietary strategy for reducing prophylactic antibiotics.

3.3 MATERIALS AND METHODS

The experimental protocol was reviewed and approved by the Animal Welfare Committee of the Università degli Studi di Milano (application No. OPBA_147_2017).

3.3.1 Animals, housing, and experimental design

The experiment was performed at the Animal Production Research and Teaching Centre of the Polo Veterinario, Università degli Studi di Milano (Lodi, Italy). The study was performed on a total of 192 weaned crossbreed Topigs piglets (average weight = 9.33 ± 1.90 kg). The trial started at the weaning of the piglets at 24 post-natal days and lasted 28 days in total. The weaning of the piglets was considered day 0 of the trial. The pigs were housed in two experimental rooms with 24 pens each and 4 pigs/pen. Each pen had plastic slatted floors and was fitted with an adjustable stainless-steel feeder and two nipple waterers. The rooms were lit by a combination of daylight through skylights and artificial light. The temperature, humidity, CO_2, and ammonium concentration of the air was automatically controlled. Ventilation was achieved by single, variable-speed fans linked to temperature sensors. The temperature inside the building was approximately 28 °C at the start of the trial and adjusted weekly until a final temperature of 24–25 °C was obtained. The relative humidity was controlled at 60–70%. The pigs had water and feed, provided in meal form, available *ad libitum*. All piglets were fed a standard commercial diet formulated to meet or exceed nutrient requirements for post-weaning piglets (NRC 2012) (Table 4).

Tab. 4 - Ingredients and chemical compositions of the prestarter (administrated from 0 to 14 study days) and starter (administrated from 15 to 28 study days) basal diets fed to post-weaning piglets during the trial (as-fed basis, %).

	Basal diets	
	Prestarter (0–14 study days)	Starter (15–28 study days)
Ingredients (%)		
Barley meal	20.71	20.00
Wheat meal	16.08	17.42
Sweet whey	10.00	5.00
Maize meal	8.32	22.00
Soycomil R	6.50	4.50
Flacked wheat	6.00	3.00
Dextrose	5.00	2.50
Flacked maize	5.00	3.50
Flacked barley	4.00	--
Soybean meal 48% CP	4.00	9.00
Wheat middlings	3.00	5.00
Herring meal	2.50	1.50
Plasma AP 820	2.50	0.50
Soybean oil	3.00	1.40
Cellulose	1.00	0.75
L-Lysine	0.55	0.45
Dicalcium phosphate	0.50	0.60
Calcium carbonate	0.40	0.60
L-Threonine	0.25	0.15
Vitamins+trace elements[1]	0.25	0.25
DL-Methionine	0.23	0.10
Salt	0.15	0.25
L-Tryptophan	0.06	0.03
Animal fat	--	1.50
Analysed composition		
DM, %	90.29	89.34
CP, %	18.27	16.63
EE, %	4.95	5.04
CF, %	2.94	3.17
Ash, %	4.47	4.34
Calcium, %	0.57	0.61
Phosphorus, %	0.55	0.53
Calculated composition		
DE, kcal/kg	3500	3420
NE, kcal/kg	2480	2440
Lysine, %	1.37	1.13
Methionine +Cystine, %	0.87	0.70
Threonine, %	0.96	0.78
Tryptophan, %	0.27	0.22

CP: crude protein; DM: dry matter; EE: ether extract; CF: crude fibre.
[1]Vitamin-mineral premix supplies per kg as fed: Vitamin A: 10 000 IU; Vitamin D3: 1.000 IU; Vitamin E: 50 mg; Vitamin B1: 1.0 mg; Vitamin B2: 3.0 mg; Vitamin B12: 0.02 mg; Vitamin B6: 3.0 mg; Pantothenic acid: 10 mg; Nicotinic acid: 15 mg; Biotin: 0.06 mg; Vitamin PP: 0.35 mg; Folic acid: 0.99 mg; Vitamin K3: 2 mg; Choline: 300 mg; Fe: 100 mg; Cu: 20 mg; Co: 0.75 mg; Zn: 100 mg; Mn: 10 mg; I: 0.75 mg; Se: 0.4 mg; Ethoxyquin: 150 mg.

The basal diet was divided into pre-starter (administered from 0 to 14 days of the trial) and starter (administered from 15 to 28 days of the trial). The piglets were randomly allocated to 1 of 3 dietary treatments with 16 replicate pens per treatment. The experimental unit was the pen. Dietary treatments were as follows: the basal diet (CTR), ANT diet (CTR + 400 mg/kg

amoxicillin), and C12-Ca diet (CTR + 1 g/kg C12-Ca). A C12-Ca soap (DRAX S) product containing 30mg/kg of zinc oxide (0.03 g/kg) by DC Practical Solution (Via Petrarca 4, 20123, Milan, Italy) was used. The C12-Ca soaps were created by saponification, combining 750 g/kg lauric acid and 100 g/kg calcium hydroxide.

3.3.2 Animal health and therapeutic treatments

The veterinary doctor responsible for animal welfare during the trial made the diagnoses of *Streptococcus suis* and diarrhea and decided upon the antibiotic therapy. The carcasses of the dead animals were sent to the Pathological Anatomy Laboratory of the University of Milan for necropsy analyses. Piglets eventually treated with antibiotics were excluded from the sample collection.

3.3.3 Measurements and samples

Pigs and feeders were weighed at 0, 14, and 28 days of the trial to determine their body weight (BW), average daily gain (ADG), average daily feed intake (ADFI), and feed efficiency (FE). Mortality and pathologies were recorded daily. Faecal samples were collected directly from the rectum of one piglet per replicate for each treatment (n=16) at weaning (0 day of the trial), at diet change (14 days of the trial), and at the end of the trial (28 days of the trial). Aliquots of faecal samples were immediately transported to the laboratory in a refrigerated container (4 °C) for microbial analysis, and other aliquots were stored at -20 °C for their immunomodulatory and anti-inflammatory parameters. At the end of the trial (28 days of the trial), six piglets from different cages were randomly chosen from each dietary treatment and slaughtered in accordance with current regulations at a local commercial slaughterhouse to collect the intestinal tissues and digesta content samples. The entire intestinal tracts were removed, and different sections of the duodenum, jejunum, ileum, and caecum were obtained from each animal. Approximately 1 cm segment each of the duodenum, jejunum, and ileum was promptly fixed in 10% paraformaldehyde in 0.01 M phosphate-buffered saline, pH 7.4, for 24 hours at 4 °C for histological measurements (n=6). Segments of 1 cm were also obtained from each tract of the small intestine to analyse the antioxidant parameters (n=6). Other sections from the duodenum, ileum, and caecum were emptied of their intestinal contents by squeezing them directly into 50 ml Falcon tubes, immediately frozen and stored at -80 °C to determine their fermentative (5 ml of intestinal content) parameters (n=6). The final set of sections from the duodenum, ileum, and caecum were refrigerated (4 °C) and transported to the laboratory to

analyse their microbial populations (1 g of intestinal content) (n=6).

3.3.4 Morphological analysis of the small intestine

Following 24 hours of fixation in 10% paraformaldehyde, the segments of the duodenum, jejunum, and ileum were dehydrated in a series of solutions with increasing alcohol content, immersed in a solution of xylene, and finally encased in paraffin. Paraffinic blocks were cut into 5 μm sections using a rotating microtome (Microm HM335E). The sections were collected, placed on slides, and then stained using haematoxylin-eosin for analysis. The slides were evaluated with a Nikon Eclipse E600 microscope equipped with a Ds-fi2 camera. The images of the sections were observed at 200x magnification and processed using the NIS-Elements software. The morphometry was quantified by measurements of the villi and crypts. A minimum of twenty well-oriented and intact villi and crypts were selected per image. The villus length was measured from the tip of the villus to the villus–crypt junction. Crypt depth was defined as the depth of the invagination between adjacent villi. The measurements were made with the ImageJ software.

3.3.5 Count of the microbial population in the intestinal contents and faeces

Bacterial counts (viable counts; log10 CFU/g) in the contents of the duodenum, ileum, caecum, and faeces were performed using the ring-plate technique (Michiels et al., 2010). Seven serial 10-fold dilutions were made from fresh digesta and faeces in a sterilized peptone solution (peptone, 1 g/L; agar, 0.4 g/L; NaCl, 8.5 g/L; and cysteine, 0.7 g/L) and plated onto selective media (Oxoid) to count the following bacterial groups: *Escherichia coli* (Tryptone bile x-glucuronide agar, followed by incubation for 72 hours at 30 °C under 90% N_2 and 10% CO_2), coliform bacteria (violet red bile lactose agar, followed by incubation for 24 hours at 37 °C under 90% N_2 and 10% CO_2), anaerobic sulphite reducer (iron sulphite agar, followed by incubation for 24–48 hours at 37 °C under 90% N_2 and 10% CO_2), Lactobacillus (De Man, Rogosa and Sharp agar, followed by incubation for 72 hours at 30 °C under 90% N_2 and 10% CO_2), Enterococcus and Streptococcus (Slanetz and Bartley agar, followed by incubation for 24 hours at 44 °C under 90% N_2 and 10% CO_2), Enterobacteriaceae (violet red bile glucose agar, followed by incubation for 24 hours at 37 °C under 90% N_2 and 10% CO_2), and total viable count (TVC) (plate count agar, followed by incubation for 72 hours at 30 °C).

3.3.6 Fermentative parameters in intestinal contents

Volatile fatty acids (VFAs) were determined using gas chromatography equipped with a capillary column according to the methods described by Erwin et al. (1961). Lactic acid concentrations were determined by the method described by Raeth-Knight et al. (2007). Briefly, 1 mL of 25% metaphosphoric acid was mixed with 5 mL of fresh digesta in a 15 mL centrifuge tube, and the mixture was frozen overnight. Samples were then thawed, neutralized with 0.4 mL of 25% NaOH, and vortexed. Thereafter, to condition the column, 0.64 mL of 0.3 M oxalic acid was added to the VFA and lactic acid standards and samples. After vortexing, the samples were centrifuged for 20 min at 3,000 × g and 4 °C, and 2 mL of supernatant was transferred into gas chromatography vials for analysis.

3.3.7 Pro- and anti-inflammatory cytokines and immunoglobulins in faeces

Pro- and anti-inflammatory cytokines, namely, Interleukin 6 (IL-6) and Interleukin 10 (IL-10), were evaluated in the faecal samples collected directly from the rectum of one piglet per pen for each treatment (n=16) at 0, 14, and 28 days of the trial. The analyses were performed by an enzyme immunoassay (Swine IL-6 ELISA Kit, Thermo Fisher; Swine IL-10 ELISA Kit, Thermo Fisher) following the manufacturer's instructions. Immunoglobulins IgG (specific for *Streptococcus suis*) and IgA were also analysed by ELISA kits (Porcine *Streptococcus suis* Antibody ELISA Test Kit, Krisghen Biosystems; Immunoglobulin A ELISA Kit, -R-Biopharm AG) according to the manufacturers' instructions. Only faeces were used to analyse the interleukins and immunoglobulins because blood sampling was considered a stressful procedure for the animals. Faecal sampling is a non-invasive technique that involves less stressful handling of the piglets.

3.3.8 Total antioxidant capacity (TAOC) and Malondialdehyde (MDA) in intestinal tissue

Frozen intestinal tissues samples (0.1 g) of the duodenum, ileum, and caecum were homogenised using a ultraturrex with cold physiological saline solution, maintained in ice, and centrifuged (3,000 × g for 15 min, 4 °C). Supernatants were then collected for analysis. Malondialdehyde (MDA) is one of most extensively studied end-products of polyunsaturated-lipid peroxidation under radical-induced oxidative stress conditions. The MDA concentration in the intestinal tissue was calculated via the acid extraction of homogenized tissue for 2 hours

under room temperature and protected from light. Subsequently, the derivatization of the extract to facilitate liquid chromatographic separation was carried out with 2.4-dinitrophenylhydrazine. After derivatization, MDA was measured via LC-MS/MS using an internal method (Laboratorio Vailati srl, Via S. Rocco, 25020 San Paolo, Brescia, Italy). The total antioxidant capacity (TAOC) activity was determined by commercial kits (ab65329, Abcam) following the manufacturer's instructions.

3.3.9 Statistical analysis

A completely randomized design was used. Growth performance was analysed using the Statistical Analysis System software (SAS version 9.4) applying a MIXED procedure for repeated measurements and accounting for the effects of treatment, time, and treatment × time interaction. The average daily gain (0–28 study days), average daily feed intake (0–28 study days), feed efficiency ratio (0–28 study days), fermentative parameters, fecal microbiology, IgA, IL-6, TAOC, and MDA, and intestinal morphology were analysed using a one-way analysis of variance (ANOVA) to compare the means of the three groups using the GLM procedure in SAS. For the statistical analysis of mortality and antibiotic treatment, Fisher's exact test was used. The pen represented the experimental unit for the growth performance parameters, while each pig represented the experimental unit for mortality and antibiotic treatment, fermentative parameters, fecal microbiology, IgA, IL-6, TAOC, and MDA. Intestinal morphology was analysed using a one-way analysis of variance (ANOVA) followed by a post hoc Tukey's test in SAS. All numerical data in the tables are presented as the least-square means (LSMeans) accompanied by the standard error of the mean (SEM) values. Differences between groups were considered statistically significant at $P<0.05$.

3.4 RESULTS

3.4.1 Animal performance and health

Table 2 summarizes the piglet growth performance data. No differences in BW, ADG, or ADFI were observed among groups. Conversely, the FE from 15 to 28 days was significantly ($P<0.05$) higher in the C12-Ca soap and ANT groups than in the CTR group. Considering the 0–28 day study period, the C12-Ca diet showed a significantly ($P<0.001$) higher FE compared to the CTR group but a lower FE compared to the ANT group ($P<0.001$).

Tab. 5 - Growth performance parameters of post-weaning piglets fed a basal diet alone (CTR) or supplemented with amoxicillin (ANT, 400 mg/kg) or lauric acid saponified with calcium (C12-Ca soaps, 1 g/kg) for 28 days[1].

	Dietary treatment				*p*-value		
Parameter	CTR	ANT	C12-Ca	SEM	Tr.	Tm.	Tr*Tm
No. pigs/treat	64	64	64				
BW (kg)							
Day 0	9.3	9.3	9.3	0.34	0.007	<0.001	0.198
Day 14	13.9	14.9	14.2				
Day 28	22.0	23.6	22.7				
ADG (g/d)							
Days 0-14	335	398	344	173.07	0.005	<0.001	0.456
Days 15-28	572	626	614				
Days 0-28	453	512	479	14.77	0.059		
ADFI (g/d)							
Days 0-14	510	542	535	309.12	0.474	<0.001	0.988
Days 15-28	964	1006	994				
Days 0-28	737	774	765	39.47	0.628		
FE							
Days 0-14	0.64[b]	0.73[a]	0.65[b]	0.01	0.001	<0.001	0.023
Days 15-28	0.57[a]	0.62[b]	0.62[b]				
Days 0-28	0.60[c]	0.66[a]	0.63[b]	0.01	0.001		

All results are presented as mean ± SEM. BW: body weight; ADG: average daily gain; ADFI: average daily feed intake; FE: feed efficiency; Tr: treatment; Tm: time; Tr*Tm: treatment*time
[1]Pigs 24 post-natal days of age at the beginning of the trial
[a,b]Within rows, different superscript letters indicate a significant difference

3.4.2 Mortality and antibiotic treatment

As shown in Table 3, during the trial, ten piglets died: 7 in the CTR group and 3 in the C12-Ca group. The ANT group suffered no mortality with a significant difference compared to the CTR group ($P<0.05$). Eight piglets were treated against *Streptococcus suis*—5 in the CTR group and 3 in the C12-Ca group—with individual injections of Amoxicillin, but there were no significant differences between groups. Notably, the three treated piglets in the C12-Ca group completely recovered. One piglet in the CTR group and nine in the ANT group were treated for diarrhoea with individual injections of Enrofloxacin, whereas no piglets in the C12-Ca group were affected by diarrhoea. Therefore, the diarrhoea cases were significantly ($P<0.05$) higher in the ANT group than in the C12-Ca and CTR groups.

Tab. 6 - Mortality and antibiotic treatment of post-weaning piglets fed a basal diet alone (CTR) or supplemented with amoxicillin (ANT, 400 mg/kg) or lauric acid saponified with calcium (C12-Ca soaps, 1 g/kg) for 28 days[1].

	Dietary treatment			*p*-value		
Parameter	CTR	ANT	C12-Ca	CTR vs ANT	CTR vs C12-Ca	ANT vs C12-Ca
No. Pigs/Treat.	64	64	64			
Dead	7[a]	0[b]	3[ab]	0.013	ns[3]	ns
Treated with antibiotics:						
Streptococcus suis	5	0	3	ns	ns	ns
Diarrhoea	1[b]	9[a]	0[b]	0.017	ns	0.003

Data are presented as Fisher test and chi-square tests results. ns: not significant
[1]Pigs 24 post-natal days of age at the beginning of the trial
[a,b]Within rows, different superscript letters indicate a significant difference

3.4.3 Fermentative parameters

Table 4 shows the data on acetic and lactic acid in the intestinal contents of the duodenum, ileum, and caecum. The C12-Ca group displayed the significantly (P<0.001) highest concentration of lactic acid in the small intestine and caecum. The acetic acid concentration in the C12-Ca and ANT groups significantly (P<0.001) decreased compared to the CTR group in the duodenum, ileum, and caecum. The propionic, butyric, and valeric acid results were under <0.1 g/100 g.

Tab. 7 - Acetic and lactic acid concentration in the duodenum, ileum, and caecum content in post-weaning piglets fed a basal diet alone (CTR) or supplemented with amoxicillin (ANT, 400 mg/kg) or lauric acid saponified with calcium (C12-Ca soaps, 1 g/kg) for 28 days[1].

	Dietary treatment				
Parameter	CTR	ANT	C12-Ca	SEM	*p*-value
No. Pigs/Treat.	6	6	6		
Acetic acid (mmol/kg)					
Duodenum	25.64[b]	21.65[a]	20.15[a]	0.005	<0.001
Ileum	42.96[a]	38.63[b]	36.97[b]	0.006	<0.001
Caecum	33.47[a]	29.47[b]	26.81[c]	0.007	<0.001
Lactic acid (mmol/kg)					
Duodenum	12.21[c]	14.10[b]	20.98[a]	0.006	<0.001
Ileum	22.87[c]	26.64[b]	33.41[a]	0.009	<0.001
Caecum	11.43[c]	12.54[b]	20.87[a]	0.007	<0.001

All results are presented as mean ± SEM
[1]Pigs 24 post-natal days of age at the beginning of the trial
[a,b,c]Within rows, different superscript letters indicate a significant difference

3.4.4 Intestinal content and faeces microbial populations

As shown in Table 5, C12-Ca soap treatment did not affect the microbial population in the faeces. Similarly, no significant differences in microbial populations were found between

groups in the duodenum, ileum, and caecum content (data not shown).

Tab. 8 - Faecal microbial populations (Log10 CFU/g) of the post-weaning piglets fed a basal diet alone (CTR) or supplemented with amoxicillin (ANT, 400 mg/kg) or lauric acid saponified with calcium (C12-Ca soaps, 1 g/kg) for 28 days[1].

	Dietary treatment				
Parameter	CTR	ANT	C12-Ca	SEM	*p*-value
No. Pigs/Treat.	16	16	16		
Faeces (Study days 0)					
Escherichia coli	3.4	4.2	4.1	0.22	ns
Coliforms	3.4	5.6	4.8	0.23	ns
Anaerobic sulphite reducers	4.4	5.8	5.6	0.14	ns
Lactobacillus	6.6	6.7	5.6	0.11	ns
Enterococcus/Streptococcus	3.1	5.2	5.0	0.18	ns
Enterobacteriaceae	4.1	4.5	3.7	0.15	ns
TVC	5.7	6.1	5.6	0.28	ns
Faeces (Study days 14)					
Escherichia coli	2.8	3.1	4.2	0.25	ns
Coliforms	2.1	3.6	4.1	0.23	ns
Anaerobic sulphite reducers	2.2	2.7	3.0	0.14	ns
Lactobacillus	6.1	6.1	7.4	0.22	ns
Enterococcus/Streptococcus	3.3	3.3	4.5	0.18	ns
Enterobacteriaceae	2.9	3.9	3.1	0.30	ns
TVC	6.8	5.3	5.4	0.43	ns
Faeces (Study days 28)					
Escherichia coli	2.1	3.1	5.0	0.14	ns
Coliforms	2.1	3.4	6.1	0.23	ns
Anaerobic sulphite reducers	2.5	3.2	3.0	0.14	ns
Lactobacillus	6.9	7.4	7.2	0.14	ns
Enterococcus/Streptococcus	3.6	3.1	4.6	0.18	ns
Enterobacteriaceae	2.4	2.7	3.3	0.25	ns
TVC	5.8	5.7	6.1	0.40	ns

All results are presented as mean ± SEM. TVC: total viable count; ns: not significant.
[1]Pigs 24 post-natal days of age at the beginning of the trial

3.4.5 Antioxidant and anti-inflammatory status

Regarding the IL-6, IL-10, IgA, and *Streptococcus suis* IgG results, no significant differences in IgA and IL-6 were found between the three groups. In particular, the average IgA content in faeces was 36.30±4.41 µg/g, 36.16±6.54 µg/g and 33.73±2.63 µg/g, while the average IL-6 concentration was 689.34±88.83 pg/g, 445.81±47.67 pg/g, and 294.96±32.15 pg/g at 0, 14, and 28 study days of the trial, respectively. The IL-10 values were below the limit of detection (<7.80pg/g). The quantitative analysis of IgG specific for *Streptococcus suis* in faeces was negative for all the samples analysed. In contrast, the results of the antioxidant parameters in the duodenum, ileum, and caecum tissue (Table 6) showed that the addition of C12-Ca soaps led to an improvement ($P<0.001$) in total antioxidant capacity of 13% and a reduction in malondialdehyde levels ($P<0.001$) of 17% in the duodenum and ileum compared to the CTR

and ANT groups. MDA was also reduced in the caecum in pigs fed C12-Ca and ANT compared to CTR (P<0.001).

Tab. 9 - Concentration of TAOC and MDA in the duodenum, ileum, and caecum tissue in post-weaning piglets fed a basal diet alone (CTR) or supplemented with amoxicillin (ANT, 400 mg/kg) or lauric acid saponified with calcium (C12-Ca soaps, 1 g/kg) for 28 days[1].

	Dietary treatment				
Parameter	CTR	ANT	C12-Ca	SEM	*p*-value
No. Pigs/Treat.	6	6	6		
MDA (nmol/mg)					
Duodenum	2.53[a]	2.08[b]	1.86[c]	0.10	<0.001
Ileum	2.51[a]	2.13[b]	1.87[c]	0.10	<0.001
Caecum	2.51[a]	2.10[b]	1.90[b]	0.10	<0.001
TAOC (nmol/µl TROLOX eq.)					
Duodenum	4.27[c]	4.58[b]	5.13[a]	0.14	<0.001
Ileum	5.98[b]	6.21[b]	6.93[a]	0.13	<0.001
Caecum	6.23[b]	6.59[ab]	7.15[a]	0.11	<0.001

All results are presented as mean ± SEM. MDA: malondialdehyde; TAOC: total antioxidant capacity.
[1]Pigs 24 post-natal days of age at the beginning of the trial
[a,b,c]Within rows, different superscript letters indicate a significant difference

3.4.6 Duodenum, jejunum, and ileum morphology

Table 7 shows the data for the morphology of the small intestine. No statistical differences were found in the duodenum for villus height (VH), villus width (VW), or crypt depth (CD). Only the villus height: crypt depth ratio (VH:CD) was significantly (P<0.01) higher in the CTR than the C12-Ca treatment group in the duodenum. In the jejunum, VH significantly (P<0.001) increased in the ANT group compared to the CTR and C12-Ca groups. VW was significantly (P<0.05) higher in the ANT group compared to the CTR group, whereas CD decreased (P<0.001) in the C12-Ca group compared to the CTR and ANT groups in the jejunum. In contrast, VH:CD increased (P<0.05) in the pigs supplemented with C12-Ca soap compared to the control group in the jejunum. However, C12-Ca soap ameliorated the villus morphology in the ileum, where VH and VW were significantly (P<0.001) higher in the animals fed C12-Ca soap compared to the CTR and ANT diets. Crypt depth increased in the CTR and C12-Ca groups compared to ANT, while the VH:CD ratio was greater in the ANT treatment group (P<0.001).

Tab. 10 - Intestinal morphology parameters of the duodenum, jejunum, and ileum of the post-weaning piglets fed a basal diet alone (CTR) or supplemented with amoxicillin (ANT, 400 mg/kg) or lauric acid saponified with calcium (C12-Ca soaps, 1 g/kg) for 28 days[1].

	Dietary treatment				
Parameter	CTR	ANT	C12-Ca	SEM	*p*-value
No. Pigs/Treat.	6	6	6		
Villus height (μm)					
Duodenum	233	226	211	10.39	ns
Jejunum	192[b]	225[a]	172[b]	7.86	<0.001
Ileum	172[b]	173[b]	211[a]	6.74	<0.001
Villus width (μm)					
Duodenum	125	128	105	7.35	ns
Jejunum	92[b]	117[a]	113[ab]	5.36	<0.05
Ileum	96[b]	100[b]	128[a]	5.04	<0.001
Crypt depth (μm)					
Duodenum	111	134	134	7.79	ns
Jejunum	147[a]	127[a]	99[b]	7.61	<0.001
Ileum	151[a]	78[b]	146[a]	6.19	<0.001
VH:CD ratio					
Duodenum	2.3[a]	2.0[ab]	1.7[b]	0.99	<0.01
Jejunum	1.6[b]	1.9[ab]	2.0[a]	0.13	<0.005
Ileum	1.3[b]	2.6[a]	1.5[b]	0.13	<0.001

All results are presented as mean ± SEM. VH:CD ratio: Villus height: crypt depth ratio; ns: not significant
[1]Pigs 24 post-natal days of age at the beginning of the trial
[a,b]Within rows, different superscript letters indicate a significant difference.

