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The impact of glutamine supplementation on morphological gut development, immune cells, tissue and digesta amino acid profiles, microbiota and bacterial metabolites in low birthweight and normal birthweight male suckling piglets

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Meiner Familie

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List of abbreviations

AA	Amino acid
Ala	Alanine
Arg	Arginine
BiW	Birthweight
BW	Bodyweight
GI	Gastrointestinal
GIT	Gastrointestinal tract
GLS	Glutaminase
Gln	Glutamine
Glu	Glutamate
GS	Glutamine synthetase
IEL	Intraepithelial lymphocyte
IgA	Immunoglobulin A
IGF - 1	Insulin – like – growth factor 1
IUGR	Intrauterine growth restriction
LBW	Low birthweight
LPS	Lipopolysaccharide
mTOR	Mammalian target of rapamycin
NBW	Normal birthweight
NEAA	Non-essential amino acid
SCFA	Short – chain - fatty acid
SI	Small intestine

1 General introduction

Neonatal mortality has become a more visible problem in pig husbandry (Huting et al. 2021). Genetic selection of hyperprolific sows increases the number of physiological and immunological immature piglets at birth, which are easily detectable by a low birthweight (LBW), possibly induced by intrauterine growth restriction (IUGR) (Hales et al. 2013). High neonatal mortality, especially that of male LBW piglets (Baxter et al. 2012), is an ethically (Marchant et al. 2000) and commercially (Beaulieu et al. 2010) highly debated topic, which should be regarded with the highest priority. Sow management and nutrition, before and during gestation, as well as around farrowing, have been shown to affect piglets. Furthermore, early colostrum intake plays a crucial role in improving piglet survival (Farmer and Edwards 2021). In sow milk, glutamine (Gln) and glutamate (Glu) have been detected as the most abundant peptide bound amino acids (AA), whereas free Gln showed an increase during lactation and becomes the most abundant free AA during lactation (Wu and Knabe 1994). For this reason Gln supplementation to neonatal suckling piglets might be a new assessment strategy to reduce neonatal mortality in LBW piglets, who have access to sow milk later after birth and to teats in general due to the poorer assertiveness caused by their weakness (Edwards and Baxter 2015). Glutamine is a primary energy source for neonatal porcine enterocytes (Darcy-Vrillon et al. 1994) and promotes protein synthesis, immune response and antioxidative defence for mucosa cells of the gastrointestinal tract (GIT) (Jacobi and Odle 2012). However, most studies supplementing Gln to pigs have been conducted in weaning or weaned piglets (Wu et al. 1996b; Wang et al. 2008; Cabrera et al. 2013), which must be considered physiologically different from suckling piglets. Therefore, it is evident that the strategic assessment to improve the physiological and immunological impairment of LBW piglets by Gln supplementation needs further research because our current knowledge is inadequate, and a lot needs to be clarified to reduce neonatal mortality in piglets significantly.

Thus, this study compared gastrointestinal (GI) development and immunity of LBW and normal birthweight (NBW) male neonatal piglets. The weight of male littermates was assessed at birth, and they were assigned to an oral Gln supplementation group or an isonitrogenous alanine (Ala) supplementation group, serving as a control group. As a possible absorption side of Gln, the jejunum was investigated for the effects of oral Gln supplementation on gut morphology, immune cells, AA metabolism, and anti–oxidative defence in neonatal suckling piglets with different birthweights (BiW). As the fermentative chamber of the pig, the colon was examined for effects of oral Gln supplementation on suckling piglets with LBW or NBW on morphometry, immunohistochemistry, bacterial composition, and bacterial metabolites.

1.1 Aims and Objective of the Thesis

Glutamine supplementation has shown to comprise a wide variety of beneficial effects on the host. Among these effects, the benefit of Gln for the health of the host and as an energy substrate for the GIT, which seems to be impaired in LBW piglets, could reduce the high rate of neonatal mortality in those piglets. Furthermore, it is unknown exactly where Gln can exhibit effects in the GIT. As known from studies *in vitro* or weaned piglets, Gln might affect the GI microbiota, morphometry, immunology, antioxidative defence and AA absorption. There is knowledge about the neonatal development of the listed parameters, but in parts, it seems that they need further investigations to provide a clear picture. Additionally, this study could be adapted for the use in human nutrition since the pig is considered an excellent animal model for humans. Existing literature does not provide sufficient information about the impact of Gln supplementation on GI development and its influence on bacterial composition in neonatal piglets in the first two weeks of life. Thus, it was the aim of this thesis to identify if:

I. Gln beneficially affects the intestinal epithelium in pre-weaning NBW and LBW piglets but more distinctly in LBW piglets.
II. Gln affects piglet jejunal development, jejunal epithelium characteristics, and immunological development patterns in suckling piglets.
III. Gln affects piglet colonic development, colonic epithelium characteristics and immunological development, microbial composition and bacterial metabolites.

1.2 Hypothesis

It was the hypothesis of this thesis that Gln supplementation improves GI development, immune characteristics of the jejunum and colon and furthermore the bacterial composition as well as bacterial metabolites in the colon digesta of LBW suckling piglets.

2 Literature Review

2.1 Low birthweight piglets

The term low birthweight (LBW) piglet can be used irrespective of time of delivery (De Vos et al. 2014) and can be defined as newborns having a birthweight under 10% of the mean BiW of the litter, or a BiW less than the mean BiW minus up to two times the standard deviation (Cooper 1975). The breeding goal of hyperprolific sows leads to increased litter size and an increased number of LBW piglets (De Boo et al. 2005; De Vos et al. 2014). A study by Martineau and Badouard (2009) reported that when the litter gets larger, the proportion of LBW piglets in these litters increases. Pigs have a higher number of LBW offspring compared to other livestock species (Cooper 1975), an associated consequence of IUGR (De Boo et al. 2005; De Vos et al. 2014). Approximately 10-20% of piglets per litter are underweight or influenced by IUGR (De Boo et al. 2005) because of placental insufficiency (Wu et al. 2006; Hales et al. 2013). Sows with high ovulation estimates have an increased risk of giving birth to piglets influenced by IUGR because of reduced uterine space. Due to the high number of fetuses, this will lead to reduced fetal size and low body weight (Foxcroft et al. 2006). Low birthweight or piglets influenced by IUGR piglets are characterised by typical phenotypical appearances like a long thin shape and a ´dolphin like´ head shape (Farmer and Edwards 2021). In addition, they have impaired brain, heart, GIT and muscle development, which impedes the uptake and absorption of colostrum (Farmer and Edwards 2021). Vast numbers of LBW piglets have a significant influence on the economy of the pig industry, because these piglets show a high percentage of neonatal mortality and impaired performance, followed by reduced carcass and meat quality at slaughter (Beaulieu et al. 2010). It was shown that piglets with a BiW lower than 1 kg have preweaning mortality rates of 40%, whereas for piglets weighing 1.0 – 1.2 kg preweaning mortality was 15%, and for piglets over 1.6 kg, 7% (Roehe and Kalm 2000). Previously another study observed an increased mortality rate of piglets with a weight of less than 0.95 kg during the period from birth to slaughter (Calderón Díaz et al. 2017).

2.1.1 Interventions on sow side to prevent high numbers of LBW piglets

Nutritional interventions during gestation and transition or management strategies implemented on the sow, to control the number and mortality of LBW piglets, have been already studied and described in detail (Farmer and Edwards 2021).

Increasing the amount of feed during gestation to gilts or sows has shown different effects on the BiW of piglets. Either an increase in the BiW of piglets was observed in gilts and sows (Cromwell et al. 1989) or an increase in the BiW of piglets born to gilts and a decrease in those

born to sows, was reported (Shelton et al. 2009). At the same time, other studies did not observe an influence of feeding different amounts of feed during different time points of gestation (Nissen et al. 2003; Rehfeldt and Kuhn 2006). The effect of low energy intake of sows on piglet BiW showed no precise results. Buitrago et al. (1974) reported a reduction of piglet's BiW by low energy intake of sows. Feeding dams with extra energy appears to have no observable effect on offspring BiW (Gatlin et al. 2002). Supplementing sows with oil during gestation has been associated with a higher percentage of surviving LBW piglets. However, the kind of lipid and the time point of supplementation appear to be just as important (Laws et al. 2009a). Feeding monounsaturated fatty acids (18:1 n-9) throughout the first trimester of pregnancy has been shown to reduce the number of LBW piglets, potentially due to improved placental growth (Laws et al. 2009a; Laws et al. 2009b). In contrast supplementation with polyunsaturated fatty acids has been shown to increase the number of LBW piglets (Laws et al. 2009b). The optimal supply of protein to gestating sows is more critical for fetal growth than the energy source. Feeding an adequate amount and a suitable composition of dietary AA's improves the live-birth rate and development of the offspring (De Vos et al. 2014). Supplementing low-protein diets to gestating gilts, has been associated with increased numbers of LBW piglets and decreased growth performance (Rehfeldt et al. 2011). Also, high protein diets during pregnancy appear to increase the number of LBW piglets (Rehfeldt et al. 2011; Mickiewicz et al. 2012). Adding functional AA like arginine (Arg) (Wu et al. 2010a) or Gln (Wu et al. 2011b) to the sows diet can have beneficial effects on the gestating sows and piglets, such as increased offspring BiW and producing litters with mean BiW that were more equal. Supplementing carnitine appears to prevent the incidence of LBW piglets as well (Musser et al. 2007; Ramanau et al. 2008).

2.1.2 Nutritional interventions on piglets to prevent the high mortality of LBW piglets

It is well known that colostrum, which is rich in bioactive molecules, nutrients and energy, is crucial for neonatal survival (Devillers et al. 2004), especially for LBW piglets, who are less active, which prevents them from moving towards the udder and competing with heavier littermates (Tuchscherer et al. 2000). Nutrition interventions such as booster preparations or colostrum substitutes containing fat, lactose, immunoglobulins and growth factors are widely used, but peer-reviewed studies regarding their efficacy are scarce (De Vos et al. 2014). Conflicting results are reported by several authors supplementing neonatal piglets with medium-chain triglycerides, with either no effect on survival (Lee and Chiang 1994) or positive effects on survival rates when mixes of medium and long-chain fatty acids were supplemented orally (Casellas et al. 2005). Additionally, studies with milk replacers and milk-based creep feed diets have been conducted. The problem with creep feed diets is that their intake before

weaning is highly variable, and very low before four weeks of age (Bruininx et al. 2002). Furthermore, supplementation of liquid milk replacers, typically consisting of bovine milk products, have been shown to increase growth rates and weaning weights (Wolter et al. 2002). Nevertheless, liquid milk replacers do not appear to reduce the mortality rate, or weight variation of LBW compared to average BiW fattening pigs at slaughtering (Wolter et al. 2002). Liquid milk replacers may not improve LBW piglet survival, as the majority of LBW individuals die within the first three days of life, a period when voluntary milk replacer intake is minimal. Forced feeding of liquid feeds did not seem to have beneficial effects on performance and survival of piglets (Van Tichelen et al. 2022). Therefore, a developmental comparison of LBW and NBW piglets is necessary to clarify their nutritional needs (De Vos et al. 2014).

2.1.3 Supplementation studies improving the gastrointestinal development of piglets

The small intestine (SI), with its essential role in terminal digestion and absorption of nutrients, is necessary for the survival and growth of LBW piglets. Intrauterine growth restriction has been associated with impaired SI development and reduced feed intake (Wang et al. 2005; D'inca et al. 2010b; Wang et al. 2010), and a wide-range of supplemental studies have been investigated to improve SI development in LBW piglets.

Oral insulin-like-growth factor 1 (IGF-1) supplementation has been shown to stimulate SI growth and maturation (Burrin et al. 1996; Houle et al. 1997). Additionally, supplementing IGF-1 to neonatal formula-fed piglets has been shown to increase crypt cell proliferation (Xu et al. 1994a), villus height (Burrin et al. 1996; Houle et al. 1997) and intestinal brush border enzyme activity (Houle et al. 1997). Administration of exogenous epidermal growth factor showed elevated intestinal brush border enzyme activity and healing effects on intestinal tissue damage of rotavirus-infected piglets (Zijlstra et al. 1994). Post-natal supplementation of IGF-1 to piglets influenced by IUGR, with an osmotic minipump, led to growth at normal levels (Schoknecht et al. 1997). Another supplement used as an additive to the diet of formula-fed piglets, during the first week of life, is leptin, which was reported to increase SI length, decrease villus height and brush border enzyme activity, with no positive effect on weight gain (Woliński et al. 2003). Supplementation with AA, including Gln (Wu et al. 1996c), in weaning piglets was reported to increase the mitosis to apoptosis ratio and decrease in villus atrophy in the jejunum. Arginine is the first limiting AA in sow's milk and milk replacers, and the endogenous synthesis in suckling piglets is insufficient (Wu et al. 2004). From sow milk, the piglet is provided ≤ 1.01 g Arg/d, which does not fulfil the reported requirements for growth and metabolic function in 7-day old piglets (≥ 2.7 g/day) (Wu et al. 2004). Arginine supplementation to piglets influenced

by IUGR led to improved GI development (Wang et al. 2012). Glutamine, a precursor of Arg, orally supplemented (1g/kg BW/d) to piglets between 0 and 21 days of age, was associated with increased weight gain and reduced weaning mortality (Wu et al. 2011). Research showed that substances originating from sow milk, lactose, fat, immunoglobulins, growth factors and milk oligosaccharides, improve the growth and maturation of the GIT in LBW piglets (De Vos et al. 2014).

2.2 Aspects of gut development in piglets before weaning

To ensure that newborns have a healthy start in life, the GIT must be able to cope with a change from parenteral nutrition through the placenta before birth, to oral intake of colostrum and milk, after birth. For this reason, the GIT, influenced by luminal and humoral factors, grows and matures very quickly during this phase (Sangild et al. 2000). During the first days of life, newborn piglets show an increase in SI weight of 72%, mainly due to a large increase in the weight of the mucosa, but also due to an increase in length. This weight gain on the first days is probably due to temporary swelling of epithelial cells caused by an intracellular accumulation of proteins (Burrin et al. 1992). The swelling of the enterocytes, is accompanied by the growth of the intestine (Xu et al. 2002). The size of a single enterocyte changes significantly from the unfed newborn piglet to the 24-hour or seven-day-old piglet. A three to the fourfold difference in intestinal protein synthesis was observed between piglets fed colostrum or milk replacer and piglets fed water. Differences in intestinal protein mass appear to result from IgG accumulation and retention because it was significantly increased in piglets fed colostrum (Burrin et al. 1992). Likewise, an acceleration of the cell proliferation rate from up to 20 days in fetal intestinal cells to 2-3 days in the intestinal tissue of the newborn piglets has been observed.

2.2.1 Development of small intestinal morphology of postnatal piglets

It has been shown that jejunal villus height decreases during the suckling period, with the most significant decrease observed on the third and seventh day after weaning. The jejunal villi of weaned piglets, compared to those of suckling piglets at the same age were much shorter. The villi appearance of two and ten-day-old suckling piglets is similar to a long, thin finger and changes its appearance until the age of 35 days, when the villi become shorter and wider (Skrzypek et al. 2005). At weaning, the villi show a close juxtaposition, which leads to a smoother intestinal lining (Cera et al. 1988). In another experiment, the villus height of the SI did not change with age or BiW, but the villus width and crypt depth gradually increased with age (Wiyaporn et al. 2013). No significant difference in cell proliferation was observed between NBW and LBW piglets; the highest proliferation rate was observed in piglets at 28 days of age. Cell proliferation in the SI and differs between “mature” and newborn piglets. Differences in the

number of apoptotic cells have been reported, with newborn piglets having significantly fewer apoptotic cells than piglets at ten days of life (Godlewski et al. 2005). In LBW piglets, intestinal barrier function does not appear to be impaired compared to NBW piglets (Huygelen et al. 2015). However age does have a significant impact, with the intestinal barrier becoming tighter, and increasing crypt depth (40%) and decreasing villus length (35%) (Marion et al. 2002). In addition to intensive growth, the SI undergoes an essential maturation process at an early stage after birth. There is increased proliferation in the crypts, which is associated with significant structural and functional transformation (Baintner 2002), as the "fetal type" enterocytes, which contain large lysosomal vacuoles, are replaced by "adult-type" enterocytes, which do not have these vacuoles. Fetal type enterocytes are divided into two categories of lysosomal vacuoles, the transport and digestive vacuoles. The transport vacuoles are visible along the entire length of the small intestine, but only in the first two days after birth. They are responsible for transporting macromolecules from the intestinal lumen, through the enterocytes, into the bloodstream. The transport vacuoles allow biologically active colostrum molecules, including immunoglobulins, to pass through the intestinal mucosa without affecting their activity. On the other hand, the lysosomal vacuoles are located in the distal half of the SI and appear to be responsible for the intracellular digestion of nutrients and controlling intestinal lumen pH (Baintner 1994). A few days after birth there is a functional change where the activity of brush border lactase, aminopeptidase A and N and dipeptidase IV is significantly reduced (Zhang et al. 1997), and the open "gut barrier", which is associated with the high absorption rate of large molecules, disappears (Teichberg et al. 1992).

2.2.2 Development of colonic morphology of postnatal piglets

Immediately after birth, the epithelium of the colon is still immature (Montedonico et al. 2006). The first day after birth, the weight of the colon increases by 30%, and then doubles by the third day of life, due to mucosal and non-mucosal tissue growth (Bach and Carey 1994). In addition, villus-like structures can be seen in the caecum and proximal colon, but only on the first day after birth (Xu et al. 1992). It is assumed that these structures are also there in late gestation (Everaert et al. 2017). In another study, no morphological difference was found between LBW and NBW piglets at the same age, but changes were observed in both BiW groups as the piglets aged (Wiyaporn et al. 2013). Colonic enterocytes are also capable of absorbing AA until the second week after birth (Xu et al. 1992). It is assumed that neonatal colonic enterocytes are part for part replaced by enterocytes lacking in this function (Sepúlveda and Smith 1979). The function to transport AA seems to be affected by neurohormonal status and food passage (Wooding et al. 1978).

2.3 Function and immune response of the neonatal pig gut

At birth, the piglet is highly immunocompromised and depends on the supply of specific and non-specific immune factors from colostrum and milk to protect its health. In between 24 to 48 hours after birth, the piglet can absorb macromolecules from the intestinal lumen in a non-selective way. Permeability decreases strongly from 24 to 48 hours after birth. The piglets absorb immunoglobulins, such as IgG, from the sow´s colostrum via the enterocytes (Rothkotter et al. 1991). The migration of lymphocytes into the mammary gland leads to the production of secretory immunoglobulin A (IgA), which is released into the sow's milk to maintain the humoral immunity of the piglets (Van Niel et al. 2001). The so-called functional immaturity of the newborn piglet's cellular and secretory immune system is limited to the fact, that they can only carry out limited T and B cell responses when confronted with pathogens. This contributes to their immunocompromised status (Seiler and Berendonk 2012). The development of immune competence must be present for optimal growth. Adaption of the organism towards the exposed wide range of antigens associated with pathogens, commensal bacteria and food is needed to adjust the responses to antigens accordingly. This refers to developing tolerance to food and commensal bacterial antigens and active immune responses to pathogens (Bailey et al. 1994). It is assumed that the immune system needs some stimulation at specific points in early postnatal development to ensure optimal functioning of the immune system. (Mclamb et al. 2013). In pigs and other animals, large amounts of protein are absorbed immunologically intact by the intestinal mucosa (Brown et al. 2013). Immune responses to harmless feeding components must be regulated to prevent tissue damage and a deteriorated absorption of macromolecules. A systemic tolerance or "oral tolerance" has been shown in pigs (Butler et al. 2002). A change in the morphology of the intestinal mucosa and intestinal immune cells depends on the feed composition and the presence of oral feed components (Holmgren and Svennerholm 2012; Mowat and Agace 2014). Maintaining the integrity of the epithelial surface is essential to prevent infections that can develop through the GIT because a disease will only develop if the microorganisms or its toxins attach themselves to the epithelial cells and gain access through the epithelium. Therefore, it is not surprising that the mucosal immunity mechanism tends to be non-inflammatory and is initially designed to keep potentially harmful antigens in the lumen of the intestine, where they are effectively removed by peristalsis and continuous flow of the digesta. IgA antibodies, which cannot elicit a complement reaction, perform this function. In healthy individuals, epithelial cells and mucus, together with IgA, provide an effective barrier against potentially harmful bacteria. When this barrier is breached, other protective mechanisms of the innate and acquired immunity start to work to protect the intestine (Stokes et al. 2004).

2.3.1 Mucins and their tasks in innate barrier functions in the gastrointestinal tract

Intestinal epithelial cells play a significant role in innate immunity by forming a highly specialised physical and functional barrier against feeding and microbial antigens. Large, highly glycosylated glycoproteins called mucins (Sheehan et al. 2000), play a major role in innate barrier function and immunity. There are two types of mucins, membrane-bound and secreted mucins. Membrane-bound mucins have a hydrophilic C-terminal transmembrane domain (Andrianifahanana et al. 2006), are monomeric, apically located on the surface of mucosal epithelial cells and participate in the protection of the mucosa by processing cellular signals, thus providing a response to pathogens and intestinal inflammation (Lindén et al. 2008). Secreted mucins, are produced and secreted in the GIT by mucus cells of the glandular tissue and special epithelial cells called goblet cells (Verdugo 1990). They are involved in the formation of mucus gel, and are characterised by a high proportion of O-linked glycosylation patterns (Roussel and Delmotte 2004). The glycosylation patterns depend on the presence of glycosyltransferases in the cell, which are determined by the genetics and localisation of the tissue (Rini and Esko 2015). Every individual animal differs in the mucin oligosaccharide terminal structure and within tissue locations (Lindén et al. 2010; Venkatakrishnan et al. 2017). Infections and inflammatory factors have been shown to change the glycosylation pattern of mucins. Therefore, glycosylation is considered a protective mechanism against mucosal pathogens (Jentoft 1990). The main function of GI mucins is due to the high level of glycosylation. It protects and lubricates the cell surface and prevents the degradation of the protein backbone by proteases (Corfield 2015). Mucins are crucial as the first defence barrier against physical and chemical attacks by ingested food, microbes and microbial products. Furthermore, they are involved in epithelial cell renewal and modulation of cell adhesion, serving as ligands for cell surface receptors, participating in host-pathogen interactions and acting as energy sources for both commensal and pathogenic microorganisms. (Andrianifahanana et al. 2006; Skoog et al. 2017)

2.3.2 Immunological mucosal development of the gastrointestinal tract

Cells and structures involved in the mucosal immune response are initially absent shortly after birth and then rapidly populate the piglet intestinal tract (Mowat 2003). Four phases of mucosal immunological architecture development have been determined (Stokes et al. 2004). In the first phase, the newborn piglet has very few lymphocytes in the epithelium of the intestine and the lamina propria. Lymphocyte accumulations occur in the areas of the mucosa, which then develop into Peyer's plates (Salmon et al. 2009). However, these accumulations have no clear immunological structure. The second phase is initiated when lymphocytes colonise the intestine during the first two weeks of life that express CD2 surface markers but do not coexpress CD4 or CD8 surface markers. Peyer's patches begin to organise themselves and acquire an almost mature structure during this period. At the beginning of the third phase, $CD4^+$ T cells colonise the intestinal mucosa of 2-4 weeks old piglets, primarily the lamina propria. Most of the $CD8^+$ cells are absent, and only a small number of B-cells express mainly IgM. The fourth phase, which starts at the age of five weeks, is characterised by the appearance of $CD8^+$ cells in the intestinal epithelium and around the epithelial basement membrane. In the areas of the crypts, many IgA positive B cells show up. By reaching seven weeks, the intestine structure is comparable to that of an adult pig. As a result, at three weeks of age, the piglet can exert an active immune response to live viruses and feed components. However, the quantity and quality of these immune responses differ from those of older pigs, as also shown in the study of Bailey et al. (2004). More specifically, the three-week-old piglet cannot make the critical choice between pathogenic and non-dangerous antigens and of developing tolerance to the non-dangerous ones (Butler et al. 2002). A tolerance to continuously fed proteins does not seem to be fully achieved by eight weeks (Butler et al. 2002).