3.5 DISCUSSION

Weaning is one of the most stressful events in the lifecycle of piglets and results in reduced pig health, growth, and feed intake. In the past, antibiotics have been widely used to address piglet weaning problems, with the consequence of increasing antibiotic bacterial resistance. For decades, research has focused on developing nutritional strategies and/or alternative solutions with effects comparable to antibiotics (Jiang at al. 2015; Bissonnette et al. 2016; Jiang et al. 2017). Medium chain fatty acids are among these new solutions due to their ability to improve growth performance and prevent the negative effects associated with weaning in piglets (Hanczakowska et al., 2013).

MCFAs have specific nutritional and metabolic effects, including rapid digestion and passive absorption, making them particularly interesting for the nutrition of young animals (Baltić et al. 2017). MCFAs can be utilized directly by enterocytes as an energy source and thereby help support the integrity of the intestines in young piglets. Moreover, MCFAs are absorbed more efficiently by the gastrointestinal tract than other fatty acids. Pigs can partially absorb MCFAs even in their stomachs (Liu 2015). Medium chain triglyceride molecules are rapidly broken down by lipase in the stomach and duodenum in glycerol, and MCFAs are

absorbed via portal circulation and transported to the liver where they undergo fast oxidation (Li et al. 2015).

The finding that dietary supplementation with C12-Ca soaps did not result in significant differences in BW, ADG, and ADFI is comparable to the results obtained by Lopez-Colom et al. in 2019. The authors concluded that the administration of a mixture of sodium salt from FAs obtained from coconut oil distilled with lauric acid content did not influence growth performance. The FE results showed a better use of feed by the antibiotic-treated group from 0 to 14 days of the trial and over the whole duration of the experiment (0–28 study days). However, when the 15- to 28-day period of the trial is considered, the FE in pigs supplemented with C12-Ca soaps was comparable to that of the pigs receiving dietary antibiotics.

Dietary supplementation of lauric acid saponified with calcium in post-weaning piglet numerically reduced the therapeutic treatments against *Streptococcus suis*, a serious issue in pig farming (Fittipaldi et al. 2012). However, the differences were not significant between treatments. It is well known that diarrhoea is another major challenge for post-weaning piglets and results in increased morbidity and mortality and causes large economic losses in the global swine industry (Vondruskova et al. 2010; Yang et al. 2014). In our study, piglets supplemented with C12-Ca soap were not affected by diarrhoea in contrast with pigs given the antibiotic treatment. However, no significant difference was found between the controls for diarrhoea incidence.

Dietary MCFA supplementation also affects intestinal VFA concentrations by decreasing propionic, butyric, and valeric acid and increasing acetic acid concentrations in the small intestines of weanling piglets (Zentek et al. 2012; Lai et al. 2014). In our study, the piglets supplemented with C12-Ca soaps displayed a significantly higher concentration of lactic acid in the duodenum, ileum, and caecum. On the other hand, the acetic acid concentration significantly decreased in all intestinal segments analysed in the C12-Ca soap group compared to the CTR group. The acetic acid concentration in the caecum was also lower in the piglets fed a diet supplemented with C12-Ca soaps compared to the ANT group. These results are partially in agreement with the work of Lopez-Colom et al. (2019), who reported that, in the ileum, the major product of fermentation is lactic acid, while acetic acid is found only at very low levels; however, the differences were not significant. On the other hand, Zentek et al. (2012), determined that the administration of encapsulated medium chain fatty acids leads to a numerically higher acetic acid concentration in the small intestine, while lactic acid concentration is not significantly affected. The supplementation of C12-Ca soaps might result in the selection of certain favourable microbial flora, such as lactic acid bacteria, which would

produce more lactic acid and, therefore, improve the gut environment. The positive effects of lactic acid have been reported to produce a reduction in gastric pH and decrease the multiplication of unwanted microorganisms (Thompson and Lawrence 1981). Low pH also increases the digestibility of nutrients through changes in the gut morphology of the small intestine in young piglets (Suiryanrayna and Ramana 2015). However, the decrease in acetic acid concentration in the small intestine in our study did not operate in the same direction. Indeed, the increase in acetic acid concentration is positive for the gut bacterial ecosystem because it can be absorbed and used as an energy source by the intestinal cells and can decrease pH, thereby avoiding the proliferation of harmful bacteria, as well as lactic acid (Montagne et al. 2003; Roberfoid 2007;).

However, we did not find significant evidence in the bacterial count to determine a clear effect of C12-Ca soaps on intestinal microbiology and correlate those results with VFA concentration. Nevertheless, the literature contains numerous studies on the effect of FAs on the concentration of VFA in pigs, but it is difficult to compare these various experimental studies due to their use of different substances (pure acids or salts, individually or in combination) and also because of other factors, such as the intestinal segments chosen for analysis and the composition of the diet (Tugnoli et al. 2020).

The antibacterial activity of MCFA is well known; moreover, lauric acid seems to have the highest antibacterial activity among MCFAs (Kabara et al. 1972). However, the microbial populations in the faeces and intestinal contents of the piglets did not differ among the groups, which agrees with Zentek et al. (2013) (Zentek et al. 2013). In the literature, it is commonly reported that MCFAs are particularly effective against Gram-positive bacteria (Dayrit 2015). In particular, lauric acid has remarkable bactericidal action against Streptococci (Bergsson et al. 2001). Streptococcosis is a disease that causes significant economic losses in the pig industry (Fittipaldi et al. 2012). It is generally accepted that the main route of infection is through the respiratory system. *Streptococcus suis* is transferred from sows' vaginal secretions into the oral–nasal cavities of piglets during parturition and colonizes the tonsils immediately after birth. According to Segura et al. (2017), the gastrointestinal tract cannot be excluded as a secondary site of infection in piglets. After weaning, it was found that the relative intestinal concentrations of *Streptococcus suis* increased or remained constant (Su et al. 2008; Wijtten et al. 2011).

The present study did not show a significant reduction in mortality due to *Streptococcus suis* in all dietary treatments; however, the pigs of the C12-Ca group that showed disease, once adequately treated, quickly recovered, unlike the control group pigs that died.

Weaning stress causes growth reduction and gut dysfunction in piglets and may lead to intestinal inflammation and minor immune responses (Bomba et al. 2014; Bhat et al. 2015). However, the results of the anti-inflammatory parameters in faeces showed that supplementation with C12-Ca soaps did not result in significant differences compared to the other groups.

Oxidative stress is defined as an imbalance between the production of reactive oxygen species (ROS) and their elimination through antioxidative mechanisms (Yin et al. 2014; Castellani et al. 2014). Weaning pigs with an imbalance and an immature antioxidant system in their intestines are easily attacked by oxidative stress. In the present study, TAOC levels in the intestine, which reflect the non-enzymatic antioxidant defence system, were significantly higher in the animals treated with C12-Ca soaps. Furthermore, the levels of MDA (a marker of lipid peroxidation) were lower. This finding indicates that the integration of C12-Ca soaps played an important role in the prevention of the peroxidation and oxidation of endogenous lipids, thereby promoting the health of the intestinal barrier.

This result can also be considered as an indirect indicator of intestinal inflammatory status. Indeed, several human studies have shown that inflammation and oxidation are related (Mittal et al. 2014; Biswas 2016) and that both participate in the pathogenesis of many chronic diseases (Dierick et al. 2002; Cao et al. 2018). Our results show a positive intestinal oxidative state in the animals treated with C12-Ca soaps, which is supported by the increase in TAOC and the decrease in MDA. However, no statistical differences were found for the anti-inflammatory parameters between treatments.

The stressful events related to weaning cause changes in the gut morphology such that villus length is reduced, and the crypt depths are increased (Pluske et al., 1997). Dierick et al., (2004) reported that feeding MCFAs to weaning pigs positively influenced their intestinal morphology, resulting in a significant increase in villus length and a decrease in crypt depth in the small intestine. However, other authors found no evidence of differences in intestinal morphology after the dietary administration of MCFAs (Hanczakowska et al., 2016). In this study, some favourable results were found with the use of C12-Ca soaps in the pig diets, particularly in the jejunum and ileum. In the jejunum, the results obtained showed a lower CD in pigs fed the C12-Ca diet compared to both the CTR and ANT treatments. Moreover, the VH:CD ratio was higher in the C12-Ca pigs with an effect comparable to the ANT treatment. On the other hand, in the ileum, pigs fed the diet with C12-Ca soaps had increased VH and VW compared to the antibiotic and control diets, thereby modulating the intestinal absorption surface. However, CD decreased and VH:CD increased in the ileum in piglets fed the ANT diet compared to those fed

the C12-Ca soaps and the control treatments. There were no significant differences in duodenum morphology between the groups for WH, CD, and VW, but VH:CD decreased in the C12-Ca soap group compared to CTR.

Considering the results obtained, we hypothesized that C12-Ca may not dissociate in the stomach, meaning that lauric acid in the form of Ca-laurate cannot be absorbed in the proximal regions of the small intestine and can reach the distal regions of the small intestine (i.e., ileum) and perhaps even further (i.e., caecum). This hypothesis is also supported by the effects of C12-Ca on the concentrations of lactic and acetic acid in the caecum. However, the intestinal tract is difficult to reach by MCFAs due to their rapid absorption in the stomach and duodenum (Liu 2015; Ferrara et al. 2017).

3.6 CONCLUSION

Our results suggest that supplementing a basal diet with C12-Ca soaps (1 g/kg) may have beneficial effects on intestinal antioxidant status and some intestinal morphology parameters in the jejunum and ileum. The present contribution of C12-Ca on intestinal microbial populations, anti-inflammatory, and immunomodulatory properties are not conclusive and justify further investigation. Furthermore, the data obtained from this study do not support a clear-cut improvement in the growth performance and gut health of post-weaning piglets. However, this supplementation may represent a promising approach for reducing the use of prophylactic antibiotics.

3.7 REFERENCES

Baltić B, Starčević M, Đorđević J, Mrdović B, Marković R. 2017. Importance of medium chain fatty acids in animal nutrition. Int J Earth Sci. 85, 012048.

Bergsson G, Arnfinnsson J, Steingrimsson O, Thormar H. 2001. Killing of gram- positive cocci by fatty acids and monoglycerides. Acta Pathol Microbiol Immunol Scand. 109, 670-678.

Bhat AH, Dar KB, Anees S, Zargar MA, Masood A, Sofi MA, Ganie SA. 2015. Oxidative stress, mitochondrial dysfunction and neurodegenerative diseases; a mechanistic insight. Biomed Pharmacother. 74, 101–110.

Bissonnette N, Jiang XR, Matte JJ, Guay F, Talbot G, Bontempo V, Gong J, Wang Q, Lessard M. 2016. Effect of a post-weaning diet supplemented with functional feed additives on ileal transcriptome activity and serum cytokines in piglets challenged with lipopolysaccharide. Vet Immunol Immunopathol. 182, 136-149.

Biswas SK. 2016. Does the interdependence between oxidative stress and inflammation explain the antioxidant paradox? Oxid Med Cell Longev. 1-9.

Bomba L, Minuti A, Moisa SJ, Trevisi E, Eufemi E, Lizier M, Chegdani F, Lucchini F, Rzepus M, Prandini A. 2014. Gut response induced by weaning in piglet features marked changes in immune and inflammatory response. Funct Integr Genomics. 14, 657–671.

Cao ST, Hang CC, Wu H, Zhang QH, Jiao LF, Hu CH. 2018. Weaning disrupts intestinal antioxidant status, impairs intestinal barrier and mitochondrial function, and triggers mitophagy in piglets. J Anim Sci. 96, 1073- 1083.

Castellani P, Balza E, Rubartelli A. 2014. Inflammation, DAMPs, tumor development, and progression: a vicious circle orchestrated by redox signalling. Antioxid Redox Sign. 7, 1086–1097.

Czaplewski L, Bax R, Clokie M, Dawson M, Fairhead H, Fischetti VA, Rex JH, et al. 2016. Alternatives to antibiotics – a pipeline portfolio review. Lancet Infect Dis. 16, 239-251

Dayrit FM. 2015. The Properties of Lauric Acid and Their Significance in Coconut Oil. J Am Oil Chem Soc. 92, 1-15.

Desbois A, Smith V. 2010. Antibacterial free fatty acids: activities, mechanisms of action and biotechnological potential. Appl Microbiol Biotechnol. 85, 1629-1642.

Dierick N, Decuypere J, Molly K, Van Beek E, Vanderbeke E. 2002. The combined use of triacylglycerols (TAGs) containing medium chain fatty acids (MCFAs) and exogenous lipolytic enzymes as an alternative to nutritional antibiotics in piglet nutrition. II. In vivo release of MCFAs in gastric cannulated and slaughtered piglets by endogenous and

exogenous lipases: effects on the luminal gut flora and growth performance. Livest Prod Sci. 76, 1-16.

Dierick N, Michiels J, Van Nevel C. 2004. Effect of medium chain fatty acids and benzoic acid, as alternatives for antibiotics, on growth and some gut parameters in piglets. Commu Agr Appl Biol Sci. 69, 87-90.

Erwin E, Marco G, Emery E. 1961. Volatile fatty acid analyses of blood and rumen fluid by gas chromatography. J Dairy Sci. 44, 1768 – 1771.

Fittipaldi N, Segura M, Grenier D, Gottschalk M. 2012. Virulence factors involved in the pathogenesis of the infection caused by the swine pathogen and zoonotic agent Streptococcus suis. Future Microbiol. 7, 259-79.

Hanczakowska E, Szewczyk A, Swiatkiewicz M, Okon´ K. 2013. Short-and medium chain fatty acids as a feed supplement for weaning and nursery pigs. Pol J Vet Sci. 16, 647–654.

Hanczakowska E. 2017. The use of medium-chain fatty acids in piglet feeding – a review. J Anim Sci. 4, 967-977.

Jensen RG. 2002. The composition of bovine milk lipids: January 1995 to December 2000. J Dairy Sci. 85, 295–350.

Jiang XR, Agazzi A, Awati A, Vitari F, Bento H, Ferrari A, Alborali GL, Crestani M, Domeneghini C, Bontempo V. 2015. Influence of a blend of essential oils and enzyme combination on growth performance, microbial counts, ileum microscopic anatomy and the expression of inflammatory mediators in weaned piglets following an Escherichia Coli infection. An Feed Sci Technol. 209, 219-229.

Jiang XR, Li XL, Awati A, Bento H, Zhang HJ, Bontempo V. 2017. Effect of an essential oils blend on growth performance, and selected parameters of oxidative stress and antioxidant defence of Escherichia Coli challenged piglets. J Anim Feed Sci. 26, 38-43.

Kabara JJ, Swieczkowski DM, Conley AJ, Truant JP. 1972. Fatty acids and derivatives as antimicrobial agents. Antimicrob Agents Chemother. 2, 23–28.

Kuang Y, Wang Y, Zhang Y, Song Y, Zhang X, Lin Y, Che L, Xu S, Wu D, Xue B, Fang Z. 2015. Effects of dietary combinations of organic acids and medium chain fatty acids as a replacement of zinc oxide on growth, digestibility and immunity of weaned pigs. Anim Feed Sci Tech. 208, 145-57.

Lai WK, Yen HC, Lin CS, Chiang SH. 2014. The effects of dietary medium chain triacylglycerols on growth performance and intestinal microflora in young pigs. J Anim Feed Sci, 23, 331-336.

Li Y, Zhang H, Yang L, Wang T. 2015. Effect of medium-chain triglycerides on growth performance, nutrient digestibility, plasma metabolites and antioxidant capacity in weanling pigs. J Anim Nutr. 1, 12–18.

Lieberman S, Enig MG, Preuss HG. 2006. A Review of Monolaurin and Lauric Acid: Natural Virucidal and Bactericidal Agents. J Altern Complem Ther. 12, 310-314.

Liu Y. 2015. Fatty acids, inflammation and intestinal health in pigs. A review. J Anim Sci Biotechnol. 41, 1-9.

López-Colom P, Castillejos L, Rodríguez-Sorrento A, Puyalto M, Mallo JJ, Martin-Orue SM. 2019. Efficacy of medium-chain fatty acid salts distilled from coconut oil against two enteric pathogen challenges in weanling piglets. J Anim Sci Biotechnol. 10, 89

Michiels J, Missotten JAM, Van Hoorick A, Ovyn A, Fremaut D, De Smet S, Dierick NA. 2010. Effects of dose and formulation of carvacrol and thymol on bacteria and some functional traits of the gut in piglets after weaning. Arch Anim Nutr. 64, 136–154.

Mittal M,Siddiqui MR, Tran K, Reddy SP, Malik AB. 2014. Reactive oxygen species in inflammation and tissue injury. Antioxid Redox Sign. 7, 1126–1167.

Montagne L, Pluske JR, Hampson DJ. 2003. A review of interactions between dietary fibre and the intestinal mucosa, and their consequences on digestive health in young non ruminant animals. Anim Feed Sci Technol. 108, 95-117.

Pluske JR, Hampson DJ, Williams IH. 1997. Factors influencing the structure and function of the small intestine in the weaned pig—a review. Livest Prod Sci. 51, 215-236.

Roberfroid M. 2007. Prebiotics: The concept revisited. J Nutr. 137, 830S-837S.

Schumann K, Siekmann K. 2005. Soaps. In Ullmann's Encyclopedia of Industrial Chemistry, Wiley-VCH, Weinheim.

Segura M, Fittipaldi N, Calzas C, Gottschalk M. 2017. Critical Streptococcus suis virulence factors: are they all really critical? Trends Microbiol. 7, 585- 599.

Su Y, Yao W, Perez-Gutierrez ON. Smidt, H; Zhu, W.Y. 2008. Changes in abundance of Lactobacillus spp. and Streptococcus suis in the stomach, jejunum and ileum of piglets after weaning. FEMS Microbiol Ecol. 66, 546-55.

Suiryanrayna MV, Ramana JV. 2015. A review of the effects of dietary organic acids fed to swine. J Anim Sci Biotechnol. 21, 6-45.

Thompson JL, Lawrence TLJ. 1981. Dietary manipulation of gastric pH in the profilaxis of enteric disease in weaned pigs. Some field observations. Vet Rec. 109, 120-2.

Tugnoli B, Giovagnoni G, Piva A, Grilli E. 2020. From acidifiers to intestinal health enhancers: how organic acids can improve growth efficiency of pigs. Animals. 10, 134

Vondruskova H, Slamova R, Trckova M, Zraly Z, Pavlik I. 2010. Alternatives to antibiotic growth promoters in prevention of diarrhoea in weaned piglets: a review. Vet Med-Czech. 55, 199–224.

Wijtten PJA, van der Meulen J, Verstegen MWA. 2011. Intestinal barrier function and absorption in pigs after weaning: a review. Brit J Nutr. 105, 967–981.

Yang KM, Jiang ZY, Zheng CT, Wang L, Yang XF. 2014. Effect of Lactobacillus plantarum on diarrhea and intestinal barrier function of young piglets challenged with enterotoxigenic Escherichia coli K88. J Anim Sci. 92, 1496-1503.

Yin J, Wu MM, Xiao H, Ren WK, Duan JL, Yang G, Li TJ, Yin YL. 2014. Development of an antioxidant system after early weaning in piglets. J Anima Sci. 92, 612–619.

Yoon B, Jackman J, Valle-González E, Cho NJ. 2018. Antibacterial free fatty acids and monoglycerides: biological activities, experimental testing, and therapeutic applications. Int J Mol Sci. 19, 1114.

Zentek J, Buchheit-Renko S, Ferrara F, Vahjen W, Van Kessel AG, Pieper R. 2011. Nutritional and physiological role of medium-chain triglycerides and medium-chain fatty acids in piglets. Anim Health Res Revi. 12, 83–93.

Zentek J, Buchheit-Renko S, Männer K, Pieper R, Vahjen W. 2012. Intestinal concentrations of free and encapsulated dietary medium-chain fatty acids and effects on gastric microbial ecology and bacterial metabolic products in the digestive tract of piglets. Arch Anim Nutr. 66, 14–26.

Zentek J, Ferrara F, Pieper R, Tedin L, Meyer W, Vajen W. 2013. Effects of dietary combinations of organic acids and medium chain fatty acids on the gastrointestinal microbial ecology and bacterial metabolites in the digestive tract of weaning piglets. J Anim Sci. 91, 3200–3210.

CHAPTER 4 –

Specific blend of esterified fatty acids improves zootechnical and gut health parameters of weaned piglets

4.1 ABSTRACT

Short- (SCFA) and medium-chain fatty acids (MCFA) have been extensively studied due to their supporting effect on piglet's growth and gut health. In this study, 192 weaned Topigs piglets (24 post-natal days, average weight 8.41 ± 1.90 kg) were used to investigate the dietary effects of a mixture of esterified Tributyrin (TB) and Monolaurin (ML) on growth performance and gut health. Piglets were randomly allocated to one of three dietary treatments with 16 replicates per treatment (4 piglets/replicate), as follows: the basal diet with no additive (CTR), the basal diet plus amoxicillin (ANT) and the basal diet plus a mixture of Tributyrin and Monolaurin (TB+ML). The feeding scheme was divided in a prestarter (0-14 days of trial) and starter phase (15-42 days of trial). ANT diet was only administered during the prestarter phase. Body weight (BW), average daily gain (ADG), and average daily feed intake (ADFI) were measured, and feed efficiency (FE) was calculated. Intestinal tissue samples of duodenum, jejunum and ileum were used to investigate the potential impact of TB+ML on gut morphology and to evaluate the total antioxidant capacity (TAOC) and Malondialdehyde (MDA). Similarly, IL-10, IL-6 and SIgA analysis were performed on intestinal tissues. BW, ADFI and ADG increased in TB+ML compared to CTR and ANT ($P<0.05$). On the contrary, the supplementation with TB+ML did not significantly affect FE. Morphometric analysis indicated that the supplementation with TB+ML reduced the villi length in duodenum, while the villi width decreased in duodenum but increased in jejunum and ileum ($P<0.05$). However, in the ileum the villus length increased ($P<0.05$). Dietary supplementation with TB+ML decreased LFs area compared to CTR ($P<0.05$). Moreover, TB+ML increased muscularis external thickness in all of its layers ($P<0.05$). TAOC was significantly higher in TB+ML ($P<0.05$) in all the intestinal tract, whereas no significant effect on IL-10, IL-6, SIgA and MDA was observed. In conclusion, TB+ML has a positive effect on the porcine gut health, protecting the intestinal epithelium from oxidative stress caused by weaning. Moreover, TB+ML enhanced piglet's growth performance, supported by the increase of the ileal villi length, which enlarges the nutrient absorption surface, and muscolaris externa thickness promoting intestinal peristalsis.

Keywords: Tributyrin, Monolaurin, esterification, piglets, post-weaning, gut health

4.2 INTRODUCTION

In recent years, there is a wide interest in finding feeding strategies to stimulate gut development and health in young pigs. The ultimate goal of these strategies is to improve pig productivity around weaning, while minimizing the use of antibiotics (De Lange *et al*., 2010). The effects of various feed additives have been extensively studied after the prohibition on the use of antibiotics as growth promoters, due the onset antibiotics-induced bacterial resistance and cross-contamination to humans (Heo et al., 2013; Czaplewski et al., 2016). Finding an individual alternative that embodies all the functions of classic antibiotics and, at the same time improves gut health and pig's growth, is not easy. One potential solution is to select multiple products which work synergistically (Allen et al., 2013).

The antibacterial effect and gut health supporting role of short- (SCFA) and medium chain fatty acids (MCFA) have been extensively studied due to their numerous positive effects on pigs that can be seen when the health of the animals is endangered, especially at weaning. SCFAs have been shown to play an important role on improving gut health and limiting intestinal inflammation in pigs (Liu, 2015). The beneficial effect of SCFA is closely correlated with increasing proliferation and decreasing apoptosis of enterocytes. In fact, SCFA can act as a major source of energy for intestinal epithelium, stimulating cell proliferation and the growth of the small intestine. Similarly, MCFAs have specific nutritional and metabolic effects, including rapid digestion, passive absorption and obligatory oxidation. MCFAs can be utilized directly by the enterocytes for energy production and thereby help to support the integrity of the intestine (Liu, 2015).

The therapeutic effects of SCFA and MCFA also include antibacterial, antimicrobial and bacteriostatic properties which may improve intestinal microecology, increase the energy and nutrient digestibility and thus improve pig's performance (Hanczakowska et al. 2013; Kuang at al., 2015). The exact mechanism is not known yet, but it is considered that SCFA and MCFA together have a synergistic effect on bacteria. Research suggests that MCFA are able to penetrate the microorganism's cell walls, due to their specific amphipathic chemical structure, which damages the bacterial cell membrane, solubilizing the lipids and phospholipids in the envelope of the pathogen leading to leakage of intracellular material and thus allowing SCFA access into the bacterial cytoplasm. Once inside the microbial cell, SCFA dissociates in the more alkaline environment of the cytoplasm, resulting in a decrease of bacterial intracellular pH. The change in pH influences the cellular metabolism, interrupting or modifying the vital processes of the bacterial cell limiting its growth or even causing death (Batovska et al., 2009;

Desbois and Smith, 2010; Kim and Rhee, 2013). Thus, the use of SCFA together with MCFA extends their antimicrobial spectrum (Kim and Rhee, 2013). However, the effectiveness of fatty acids (FA) may be limited due to prompt absorption and metabolism in the stomach and in the duodenum, which limits the amount that reaches the lower gut and therefore, their ability to modulate intestinal structure, flora and the immunity state.

The effectiveness of SCFAs and MCFAs could be enhanced by protecting the active compound by esterification with glycerol to dissolve them slowly as they pass along the GIT (Decuypere and Dierick, 2003). TB and ML are triglyceride and monoglyceride of butyric acid and lauric acid, respectively. They are made up of glycerol molecules plus the FA. This form protects butyric and lauric acid from the total absorption in the stomach and duodenum and allow them to be released along the entire GIT. The free form of the two FAs is released from the glycerol through the action of lipase and then, butyric and lauric acid could affect the intestinal epithelium development, the immune response and they can perform their bactericidal effect in pigs' gut (Mallo et al., 2012). In particular, TB exerts a major action on intestinal morphology, improves growth performance during the 3-4 weeks after weaning and mitigates adverse changes in the intestinal structure associated with weaning stress. Moreover, dietary supplementation with TB alleviated intestinal injury by inhibiting apoptosis, promoting tight junction formation and activating epidermal growth factor receptor signaling in piglets (Wang et al., 2019). Its action was studied in a work of Hou *et al*. (2006) who tested how the administration of TB has improved growth performance, small intestine morphology and enzymatic activity in post weaning piglets. Furthermore, in a more recent study, Hou *et al*. (2014) argued that dietary supplementation with TB alleviated intestinal damage in piglets undergoing intra-rectal administration of acetic acid, reducing ulcerative colitis. Instead, ML is the MCFA with the highest antibacterial capacity as assessed in literature. In fact, ML seems to have a predominantly antibacterial effect, particularly towards the Gram-positive (Bergsson et al., 2001; Lieberman et al., 2006). It is widely recognized that monoglycerides are more effective than their FAs: ML is more biologically active than lauric acid in killing bacteria (Kabara et al., 1972; Batovska et al., 2009; Zhang et al., 2009). Moreover, medium chain monoglycerides are reported to be active in the entire GIT and the blood stream and pathogens cannot develop resistance against them (Antongiovanni et al., 2006).

Therefore, the choice to use tributyrin (TB) and monolaurin (ML) together, arises from the need to deepen the synergistic effects that they may have on weaning piglet's growth and gastrointestinal functions and health.

4.3 MATERIAL AND METHODS

The experimental protocol was reviewed and approved by the Animal Welfare Committee of the Università degli Studi di Milano (application No. OPBA_148_2017).

4.3.1 Animal, housing and management

The experiment was performed at the Animal Production Research and Teaching Centre of Università degli Studi di Milano (Lodi, Italy). The study was performed on a total of 192 crossbreed Topigs weaned piglets (average weight of 8.41±1.90 kg). The trial started at 24 days of piglet age (day 0 of trial) and lasted until 70 days of age (41 days of trial). The pigs were housed in two experimental rooms with 24 pens each and 4pigs/pen. Each pen had plastic slatted floors and was fitted with an adjustable stainless-steel feeder and two nipple waterers. The rooms were lit by a combination of daylight (through skylights) and artificial light (non-programmable). The temperature, humidity, CO_2 and ammonium concentration of the air was automatically controlled. Ventilation was achieved by single, variable-speed fans linked to temperature sensors. The temperature inside the building was approximately 28 ºC at the start of the trial and adjusted weekly until a final temperature of 24-25 ºC was obtained. The relative humidity was controlled at 60–70%. The pigs had water and feed, provided in meal form, available *ad libitum*. The piglets were obtained from sows of a single weaning. A randomization process was used to allocate piglets to replicates, such that each treatment consists of an equal number of homogeneous replicates. The veterinary doctor responsible for animal welfare during the trial, checked the piglets when they were put on trial and only healthy piglets were allowed to the trial. No prophylactic antibiotic treatment was given. The carcasses of the dead animals were sent to the Pathological Anatomy Laboratory of the University of Milan for necropsy analyses.

4.3.2 Experimental design and Diet

The piglets were randomly allocated to 1 of 3 dietary treatments with 16 replicate pens per treatment and 4 piglets per replicates. The experimental unit was the pen. The basal diet was a typical commercial diet, divided in Pre-starter (administered from 0 to 14 days of trial) and Started (administered from 15 to 41 days of trial) and was based on barley meal, wheat meal and maize meal. It was formulated to exceed the crude protein (CP) requirements recommended for pigs (19% CP pre-starter, 18% CP starter). The composition and the calculated analysis of the diets according to NRC (2012) are presented in Table 11. The feeds were manufactured before the start of the trial, to allow time for nutritional homogeneity and analysis and eventual second delivery in case of non-conformity. Dietary treatments were as follow: the basal diet (CTR), ANT diet (CTR + 400 mg/kg Amoxicillin) and TB+ML diet (CTR + 5 kg/ton butyric and monolaurin). Amoxicillin was administered only for the first two weeks of trial, as under normal farming conditions.

Tab. 11 - Ingredients and chemical composition of pre-starter (administrated from 0 to 14 study days) and starter (administrated from 15 to 41 study days) basal diets fed to post-weaning piglets during the trial (as-fed basis, %).

	Basal diets	
Ingredients (%)	Prestarter (0-14 days)	Starter (15-41 days)
Barley meal	20.71	22.00
Wheat meal	17.08	20.42
Sweet whey	10.00	5.00
Maize meal	5.32	12.00
Soycomil R	8.50	7.00
Flacked wheat	7.50	5.00
Dextrose	5.00	2.50
Flacked maize	5.00	3.50
Flacked barley	4.00	--
Soybean meal 48% CP	5.00	10.00
Wheat middlings	3.00	5.00
Herring meal	2.50	1.50
Soybean oil	3.00	1.40
Cellulose	1.00	0.75
L-Lysine	0.55	0.45
Dicalcium phosphate	0.50	0.60
Calcium carbonate	0.40	0.60
L-Threonine	0.25	0.15
Vitamins+trace elements[1]	0.25	0.25
DL-Methionine	0.23	0.10
Salt	0.15	0.25
L-Tryptophan	0.06	0.03
Animal fat	--	1.50
Analysed composition (%)		
Dry matter	90.29	89.34
Crude protein	19.50	18.40
Crude fat	4.95	5.04
Crude fibre	3.05	3.30
Ash	4.47	4.34
Calcium	0.57	0.61
Phosphorus	0.55	0.53
Calculated composition		
DE, kcal/kg	3490	3430
NE, kcal/kg	2460	2410
Lysine	1.35	1.20
Methionine +Cystine	0.87	0.70
Threonine	0.96	0.78
Tryptophan	0.27	0.22

[1]Vitamin-mineral premix supplies per kg as fed: Vitamin A: 10.000 IU; Vitamin D3: 1.000 IU; Vitamin E: 50 mg; Vitamin B1: 1.0 mg; Vitamin B2: 3.0 mg; Vitamin B12: 0.02 mg; Vitamin B6: 3.0 mg; Pantothenic acid: 10 mg; Nicotinic acid: 15 mg; Biotin: 0.06 mg; Vitamin PP: 0.35 mg; Folic acid: 0.99 mg; Vitamin K3: 2 mg; Choline: 300 mg; Fe: 100 mg; Cu: 20 mg; Co: 0.75 mg; Zn: 100 mg; Mn: 10 mg; I: 0.75 mg; Se: 0.4 mg; Ethoxyquin: 150 mg.

4.3.3 Measurements and Samples

Pigs and feeders were weighed at 0, 14, 28 and 41 days of trial to determinate their body weight (BW), average daily gain (ADG), average daily feed intake (ADFI) and feed efficiency (FE). Mortality and pathologies were recorded daily. At the end of the trial (41 days of the trial), ten piglets from different cages were randomly chosen from each dietary treatment and slaughtered

in accordance with current regulation at a local commercial slaughterhouse to collect intestinal tissue samples. The entire intestinal tracts were removed, and different sections of duodenum, jejunum and ileum were obtained for each animal. Approximately 2 cm segment each of duodenum, jejunum and ileum was promptly fixed in 10% paraformaldehyde in 0.01 M phosphate buffered saline (PBS) pH 7.4 for 24 hours at 4◦C for histological measurements (n=10). Segments of 2 cm were also obtained from each tract of the small intestine to analyse the antioxidant and inflammatory parameters (n=10).

4.3.4 Morphological analysis of duodenum, jejunum and ileum

Following 24 h fixed in 10% paraformaldehyde, the segments of duodenum, jejunum and ileum were dehydrated in a series of solutions of increasing alcohol content, immersed in a solution of xylene and finally included in paraffin. Paraffinic blocks were cut into 5 µm sections, with rotating microtome (Microm HM335E). The sections were collected and placed on slides, then stained using hematoxylin-eosin for analyses. The slides obtained were evaluated with a Nikon Eclipse E600 microscope equipped with a Ds-fi2 camera. The images of the sections were observed with 200x magnification and processed using the NIS-Elements software. The morphometry was quantified by measurements of the villi and crypts. A minimum of twenty well-oriented and intact villi and crypts were selected per image. The villus length was measured from the tip of the villus to the villus–crypt junction. Crypt depth was defined as the depth of the invagination between adjacent villi. The measurements were made with the ImageJ software. The lymphatic follicles area (LFs) and the thickness of *muscularis externa* of ileum were also determined. For this purpose, the LFs were divided into their defined compartments: the cortical region, the germinal center, the dome region, according to Makala et al. (2000). Such compartment areas were measured (μm^2) in five LFs area in randomly selected field of each tissue sample. Measurements of LFs were made for each animal for each intestinal section (one slide per animal, per section). Each animal had about 4 fields on each slide, and 5 random follicles were measured in each field. Both lymphatic follicles area and thickness of *muscularis externa* were quantified using ImageJ software.

4.3.5 Pro- and anti-inflammatory cytokines and immunoglobulins in intestinal tissues

Pro- and anti-inflammatory cytokines, namely, Interleukin 6 (IL-6) and Interleukin 10 (IL-10), were evaluated in intestinal tissues of duodenum, jejunum and ileum. The analyses were

performed by an enzyme immunoassay (Swine IL-6 ELISA Kit Thermofisher; Swine IL-10 ELISA Kit Thermofisher), following manufacturer's instructions. Secretory immunoglobulins A (SIgA) were also determined by ELISA kit (Immunoglobulin A ELISA Kit -R-Biopharm AG), following manufacturer's instructions.

4.3.6 Total antioxidant capacity (TAOC) and Malondialdehyde (MDA) in intestinal tissues

Frozen intestinal tissue samples (0.1 g) of duodenum, jejunum and ileum were homogenised using a untraturrex with cold physiological saline solution, maintained in ice, and centrifuged (3,000 × *g* for 15 min, 4°C). Supernatants were then collected for analysis. Malondialdehyde (MDA) is one of most extensively studied end-products of polyunsaturated-lipid peroxidation under radical-induced oxidative stress conditions. The MDA concentration in the intestinal tissue was calculated via the acid extraction of homogenized tissue for 2 hours under room temperature and protected from light. Subsequently, the derivatization of the extract to facilitate liquid chromatographic separation was carried out with 2.4-dinitrophenylhydrazine. After derivatization, MDA was measured via LC-MS/MS using an internal method (Laboratorio Vailati srl, Via S. Rocco, 25020 San Paolo, Brescia, Italy). The total antioxidant capacity (TAOC) activity was determined by commercial kits (ab65329, Abcam) following the manufacturer's instructions.

4.3.7 Statistical analysis

A completely randomized design was used. Growth performance was analysed using the Statistical Analysis System software (SAS version 9.4; SAS Institute Inc., Cary, NC, USA) applying a MIXED procedure for repeated measurements and accounting for the effects of treatment, time, and treatment × time interaction. The average daily gain (0–41 study days), average daily feed intake (0–41 study days), feed efficiency ratio (0–41 study days), SIgA, IL-10, IL-6, TAOC, and MDA, and intestinal morphology were analysed using a one-way analysis of variance (ANOVA) to compare the means of the three groups using the GLM procedure in SAS. For the statistical analysis of mortality and antibiotic treatment, Fisher's exact test was used. The pen represented the experimental unit for the growth performance parameters, while

each pig represented the experimental unit for mortality and antibiotic treatment, SIgA, IL-10, IL-6, TAOC, and MDA. Intestinal morphology was analysed using a one-way analysis of variance (ANOVA) followed by a post hoc Tukey's test in SAS. All numerical data in the tables are presented as the least-square means (LSMeans) accompanied by the standard error of the mean (SEM) values. Differences between groups were considered statistically significant at $P<0.05$.

4.4 RESULT

4.4.1 Growth performance

Table 2 summarizes the piglet growth performance. As for BW, the animals belonging to the group fed with TB+ML showed higher BW than those of CTR group ($P<0.05$) at 28 days of trial, whereas, at the end of trial (41 days of trial), the supplementation with TB+ML showed higher BW compared to both CTR and ANT groups ($P<0.05$). ADG was higher in animals of TB+ML group in the period from 15 to 28 days of trial, as well as from 29 to 41 days of trial and for the whole duration of the trial compared to CTR and ANT groups ($P<0.05$). ADFI was higher in piglets of TB+ML group compared to CTR ($P <0.05$) in the periods from 15 to 28 days, from 29 to 41 days and throughout all duration of the trial. No differences in FE were observed among groups. In general, health conditions were good throughout the whole process. No episodes of diarrhea occurred.

Tab. 12 - Growth performance parameters of post-weaning piglets fed a basal diet alone (CTR) or supplemented with amoxicillin (ANT, 400 mg/kg) or tributyrin and monolaurin (TB+ML, 5 kg/ton) for 41 days[1].

	Dietary treatment				*p*-value		
Item	CTR	ANT	C12-Ca	SEM	Tr.	Tm.	Tr*Tm
No. pigs/treat	64	64	64				
BW (g)							
Day 0	8408	8418	8416	439.32	0.0004	<0.0001	0.051
Day 14	12.21	12.36	12.63				
Day 28	17.68[b]	18.47[ab]	19.47[a]				
Days 41	25.05[b]	26.04[b]	27.76[a]				
ADG (g/d)							
Days 0-14	271.93	286.67	299.52	15.78	<0.0001	<0.001	0.245
Days 15-28	397.26[b]	432.22[b]	492.41[a]				
Days 29-41	574.58[b]	582.32[b]	637.95[a]				
Days 0-41	405.30[b]	428.10[b]	470.70[a]	14.74	0.001		
ADFI (g/d)							
Days 0-14	396.88	409.69	428.45	28.52	0.016	<0.0001	0.800
Days 15-28	747.12[b]	787.56[ab]	834.14[a]				
Days 29-41	1107.09[b]	1152.99[ab]	1207.68[a]				
Days 0-41	741.66[b]	774.83[ab]	814.06[a]	32.34	0.092		
FE							
Days 0-14	0.67	0.68	0.64	0.02	0.897	<0.0001	0.487
Days 15-28	0.52	0.53	0.56				
Days 29-41	0.50	0.50	0.51				
Days 0-41	0.51	0.52	0.55	0.02	0.351		

All results are presented as mean ± SEM. BW: body weight; ADG: average daily gain; ADFI: average daily feed intake; FE: feed efficiency; Tr: treatment; Tm: time; Tr*Tm: treatment*time
[1]Pigs of 24 post-natal days of age at the beginning of the trial
[a,b] Within rows, different superscript letters indicate a significant difference

4.4.2 Duodenum, jejunum and ileum morphometry

Table 3 presents the data for morphology of duodenum, jejunum and ileum. Piglets receiving the diet supplemented with TB+ML had shorter villi (P<0.05) and smaller width (P<0.01) in the duodenum compared to piglets in the CTR and ANT groups. Jejunum morphology shows bigger villus width (P<0.05) in the group supplemented with ANT and TB+ML compared to CTR group. In the ileum, dietary supplementation with TB+ML led to higher villus length (P<0.01) and lower villus width (P<0.05) as well as in ANT group compared to CTR. The crypts depth was lower (P<0.05) in piglets supplemented with ANT compared to CTR and TB+ML groups.

Tab. 13 - Intestinal morphology parameters of duodenum, jejunum and ileum of post- weaning piglets fed a basal diet alone (CTR) or supplemented with amoxicillin (ANT, 400 mg/kg) or Tributyrin and Monolaurin (TB+ML, 5 kg/ton) for 41 days[1].

	Treatment				
Item	CTR	ANT	TB+ML	SEM	P-value
No. Pigs/Treat.	10	10	10		
Duodenum					
Villus height, µm	275.65[a]	263.50[a]	230.62[b]	10.11	0.01
Villus width, µm	108.43[a]	104.40[a]	93.70[b]	6.50	0.0007
Crypt depth, µm	115.49	116.23	119.93	7.40	ns
VH:CD ratio	2.86	2.71	2.33	0.17	ns
Jejunum					
Villus height, µm	209.64	209.65	192.40	9.05	ns
Villus width, µm	90.36[b]	104.9[a]	97.41[a]	5.60	0.019
Crypt depth, µm	120.5	121.32	109.77	7.50	ns
VH:CD ratio	1.96	2.06	2.05	0.14	ns
Ileum					
Villus height, µm	161.23[b]	179.54[a]	183.93[a]	5.31	0.004
Villus width, µm	122.00[a]	110.70[b]	107.50[b]	5.06	0.05
Crypt depth, µm	102.79[a]	83.06[b]	115.93[a]	7.55	0.05
VH:CD ratio	1.49	1.60	1.40	0.08	ns

All results are presented as mean ± SEM. VH:CD ratio: Villus height: crypt depth ratio; ns: not significant
[1]Pigs 24 post-natal days of age at the beginning of the trial.
[a,b]Within rows, different superscript letters indicate a significant difference.

4.4.3 Antioxidant and ant-inflammatory status

Il-6, IL-10 and SIgA results are presented in Table 4. No significant differences in SIgA, IL-10 and IL-6 were found between the three groups.

Tab. 14 - Concentration of SIgA, IL-10 and IL-6 in duodenum, jejunum and ileum tissue in post-weaning piglets fed a basal diet alone (CTR) or supplemented with amoxicillin (ANT, 400 mg/kg) or Tributyrin and Monolaurin (TB+ML, 5 kg/ton) for 41 days[1].

	Treatment				
Item	CTR	ANT	TB+ML	SEM	*P*- value
No. Pigs/Treat	10	10	10		
Duodenum					
SIgA (µg/g)	286.50	299.28	339.71	19.35	ns
IL-10 (pg/g)	11.78	10.35	9.85	2.76	ns
IL-6 (pg/g)	43.38	51.91	63.57	16.66	ns
Jejunum					
SIgA (µg/g)	330.40	297.80	343.41	52.50	ns
IL-10 (pg/g)	9.56	10.34	34.43	23.96	ns
IL-6 (pg/g)	115.77	91.23	53.73	73.69	ns
Ileum					
SIgA (µg/g)	295.02	303.54	295.02	16.12	ns
IL-10 (pg/g)	17.54	14.53	16.64	3.25	ns
IL-6 (pg/g)	69.36	65.23	50.26	24.27	ns

All results are presented as mean ± SEM. SIgA: secretory IgA; IL-10: interleukin 10; il-6: interleukin 10; ns: not significant. [1]Pigs 24 post-natal days of age at the beginning of the trial. [a,b,c]Within rows, different superscript letters indicate a significant difference

Similarly, the results of antioxidant parameters on duodenum, jejunum and ileum (Table 5) show no significant differences in MDA between the groups. On the other hand, animals in TB+ML group have significantly higher TAOC in all the intestinal districts analysed than animals in the CTR and ANT groups (P<0.01).

Tab. 15 - Histometric analyses related to lymphatic follicle and muscular external measurement in ileum of post-weaning piglets fed a basal diet alone (CTR) or supplemented with amoxicillin (ANT, 400 mg/kg) or lauric acid saponified with calcium (C12-Ca soaps, 1 g/kg) for 28 days[1].

	Treatment				
Item	CTR	ANT	TB+ML	SEM	*P*- value
No. Pigs/Treat.	10	10	10		
Ileum					
Lymphatic follicle area (μm^2)	536.45^a	438.35^b	387^b	77.91	0.003
Muscularis externa width (μm)	333.59^b	314.34^b	434.47^a	35.66	<0.001
Muscularis externa inner circular layer (μm)	158.91^{ab}	130.69^b	167.34^a	19.18	0.02
Muscularis externa outer longitudinal layer width (μm)	170.72^b	175^b	238.83^a	21.30	0.001

All results are presented as mean ± SEM
[1]Pigs 24 post-natal days of age at the beginning of the trial
[a,b]Within rows, different superscript letters indicate a significant difference.

4.4.4 LFs area and muscularis externa measurements in ileum

Data for LFs and muscularis externa measurements results are present in Table (6). Dietary supplementation with TB+ML and ANT decreased LFs area compared to CTR (P<0.05). On the contrary, TB+ML increased muscularis external thickness in general and of its outer longitudinal layer compared to both CTR and ANT group (P<0.05). Regarding the thickness of muscularis externa inner circular layer, the supplementation with TB+ML increased the thickness of the inner layer compared with ANT (P<0.05) but not difference was found with CTR group.

Tab. 16 - Concentration of TAOC and MDA in duodenum, jejunum and ileum tissue in post-weaning piglets fed a basal diet alone (CTR) or supplemented with amoxicillin (ANT, 400 mg/kg) or Tributyrin and Monolaurin (TB+ML, 5 kg/ton) for 41 days[1].

	Treatment				
Item	CTR	ANT	TB+ML	SEM	*P*- value
No. Pigs/Treat.	10	10	10		
Duodenum tissue					
MDA (nmol/mg)	10.60	10.30	8.00	0.94	ns
TAOC (nmol/µl TROLOX eq.)	3.96[c]	4.78[b]	5.95[a]	0.12	<0.001
Jejunum tissue					
MDA (nmol/mg)	10.60	9.20	6.30	2.10	ns
TAOC (nmol/µl TROLOX eq.)	3.52[c]	4.65[b]	5.35[a]	0.10	<0.001
Ileum tissue					
MDA (nmol/mg)	14.20	13.00	12.00	1.84	ns
TAOC (nmol/µl TROLOX eq.)	3.54[c]	4.19[b]	4.60[a]	0.15	<0.001

All results are presented as mean ± SEM. MDA: malondialdehyde; TAOC: total antioxidant capacity.
[1]Pigs 24 post-natal days of age at the beginning of the trial
[a,b,c]Within rows, different superscript letters indicate a significant difference

4.4.5 Mortality and antibiotic treatment

As shown in Table 7, during the trial, seven piglets died: 2 in the CTR group, 3 in the ANT group and 2 in the TB+ML group. Anatomo-pathological examination has diagnosed that the cause has always been the infection with *Streptococcus suis* but there were no significant differences between groups. One piglet was treated against *Streptococcus suis* in TB+ML group with two injections of 1 ml of amoxicillin, one after 48 hours from the other, according to veterinarian's indication. The animal has completely recovered and has not been included in the mortality count. No significant differences occurred between groups.

Tab. 17 - Mortality and antibiotic treatment of post-weaning piglets fed a basal diet alone (CTR) or supplemented with amoxicillin (ANT, 400 mg/kg) or tributyrin and monolaurin (TB+ML, 5 kg/ton) for 41 days[1].

	Treatment			*P*-value		
Item	CTR	ANT	TB+ML	CTR vs ANT	CTR vs TB+ML	ANT vs TB+ML
No. Pigs/Treat.	64	64	64			
Dead	2	3	2	ns	ns	ns
Treated with antibiotics:						
Streptococcus suis	0	0	1	ns	ns	ns

Data are presented as Fisher test and chi-square tests results. ns: not significant
[1]Pigs 24 post-natal days of age at the beginning of the trial
[a,b]Within rows, different superscript letters indicate a significant difference

4.5 DISCUSSION

Feeding strategies are one of the most commonly used management factors for the improvement of gut health and function in weaned pigs. A wide range of substances has been proposed as tools to help reduce the incidence and severity of digestive problems associated with weaning and to maximize growth performance. Among them, SCFAs and MCFAs have been extensively studied (Hanczakowska et al., 2013; Zhang et al., 2019).

The literature reports that the use of SCFAs and MCFAs in piglet diet has a positive effect on gut health and on the growth of animals, thanks to the different properties of the two types of fatty acids (Liu, 2015). Therefore, considering that the mechanism and site of action of these fatty acids can differ, a combined supplementation could result in a synergistic action. However, when administered in free form, SCFAs and MCFAs are rapidly absorbed in the first tract of the intestine, reducing or nullifying their effects. A longer retention time in the gut may allow more extended effects on piglets' gut health. Thus, SCFAs and MCFAs can be protected by a glycerol esterification; in this way, it is possible to delay their absorption to prolong their effectiveness along the GIT (Decuypere and Dierick, 2003).

In this experiment the effect of a SCFA, in the form of TB, and of a MCFA, in the form of ML, on growth performance and gut health of weaning piglets was studied. Hanczakowska et al. (2013) proved that the combination of SCFAs (propionic C_3 and fumaric C_4) together with MCFAs (caprylic C_8 and Capric C_{10}) significantly improves performance and gut health of piglets. Similarly, Kuang at al. (2015) showed that adding a mixture of SCFAs and MCFAs in feed contributed to growth of weaning pigs under commercial conditions. Our results are in line with what is reported in literature. From the data collected it emerged that TB + ML supplementation improved growth performance of weaned piglets. During the 2 weeks of adaptation after weaning (from 0 to 14 days of trial), animals from the different diets displayed similar BW, with no influence of TB+ML respect other groups. At 28 days of trial, the piglets fed TB + ML have grown more than the controls and at the end of the trial, their BW was greater even compared to the antibiotic group. In addition, animals treated with TB + ML had a higher ADG starting from day15 of trial and considering the whole duration of it (from 0 to 41 days of trial) compared both to CTR and ANT. Moreover, the combination of TB and ML seems to have had no negative effects on feed palatability. In fact, in practical application, SCFAs and MCFAs are not easy to include in piglet feeds due to their different solubility rates and unpleasant odors (Croxen et al., 2013) that could stimulate the release of cholecystokinin and reduce the feed intakes of animals (Yang et al., 2014).

Our data showed that piglets fed TB + ML have higher ADFI than CTR diet, testifying that the inclusion dose of TB+ML did not compromise piglets feed intake.

Lee et al., (2007) and Zentek et al., (2013) have shown that a mixture of MCFAs and SCFAs is an excellent source of energy for intestinal cells and can mitigate the negative effects of weaning on intestinal morphology, increasing the growth performance. The small intestine is the major site for digestion and absorption of nutrients, and intestinal mucosa plays an important role in this process. Villus height, crypt depth and villus high: crypt depth ratio are indicators reflecting gut health status in piglets, and commonly used for evaluation of intestinal morphology (Liu et al. 2012; Han et al. 2013). During weaning, the intestinal structure of piglets will undergo profound changes, including villous atrophy and crypt elongation, which reduce the nutrient absorption (Barszcz and Skomiał 2011; Pluske 2013).

Previously, it has been shown that TB can improve the health status of the intestinal mucosa in piglets gut (Hou et al., 2006) reported that feeding of MCFAs to weaning pigs influenced the intestinal morphology, resulting in a significant increase in the length of the villi in the small intestine combined with a lower crypt depth and a lower number of intraepithelial lymphocytes. Moreover, a recent digestion experiment in vitro found that tributyrin only released a small amount of butyrate (<5%) during gastric stage and released approximately 75% of total butyrate in the small intestinal phase (Donovan et al., 2017), which showed that tributyrin had a higher utilization efficiency in the intestine. In our study, TB+ML revealed its trophic effect especially on the ileum, where the villus length was higher. By increasing the length of the ileal villi, TB+ML enlarges gut absorptive surface thereby favorable influencing the absorption of nutrients from the gut lumen. In contrast, no corresponding changes were observed in the duodenum and jejunum, maybe due the quick chime flow through this segment of the gut. Furthermore, the findings regarding the crypt depth, villus width and villus height: crypt depth ratio in all the intestinal districts analysed, are not conclusive and will require further investigation. Therefore, the assumption that dietary supply with TB+MN would affect intestinal morphology could be confirmed only in part, especially in the ileum.