2.4 Amino acids characterization and function in young pigs

By forming short polymer chains, peptides or polypeptides, AA are the building blocks of proteins. There are 20 proteinogenic AA and they are required to maintain an animals' normal physiological function, nutritional status improve health and growth (Rezaei et al. 2013). Traditionally, AA have been categorised as nutritionally essential and non-essential. The essential AA are histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine (Rezaei et al. 2013), as they must be added to the diet in sufficient amounts because their carbon skeletons are not synthesised in vivo (Kim et al. 2005). The non-essential AA (NEAA) are Ala, asparagine, Arg, aspartate, cysteine, Glu, Gln, glycine, proline, serine and tyrosine, as the inter-organ AA metabolism in the body leads to their de novo synthesis (Wu 2009). For NEAA and their metabolites, many physiological functions are described. Glutamine and Glu, for example, get their α–amino nitrogen from carbon

skeletons of branched-chain AA, which cannot be formed in the body. (Rezaei et al. 2013). Studies have shown that not enough NEAA are synthesised by pig's during specific developmental phases or under particular feeding circumstances, to sustain growth, development, lactation and reproduction performance (Kim and Wu 2007). Therefore Arg, cysteine, Glu, Gln, glycine, proline and tyrosine are considered conditionally essential AA (Rezaei et al. 2013). Feed proteins, which are the nutrients with the highest costs in pig production, are transformed into tissue proteins via complex biochemical and physiological processes (Wu 2009). It is known that Glu, Gln and aspartate are extensively metabolised in the SI. At the same time, other AA are primarily used for protein accretion in immature pigs. The utilisation of dietary proteins remains suboptimal with regard to the pig. A previous study showed that in young piglets, only 50 to 70% of dietary AA are transformed into tissue protein (Wu et al. 2010a). The degradation end product of the surplus AA is excreted in urine and faeces, contributing to environmental pollution. Next to the synthesis of tissue proteins, some AA function as signalling molecules that adjust mRNA translation. For instance, the essential AA leucine can trigger protein synthesis in cells by ameliorating the phosphorylation of mammalian target of rapamycin (mTOR) and its downstream target proteins (Anthony et al. 2000). Furthermore, almost all AA affect direct or indirect immune function. Additionally, some AA work as precursors for synthesizing neurotransmitters (Li et al. 2007) and particular hormones (Wu 2009).

2.4.1 Glutamine

Glutamine ($C_5H_{10}N_2O_3$) is an α-AA synthesised by diverse tissues in the body, especially by the skeletal muscle. The enzymatically easily removed side-chain amide group makes Gln hydrophilic and a favourite substrate for cells requiring a source of Glu or NH_3 for physiological functions (Young and Ajami 2001). Many enzymes are connected to Gln metabolism, but the major ones are Gln synthetase (GS) and glutaminase (GLS). Glutamine synthetase promotes Gln synthesis out of Glu and NH_3 in an ATP-dependent process (Meynial-Denis 2017). The hydrolysis of Gln results through the enzyme GLS, setting NH_4^+ free (Newsholme et al. 2003). Depending on the factors like metabolic and energy status, various organs show GLS and GS functions. Liver Gln functions as an essential transporter for nontoxic transport of NH_3 since the liver is the major site of nitrogen metabolism in the body and the tissue that shows the highest capacity for Gln synthesis (Newsholme et al. 2003). Additionally, certain cell types and tissues, like immune, kidney and intestinal cells, are predominantly Gln consuming tissues (Van De Poll et al. 2004). Glutamine has been shown to exhibit effects on cell division, function, and maintenance of intermediary metabolism in Hela cells, lymphocytes, macrophages and

enterocytes. On the other hand, Gln also promotes NADPH and CO_2 production. Furthermore, Gln contributes nitrogen atoms for the macromolecular synthesis of purines, pyrimidines and amino sugars (Newsholme et al. 2003). Because Gln can be synthesised and released from various tissues, it is considered to be nutritionally dispensable. Nevertheless, in certain conditions, such as sepsis, rehabilitation of burns, or surgery, Gln availability can be lowered due to increased requirements by catabolic or inflammatory processes and oxidative stress in humans (Rodas et al. 2012) and animals (Cruzat et al. 2010; Stavrou et al. 2018), considering Gln as a conditionally essential AA (Newsholme et al. 2011).

2.4.2 Functions of glutamine in the pig

It is known that the colostrum and milk of sows are rich in Glu and Gln, whereas the amounts of ornithine and citrulline are quite low (Wu and Knabe 1994). In the SI, Gln is utilised by the enterocytes as a major energy substrate (Wu et al. 1994b). For example, Gln provided more ATP to the enterocytes of pigs than glucose or fatty acids (Wu et al. 1995c). Additionally, it has been described that Gln acts as a substrate for the synthesis of citrulline and Arg in enterocytes of piglets during the first week of life. This is important because, during the neonatal period, the requirements for Arg are much higher than its provision from milk, as the endogenous synthesis of Arg is crucial for optimal growth and development during the first week of life (Wu et al. 1995c). Furthermore, Gln is an important substrate for the production of glucosamine-6-phosphate, which is used for building amino sugars and glycoproteins. Also the function of monocytes, macrophages, lymphocytes and neutrophils is dependent on Gln (Alverdy 1990). High concentrations of Gln in plasma benefit piglets in maintaining the regular activity of lymphoid organs and the immune system. This is why Gln is considered a nutritionally essential AA in young pigs (Wu 2010).

2.4.3 Effects of glutamine supplementation on the gastrointestinal tract

The two NEAA Gln and Glu cannot be considered separately due to their partially identical metabolic pathways in the function and metabolism of the healthy intestine or pathological events (Blachier et al. 2009). In cells where the enzyme GLS is present, such as the enterocytes, Gln can be converted to Glu and NH_4^+. Conversely, cells in which a high GS activity prevails, as in colonocytes (Andriamihaja et al. 2010), Gln can be produced from Glu and ammonia (Eklou-Lawson et al. 2009). However, only Gln is used to synthesise purines and pyrimidines (Newsholme and Carrie 1994), and N-acetylglucosamine and N-acetylgalactosamine, which are required for intestinal mucin synthesis (Reeds and Burrin 2001). Glutamine is also the preferred energy source for rapidly dividing cells, such as epithelial cells and immune cells, such as lymphocytes. Almost 60% of the absorbed Gln is

oxidised in the SI to provide energy (Windmueller and Spaeth 1978; Blachier et al. 2009). The epithelial cells of the colon are supplied with Gln by the basolateral side via the bloodstream. In addition, colonocytes can use short-chain fatty acids such as acetate, propionate and butyrate as energy substrates, which are released by bacteria from Glu and other AA (Blachier et al. 2007). The transport of Gln in the intestine requires sodium as a co-transporter, and Gln has an additional effect on sodium absorption comparable to that of glucose. Glutamine enters the enterocytes mainly through the sodium-dependent AA transporters ATB0/ASCT2 (Avissar et al. 2004). Under hypersecretory conditions, Gln supplementation improves absorption (Coeffier et al. 2005), suggesting that Gln may positively affect various intestinal diseases associated with diarrhoea. Glutamine appears to be the preferred fuel for immune cells, regulating lymphocyte proliferation and cytokine release (Yaqoob and Calder 1998), and a lack of Gln leads to a deteriorated immune response under various pathophysiological conditions (Wernerman 2008). Glutamine might improve the intestinal barrier function by acting on cell proliferation and apoptosis balance, protein synthesis and degradation, and specific signalling pathways (Rhoads and Wu 2009). It has also been shown to enhance mucosal growth and improve gut barrier function during certain situations. Windmueller (1982) demonstrated that Gln provides a major portion of the energy required by the enterocytes, and Ardawi and Newsholme (1983) showed similar effects in colonocytes. Rhoads et al. (1997) demonstrated that Gln activates a variety of early response genes, essential to the proliferative response of the enterocyte (Kandil et al. 1995). In addition, Gln enhances the effect of growth factors on enterocyte DNA synthesis (Jacobs et al. 1988) and stimulates ornithine decarboxylase activity in a dose- and time-dependent manner. This latter enzyme regulates the rate-limiting step in polyamine biosynthesis, which is critical for intestinal cell generation and repair. Glutamine also improves the protein metabolism of the intestinal mucosa (Coeffier et al. 2003), via the regulation of mTOR signaling. In contrast, Gln deficiency stops protein synthesis via the GCN2 signalling pathway (Boukhettala et al. 2012). In vitro studies also show that Gln depletion is associated with increased intestinal permeability (Boukhettala et al. 2012), potentially via reduced expression of the tight junction proteins, occludin, claudin-1 and ZO-1. (Li et al. 2004). In addition, Gln also has cytoprotective effects in the gut, due to its ability to induce heat shock proteins (Meynial-Denis 2017)

2.4.4 Metabolism of Glutamine in Enterocytes

Two types of Na^+-independent transporters are connected to the brush border membranes of jejunal enterocytes (Fan et al. 1998). The Na^+-dependent B° transport system serves as the primary transporter of luminal Gln. Furthermore, it is essential to emphasise that enterocytes

can take up Gln from the bloodstream (Meynial-Denis 2017). This supply of Gln through the bloodstream is especially relevant for epithelial cells of the colon as they transfer very little AA from the intestinal lumen into the blood, except for a short period directly after birth (Darragh et al. 1994), and are therefore dependent on the supply of Gln as an oxidative substrate for energy supply (Darcy-Vrillon et al. 1993). The mitochondrial phosphate-dependent enzyme GLS degrades Gln from the intestinal lumen or blood to Glu or ammonia (Wu et al. 1994a; Wu et al. 1995b). Glutamate enters the cytosol and is transaminated with oxaloacetate to form alpha-ketoglutarate and aspartate (Blachier et al. 2009) or can be transaminated in the presence of pyruvate to produce Ala and alpha-ketoglutarate (Wu et al. 1995b). Aspartate, which is endogenously produced by Gln or Glu, derives from the intestinal contents and can be used by the mitochondria for oxidative metabolism (Windmueller and Spaeth 1978). Since enterocytes have only a limited capacity to convert Glu via the enzyme glutamate dehydrogenase into alpha-ketoglutarate and ammonia, most of the conversion occurs via transamination (Madej et al. 2002). In the mitochondria, alpha-ketoglutarate is then used for the Krebs cycle (Duee et al. 1995). Glutamine and Glu may also be converted to other AA such as ornithine (Blachier et al. 1992), citrulline and proline (Wu et al. 1994a). The synthesis of glutathione from the AA cysteine, glycine and Glu also occur in the enterocytes (Reeds et al. 1997). The metabolism of Gln and Glu in colonocytes is similar to that of enterocytes (Darcy-Vrillon et al. 1994). The enzyme GLS is also expressed in colonocytes, but the activity of the enzyme GS appears to be higher in colonocytes than in enterocytes (Eklou-Lawson et al. 2009). The compression of Glu and ammonia to form Gln may depend on the high ammonia concentration in the colon (Mouille et al. 2004), and the inhibitory effect this high concentration has on colonocyte respiration (Mouille et al. 2004; Andriamihaja et al. 2010).

Table 2.1 Distribution of glutamine and general effects of glutamine supplementation

	Main findings	References
Physiological fluids and tissue proteins	Free Gln in porcine milk ↑ from 0.1 to 4 mM between 1 and 28 days of lactation	(Wu and Knabe 1994)
	Gln and Glu make up to 20% of total peptide bound AA in the colostrum and milk	(Haynes et al. 2009)
	Gln in the plasma of fetal pigs goes from 0.8 to 1.4 mM during gestation	(Wu et al. 1995a; Wu et al. 1996a)
	Between 30 and 40 days of gestation Gln concentration in amniotic and allantoic fluids of sows increased from 0.64 to 1.3 and 0.69 to 3.5 mM	(Wu et al. 1996a; Wu et al. 1998)
	Between 30 and 40 days of gestation Gln concentration in amniotic and allantoic fluids of sows increased from 0.64 to 1.3 and 0.69 to 3.5 mM Gln in porcine uterine flushing's ↑ at 10 and 15 days of pregnancy	(Gao et al. 2009; Wu et al. 2010a)
	Gln represents ca 5.2% of total AA in body proteins	(Wu et al. 1999; Wu et al. 2010a)
	Skeletal muscle is quantitatively the most important tissue for Gln storage	(Wu et al. 2006; Manso et al. 2007; Li et al. 2009a)
Physiological functions	Multiple metabolic pathways, regulating gene expression and signal transduction Major energy substrate for rapidly dividing cells, providing ATP for intracellular protein turnover, nutrient transport through the plasma membrane, cell growth and migration and maintaining the integrity of cells.	(Curi et al. 2005; Wang et al. 2008)
	Regulation of the acid-base balance	(Curthoys and Watford 1995)
	Precursor of purines and pyrimidines	(Wu 1998)
	Major AA for endogenous synthesis of citrulline and Arg via the intestinal renal axis	(Wu and Morris 1998)
	Formation of N-acetylglucosamine	(Wu et al. 2011b)
	Antioxidant in cells	(Reeds et al. 1997; Stipanuk et al. 2009)
	Gln modulates gene expression of ornithine decarboxylase, heat stock proteins, and nitric oxide synthetase	(Kwon et al. 2004; Rhoads and Wu 2009)
	Gln ↑ intestinal expression of genes that are necessary for cell growth and removal of oxidants, while ↓ expression of genes that promote oxidative stress and immune activation	(Wang et al. 2008)

Physiological functions	Gln activates mTOR	(Curi et al. 2005; Xi et al. 2011)
	Stimulation of protein synthesis and protection of skeletal muscle and enterocytes from proteolysis by ↑extracellular concentrations of Gln	(Wu and Thompson 1990; Xi et al. 2011)
	Gln affects adenosine monophosphate-activated protein kinase, extracellular signal-related kinase, Jun kinase and mitogen activated protein kinase	(Curi et al. 2005; Wu et al. 2007a)
	Adjusts the production of NO and CO for gaseous signaling	(Li et al. 2009c; Wu 2009)
Digestion and absorption	Extensively catabolized by the small intestine / also takes up Gln from arterial blood	(Wu et al. 1994c)
	Free Gln is stable in the stomach and the duodenum	(Wu et al. 1996b)
	Small peptides are hydrolyzed by mucosal peptidases / di- and tripeptides that can be directly taken up into enterocytes via PepT1 to form free Gln in small intestine	(Daniel 2004; Haynes et al. 2009)
	Via Na^+-dependent system N transporters free luminal Gln of the small intestine is absorbed by enterocytes	(Bode 2001)
	Small peptides with Gln and free Gln can be absorbed by luminal bacteria via transporters	(Ling and Armstead 1995)
	The jejunum takes up most of the dietary Gln, the ileum and duodenum come after the jejunum in absorption of Gln	(Haynes et al. 2009)
	Almost 67% of dietary Gln is exploit by the small intestine in pigs, while the rest enters the portal circulation	(Stoll and Burrin 2006; Wu et al. 2010b)
Usage of dietary and aterial blood Gln	The organs of pigs in all age groups use Gln, especially the SI	(Wu et al. 2010b)
	In young pigs the flow of Gln in arterial plasma is 3.27 g/kg of BW	(Bertolo and Burrin 2008)
	Stomach, spleen, pancreas, kidneys skeletal muscle, lymphoid organs and vascular endothelia use the majority of arterial Gln The GIT acquires Gln from feed and blood	(Wu et al. 2011b)
	Out of arterial blood Gln is the only AA that can be absorbed by the SI of swine	(Wu and Knabe 1994)
Endogenous synthesis of Gln in pigs	The majority of the circling Gln is acquired by de novo synthesis from Glu and ammonia by Gln synthetase in serval tissues like skeletal muscle, mammary gland (lactating) and adipose tissue	(Li et al. 2009b; Wu 2009)
	The Gln in sow milk is synthesized from branch-chained AA and α-ketoglutarate in the lactating teats	(Li et al. 2009a)
	Via catabolism of essential AA the Ammonia utilized for Gln synthesis is formed	(Wu et al. 2010b)
	Through insignificant action of Gln synthetase in mucosal cells of neonatal, growing, and lactating pigs only a small amount of Gln is synthesized in the small intestine in contrast to the high usage of Gln by the intestine.	(Haynes et al. 2009; Li et al. 2009a)

Table 2.2 Basic effects of glutamine supplementation on the sow, piglets before and after weaning

	Supplementation	Main findings	Reference
Preweaning piglets			
Glutamine concentration in plasma of 1 day old suckling piglets	Sow milk	• NBW: 0.53 ± 0.04 m*M* • IUGR: 0.36 ± 0.03 m*M*	(Wu et al. 2011b)
Growth Performance	Sows diet + 2.5% crystalline Gln	• No influence on growth performance of piglets	(Kitt et al. 2004)
	1 g Gln/kg BW	• ↓ daily weight gain by 19% in 7 to 21 day old piglets	(Haynes et al. 2009)
	3.42 mmol/kg BW Gln; Ala-Gln twice daily	• healthy sow-reared piglets intestinal and whole-body growth ↑ • No effect on milk intake • Growth performance ↑ after LPS-challenge	(Haynes et al. 2009)
	4.87 g Gln per litre of whole milk Intake of 4.45 g Gln per day	• IUGR piglets growth and milk intake ↑, preweaning mortality ↓	(Wu et al. 2010b)
	1 g/kg BW of Gln per day	• IUGR piglets growth ↑, • milk intake ↑, • preweaning mortality ↓	(Wu et al. 2011b)
	1 % of Gln as fed basis	• ↑ growth performance • ↑ increased villus height/villus height : crypt depth ratio in duodenum	(Yang et al. 2018)
Physiological Parameters	3.42 mmol/kg BW Gln; Ala-Gln twice daily	• ↑ concentration of Gln in plasma, more effective ↑ by Ala-Gln • Plasma concentrations of adenyl purines and jejunal tissue concentrations of ATP, AMP, adenosine and cAMP normalised • Lipopolysaccharide (LPS) - induced increase in rectal temperature was reduced by 0.5°C	(Haynes et al. 2009)

Preweaning piglets			
Physiological Parameters	1 g/kg BW of Gln per day	• ↓ ammonia concentration in plasma of IUGR piglets	(Wu et al. 2011b)
	1 g/kg BW of Gln	• Stimulation of cell proliferation in muscle tissue • Larger muscle fibers • Slightly altered abundance of myosin heavy chain isoforms	(Zhao et al. 2020; Zhao et al. 2021)
Immune response	3.42 mmol/kg BW Gln; Ala-Gln twice daily	• LPS-induced jejunal atrophy, cell death and oxidative stress ↓ • Intestinal expression of Toll-like receptor-4, caspase-3, and NF*k*B ↓	(Haynes et al. 2009)
Postweaning piglets			
Findings		• In early weaned piglets reduced feed intake and intestinal epithelial damage occurs linked to ↓ intake of Gln by the diet • ↓synthesis of Gln from glucose branch-chained AA and other AA	(Wu et al. 1996b; Wang et al. 2008)
		• Small intestine requirement of Gln in 21–35-day old sow reared piglets (965 mg/kg BW per day) • Supply of Gln from the diet + arterial blood is only 618 mg/kg of BW per day • Need of Gln supplementation of 347 mg/kg of BW	(Wu et al. 2010b)
Benefits		• No additionally protein in the diets → N excretion/incidence of diarrhea ↓	(Lalles et al. 2007)
		• Gln supplementation fits to the physiological needs of the SI and can reduce conversion of essential AA and others for Gln syntheses by extraintestinal tissue	(Wu et al. 2011b)
Growth performance	Diets with 0.2, 0.6 or 1.0% free Gln	• ↑ feed efficiency by 25% • Prevention of villus atrophy	(Wu et al. 1996b)
	Sows diet + 2.5% crystalline Gln	• LPS challenged and unchallenged piglets from sows supplemented with glutamine had lower weight gain, ↓ feed intake and ↓small intestine weights	(Kitt et al. 2004)
	2% as fed basis Gln	• Improvement of growth performance and small intestinal morphology in Escherichia coli or LPS-challenge	(Yi et al. 2005)
	Corn-soybean meal diet supplemented with 1% free Gln	• ↓ lower incidence of diarrhea and shorter duration • First ten days after weaning ↓ feed:gain ratio • Second ten days ↑ daily gain	(Zou et al. 2006)

Preweaning piglets			
Growth performance	1% free Gln in diets	• ↑ BW gain • Prevention of jejunal atrophy • ↑ small intestine growth	(Wang et al. 2008)
	0.8% Gln	• ↑ average daily gain • ↑ villus height and villus height : crypt depth ratio	(Zhang et al. 2017)
	1% Gln or 1% glutamic acid	• No effects on performance	(Amorim et al. 2018)
	0.8% Gln	• ↑ expression of jejunal glutathione peroxidase and total superoxide dismutase • ↓ malonaldehyde concentration • ↑ expression of occluding mRNA	(Zhang et al. 2017)
Physiological parameters	Diets with 0.2, 0.6 or 1.0% free Gln	• ↑ plasma concentrations of asparagine, Glu and Ala • ↓ drop in plasma taurine	(Wu et al. 1996b)
	Diet supplemented with 1% free Gln	• ↓ serum urea nitrogen for the first 10 days • ↑ total protein in serum	(Zou et al. 2006)
	1% Gln in creep feed or 0.88% Aminogut	• Best feed:gain ratio • ↑ villus height and proliferating cells	(Cabrera et al. 2013)
	0.8% Gln	• ↑ concentrations of Gln, Glu, Arg, citrulline in plasma and protein	(Zhang et al. 2017)
	1% Gln or 1% glutamic acid	• acceleration of carbon turnover	(Amorim et al. 2018)
Gestating Gilts			
Findings		• 60% of fetal growth happens in the last three weeks of gestation → higher need of Gln by the fetus • Sows plasma concentrations of Gln significantly ↓ in late gestation	(Wu et al. 1999)

Gestating Gilts			
Findings	1% Gln to diets of gilts during 90 and 114 days	• ↓amount of IUGR piglets, deviation in BiW and preweaning mortality • ↑ mean BiW and litter BiW of alive born piglets	(Wu et al. 2011b)
Lactating sows			
Findings		• On day 19 of lactation the mammary gland takes up 16 g of Gln/d from arterial circulation • Mammary gland secretes 36 g of Gln/d in milk	(Trottier et al. 1997) (Haynes et al. 2009)
		• Supplementation contributes Gln for tRNA-Gln creation and saves BCAA for other metabolic pathways, like synthesis of milk proteins, by working as substrates and activators of mTOR	(Curi et al. 2005)
		• Gln modulates cell signalling via extracellular signal-related kinase, Jun kinase, mitogen-activated protein kinase, and NO, which in turn regulates milk production in the lactating mammary gland	(Wu et al. 2007b)
		• Increased requirements for Gln	(Kim and Wu 2009)
		• Possible triggering of ornithine decarboxylase expression in the lactating mammary tissue	(Rhoads and Wu 2009)
		• For synthesis of milk proteins there is inadequate uptake of Gln by the mammary gland • Branch - chained AA play an important role in Gln synthesis by the lactating porcine mammary gland	(Li et al. 2009a)
Physiological Parameters	Corn-soybean meal + 2.5% crystalline Gln	• ↑ milk and plasma Gln levels	(Kitt et al. 2004)
	1% Gln in diets	• ↑ concentrations of Gln in milk	(Manso et al. 2007)
	1% Gln in diets 0 to 21 days of lactation	• ↑ concentrations of Gln in plasma, skeletal muscle and milk	(Wu et al. 2011b)
	1% Gln in diets 0 to 21 days of lactation	• ↑ milk yield at 14 and 21 days of lactation • ↑ total protein and urea nitrogen in serum at 14 days of lactation • ↑ concentration of Gln in plasma and milk • ↑ Branch – chained AA concentration in plasma	(Yang et al. 2018)

Safety towards swine			
Findings		• Gln is abundant in culture medium for all cell types	(Curi et al. 2005)
		• High concentrations in foetal fluids	(Wu et al. 1995c; Wu et al. 1996b; Kwon et al. 2004; Gao et al. 2009)
		• Short half-life in blood of neonatal, gestating and lactating pigs	(Wu et al. 2010b)
		• Supplementation of 1% Gln in the diet can be regarded safe	(Manso et al. 2007; Wu et al. 2010b)
		• In connection with other nutrients higher concentrations of Gln may lead to an unwanted side-effect because of AA imbalances and increased amounts of NH3 in Plasma	(Suryawan et al. 2009; Wu et al. 2010b)
		• proposed use level (25 mg/kg feed) when used as sensory additive (flavouring compound) is safe for all animal species	(Bampidis et al. 2020)

2.5 Development of microbiota during the suckling period in piglets

2.5.1 Development of microbiota in the jejunum and the colon during suckling period

In the first few days after birth, the microbiota changes and becomes more diverse, as bacteria from the environment enter the GIT. From birth, the GI microbiota develops in a rapidly evolving environment where intestinal physiology and innate immunity develop simultaneously (Kim et al. 2011). At birth, the piglet GIT is mainly oxic, and colonised by aerobic bacteria like lactic acid bacteria, enterobacteria and streptococci (Lalles et al. 2004). During the first week of life, the metabolic activity of these early colonizing microbiota leads to oxygen depletion and an increase in the number of anaerobic microbes (Jost et al. 2012), with other bacteria such as *Bacteroides, Lactobacillus, Bifidobacterium* and *Clostridium* starting to colonise the GIT (Petri et al. 2010). It has been observed that the microbial composition in pigs is fairly constant during the first three weeks of life. Mainly consisting of *Enterobacteriaceae, Lachnospiraceae, Bacteroidaceae, Clostridiaceae* and *Lactobacillaceae* (Frese et al. 2015). The environmental changes lead to an ecosystem with a quick changing structure which shows different age depended shifts along the whole GIT (Isaacson and Kim 2012). The intake of colostrum / milk also impacts the development of the gut microbiota in neonatal piglets, and as a result, the microbiome becomes “milk orientated” (Frese et al. 2015). There is continuing debate about which are the two main phyla in the GIT of piglets. Several studies show that *Firmicutes* and *Bacterioidetes* are the main phyla (Kim et al. 2011; Gresse et al. 2017), but another study indicates that *Firmicutes* and *Proteobacteria* in the jejunum and ileum are the predominant phyla (Yang et al. 2016). In the colon, *Firmicutes* and *Bacteroidetes* are described as the phyla with the main abundance during suckling (Arnaud et al. 2020; Qi et al. 2021). The first microbes to which piglets are exposed during birth come from the sow's vagina. Shortly afterwards, factors such as hygiene conditions (Montagne et al. 2010), the genotype of the sow, her milk (Frese et al. 2015), parity (Carney-Hinkle et al. 2013) and the housing environment (Kubasova et al. 2017), have an impact on microbial composition. Of all these factors age, location of the gut microbiota and the gut segment seem to have the greatest influence on microbiota composition (Wang et al. 2019). Since the development of intestinal microbiota depends on continuous exposure to microbes, exposure to microbes in the early postnatal period should not be considered harmful (Schmidt et al. 2011).

2.5.2 Function of bacterial metabolites in neonatal piglets

It is assumed that the gut of neonatal piglets prior to birth is not colonised with bacteria. Shortly after birth, there is a massive shift from an actual germ-free state to a dense microbial population (Isaacson and Kim 2012). The number and diversity of bacteria increase from the

stomach and jejunum toward the colon (Shirkey et al. 2006). The primary substrates for microbial fermentation are carbohydrates. Through the breakdown of easily fermentable carbohydrates, lactate is produced by lactobacilli, especially in the stomach and small intestine (Sakata et al. 1999). Fermentation of non-starch polysaccharides and oligosaccharides, primary in the colon, leads to the production of short-chain fatty acids (SCFA). It is important to know that sow milk is rich in oligosaccharides, especially at the beginning of lactation. During the previously described fermentation process, a huge amount of acetic acid, propionic acid and butyric acid arise from the fermentation of these milk oligosaccharides (James et al. 2003). With small amounts of fermentable carbohydrates, there is bacterial utilisation of AA in addition to carbohydrate degradation. As a result, the SCFA iso-butyric acid and iso-valeric acid are increasingly produced. An increased proportion of these branched fatty acids indicates an increased bacterial utilisation of proteins (Blaut and Clavel 2007). The SCFA butyrate is increasingly metabolised in colonic epithelial cells and utilised by them as a major source of energy. Butyrate also has various health-promoting effects at the cellular level, such as inducing epithelial cell differentiation. It reduces proliferation, strengthens the epithelial barrier and suppresses tumorigenesis (James et al. 2003). Sow milk is also an essential source of AA and biogenic amines produced by AA degradation from gut bacteria. Members of the family of *Enterobacteriaceae* are known to produce biogenic amines. Nevertheless, gut-health promoting effects of biogenic amines are difficult to predict. Both protective and toxic dose-dependent effects have been described (Schokker et al. 2018)

2.5.3 Influence of the colonic microbiota on the development and intestinal immunity in the GI-tract

As the fermenting chamber, the colon plays a vital role in breaking down feed components by anaerobic bacteria that can be absorbed and used by the piglet, such as SCFA, which serve as an energy source for pigs (De Vries and Smidt 2019). The transport of water in the colon is dependent on SCFA originating from colonic bacteria, and the intestinal bacteria also provide exogenous alkaline phosphatases (Yolton and Savage 1976). The production of vitamin K (Ramotar et al. 1984) and the recycling of bile salts (Gilliland und Speck 1977) impact GI health. The first contact line for gut microorganisms are the epithelial cells. Intestinal epithelial barrier function is activated by commensal gut microorganisms and reacts with tolerance toward their presence (Sharma et al. 2010). Many mammalian studies observed an essential effect on immune system development combined with microbial colonisation (Hooper et al. 2012). Also, different bacteria positively influence BW because of their interaction with the host's immune system. These taxonomic groups of bacteria are *Turicibacter, Clostridiaceae, Streptococcaceae* and *Lactobacillaceae*. *Lactobacillus mucosae* is a bacteria suggested to

help piglet BW gain due to its ability to improve epithelial barrier function and bind mucus (Wang et al. 2019). As described by Dou et al. (2017), 7-day old piglets with a higher abundance of *Prevotellaceae, Lachnospiraceae, Ruminococcaceae* and *Lactobacillaceae* showed a lower incidence of post weaning diarrhoea.

2.5.4 The effects of glutamine supplementation on the gastrointestinal development of microbiota and bacterial metabolites

Currently, there is very little literature describing the potentials effects of Gln supplementation on GI microbiota and their metabolites. Some studies suggest that SI bacteria are involved in the synthesis and catabolism of the AA lysine, threonine, Arg, Glu and Gln (Metges and Petzke 2005; Dai et al. 2010; Dai et al. 2012b). Glutamine is regulating the bacterial metabolism, especially the metabolism of nitrogenous compounds. For this reason Gln is regarded as a key regulator of the survival and growth of bacteria (Forchhammer 2007) and additionally Gln is quickly utilised and metabolised by bacteria in the intestine. As described by Dai et al. (2012b), up to 36% of Gln is utilised by SI bacteria, but only 10% of it is used for protein synthesis. The exact metabolic routes and the impact of Gln on intestinal bacteria is not clear. One possible metabolic pathway for AA by bacteria, especially for Gln, could be deamination. Small intestine bacteria use Gln or Glu, and differences in utilisation was observed to be species and gut section dependent (Dai et al. 2010; Dai et al. 2011; Dai et al. 2012a). There is also a difference in utilisation of AA between luminal and mucosa attached bacteria. Luminal bacteria of jejunal digesta use Gln more intensively than tightly attached bacteria (Yang et al. 2014). In mice, Gln supplementation leads to decreased abundance of *Firmicutes* and higher mRNA levels for antibacterial factors in the gut (Ren et al. 2014). In rats, Gln supplementation increased the abundance of *Lactobacillus*, *Comamonas*, *Enterobacter*, *Peptostreptococcaceae, Acinetobacter*, *Enterococcus* and *Wohlfahrtimonas* (Xu et al. 2012). Furthermore, dietary Gln supplementation to weaned rabbits led to lower mortality and modified the gut microbiota (Chamorro et al. 2009). In humans, Gln supplementation has been shown to affect the *Firmicutes:Bacteroidetes* ratio in the gut of obese patients (Zambom De Souza et al. 2015), and decreased infectious morbidity with no associated alterations in the prevalence of *Bifidobacteria*, *Lactobacilli*, *E.Coli*, *Streptococci*, and *Clostridia* in LBW infants (Van Den Berg et al. 2007). Supplementation of Arg or Gln to mice also led to a decreased colonisation of the SI by pathogenic *E. coli* and increased IgA secretion (Liu et al. 2017). Glutamine supplementation seems to reduce the severity of intestinal infection related to weaning in pigs. In *ex vivo* experiments, lower mucosal cytokine response and an increased intestinal barrier function was observed. However, it is not clear if the observed findings are the effects of Gln on the microbiota or the host (Ewaschuk et al. 2011). Glutamine affects the microbiota by

intestinal secretion of immunoglobulin through bacteria by T cell-dependent and independent pathways (Wu et al. 2016). This is why the effect of Gln supplementation on GI bacteria might be a secondary immune- and receptor-mediated process (Husted et al. 2017).

2.6 Gut maturation and colonization in low birthweight piglets

No differences have been reported in intestinal morphology and barrier function between LBW and NBW piglets during the suckling period (Huygelen et al. 2014; Huygelen et al. 2015). However, many authors described a lower villus height and crypt depth, indicating a smaller intestinal absorptive area, in piglets affected by IUGR (D'inca et al. 2010b; D'inca et al. 2011; Mickiewicz et al. 2012). There seems to be an altered proliferation-apoptosis homeostasis, possibly associated with a changed gene expression pattern of growth-related proteins in piglets influenced by IUGR, which leads to a decrease in surface area (Wang et al. 2005; D'inca et al. 2010b). The smaller intestinal surface area in piglets affected by IUGR leads to lower activity of brush border enzymes, notably lactase, and affects the gut barrier function, showing an increased permeability (D'inca et al. 2010b; D'inca et al. 2011; Ferenc et al. 2014). Another finding in piglets influenced by IUGR is, that transcellular and paracellular permeability are transiently increased (Wang et al. 2016), indicating an impaired barrier function of the gut. For example, the higher paracellular permeability might explain the increased translocation of antigens and microorganisms in neonatal piglets affected by IUGR (D'inca et al. 2011).
Interestingly the structural and functional changes are not visible anymore in piglets influenced by IUGR that have survived the first days of life (Mickiewicz et al. 2012; Huygelen et al. 2014; Wang et al. 2016). At the same time, proteomic analysis detected that protein related to absorption, digestion, transport, apoptosis, metabolism and redox homeostasis are still impaired during the suckling period (D'inca et al. 2010b; Wang et al. 2010). Since impaired gut maturation and colonisation are apparent, differences in gut colonisation also occur because of differences in BiW (D'inca et al. 2010b; Li et al. 2019). In piglets affected by IUGR, differences in the adherent bacteria flora in the neonatal period are shown (D'inca et al. 2010b). In contrast, similar to the disappearance of structural and functional changes between piglets influenced by IUGR and NBW piglets, differences in the gut colonisation and composition are not present in piglets affected by IUGR, if they are older than one week of age (D'inca et al. 2010b). In contrast, recent studies comparing the microbial composition of LBW and NBW piglets observed minor changes in microbial composition and microbial genes at 7 and 21 days during the suckling period (Li et al. 2019).

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RESEARCH ARTICLE

Effects of oral glutamine supplementation on jejunal morphology, development, and amino acid profiles in male low birth weight suckling piglets

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Abstract

Background

It has been shown that small intestine development in low birth weight **(LBW)** piglets is impaired. Glutamine **(Gln)** has been reported to improve piglet health and intestinal function in weaned piglets, but data is scarce in suckling piglets. This study was conducted to investigate the effects of oral Gln supplementation compared to Alanine (**Ala**) on jejunal development and function in 5 and 12 d old male LBW and normal birth weight **(NBW)** suckling piglets.

Results

Gln had no effect on the jejunal morphology, development, tissue and digesta amino acid profiles and mRNA abundance of genes involved in amino acid transport, metabolism, glutathione synthesis in LBW piglets when compared to Ala supplementation and birth weight controls at 5 and 12 d. Only the concentration of Gln in jejunal tissue was higher in NBW piglets supplemented with Gln compared to Ala at 5 d (P Ā 0.05). A comparison of the birth weight groups showed no differences between LBW and NBW piglets at 5 and 12 d in any parameter. Jejunal crypt depth, villus height / width, tunica muscularis thickness, number of goblet and IgA positive cells, the ratio of jejunal RNA to DNA and the concentration of DNA, protein and RNA changed (P Ā 0.05) from 5 compared to 12 d. The concentrations of several free, and protein bound amino acids as well as amino metabolites differed between age groups in jejunal tissue but the digesta concentrations were affected to a lesser extent.

Conclusions

Oral Gln supplementation to suckling male piglets over the first 12 d of life was not associated with changes in jejunal parameters measured in this study. The absence of effects may

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Abbreviations: AA, Amino Acid; Aad, α-Aminoadipic acid; Ala, Alanine; BiW, Birth weight; CD, Crypt depth; FAA, Free amino acids; FPSR, Fractional protein synthesis rate; Gln, Glutamine; LBW, Low birth weight; LSM, Least squares means; NBW, Normal birth weight; PBAA, Protein-bound amino acids; SE, Standard error; SI, Small intestine; Suppl, supplementation group; TAA, Total amino acids; TuM, Tunica muscularis thickness; VH, Villus height; VW, Villus width.

indicate that Gln is absorbed as well as metabolized in the upper intestinal tract and thus could benefit intestinal development at a more proximal location.

Introduction

Increasing litter sizes in modern pig production have led to higher numbers of LBW piglets [1]. Low birth weight is accompanied by an increased risk of disease, impaired organ development, and higher mortality [2–4]. In terms of animal welfare, the high rate of mortality in LBW piglets, especially in male piglets [5], is ethically debated [6] and results in significant economic losses [7]. The pig is additionally interesting because it is considered as an excellent animal model for human nutrition [8]. Underweight infants often have problems related to immature development of the intestinal tract [9].

The small intestine (**SI**) has digestive, absorptive as well as immunological functions and grows rapidly in the early neonatal period [10]. This rapid growth is fueled by colostrum and milk intake, which provides not only energy and essential nutrients, but also different bioactive compounds such as growth hormones [11]. The jejunum is the largest section of SI [12] and during the neonatal period the morphology, several metabolic pathways and immunological functions are constantly changing [13–15]. Previous studies in piglets show that jejunal morphology, development and function is impaired in LBW individuals [16–18]. To overcome this impairment several nutritional strategies have been developed [1, 19]. These include supplementations with colostrum [20] or bovine whey protein [21], nucleotides [22], short-chain fatty acids [23] and specific amino acids (**AA**) [24].

Glutamine **(Gln)** and glutamate (**Glu**) are the most abundant protein bound amino acids **(PBAA)**, whereas free glutamine in sow milk increases during lactation and becomes the most abundant free amino acid **(FAA)** [25]. *In vitro* studies have shown that Gln is a primary energy source for neonatal porcine enterocytes [26–28]. It is assumed that Gln promotes protein synthesis, immune response and oxidative status in mucosal cells of SI [29]. In enterocytes, Gln can be transformed to Glu, which is a precursor of glutathione, a key anti-oxidative defense molecule [30]. Gln metabolism by the jejunum has been investigated in several species, including pigs [27, 31, 32]. However, the majority of the Gln supplementation studies in pigs have been conducted in weaned piglets [29, 33–36], which are, from a physiological perspective, different from suckling piglets. In these studies Gln has been supplemented as single AA [34–36] or as dipeptide [29, 33], with most of the control groups supplemented with isonitrogenous amounts of alanine (Ala). Studies supplementing Gln to piglets during the later suckling period, analyzing parameters after weaning, have been conducted as well [33, 37–39]. Studies with lactating sows suggest that the Gln provided by milk might be limiting for protein synthesis of piglets [39]. Hence, it was also investigated whether supplementation of maternal diet with Gln either during pregnancy or lactation [40] was beneficial, but the effects on piglet growth were inconclusive.

To the best of our knowledge, this is the first study to investigate the effect of oral Gln supplementation on jejunal morphology, development and AA profiles in tissues and digesta in sow reared piglets. Since jejunal morphology as well as development is impaired in LBW compared to NBW piglets [18], we hypothesized that Gln supplementation would improve these parameters in LBW piglets compared to their Ala supplemented control littermates. In addition, changes in jejunal AA profiles could provide insights into jejunal Gln metabolism and its potential role in improving LBW jejunal morphology and development. The aim of this study

was to investigate the effects of oral Gln supplementation to suckling piglets with different birth weights on jejunal characteristics including morphology, AA-metabolism and anti-oxidative-defense.

Methods

Animals, experimental design and sample collection

All experimental procedures were approved by the licensing authority State Office for Agriculture, Food Safety and Fishery Mecklenburg-Western Pomerania, Germany (permission No. 7221.3-1-026/16), and performed according to the German Animal Welfare Act following the Directive 2010/63/EU (European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes). Healthy German Landrace gilts were bred and gave birth at the Research Institute for Farm Animal Biology experimental pig facility, where the entire study was conducted [41].

The trial design has been previously described in detail [42]. Briefly, male LBW with a mean birth weight **(BiW)** of 1.1 ± 0.04 kg (n = 48; below the lowest BiW quartile of the experimental pig facility) [42] and NBW (1.49 ± 0.04 kg; n = 48; represents the middle 50th percentile of piglets born at the experimental pig facility) littermates selected at birth. Within 24 h post farrowing, litter sizes were standardized to 12 piglets and experimental piglets assigned to either Gln (1 g/kg BW/d; n = 48) or Ala (1.22 g/kg BW/d; isonitrogenous to Gln; n = 48) supplementation. Each LBW or NBW sibling was assigned to a supplementation (Ala, Gln) or age-group (5 or 12 d) in order to obtain similar mean birth weights of LBW (5 or 12 d; LBW-Ala vs. LBW-Gln) or NBW (5 or 12 d; NBW-Ala vs. NBW-Gln) supplementation pairings. Not more than three piglet pairs per sow were selected. Experimental piglets remained with, and were suckled by their respective dam throughout the study, which was performed across 17 experimental blocks. Approximately 24 h post birth, experimental piglets were orally supplemented with Gln or Ala as described [42]. Piglets were supplemented 3 times daily (07:00, 12:00 and 17:00) with 1/3 of the calculated daily dose using disposable syringes. The procedure used to orally dose the piglets with the supplemental amino acids is described in the S1 File. Exclusion criteria for pairs of piglets in this study were loss of body weight for more than two consecutive days, sickness or lack of mobility of already one of the paired piglets. During the experimental period 5 pairs of LBW and NBW piglets were excluded accordingly. Excluded pairs were replaced by matching pairs of piglets to reach the total sample size (n = 96). In addition, no blinding was conducted during the study, with all participants knowing the experimental group allocations from birth.

At 5 and 12 d, piglets were transported to the Research Institute for Farm Animal Biology slaughterhouse 2.5 h prior to euthanasia. Two h before euthanasia each piglet received 33% of their respective daily AA supplement in 6 mL milk replacer (150 g/L water at 45˚C; 16.5 MJ of metabolizable energy (ME)/kg, 20.5% crude protein, 10% crude fat, 0.2% crude fiber; Neopigg Rescuemilk 2.0, Provimi, Netherlands). Piglets were electro-stunned and euthanized by exsanguination. Within 5 min post-euthanasia a 35 (5 d) or 40 cm (12 d) jejunal tissue section was sampled from a defined anatomical site in each animal and age group (5 d; ~40 cm, 12 d; ~60 cm prior to the ileocecal junction). Digesta was collected, snap-frozen in liquid N_2, and stored at -80˚C for subsequent analysis. The jejunal tissue was then washed with physiological saline and a 5 cm section (most proximal to the ileocecal junction) put into Roti-Histofix (4% paraformaldehyde, Histofix, Roth, Karlsruhe, Germany) for histological analysis. The remaining tissue was diced into small pieces, snap frozen in liquid nitrogen and stored at -80˚C for subsequent analysis.

Jejunal morphometry, histochemistry and immunohistochemistry

Histo-fixed jejunum samples were cut with a feather-trimming blade (FEATHER, No.130 Type(S)) into 1 large and 2 smaller pieces and prepared as previously described [43]. A microtome (Type 1400 Fa. Leitz Wetzlar, Germany) was used for cutting 5 μm sections from the paraffin blocks. For mucosal morphometry measurements and differentiation of diverse mucin types defined by the carbohydrates displayed, the Alcian blue pH 2.5 -periodic acid Schiff staining method described by Liu et al. (2014) [44], was used. The measurements were investigated using a microscope (Photomicroscope BX43F, Olympus, Tokyo, Japan) equipped with a digital camera (Olympus DP72, Tokyo, Japan). Pictures were examined with the cellSens imaging software (v. 1.4, Olympus). Five villi and corresponding crypts were randomly chosen from various well-orientated parts of at least four sections. Sections with undamaged villi and crypts were cut longitudinally. The distance from the tip of the villi to the bottom of the crypts was measured. Morphometric measurements included villus height **(VH)** (from the tip of the villus to the crypt mouth), villus width **(VW)**, crypt depth **(CD)** (from the crypt mouth to the base of the crypt), villus height to crypt depth ratio and tunica muscularis thickness **(TuM)** [44].

For quantification of Immunoglobulin-A (IgA) secreting cells, 5 μm jejunal paraffin sections were mounted on glass slides. After deparaffinization and rehydration, the slides were boiled in 0.1 M sodium citrate buffer (pH 6.0). Endogenous peroxidase was inhibited with 1% aqueous hydrogen peroxide solution for 30 min at room temperature. Slides were then incubated in a humid chamber for 1 h in PBS containing 10% normal horse serum to avoid non-specific antibody binding. Afterwards, sections were incubated over night at 4˚C with the following antibody: goat anti-porcine IgA 1:4000 (NB724, Novus Biologicals, Abingdon, UK). Subsequently washed sections were incubated for 1 h with biotinylated horse anti goat IgG 1:500 (Cat. NO: BA-9500, Vector Laboratories) and then administered with ABC complex (Vectastain elite ABC peroxidase Kit, Standard, Vector Laboratories). To visualize the immune reaction, a 3,3´-diaminobenzidine chromogen solution (DAB Substrate kit, Vector Laboratories) was applied [45]. An isotype control with a non-specific antibody (goat IgG, AB-108-c, R&D Systems) was conducted to avoid nonspecific binding of the Fc part of the primary antibody. IgA positive cells were counted in the jejunal lamina propria in three areas in five eye fields from four sections per animal according to Waly et al. (2001) [46]. The areas were delineated with cellSens imaging software (v. 1.4, Olympus), ignoring the epithelium, large blood vessels and artefacts. In each area, stained cells were counted and the results were given as positive cells per 10,000 μm^2 of lamina propria tissue [47].

The detection of CD3-positive intraepithelial lymphocytes was performed as described previously [48]. Briefly, for antigen retrieval, slides were heated in 0.1 M sodium citrate buffer (pH 6.0) in a microwave oven until boiling for 30 min. Afterwards the primary antibody PPT3 (mouse anti porcine CD3 epsilon, CAT NO 4510–01, Southern Biotech) was applied to the slices. An isotype control with a non-specific antibody (mouse IgG, CAT NO 0102–01, Southern Biotech) was included, to control nonspecific binding of the Fc part of the primary antibody. For visualization of the primary antibody, a two-step indirect method was used (mouse and rabbit Specific HRP/DAB IHC Detection Kit, ab236466, ABCAM). The secondary antibody was conjugated with horseradish peroxidase (HRP) labelled micro-polymer (goat anti-rabbit HRP Conjugate, 58009 ABCAM). The whole immunohistochemistry protocol was performed according to a published procedure [49]. To evaluate the stained sample a double-blind quantification of CD3-positive intraepithelial lymphocytes was performed. Only complete and intact villi (two slices per animal, five villi per slice) were evaluated and cell counts were expressed per 100 enterocytes (Fig 1).