Inflammation is a fundamental aspect when considering the functioning of the GIT. In addition, inflammation induced by stresses such as weaning has also a substantial impact on gut health (Piè et al., 2004). MCFAs and SCFAs have been suggested to have an effect on gut inflammatory conditions in pigs (Liu, 2015). Tributyrin, as a precursor of butyric acid, is well-known for its anti-inflammatory properties (Guilloteau et al., 2010). The treatment with TB has been associated with various beneficial effects at intestinal and systemic level: TB protected the gut integrity through an anti-inflammatory action in a murine ethanol-induced intestinal

injury model (Cresci et al., 2014) and in acetic acid-induced colitis in pigs (Leonel et al., 2013; Hou et al., 2014). A study of Tugnoli et al., (2020) has also highlighted how Tributyrin also regulates inflammatory markers and modulates T goblet cells number along the intestinal tract segments of weaning pigs. Regarding anti-inflammatory activity of MCFAs, some studies have been made on rats and reported that diets enriched with MCFAs decreased IL-6, IL-8, intercellular adhesion molecule-1 (ICAM-1) levels and glutathione S-transferase (GST) activity in TNBS colitis and decreased the expression of proinflammatory cytokines and chemokines in the ileum and Peyer's patches in a sepsis model of rat (Kono et al., 2004; Papada et al., 2014). In our study, the results concerning pro (IL-6), anti-inflammatory (IL-10) and secretory IgA (SIgA) cytokines showed no significant differences between groups. Cytokines participate in immune and inflammatory responses and regulation of intestinal barrier integrity and are produced in the acute phase of inflammation (AlSadi et al. 2009).

Many pro-inflammatory cytokines, like IL-6, have adverse effects on intestinal mucosal integrity and epithelial function (AlSadi and Ma 2007; Liu et al. 2008). On the other hand, anti-inflammatory cytokines, such as IL-10, play an important role in protecting intestinal barrier function by attenuating the defects in tight junction permeability (Madsen et al. 1997; Howe et al. 2005). SIgA has long been considered to be the first protective barrier against adhesion and invasion of pathogenic microorganisms to the mucosa (Pabst 2012). SIgA production is driven largely in response to mucosal antigens encountered by gut-associated lymphoid tissue (GALT). In this regard, Hu et al. (2013) showed that weaning stress caused in piglets, especially in the first 7 days, an activation of the intestinal inflammatory processes, demonstrated by the increase in IL-6 mRNA expression in jejunum. Our results shown that the tissue concentration of IL-6 appears to be in line with the data measured by Huang et al. (2012) in healthy piglets. Therefore, it can be assumed that, at the time of the measurement carried out at the end of the trial, the acute inflammatory phase caused by weaning had already been passed, and that acute inflammatory processes were not in progress. On the other hand, histometric analyses highlighted that TB+ML and ANT group had a smaller lymphatic follicle area compared with control group. The lymphatic follicle is a mucosal structure composed of lymphatic tissue, which is found in ileum individually or in the form of aggregates (Peyer's plaques), in which lymphocytes are present; therefore, lymphatic follicles play a critical role in the humoral immune response (Inoue et al., 2015; Jiang et al, 2015). Generally, measurements are made on this structure in order to highlight if there is a possible inflammatory state in the gut. Large lymphatic follicles usually indicate a possible inflammation state as there is increased lymphocyte stimulation and an increase in the number of white blood cells (Makala, 2001). Our

results indicate that the CTR group, which features the lymphatic follicle with the largest area, may have had an altered intestinal inflammatory status compared to the TB + ML and ANT group. However, considering the lack of significant differences in the level of IL-6, IL-10 and SIgA between the three groups, the correlation between the minor area of the LFs and the reduction of the inflammatory state following treatment with TB + ML cannot be ascertained and will require further investigation.

In order to have a clear situation of piglets' gut health and functionality during our trial, we also analysed the thickness of tunica muscularis externa, an intestinal muscular layer involved in peristalsis. In fact, according to the studies of Redlich et al., (1997) and Flores et al., (2018), if there is atrophy in the tunica muscularis externa, it means that there are problems of intestinal peristalsis. On the other hand, if the muscle layer is well developed, there will be a proper functionality of intestinal peristalsis which results in better digestion and absorption of nutrients. Our results show that the thickness of the tunica muscularis externa in general and the thickness of the muscularis external outer layer was greater in piglets treated with TB+ML compared to CTR and ANT groups. Regarding muscularis externa inner circular layer width, piglets fed TB+ML had a higher thickness compared to ANT group, but no differences were found with CTR group. These results demonstrate how TB + ML treatment has a positive effect on the intestinal functionality of piglets. Furthermore, these results support the data concerning the best growth performance of piglets fed with TB + ML.

As regards the antioxidant state, our data show how the levels of TAOC in the TB+ML group are statistically higher in all the intestinal portion of the small intestine. This indicated a good oxidative state of the piglet's intestinal environment. Wang et al. (2019) showed an interesting direct antioxidant effect of Tributyrin in pigs treated with Diquat, a substance capable of causing oxidative stress. The treatment resulted in an increase in TAOC, the activity of superoxidismutase (SOD) and a reduction in MDA. The antioxidant effect of MCFA was also detected by Lee and Kang (2017), both in vitro (on IPEC-J2 cells) and in vivo on pigs, after the administration of cyclophosphamide. According to the authors, the anti-inflammatory action of Capric acid would occur through both the inhibition of the activity of NK-kb (Huang et al., 2014), but also through the link with another family of nuclear transcription factors, the Peroxisome Proliferator-Activated Receptors (PPARs), and in particular with the PPAR-γ. In addition, PPAR-γ, together with another transcription factor, the Nuclear factor erythroid 2-related factor 2 (Nrf2), plays a key role in the expression of genes in cellular antioxidant systems. The binding of PPAR-γ and Nrf2 with specific molecules activates their translocation at the cell nucleus level, where the expression of the genes of enzymes with antioxidant action such

as CAT, SOD, GPx and Eme-Oxygenase is stimulated (HO-1) (Lee, 2017). Among the possible activators of these transcription factors, 3-O-Laurylglyceril Ascorbate is of particular interest. This amphipathic compound, obtained from Monolaurin linked to Ascorbic acid, has demonstrated a direct antioxidant effect of neutralizing ROS but also indirectly through the stimulation of both PPAR-γ and Nrf2, thanks also to the alkyl chain of monolaurin (Katsuyama et al., 2016). Therefore, the improvement in TAOC shown in the group of pigs fed with TB+ML would suggest the effectiveness of the treatment in improving the oxidative state of the intestinal membrane.

In the final analysis, considering the mortality rating among the different groups, it emerges that mortality was homogeneous. The only treated piglet we had during the trial was affected by *Streptococcus suis* and after the antibiotic treatment completely recovered and has not been included in the mortality count. In general, no significant differences occurred between groups regarding both mortality and antibiotic treatment.

4.6 CONCLUSION

Our results suggest that supplementing a basal diet with TB+ML (5 kg/ton) has beneficial effects on the porcine gut health, protecting the intestinal epithelium from oxidative stress caused by weaning. Moreover, TB+ML enhanced piglet's growth performance, supported by the increase of the ileal villi length which enlarged the nutrient absorption surface.

4.7 REFERENCES

Al-Sadi RM, Boivin M and Ma TY. 2009. Mechanism of cytokine modulation of epithelial tight junction barrier. Front Biosci (Landmark Ed) 14:2765–2778.

Al-Sadi RM and Ma TY. 2007. IL-1beta causes an increase in intestinal epithelial tight junction permeability. J Immunol 178:4641–4649.

Antongiovanni M, Buccioni A, Petacchi F, Sargentini C, Minieri S. 2006. Mixtures of mono-, di- and tri-glycerides as energy supplements to broilers 'diets. Ital J Anim Sci 5:55-62.

Allen HK, Levine UY, Looft T, Bandrick M, Casey TA. 2013. Treatment, promotion, commotion: Antibiotic alternatives in food-producing animals. Trends Microbiol 21:114–119.

Batovska DI, Todorova IT, Tsvetkova IV, Najdenski HM. 2009. Antibacterial study of the medium chain fatty acids and their 1-monoglycerides: individual effects and synergistic relationship. Pol J Microbiol 58:43-47.

Barszcz M and Skomiał J. 2011. The development of the small intestine of piglets-Chosen aspects. J Anim Feed Sci 20:3–15.

Bergsson G, Arnfinnsson J, Steingrimsson O, H. Thormar H. 2001. Killing of gram- positive cocci by fatty acids and monoglycerides. Acta Pathol Microbiol Immunol Scand 109:670-678.

Cresci GA, Bush K and Nagy LE. 2014. Tributyrin supplementation protects mice from acute ethanol-induced gut injury. Alcohol Clin Exp Res 38:1489–1501.

Croxen MA, Law RJ, Scholz R, Keeney KM, Wlodarska M, Finlay BB. 2012. Recent advances in understanding enteric pathogenic Escherichia coli. Clinic Microbiol Rev 26:822-880.

Czaplewski L, Bax R, Clokie M, Dawson M, Fairhead H, Fischetti VA, Rex JH et al. 2016. Alternatives to antibiotics-a pipeline portfolio review. Lancet Infect Dis 16:239–251

De Lange CFM, Pluske J, Gong J, Nyachoti CM. 2010. Strategic use of feed ingredients and feed additives to stimulate gut health and development in young pigs. Livest Sci 134:124–134.

Decuypere JA and Dierick NA. 2003. The combined use of triacylglycerols containing medium-chain fatty acids and exogenous lipolytic enzymes as an alternative to in-feed antibiotics in piglets: concept, possibilities and limitations. An overview. Nutr Res Rev 16:193–210.

Desbois A and Smith V. 2010. Antibacterial free fatty acids: activities, mechanisms of action and biotechnological potential. Appl Microbiol Biotechnol. 85:1629-1642.

Donovan JD, Bauer L, Fahey GC and Lee Y. 2017. In vitro digestion and fermentation of microencapsulated tributyrin for the delivery of butyrate. J Food Sci 82: 1491–1499.

Flores TJ, Nguyen VB, Widdop RE, Sutherland MR, Polglase GR, Abud HE, Black MJ. 2018. Morphology and Function of the Lamb Ileum following Preterm Birth. Front Pediatr 6: 8.

Guilloteau P, Martin L, Eeckhaut V, Ducatelle R, Zabielski R, Van Immerseel F. 2010. From the gut to the peripheral tissues: the multiple effects of butyrate. Nutr Res Rev 23: 366–384.

Han F, Hu L, Xuan Y, Ding X, Luo Y, Bai S, He S, Zhang K and Che L. 2013. Effects of high nutrient intake on the growth performance, intestinal morphology and immune function of neonatal intrauterine growth-retarded pigs. Br J Nutr 110:1819–1827.

Hanczakowska E, Szewczyk A, Swiatkiewicz M and Okon´ K. 2013. Short-and medium chain fatty acids as a feed supplement for weaning and nursery pigs. Pol J Vet Sci 16: 647–654.

Heo JM, Opapeju FO, Pluske JR, Kim JC, Hampson DJ, Nyachoti CM. 2013. Gastrointestinal health and function in weaned pigs: a review of feeding strategies to control post-weaning diarrhoea without using in-feed antimicrobial compounds. J Anim Physiol Anim Nutr (Berl) 97:207–237.

Howe KL, Reardon C, Wang A, Nazli A and McKay DM. 2005. Transforming growth factor-beta regulation of epithelial tight junction proteins enhances barrier function and blocks enterohemorrhagic Escherichia coli O157:H7- induced increased permeability. Am J Pathol 167:1587–1597.

Hou YQ, Liu YL, Hu J and Shen WH. 2006. Effects of lactitol and tributyrin on growth performance, small intestinal morphology and enzyme activity in weaned pigs. Asian-Australasian J Anim Sci 19:1470–7.

Hou YQ, Wang L, Yi D, Ding B, Chen X and Wang Q. 2014. Dietary supplementation with tributyrin alleviates intestinal injury in piglets challenged with intrarectal administration of acetic acid. Brit J Nutr 111:1748–58.

Hu CH, Xiao K, Luan ZS and Song J. 2013. Early weaning increases intestinal permeability, alters expression of cytokine and tight junction proteins, and activates mitogen-activated protein kinases in pigs. J Anim Sci 91:1094–1101.

Huang Y, Li YL, Huang Q, Cui ZW, Yu DY, Rajput IR, Hu CH and Li WH. 2012. Effect of orally administrated Enterococcus faecium EF1 on intestinal cytokines and chemokines production of suckling piglets. Pak Vet J 32:81-84.

Huang WC, Tsai TH, Chuang LT, Li YY, Zouboulis CC and Tsai PJ. 2014. Anti-bacterial and anti-inflammatory properties of capric acid against Propionibacterium acnes: A comparative study with lauric acid. J Dermatol Sci 73: 232-240.

Inoue R, Tsukahara T, Nakatani M, Okutani M, Nishibayashi R, Ogawa S, Harayama T, Nagino T, Hatanaka H, Fukuta K, Romero-Pérez GA, Ushida K and Kelly D. 2015. Weaning Markedly Affects Transcriptome Profiles and Peyer's Patch Development in Piglet Ileum. Front Immunol 6:630.

Jiang XR, Agazzi A, Awati A, Vitari F, Bento H, Ferrari A, Alborali GL, Crestani M, Domeneghini C and Bontempo V. 2015. Influence of a blend of essential oils and enzyme combination on growth performance, microbial counts, ileum microscopic anatomy and the expression of inflammatory mediators in weaned piglets following an Escherichia Coli infection. An Feed Sci Technol 209: 219-229.

Kabara JJ, Swieczkowski DM, Conley AJ and Truant JP. 1972. Fatty acids and derivatives as antimicrobial agents. Antimicrob Agents Chemother 2:23–28.

Katsuyama Y, Tsuboi T, Taira N, Yoshioka M and Masaki H. 2016. 3-O-Laurylglyceryl ascorbate activates the intracellular antioxidant system through the contribution of PPAR-g and Nrf2. J Dermatol Sci 82: 189-196.

Kim SA and Rhee MS. 2013. Marked synergistic bactericidal effects and mode of action of medium-chain fatty acids in combination with organic acids against Escherichia coli O157:H7. Appl Environ Microbiol 79: 6552-6560.

Kono H, Fujii H, Asakawa M, Maki A, Amemiya H, Hirai Y, et al. 2004. Medium-chain triglycerides enhance secretory IgA expression in rat intestine after administration of endotoxin. Am J Physiol Gastrointest Liver Physiol 286:1081–9.

Kuang Y, Wang Y, Zhang Y, Song Y, Zhang X, Lin Y, Che L, Xu S, Wu D, Xue B, Fang Z. 2015. Effects of dietary combinations of organic acids and medium chain fatty acids as a replacement of zinc oxide on growth, digestibility and immunity of weaned pigs. Anim Feed Sci Tech. 208:145-57.

Lee SI and Kang KS. 2017. Function of capric acid in cyclophosphamide-induced intestinal inflammation, oxidative stress, and barrier function in pigs. Sci Rep 7:16530.

Lee DN, Liu SR, Chen YT, Wang RC, Lin SY and Weng CF. 2007. Effects of diets supplemented with organic acids and nucleotides on growth, immune responses and digestive tract development in weaned piglets. J Animal Physiol Anim Nutr 91:508-518.

Leonel AJ, Teixeira LG, Oliveira RP, Santiago AF, Batista NV, Ferreira TR, Santos RC, Cardoso VN, Cara DC, Faria AM and Alvarez-Leite J. 2013. Antioxidative and

immunomodulatory effects of tributyrin supplementation on experimental colitis. Br J Nutr 109: 1396–1407

Lieberman S, Enig MG, Preuss HG. 2006. A Review of Monolaurin and Lauric Acid: Natural Virucidal and Bactericidal Agents. Altern Complement Ther 12:310-314.

Liu YL, Huang JJ, Hou YQ, Zhu HL, Zhao SJ, Ding BY, Yin YL, Yi GF, Shi JX and Fan W. 2008. Dietary arginine supplementa- tion alleviates intestinal mucosal disruption induced by Escherichia coli lipopolysaccharide in weaned pigs. Br J Nutr. 100:552–560.

Liu Y, Chen F, Odle J, Lin X, Jacobi SK, Zhu H, Wu Z and Hou Y. 2012. Fish oil enhances intestinal integrity and inhibits TLR4 and NOD2 signaling pathways in weaned pigs after LPS challenge. J Nutr 142:2017–2024.

Liu Y. 2015. Fatty acids, inflammation and intestinal health in pigs. A review. J Anim Sci Biotechnol 41:1-9.

Madsen K, Lewis SA, Tavernini MM, Hibbard J and Fedorak RN. 1997. Interleukin 10 prevents cytokine-induced disruption of T84 monolayer barrier integrity and limits chloride secretion. Gastroenterol 113:151–159.

Makala LHC, Kamada T, Nishikawa Y, Nagasawa H, Igarashi I, Fujisaki K, Suzuki N, Mikami T, Haverson K, Bailey M, Stokes CR, Bland PW. 2000. Ontogeny of pig discrete Peyer's patches: distribution and morphometric analysis. Pathobiol 68:275–282.

Mallo JJ, Balfagon A, Gracia MI, Honrubia P and Puyalto M. 2012. Evaluation of different protections of butyric acid aiming for release in the last part of gastrointestinal tract of piglets. J Anim Sci 90: 227-229.

Pabst O. 2012. New concepts in the generation and functions of IgA. Nat Rev Immunol 12:821–832.

Papada E, Kaliora AC, Gioxari A, Papalois A, Forbes A. 2014. Anti-inflammatory effect of elemental diets with different fat composition in experimental colitis. Br J Nutr 1213–20.

Pié S, Lallès JP, Blazy F, Laffitte J, Sève B and Oswald IP. 2004. Weaning is associated with an upregulation of expression of inflammatory cytokines in the intestine of piglets. J Nutr 134:641–7.

Pluske JR. 2013. Feed- and feed additives-related aspects of gut health and development in weanling pigs. J Anim Sci Biotechnol 4, 1.

Redlich J, Souffrant WB, Laplace JP, Hennig U, Berg R and Mouwen JMVM. 1997. Morphometry of the Small Intestine in Pigs with Ileo-Rectal Anastomosis. Can J Vet Res. 61: 21-27.

Tugnoli B, Piva A, Sarli G and Grilli E. 2020. Tributyrin differentially regulates inflammatory markers and modulates goblet cells number along the intestinal tract segments of weaning pigs. Livest Sci. doi.org/10.1016/j.livsci.2020.103996.

Wang C, Cao S, Zhang Q, Shen Z, Feng J, Hong Q, Lu J, Xie F, Peng Y and Hu C. 2019. Dietary Tributyrin attenuates intestinal inflammation. Enhances mitochondrial function and induces mitophagy in piglets challenged with Diquat. J Agri Food Chem 67: 1409-1417.

Wang C, Shen Z, Cao S, Zhang Q, Peng Y, Hong Q, Feng J and Hu C. 2019. Effects of tributyrin on growth performance, intestinal microflora and barrier function of weaned pigs. Anim Feed Sci Technol. doi.org/10.1016/j.anifeedsci.2019.114311.

Yang J, Jiang ZY, Zheng CT, Wang L, Yang XF. 2014. Effect of Lactobacillus plantarum on diharrea and intestinal barrier function of young piglets challenged with enterotoxigenic Escherichia coli K88. J Anim Sci 92:1469-1503.

Zentek J, Ferrara F, Pieper R, Tedin L, Meyer W and Vajen W. 2013. Effects of dietary combinations of organic acids and medium chain fatty acids on the gastrointestinal microbial ecology and bacterial metabolites in the digestive tract of weaning piglets. J Anim Sci 91:3200–3210.

Zhang H, Wei H, Cui Y, Zhao G, Feng F. 2009. Antibacterial interactions of monolaurin with commonly used antimicrobials and food components. J Food Sci 74:M418-M421.

Zhang S, Xiong J, Lou W, Ning Z, Zhang D, Yang J. 2019. The key lethal effect existed in the antibacterial behavior of short, medium and long chain fatty acid monoglycerides on Escherichia coli. BioRxiv 339309.

CHAPTER 5 -

Lauric acid saponified with calcium in post-weaning piglets born from sows fed a diet supplemented with medium-chain fatty acids: effects on growth performance and gut health

5.1 ABSTRACT

Across species, mother feeding is known to have an effect on the development and health of the offspring. The objective of the study was to evaluate the effects of dietary lauric acid saponified with calcium (C12-Ca soap) starting from sows' late pregnancy, on offspring growth and health. Additionally, the digestion to which the product is subjected in the stomach was simulated to assess whether C12-Ca soaps is able to reach the small intestine of pigs. At 3 weeks before parturition, 16 sows were randomly assigned to two dietary treatment: basal diet (CTR) or basal diet with 1 kg/ton C12-Ca soap (T). At weaning 96 piglets were chosen from the litters and distributed in 4 post-weaning dietary treatments, considering the mother group of origins, namely: CTR-CTR and T-CTR fed basal diet and CTR-T and T-T fed basal diet plus C12-Ca soaps (1 kg/ton). Each treatment consisted of six replicates, being the pen with 4 animals considered as experimental unit. Body weight (BW), average daily gain (ADG), and average daily feed intake (ADFI) were measured, and feed efficiency (FE) and was calculated. Fecal samples were taken from one piglet per replicate a 14 and 26 days of trial to evaluate Calprotectin (CALP) concentration. Intestinal mucosa samples of duodenum and ileum were used to investigate the impact of C12-Ca soaps on the total antioxidant capacity (TAOC) and SIgA concentration. The results showed improved BW in CTR-T pigs compared to CTR-CTR ($P<0.05$) at 26 trial days, whereas no differences were found for ADG. Similarly, ADFI was higher in piglets of CTR-T group compared to CTR-CTR and T-T ($P <0.05$) from 15 to 26 days and for the whole trial (0-26 days). On the contrary, the results concerning FE showed a better use of the feed in CTR-CTR and T-T ($P<0.05$) compared with other groups from 15 to 26 days and for the whole trial (0-26 days). No differences in mortality and antibiotic treatments were found. CALP concentration at 14 days was lower in T-CTR and T-T compared with the other groups ($P<0.05$), whereas no differences were observed for TAOC in both duodenal and ileum mucosa. SIgA concentration in duodenum was lower in T-T group compared to CTR-CTR and CTR-T groups ($P<0.05$) but no corresponding differences were found in ileum. Furthermore, it has been shown that C12-Ca soaps at pH 4 do not dissociate. Although the administration of C12-Ca soaps in the diet of the mothers did not show clear effects on the growth of the offspring, our findings suggest that the administration of C12-Ca soaps modulates intestinal inflammatory status during the first two weeks after weaning, improving an important aspect of the gut health.

Keywords: Lauric acid, saponification, piglets, sows, growth performance, gut health

5.2 INTRODUCTION

Inadequate maternal nutrition and stress during gestation can affect physiological development of the offspring and may increase their susceptibility to diseases later in life (Tuchscherer et al., 2012). Across species, pigs included, it is well known that the diet of the mother has an effect on the growth and health of the offspring. The reproductive performance of sows and growth performance of the offspring is fundamental to the development of pig farming. However, supplementation of the diet of sows during late pregnancy and lactation is one of the less popular ways to positively affect the health status of offspring. Three-quarters of fetal weight is gained in the last quarter of pregnancy, during which sufficient energy is required to meet sows' nutritional needs. Therefore, the choice of an appropriate nutritional strategy for sows during gestation, and the use of additives with positive effects on the health and growth of the offspring, is recommended.

In the last years, studies have found that medium chain fatty acids (MCFAs) act as a source of energy, and have several unique roles, such as metabolic regulation, antibacterial activity, and anti-inflammatory effects on pig's health and growth (Zentek et al., 2011; Liu, 2015; Jackman et al., 2020). However, little is known about the effects that supplementing MCFAs in the late gestation phase of sows may have on the growth and gut health of piglets at weaning.

Weaning is the most critical period in the life cycle of piglets. In fact, during this time, they have not yet fully developed their intestinal tract and immune system (Pluske et al., 1997; Bailey et al., 2005; Campbell et al., 2013). Therefore, they are an easy target for pathogenic microorganisms causing gastrointestinal diseases (Castillo et al., 2006). They also have to adapt to new stressful conditions, which results in reduction of feed consumption, temporary malnutrition, and growth retardation (Lalles et al., 2004). The reduction of villus length and increased crypt depth and enzyme activity are often observed during the first 3–5 days post-weaning, which predispose the piglets to gastrointestinal disorders and consequently lead to much slower growth rate during the post-weaning period (Ferrara et al., 2016). It is known that ileum, especially the Peyer's patches located in its terminal region, plays a crucial role in targeting antigens and act as a first line of blockage of pathogens in the small intestine (Cappai et al., 2020).

Antibiotic growth promoters were used to prevent these issues, but they have been banned by the European Union due to the development of bacteria resistance against antibiotics (Anadon, 2006). Moreover, today, consumers are demanding more antibiotic-free pigs, so the search is on for alternatives. Various nutritional approaches have been proposed to improve the

gut health status and prevent gastrointestinal disorders in piglets without the use of antibiotics (Jiang et al., 2015; 2016). One of the suitable solutions include MCFAs. MCFAs can provide a rapid supply of energy for the body because they can be rapidly digested, absorbed, and oxidatively metabolized even by young animals (Baltic et al., 2017). As intestinal epithelium cells are the main site of nutrient absorption, provision of such easily absorbable nutrients should improve their structure and function. They have also strong antibacterial activity and can improve post-weaning gut development (Kabara, 1972; Skrivanova et al., 2009).

Previous research indicated that dietary provision of MCFAs to weanling pigs improved growth performance and gut health (Liu, 2015; Hanczakowska, 2017). In a previous study conducted by our research group (Giorgi et al., 2020 s*ubmitted*), was shown that the administration of lauric acid saponified with calcium (C12-Ca soaps) in post-weaning pigs diet had positive effects on some gut health parameters. However, the data obtained from the study did not support a marked improvement in the growth performance and intestinal health of the piglets after weaning. Furthermore, studies investigating the application of MCFAs in general, and the administration of C12-Ca soaps in particular, in the maternal diet and corresponding effects on weaned pig performance and gut health aspects are limited (Azain, 1993; Chen et al., 2019; S´wiatkiewicz et al., 2020).

For this reason, the purpose of this study was to evaluate the effect of C12-Ca soaps on the growth performance and gut health of piglets born from mothers to which the same product was administered or not, in the last phase of gestation. This new study has the ultimate aim of responding to the hypothesis that emerged at the end of the previous work, namely: what could be the effect of C12-Ca soaps on the health of piglets, if its administration had started in the prenatal phase?

5.3 MATERIAL AND METHODS

All procedures were approved by the Animal Welfare Committee of the Università degli Studi di Milano (application No. OPBA_45_2018)

5.3.1 Animals, housing, and experimental design

The experiment started in a commercial pig farm (Azienda Agricola Arioli&Sangali). At 3 weeks before parturition, 16 sows were chosen and divided in two dietary treatment: CTR: fed basal diet and T: fed basal diet supplemented with 1 kg/ton C12-Ca soap. At weaning (28 days of age), 96 piglets (48 from control sows and 48 from treated sows) of average weight (7,24 ± 0,15 kg) were chosen and transferred to Centro Zootecnico Didattico Sperimentale (CZDS) of the University of Milan (Lodi) for the post-weaning period. The weaning of the piglets was considered day 0 of the post-weaning period trial part. The post-weaning phase lasted 26 days in total. Once arrived to CZSD, the pigs were housed in one experimental room with 24 pens. Each pen had plastic slatted floors and was fitted with an adjustable stainless-steel feeder and two nipple waterers. The rooms were lit by a combination of daylight through skylights and artificial light. The temperature, humidity, CO_2, and ammonium concentration of the air was automatically controlled. Ventilation was achieved by single, variable-speed fans linked to temperature sensors. The temperature inside the building was approximately 28 °C at the start of the trial and adjusted weekly until a final temperature of 24–25 °C was obtained. The relative humidity was controlled at 60–70%. The pigs had water and feed, provided in meal form, available *ad libitum*. All piglets were fed a standard commercial diet formulated to meet or exceed nutrient requirements for post-weaning piglets (NRC 2012) (Table 18).

Tab. 18 - Ingredients and chemical compositions of the basal diet fed to post-weaning piglets during the trial (as-fed basis, %).

Ingredients (%)	Basal diet
Barley meal	20.40
Wheat meal	16.75
Maize meal	15.16
Soycomil R	5.50
Flaked wheat	4.50
Dextrose	3.75
Flacked maize	4.25
Flacked barley	2.00
Soybean meal 48% CP	6.50
Wheat middlings	4.00
Herring meal	2.00
Plasma AP 420	1.50
Soybean oil	2.20
Cellulose	0.87
L-Lysine	0.50
Dicalcium phosphate	0.55
Calcium carbonate	0.50
L-Threonine	0.20
Vitamins+trace elements[1]	0.25
DL-Methionine	0.16
Salt	0.17
L-Tryptophan	0.04
Animal fat	0,75
Analysed composition (%)	
Dry matter	90.29
Crude protein	17.27
Crude fat	4.95
Crude fibre	3.05
Ash	4.47
Calcium	0.60
Phosphorus	0.55
Calculated composition	
DE, kcal/kg	3450
NE, kcal/kg	2460
Lysine	1.25
Methionine +Cystine	0.75
Threonine	0.85
Tryptophan	0.23

[1]Premix Mineral-vitaminico aggiunto per kg di tal quale: Vitamin A: 10,000 IU; Vitamin D3: 1,000 IU; Vitamin E: 50 mg; Vitamin B1: 1.0 mg; Vitamin B2: 3.0 mg; Vitamin B12: 0.02 mg; Vitamin B6: 3,0 mg; Pantotenic acid: 10 mg; Nicotinic acid: 15 mg; Biotin: 0.06 mg; Vitamin PP: 0,35 mg; Folic acid: 0,99 mg; Vitamin K3: 2 mg; Choline: 300 mg; Fe: 100 mg; Cu: 20 mg; Co: 0.75 mg; Zn: 100 mg; Mn: 10 mg; I: 0.75 mg; Se: 0.4 mg.

There, piglets were distributed in a randomized block design considering the two maternal dietary treatments during gestation and lactation (CTR diet and T) and two dietary treatments for piglets at weaning (CTR diet and T), totaling 4 treatments. The piglets were randomly allocated to 1 of the 4 dietary treatments with 6 replicate pens per treatment. The experimental unit was the pen with 4 piglets per pen. Dietary treatments were as follows: CTR-CTR piglets coming from CTR sows receiving basal diet, CTR-T piglets coming from CTR sows receiving basal diet supplemented with C12-Ca soap (1 kg/ton), T-CTR piglets coming from T sows

receiving basal diet and T-T piglets coming from T sows receiving basal diet supplemented C12-Ca soap (1 kg/ton). A C12-Ca soap (DRAX S) product containing 30mg/kg of zinc oxide (0.03 g/kg) by DC Practical Solution (Via Petrarca 4, 20123, Milan, Italy) was used both in sows and piglets treated diet. The C12-Ca soaps were created by saponification, combining 750 g/kg lauric acid and 100 g/kg calcium hydroxide.

5.3.2 Animal health and therapeutic treatments

The veterinary doctor responsible for animal welfare during the trial, checked the piglets when they were put on trial and only healthy piglets were allowed to the trial. No prophylactic antibiotic treatment was given. The veterinary doctor responsible for animal welfare during the trial made also the diagnoses of *Streptococcus suis* and decided upon the antibiotic therapy. The carcasses of the dead animals were sent to the Pathological Anatomy Laboratory of the University of Milan for necropsy analyses. Piglets treated with antibiotics were excluded from sample collection.

5.3.3 Measurements, samples and product characterization

At 0, 14 and 26 days after weaning the piglets were weighed, as were the amount of feed supplied, the feeders and the residue contained in them. From the data obtained, performance parameters were analyzed regarding average daily feed intake (ADFI), average daily gain (ADG) and feed efficiency (FE). Mortality and pathologies were recorded daily. Fecal samples were collected directly from the rectum of one piglet of average weight per replicate for each treatment (n=24) at 14 and 26 post-weaning days of trial. Two aliquots of fecal samples were stored at -20 °C for the determination of Calprotectin, a non-invasive marker of intestinal inflammation. At the end of the trial (26 days of the trial), the same piglets used for fecal sampling were slaughtered in accordance with current regulations at a local commercial slaughterhouse to collect the intestinal mucosa samples. The entire intestinal tract was removed, and different sections of the duodenum (n=24) and ileum (n=24) were obtained from each animal. Approximately 5 cm segment were obtained from duodenum and ileum and emptied of their intestinal contents by squeezing them. Subsequently, the mucosa was scrapped and collected in 15 ml Falcon tubes and immediately frozen and stored at -80 °C to assess the total antioxidant capacity and the concentration of secretory immunoglobulins A (SIgA). An *in vitro* experiment was also conducted on the product used in this study (C12-Ca soaps) to assess

whether the product dissociates in an acid environment and therefore is able to reach the small intestine.

5.3.4 Fecal calprotectin

Frozen fecal samples (0.1 g) were homogenized with 1ml of cold physiological saline solution, maintained in ice, and centrifuged (3,000 × *g* for 15 min, 4°C). Supernatants were then collected to perform the analysis. The concentration of calprotectin was determined by commercial kits (Cusabio – CSB-EQ013485PI). The assay employed the competitive inhibition enzyme immunoassay technique, and it was performed following the manufacturer's instructions.

5.3.5 Total antioxidant capacity (TAOC) and secretory immunoglobulins A (SIgA) concentration in intestinal mucosa

Frozen intestinal mucosa samples (0.2 g) of duodenum and ileum were homogenized using a ultraturrex with 2ml of cold physiological saline solution, maintained in ice, and centrifuged (3,000 × *g* for 15 min, 4°C). Supernatants were then collected and divided in two aliquots to perform the analysis. The total antioxidant capacity (TAOC) activity was determined by ABTS method. The choice of this method was motivated by the fact that it is one of the most widespread analytical method to evaluate the TAOC of a sample. In summary, the ABTS test evaluates the antioxidant capacity through the ability of radical removal by the radical cation ABTS (acronym of 2,2'-azinobis (3-eylbenzothiazoline-6-6 sulfonic)). A standard solution of Trolox (a compound analogous to vitamin E) was used for carrying out this analysis. The absorbance of the samples was measured at a wavelength of 734 nm using a spectrophotometer. The concentration of SIgA was determined by commercial kits (Cusabio – CSB-E12063p) following the manufacturer's instructions.

5.3.6 Product characterization

To simulate the physiological digestion that C12-Ca soaps undergoes in the stomach, 0.1 g of the product was weighed and then diluted in distilled water and in an acid solution with hydrochloric acid (HCL) of pH 4, 3 and 2. The release of calcium (Ca) in the different solutions was evaluated immediately after the addition of the product in the solvent (time 0) and after 2 and 3 hours. The Ca content of the solutions was determined by commercial kits (Elabscience

– E-BC-K103-M) following the manufacturer's instructions. The principle of the kit was that the calcium ion in the sample, bound to the Methyl Thymol Blue (MTB) in an alkaline solution, forming a blue complex. The calcium content was then calculated by measuring the OD value at 610 nm.

5.3.7 Statistical analysis

A completely randomized design was used. Growth performance were analysed using the Statistical Analysis System software (SAS version 9.4; SAS Institute Inc., Cary, NC, USA) applying a MIXED procedure for repeated measurements and accounting for the effects of treatment, time, and treatment × time interaction. Tukey-test was considered for differences among treatments. For the statistical analysis of mortality and antibiotic treatment, Fisher's exact test was used. Regarding the laboratory analyzes performed on feces, on the intestinal mucosa samples of the duodenum and ileum and on the product, the statistical analysis was carried out using the GraphPad-Prism (version 8) software package. The data were subjected to variance analysis using the one-way ANOVA procedure and the differences between the means were compared using the Tukey-test. The pen represented the experimental unit for the growth performance parameters, while each pig represented the experimental unit for mortality, antibiotic treatment and laboratory analyses. All numerical data in the tables are presented as the least-square means (LSMeans) accompanied by the standard error of the mean (SEM) values. Differences between groups were considered statistically significant with $P<0.05$.

5.4 RESULTS

5.4.1 Growth performance

Table 19 summarizes the piglet growth performance. As for BW, the animals belonging to the group CTR-T showed higher BW than those of CTR-CTR group ($P<0.05$) at 26 days of trial, whereas, no significant differences were found between groups at 0 and 14 post-weaning days. Regarding ADG no differences were found between groups. On the other hand, ADFI was higher in piglets of CTR-T group compared to CTR-CTR and T-T ($P <0.05$) in the period from 15 to 26 days and for the whole trial (0-26 days). On the contrary, the results concerning FE showed a better use of the feed in groups CTR-CTR and T-T ($P<0.05$) compared with other

groups from 15 to 26 days and for the whole trial (0-26 days). In general, health conditions were good throughout the whole trial. No episodes of diarrhea occurred.

Tab. 19 - Growth performance parameters of post-weaning piglets fed a basal diet alone (CTR-CTR and T-CTR) or supplemented with lauric acid saponified with calcium (CTR-T and T-T) for 26 days[1].

	Dietary treatments					*p*-value		
Parameter	1	2	3	4	SEM	Tr.	Tm.	Tr*Tm
No. pigs/treat	24	24	24	24				
BW (kg)								
Day 0	7.13	7.14	7.32	7.33	0.34	0.109	<0.001	0.598
Day 14	10.35	11.07	10.74	10.47				
Day 26	16.30^{b}	17.61^{a}	16.82^{ab}	16.75^{ab}				
ADG (g/d)								
Days 0-14	0.229	0.281	0.244	0.224	0.022	0.034	<0.001	0.989
Days 15-26	0.496	0.544	0.506	0.521				
Days 0-26	0.352	0.402	0.365	0.365				
ADFI (g/d)								
Days 0-14	0.386	0.466	0.402	0.381	0.037	0.0008	<0.001	0.922
Days 15-26	0.721^{b}	0.867^{a}	0.822^{ab}	0.724^{b}				
Days 0-26	0.541^{b}	0.651^{a}	0.596^{ab}	0.539^{b}				
FE								
Days 0-14	0.59	0.60	0.60	0.59	0.056	0.0001	<0.001	0.0034
Days 15-26	0.69^{a}	0.63^{b}	0.62^{b}	0.72^{a}				
Days 0-26	0.65^{a}	0.62^{b}	0.61^{b}	0.67^{a}				

All results are presented as mean ± SEM

1: CTR-CTR; 2: CTR-T; 3: T-CTR; 4: T-T; BW: body weight; ADG: average daily gain; ADFI: average daily feed intake; FE: feed efficiency; Tr: treatment; Tm: time; Tr*Tm: treatment*time

[1]Pigs 28 post-natal days of age at the beginning of the trial

[a,b]Within rows, different superscript letters indicate a significant difference

5.4.2 Mortality and antibiotic treatment

As shown in Table 20a and 20b, during the trial, only one piglet died in T-T group due to *Streptococcus suis* infection. Seven piglets were treated against *Streptococcus suis*—1 in the CTR-T group, 3 in T-CTR group and 3 in T-T group—with individual injections of Amoxicillin (1ml/10kg), but there were no significant differences between groups.

Tab. 20a Mortality and antibiotic treatment of post-weaning piglets fed a basal diet alone (CTR-CTR and T-CTR) or supplemented with lauric acid saponified with calcium (CTR-T and T-T) for 26 days[1].

	Dietary treatments			
	CTR-CTR	**CTR-T**	**T-CTR**	**T-T**
Treated	0	1	3	3
Dead	0	0	0	1

Tab. 20b - Fisher exact test mortality and antibiotic treatments results.

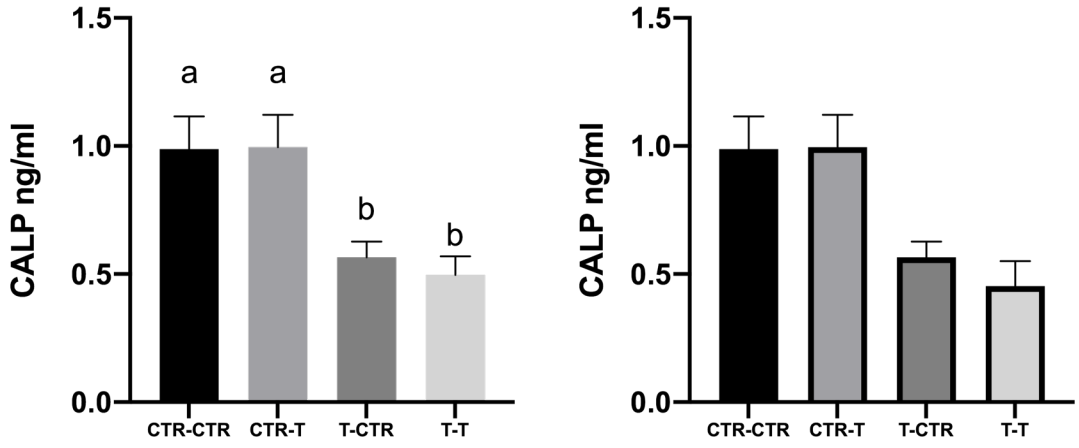
	P-value					
	CTR-CTR vs CTR-T	**CTR-CTR vs T-CTR**	**CTR-CTR vs T-T**	**CTR-T vs T-CTR**	**CTR-T vs T-T**	**T-CTR vs T-T**
Treated	ns	ns	ns	ns	ns	ns
Dead	ns	ns	ns	ns	ns	ns

Data are presented as Fisher test and chi-square tests results. ns: not significant

5.4.3 Calprotectin (CALP) concentration in faeces

The CALP concentration in the feces of post-weaning piglets at 14 and 26 days is shown in the graphs below (Graph 1 and 2) As shown, the CALP concentration in feces at 14 days was remarkably lower in T-CTR and T-T groups compared to CTR-CTR and CTR-T groups ($P<0.05$). On the contrary, no differences between groups were found for CALP concentration in feces at 26 days of trial.

Graph 1 and 2 – CALP concentration in feces at 14 and 26 post-weaning days. [a,b]different letters indicate a significant difference.



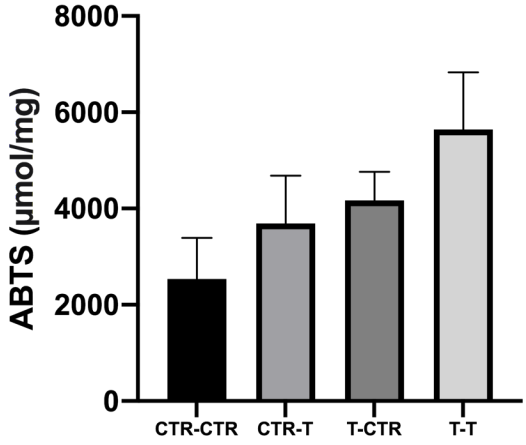
Graph 1 **Graph 2**

5.4.4 TAOC and SIgA concentration in duodenum and ileum mucosa

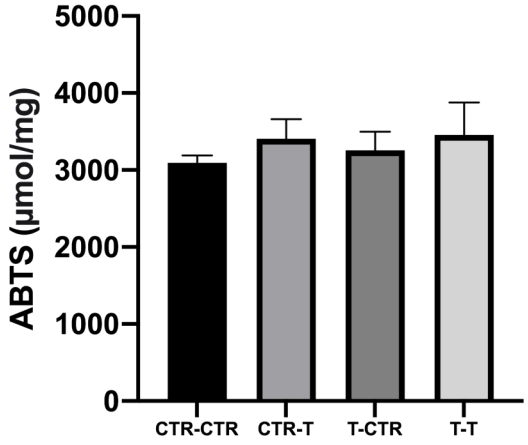
TAOC results are presented in Graph 3 and 4. As shown, no significant differences were found between groups in both duodenal and ileum mucosa. However, in the graph 4, despite the high

variability visible in the results, it was observed that in the T-T group, the TAOC was numerically higher than in the other groups.

Graph 3 and 4 – TAOC in duodenum and ileum mucosa of post-weaning piglets



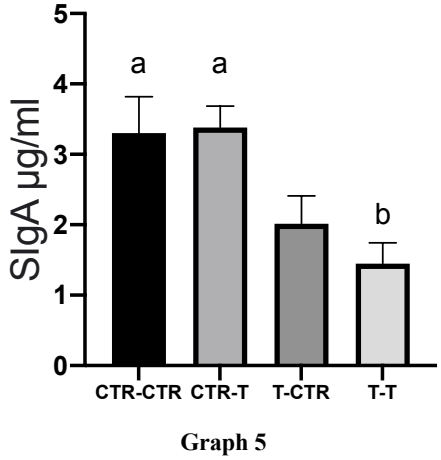
Graph 3



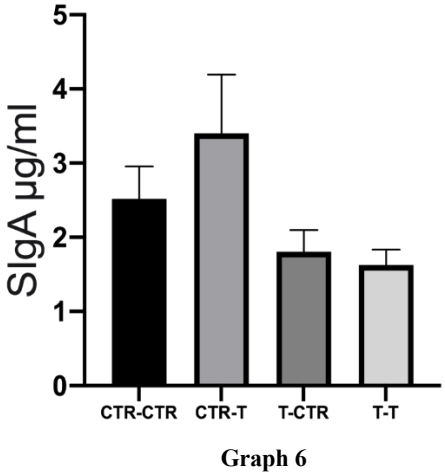
Graph 4

Regarding SIgA (Graphs 5 and 6) concentration in duodenum and ileum mucosa, results have shown that in duodenum, the SIgA concentration was significantly lower in T-T group compared to CTR-CTR and CTR-T groups ($P<0.05$). On the other hand, no significative differences were found between groups in ileum mucosa, even if the SIgA concentration in CTR-T group was numerically higher compared with other groups.

Graph 5 and 6 – SIgA concentration in duodenum and ileum mucosa of post-weaning piglets. [a,b]different letters indicate a significant difference.



Graph 5

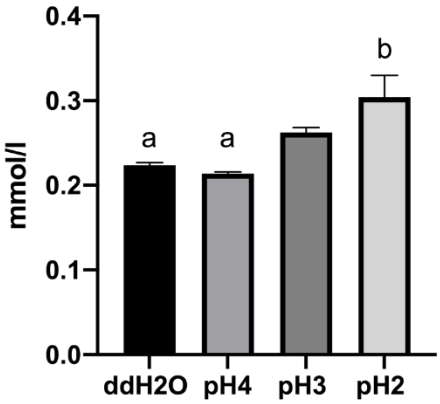


Graph 6

5.4.5 In vitro release of Calcium from C12-Ca soaps.

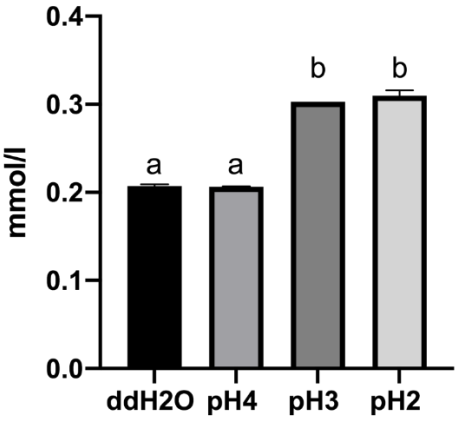
The results presented in the graphs 7, 8 and 9 shown the digestion of lauric acid saponified with calcium simulated in different pH environments and in water at different times. At time 0, the results showed a lower calcium release from the product in water and at pH 4 compared to pH 3 ($P<0.05$).

Graph 7 – In vitro release of calcium in water, pH 4, pH3 and pH 2 at time 0. [a,b]different letters indicate a significant difference.

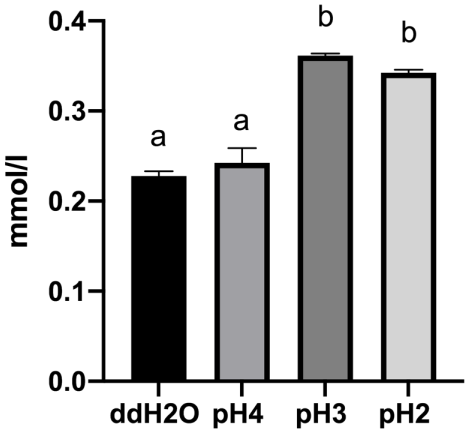


On the other hand, at time 2 (after 2 hours) and time 3 (after 3 hours), the release of calcium was significantly lower in water and at pH 4, compared to pH 3 and pH 2 ($P<0.05$).

Graph 8 and 9 – In vitro release of calcium in water, pH 4, pH3 and pH 2 after 2 hours (time 2) and after 3 hours (time 3). [a,b]different letters indicate a significant difference.



Graph 8



Graph 9

5.5 DISCUSSION

Weaning is often associated with undesirable morphological and physiological changes in the piglet's gastrointestinal environment related to a reduced voluntary feed intake, which subsequently increase susceptibility to intestinal dysfunction (Pluske et al., 1997; Lalles et al., 2004). Since the ban on antibiotics as growth promoters and with their increasing restriction, measures to alleviate weaning-associated intestinal dysfunction and growth retardation have focused on post-weaning dietary manipulations (Pierce et al., 2005; Boudry et al., 2007). In this context, dietary inclusion of medium chain fatty acids (MCFAs) to pigs' diets has received increasing attention in recent years due to their multiple positive effects on growth performance and gut health (Zentek et al., 2011; Hanczakowska et al., 2017).

Fatty acids are a major energy source, important components of the cell membrane, metabolic substrates in many biochemical pathways, cell-signaling molecules, and play a critical role as immune modulators (Calder, 2009). In particular, fatty acids with aliphatic tails of 6 to 12 carbon atoms are called MCFAs, which occur naturally as medium-chain triglycerides (MCT) in milk fat and various feed materials, especially coconut, palm oils and Cuphea seed oils (Jensen, 2002; Dayrit, 2015). Due to their many positive effects, MCFAs are great for feeding young animals, especially piglets (Odle, 1997; Baltic et al., 2017). In fact, MCFAs can be utilized directly by the enterocytes for energy production and thereby help to support the integrity of the intestine in young piglets (Guillot et al., 1993). MCFAs have also been suggested to improve gut health under inflammatory conditions. However, evidence in pigs is not always confirmed. Additionally, MCFAs have been shown to have antimicrobial and antiviral activity in gastric lining and small intestine of pigs. Zentek et al., (2012) reported that low dietary MCFAs supplementation affected gastric microbial ecology, decreased propionic, butyric and valeric acid concentrations, and increased acetic acid concentration in the small intestine of weanling piglets. MCFAs are mainly considered to be anionic surfactants, which, as a result of this property, have antibacterial effects (Mroz et al., 2006). Membrane destabilization by the incorporation of MCFAs into the bacterial cell wall and cytoplasmic membrane, as well as the inhibition of bacterial lipases, which are necessary for the colonization of the skin and the intestinal mucosa, are considered the cardinal mechanisms (Zentek et al., 2011).

Some research has indicated that maternal dietary supplementation may influence growth performance, immune status and gastrointestinal health of weanling pigs (Patterson et al., 2008;

Bontempo et al., 2004). However, until recently, nutrition of the pregnant sow has received little attention in general in the scientific community because of the length of studies and number of animals needed for statistical differences to be observed and because response criteria including birth weight, sow longevity, and offspring performance make sow gestation research complicated. For all practical purposes, this is a relatively untapped field with great potential to benefit swine production. In this context, the use of lauric acid (a MCFA) saponified with calcium (C12-Ca soaps) in the last phase of gestation of sows, may be considered an innovative nutritional strategy to improve the performance and intestinal health of piglets at weaning.