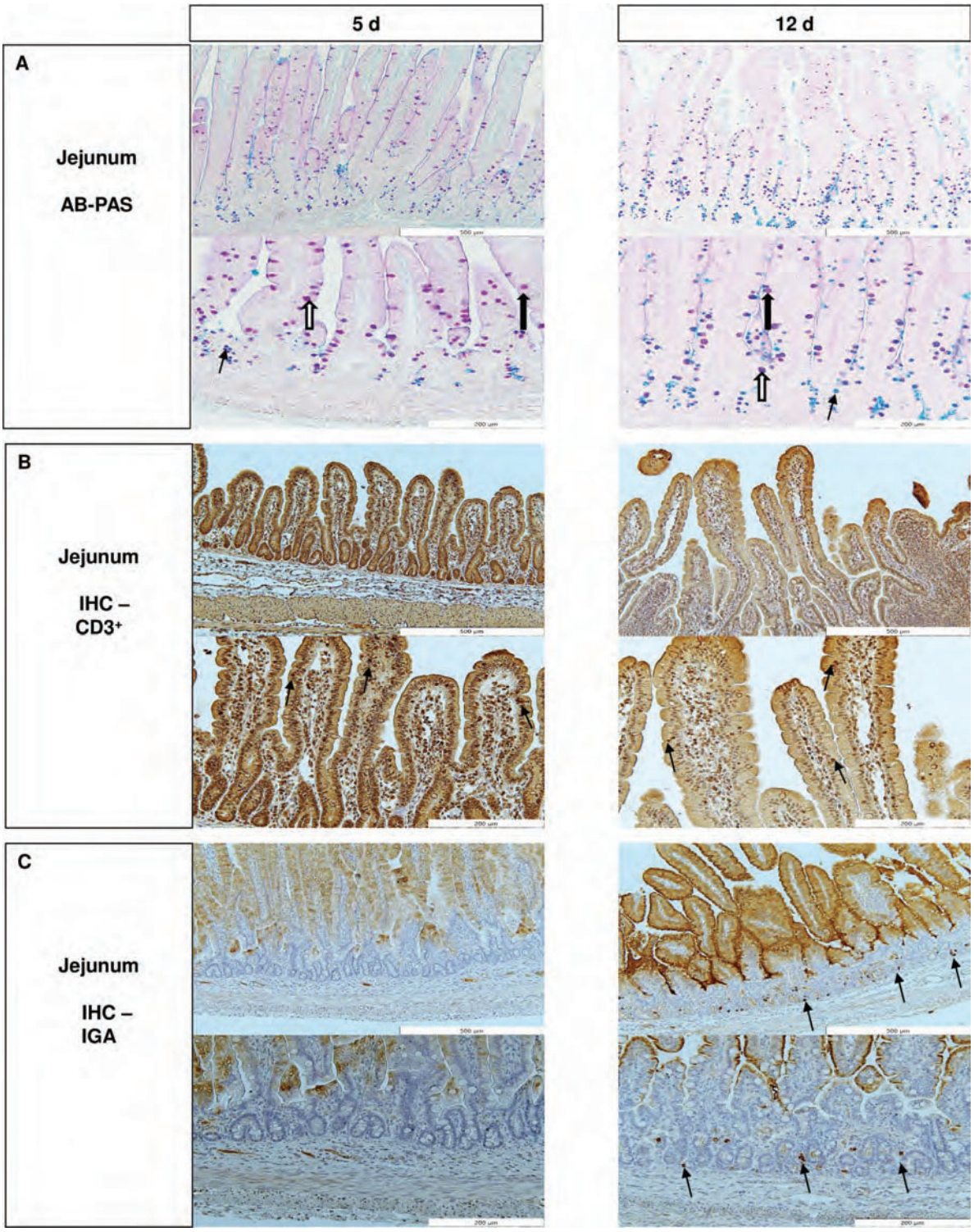

Fig 1. Jejunal Histomorphology and Immunohistochemistry (IHC) of 5 d and 12 d old male suckling piglets. A Alcian blue pH 2.5 -periodic acid Schiff stained jejunal tissue with stained goblet cells, different arrows indicating goblet cells containing different mucins. Narrow arrow = acidic mucins, wide closed arrow = neutral mucins, wide open arrow = mixed mucins. 5 d, 12 d: upper picture 100 x, lower picture 200 x magnification. **B** IHC of CD3, arrows indicating positive stained intraepithelial CD3+ cells in villi. 5 d, 12 d: upper picture 100 x, lower picture 200 x magnification. **C** IHC of IgA positive stained cells in lamina propria, no IgA positive cells detected at day 5, arrows indicating IgA positive

cells. 5 d, 12 d: upper picture 100 x, lower picture 200 x magnification. Villus tip stained probably by milk derived secretory IgA (SIgA) on the apical side of the enterocytes.

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Free and protein bound AA and AA metabolites concentration in jejunal tissue and digesta

Jejunal tissue samples were prepared as previously described [50] and tenfold diluted by ultrapure water for FAA analysis. For the assessment of total amino acids **(TAA)**, an enzymatic hydrolysis was performed [51]. Two μL of supernatant was diluted with 55 μL HEPES buffer (50 mM, pH 7.5), combined with 1 μL of pronase E (54 Units/mL) (Sigma-Aldrich, Munich, Germany), 1 μL of prolidase (250 Units/mL) (Sigma-Aldrich, Munich, Germany), and 1 μL of aminopeptidase M (25 Units/mL) (MP Biomedicals, Santa Ana, California) and incubated at 37˚C for 20 h. Samples were centrifuged (4˚C) at 16,000 *g* for 10 min, and diluted 15/100 by ultrapure water. Digesta samples were lyophilized and 5 mg was suspended in 500 μL of ultrapure water. Samples were vortexed for 15 s and centrifuged at 17,000 *g*, 4˚C, for 10 min. The supernatant was transferred to a new test tube without the lipid layer and diluted by factor 4 with ultrapure water for FAA determination. For the assessment of TAA, samples were hydrolyzed enzymatically as described above with the exception that 20 μL of supernatant and 37 μL HEPES buffer were used. Free AAs, AA metabolites and TAA were measured by HPLC as described earlier [52] using 5 μm C18 columns, 250 x 4 mm HyperClone™ 120 Å or 250 x 4.6 mm Gemini® 110 Å (both Phenomenex, Aschaffenburg, Germany). Protein bound AA were calculated by subtracting FAA from TAA concentrations.

Jejunal biochemical indices and fractional protein synthesis rate

Total RNA and DNA was extracted from ground jejunal tissue (80–120 mg) according to the manufacturer's protocol (peqGOLD TriFast; VWR International GmbH, Hannover, Germany), whereas total protein was isolated, using a lysis buffer [50] described above. Total RNA and DNA was quantified using a Nanophotometer (Implen GmbH, Munich, Germany), whilst total protein was quantified photometrically using BCA reagent (Biorad Laboratories, Feldkirchen, Germany). Biochemical indices of cell size (protein:DNA ratio), protein synthetic efficiency (protein:RNA ratio) and protein synthetic capacity (RNA:DNA ratio) were calculated as previously described [53].

Fractional protein synthesis rate **(FPSR)** was determined using the flooding dose method as described [54] with modifications. Piglets were given an intraperitoneal injection of L-$^{2}H_5$ phenylalanine (Ring-$^{2}H_5$, 99.1% atom ^{2}H; $^{2}H_5$-Phe; Euriso-Top, Saint-Aubin, France) (125 mg/kg BW) in physiological saline (Serumwerk Bernburg AG, Bernburg, Germany) one h before euthanasia, to measure the jejunal FPSR.

Fifty mg of ground jejunal tissue was suspended in 0.5 mL of 0.2 M perchloric acid kept on ice, using a sonication tip (Amplitude 80, cycle 0.5, 30 pulses), vortexed and centrifuged (4˚C) at 3,000 *g* for 10 min. The FAA containing supernatant was adjusted to pH 7 using 4 M KOH. After centrifugation (4˚C) at 3,000 *g* for 10 min, the supernatant was dried at 60˚C under nitrogen. Samples were treated with N-Methyl-N-tert-butyldimethylsilyltrifluoroacetamide to form tert-butyldimethylsilyl-derivatives. Additionally, the protein pellet was used to determine the protein-bound $^{2}H_5$-Phe enrichment. After washing the protein pellet twice with 1 mL of 0.2 M perchloric acid and with 1 mL of ultrapure water it was dried at 60˚C under nitrogen gas. The dried pellet was hydrolyzed as described [55] and the free AA were converted to tert-butyldimethylsilyl-derivatives. The abundance of $^{2}H_5$-Phe was quantified using GC-MS

(Quadrupole, GC-MS QP 2010, Shimadzu, Japan, equipped with a Zebron ZB-5HT column, 30 m × 0.25 mm × 0.25 μm column, Phenomenex, USA) as described [55]. The diagnostic ions m/z 336 (M+0) and m/z 341 (M+5) were used to calculate the enrichment as molar per cent excess of $^{2}H_{5}$-Phe. The FPSR was calculated using the following equation:

$$FPSR(\%/d) = \frac{E_{Protein}}{E_{free}} \times \frac{1}{t} \times 100$$

Where $E_{Protein}$ is the enrichment of $^{2}H_{5}$-Phe in the jejunal tissue protein and E_{free} is the enrichment of $^{2}H_{5}$-Phe in the free AA pool of the jejunal tissue at the time of sampling. The period between $^{2}H_{5}$-Phe injection and sampling is defined as t. The FPSR is expressed as the percentage of tissue protein renewed per d (%/d).

Jejunal transcript abundances related to AA transport, AA metabolism and antioxidative defense

Purification of RNA and cDNA synthesis. Total jejunal RNA (30 μg) extracted for the calculation of biochemical indices was purified using RNeasy minikits (Qiagen, Hilden, Germany) and quantified using a Nanophotometer (Implen GmbH, Munich, Germany). The RNA quality was assessed using a Bioanalyzer 2100 and RNA 6000 Nano kit (Agilent Technologies, Waldbronn, Germany), with an RNA integrity number range of 6.9 and 9.7 (mean 8.8 ± 0.8). Purified RNA (500 ng) was reverse transcribed to make cDNA using the SensiFAST™ cDNA Synthesis Kit (Bioline, Berlin, Germany) according to the manufacturer's instructions.

Primer design, real time PCR assay and data preparation. Primers were made by Integrated DNA Technologies (IDT, Antwerp, Belgium), and selected from previous studies or designed using the IDT RealTime qPCR Assay design tool. Primers were tested using serial dilutions (1/25, 1/50, and 1/100 diluted cDNA). Due to varying mRNA abundances between targets either 1/25 or 1/50 dilutions were used for quantification. Primer details are presented in (S1 Table in S1 File). Amplified cDNA samples were analyzed on 96 well plates (Roche) using the LC 96 system (Roche Diagnostics, Mannheim, Germany). Samples were analyzed in duplicate (plus five additional samples: two inter-run calibrators, a no-template and a no-enzyme control and a water control). Quantitative real time PCR was performed using the SensiFAST SYBR No-Rox Mix (Code: 98050, Bioline, Berlin, Germany), with the template (4 μM) and all reagents at half of the manufacturers recommended volume. The same reaction conditions; enzyme activation and initial denaturation (95˚C for 30 s); denaturation/annealing repeated 40 cycles (95˚C for 30 s, 60˚C for 20 s); and melting curve analysis from 65 to 98˚C with 1˚C increment every 5 s) were used for all mRNA targets analyzed. The PCR efficiency and quantification cycle values were then obtained for each sample using LinRegPCR v 2014.5 [56]. Average PCR-efficiency and quantification cycle values are reported in S1 Table in S1 File. The GeNorm applet from qBASEplus selected the reference genes from six candidates (5 d: beta actin (ACTB), ribosomal protein S18 (RPS18), and DNA topoisomerase 2-beta (TOP2B), 12 d: peptidyl-prolyl isomerase A (PPIA) and ribosomal protein S18 (RPS18)) as the most stably expressed across the BiW and Suppl used in this study. Reference genes were used to normalize target gene mRNA abundance in the qBASEplus software and the Cq-values were converted into log transformed calibrated normalized relative quantities (Log-CNRQ) values, taking into account amplification efficiencies, inter-run variations, and normalization factors. All data was reported as per the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines [57].

Data and statistical analysis

The required experimental sample size (*n*) was calculated per 2 and 3 level factor combination of (1) birth weight (LBW, NBW), (2) supplementation (Ala, Gln) and (3) treatment duration / age group (5 d and 12 d), using CADEMO for Windows ANOV version 4.03 (2000; BioMath GmbH, Rostock, Germany), and the settings $\alpha = 0.05$, $\beta = 0.20$. The primary outcome measures used to determine *n* were body weight gain and changes in intestinal villus height and abundance of mRNA molecules associated with oxidative status.

Normal distribution was assessed via Shapiro-Wilks criteria, followed by a linear mixed model analysis which was conducted for each of the 143 variables using the MIXED procedure of SAS (version 9.4; SAS Institute Inc., Cary, NC, USA) with three fixed factors: (1) birth weight (LBW, NBW), (2) supplementation (Ala, Gln) and (3) age group (5 d and 12 d). Unless otherwise indicated, the group size for each analysis performed was $n = 12$. Deviating group sizes are reflected in the footnotes of the corresponding table. Sow was defined as a random factor which allowed explicit modelling of the non-independence of littermates from the same sow and improved inference about the fixed effects. ANOVA F-tests for the three fixed effects and their interactions were carried out and the Tukey-Kramer test was applied to compare groups and correct for multiple testing. Least squares means **(LSM)** and their standard errors **(SE)** are reported, with the largest SE shown. Differences were considered significant if Tukey-Kramer test was $P \leq 0.05$.

The linear mixed model analysis revealed that the factor 'age group' had a significant effect on the analyzed set of variables. To identify the variables discriminating the two age groups (5 d and 12 d), the N-integration with Projection to Latent Structures models with Discriminant Analysis (PLS-DA) was applied, using R 4.1.0 (R Core Team, 2021) and the mixOmics package (v6.14.1; [58]). Here, so called 'blocks' of variables measured on the same samples are integrated in a holistic supervised analysis. In this study, all 143 variables were first analyzed together. Cross-validation was used to evaluate the performance of the PLS-DA model, with a 10-fold cross-validation and 1000 repeats to get an accurate estimations of the error rates. Centroid distance was chosen as it is regarded a suitable measure for the complex classification problems [58]. The quality of the PLS-DA model was verified by fold cross-validation using two performance indicators: Q^2, "goodness of prediction", or predicted variation and R^2, known as the goodness of fit [59]. All 143 variables were then assigned to ten individual blocks; morphology characteristics (n = 5), cell types (n = 13), biochemical indices (n = 7), mRNA target-molecules (n = 21), jejunal tissue (free AA; n = 20, AA-metabolites; n = 10, PBAA; n = 20) and digesta (free AA; n = 20, AA-metabolites; n = 7, PBAA; n = 20). Sample plots for each 'block' of variables are presented only to visualize the potential discriminatory ability of each component in the space spanned by the first two latent variables.

A volcano plot was generated using R 4.1.0 (R Core Team, 2021) and the effsize package (v0.8.1; [60]) and qvalue package (v2.22.0; [61]). Effect sizes (Cohen's d) were calculated for each variable based on the estimated least square means of 5 d versus 12 d. The list of p-values of the age group fixed effect estimate was then used in conjunction with a standard false discovery rate (FDR) estimation procedure to find the number of variables to be declared as different while controlling FDR at a specified level of 0.05. The FDR-adjusted p values were calculated using the Benjamini & Hochberg procedure [62].

Results

Jejunal morphology and abundance of goblet cells, intraepithelial lymphocytes, and IgA positive cells

The VH was affected by Suppl ($P = 0.041$), whereas VW ($P = 0.012$) was influenced by BiW (Table 1). Age affected VH ($P = 0.019$), VW ($P = 0.019$), TuM ($P = 0.020$) and CD ($P < 0.001$).

Table 1. Jejunal morphology characteristics in low and normal birth weight male suckling piglets.

		Ala		Gln			*P* values[1]		
Item	Age (d)	LBW	NBW	LBW	NBW	SE	BiW	Suppl	Age
Villus height (μm)	5	812	718	789	872	65.3	0.265	0.041	0.019
	12	899	855	1061	935	66.5			
Villus width (μm)	5	95.3	97.8	95.3	101	2.87	0.012	0.256	0.019
	12	99.9	105	103	107	2.93			
Crypt depth (μm)	5	113[e]	126	124	125	5.90	0.949	0.703	<0.001
	12	150[f]	145	149	142	5.90			
Villus height to crypt depth ratio	5	7.25	5.90	6.67	7.12	0.51	0.274	0.098	0.622
	12	6.17	6.00	7.11	6.78	0.52			
Tunica muscularis thickness (μm) [2]	5	107	105	122	102	12.3	0.814	0.882	0.020
	12	127	144	128	126	12.2			

[e,f] Labeled LSM within a column within one Suppl and BiW group without a common letter differ, $P < 0.05$ (Tukey-Kramer test).

Values are LSM ± SE, the largest SE is shown; n = 12/group (5, 12 d).

[1] ANOVA F test. None of the interactions of the fixed factors (Suppl x BiW; Suppl x Age; BiW x Age or Suppl x BiW x Age) were significant ($P > 0.05$).

[2] Tunica muscularis was damaged due to the thawing procedure. Therefore group size deviated from $n = 12$ for the parameter Tunica muscularis thickness. 5 d LBW-Ala, 5 d LBW-Gln, 12 d NBW-Gln, 12 d LBW-Gln, and 12 d NBW-Ala $n = 11$.

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The CD in the jejunum was higher at 12 d than at 5 d in LBW-Ala piglets ($P < 0.001$) (Table 1). Mixed mucins containing goblet cells in villi were affected by Suppl ($P = 0.007$) (Table 2). Age affected the number of mixed mucins containing goblet cells in crypts ($P = 0.025$) and villi ($P = 0.020$), as well as the total number of goblet cells in the crypt

Table 2. Number of jejunal goblet cells in low and normal birth weight male suckling piglets.

			Ala		Gln			*P* values[1]
Item[2]		Age (d)	LBW	NBW	LBW	NBW	SE	Age
Villus	Acid	5	3.97	4.23	4.65	4.83	0.59	0.588
		12	4.83	4.92	4.7.0	4.38	0.60	
	Neu	5	6.58	8.24	6.76	7.02	0.72	0.901
		12	6.71	7.41	7.07	7.10	0.74	
	NA	5	7.31	8.52	5.96	6.10	0.64	0.020
		12	5.52	6.14	5.11	5.51	0.65	
	Total	5	17.8	21.0	17.4	18.0	1.38	0.318
		12	17.1	18.5	16.9	17.0	1.41	
Crypt	Acid	5	21.7	20.0	22.2	24.5	1.85	0.081
		12	18.2	18.4	19.0	21.9	1.87	
	Neu	5	12.0	13.6	12.9	11.6	1.74	0.755
		12	11.7	13.8	12.3	14.2	1.77	
	NA	5	22.6	21.6	21.6	20.5	1.58	0.025
		12	18.3	19.0	17.9	19.3	1.59	
	Total	5	56.5	55.3	56.5	56.5	2.9	0.029
		12	48.2	51.1	49.1	55.2	2.92	

Values are LSM ± SE of goblet cells containing different mucins per 1 mm basement membrane, the largest SE is shown; n = 12/group (5, 12 d).

[1] ANOVA F test. Suppl had a significant effect on NA mucins in villus ($P < 0.01$); neither the fixed factor (BiW) nor the interactions of the fixed factors (Suppl x BiW; Suppl x Age; BiW x Age or Suppl x BiW x Age) were significant ($P > 0.05$).

[2]Acid = acidic mucins; NA = mixed neutral and acidic mucins; Neu = neutral mucins.

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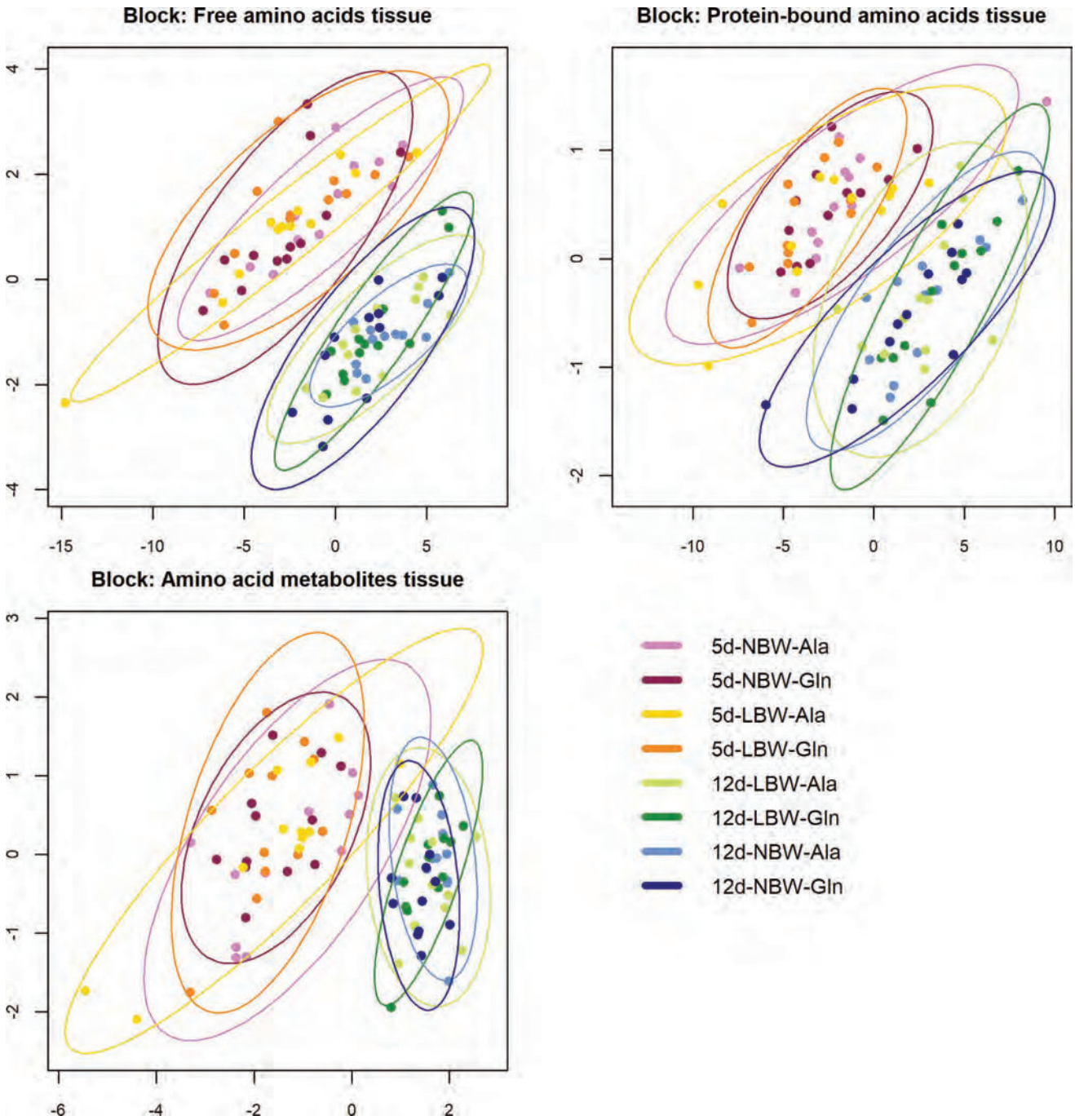

Fig 2. Partial Least-Squares Discriminant Analysis (PLS-DA). Sample plots of the block PLS-DA of all 143 jejunal variables assigned to ten variable groups ('blocks') and measured in 96 samples. Shown are the sample plots for the three blocks with the best discriminatory ability: Free amino acids tissue, Protein-bound amino acids tissue, Amino acid metabolites tissue. The other seven blocks are shown in the supplementary material (S1 Fig in S1 File). The colours indicate the eight experimental groups of the 3-factorial crossed design (birth weight: LBW/NBW, supplementation: Ala/Gln and age group: 5 d/12 d) and highlight the main comparison of the two age groups (reddish: 5 d; bluegreen: 12 d).