In the present study it was shown that C12-Ca soaps improved the BW of the CTR-T group compared to CTR-CTR at 26-day trial. On the other hand, the absence of differences in the first phase of the post-weaning period (first 14 trial days) can be justified by the stress that piglets are usually subjected at weaning. In fact, it is well known that piglets in the first 14 days after weaning lost their appetite. As a consequence, the weight gain is reduced. Therefore, taking into account the stressful situation to which all the pigs in the trial were subjected in the first 14 days, regardless of the dietary group, it can be considered natural that there were no differences in the BW of the animals. Subsequently, the pigs adapted to the change and therefore, it was possible to highlight a significant weight difference particularly in the CTR-T group at 26 days of trial. This result could lead to hypothesize a positive effect of C12-Ca soaps on the growth performance of piglets belonging to the CTR-T group, compared to those of the CTR-CTR group. However, no significant differences were found with the other groups. Furthermore, the results related to the ADG were homogeneous among the groups.

Nevertheless, the positive result that C12-Ca soaps recorded for the weight in the CTR-T group was confirmed by the results concerning the feed intake. In fact, the ADFI of piglets belonging to the CTR-T group was significantly higher compared both with CTR-CTR and T-T groups from 15 to 26 days of trial and for the whole duration of it. On the other hand, the FE results were conflicting as piglets belonging to the CTR-CTR and T-T groups showed better feed utilization than the other two groups, both from 15 to 26 days and for the whole duration of the trial (0-26 trial days).

The growth performance results in the present study are in agreement with the results obtained in our previous study (Giorgi et al., 2020 submitted) where C12-Ca soaps (1 kg/ton) was administered to post-weaning piglets compared to a basal diet and a diet supplemented with amoxicillin. The results obtained did not show a clear improvement effect on the growth performance of piglets fed with C12-Ca soaps, although some parameters were positive.

In this context, it should be considered that the health of the piglets during the trial was generally good. The percentage of mortality was very low (0.9%) with only one pig dead and antibiotic treatments were also few in number and without significant differences between the groups.

In developing management and nutritional strategies to maximize growth performance and health of pigs, it is critical to consider also the effect of inflammation on gastrointestinal (GIT) function. As we know, the GIT is not only an important organ for digestion, absorption and metabolism of dietary nutrients, but also is the largest immune organ in the body, which comprises more than 70 % of the body's immune cells (Blikslager et al., 2007). Calprotectin is an inflammatory protein released from neutrophils and activated macrophages (Lallès and Fagerhol, 2005; Lallès et al., 2007). Moreover, fecal calprotectin is considered a non-invasive marker of gut inflammation and has been widely utilized in human gastrointestinal diagnostics (Xiao et al., 2014). However, the use of porcine fecal calprotectin as a gut inflammatory marker in pigs, has not been widely used in pig samples, until recently (Lallès and Fagerhol, 2005). In our study, fecal calprotectin concentration was used to assess the intestinal inflammatory status of pigs following treatment with C12-Ca soaps. In particular, the concentration of calprotectin was significantly lower in piglets of the T-T and T-CTR groups, in particular at 14 days post-weaning, the most delicate period after weaning. Therefore, this was an expected result, as intestinal inflammation is higher in the first 14 days after weaning. This result also allows us to hypothesize that in this case, the administration of C12-Ca soaps in the last part of the gestation of the sows, had an anti-inflammatory effect on the intestines of their offspring. In fact, piglets with a lower fecal calprotectin concentration were those born from sows to which the product was administered. On the other hand, no differences were highlighted at 26 days post weaning between groups, as the most critical moment of weaning had passed. Therefore, it can be assumed that the intestinal inflammatory state had stabilized.

It is known that oxidative stress and the inflammatory process are closely related and tend to stimulate each other, through the activation of NF-kb, Nuclear-factor kb (Biswas, 2016). In this context, the confirmation that C12-Ca soaps had a positive effect on the gut health of piglets, both at an inflammatory level but also on the antioxidant state, was also highlighted by the results obtained on intestinal TAOC. In fact, the results concerning the TAOC in duodenum mucosa showed that the antioxidant status of the intestinal mucosa of the pigs of the T-T group was numerically higher compared with the other groups, although the difference was not statistically significant. On the other hand, TAOC in the ileal mucosa was homogeneous between groups. This result is partially in agreement with our previous work (Giorgi et al., 2020

submitted) in which the use of Ca-laurate (1 kg / ton) in the diet of post-weaning piglets, showed a higher TAOC in all the segments of the small intestine (duodenum, jejunum and ileum) in the group treated with C12-Ca soaps compared to the control group and the group treated with amoxicillin.

The antioxidant and anti-inflammatory effect of MCFAs has been highlighted by numerous other authors. For example, Sengupta et al., (2014), investigated the direct antioxidant effect of caprylic, capric and lauric acid, administered through a rice bran oil, to rats which were induced oxidative stress by administering arsenic. The activities of the antioxidant enzymes Catalase (CAT), superoxide dismutase (SOD) and Glutathione Peroxidase (GPx) were significantly increased in brain, liver and red blood cells. The antioxidant and anti-inflammatory effect of MCFAs was also noted by Lee and Kang (2017), both *in vitro* (on IPEC-J2 cells) and *in vivo* on pigs, after administration of cyclophosphamide. According to the authors, the anti-inflammatory action of MCFAs lies in two characteristics: the inhibition of NK-kb activity and the binding with another family of nuclear transcription factors, the Peroxisome Proliferator-Activated Receptors (PPARs), and in particular with the PPAR-γ. The improved intestinal antioxidant activity, particularly in the group of pigs born from mothers fed with C12-Ca soaps and to which the product was administered during the post-weaning phase, allows us to hypothesize that the C12-Ca soaps had a positive effect in preventing the oxidation of endogenous lipids and oxidative damage during weaning. Furthermore, the administration of C12-Ca soaps to sows may have played a role in improving the TAOC of the offspring, although the result is not statistically significant.

However, discordant results were highlighted in the concentration of SIgA on the intestinal mucosa of piglets, in particular in the duodenum. SIgA are some of the main antibodies of the intestinal mucosa. Physiologically, the IgA secreted by the plasma cells of the intestinal lamina propria binds to the Ig receptors of the enterocytes. This is followed by the endoproteolytic cleavage of the Ig receptor near the plasma membrane which releases the secretory component in the lumen for the formation of the SIgA complex (Mantis et al., 2011). This antibody has been shown to play a crucial role in the intestinal mucosal immune system as a specific first line of defense, thanks to its ability to prevent adhesion and invasion by pathogenic bacteria and neutralize the toxins produced by them. Therefore, the level of SIgA in the intestine was used as an indicator to evaluate intestinal mucosal immunity. Low concentrations of SIgA have been reported to be associated with increased bacterial adhesion to the mucosa (Shen et al. 2014; Wu et al., 2016). In the present study, the concentration of SIgA in the duodenal mucosa of piglets was significantly lower in the animals belonging to the

T-T group than in the CTR-CTR and CTR-T groups. Similarly, the SIgA concentration in ileum mucosa was numerically higher in CTR-T group compares with others, even if the difference was not significative. The low concentrations of intestinal SIgA in the T-T group may indicate a decrease in defensive capacity of the piglet's mucosal immune system. This result may be related to the only animal that died in the trial belonging to the T-T group, which, following a lower mucosal immunity, may have been more susceptible to *S. suis* infection. This hypothesis is also supported by the fact that the T-CTR and T-T group recorded more antibiotic treatments against *S. suis* than the other two groups.

It is well known that the free form of MCFAs, and of fatty acids in general, are rapidly absorbed in the first tract of the small intestine. Moreover, MCFAs are absorbed more efficiently by the gastrointestinal tract than other fatty acids. In fact, pigs can partially absorb MCFAs starting from the stomach (Liu 2015). For this reason, MCFAs are often administered in the diet of pigs in a protected form to retard their absorption. An innovative form of administration is represented by the saponification of MCFAs (Giorgi et al., 2020 submitted). In the previous study conducted by our research group, it was hypothesized that the saponification of lauric acid with calcium could delay the absorption of the fatty acid allowing it to reach the small intestine. However, the hypothesis had not been confirmed. Instead, in the present study, through an *in vitro* simulation of the digestion that C12-Ca soaps faces in the stomach of a post-weaning pig, it was possible to demonstrate how this product is able to bypass the stomach and reach the duodenum. In fact, the results obtained by simulating the digestion of C12-Ca soaps in solutions with different pH compared to distilled water, showed that calcium does not dissociate from lauric acid at pH 4 as well as in water compared to pH 2 and 3.

The pH of the piglets' stomach, after the first hours of lactation after birth, drops to around 4 to remain there until weaning and, in most cases, during the first three to four weeks after weaning. Callegari and colleagues (2015) found that at 63 days of life (40 days after weaning) the stomach pH of piglets who had received a basal diet without any supplement was 4.39 on average. Indeed, weaning pigs have a limited ability to secrete HCl, which can cause a higher gastric pH (Efird et al., 1982). It is only about 3 weeks after weaning that gastric pH gradually drops to mature levels (2 to 3) (Roth, 2000; Callegari et al., 2015). Considering that the piglets used in the trial object of this study were 54 days old, we can assume that the pH of their stomach was about 4. Therefore, it is possible to state that C12-Ca soaps was able to overcome the stomach and reach the small intestine where it exerted the positive effects that we have highlighted both in the course of this study and in the previous study (Giorgi et al., 2020 submitted).

5.6 CONCLUSION

The administration of C12-Ca soaps in the diet of post-weaning piglets born from sows fed or not with the same product in the last 3 weeks of gestation, showed positive effects on some parameters of piglets' gut health. In particular, C12-Ca soaps improved the intestinal inflammatory status of piglets born from mothers fed the product. Moreover, the intestinal TAOC was numerically increased in pigs fed C12-Ca soaps. The results relating to piglets' growth performance and to the intestinal immune effect need further study. Similarly, the maternal effect from the administration of C12-Ca soaps and the effect of the product on the mothers themselves, is to be further investigated, considering that in this study the objective was to investigate the action of C12-Ca soaps mainly on the piglet growth and health. In this study, it was also demonstrated how saponification is effective in protecting lauric acid from digestion that takes place in the stomach, allowing it to reach the small intestine.

5.7 REFERENCES

Anadón A. 2006. The EU ban of antibiotics as feed additives: Alternatives and consumer safety. J Vet Pharmacol Ther. 29: 41–44.

Azain MJ. 1993. Effects of adding medium-chain triglycerides to sow diets during late gestation and early lactation on litter performance. J Anim Sci 71: 3011-3019.

Bailey M, Haverson K, Inman C, Harris C, Jones P, Corfield G, Miller B, Stokes C. 2005. The development of the mucosal immune system pre- and post-weaning: Balancing regulatory and effector function. Proc Nutr Soc 64: 451–457.

Baltić B, Starčević M, Đorđević J, Mrdović B, Marković R. 2017. Importance of medium chain fatty acids in animal nutrition. Int J Earth Sci. 85, 012048.

Biswas SK. 2016. "Does the Interdependence between Oxidative Stress and Inflammation Explain the Antioxidant Paradox?" - Oxidative Medicine and Cellular Longevity, Vol. 2016, Article ID 5698931.

Blikslager AT, Moeser AJ, Gookin JL, Jones SL, Odle J. 2007. Restoration of barrier function in injured intestinal mucosa. Physiol Rev 87:545–64.

Bontempo V, Sciannimanico D, Pastorelli G et al. 2004. Dietary conjugated linoleic acid positively affects immunologic variables in lactating sows and piglets. J Nutr 134: 817–824.

Boudry C, Buldgen A, Portetelle D et al. 2007. Effects of oral supplementation with bovine colostrum on the immune system of weaned piglets. Res Vet Sci 83: 91–101.

Calder PC. 2009. Fatty acids and immune function: relevance to inflammatory bowel diseases. Int Rev Immunol 28:506–34.

Callegari MA, Novais AK, Olivieira ER, Dias CP, Schmoller DL, Pereira M Jr, Dario JGN, Alves JB, de Silva CA. 2015. Microencapsulated acids associated with essential oils and acid salts for piglets in the nursery phase. Ciencias Agrarias 4: 2193-2208.

Campbell JM, Crenshaw JD, Polo J. 2013. The biological stress of early weaned piglets. J Anim Scie Biotechnol 4: 19.

Cappai MG, Dimauro C, Arlinghaus C, Sander SJ, Pinna W, Kamphues J. 2020. Subluminal focal lesions in Peyer's patches in the terminal ileum of pigs fed with different physical forms of one same diet. Front Vet Sci 7: 1-8.

Castillo MSM, Martin-Orue M, Roca EG, Manzanilla I, Badiola JF, Perez J, Gasa J. 2006 The response of gastrointestinal microbiota to avilamycin, butyrate and plant extracts in early-weaned pigs. J Anim Sci 84: 2725–2734.

Chen J, Xu Q, Li Y, Tang Z, Sun W, Zhang X, Sun J, Sun Z. 2019. Comparative effects of dietary supplementations with sodium butyrate, medium chain fatty acids, and n-3 polyunsaturated fatty acids in late pregnancy and lactation on the reproductive performance of sows and growth performance of suckling piglets. J Anima Sci 10: 4256-4267.

Corino C, Pastorelli G, Rosi F et al. 2009. Effect of dietary conjugated linoleic acid supplementation in sows on performance and immunoglobulin concentration in piglets. J Anim Sci 87: 2299–2305.

Dayrit FM. 2015. The Properties of Lauric Acid and Their Significance in Coconut Oil. J Am Oil Chem Soc. 92, 1-15.

Efird RC, Armstrong WD, Herman DL. 1982. The development of digestive capacity in young pigs: effects of age and weaning system. J Anim Sci 55: 1380–1387.

Ferrara F, Tedin L, Pieper R, Meyer W, Zentek J. 2016. Influence of medium-chain fatty acids and short-chain organic acids on jejunal morphology and intra-epithelial immune cells in weaned piglets. J Anim Physiol Anim Nutr 1–10.

Giorgi S, Comi M, Ghiringhelli M, Cheli F, Rebucci R, Bontempo V. 2020. The saponification of Lauric acid with calcium soaps as an alternative to infeed antibiotics in post-weaning piglets, Italian Journal of Animal Science, Vol. V, No. 1s, ISSN: 2283-3927, submitted.

Guillot E, Vaugelade P, Lemarchal P, Rérat A. 1993. Intestinal absorption and liver uptake of medium-chain fatty acids in non-anaesthetized pigs. Br J Nutr 69: 431–42.

Hanczakowska E. 2017. The use of medium-chain fatty acids in piglet feeding – a review. J Anim Sci. 4, 967-977.

Jackman JA, Boyd RD, Elrod CC. 2020. Medium-chain fatty acids and monoglycerides as feed additives for pig production: towards gut health improvement and feed pathogen mitigation. J Animal Sci Biotechnol 11, 44.

Jensen RG. 2002. The composition of bovine milk lipids: January 1995 to December 2000. J Dairy Sci. 85, 295–350.

Jiang XR, Agazzi A, Awati A, Vitari F, Bento H, Ferrari A, Alborali GL, Crestani M, Domeneghini C, Bontempo V. 2015. Influence of a blend of essential oils and enzyme combination on growth performance, microbial counts, ileum microscopic anatomy and the expression of inflammatory mediators in weaned piglets following an Escherichia Coli infection. An Feed Sci Technol. 209, 219-229.

Jiang XR, Li XL, Awati A, Bento H, Zhang HJ, Bontempo V. 2017. Effect of an essential oils blend on growth performance, and selected parameters of oxidative stress and antioxidant defence of Escherichia Coli challenged piglets. J Anim Feed Sci. 26, 38-43.

Kabara JJ, Swieczkowski DM, Conley AJ and Truant JP. 1972. Fatty acids and derivatives as antimicrobial agents. Antimicrob Agents Chemother 2:23–28.

Lallès J, Formal P and Fagerhol M. 2007. Changes in calprotectin concentration in sow's milk throughout lactation. Paper presented at the Animal health, animal welfare and biosecurity. Proceedings of 13th International Congress in Animal Hygiene, Tartu, Estonia 1: 17-21.

Lallès JP and Fagerhol MK. 2005. Faecal Calprotectin: A non-invasive marker of inflammation in pigs? Paper presented at the Proceedings of the XIIth International Congress of the International Society for Animal Hygiene, Warsaw, Poland.

Lallès JP, Boudry G, Favier C, et al. 2004. Gut function and dysfunction in young pigs physiology. Anim Res 53: 301–316.

Lee SI, Kang KS. 2017. Function of capric acid in cyclophosphamide-induced intestinal inflammation, oxidative stress, and barrier function in pigs. 2017. Scientific RepOrTs 7: 16530 DOI:10.1038/s41598-017-16561.

Liu Y. 2015. Fatty acids, inflammation and intestinal health in pigs. A review. J Anim Sci Biotechnol 41:1-9.

Mantis NJ, Rol N, Corthésy B. 2011. Secretory IgA's complex roles in immunity and mucosal homeostasis in the gut. Mucosal Immunol 4 :603-61

Mroz Z, Koopmans SJ, Bannink A, Partanen K, Krasucki W, Overland M, et al. Carboxylic acids as bioregulators and gut growth promoters in nonruminants. 2006. In: Mosenthin R, Zentek J, Zebrowska T, editors. Biology of nutrition in growing animals. Edinburgh UK: Elsevier.

Odle J. 1997. New insights into the utilization of medium-chain triglycerides by the neonate: Observations from a piglet model. J Nutr 127: 1061–7.

Patterson R, Connor ML, Krause DO et al. 2008. Response of piglets weaned from sows fed diets supplemented with conjugated linoleic acid (CLA) to an *Escherichia coli* K88+oral challenge. Anim 2: 1303–1311.

Pierce KM, Sweeney T, Brophy PO et al. 2005. Dietary manipulation post weaning to improve piglet performance and gastro-intestinal health. Anim Sci 81: 347–356.

Pluske JR, Hampson DJ, Williams IH. 1997. Factors influencing the structure and function of the small intestine in the weaned pig: a review. Livest Prod Sci 51: 215–236.

Roth F. 2000. Acidos organicos en nutrition porcina: eficacia y modo de acción. In: Curso de especialización, 11, 2000, Barcelona. Fundacion Espanola para El Desarrollo de la nutrition animal – FEDNA 2000, pp. 169-181.

S´wiatkiewicz M, Hanczakowska E, Okon´ K, Kowalczyk P, Grela ER. 2020. Effect of Maternal Diet and Medium Chain Fatty Acids Supplementation for Piglets on Their Digestive Tract Development, Structure, and Chyme Acidity as Well as Performance and Health Status. Anim 10: 834. doi:10.3390/ani10050834

Sengupta A, Ghosh M, Bhattacharyya DK. 2014. Antioxidative effect of rice bran oil and medium chain fatty acid rich rice bran oil in arsenite induced oxidative stress in rats. J Oleo Sci 11:1117-1124

Shen J, Chen Y, Wang Z, Zhou A, He M, Mao L, Zou H, Peng Q, Xue B, Wang L, Zhang X, Wu S, Lv Y. 2014. Coated zinc oxide improves intestinal immunity function and regulates microbiota composition in weaned piglets. Brit J Anim Nutr 111: 2123–2134

Skrivanová E, Molatová Z, Skrivanová V, Marounek M. 2009. Inhibitory activity of rabbit milk and medium-chain fatty acids against enteropathogenic Escherichia coli. Vet Microbiol 135: 358–362.

Tang M, Laarveld B, Van Kessel AG, Hamilton DL, Estrada A, Patience JF. 1999. Effect of segregated early weaning on postweaning small intestinal development in pigs. J Anim Sci 77: 3191–3200.

Tuchscherer M, Otten W, Kanitz E, Gräbner M, Tuchscherer A, Bellmann O, Rehfeldt C, Metges CC. 2012. Effects of inadequate maternal dietary protein:carbohydrate ratios during pregnancy on offspring immunity in pigs. BMC Vet Res 8: 232. doi: 10.1186/174661488232.

Wu JJ, Cao CM, Ren DD, Zhang Y, Kou YN, Ma LY, Feng SB, Li Y, Wang XC. 2016. Effects of soybean antigen proteins on intestinal permeability, 5-hydroxytryptamine levels and secretory IgA distribution in the intestine of weaned piglets. Ita J Anim Sci 1: 174–180

Xiao D, Wang Y, Liu G, He J, Qiu W, Hu X, Feng Z, Ran M, Nyachoti CM and Kim SW. 2014. Effects of chitosan on intestinal inflammation in weaned pigs challenged by enterotoxigenic Escherichia coli. PLoS One. 9: e104192.

Zentek J, Buchheit-Renko S, Ferrara F, Vahjen W, Van Kessel AG, Pieper R. 2011. Nutritional and physiological role of medium-chain triglycerides and medium-chain fatty acids in piglets. Anim Health Res Rev 12:83–93

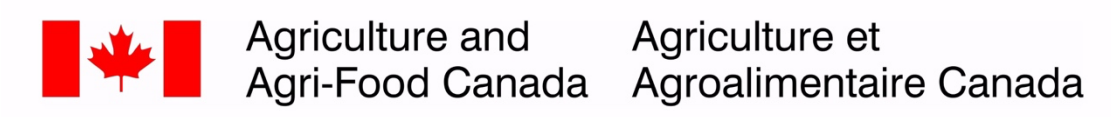
Zentek J, Buchheit-Renko S, Männer K, Pieper R, Vahjen W. 2012. Intestinal concentrations of free and encapsulated dietary medium-chain fatty acids and effects on gastric microbial

ecology and bacterial metabolic products in the digestive tract of piglets. Arch Anim Nutr. 66: 14–26.

CHAPTER 6 -

Evaluation of the effects of fatty acids and milk fractions on IPEC-J2 porcine cell line proliferation and viability

In collaboration with Sherbrooke Development and Research Centre – Agricolture and Agrifood Canada, Quebec



6.1 ABSTRACT

The fatty acids (FAs) of different chain length have been suggested as an alternative to the use of antibiotics in animal production with promising results. Moreover, the role of substances derived from bovine milk in wound healing has been investigated and there are increasing evidences showing that milk and colostrum components protect gut against injuries and promote gut development. In this study a porcine in vitro model using the porcine cell line IPEC-J2 was used to test the effects of butyric acid (C4), lauric acid (C12) and different bovine milk fraction (complete milk, milk cream and defatted milk) on intestinal cell proliferation. Cell proliferation assay (XTT test) was performed on both growing and differentiated cells using different FAs (100µmol, 50µmol and 20µmol) and milk fractions (20%, 10%, 5% and 1%) concentration. The results showed that 100µmol of C12 and C4 improved differentiated cell proliferation compared with CTR ($P<0.05$) whereas, no significative differences were found in growing cells. Regarding the milk fractions effect, results showed that the exposure of IPEC-J2 growing cell to 10% milk cream improved cell proliferation compared to complete milk and defatted milk ($P<0.05$), comparably with CTR. On the other hand, 5% cream milk showed an increase in cell proliferation compared to both CTR and cream and defatted milk. No differences were found on growing cell proliferation for 20% and 1% milk fractions compared with CTR. Similarly, no significative differences were registered on differentiated cell proliferation exposed to 20%, 10%, 5% and 1% milk fraction. In conclusion, exposure of porcine IPEC-J2 cells to different concentrations of C12 and C4 did not reveal any negative effects on cell growth. Furthermore, 100 µmol of C12 and C4 enhanced the proliferation of differentiated intestinal cells. Similarly, the effect of different milk fractions on pig's intestinal cells had no negative effects on cell growth, improving proliferation especially with 5% cream.

Keywords: fatty acids, bovine milk, IPEC-J2 cell line, cell proliferation, pig

6.2 INTRODUCTION

Gastrointestinal disorders are one of the major causes of losses in pig production. For decades, antibiotics have been successfully used to solve farming problems, especially in delicate moments of pigs' life cycle as weaning. However, there is an increasing concern regarding public health due to the rise of antibiotic-resistant bacteria besides to the impact on the environment (Kemper, 2008; Davies and Davies 2010; Heuer et al., 2011). Nowadays, high efforts are carried out in the search of alternatives to substitute or at least decrease the use of antibiotics in animal production (Allen et al., 2013; Seal et al., 2013).

Fatty acids (FAs) of different chain length have been suggested as alternatives to the use of antibiotics in animal nutrition with promising results, especially on their gut health and growth performance. Furthermore, few adverse effects of FAs have been found, which ensures their easier acceptance as animals feed additives by consumers.

Short chain fatty acids (SCFAs) are FAs fatty acids produced by the bacterial fermentation of residual carbohydrates and proteins in the colonic contents (Sakata 1989). In particular, butyric acid demonstrated to be rapidly absorbed by and provides energy to colonocytes. Moreover, its effect on the colonic tissues in pigs after increasing its production by butyrogenic dietary substrates, or after cecal administration, have been demonstrated (Kien et al. 2007). Additionally, in pig colon, butyrate inhibits apoptosis of mucosal cells in vivo through up-regulating the expression of the anti-apoptotic signal Bcl-2 as shown by Mentschel and Claus (2003). Moreover, the administration of butyrate in piglets diet led to an increase of jejunal villus length and mucosal thickness, suggesting an increase in proliferation of jejunal intestinal cells (Kotunia et al. 2004)

On the other hand, MCFAs are saturated fatty acids with 6-12 carbon atoms. They are found in many natural sources like coconut and palm kernel oil (Dayrit, 2015) and in the milk of different animal species (Breckenridge and Kuksis 1967; Jensen, 2002). Different in vitro and in vivo studies conducted on lauric acid have shown a particular antimicrobial effect against some algae, fungi, protozoa, viruses and Gram-positive bacteria (Desbois and Smith 2010) whereas results on its effect against Gram-negative are contradictory (Zentek et al., 2011). For this reason, lauric acid has the potential to replace antibiotics in feed to prevent post-weaning diarrhea and increase overall pigs' productivity. However, the effects of lauric acid on the intestinal epithelial cells remain unclear.

Nowadays, alternatives are being developed not only to reduce the use of antibiotics in animal production, but also to reduce the use of animals in scientific studies. Intestinal epithelial

cell (IEC) lines give an approach to the mechanisms of signaling pathways related with the interaction between the epithelial cell and bacteria or viruses. Compared with animal model, cell line studies are less expensive, associated with no ethical concerns, and provide highly controlled simple model to investigate isolated factors, for example diet, on the IECs response.

The IPEC-J2 cell line is one of the most often used porcine intestinal in vitro model. IPEC-J2 is a non-transformed and non-tumorigenic intestinal cell line isolated from the jejunum of an unsuckled neonatal piglet (Berschneider, 1989) and is morphological and functionally similar to IECs. This cell line has been used for morphological studies regarding the integrity of epithelial monolayer in microbiological studies (Brosnahan and Brown 2012) or the response to bacterial challenge after pre-treatment with different substances (Spitzer et al., 2016). They have also been used to study the epithelial innate immune responses by measuring the expression of epithelial and immune-related genes (Brasnahan and Brown 2012; Stoy et al., 2013). This cell line can secrete mucin, produce cytokines/chemokines, and express Toll-like receptors similar to those of the original tissue. It conserves its epithelial nature and can serve as a convenient model to simulate innate immune functions of the intestinal epithelium. Two major points demonstrate that IPEC-J2 represents a better model of normal intestinal epithelial cells than transformed cell lines: 1) they maintain their differentiated characteristics and exhibit strong similarities to primary intestinal epithelial cells and 2) IPEC-J2 cells can be an appropriate model through the advantage of direct comparison with the experimental animal (Schierack et al. 2006).

As feed additive to improve the gut health, bovine colostrum and milk are quite interesting because they contain several bioactive molecules with immune-regulatory and antimicrobial properties that are essential for development of immune system (Gill et al., 2000; Smilowitz et al., 2014; Bagwe et al., 2015). The role of substances derived from milk or colostrum in wound healing has been investigated (Velioglu Ogunc et al., 2008; Zava et al., 2009; Blais et al., 2014) and there are increasing evidences showing that milk and colostrum components protect gut against injuries and promote gut development and growth performance (Blais et al., 2014). Therefore, the use of milk and colostrum as a feed additive to promote intestinal health has been proposed, yet little is known about mechanisms implicated in its beneficial properties on intestinal epithelial cells.

For these reasons and taking into account all the positive effects that FAs and milk fractions have on the gut health of piglets, in this study the efficacy of different fatty acids and milk fractions was tested on IPEC-J2 porcine cell line proliferation and viability.

6.3 MATERIALS AND METHODS

The experiment was conducted at the Sherbrooke Research and Development Center (Agriculture and Agrifood Canada, Sherbrooke, Quebec).

6.3.1 Cell culture conditions

Non-transformed porcine intestinal epithelial IPEC-J2 cells, derived from newborn piglet jejunum, were kindly provided by Dr. Joshua Gong (Agriculture and Agri-Food Canada, Guelph, Ontario, Canada). The IPCE-J2 cells were grown in a humidified incubator at 37°C under 5% CO_2 in 25cm^2 cell culture dish (Corning Inc., Corning, NY). Cells were grown in Dulbecco's modified Eagle medium/Ham's F-12 [1:1], namely growing medium (DMEM/F12; Wisent Inc.) supplemented with 5% heat inactivated fetal bovine serum (FBS; Gibco^{TM}), 1% insulin-transferrin-selenium (ITS premix: insulin 1 mg/ml, transferrin 0.6 mg/ml and selenium 0.6 µg/ml; Corning), 5 ng/ml epidermal growth factor (EGF; Corning), 1% L-Glutamine (L-GLU; Wisent Inc.), and 1% antibiotic-antimycotic solution 100X (Multicell). The cells were seeded in 96-well plates at a density of 10^5 Cells/ml and grown for 3-4 days until they reached the confluence. Once confluence was reached, the growing medium was replaced in some of the petri dishes with differentiation medium to achieve cell differentiation. The differentiation medium had the same composition of the growing medium except for the absence of the FBS that stimulate the growth of the cells. The FBS-deprived medium induced differentiation by arresting cells growth. Cell differentiation is the process through which a cell undergoes changes to become a more specific type of cell; intestinal epithelial cells in this case. The main difference between differentiated and growing cells is that differentiated cells are specialized to perform a unique function in the body, whereas growing cells undergo proliferation and are capable of self-renewing. IPEC-J2 cells were selected precisely because are not transformed and have the potential to differentiate (Brosnahan and Brown, 2012).

The medium was replaced every 2 days to avoid nutrient depletion in both growing and differentiating cells. The differentiated cells were ready in 8-10 days after the passage from growing to differentiation of medium.

6.3.2 Proliferation and viability assay using the XTT test

The determination of cellular proliferation, viability and activation are key areas in a wide variety of cell biological approaches. Cell proliferation and viability assays are of particular importance for routine applications. Tetrazolium salts XTT are especially useful for assaying the quantification of viable cells. The assay is based on the cleavage of the yellow tetrazolium salt XTT to form an orange formazan dye by metabolic active cells. Therefore, this conversion only occurs in viable cells. The formazan dye formed is soluble in aqueous solutions and is directly quantified using a scanning multi-well spectrophotometer (ELISA reader). This ensures a high degree of accuracy, enables on-line computer processing of the data (data collection, calculation and report generation) and, thereby, allows the rapid and convenient handling of a high number of samples. Cells, grown in a 96 well tissue culture plate, are incubated with the yellow XTT solution for a minimum of 30 minutes up to a maximum of 24 h. After this incubation period, orange formazan solution is formed, which is spectrophotometrically quantified using an ELISA plate reader. An increase in number of living cells results in an increase in the overall activity of mitochondrial dehydrogenases in the sample. This increase directly correlates to the amount of orange formazan formed, as monitored by the absorbance. When the color turns red the reaction is over. To determine the appropriate dose of FAs and milk fractions for small intestinal epithelial cells used in this study, the viability of IPEC-J2 cells was monitored. Cell proliferation assay (XTT test) was performed on both growing and differentiated cells previously cultured in 96-well plates. When the cells reached confluence, they were further incubated for 24h at 37°C with the different concentrations of FAs (100µmol, 50µmol and 20µmol) and milk fractions (20%, 10% and 5%). After 24h of incubation the wells were washed two times with HBSS (Wisen Inc.). Then, 150 µl of XTT solution suspended in the Opti-MEM was added to each well following the manufacture's instruction (Cell Proliferation Kit II, Roche). Cells were further incubated for a total of 4h and the plates were read at 30 min., 60 min., 2h and 4h. The absorbance was measured at 492/690 nm using a SoftMax Pro 5 plate reader (Molecular Devices). The absorbance measured at 690 nm served as the background and was subtracted from that measured at 492 nm. In total, three independent experiments were done in triplicate. The results were compared with the mean of the negative control group to be expressed in % of absorbance.

6.3.3 Fatty acids used on IPEC-J2 cell line

During the test different type of fatty acids were used. The MCFAs tested in this trial was lauric acid (C12). Together with lauric acid, butyric acid (C4) have also been used, to better compare the possible different effects of both MCFAs and SCFAs on cells proliferation. Lauric acid was provided by Sigma Aldrich Canada whereas butyric acid was provided by Millipore. The stock solution 100Mm of lauric acid, was prepared in 13 mm × 100 mm Pyrex glass tubes with screw caps adding 100 mg FA powder in 1 ml of ethanol 100% and stirred for 1 hour at 37°C. The stock solutions 500 mM for butyric acid have been ordered ready to use and have been used since. After preparation, the stock solutions were stored at -20°C until analysis. Both lauric and butyric acid were tested on IPEC-J2 cells at final concentrations of 100µmol, 50µmol and 20µmol at 1% bovine serum albumin (BSA).

6.3.4 Milk fractions used on IPEC-J2 cell line

The milk used in this study was collected by the team of the dairy farm of the Sherbrooke Research and Development Centre (Agriculture and Agri-food Canada, Sherbrooke). The milk was collected in sterile 50 ml Falcon tubes kept immersed in ice for more than an hour, in order to freeze the fat in the milk. Subsequently, the tubes were centrifuged at 1900 x *g* for 15 min at 4°C. This centrifugation allows the milk to be separated into two layers: the fat on top and the defatted milk on the bottom. After centrifugation, the falcons were put on ice for 10/15 minutes in order to solidify the fat. Then, the fat layer was recovered from the defatted fraction. Milk cream and defatted milk were both aliquoted and stored at -20°C together with other aliquots of complete milk in order to have aliquots of cream, complete and defatted milk. The milk fractions were finally used on IPEC-J2 cells at final concentrations of 20%, 10% and 5% and 1% BSA.

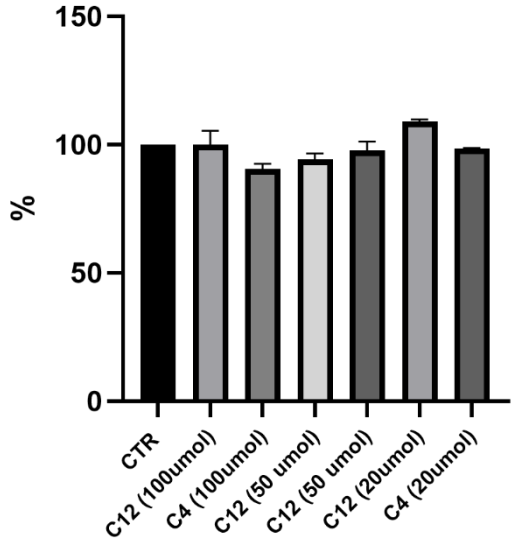
6.3.5 Statistical analysis

Data are presented as means ± SEM. Analysis was performed using GraphPad Prism Version 8 (GraphPad Software). The significant difference in assay values was evaluated using one-way ANOVA, followed by the Tuckey's multiple comparison test. A value of $P < 0.05$ was considered to be statistically significant.

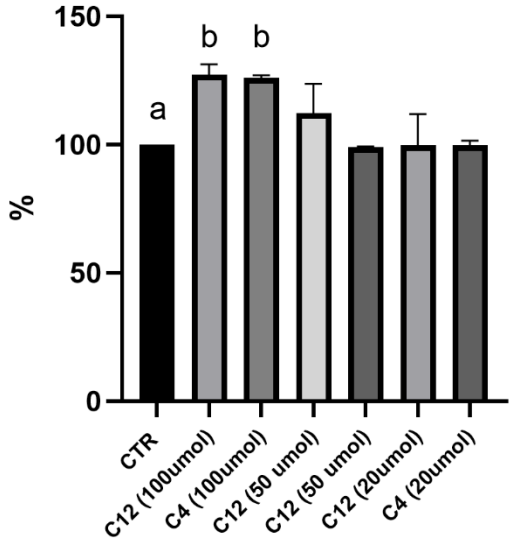
6.4 RESULTS

6.4.1 C12 and C4 effects on growing and differentiated porcine IPEC-J2 cell line proliferation

As shown in Graph 10, no significative differences were found between C12 and C4 compared with CTR in all the FAs concentration tested on growing cell proliferation. On the contrary, Graph 11 shown how the exposure of IPEC-J2 differentiated cells to 100µmol of both C12 and C4 improved cells proliferation ($P<0.05$).



Graph 10 – C12 and C4 effect on growing IPEC-J2 cells proliferation

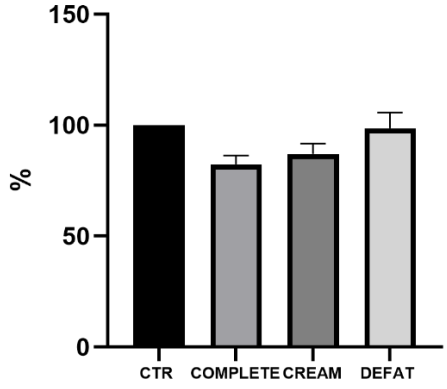


Graph 11 – C12 and C4 effect on differentiated IPEC-J2 cells proliferation. [a,b]different letters indicate a significant difference.

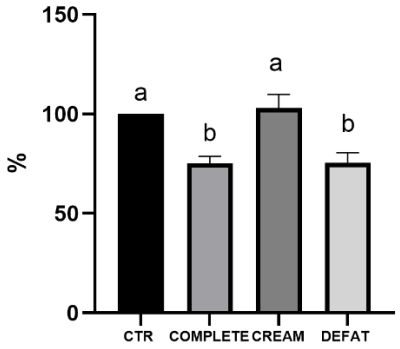
6.4.2 Milk fractions effects on growing and differentiated porcine IPEC-J2 cell line proliferation

No significative differences were found in the effect of 20% and 1% milk fractions on growing IPEC-J2 cell line (Graphs 12 and 15). On the contrary, Graph 13 showed that 10% milk cream, had an improving effect on the proliferation of growing cells, comparable to CTR, and greater than complete milk and defatted milk ($P<0.05$). Moreover, the exposure of growing IPEC-J2

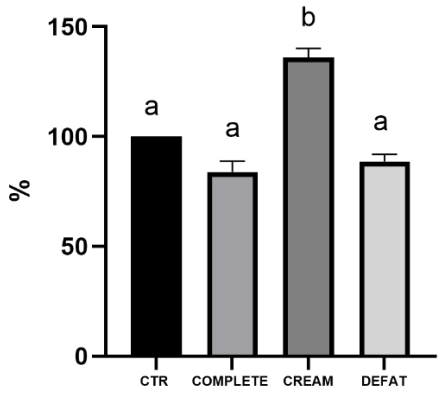
cells to 5% of milk cream (Graph 14) improved cell proliferation compared with CTR, the complete milk and defatted milk ($P<0.05$).



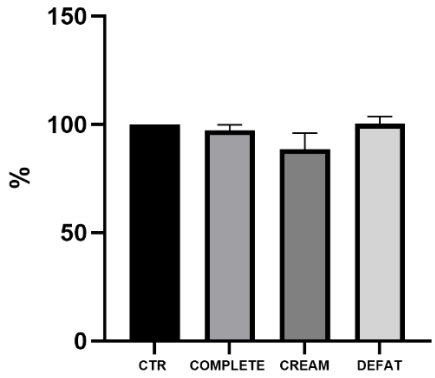
Graph 12 – 20% Milk fractions effect on growing IPEC-J2 cells proliferation



Graph 13 – 10% Milk fractions effect on growing IPEC-J2 cells proliferation. [a,b]different letters indicate a significant difference.

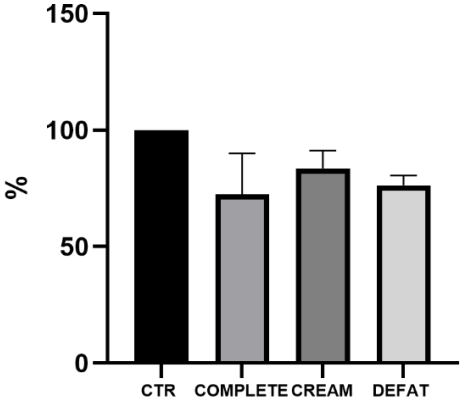


Graph 14 – 5% Milk fractions effect on growing IPEC-J2 cells proliferation. [a,b]different letters indicate a significant difference.

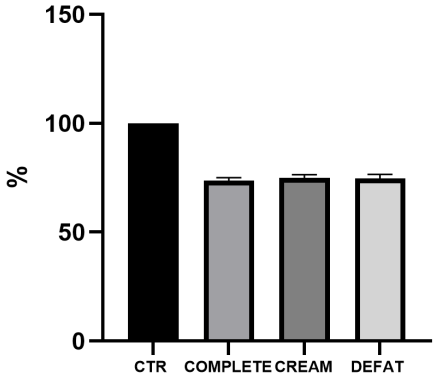


Graph 15 – 1% Milk fractions effect on growing IPEC-J2 cells proliferation

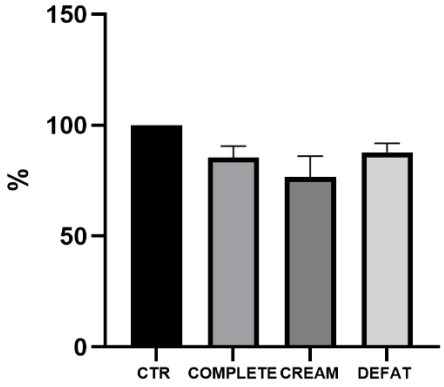
Regarding the effects of the 20%, 5%, and 1% milk fractions on the differentiated IPEC-J2 cell line, results did not show significant differences (Graphs 16, 17, 18 and 19).



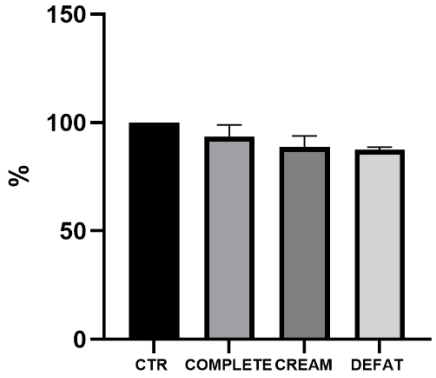
Graph 16 – 20% Milk fractions effect on differentiated IPEC-J2 cells proliferation



Graph 17 – 10% Milk fractions effect on differentiated IPEC-J2 cells proliferation



Graph 18 – 5% Milk fractions effect on differentiated IPEC-J2 cells proliferation



Graph 19 – 1% Milk fractions effect on differentiated IPEC-J2 cells proliferation

6.5 DISCUSSION

Intestinal mucosa undergoes a continual process of proliferation, differentiation, and apoptosis. In particular, intestinal epithelium displays the highest turnover of all solid organs. In pigs, intestinal epithelial cell proliferation and growing is very active and the whole population is entirely renewed every 2 to 3 days (Gelberg, 2014). This is necessary because it must maintain a constant and effective barrier function in a harsh intestinal environment and, at the same time, ensure the absorption of all the necessary nutritional principles (Barker et al., 2012).

In the present study, we used non-transformed porcine jejunum-derived IPEC-J2 cells to study the effect of butyric acid (C4), lauric acid (C12), and different milk fractions on cell proliferation. The choice of this cell line is given by the fact that it is a cellular line well known for forming confluent polarized monolayers with apical and basolateral regions, allowing an in vitro model of the intestinal epithelium.

Butyric acid already proven to have effects on cell proliferation (Blottiere et al., 2003). In general, it has proven to have many positive effects on the gut health of piglets as ease the transition of piglet weaning (de Lange et al., 2010; Heo et al., 2013; Thacker et al., 2013), improve growth performance (Piva et al., 2002a), stimulate gut maturation (Mazzoni et al., 2008; Mallo et al., 2012) and increase mucosa thickness (Kotunia et al., 2004). These studies show the potential proliferative effects of butyrate on the porcine intestine.

Similarly, lauric acid have been proposed as a potential alternative to antibiotics based on its long-known antibacterial activity (Skrivanova et al., 2006). In contrast to antimicrobial agents, lauric acid (as all the other MCFAs) have not shown evidence of acquiring resistance (Manohar et al., 2013). Besides this, lauric acid is an immediate source of energy for pigs, due to its rapid passive absorption and digestion (Li et al., 2015; Schonfeld et al., 2016). However, although the effects of lauric acid on intestinal epithelial cells have already been partially investigated (Yang et al., 2020), its real effects remain unclear.

In the present study, the exposure of porcine IPEC-J2 cell line to both C4 and C12 did not cause negative effects on cell proliferation and viability. In fact, the results were perfectly comparable to the control, so cell proliferation was not reduced. Indeed, C4 and C12 had an even better effect on differentiated cell proliferation compared with control, when their concentration was 100 μmol. Although the other concentrations used (50 μmol and 20 μmol) were also not deleterious to cell health, we can hypothesize that a higher concentration of both C12 and C4 was able to bring greater benefits to the cells. These results are in agreement with the work of Yang et al., (2020), who demonstrated that 0.25-0.1 mM of C12 promoted IPEC-J2 cell differentiation. On the contrary, at 1 mM or higher concentrations, it induced IPEC-J2

cell viability decreases, lipid accumulation, cell proliferation inhibition, and cell apoptosis. This dual effect was observed also in primary culture in the work of Blottiere et al., (2003). Indeed, at low concentrations (0.05 and 0.1 mM) butyrate mildly stimulated cell proliferation. At >1 mM butyrate dose-dependently inhibited cell proliferation.

To consider milk as a nutrient providing only necessary nutrition to postnatal development in young mammals is now an outdated concept. Milk from different species contains more than 2000 molecules, some of them exhibiting biological properties: molecules that facilitate the uptake of nutrients, antibacterial substances and enzymes, alongside hormones and growth factors (Guimont et al., 1997). In the past, many cell culture researchers have used milk fractions or by-products as components in culture media or as a substitute for fetal calf serum (FCS) due to the nutritional properties of milk or its fractions. It should be also mentioned that, in addition to the basic nutritional medium, culture media for animal cells contain growth factors, adhesion factors, and transport proteins, and that these three types of factors are naturally present in milk. Furthermore, intestinal explants incubated in media with or without colostrum revealed that it may stimulate the expression of genes involved in epithelial cell migration along the crypt–villous axis and genes bearing anti-apoptotic properties (Huguet et al., 2007). Therefore, it is possible to say that bovine milk and/or colostrum has numerous positive effects on cellular health *in vitro*.

Studies *in vivo*, have shown that bovine colostrum, when included in diets for weaning piglets, led to increased growth performance (Pluske et al., 1999). Such an effect on growth was associated with increased feed intake and the results showed that colostrum had a positive effect of up to five weeks after the end of the study (Le Huërou-Luron et al., 2004).

The results obtained in our study showed that, in general, cell proliferation was not adversely affected by exposure to milk fractions, although, especially in differentiated cells, proliferation was numerically slightly lower than control, but the difference was not significant. On the contrary, the exposure of IPEC-J2 growing cell to 10% milk cream improved cell proliferation compared to complete milk and defatted milk. This effect was totally comparably to CTR. Moreover, 5% cream milk showed an increase in cell proliferation compared to both CTR and cream and defatted milk. These results are in line with the work of Blais et al., 2014, where the authors highlighted that both colostrum and cheese whey, used on IPEC-J2 cell line to investigate their effects on mechanisms involved in the cellular wound-healing process, increased cell proliferation, with the highest values observed with the highest concentration tested (10 mg/ml).

6.6 CONCLUSION

In conclusion, lauric acid and butyric acid not reveal any negative effects on porcine intestinal cell proliferation. Furthermore, 100 μmol of both lauric acid and butyric acid enhanced the proliferation of differentiated cells. Similarly, the effect of complete milk, milk cream and defatted milk on pig's intestinal cells was not deleterious in general. Moreover, cell proliferation improved with 5% cream milk. Therefore, it is possible to state that lauric acid, butyric acid and some milk fractions have a positive effect on pig's intestinal health, improving cell proliferation without negative effects.

6.7 REFERENCES

Allen HK, Levine UY, Looft T, Bandrick M. Casey TA (2013). Treatment, promotion, commotion: antibiotic alternatives in foo-producing animals. Trends Microbiol 21:114-119.

Bagwe S, Tharappel LJ, Kaur G, Buttar HS (2015). Bovine colostrum: An emerging nutraceutical. J ComplemenT Integr Med 12:175-185.

Barker N, Van Es JH, Kuipers J, Kujala P, Van Den Born M, Cozijnsen M, Haegebarth A, Korving J, Begthel H, Peters PJ, Clevers H. 2007. Identification of steam cells in small intestine and colon by marker gene Lgr5. Nature. 449: 1003-1007.

Berschneirder H (1989). Development of normal cultured small intestinal epithelial cell lines which transport Na and Cl. Gastroenterology 96:A41.

Blais M, Pouliot Y, Gauthier S, Boutin Y, Lessard M. 2014. A gene expression programme induced by bovine colostrum whey promotes growth and wound-healing processes in intestinal ephitelial cells. J Nutr Sci 3: 1-10.

Blottiere HM, Buecher B, Galmiche JP, Cherbut C. Molecular analysis of the effect of short chain fatty acids on intestinal cell proliferation. Proceedings of the nutrition society 62: 101-106.

Breckenridfe WC, Kuksis A (1967). Molecular weight distributions of milk fat triglycerides from seven species. J Lipid Res 8:473-478.

Brosnahan AJ, Brown DR (2012). Porcine IPEC-J2 intestinal epithelial cells in microbiological investications. Vet Microbiol 156:229-237.

Davies J, Davies D (2010). Antibacterial free fatty acids: activities, mechanisms of action and biotechnological potential. Microbiol Mol Biol R 74:417-433.

Dayrit FM (2014). The Properties of Lauric Acid and Their Significance in Coconut Oil. J Am Oil Chem Soc 92:1-15.

de Lange CFM, Pluske J, Gong J, Nyachoti CM. 2010. Strategic use of feed ingredients and feed additives to stimulate gut health and development in young pigs. Livest Sci 134:124–134.

Desbois A, Smith V (2010). Antibacterial free fatty acids: activities, mechanisms of action and biotechnological potential. Appl Microbiol and Biotechnol 85:1629-1642.

Gelberg HB. 2014. Comparative anatomy, physiology, and mechanisms of disease production of the esophagus, stomach, and small intestine. Toxicol Path 42: 54–66.

Gill HS, Doull F, Rutherfurd KJ, Cross ML (2000). Immunoregulatory peptides in bovine milk. Br J Nutr, 84:S111-S117.

Heo JM, Opapeju FO, Pluske JR, Kim JC, Hampson DJ, Nyachoti CM. 2013. Gastrointestinal health and function in weaned pigs: a review of feeding strategies to control post-weaning diarrhoea without using in-feed antimicrobial compounds. J Anim Physiol Anim Nutr Berl 97:207–237.

Heuer H, Schmitt H, Smalla K (2011) Antibiotic resistance gene spread due to manure application on agricultural fields. Curr Opin Microbiol 14:236-243.

Huguet A, Sève B, Le Dividich J, Le Huërou-Luron I. 2006. Effects of a bovine colostrum-supplemented diet on some gut parameters in weaned piglets. Reproduction Nutrition Development 46: 167-178.

Jensen RG. 2002. The composition of bovine milk lipids: January 1995 to December 2000. J Dairy Sci. 85, 295–350.

Kemper N (2008). Veterinary antibiotics in the aquatic and terrestrial environment. Ecol Indic 8:1-13.

Kien CL, Blauwiekel R, Bunn JY, Jetton TL, FrankelWL, Holst JJ. 2007. Cecal infusion of butyrate increases intestinal cell proliferation in piglets. J Nutr 137:916–922

Kotunia A, Wolinski J, Laubitz D, Jurkowska M, Rome V, Guilloteau P. 2004. Effect of sodium butyrate on the small intestine development in neonatal piglets fed [correction of feed] by artificial sow. J Physiol Pharmacol 55:59–68.

Le Huërou-Luron I, Huguet A, Callarec J, Leroux T, Le Dividich J. 2004. Supplementation of a weaning diet with bovine colostrum increases feed intake and growth of weaned piglets. Journ Rech Porc, 33-38.