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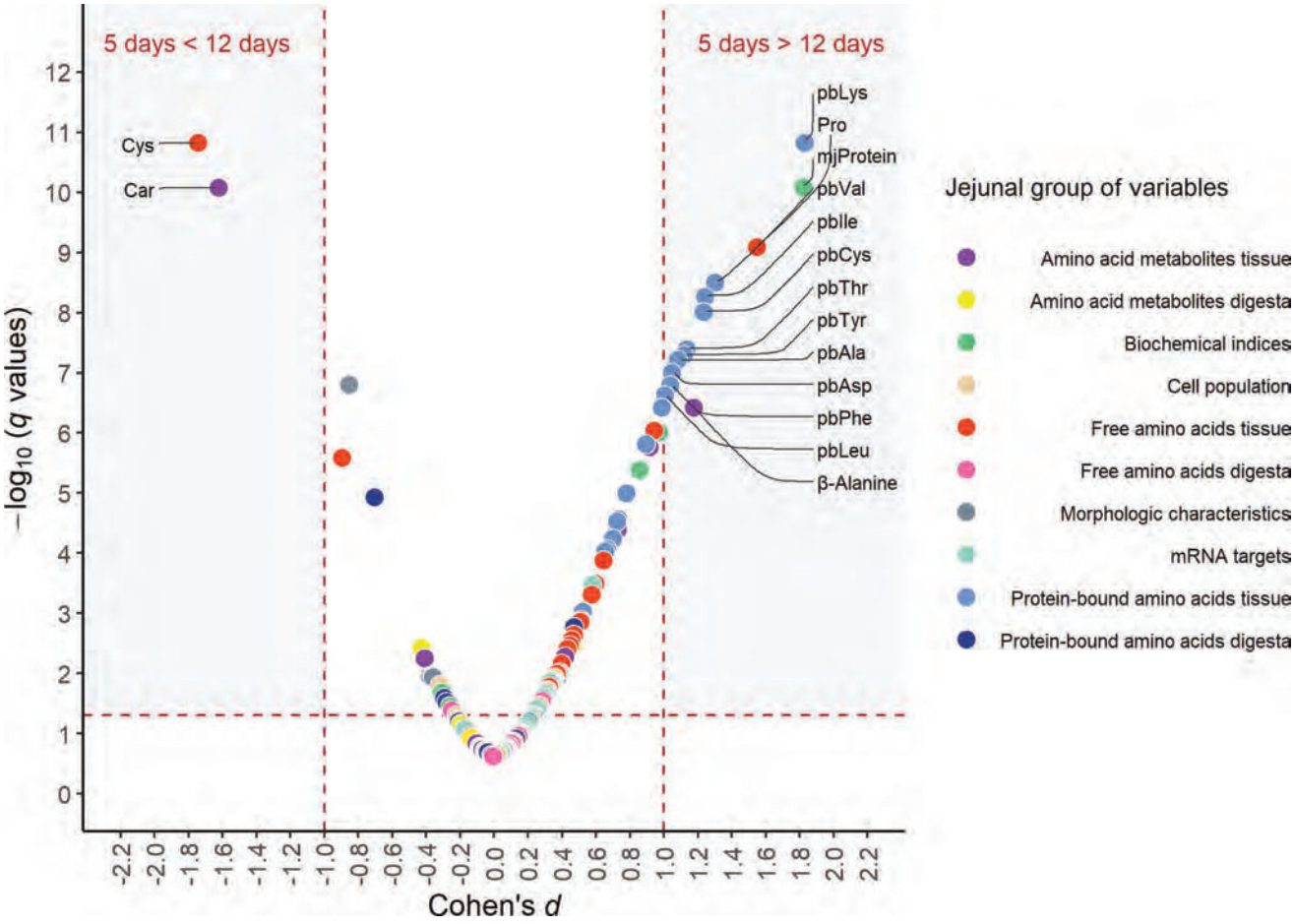

Fig 3. Volcano plot of jejunal variables analysed between 5 and 12 d old suckling piglets. Comparison of quantities of all 143 variables measured in 5 d and 12 d old suckling piglets. Q-values estimating the false discovery rate (FDR) were calculated for each variable from p-values of multiple Tukey-Kramer-tests comparing 5 d and 12 d old piglets. Effect sizes (Cohen's d) were calculated for each variable based on the differences in estimated marginal means and standard deviations of 5 d versus 12 d old piglets. Differences are classified as being substantial (grey shaded area) if FDR is limited to 0.05 ($q < 0.05$) and if the effect size of Cohen's $d < 1$ (5 d is smaller than 12 d) or Cohen's $d > 1$ (5 d is larger than 12 d). The 16 variables meeting this condition are annotated.

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($P = 0.029$). We could not observe IgA positive stained cells in the jejunal lamina propria of 5 d old piglets. In 12 d old piglets supplementation influenced the number of IgA positive cells in the lamina propria next to the crypts (Area 3) ($P = 0.048$) (S2 Table in S1 File). The number of intraepithelial lymphocytes $CD3^+$ cells in the jejunal villi (S3 Table in S1 File) and the number of $CD3^+$ cells in crypt area did not differ among groups.

Jejunal free AA and AA metabolite concentrations

There was an effect of Suppl on Gln, of BiW on Cys, and of Age on the concentration of all FAA measured in the jejunal tissue (S4 Table in S1 File), with the exception of the AA metabolites citrulline, ornithine and taurine (S5 Table in S1 File). The interaction BiW x Suppl affected α-Aminoadipic acid **(Aad)**, whereas the interaction BiW x Suppl x Age was significant for Asp, Gln, His, Ile, Met, Ser, Thr, Val, the branched-chained AA, and the Aad concentration. The concentration of Gln was higher in 5 d NBW-Gln compared to NBW-Ala piglets ($P = 0.044$). The concentration of hydroxyproline (P = 0.029) was higher in all four 5 d groups

than in the 12 d groups. Higher concentrations of Aad and Ala ($P < 0.028$) were found in the jejunal tissue of 5 d group compared to 12 d group with exception of NBW-Ala. The jejunal concentrations of Ser ($P = 0.006$), Gln, His, Ile, Thr ($P = 0.041$), and the group of dispensable AAs ($P = 0.009$) were higher in 5 d compared to 12 d LBW-Ala piglets. The concentrations of Glu and 3-Methylhistidine ($P = 0.042$) were higher in 5 d compared to 12 d LBW-Gln piglets. In NBW piglets supplemented with Gln the concentrations of Gln ($P = 0.007$), Asp, Glu, Ser, the group of dispensable AA ($P = 0.029$) were higher at 5 d compared to 12 d. In 12 d LBW-Gln ($P = 0.010$) and NBW-Ala ($P < 0.001$), the jejunal Gly concentration was higher compared to 5 d.

The Block PLS-DA showed a separation between the blocks jejunal FAA and AA metabolites (Fig 2B and 2C), probably contributing to the 5 and 12 d group separation observed in the PLS-DA analysis of all experimental blocks (Fig 2; 1 comp, $R^2 = 0.65$, $Q^2 = 0.65$). A subsequent univariate analysis (volcano plot; Fig 3) showed that four jejunal FAA and AA metabolites were different between the two age groups (Cohen's $d \geq 1$, FDR ≤ 0.05). The FAA in jejunal tissue Pro and the AA metabolite βAla were lower in 12 d compared to 5 d age groups, whereas the Cys and the AA metabolite Car were higher in the 12 d compared to the 5 d age groups (Fig 3) (S6 Table in S1 File).

Jejunal protein bound AA concentrations

There was an effect of Age on the concentration of all PBAAs (except Pro), whereas BiW affected Asn and Ile concentrations. The concentration of protein bound Glu, Ser ($P = 0.049$), the group of indispensable AA, branched-chained AA, dispensable AA and the group of total AA ($P = 0.029$) were higher in all 4 groups of 5 d piglets compared to the groups of 12 d piglets (S7 Table in S1 File). The concentration of protein bound Asn and Met ($P = 0.031$) was higher in groups of 5 d piglets compared to the respective groups of 12 d piglets except for the group of NBW-Ala. Higher concentrations of protein bound Arg and Trp ($P < 0.047$) were found in the jejunal tissue of the 5 d group in LBW-Ala and NBW-Gln compared to the respective 12 d groups. Lower concentrations of protein bound Arg and Trp ($P < 0.029$), were found in 12 d compared to 5 d LBW-Ala piglets. The concentrations of Gly ($P < 0.048$) were lower in 12 d compared to 5 d NBW-Gln piglets.

Block PLS-DA indicated that jejunal PBAA (Fig 2D) may be contributing to the 5 and 12 d group separation observed in the PLS-DA analysis of all experimental blocks (Fig 2A). A subsequent univariate analysis (volcano plot) showed that 10 variables (jejunal protein-bound Ile, Leu, Lys, Phe, Thr, Val, Ala, Asp, Cys, and Tyr) were lower in 12 than in the 5 d age groups (Cohen's $d \geq 1$, FDR ≤ 0.05), (Fig 3) (S6 Table in S1 File).

Free and protein bound AA concentrations in jejunal digesta

The concentration of the digesta γ-aminobutyric acid was influenced by Suppl, whereas the FAAs Asp and Ser were affected by BiW, while Aad, α-aminobutyric acid, and Orn were affected by Age. The interaction BiW x Suppl was significant for digesta free Glu, α-aminobutyric acid while the interaction Age x Suppl affected Arg and Asp (S8 and S9 Tables in S1 File). The concentration of digesta free Aad was lower in NBW-Gln ($P < 0.001$) at 5 d compared to 12 d, and in LBW-Gln compared to NBW-Gln piglets at 5 d ($P = 0.008$). Additionally, the concentration of free Aad was higher in NBW-Gln than in NBW-Ala at 5 d ($P = 0.006$). Age was significant for the concentration of the digesta PBAAs Lys, Gln, and Pro (S10 Table in S1 File). The concentration of protein-bound Lys increased from 5 d to 12 d, in NBW-Ala piglets ($P = 0.017$).

Table 3. Jejunal biochemical indices in low and normal birth weight male suckling piglets.

		Ala		Gln			*P* values[1]
Item	**Age (d)**	**LBW**	**NBW**	**LBW**	**NBW**	**SE**	**Age**
DNA (μg/mg $_{FM^2}$)	5	4.38	4.90^{e}	4.89^{e}	5.17^{e}	0.30	<0.001
	12	3.74	3.27^{f}	3.23^{f}	3.09^{f}	0.31	
Protein (μg/ mg $_{FM^2}$)	5	117^{e}	1084	119^{e}	114^{e}	3.27	<0.001
	12	81.9^{f}	83.4^{f}	86.7^{f}	84.0^{f}	3.28	
RNA (μg/ mg $_{FM^2}$)	5	3.97	4.41^{e}	4.15^{e}	3.99^{e}	0.18	<0.001
	12	3.23	3.24^{f}	3.10^{f}	3.16^{f}	0.19	
RNA/DNA	5	0.94	0.92	0.86	0.79	0.11	0.044
	12	0.97	1.07	1.13	1.13	0.11	
Protein/RNA	5	29.9	25.0	29.6	29.7	1.53	0.196
	12	25.9	26.3	28.6	27.5	1.53	
Protein/DNA	5	27.6	22.8	25.5	22.7	3.09	0.085
	12	25.9	28.9	31.8	29.5	3.12	
FPSR (%/d)[3]	5	60.7	69.8	72.8	71.6	7.42	0.123
	12	55.7	61.7	57.8	58.7	7.56	

[e,f] Labeled LSM within a column between one Suppl—birth weight group without a common letter differ, $P < 0.05$ (Tukey-Kramer test).

Values are LSM ± SE, the largest SE is shown; $n = 12$/group (5, 12 d).

[1] ANOVA F test. None of the other fixed factors (Suppl or BiW) or interactions of the fixed factors (Suppl x BiW; Suppl x Age; BiW x Age or Suppl x BiW x Age) were significant ($P > 0.05$).

[2] FM = Fresh matter

[3] Because of an insufficient accumulation of $^{2}H_5$-Phe in jejunal tissue, the group size deviated from $n = 12$ for jejunal FPSR. 5 d LBW-Gln, 12 d NBW-Gln, 12 d LBW-Gln $n = 11$.

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Jejunal biochemical indices and fractional protein synthesis

Protein, RNA, DNA ($P < 0.001$) concentrations and the RNA/DNA ratio ($P < 0.044$), reflecting protein synthetic capacity, in jejunal tissue were affected by Age. The concentration of DNA ($P = 0.005$) and RNA ($P = 0.050$) was higher in 5 d compared to 12 d LBW-Gln, NBW-Ala and NBW-Gln piglets (Table 3). Jejunal FPSR was unaffected by any of the main factors (Table 3). Subsequent univariate analysis (volcano plot) showed that the variable protein concentration was lower (FDR $P < 0.001$) in the 12 d compared to 5 d age group (Fig 3) (S6 Table in S1 File).

Jejunal transcript abundance related to AA transport, AA metabolism and antioxidative defense

The BiW class affected AST-2 ($P = 0.020$) whereas Age influenced the mRNA abundance of solute carrier family 1 member 5 (SLC1A5), solute carrier family 1 member 4 variant 1 (SLC1A4V1), aspartate aminotransferase 2 (AST-2), Glu cysteine ligase (GCL), glutathione synthetase (GSS) ($P < 0.05$), and succinate dehydrogenase complex, subunit A (SDHA) ($P < 0.001$) (S11 Table in S1 File). The BiW x Age interaction affected PSMC3 ($P = 0.039$). The mRNA abundance of succinate dehydrogenase complex, subunit A (SDHA) was higher in 5 d compared to 12 d LBW-Ala piglets ($P = 0.009$).

Discussion

The SI of LBW piglets is developmentally and functionally compromised compared to NBW individuals [63, 64]. Oral Gln supplementation has been previously shown to be beneficial for

the jejunal development and function of piglets around weaning [34, 35], however only few studies examined effects of Gln supplementation in piglets during the suckling phase [33, 38, 65]. While most studies have looked at weaned piglets, the present work is focused on the early suckling period, which to our knowledge has not been studied in this species. The jejunum is critical for the digestion of milk and absorption of nutrients. Therefore, in this work we focused on this section of the intestine and used a wide range of analytical methods to characterize potential effects of glutamine or alanine supplementation.

Our outgoing hypothesis was that the jejunal morphology and development of LBW compared to NBW male piglets benefits from Gln as compared to Ala supplementation. In addition, changes in jejunal AA profiles could provide insights into Gln jejunal metabolism and its potential role in improving LBW jejunal morphology and development.

Comparison among supplementation groups

Oral Gln supplementation to LBW suckling piglets was not associated with changes in any of the jejunal parameters measured, when compared to LBW-Ala or NBW-Gln control groups, at 5 d or 12 d of life. In addition, no effects were observed when Gln supplementation was assessed within each age group irrespective of BiW. *In vitro* studies in intestinal porcine enterocytes have shown that media supplemented with 2 mM Gln increased FPSR [66] and cell growth [67], both of which were unaffected in this study. However, this is a concentration 2–8 times higher than that reported in piglet plasma [38, 68] and thus translation of results is difficult. Furthermore, more recent *in vitro* studies on intestinal porcine enterocytes have shown effects of Gln on ATP production and apoptosis [28, 69]. In LPS-challenged suckling piglets, Haynes et al. (2009) showed that oral Gln supplementation prevented endotoxin related villus atrophy [38]. *In vivo* studies investigating effects of Gln supplementation on intestinal physiology have been conducted in piglets at the end of the suckling period, but different parameters were evaluated [65]. However, the majority of studies were conducted in weaned piglets [29]. Their physiological conditions are very different from that of the suckling piglets used in this study as intestinal AA-metabolism, local immunity and cellular population are changing [15]. It has been reported that Gln supplementation during weaning improved growth performance and intestinal health by preventing villus atrophy and reducing antioxidative stress [33–35, 70]. Hsu et al. (2012) [71] observed increased tunica muscularis thickness in jejunum and ileum in weaned piglets upon Gln supplementation. In an infection study with pathogenic *E. coli* Gln supplementation of weaned piglets inhibited villus atrophy [72]. Thus, it appears that Gln may have a protective effect on the SI under stressful conditions such as infection [38, 72] and weaning [33–35, 70]. Another study reported that Gln supplementation had similar effects on growth performance and plasma concentration of TNF-alpha in weaned piglets as antibiotic treatment [36]. Although we observed in a companion study with the same experimental animals that plasma Gln concentrations were higher 2 h after oral Gln supplementation in the 5 d and 12 d old piglets compared to Ala supplementation [42], in jejunal digesta and tissue the FAA and PBAA Gln concentrations and that of its metabolite Glu were not different between the Ala and Gln piglets. This may indicate that the Gln dose was absorbed in the proximal SI (duodenum and/or proximal jejunum) [29].

It has been reported that excess Gln is stored in the skeletal muscle [73] and that skeletal muscle is one of the main locations of Gln synthesis [74]. Glutamine is released under stressful conditions such as starvation or infection from the skeletal muscle, and the synthesis of Gln increases under such conditions [74]. A companion paper, using the same animals as in this study, showed no difference in free Gln concentrations in the *M*.

longissimus dorsi of LBW-Gln when compared to LBW-Ala or NBW-Gln groups, at 5 d or 12 d of life [75]. However, the concentration of Ala in *M. longissimus dorsi* in the Ala supplemented NBW and LBW piglets was higher than in the Gln littermates at 5 and 12 d of life. A study by Stoll et al. (1998) [76], using ^{13}C labelled AAs in suckling pigs, showed that Gln has a negative portal balance, indicating that Gln is utilized intensively by the SI. Thus, our observations, together with FAA profiles from the duodenum of these animals that show higher Gln concentrations in LBW-Gln and NBW-Gln compared to their Ala birth weight companions (unpublished data) suggest, that the supplemental Gln is already absorbed in more proximal regions of the SI. Additionally, it seems that the skeletal muscle as a Gln storage tissue [73], may not be relevant within the 2 h time period between Gln administration and sampling in this study.

Comparison between birth weight groups

A possible reason for the absence of differences in jejunal development between LBW-Gln and LBW-Ala or NBW-Gln piglets could be linked to the birth weight range of LBW piglets in this study (0.8–1.2 kg vs. 1.4–1.8 kg). In fact, the range of BiW reported for LBW piglets is rather wide [24, 64, 77]. Apparently, differences in intestinal development and function between low and normal BiW piglets, were reported mostly in piglets with much lower body weights than used here [77–79]. For example, Xu et al. (1994) observed reduced jejunal VH, CD, intestinal thickness, total DNA, RNA and protein content in very low birth weight piglets (0.59 ± 0.34 kg) at birth (prior to suckling) compared to normal BiW (1.32 ± 0.47 kg) littermates. Another study investigating newborn low birth weight piglets (0.83 ± 0.04 kg) and normal birth weight piglets (1.66 ± 0.07 kg) showed decreased length and weight of the SI, decreased VH:CD ratio and reduced expression of genes related to oxidative defense in low birth weight piglets [80]. In contrast, neither Wang et al. (2016) [79] nor Wiyaporn et al. (2013) [81] did observe differences in proximal jejunum VH, or CD between newborn un-suckled LBW piglets (0.81 ± 0.02 kg; and 0.88 ± 0.02 kg) compared to normal littermates (1.30 ± 0.03 kg; and 1.47 ± 0.03 kg). Similarly, small intestinal villus height, width and depth did not differ according to BiW (Huygelen et al., 2015). Additionally, Thongsong et al. (2019) [82], utilizing the same experimental piglets as Wiyaporn et al. (2013), did not find an effect of BiW on mRNA abundance of jejunal glucose, peptide and AA transporters including SLC7A8, which we determined in the present study. In the present study we did not determine jejunal parameters in newborn un-suckled piglets, thus it is not known whether the jejunal parameters measured differed at birth in our piglets. Interestingly, Wang et al. (2016) reported that in un-suckled low BiW piglets' jejunal permeability and tight junction (OCLN) mRNA abundance were higher, and antioxidant scavenger (Gpx1, CAT) mRNA abundance was lower compared to normal BiW littermates. Yet by 3 d of life, the differences were no longer present. The absence of difference on mRNA level related to oxidative defense in our study might indicate that LBW were not challenged by additional oxidative stress. Also Huygelen et al. (2015), did not observe differences in SI cell proliferation and in intestinal barrier function between low and normal BiW piglets at birth and after 3, 10, and 28 d of suckling. These results suggest three possibilities, (1) that the intestinal parameters measured in our study do not differ between low and normal BiW piglets, or (2) that differences observed at birth prior to suckling might have already disappeared during postnatal development if the nutritional requirements of piglets are met as reviewed by Everaert et al. (2017). Thirdly, it cannot be excluded that low birth weight piglets surviving the first 3 days of life are more vital and less comparable to the very low birth weight piglets with compromised intestinal development, which leads to a bias of selection of these piglets.

Comparison of piglet age groups

The development of the SI during the suckling phase is characterized by rapid growth, both on a macroscopic [10] and microscopic [77] scale. Several studies indicate that SI maturation is accompanied with changes in enterocyte metabolism [15, 26, 83] and nutrient absorption kinetics [84]. In the current study, jejunal morphological and immunological markers as well as nucleic acid, protein and AA profiles were compared between 5 d and 12 d old piglets, irrespective of the Suppl or BiW group. Morphologically, higher CD, VH, VW and TuM were observed in the 12 d compared to the 5 d group, consistent with previous studies conducted in sow-reared piglets [10, 19, 77, 85]. Longer and wider villi, as well as deeper crypts observed in 12 d compared to 5 d old piglets reflect an increased absorptive area. An increasing CD also indicates a higher crypt cell production and is an indicator for maturation of villous enterocytes [85]. The higher TuM observed in the older age group indicates jejunal cell proliferation and maturation. It should be noted that conflicting results in regard to the development of VH with age have been reported [19, 77, 86], which appear to be related to differences in piglet age, SI segment, villus atrophy, creep feed consumption and milk intake [12, 86]. Generally, the shape and length of the villi in the small intestine changes with weaning [12]. We observed lower staining of mixed mucins containing goblet cells in the villi and crypts and lower total number of crypt goblet cells in piglets of the 12 d compared to the 5 d group. Goblet cells containing different mucin types act as an innate defense mechanism, where the mucins protect the gastrointestinal tract by acting as a diffusion and micro-ecological barrier [87]. The observed decrease may suggest that at 12 d of age the mucosal barrier function built by mucins is changing due to immune system maturation, or may indicate changing luminal bacteria composition [88]. In addition, the abundance of IgA positive cells in the lamina propria was assessed. These IgA positive cells are B-cells that are derived from the antigen-specific IgA-committed B cells in Peyer's patches, which migrate to the lamina propria and function as part of the innate immune defense [89]. Consistent with previous studies [90, 91] our results show that IgA positive cells were mainly located in the lamina propria and were detected only in the 12 d group. The abundance of IgA positive plasma cells have been shown to be influenced by age [90, 92], commensal microbiota [93], and diet [94]. Taken together, these results indicate that the jejunum of the piglets in this study matures morphologically and immunologically from 5 d to 12 d of age and neither differences in maturation due to BiW nor to AA supplementation were observed.

Multivariate analysis via block PLS-DA showed that jejunal FAA, PBAA and amino-metabolites were the only variable blocks significantly affected by piglet age. Univariate analysis of the individual variables within each block confirmed this observation, revealing altered jejunal concentrations for almost all of the individual and grouped FAA and PBAA when 12 d were compared to 5 d piglets. A subsequent, more stringent univariate analysis (volcano plot) was performed to identify highly significant variables contributing to the age group separation in these blocks. Identified were Pro and β-Alanine, which were lower in the 12 d animals compared to 5 d, whilst the opposite was observed for Cys and Carnosine. The importance of these AA and amino-metabolites for the age-dependent development of the porcine intestine are not fully understood. It has been previously reported [84] that the capacity to absorb AA per length unit of intestine decreases, as the total length of intestine increases, potentially explaining the decrease for several AA concentrations observed in the older piglet group used in this study. Moreover it was shown that AA metabolism in the jejunum of piglets changes within the different periods of the suckling phase [83]. Why this occurs is currently not understood, but it could be linked to differences in the intestinal microbiota [95], changes in cell structure and function, or in AA metabolism [83] and absorption [11, 96].

In the present study, lower jejunal protein, RNA and DNA concentrations and increased RNA to DNA ratio, a measure of ribosomal RNA content or protein synthetic capacity was observed in piglets of the 12 d compared to the 5 d group. The DNA concentration is a marker of cell number, and lower concentrations in the 12 d group indicates that the numbers of cells per mg of jejunal tissue is lower potentially explaining why the protein, RNA, FAA and PBAA concentrations are decreased. Why the cell number decreased from 5 d to 12 d is not fully understood, but it may be linked to reappearing rise in apoptosis, after an enhanced mitosis accompanied by a reduction of apoptosis during the first days after birth [97]. In addition it should not be overlooked, that intestinal cell turnover is affected by nutrition and specific nutrients [15]. Whilst protein synthetic capacity increased, there was no effect of age on FPSR or the ratio of protein to DNA. Thus, in terms of protein synthesis, there appears to be no effect of age in these very young piglets.

Furthermore, the mRNA abundance of genes related to Gln/Ala-uptake and metabolism and glutathione production was assessed. We observed that the mRNA abundance of one Gln (SLC1A5) and one Ala (SLC1A4 transcript variant 1) transporter, two enzymes involved in Gln metabolism (AST-1, GLUD-1, SDHA) and three involved in the glutathione synthesis pathway (GCL, GSS and GPX4) were lower in piglets from the 12 d compared to the 5 d group. The GCL encodes the rate-limiting enzyme for the glutathione production, whilst GSS encodes the enzyme involved in the subsequent step and our results may indicate that oxidative defense via glutathione production was lower in piglets from the 12 compared to 5 d group. This observation is similar as in a previous study which showed a downwards trend of GPX4 expression, an enzyme converting glutathione to glutathione-disulfide in the presence of radical oxygen species, in the jejunum of suckling piglets after the age of d 14 [98]. The mRNA abundance of antioxidative enzymes is not only dependent on the age of the individual piglet, but on the sampled tissue as well [99]. Thus, within the context of earlier studies on ontogenetic development of the jejunum in suckling piglets, the results from this study are consistent with an adequate physiological development independent of BiW or Gln supplementation.