Li Y, Zhang H, Yang L, Zhang L, Wang T. 2015. Effect of medium-chain triglycerides on growth performance, nutrient digestibility, plasma metabolites and antioxidant capacity in weanling pigs. Anim Nutr 1:12–18.

Mallo J, Balfagon A, Gracia M, Hunrubia P, Puyalto M. 2012. Evaluation of different protections of butyric acid aiming for release in the last part of the gastrointestinal tract of piglets. J Anim Sci 90:227–229.

Manohar V, Echard B, Perricone N, Ingram C, Enig M, Bagchi D, et al. 2013. *In vitro* and *in vivo* effects of two coconut oils in comparison to monolaurin on *Staphylococcus aureus*: rodent studies. J Med Food 16:499–503.

Mazzoni M, Le Gall M, De Filippi S, Minieri L, Trevisi P, Wolinski J. 2008. Supplemental sodium butyrate stimulates different gastric cells in weaned pigs. J Nutr 138:1426–1431.

Mentschel J, ClausR. 2003. Increased butyrate formation in the pig colon by feeding raw potato starch leads to a reduction of colonocyte apoptosis and a shift to the stem cell compartment. Metabolism 52:1400–1405

Piva A, Morlacchini M, Casadei G, Gatta PP, Biagi G, Prandini A. 2002. Sodium butyrate improves growth performance of weaned piglets during the first period after weaning. Ital J Anim Sci 1:35–41.

Pluske JR, Pearson G, Morel PCH, King MR, Skilton G, Skilton R. 1999. A bovine colostrum product in a weaner diet increases growth and reduces days to slaughter. Manipulating Pig Production VII, 256.

Sakata T. 1989. Stimulatory effect of short-chain fatty acids on epithelial cell proliferation of isolated and denervated jejunal segment of the rat. Scand J Gastroenterol 24:886–890

Schierack P, Nordhoff M, Pollmann M, Weyrauch KD, Amasheh S, Lodemann U, Jores J, Tachu B, Kleta S, Blikslager A, Tedin K, Wieler LH (2006). Characterization of a porcine intestinal ephitelial cell line for in vitro studies of microbial pathogenesis in swine. Histochem Cell Biol 125:293-305.

Schönfeld P, Wojtczak L. 2016. Short- and medium-chain fatty acids in energy metabolism: the cellular perspective. J Lipid Res 57: 943–954.

Seal BS, Lillehoj HS, Donovan DM, Gay CG (2013). Alternatives to antibiotics: a symposium on the challenges and solutions for animal production. Anim Health Res Rev 14:78-87.

Skrivanová E, Marounek M, Benda V, Brezina P. 2006. Susceptibility of *Escherichia coli*, *Salmonella* sp. and *Clostridium perfringens* to organic acids and monolaurin. Vete Med 51:81–88.

Smilowitz JT , Lebrilla CB, Mills DA, German JB, Freeman SL (2014). Breast milk oligosaccharides: Structure–function relationships in the neonate. Annu Rev Nutr 34:143–169.

Stoy ACF, Heegaard PMH, Sangild PT, Ostergaard MV, Skovgaard K (2013). Gene expression analysis of the IPEC-J2 cell line: a simple model for the inflammation-sensitive preterm intestine. ISRN Genom Article ID 980651. Doi:10.1155/2013/980651.

Thacker PA. 2013. Alternatives to antibiotics as growth promoters for use in swine production: a review. J Anim Sci Biotechnol 4: 78.

Velioglu Ogünç A, Manukyan M, Cingi A, Eksioglu-Demiralp E, Ozdemir Aktan A, Süha Yalçin A (2008). Dietary whey supplementation in experimental models of wound healing. Int J Vitam Nutr Res 78:70-73.

Yang Y, Huang J, Li J, Yang H, Yin Y. 2020. The effects of lauric acid on IPEC-J2 cell differentiation, proliferation and death. Current Molecular Medicine 7: 572-581.

Zava S, Barello C, Pessione A, Perono Garoffo L, Fattori P, Montorfano G (2009). Mare's colostrum globules stimulate fibroblast growth in vitro: a biochemical study. J Med Food 12:836-845.

Zentek J, Buchheit-Renko S, Ferrara F, Vahjen W, Van Kessel AG, Pieper R (2011). Nutritional and physiological role of medium-chain triglycerides and medium-chain fatty acids in piglets. Anim Health Res Rev 12:83–93.

CHAPTER 7 – General discussion and Conclusion

7.1 GENERAL DISCUSSION

It is well known that nutrition, is one of the main factors influencing animal health and performance. Nowadays, in the context of livestock production, the use of nutritional strategies to improve growth performance and animal health has assumed a fundamental role in the search for possible substitutes of antibiotics, which were widely used in the past to overcome farming problems, especially in the most delicate phase of the life cycle of the animals, as piglets weaning.

The main objectives of this thesis were to evaluate innovative and sustainable nutritional strategies to improve the gut health of monogastric animals, and therefore, propose possible alternatives to the use of antibiotics. In particular, this thesis has focused on the effects of SCFAs and MCFAs in the pigs' diet. Specifically, it highlighted the different effects of an innovative form of administration of lauric acid (lauric acid saponified with calcium) and a blend of esterified tributyrin and monolaurin as dietary treatments in pigs, in the most delicate moment of their life: the post-weaning period.

The choice of SCFAs and MCFAs was driven by the fact that these fatty acids have been shown to be truly effective for improving the health and growth of young animals, such as piglets. The gut health supporting role of SCFAs and MCFAs have been extensively studied due to their numerous positive effects on pigs that can be seen especially when health of the animals is endangered, as in the post-weaning period. In particular, the beneficial effect of SCFAs is closely correlated with increasing proliferation and decreasing apoptosis of intestinal cells. In fact, SCFAs can act as a major source of energy for intestinal epithelium, stimulating enterocyte proliferation. Likewise, MCFAs have specific nutritional and metabolic effects, including rapid digestion and passive absorption that make them directly usable by enterocytes for energy production, by promoting and improving intestinal integrity. SCFAs and MCFAs are also able to improve the intestinal inflammatory and oxidative status of pigs.

The therapeutic effects of SCFAs and MCFAs also include antibacterial, antimicrobial and bacteriostatic properties that can improve intestinal microecology and protect pigs from bacterial infections, both against Gram-positive and Gram-negative bacteria. As has already been pointed out, the exact mechanism is not known yet, but it is considered that SCFAs and MCFAs together have a synergistic effect on bacteria. Research suggests that MCFAs are able to penetrate the microorganism's cell walls, due to their specific amphipathic chemical structure, which damages the bacterial cell membrane, solubilizing the lipids and phospholipids in the envelope of the pathogen (of Gram-positive in particular) leading to leakage of intracellular

material and thus allowing SCFAs access into the bacterial cytoplasm. Once inside the microbial cell, SCFAs dissociates in the more alkaline environment of the cytoplasm, resulting in a decrease of bacterial intracellular pH. The change in pH influences the cellular metabolism, interrupting or modifying the vital processes of the bacterial cell. However, the effectiveness of both SCFAs and MCFAs is limited due to prompt absorption and metabolism in the stomach and in the duodenum, which limits the amount that reaches the lower gut and therefore, their ability to modulate the intestinal health.

For this reason, in the first study presented in this thesis, the effect of an innovative form of administration of lauric acid (a MCFA) in the diet of weaning pigs, namely lauric acid saponified with calcium, was evaluated. The use of this product, although it has not shown a clear improvement in the growth performance of piglets, has nevertheless shown to have a promoting effect on gut health, increasing the antioxidant capacity of the intestine and also acting on some parameters of intestinal morphology. However, the antibacterial, anti-inflammatory and immune activity was not relevant and would justify a study focusing only on the antibacterial effect of this product.

In the second study included in this thesis, the focus was instead on a further form of administration of fatty acids in post-weaning pigs' diet, namely a product based on esterified tributyrin (a SCFA) and monolaurin (a MCFA). Currently, considering that esterification is one of the most widely used forms of fatty acid administration in pig nutrition, and that the synergistic effect of SCFAs and MCFAs is able to amplify the positive effects on animal health and growth, the use of this type of product was very promising. Indeed, from the results obtained it was possible to highlight how the administration of esterified tributyrin and monolaurin has beneficial effects on pigs' gut health, protecting the intestinal epithelium from oxidative stress caused by weaning. In addition, the product improved the growth performance of piglets, supported by the increase in ileal villous length. However, even in this case, the effect on the intestinal inflammatory and immune status and the antibacterial activity was not relevant.

In this context, it should be considered that the effect of fatty acids, both short- and medium chain, is strongly linked to numerous factors, such as: the concentration, the duration of administration, the form of administration, the matrix on which the analysis are performed and obviously, the timing with which the samples are taken during the trials. For this reason, the effect of SCFAs and MCFAs on intestinal inflammatory and immune status and antibacterial activity is not always confirmed in scientific studies.

The third study presented in this thesis aimed to further investigate the effect of lauric acid saponified with calcium in post-weaning piglet diets. To better evaluate the improvement effect on the growth performance and gut health of the piglets, the product was administered both to sows in the last phase of pregnancy and their offspring after weaning. Also, in this case, the effect of lauric acid saponified with calcium on piglet growth performance was not clear, in agreement with the results obtained in the first trial. However, an ameliorative effect of the intestinal inflammatory state of piglets fed the product was shown in this study, particularly in the first two weeks after weaning. Similarly, although the results were not statistically significant, the antioxidant capacity of the intestine was also higher. On the contrary, the intestinal immune status showed conflicting results, still in line with the results obtained both in the first study examined but also in the second. In fact, in none of the previous works covered by this thesis, it was possible to highlight a clear positive effect of both products on the intestinal immune status of pigs.

Ultimately, to conclude this thesis, the aim of the fourth study was to investigate the effect of lauric acid, butyric acid and some milk fractions in vitro on porcine IPEC-J2 cell line. The results showed that both fatty acids and milk fraction not have any negative effects on porcine intestinal cells proliferation. Furthermore, cell proliferation improved, especially when cells were exposed to 100 μmol of both lauric acid and butyric acid and to 5% milk cream.

Therefore, it was also possible to prove in vitro how both short- and medium chain fatty acids, but also some milk fractions, have a positive effect on the gut health of the pig, improving cell proliferation without negative effects.

7.2 CONCLUSION

In conclusion, I believe that this thesis can improve the knowledge on the beneficial effects of short- and medium chain fatty acids included in pigs' diet, especially during a delicate moment as weaning. The dietary inclusion of these substances is an innovative nutritional approach that improves piglet's production and health without negative effects on the animals, helping them to face the post-weaning period. Furthermore, to the best of our knowledge, the use of lauric acid saponified with calcium and the blend of esterified tributyrin and monolaurin in pig diets, has never been investigated before in the scientific literature.

Certainly, further studies will be needed to better understand their effects on pigs, considering that not all the results we have obtained have shown a clear improvement effect on pig health and growth. In particular, the following topics may be of interest to better investigate the effects of SCFAs and MCFAs in the zootechnical field:

- Evaluate the efficacy of lauric acid saponified with calcium and the blend of tributyrin and monolaurin at different inclusion levels, in different species and in different physiological periods aimed to enhancing animal health and productivity, not only in pigs post-weaning period;
- Further investigate the antibacterial effect of lauric acid saponified with calcium, perhaps in a challenge trial with *S. suis*, one of the most deleterious pathogens for the swine industry.
- Check the ability of lauric acid saponified with calcium to reach intact not only the duodenum but also other intestinal tracts as jejunum and ileum.
- Better investigate the effect that these products can also have on the health and productive performance of mothers to better understand the possible effect on the offspring.
- Deepen the knowledge about the action of short- and medium chain fatty acids and milk and / or colostrum on pig intestinal cells by focusing on wound-healing, TEER and the gene expression of particular genes useful to better clarify the effect of these substances on the gut health of the pig.

CHAPTER 8 -

List of Tables, Figures and Graphs

8.1 List of Tables

- **Table 11 -** Ingredients and chemical composition of pre-starter (administrated from 0 to 14 study days) and starter (administrated from 15 to 41 study days) basal diets fed to post-weaning piglets during the trial (as-fed basis, %).
- **Table 12 -** Growth performance parameters of post-weaning piglets fed a basal diet alone (CTR) or supplemented with amoxicillin (ANT, 400 mg/kg) or tributyrin and monolaurin (TB+ML, 5 kg/ton) for 41 days[1].
- **Table 13 -** Intestinal morphology parameters of duodenum, jejunum and ileum of post-weaning piglets fed a basal diet alone (CTR) or supplemented with amoxicillin (ANT, 400 mg/kg) or Tributyrin and Monolaurin (TB+ML, 5 kg/ton) for 41 days[1].
- **Table 14 -** Concentration of SIgA, IL-10 and IL-6 in duodenum, jejunum and ileum tissue in post-weaning piglets fed a basal diet alone (CTR) or supplemented with amoxicillin (ANT, 400 mg/kg) or Tributyrin and Monolaurin (TB+ML, 5 kg/ton) for 41 days[1].
- **Table 15 -** Histometric analyses related to lymphatic follicle and muscular external measurement in ileum of post-weaning piglets fed a basal diet alone (CTR) or supplemented with amoxicillin (ANT, 400 mg/kg) or lauric acid saponified with calcium (C12-Ca soaps, 1 g/kg) for 28 days[1].
- **Table 16 –** Concentration of TAOC and MDA in duodenum, jejunum and ileum tissue in post-weaning piglets fed a basal diet alone (CTR) or supplemented with amoxicillin (ANT, 400 mg/kg) or Tributyrin and Monolaurin (TB+ML, 5 kg/ton) for 41 days[1].
- **Table 17 -** Mortality and antibiotic treatment of post-weaning piglets fed a basal diet alone (CTR) or supplemented with amoxicillin (ANT, 400 mg/kg) or tributyrin and monolaurin (TB+ML, 5 kg/ton) for 41 days[1].
- **Table 18 -** Ingredients and chemical compositions of the basal diet fed to post-weaning piglets during the trial (as-fed basis, %).
- **Table 19 -** Growth performance parameters of post-weaning piglets fed a basal diet alone (CTR-CTR and T-CTR) or supplemented with lauric acid saponified with calcium (CTR-T and T-T) for 26 days[1].
- **Table 20a -** Mortality and antibiotic treatment of post-weaning piglets fed a basal diet alone (CTR-CTR and T-CTR) or supplemented with lauric acid saponified with calcium (CTR-T and T-T) for 26 days[1].
- **Table 20b -** Fisher exact test mortality and antibiotic treatments results.

8.2 List of Figures

8.3 List of Graphs

- **Graph 11** - C12 and C4 effect on differentiated IPEC-J2 cell proliferation. [a,b]different letters indicate a significant difference.
- **Graph 12 –** 20% Milk fractions effect on growing IPEC-J2 cells proliferation.
- **Graph 13 –** 10% Milk fractions effect on growing IPEC-J2 cells proliferation. [a,b]different letters indicate a significant difference.
- **Graph 14** – 5% Milk fractions effect on growing IPEC-J2 cells proliferation.
- **Graph 15** - 1% Milk fractions effect on growing IPEC-J2 cells proliferation. [a,b]different letters indicate a significant difference.
- **Graph 16** – 20% Milk frations effect on differentiated IPEC-J2 cells proliferation.
- **Graph 17** – 10% Milk frations effect on growing IPEC-J2 cells proliferation.
- **Graph 18** – 5% Milk frations effect on differentiated IPEC-J2 cells proliferation.
- **Graph 19** - 1% Milk frations effect on differentiated IPEC-J2 cells proliferation.

CHAPTER 9 – Acknowledgment / Ringraziamenti

9.1 ACKNOWLEDGMENTS

Acknowledgments are always the most difficult but important part of a thesis. They determine the end of a path and force you to really think about the people who have been part of it and who have enriched it in some way, positively, or even negatively. But leaving out the negative, which certainly does not deserve a deepening in the acknowledgments, at the end of these 3 years, I realized that there are so many people I have to thank for achieving this goal. So, where should I start?

To my tutor, Professor Valentino Bontempo, whose guidance allowed me to achieve this important goal.

To my family: my mommy, my daddy, my sister and my grandparents. My personal army. With you by my side no battle is impossible to win. But let's go in order…

To my parents, my two pillars, the cornerstones on which my whole life is based. Without you, without your unwavering support, advice, laughs, and the way you taught me to see the world, I wouldn't be who I am today. You gave the world to me, literally, and there will never be adequate words to thank you for this. I have learned to walk alone in these 3 years, but my steps have been well directed. You are my personal heroes and although this is an achievement that I hope will make you proud, I can say that I am proud of you too, for everything.

To my sister, definitely the best part of me. There are few certainties in life, perhaps not even one, but I am a lucky girl and I can say I have a certainty: my Patrik. My certainty that whatever may happen in my life, I will never be alone. There will always be a philadelphia crouton eaten at midnight to make me laugh, a quote from some movie we both know by heart to make me stop crying, a shared thought exchanged with a single glance or an evening in bed watching trash programs and eating junk foods to be happy. Everyone should have a Patrik in their life, so thank you for being mine.

To my grandfather. You left when this new path had just begun and the emptiness you left is comparable only to the love you felt for us, your family. I just hope I made you proud. Throw an eye down every now and then, I'm sure you'll enjoy seeing how I make my way through this life. I miss you every single day.

To Andrea, my partner in life and in the passions that bind us. You were crazy enough to follow me to Canada and thus gave us the opportunity to have an incredible experience, the sharing of which will accompany us forever. Thank you for being by my side every day, at all times, and for accepting even those parts of me that may not be exactly the best. Thank you for

these years together and for those to come, both as a couple and as part of the pack with those 5 furious mad dogs of Jupiter, Kora, Antares, Kimi and Nanouk (Obviously "mad dogs" is tied for at least 90% to Nanouk, mind you).

To my dear friends, Giulia, Valeria and Martina. You are “my person” and I am very, too lucky to have you in my life. Thank you for being there and for always having been there for me, even at a distance of 6000km.

In Madison, my first family in Canada. One of the funniest and kindest people I know, and definitely, the best roommate I could ever desire. Some of the best laughs of my life happened with you. Thank you for being and still being my friend on the other side of the ocean.

To Adam, the most pedantic and annoying Canadian on earth. But also, the best friend that an Italian girl who landed in Québec could have. One of those people who, seeing you alone at a party, approaches you to not make you feel excluded because you still don't speak French. And then the evening turns into the party of the year, followed by dinners, parties, trekking, shopping days and hours on the phone. One of those friendships that are born by chance but that you carry inside forever. I miss you Pablo and I almost miss being called “ciciona”!

To Martin and Mylene, my Canadian tutors. Two wonderful people who not only welcomed me into their research group but also into their lives. You have been two exceptional teachers. Thank you for all you have taught me, in and out of the research centre. Especially Martin, who reminded me of the pleasure of a healthy tennis match with a friend.

To David, Jason, Laura, Alison, Noemi, Sam, Aline, Carol & Leopold, Pierre & Linda, Marc, Pierluc, Benoit and all the fantastic people I had the honour and pleasure of meeting in Canada and at Sherbrooke. You changed my life and I really hope to be able to return someday.

To all those fantastic and half-mad Italians in Canada: Luca, Luigi, Claudia, Veronica, Nicoletta, Adrien (Italian for pretend). My little Italy on the other side of the world. My experience in Canada would never have been the same without you.

Thanks to Canada then. The country of my dreams in which I found myself and I really understood what I want from life. Even if I fail to return, I will cherish these memories as the most precious of treasures. “Je me souviens”.

To Marco, Eleonora, Nicoletta and Abdel, the colleagues that everyone dreams of. Thanks to you, going to work has been a pleasure in the last year.

To Alice, my partner in university life first and then in the PhD. Thank you for sharing with me the joys and pains of this journey.

To my dogs, my greatest joy. The love I feel for you is an incredible strength that has supported me many times in difficult moments. You are a constant source of happiness capable of straightening even the worst days.

And last, but not least, thanks to myself. A pat on the back from yourself to yourself: you deserve it all.

9.2 RINGRAZIAMENTI

I ringraziamenti sono sempre la parte più difficile ma importante di una tesi. Sanciscono la fine di un percorso e ti costringono a pensare davvero alle persone che ne hanno fatto parte e che lo hanno arricchito in qualche modo, in positivo, o anche in negativo. Ma tralasciando il negativo, che non merita certo un approfondimento in tema di ringraziamenti, alla fine di questi 3 anni, mi sono resa conto che sono davvero tante le persone che devo ringraziare per il raggiungimento di questo traguardo. Quindi, da dove cominciare?

Al mio tutor, il Professor Valentino Bontempo, la cui guida mi ha permesso di raggiungere questo importante obiettivo.

Alla mia famiglia: la mia mamma, il mio papà, mia sorella e i miei nonni. Il mio esercito personale. Con voi al mio fianco nessuna battaglia è impossibile da vincere.

Ma andiamo con ordine:

Ai miei genitori, i miei due pilastri, i punti fermi sulla quale si basa tutta la mia vita. Senza di voi, senza il vostro sostegno incrollabile, i consigli, le risate, e il modo in cui mi avete insegnato a vedere il mondo, oggi non sarei la persona che sono. Voi me lo avete dato il mondo, letteralmente, e non ci saranno mai parole adeguate a ringraziarvi per questo. Ho imparato a camminare con le mie gambe in questi 3 anni ma i miei passi sono stati ben indirizzati. Siete i miei eroi personali e sebbene questo sia un traguardo che spero vi renderà orgogliosi, anche io posso dire di essere fiera di voi, per tutto.

A mia sorella, sicuramente la parte migliore di me. Di certezze nella vita se ne hanno poche, forse nemmeno una, ma sono una ragazza fortunata e io una certezza posso dire di averla: il mio Patrik. La mia certezza che qualsiasi cosa possa accadere nella mia vita, non sarò mai sola. Ci sarà sempre un crostino con il philadelphia mangiato a mezza notte per farmi ridere, una citazione di qualche film che sappiamo entrambe a memoria per farmi smettere di piangere, un pensiero condiviso scambiato con una sola occhiata o una serata a letto a guardare

programmi improbabili e mangiando schifezze per essere felice. Tutti dovrebbero avere un Patrik nella loro vita, quindi grazie di essere il mio.

A mio nonno. Te ne sei andato che questo nuovo percorso era appena cominciato e il vuoto che hai lasciato è paragonabile solo all'amore che provavi per noi, per la tua famiglia. Spero solo di averti reso orgoglioso. Butta un occhio giù ogni tanto, sono sicuro che ti divertirai a vedere come mi faccio strada in questa vita. Mi manchi ogni giorno.

Ad Andrea, il mio compagno nella vita e nelle passioni che ci legano. Sei stato tanto matto da seguirmi fino in Canada e ci hai dato così la possibilità di fare un'esperienza incredibile, la cui condivisione ci accompagnerà per sempre. Grazie per essere al mio fianco ogni giorno, in ogni momento, e per accettare anche quelle parti di me che magari non sono proprio il massimo. Grazie per questi anni insieme e per quelli che verranno, sia come coppia, sia come parte del branco con quei 5 pazzi furiosi di Jupiter, Kora, Antares, Kimi e Nanouk (Ovviamente "pazzi furiosi" è legato per almeno un 90% a Nanouk, sia chiaro).

Alle mie amiche, Giulia, Valeria e Martina. Siete le mie persone e io sono molto, troppo, fortunata ad avervi nella mia vita. Grazie di esserci e di esserci sempre state per me, anche a 6000km di distanza.

A Madison, la mia prima famiglia in Canada. Una delle persone più spassose e gentili che io conosca, e sicuramente, la migliore coinquilina che io potessi mai desiderare. Alcune delle migliori risate della mia vita le ho fatte con te. Grazie per essere stata ed essere tutt'ora mia amica anche dall'altra parte dell'oceano.

Ad Adam, il più saccente e irritante canadese esistente. Ma anche il migliore amico che una ragazza italiana sbarcata in Québec potesse avere. Una di quelle persone che vedendoti da sola a una festa, ti si avvicina per non farti sentire esclusa perché ancora non parli il francese. E poi la serata si trasforma nel party dell'anno, a cui seguono cene, feste, trekking, giornate di shopping e ore al telefono. Una di quelle amicizie che nasce per caso ma che ti porti dentro per sempre. Mi manchi Pablo e quasi mi manca anche sentirmi chiamare "ciciona"!

A Martin e Mylene, i miei tutor canadesi. Due persone meravigliose che non solo mi hanno accolto nel loro gruppo di ricerca ma anche nelle loro vite. Siete stati due insegnanti eccezionali. Grazie per tutto quello che mi avete insegnato, dentro e fuori dal centro di ricerca. Soprattutto Martin, che mi ha ricordato il piacere di una sana partita a tennis con un amico.

A David, Jason, Laura, Alison, Noemi, Sam, Aline, Carol & Leopold, Pierre & Linda, Marc, Pierluc, Benoit e tutte le fantastiche persone che ho avuto l'onore e il piacere di incontrare in Canada e a Sherbrooke. Mi avete cambiato la vita e spero davvero di poter tornare un giorno.

A tutti quei fantastici e mezzi matti italiani in Canada: Luca, Luigi, Claudia, Veronica, Nicoletta, Adrien (italiano per finta). La mia piccola Italia dell'altra parte del mondo. La mia esperienza in Canada non sarebbe mai stata la stessa senza di voi.

Grazie al Canada quindi. Il paese dei miei sogni nella quale ho ritrovato me stessa e ho capito davvero cosa voglio dalla vita. Se anche non riuscissi a tornare, custodirò questi ricordi come il più prezioso dei tesori. "Je me souviens".

A Marco, Eleonora, Nicoletta e Abdel, i colleghi che tutti sognano. Grazie a voi, andare a lavorare è stato un piacere nell'ultimo anno.

Ad Alice, mia compagna della vita universitaria prima e nel dottorato poi. Grazie per aver condiviso con me le gioie e i dolori di questo percorso.

Ai miei cani, la mia gioia più grande. L'amore che provo per voi è una forza incredibile che mi ha sostenuto tante volte nei momenti difficili. Siete una fonte di felicità costante capace di raddrizzare anche le giornate più storte.

Ed ultima, ma non meno importante, grazie a me stessa. Una pacca sulla spalla da te stessa a te stessa: te la meriti tutta.

"I se e i ma sono la patente dei falliti. Nella vita si diventa grandi nonostante…"