Conclusion

This study is the first to investigate the effect of oral Gln supplementation on jejunal development and AA profiles in suckling low and normal birth weight piglets. Results show that Gln as compared to Ala supplementation and BiW appears to have only small effects on the measured jejunal parameters, whereas the effect of age was significant. These novel findings suggest that oral Gln supplementation might not be an appropriate way to stimulate the development of jejunum in the suckling period. However, it is conceivable that Gln might be beneficial in a more challenging environment. Thus further research is warranted to investigate more proximal sections of the GIT, or cellular proliferation, microbial composition and the abundance of tight junction proteins during jejunal development.

Supporting information

S1 File. Supplementation procedure.
(DOCX)

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Table 3.4 Anteilerläuterung von Publikation mit geteilter Erstautorenschaft

Name	Bezeichnung Autor	Erläuterung Anteil Leistung
Schregel, Johannes	1. Erstautor	Anteil an Erstellung und Verfassung des Artikels fünfzig prozent (50%) Sammlung und Auswertung der Daten; Durchführungen von Untersuchungen (Abschnitt: Free and protein bound AA and AA metabolites concentration in jejunal tissue and digesta; Jejunal biochemical indices and fractional protein synthesis rate; Jejunal transcript abundances related to AA transport; AA metabolism and antioxidative defense); statistische Analysen Erstellung des Manuskripts
Schulze Holthausen, Johannes	2. Erstautor	Anteil an Erstellung und Verfassung des Artikels fünfzig prozent (50%) Sammlung und Auswertung der Daten Durchführungen von Untersuchungen (Abschnitt: Jejunal morphometry; histochemistry and immunohistochemistry) Erstellung des Manuskripts:
Sciascia, Quentin L.	Co. Autor	Sammlung und Auswertung von Daten; Durchführung von Untersuchungen; statistische Analysen; Überprüfung und Bearbeitung des Manuskripts
Li, Zeyang	Co. Autor	Sammlung von Daten
Görs, Solvig	Co. Autorin	Durchführung von Untersuchungen
Eggert, Anja	Co. Autorin	Statistische Analysen; Visualisierung
Tuchscherer, Armin	Co. Autor	Statistische Analysen
Zentek, Jürgen	Co. Autor	Konzeptionierung; Betreuung; Akquisition von Finanzmitteln; Überprüfung und Bearbeitung des Manuskripts;
Cornelia C. Metges	Co. Autorin	Konzeptionierung; Betreuung; Akquisition von Finanzmitteln; Überprüfung und Bearbeitung des Manuskripts;

microorganisms

MDPI

Article

Effects of Oral Glutamine Supplementation, Birthweight and Age on Colonic Morphology and Microbiome Development in Male Suckling Piglets

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Abstract: Mortality, impaired development and metabolic dysfunctions of suckling low-birthweight piglets may be influenced by modulating the intestinal microbiome through glutamine supplementation. Therefore, this study examined whether glutamine supplementation may affect the colonic development and microbiome composition of male low- and normal-birthweight piglets at 5 and 12 days of age. Suckling piglets were supplemented orally with glutamine or alanine. Colonic digesta samples were obtained for 16S rDNA sequencing, determination of bacterial metabolites and histomorphological tissue analyses. Glutamine-supplemented piglets had lower concentrations of cadaverine and spermidine in the colonic digesta ($p < 0.05$) and a higher number of $CD3^+$ colonic intraepithelial lymphocytes compared to alanine-supplemented piglets ($p < 0.05$). Low-birthweight piglets were characterised by a lower relative abundance of *Firmicutes*, the genera *Negativibacillus* and *Faecalibacterium* and a higher abundance of *Alistipes* ($p < 0.05$). Concentrations of cadaverine and total biogenic amines ($p < 0.05$) and $CD3^+$ intraepithelial lymphocytes ($p < 0.05$) were lower in low- compared with normal-birthweight piglets. In comparison to the factor age, glutamine supplementation and birthweight were associated with minor changes in microbial and histological characteristics of the colon, indicating that ontogenetic factors play a more important role in intestinal development.

Keywords: glutamine; colon; suckling piglets; low birthweight; intestinal morphometry; microbiota; bacterial metabolites

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1. Introduction

After birth, the neonatal piglet must adapt to a nonsterile environment and transition from uterine nutrition to colostrum and milk. This transition initiates the rapid development towards a maturing digestive and immune system. By suckling milk, piglets are provided with essential nutrients, such as lactose and proteins, as well as immunoglobulins and oligosaccharides [1]. Low-birthweight piglets (LBW), often born to sows with a high reproductive performance, have a higher risk of neonatal mortality and digestive disease, lower body weight (BW) gain [2] and impaired gastrointestinal (GIT) development [3].

There is increasing evidence that host–microbiota interactions are associated with nutrient uptake and metabolism, development of host immune functions and disease disposition [4]. It is known that the GIT microbiota are unstable in the first days of life [5]

and influenced by the maternal and solid diet [6], as well as by the environment [7]. Neonates with intestinal microbial dysbiosis may be more susceptible to intestinal diseases [8]. The majority of studies investigating the development of GIT microbiota in newborn piglets report data from normal-birthweight (NBW) piglets [5,9,10], whereas few studies have looked more closely at the development of the colonic microbiota of LBW piglets [11]. Studies in neonatal intrauterine-growth-restricted piglets show that mucosa-associated bacterial colonisation was increased compared to NBW piglets [12,13]. Early age is characterised by a rapidly changing colonic [11] and faecal [14] microbiome. Microbial metabolites are considered as important factors for the microbiota–host cross-talk [10], impacting many physiological and immunological traits of the host. Short-chain fatty acids (SCFAs) and lactate contribute to meeting the energy needs in pigs, but also have an important signalling function [15]. SCFAs and biogenic amines, which control cell metabolism and may have neuromodulatory effects in animals [16], are considered important bacterial metabolites with physiological and immune-modulating functions [10]. Increasingly, data are available, showing that the mechanisms involve complex signalling systems and molecular cascades [15].

In sow milk, Gln and glutamate are highly concentrated peptide-bound amino acids. The free Gln concentration increases during lactation [17]. Glutamine is an important energy source for enterocytes of neonatal piglets [18]. Studies investigating the effect of enteral Gln on improving GIT development have mainly focused on weaned piglets, indicating its importance in numerous metabolic processes essential for the morphological development and function of the small intestine (SI) [19]. In addition, Gln has been shown to affect the bacteria of the SI and their AA utilisation pattern [20]. In suckling piglets, little is known about the effect of Gln supplementation on the colonic microbiome and important fermentation products such as SCFAs and biogenic amines.

Therefore, this study used a pig model with different birthweights (BiWs; LBW vs. NBW) and oral AA supplementation (Gln vs. Ala) across two different age groups (5 and 12 days old) to investigate their potential impact on colon development, the colon microbiome and targeted metabolites. Alanine was used as the control supplementation to balance for the nitrogen content of Gln supplementation [19,21–23].

2. Materials and Methods

2.1. Animals, Experimental Design and Sample Collection

All experimental procedures were performed according to the German Animal Welfare Act following Directive 2010/63/EU and were approved by the State Office for Agriculture, Food Safety and Fisheries, Mecklenburg-Vorpommern, Germany (permission no. 7221.3-1-026/16). German Landrace gilts were kept at the Research Institute for Farm Animal Biology. A detailed description of the experiment was published previously [17]. To remove sex-specific effects, only male piglets were chosen. In brief, LBW (0.8–1.2 kg; n = 48; with BiW below the lowest BiW quartile of the experimental pig farm) and NBW (1.4–1.8 kg; n = 48; with BiW reflecting the middle 50th percentile of the BiW of piglets born on the experimental pig farm) male littermate piglets born to gilts were observed until 5 or 12 days (d) of age. Within 24 h post-farrowing, litters were standardised to 12 piglets, and the LBW and NBW piglets were assigned to either Gln (1 g/kg BW/d; n = 48) or isonitrogenous Alanine (Ala, 1.22 g/kg BW/d; n = 48) supplementation groups (Supp), with up to three piglet pairs per sow being involved in the study. In a three-factorial design (Supp, BiW, Age), 4 experimental groups (Gln-LBW, Gln-NBW, Ala-LBW, Ala-NBW: n = 24/age-group) were investigated at 5 or 12 d of age. The supplementation of Gln and Ala was performed as described [17].

Two hours (h) before sampling, each piglet received 33% of the respective daily AA supplement and 6 mL of milk replacer (150 g/L water at 45 °C; 16.5 MJ metabolisable energy (ME)/kg, 20.5% crude protein, 10.0% crude fat, 0.2% crude fibre; Neopigg Rescuemilk 2.0, Provimi, The Netherlands).

Colonic tissue and digesta were sampled from the ascending colon. After collection, the digesta was snap-frozen in liquid nitrogen and then stored at −80 °C until subsequent analysis. A section of the sampled colonic tissue was rinsed with 0.9% physiological saline, and preserved in Roti-Histofix (4% paraformaldehyde, Histofix, Roth, Karlsruhe, Germany) for histological analysis.

2.2. Colonic Morphometry, Histochemistry and Immunohistochemistry

Histo-fixed colonic tissue samples were processed as previously described [24]. From paraffin blocks, 5 μm sections were cut with a sledge microtome (Type 1400, Leitz Wetzlar, Germany). The Alcian blue pH 2.5–periodic acid–Schiff (AB-PAS) staining method was used for morphometry and for the quantification of neutral, acidic and mixed mucin types [25]. Measurements were carried out using a Photomicroscope BX43F (Olympus, Tokyo, Japan) with an attached digital camera (Olympus DP72, Tokyo, Japan). Pictures were examined with cellSens imaging software (v. 1.4, Olympus). Ten well-orientated crypts were randomly chosen. Morphometric measurements included crypt depth (CD) (from the crypt mouth to the bottom of the crypts) and crypt area (CA) [26]. Mucins in goblet cells were differentiated by AB-PAS staining [26].

For quantification of IgA secreting cells, slides were boiled in sodium citrate buffer (pH 6.0) in a microwave. Endogenous peroxidase was inactivated at room temperature for 30 min with 1% aqueous hydrogen peroxide solution. Slides were incubated for 1 h in a humid chamber with PBS and 10% normal horse serum to block nonspecific antibody binding. Sections were incubated overnight at 4 °C with goat anti-porcine IgA 1:4000 antibody (NB724, Novus Biologicals, Abingdon, UK), washed and incubated for 1 h with biotinylated horse anti-goat IgG 1:500 (Cat. NO: BA-9500, Vector Laboratories), treated with ABC complex (Vectastain Elite ABC peroxidase Kit, Standard, Vector Laboratories) and a DAB Substrate Kit (Vector Laboratories) [27]. Isotype control was produced with a nonspecific antibody (goat IgG, AB-108-c, R&D Systems). To quantify IgA-positive stained cells, 3 areas of lamina propria on each section were chosen [28]. The areas were delineated with cellSens imaging software (v. 1.4, Olympus), ignoring the epithelium, large blood vessels and artefacts. Positive stained cells were counted and expressed per 10,000 μm^2 of lamina propria tissue [29].

The analysis of $CD3^+$ intraepithelial lymphocytes (IELs) was performed as described previously [30], and the number of $CD3^+$ IELs in the lamina propria next to the crypts was evaluated. Slides were heated for 30 min in boiling citrate buffer using a microwave. Slides were then cooled and incubated with a primary antibody PPT3 (mouse anti-porcine CD3 epsilon, CAT NO 4510-01, Southern Biotech) and an isotype control with a nonspecific antibody (mouse IgG, CAT NO 0102-01, Southern Biotech). The visualisation was achieved with the mouse and rabbit Specific HRP/DAB IHC Detection Kit (ab236466, ABCAM), and the secondary antibody was visualised with horseradish peroxidase (HRP)-labelled micropolymer (goat anti-rabbit HRP Conjugate, 58009 ABCAM) [31]. A double-blinded quantification of CD3-positive IELs was performed in well-orientated complete crypts (two slices per animal, ten crypts per slice). The $CD3^+$ IELs were expressed per 100 enterocytes and $CD3^+$ in the lamina propria per 10,000 μm^2.

2.3. Chemical Analyses

Colon digesta SCFA and biogenic amines were quantified as described previously [32,33]. Briefly, SCFA analysis of digesta was performed by acidifying the samples with oxalic acid, followed by centrifugation for 3 min at 14,000 g and adding the internal standard (caproic acid). A gas chromatograph (Agilent Technologies 6890N, autosampler G2614A and injection tower G2613A; Network GC Systems, Böblingen, Germany) was used. Ion exchange chromatography was performed with a Biochrom 30 Amino Acid Analyzer (Biochrom) to analyse biogenic amines (putrescine, cadaverine, spermidine, spermine, propylamine, tyramine). Trichloroacetic acid (10%) was added to the digesta samples. After homogenisation and filtering (0.2 μm pore size), samples (25 μL) were

injected onto a 10 cm polyamine ion-exchange column (Laborservice Onken GmbH, Gründau, Germany). The eluent was sodium citrate buffer (pH 7.2). Amines were quantified after post-column ninhydrin derivatisation by photometric detection at 570 nm [33].

2.4. DNA Extraction and 16S rDNA Sequencing

Bacterial genomic DNA was extracted from 250 mg digesta using a commercial kit, NucleoSpin Tissue Mini Kit for DNA from cells and tissue (NucleoSpin, Macherey & Nagel, Düren, Germany) according to the manufacturer's instructions with the following exceptions: bead beating of 250 mg digesta in 1 mL of pre-lysis solution was carried out on a FastPrep-24™ 5G homogeniser (MP Biomedicals, LLC, Santa Ana, CA, USA) at a speed of 6 m/s for 10 min (4 times 5 × 30 s and 15 s cooling pause); Proteinase K treatment lasted for 30 min at 56 °C. The following steps were performed as described by the manufacturer, but the volume of the elution buffer was doubled to increase DNA yield. According to the manufacturer's instructions, DNA concentration was determined using Promega QuantiFluor® dsDNA System (Promega, Corporation, Madison, Wisconsin, USA). DNA extracts were subjected to amplicon sequencing using an Illumina NextSeq500 sequencer (LGC, Berlin, Germany) with 150 bp-paired reads using 16S rDNA primers 341f and 785r. Demultiplexing was achieved with Illumina bcl2fastq (v. 2.17.1.14); paired reads were combined with BBMerge (v. 34.48).

2.5. Data Evaluation and Statistical Analysis

A multivariate approach was used for statistical analyses of histological data, biogenic amines and SCFAs. Linear mixed model analysis was conducted using the ANOVA procedure of the IBM SPSS Statistics software Version 25 (IBM, Chicago, Illinois, USA). The three fixed-effects Supp (Ala, Gln), BiW (LBW, NBW) and Age (5 d and 12 d) and their interactions were tested, and the Tukey test was used for groupwise comparisons. Means and their standard errors are shown. Differences were considered statistically significant at $p < 0.05$ and as trends at $p \leq 0.1$.

The 16S-rDNA sequences were analysed using the QIIME2 pipeline [34] and the SILVA SSU database [35]. Quality control and determination of sequence counts were performed using the DADA2 database software [36]. Further details were previously described [37]. The bacterial alpha-diversity measures Richness, Shannon Index and Evenness were calculated from ASV-level data. The Kruskal–Wallis test was used to test the effects of the main factors Supp, BiW and Age and their interactions on the bacterial abundance in the colon . A level of 95% was deemed as significantly different. Principal component analysis (PCA) and hierarchical clustering, using *Clustvis* [38], were used to visualise Supp, BiW and Age differences (Figure S1).

3. Results

3.1. Morphology of the Colon and Frequency of Goblet Cells, Intraepithelial Lymphocytes and IgA-positive Cells

Colon tissue from Gln-supplemented piglets had a higher number of $CD3^+$ IELs ($p = 0.028$) and showed a trend for an increase in the lamina propria ($p = 0.054$) (Table 1). A higher number of $CD3^+$ IELs was observed in NBW compared to LBW piglets ($p = 0.047$).

In piglets that were 12 d-old, CD and CA and the number of $CD3^+$ IELs and $CD3^+$ lymphocytes in the lamina propria ($p < 0.001$) were higher than at 5 d. However, the total numbers of goblet cells ($p = 0.001$) with neutral ($p = 0.006$) or mixed mucins ($p < 0.001$) were lower at 12 compared to 5 d of age. IgA-positive cells were absent in the colonic lamina propria in 5 d-old piglets, and were detected in all piglets at 12 d (Table 1, Figure 1)

The interactions of Supp x Age and BiW x Age were associated with changes in CD ($p = 0.001$; $p < 0.001$) and CA ($p = 0.010$; $p = 0.002$), respectively, and the interaction of Supp × BiW × Age was associated with changes in CD ($p = 0.026$), CA ($p = 0.008$) and $CD3^+$ IELs ($p = 0.043$) and a trend for the number of mixed mucins ($p = 0.080$) (Tables S1 - S3).

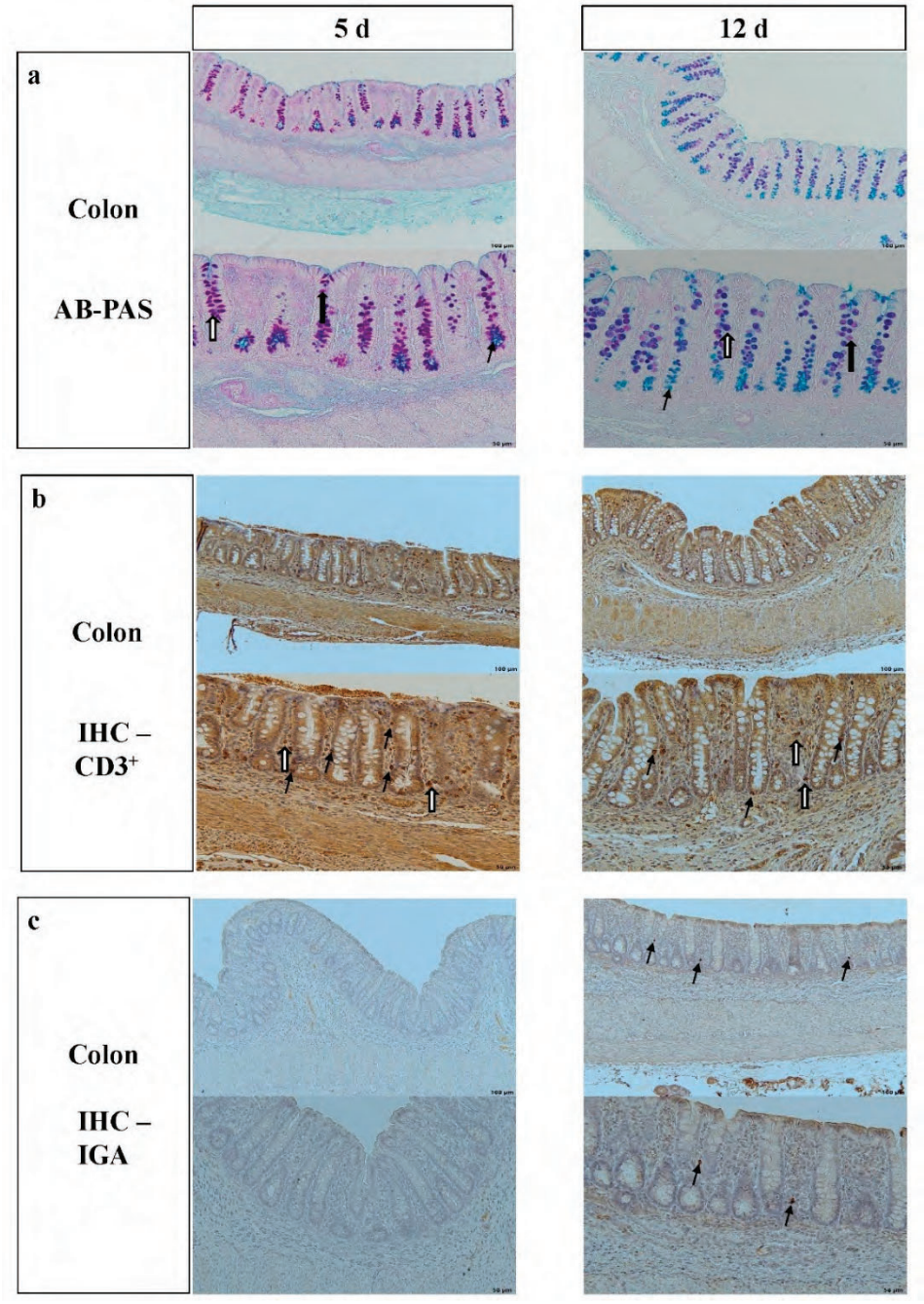

Figure 1. Histomorphology and immunohistochemistry of the colon of 5 d- and 12 d-old male suckling piglets. (**a**) Alcian blue pH 2.5–periodic acid–Schiff-stained colonic tissue with stained goblet cells, with different arrows indicating goblet cells containing different mucins, white arrow with black border = acidic mucins, black arrow = neutral mucins, white arrow = mixed mucins, 100× (upper pictures), 400× magnification (lower pictures); (**b**) IHC of CD3, with black arrows indicating positive stained intraepithelial CD3+ cells in colon, white arrows indicating positive stained CD3+ cells in lamina propria 100× (upper pictures), 400 x (lower pictures) magnification; (**c**) IHC of IgA-positive stained cells in lamina propria, no IgA-positive cells detected at day 5, with arrows indicating IgA-positive cells,100× and 400× magnification.

Table 1. Morphometric and immunohisto-morphometric measurements of the colon of 5- and 12-day-old male suckling piglets [1].

	Supp		BiW		Age			*p* Values [5]						
Item	**Gln**	**Ala**	**LBW**	**NBW**	**5 d**	**12 d**	**SEM**	**Supp**	**BiW**	**Age**	**Supp × BiW**	**Supp × Age**	**BiW × Age**	**Supp × BiW × Age**
Morphometry														
CD, µm	237	236	234	238	210	263	2.13	0.730	0.197	**<0.001**	0.490	**0.001**	**<0.001**	**0.026**
CA, µm²	10950	10993	10964	10979	9389	12554	144	0.842	0.947	**<0.001**	0.259	**0.010**	**0.002**	**0.008**
AB—PAS staining of Goblet cells [2]														
Acid	21.9	24.7	22.7	23.8	21.7	24.8	1.07	0.196	0.609	0.145	0.234	0.684	0.244	0.681
Neutral	67.1	69.2	70.9	65.4	75.4	60.9	2.64	0.686	0.280	**0.006**	0.296	0.915	0.527	0.128
Mixed	84.1	87.1	89.2	82.0	96.4	74.8	2.60	0.513	0.130	**<0.001**	0.330	0.759	0.303	***0.080***
Total	173	181	183	171	194	161	5.25	0.430	0.248	**0.001**	0.213	0.911	0.578	0.130
CD3⁺ lymphocytes [3]														
CD3⁺ IEL	1.97	1.76	1.77	1.96	1.15	2.60	0.07	**0.028**	**0.047**	**<0.001**	0.255	0.107	0.165	**0.043**
CD3⁺ lamina propria	8.70	8.28	8.83	8.65	5.90	11.2	0.18	**0.054**	0.109	**<0.001**	0.911	0.495	0.514	0.603
IgA-positive cells in lamina propria [4]														
IgA-positive cells	8.17	8.71	8.04	8.83	n.d	8.44	0.54	0.627	0.479	n.a	0.911	n.a	n.a	n.a

[1] Values are means, the SE of all groups is shown; n = 48/group. Colon samples were obtained 2 h after oral supplementation of Gln or Ala and fixed in Formalin. [2] Number of Alcian blue–periodic acid–Schiff (AB-PAS)-positive goblet cells per 1 mm basal membrane. [3] Number of CD3⁺-positive lymphocytes per 100 enterocytes and per 10 000 µm² lamina propria next to the crypts. [4] Number of IgA-positive cells per 10,000 µm² of lamina propria. [5] ANOVA F test; significant differences ($p < 0.05$) are marked in bold, trends ($p < 0.1$) are marked in italic and bold. Ala = Alanine; Acid = acidic mucins; BiW = birthweight; CA = crypt area; CD = crypt depth; Gln = Glutamine; IELs = intraepithelial lymphocytes; LBW = low birthweight; n.a = not available (no IgA-positive cells detectable at 5 d); NBW = normal birthweight; n.d = not detectable; Mixed = mixed neutral and acidic mucins; SE = standard error; Supp = supplementation group; Total = total number of AB-PAS-positive goblet cells.

3.2. Bacterial Metabolites in the Colon Digesta

Glutamine-supplemented piglets had lower concentrations of cadaverine ($p = 0.036$) and spermidine ($p = 0.020$) and tended to have lower tyramine concentrations ($p = 0.087$) in the colonic digesta compared to Ala piglets (Table 2).

Normal-birthweight piglets had higher concentrations of cadaverine ($p = 0.026$) and total biogenic amines ($p = 0.011$) and a trend for increased concentrations of tyramine ($p = 0.057$) compared to LBW piglets (Table 2).

At 12 d, colonic digesta had higher tyramine ($p = 0.019$), putrescine ($p = 0.018$) and total biogenic amines ($p < 0.001$) and lower spermidine ($p < 0.001$) concentrations than at 5 d (Table 2).

The interaction of Supp x BiW showed a trend towards an effect on concentrations of propionic acid ($p = 0.071$), total SCFA ($p = 0.074$) (Table 3) and concentrations of cadaverine ($p = 0.023$), tyramine ($p = 0.053$), spermidine ($p = 0.099$) and total biogenic amines ($p = 0.061$).

The interaction of Supp × Age showed two trends, BiW × Age, with one significant effect and two trends and Supp × BiW × Age, with one significant effect and one trend for an effect on the concentration of biogenic amines (Tables 2 and S5). The interaction of Supp × BiW × Age tended to affect the concentration of butyric acid ($p = 0.090$) (Tables 3 and S6).

Table 2. Concentrations of biogenic amines in the colon digesta of 5- and 12-day-old male suckling piglets [1].

	Supp		**BiW**		**Age**			***p* Values [2]**						
Item, μmol/g Wet Weight	**Gln**	**Ala**	**LBW**	**NBW**	**5 d**	**12 d**	**SEM**	**Supp**	**BiW**	**Age**	**Supp × BiW**	**Supp × Age**	**BiW × Age**	**Supp × BiW × Age**
Spermine	0.02	0.03	0.03	0.03	0.03	0.03	0.00	0.275	0.534	0.764	0.351	0.840	0.744	***0.076***
Cadaverine	0.05	0.26	0.04	0.27	0.04	0.27	0.07	**0.036**	**0.026**	0.253	**0.023**	0.163	0.189	0.258
Tyramine	0.06	0.17	0.05	0.17	0.05	0.17	0.05	***0.087***	***0.057***	**0.019**	***0.058***	0.152	***0.086***	**0.049**
Propylamine	0.06	0.03	0.04	0.05	0.04	0.05	0.01	0.111	0.676	0.198	0.931	***0.067***	0.672	0.712
Histamine	0.07	0.09	0.07	0.09	0.07	0.09	0.02	0.698	0.611	0.546	0.192	0.725	0.901	0.319
Spermidine	0.29	0.39	0.36	0.33	0.36	0.33	0.03	**0.020**	0.205	**<0.001**	***0.099***	0.485	**0.034**	0.427
Putrescine	0.58	0.46	0.46	0.58	0.46	0.58	0.07	0.152	0.208	**0.018**	0.470	***0.074***	0.189	0.819
Total biogenic amines	1.14	1.43	1.05	1.52	1.05	1.52	0.15	0.130	**0.011**	**<0.001**	***0.061***	0.816	***0.098***	0.188

[1] Values are means, the SE of all groups is shown; $n = 10$ group (not enough colonic digesta of all piglets available for analyses). Colon digesta samples were obtained at 2 h after oral supplementation with Gln or Ala and milk replacer, and snap-frozen in liquid nitrogen. [2] ANOVA *F* test; significant differences ($p < 0.05$) are marked in bold, trends ($p < 0.1$) are marked in italic and bold. Ala = Alanine; BiW = birthweight; Gln = Glutamine; LBW = low birthweight; NBW = normal birthweight; SE = standard error; Supp = supplementation group.

Table 3. Short chain fatty acid concentrations in the colon digesta of 5- and 12-day-old male suckling piglets [1].

	Supp		BiW		Age			*p* Values [2]						
Item, mmol/L	**Gln**	**Ala**	**LBW**	**NBW**	**5 d**	**12 d**	**SEM**	**Supp**	**BiW**	**Age**	**Supp × BiW**	**Supp × Age**	**BiW × Age**	**Supp × BiW × Age**
Acetic Acid	26.2	27.2	26.8	26.6	27.1	26.2	1.39	0.726	0.948	0.753	0.173	0.804	0.531	0.621
Propionic acid	7.94	9.49	8.19	9.24	8.07	9.36	0.83	0.359	0.532	0.445	***0.071***	0.737	0.893	0.222
Isobutyric acid	1.39	1.49	1.38	1.49	1.39	1.48	0.10	0.645	0.594	0.671	0.252	0.627	0.894	0.597
Butyric acid	3.00	3.60	3.03	3.57	2.86	3.73	0.39	0.436	0.487	0.263	0.101	0.520	0.870	***0.090***
Isovaleric acid	1.25	1.39	1.23	1.41	1.23	1.40	0.09	0.451	0.328	0.372	0.363	0.417	0.616	0.641
Valeric acid	1.13	1.19	1.13	1.18	1.07	1.24	0.09	0.718	0.767	0.325	0.174	0.203	0.865	0.132
Total SCFA	40.85	44.34	41.73	43.46	41.77	43.41	2.43	0.486	0.728	0.742	***0.074***	0.649	0.738	0.289

[1] Values are means; the SE of all groups is shown; n = 24/group (not enough colonic digesta of all piglets available for analyses). Colon digesta samples were obtained at 2 h after oral supplementation with Gln or Ala and milk replacer, and snap-frozen in liquid nitrogen. [2] ANOVA F test; significant differences ($p < 0.05$) are marked in bold, trends ($p < 0.1$) are marked in italic and bold. Ala = Alanine; BiW = birthweight; Gln = Glutamine; LBW = low birthweight; NBW = normal birthweight; SE = standard error; Supp = supplementation group.

3.3. Impact of AA Supplementation on the Colonic Microbiota

Trends for lower relative abundances of *Planctomycetes* on the phylum level (Table 4) and on the order level and a trend for lower abundances of an unknown *Firmicutes* were detected in colonic digesta of Gln- compared to Ala-supplemented piglets ($p = 0.054$) (Table S7).

At the genus level, the relative abundances of *Phascolarctobacterium* ($p = 0.086$) and *Peptococcus* ($p = 0.081$) showed a trend of being higher (Table S8), and relative abundances of several unknown genera from the families of *Clostridiaceae 1* ($p = 0.091$), *Carnobacteriaceae* ($p = 0.055$) and *Streptococcaceae* ($p = 0.053$) tended to be lower in colonic digesta of Gln- compared to Ala-supplemented piglets.

Table 4. Microbial diversity indices and relative abundance of bacterial phyla in the colon digesta of male suckling piglets [1].

	Supp		BiW		Age			*p* Values[2]						
Item, %	**Gln**	**Ala**	**LBW**	**NBW**	**5 d**	**12 d**	**SEM**	**Supp**	**BiW**	**Age**	**Supp × BiW**	**Suppl × Age**	**BiW × Age**	**Suppl × BiW × Age**
Richness [3]	179	167	172	173	164	181	5.56	0.391	0.974	0.114	0.794	0.343	0.204	0.550
Shannon.Index [3]	3.73	3.72	3.72	3.72	3.67	3.77	0.05	0.792	0.956	0.389	0.694	0.763	0.657	0.633
Evenness [3]	0.72	0.73	0.72	0.73	0.72	0.73	0.01	0.613	0.801	0.621	0.867	0.775	0.959	0.695
Firmicutes	68.8	66.6	64.4	71.1	65.1	70.4	1.82	0.429	**0.049**	0.156	**0.009**	0.429	0.105	**0.032**
Bacteroidetes	22.1	21.9	23.6	20.4	23.8	20.2	1.40	0.301	0.423	***0.052***	0.412	0.175	0.180	0.420
Fusobacteria	3.75	6.42	5.71	4.35	6.89	3.17	1.17	0.538	0.374	0.362	0.371	0.670	0.625	0.635
Proteobacteria	3.07	3.77	4.01	2.80	3.65	3.16	0.57	0.322	0.258	0.307	0.504	0.510	0.486	0.804
Verrucomicrobia	0.78	0.00	0.81	0.00	0.00	0.81	0.40	0.973	0.360	**0.002**	0.741	**0.025**	**0.009**	***0.098***
WPS-2	0.42	0.10	0.06	0.47	0.00	0.53	0.17	0.179	0.138	0.173	0.233	0.281	0.256	0.473
Actinobacteria	0.33	0.24	0.32	0.26	0.36	0.21	0.06	0.524	0.904	0.869	0.779	0.796	0.717	0.836
Spirochaetes	0.29	0.32	0.43	0.17	0.05	0.55	0.11	0.380	0.917	**0.003**	0.784	**0.024**	**0.019**	0.121
Planctomycetes	0.27	0.17	0.26	0.18	0.07	0.38	0.06	***0.054***	0.248	0.113	0.166	*0.074*	0.293	0.309
Tenericutes	0.12	0.04	0.10	0.06	0.00	0.16	0.05	0.590	0.582	**0.002**	0.897	**0.022**	**0.023**	0.198
Epsilonbacteraeota	0.03	0.23	0.19	0.06	0.03	0.23	0.08	0.465	0.819	**< 0.001**	0.896	**0.004**	**0.006**	***0.054***
Kiritimatiellaeota	0.02	0.11	0.06	0.07	0.00	0.12	0.03	0.396	0.973	**0.021**	0.757	**0.037**	0.127	0.242
Lentisphaerae	0.01	0.04	0.02	0.03	0.05	0.01	0.01	0.937	***0.080***	0.230	0.145	0.499	***0.083***	0.191

[1] Values are means of relative abundance; the SE for all groups is shown; n = 22/group (not enough colonic digesta of all piglets available for analyses). Colon digesta samples were obtained at 2 h after

oral supplementation of Gln or Ala and milk replacer, and snap-frozen in liquid nitrogen. [2] Kruskal–Wallis test, asymptotic significance; significant differences ($p < 0.05$) are marked in bold, trends ($p < 0.1$) are marked in italic and bold [3] Calculated on amplicon sequence variant (ASV) level. Ala = Alanine; BiW = birthweight; Gln = Glutamine; LBW = low birthweight; NBW = normal birthweight; SE = standard error of the mean; Supp = supplementation group.

3.4. Impact of Birthweight on the Colon Microbiota

Low-birthweight piglets were characterised by a lower abundance of the phylum Firmicutes ($p = 0.049$) and showed a trend for a decrease in the phylum *Lentisphaerae* ($p = 0.080$) compared to NBW piglets (Table 4). The order *Actinomycetales* tended to be increased in LBW compared to NBW piglets ($p = 0.062$) (Table S7)

On the genus level, LBW piglets had a higher relative abundance of *Alistipes* ($p = 0.043$) and a trend for slightly higher relative abundances of *Peptostreptococcus* ($p = 0.087$), *Mannheimia* ($p = 0.075$) and unknown *Desulfovibrionaceae* ($p = 0.095$) compared to NBW piglets (Table S8). In comparison to LBW piglets, the relative abundance of *Negativibacillus* ($p = 0.020$) and *Faecalibacterium* ($p = 0.039$) was higher and showed a trend for a slightly higher abundance of the genera *Dorea* ($p = 0.066$) and unknown *Prevotellaceae* ($p = 0.063$) in the colon digesta of NBW piglets.

3.5. Impact of Age on the Colon Microbiota

At the level of the phyla, a lower relative abundance of Verrucomicrobia ($p = 0.002$), Spirochaetes ($p = 0.003$), Tenericutes ($p = 0.002$), Epsilonbactereota ($p < 0.001$) and Kritimatiellaeota ($p = 0.021$) and a trend for slightly higher abundances of Bacteriodetes ($p = 0.052$) were detected in the colon digesta of piglets at 12 d compared to 5 d of age (Table 4).

At the level of bacterial order, the abundance of *Victivallales* ($p = 0.037$) and *Coriobacteriales* ($p = 0.003$) was lower at 5 than at 12 d (Table S7). *Lactobacillales* ($p = 0.063$), and *Bacteroidales* ($p = 0.052$) showed a trend for a higher abundance at 5 compared to 12 d. The relative abundance of unknown *WPS-2* ($p = 0.002$), *Desulfovibronionales* ($p = 0.002$), *Betaproteobacteriales* ($p = 0.002$), *Corynebacteriales* ($p = 0.005$) and *Campylobacterales* ($p = 0.021$) was lower at 5 than at 12 d, and a similar trend was found for *Spirochaetales* ($p = 0.054$), *Mollicutes* RF39 ($p = 0.058$) and *Micrococcales* ($p = 0.092$).

At the genus level, the relative abundances of 28 genera were higher ($p < 0.05$), and 8 tended to be higher ($p < 0.1$) in the colon digesta of 12 d- compared to 5 d-old piglets. Furthermore, the relative abundances of 26 genera were lower ($p < 0.05$), and another 8 genera tended to be lower ($p < 0.1$) in the colon digesta of 12 d- compared to 5 d-old piglets (Table S8). Of the dominating genera with a mean abundance > 1%, unknown *Muribaculaceae* ($p = 0.001$), unknown *Lachnospiraceae* ($p = 0.013$), *Lachnoclostridium* ($p = 0.022$) and *Parabacteroides* ($p < 0.001$) were lower at 12 d than 5 d, and the relative abundance of *Fusobacterium* ($p = 0.052$) and *Prevotellaceae* NK3B31 groups ($p = 0.091$) showed a trend for lower values at 12 d than at 5 d. The genera *Rombutsia* ($p = 0.010$), *Ruminococcaceae* UCG-002 ($p = 0.004$), *Ruminococcaceae* UCG-005 ($p < 0.001$), *Alloprevotella* ($p = 0.024$), *Christensenelllaceae* R-7 group ($p < 0.001$) and unknown F082 ($p < 0.001$) showed an age-dependent increase in colon digesta from 5 to 12 d of age (Table S8).

3.6. Interaction of Supplementation, Birthweight and Age Effects on Bacterial Phyla, Order and Genera

The interaction of Supp x BiW ($p = 0.009$) and Supp x BiW x Age ($p = 0.032$) influenced the abundance of *Firmicutes*. Several other significant and trends for interactions for bacterial phyla with a relative abundance < 1%, mainly influenced by the factor Age, are shown in Table 4.

The interaction of Supp x BiW influenced the relative abundance of *Bradymondales* ($p = 0.024$) and showed a trend for an influence on the relative abundance of *WCHB1*-41 ($p = 0.096$). In total, seven significant and two trends for the interaction of Supp × BiW, six

significant and one trend for the interaction of BiW × Age and three trends for the interaction of Supp × BiW × Age of bacterial abundances < 1% on the order level are shown in Tables S7 and S11.

The interaction of Supp × BiW affected the relative abundances of unknown *Bradymondales* ($p = 0.024$), unknown *Prevotellacae* ($p = 0.049$), *Alistipes* ($p = 0.030$), *Staphylococcus* ($p = 0.028$) and *CAG-873* ($p = 0.015$) in colonic digesta. Moderate interactions of Supp x BiW and effects of the other interactions (Supp × Age, BiW × Age; Supp × BiW × Age), again mainly influenced by the factor Age, were found and are shown in Tables S8 and S12.

3.7. Quantitative Analysis, Ecological Indices and Principal Component Analysis of the Colonic Microbiota

The main abundant phyla in the colon digesta of male suckling piglets were *Firmicutes* and *Bacteroidetes*, followed by *Fusobacteria* and *Proteobacteria* (Table 4). On the order level, *Clostridiales*, *Lactobacillales* and *Bacteroidales* were most abundant (Table S1). Regardless of Supp, BiW and Age, the dominating bacterial genera were *Lactobacillus* and *Clostridium sensu stricto* 1 (Table S2). Neither Supp nor BiW or Age affected Richness, Evenness or Shannon Indices (Table 4). Principal component analysis of all bacterial genera revealed no separation between Supp or BiW (Figure 2 a,b); however, Age did, with 5 d-old piglets clustered separately from 12 d-old piglets (Figure 2c,d).

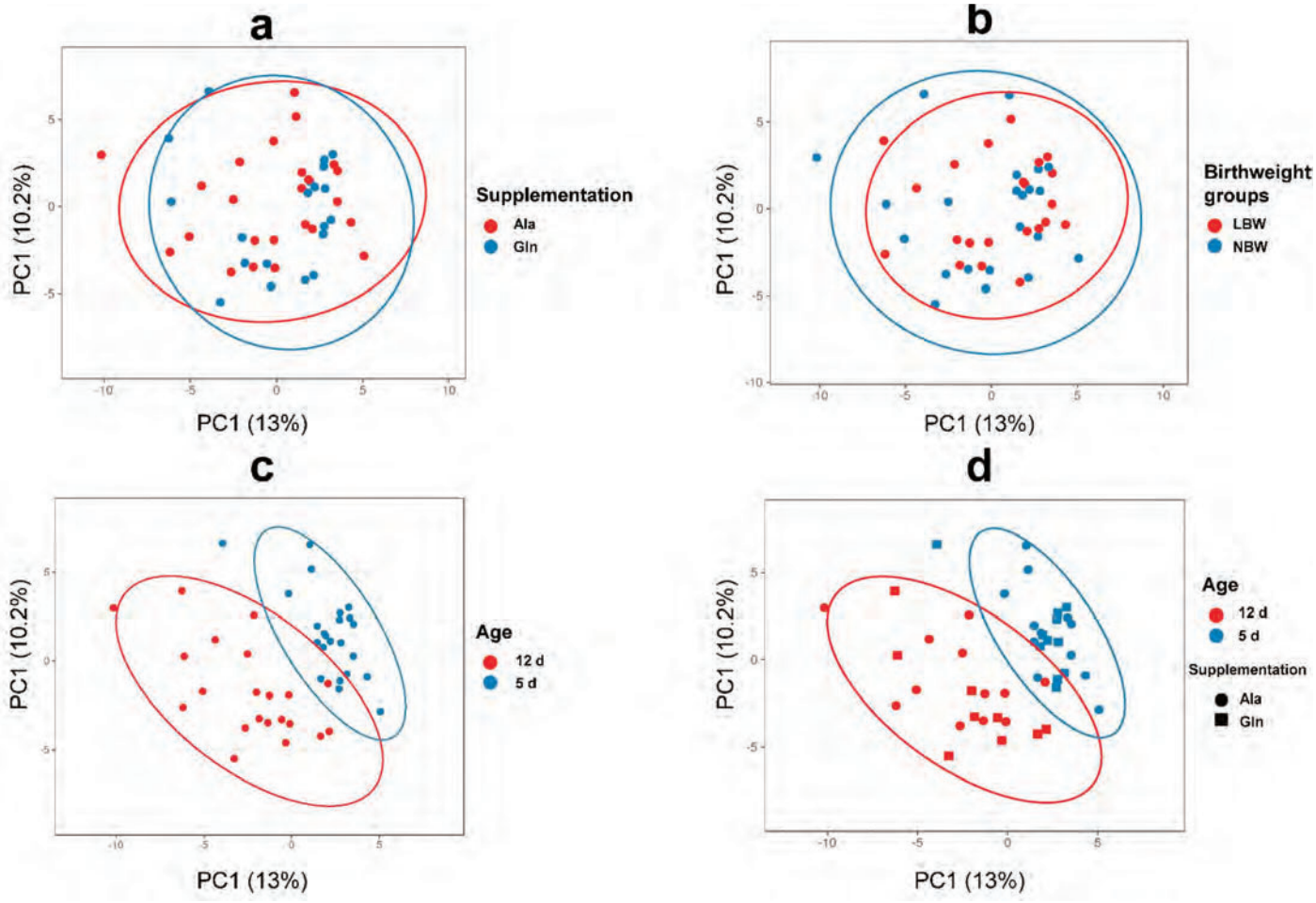

Figure 2. Principal component analysis (PCA). Principal component analysis (PCA) showing the effect of (**a**) supplementation; (**b**) birthweight; (**c**) age; (**d**) age and supplementation × birth weight on bacterial genus level. Ala = Alanine, Gln = Glutamine, LBW = low birthweight, NBW = normal birthweight. PCA was performed with Clustvis.

3.8. Correlations between Microbiota and Bacterial Metabolites in Colon Digesta

A link between the colonic metabolites and the major genera was investigated by Spearman correlation. The Ala and Gln Supp groups were taken together because the bacterial abundance and the metabolite concentrations did not show major significant changes between groups. Figure 3 a,b show the correlations between SCFAs and major bacteria genera (mean abundance > 0.5%) in the colon digesta of 5 d- and 12 d-old piglets. Total SCFAs and acetic and propionic acid positively correlated with the abundance of *Lactobacillus* and *Alloprevotella* at 5 d, respectively ($p < 0.05$). Butyric acid showed a significant negative correlation with the abundance of *Lactobacillus* and *Streptococcus* at 5 d, whereas the genus *Alloprevotella showed a* positive correlation ($p < 0.05$). At 12 d of age, total SCFAs negatively correlated with the abundance of *Fusobacterium* and *Actinobacillus*, whereas they positively correlated with the genera *Rikenellaceae RC9 gut group*. Propionic acid also showed a negative correlation with *Fusobacterium* and *Actinobacillus* and a positive correlation with the genera *Phascolarctobacterium*, *Ruminiclostridium 9* and *Dorea*. Butyric acid was negatively correlated with the abundance of *Fusobacterium*, *Prevotellaceae* NK3B31 group and *Terrisporobacter* and positively correlated with the *Rikenellaceae* RC9 gut group, *Ruminococcaceae* UCG 002/UCG 005 and unknown *Clostridiales vadin* BB60 group. Few correlations between the biogenic amines and major bacteria genera (mean abundance > 0.5%) at 5 d (Figure 3 c**)** and 12 d (Figure 3 d) could be detected. We found the most positive correlations between different bacterial genera and histamine at 5 d of age. At 12 d of age, most positive correlations between different bacterial genera and propylamine were detected. Cadaverine showed a negative correlation with the genera *Actinobacillus* and *Prevotellaceae* NK3B31 group in 12 d-old piglets.

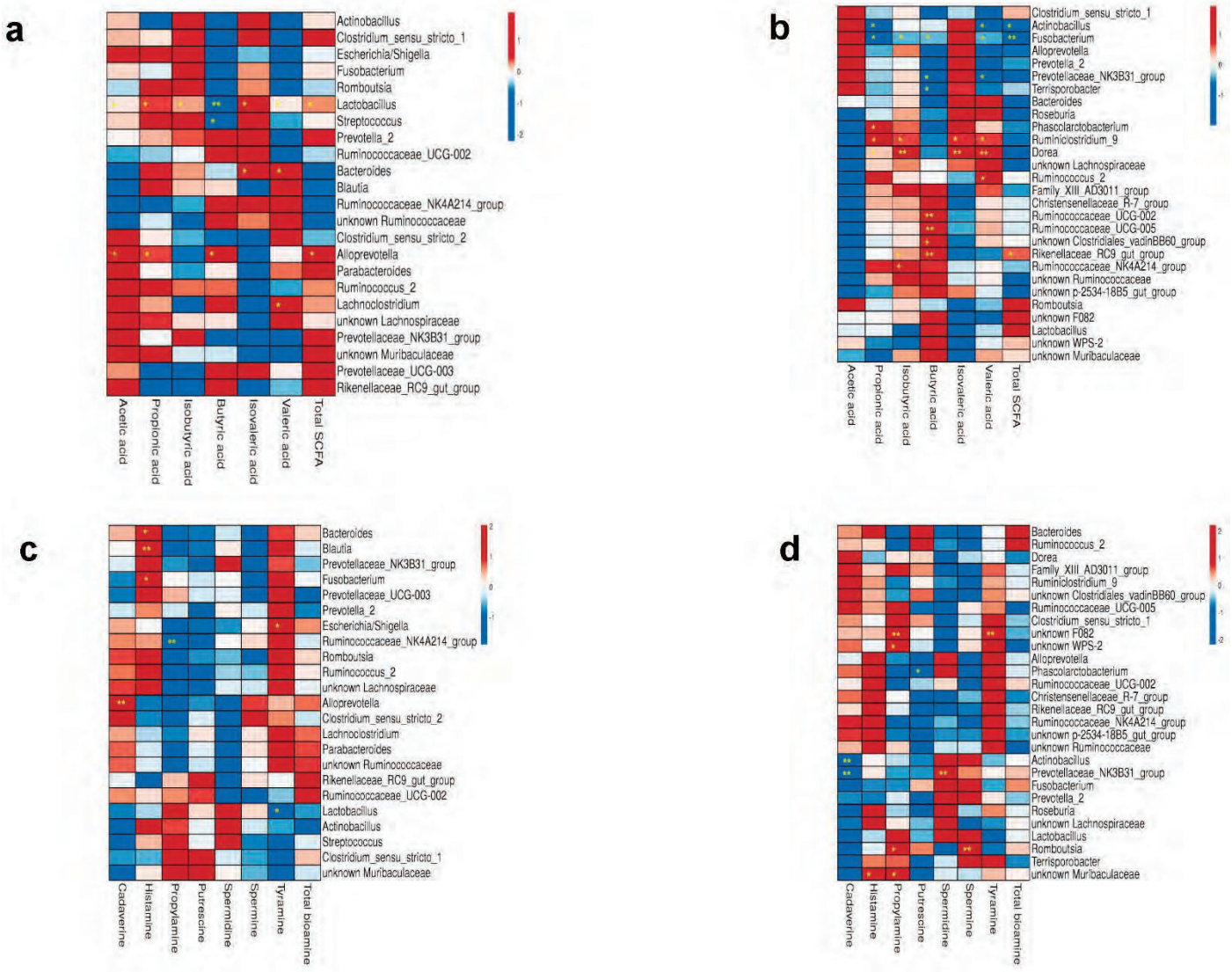

Figure 3. Concentration of bacterial metabolites and their correlation with bacteria having an abundance > 0.5%. The data are presented as mean values, $n = 5$ for SCFAs between groups; n = 2 for biogenic amines at 5 d of age and $n = 4$ at 12 d of age; the colours range from blue (negative correlation) to red (positive correlation). Significant correlations are marked by ** $p < 0.01$ and * $p < 0.05$; (**a**) Spearman's correlation between colonic microbiota with a general abundance > 0.5% and SCFAs at day 5; (**b**) Spearman´s correlation between colonic microbiota with a general abundance > 0.5% and SCFAs at day 12; (**c**) Spearman´s correlation between colonic microbiota with a general abundance > 0.5% and biogenic amines at day 5; (**d**) Spearman´s correlation between colonic microbiota with a general abundance > 0.5% and biogenic amines at day 12. The colours range from blue (negative correlation) to red (positive correlation). Significant correlations are marked by ** $p < 0.01$ and * $p < 0.05$.

4. Discussion

The objective of the study was to follow up on the results of a previous study in which Gln was administered to neonatal suckling piglets. That study showed improved growth, milk intake and lipid metabolism in LBW pigs, and associations with AA metabolism in NBW piglets [17]. Therefore, in the present study, we further investigated the impact of Gln, BiW and age on the colonic microbiota composition, microbial metabolites, mucosal morphology and immune cell density. We hypothesised that Gln supplementation from 1 to 12 d of age is associated with changes in the intestinal microbiota and microbial metabolites, also impacting the lower intestinal tract. Our results show some interesting effects in Gln-supplemented piglets. However, age had the most profound influence.

To the best of our knowledge, this is the first study investigating the effects of Gln supplementation in the ascending colon of piglets during the early suckling phase. Few studies with suckling piglets have investigated histological and immunologic parameters, and the microbiota and the metabolites of the colon [9–11]. However, the majority of studies investigated Gln and its relation to the development and function of the SI after weaning [19,23], while this was rarely explored in suckling piglets [21,39]. A possible downstream transfer of beneficial effects of Gln supplementation from proximal gut segments to the large intestine has not been characterised.

4.1. Effects of Gln Supplementation

Glutamine is an important energy source for immune cells in the GIT of piglets [23]. Within the adaptive immune system, T lymphocytes in the intestinal epithelium play a significant role in the gut barrier function, defence and tolerance mechanisms. Intraepithelial lymphocytes have a primary function for maintaining gut health in early life. They are one of the first cells with an immunological function protecting the intestinal epithelium [40]. Therefore, a higher number of $CD3^+$ IELs in Gln- compared to Ala-supplemented piglets might indicate a more maturate intestinal immune system. The observed difference in $CD3^+$ IELs could be related to T-cell-dependent pathways via a direct effect of Gln supplementation on the intestinal microbiota in more proximal gut segments [41]. An explanation for the absence of effects of Gln supplementation on most of the examined morphological colonic parameters could be due to the metabolism and absorption of the supplemented Gln in proximal parts of the SI with only minor changes in AA concentrations in jejunal digesta and tissue [42] and possibly only little carry-over effects of proximal metabolic products into distal gut segments [43].

In our study, colonic SCFA concentrations remained largely unaffected by Gln supplementation. The relative abundance of *Lactobacillus* spp. was quite similar between Gln- and Ala-supplemented suckling piglets. There was obviously no promotion of SCFA production by the presence of *Lactobacillus* spp., which may also be due to the fact that 5 d- or 12 d-old animals still show extreme fluctuations in the microbiome, which is not as stable as after weaning [10]. Biogenic amines are products of bacterial AA decarboxylation, whose biological importance has been increasingly recognised for both the microbiota and the intestinal tissue [44]. A lower pH is important for the AA decarboxylation activity, and *Lactobacillus* ssp. are mainly responsible for the synthesis of biogenic amines [45]. This is why decreased concentrations of cadaverine and spermidine in the Gln-supplemented piglets in our study might reflect different microbial abundances and microbial fermentation profiles in the colon or more proximal gut segments. Putrescine, spermidine and cadaverine influence gut maturation in weaned piglets [46]. Spermidine is believed to contribute to gut maturation in young piglets. Therefore, it could be assumed that a lower concentration of spermidine in the colon might indicate a more immature gut with reduced autophagic activity [46]. All in all, knowledge on the effects of intestinal biogenic amines in suckling piglets is scarce, but it is important to note that the function of biogenic amines is probably dependent on their concentration and the condition of the host [47].

Glutamine is extensively catabolised by bacteria in the SI of pigs and mice [22,43]. It was shown that 1% Gln supplementation in six week old mice had an influence on microbial composition in the jejunum and ileum, and activated proinflammatory processes through TLR4-nuclear factor κB (NF-κB), mitogen-activated protein kinases (MAPKs), and phosphoinositide-3 kinases (PI3Ks)/PI3K-protein kinase B (Akt) signalling pathways [43]. Even if Gln is mainly utilised in the SI, bacteria and metabolic products might reach the large intestine and influence the microbial composition and metabolic activity. Studies investigating effects of Gln or AA blends on the colonic microbiota have not been reported in suckling piglets. In weaned pigs, blends of Glu, Gln, glycine, arginine and N-acetylcysteine, added at 1% in the diet, increased *Lactobacillus* and *Bifidobacterium* spp. in mid colonic digesta [48]. In our study, the microbial abundance in colonic digesta was only moderately affected by Gln supplementation, similar to findings in rats [43], rabbits [49] and

in faecal samples of underweight infants [50]. Effects of AA supplementation might be influenced by individual microbial composition in the colon of weaned piglets [51]. The individual variability in microbial AA degradation in suckling piglets, on the other hand, is not known.

4.2. Effects of Birthweight

Intraepithelial lymphocytes are components of the gut-associated lymphoid tissue, and are a first line of defence against infection [40]. In the current study, we show that LBW was associated with a lower number of CD3+ IELs in the colon compared to NBW, possibly indicating a better adaptive immune response of NBW piglets. A connection between the number of IELs and BW has been described in other animal species. For example, it was shown in mice that IELs have an important function in promoting weight gain [52]. This is possibly associated with the protective function of IELs in the gut [40].

Dietary protein has been reported to be mainly responsible for the concentration of biogenic amines in the colon of pigs [53]. While branched-chain fatty acids are produced by the deamination of valine, leucine and isoleucine, amines are produced by the decarboxylation of different AAs [53]. The digestive system of piglets in the current study can be considered as rapidly developing. Higher milk intake and the rapidly changing intestinal microbiome might lead to higher total biogenic amine and cadaverine concentrations in the colonic digesta of NBW piglets. It is known that the diamine cadaverine is almost only synthesised by bacteria [54].

Normal-birthweight piglets have different abundances of bacteria in the faeces [14,55], ileum and colon [11] during suckling and weaning compared with LBW piglets. At 3 and 7 d after birth, it has been reported that LBW suckling piglets (0.75–0.95 kg BiW) have a lower faecal abundance of *Firmicutes* than NBW (1.35–1.55 kg BiW) [14]. The higher relative abundance of *Firmicutes* in NBW piglets observed in the current study might relate to similar findings in obese minipigs [56]. Thus, a higher BW in NBW compared to LBW piglets [17] could be associated with a higher abundance of *Firmicutes.* However, overall, the abundance of the major bacterial genera was similar between LBW and NBW piglets. Most changes have been reported to occur in the minor bacterial genera in the colon and faeces of suckling piglets [11,14,55], which is in line with the current study. The faecal microbiota of LBW piglets were characterised by a lower relative abundance of *Lactobacillus*, *Streptococcus* and *Faecalibacterium* spp. and a higher proportion of *Fusobacterium* spp. at 3 and 7 d of age [14]. Piglets with a low daily BW gain at the ages of 4, 8 and 14 d have been reported to have lower faecal abundances of *Lactobacillus*, unclassified *Prevotellaceae* and *Ruminococcaceae* UCG-005 [55]. To the best of our knowledge, there appears to be only one other study [11] comparing the colonic microbiota of LBW and NBW suckling piglets. In the current study, LBW piglets had lower abundances of colonic *Alistipes*, *Lachnospiraceae*, *Ruminococcaceae* and *Prevotellaceae* and *Faecalibacterium* spp. Piglets harbouring increased levels of *Faecalibacterium* in the GIT showed a lower risk for diarrhoea after weaning [57]. In humans, this genus is associated with a lower incidence of inflammatory bowel disease and colorectal cancer [58]. In contrast to previous studies [11,55], we did not observe lower microbial abundances of *Bacteroidetes*, *Bacteroides* and *Ruminococcaceae*, especially *Ruminococcaceae* UCG-005 in LBW piglets. This relative stability of the microbiota might explain the similar SCFA concentrations in the colonic digesta. Interestingly, we observed a higher relative abundance of the genus *Alistipes* in colon digesta of LBW compared to NBW piglets. Low birthweight is associated with increased body fat accumulation [59], hepatic lipid droplets, the rate of lipolysis in the liver [60] and the number of intramyocellular lipid droplets in juvenile pigs [61]. *Alistipes finegoldii* belonging to the *Bacteroidetes* phylum has been shown to be a resident in the human gut microbiome and is involved in lipid metabolism via the bacterial type II fatty acid biosynthesis system [62]. The genus *Alistipes* has been associated with increased porcine back fat mass, intramuscular fat accumulation [63] and lean body mass in pigs [64], while in humans, health-

protective effects have been reported to be related to liver fibrosis, colitis, cancer and gut dysbiosis [65].

As described previously [55], only minor effects of BiW on the microbiota, bacterial metabolites and histomorphomometric traits may have been observed, due to the higher BiW of selected LBW piglets compared to other studies [11–14]. Since studies on the histomorphological development of the large intestine in suckling piglets are scarce, especially those comparing LBW and NBW piglets, it should be mentioned that in this and in previous studies, using intrauterine-growth-restricted (BiW < 1.15 kg) and NBW piglets (1.25–1.70 kg BiW), no major morphological differences in the colon were detected in piglets at 5 d of age or earlier [12,13]. In addition, the lack of differences observed between LBW and NBW piglets in the relative abundance of the colonic microbiota reported in this study may also explain the lack of differences in bacterial metabolites concentrations. A possible explanation could be that biogenic amines and SCFAs are primarily produced by the dominant (>1% relative abundance) bacteria [10], and all other bacteria probably do not contribute strongly to the production of metabolites. Another explanation could be that possible birthweight-dependent differences may have been reduced during postnatal development when milk intake was high [66]. Furthermore, if the piglets survive the first 3 days of life, and suckle enough sow milk including immunoglobulins, antibodies and milk oligosaccharides [67], they become less comparable to very low birthweight piglets, which have been shown to differ in intestinal development, gene expression and bacterial profiles compared to NBW piglets immediately after birth [3,12,13,23].

4.3. Comparison of Age Groups

In the present study, the CD and CA of the colon and numbers of CD3+ IELs, CD3+ lymphocytes in the lamina propria and the number of IgA-positive cells as markers of immunological development increased with age, indicating a highly dynamic gut development in suckling piglets. Comparable results were obtained in piglets from 1 to 42 d of life observing an increased CD and increased expression of Toll-like receptors 2 and 9, indicating a better immune protection against pathogens [10]. The increase in CD was also confirmed in 0 d- compared to 28 d-old pigs [68]. The increases in CD are associated with increases in the absorptive surface and mucosal mass and could be related to factors such as intake of sow´s faeces and spilled feed or the developing intestinal microbiota. The influence of the intestinal microbiota on the intestinal architecture has already been shown. For example, the SI of germfree mice had shorter crypts [69], and similar findings, such as reduced mitotic index and cell turnover rate in the intestinal epithelium of colon and ileum of rats, were reported [70]. A decrease in the abundance of goblet cells containing different mucins in colonic crypts could also be dependent on the interaction with luminal and mucosal gut bacteria and the changing immune system due to maturation as was assumed earlier [9]. Goblet cells provide mucins for the mucus layers found in the colon. The outer layer, mainly consisting of MUC2, is permeable to bacteria. The tightly adherent inner layer, including different mucins impermeable to bacteria, contributes to the strong barrier function in the colon [71]. Decreasing densities of goblet cells in our study from 5 to 12 d of age are in accordance with a previous study observing a decrease in goblet cell density from 0 to 7 days of age and an increase at 14 d of age [9]. Examination of mucin composition revealed a relatively constant number of goblet cells containing acidic mucins from 5 d- and 12 d-old piglets, mostly located in the bottom of the crypts. The location of goblet cells containing acidic mucins was also seen in colonic crypts of piglets after weaning [26]. The balance between the major commensal bacteria leads to colonic epithelial homeostasis due to their effect on mucus production [72]. *Bacteroides* spp. positively affect mucus production, reducing neutral and mixed goblet cells in piglets [72]. In addition, it could be assumed that different bacteria ferment mucins. Therefore, the general bacterial interaction could be responsible for the abundance of mucins [72].

A higher frequency of CD3+ cells might indicate a more mature immune system in 12 d- compared to 5 d-old piglets. It is known that an adequate density of CD3+ IELs

sustains epithelial barrier function [73]. The lower number of neutral, mixed and total goblet cells at 12 d of life may indicate that other factors contribute to an efficient barrier function. Due to the increasing milk intake of piglets in the first two weeks of life, it could be that sow milk including immunoglobulins, antibodies and milk oligosaccharides could lead to adequate protection. Therefore, the described protecting effect of milk oligosaccharides on the intestine [67] might lead to lower mucus production as the intestinal barrier function of the gut is intact and improved. At this point, it is of interest that milk oligosaccharides have a similar structure to mucin glycans, possibly having similar tasks in barrier function next to the known immunomodulatory and microbial effects [67]. Therefore, the intestinal barrier function in the colon might be supported by maternal milk and the higher number of CD3+ IELs.

IgA-positive B cells function as part of the innate immune defence after migrating from the Peyer´s patches into the lamina propria [74]. Factors such as age [75], the composition of the microbiota [76] and diet [77] seem to influence the abundance of IgA-positive B cells. The lack of IgA-positive cells at 5 d of age is in line with previous studies [75]. The emergence of an active immune system in the colon at the second week of life was demonstrated in the current study by the detection of IgA-positive cells in the lamina propria.

The primary site of lactate production is the upper GIT, where it is mainly produced by *Lactobacillus* spp., having beneficial effects on gut health, while acetate, proprionate and butyrate are mainly produced by specific microbial communities in the colon [15]. Arnaud et al. [9] reported increases in colonic SCFAs in the early postnatal period (7 to 14 d of age), whereas Li et al. [11] (7 to 21 d of age) and Qi et al. [10] (7 to 14 d of age) did not observe age-dependent changes, which is in line with the current study.

Polyamines have been reported to have concentration-dependent protective effects [46], and it has been suggested that putrescine, spermidine and cadaverine have an influence on gut maturation in weaned piglets. In contrast to Qi et al. [10], we observed a lower spermidine concentration and a higher total biogenic amine concentration in colon digesta in 12 d- compared to 5 d-old piglets. A decrease in putrescine concentrations in colon digesta of 7 d- compared to 14 d-old piglets has been previously reported [10]. The lack of agreement between the studies could be due to creep feeding (not in the current study) or the physiologically decreasing protein content of sow milk during lactation [78]. Higher tyramine and total biogenic amine concentrations in the colonic content of piglets could be due to a higher intake of indigestible nutrients and immature digestive function. Tyramine and other biogenic amines are produced by gut microbiota degradation of AAs [44]. A decrease in tyramine was observed in piglets right after weaning [79], which could be related to lower or no feed intake. In this study, piglets consumed more nutrients with increasing age, which might have led to an increase in tyramine and other biogenic amines.

The core microbiota of the colon and faeces are the *Firmicutes* and *Bacteroidetes* [9–11,14,55], and we observed here that they are the most abundant microbial phyla in the colonic digesta of neonatal piglets. The abundance of the genus *Lactobacillus* did not change in the colon digesta between 5- and 12-day-old piglets. Most of the initially less abundant bacterial phyla increased in number with age in the colon digesta, in line with previous observations [10,55]. Unknown *Muribaculaceae*, the fourth most abundant genus, decreased in abundance from 5 to 12 d. The functional role is not yet clear, but *Muribaculaceae* can degrade carbohydrates, although lower abundances were observed in mice and rats fed carbohydrate-enriched diets [80]. The observed increased abundance in the *Ruminococcaceae* family with age has also been described in previous studies [11,14,55]. *Ruminococcacae* can ferment dietary fibre, produce SCFAs and are considered as a dominant part of the microbiota of the pig colon [55]. In our study, *Ruminococcacceae* abundance was positively correlated with butyric acid concentration. It is not known whether bacteria belonging to the *Ruminococcaceae* family are involved in the fermentation of milk oligosaccharides to produce SCFAs, as has been observed for *Lactobacillus* and *Bifidobacterium* [67].

However, unknown interactions with the other members of the colon microbiota might also increase the abundance of *Ruminococcaceae*. These findings might indicate the maturation of the intestinal microbiota and the immune system of neonatal piglets towards increasing protection of the intestine in the first weeks of life or the protective function of milk oligosaccharides against possible pathogens [67].

Changes in the diversity of the colonic microbiota were identified in the first week of life and after 21 d [10] or 28 d [9] of life in piglets with no major changes in-between. Additionally, the bacterial abundance depends on the location and type of the sample as well as on nutritional and environmental factors [43] Another explanation for minor differences in bacterial abundances of the colon between 5 d- and 12 d-old suckling piglets could be that the colonic microbial composition of suckling piglets during the first two weeks of life is more likely driven by the milk oligosaccharides in sow milk [67] or by the sow–piglet relationship [81].

5. Conclusions

Glutamine supplementation, compared to Ala, and birthweight had minor effects on colonic development, microbial composition and microbial metabolites in piglets, whereas most of the observed effects were age-dependent. Glutamine supplementation increased the number of CD3+ IELs in the colon as well as the concentration of some biogenic amines in the colonic digesta. Our data suggest the effects of Gln supplementation are less distinct in distal parts of the gastrointestinal tract in neonatal piglets.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/microorganisms10101899/s1. Figure S1: Hierarchical clustering of bacterial genera in colon of male suckling piglets; Table S1: Morphometric measurements of the colon mucosa of 5 and 12 – day old suckling piglets splitted; Table S2: Immunohistomorphometric measurements in the colon of 5 and 12-day old male suckling piglets splitted; Table S3: Goblet cells in colon of 5 and 12-day old male suckling piglets splitted; Table S4: IgA positive cells in the colon lamina propria of 12-day old male suckling piglets; Table S5: Concentrations of biogenic amines in the colon digesta of 5 and 12-d suckling piglets splitted; Table S6: SCFA concentrations in the colon digesta of 5 and 12-d old suckling piglets splitted; Table S7: Relative abundance of bacterial order in colon digesta of male suckling piglets; Table S8: Relative abundance of bacterial genera in colon digesta of male suckling piglets; Tabel S9: Diversity of bacterial abundance in the colon digesta of 5 and 12-d old suckling piglets splitted; Table S10: Relative abundance of bacterial phyla in the colon digesta of male suckling piglets splitted; Table S11: Relative abundance of bacterial order in the colon digesta of male suckling piglets splitted; Table S12: Relative abundance of bacterial genera in the colon digesta of male suckling piglets splitted.

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Tabelle 4.5 Anteilerläuterung von Publikation mit alleiniger Erstautorenschaft

Name	Bezeichnung Autor	Erläuterung Anteil Leistung
Schulze Holthausen, Johannes	Erstautor	Methodik; Überprüfung; statistische Analysen; Sammlung von Daten und Durchführung von Untersuchungen; Erstellung des Manuskripts; Visualisierung
Schregel, Johannes	Co. Autor	Methodik; Sammlung von Daten und Untersuchungen;
Sciascia, Quentin L.	Co. Autor	Sammlung von Daten; Überprüfung und Bearbeitung des Manuskripts
Li, Zeyang	Co. Autor	Methodik; Sammlung von Daten
Vahjen, Wilfried	Co. Autor	Statistische Analysen; Überprüfung und Bearbeitung des Manuskripts
Tuchscherer, Armin	Co. Autor	Statistische Analysen
Metges, Cornelia	Co. Autorin	Konzeptionierung; Methodik Betreuung; Akquisition von Finanzmitteln Überprüfung und Bearbeitung des Manuskripts;
Zentek, Jürgen	Co. Autor	Konzeptionierung; Methodik; Betreuung; Akquisition von Finanzmitteln; Überprüfung und Bearbeitung des Manuskripts;

5 Discussion

The aim of our study was to provide insight into the GI development of suckling normal and LBW piglets, supplemented with Gln or Ala. In the jejunal tissue, morphometric data, immune cell colonization, and AA profiles were investigated, together with genes involved in AA metabolism. In the digesta of jejunum, AA profiles and in digesta of the colon the composition of the microbiota and the concentration of microbial metabolites were used as indicators of early microbial development. In contrast to reports in post weaning piglets, Gln and BiW had only a minor influence in gut development, whereas age had a major effect on the intestinal characteristics.

The problem of large litter sizes in hyperprolific sows with increased numbers of LBW piglets is well known (Foxcroft et al. 2006). Since LBW have high mortality in the first days of life, it is a relevant topic to develop solutions for this problem (Marchant et al. 2000), which goes along with high economic losses (Beaulieu et al. 2010). Different feeding trials of sows and piglets to reduce mortality of LBW piglets, using other AA, nucleotides, bovine milk replacers and SCFA, next to modulating the housing and the environment, have been already conducted (Farmer and Edwards 2021; Huting et al. 2021). Therefore, the supplementation of Gln, which is known to be an important energy substrate for enterocytes (Darcy-Vrillon et al. 1994; Wu et al. 1995c), to LBW suckling piglets is a promising supportive strategy. In previous trials mainly conducted in weaned piglets Gln supplementation showed beneficial effects on performance and GI development (Wu et al. 1996b; Wang et al. 2008; Cabrera et al. 2013) which was also conformed in LPS treated suckling piglets (Haynes et al. 2009). The problem of LBW is also seen in infants. Using a pig model which can be transferred to humans was not the main aim but additionally justifies this basic research study.

A critical aspect is the selection of piglets, which was handled very differently in the previous studies (Cooper 1975; De Vos et al. 2014; Feldpausch et al. 2019). Selection criteria and the high mortality makes it difficult to compare all effects between different studies. The selection criteria for LBW in our study, with LBW piglets defined by a BiW of 0.8 – 1.2 kg is rather high. It can be explained by the high mortality of piglets with lower BiW. However, it is a certain limitation, results could be different in severely underweight piglets. Therefore, it should be tried in further studies to focus on piglets with BiW below 0.8 kg, to characterize the potentially beneficial effect of Gln supplementation. Another interesting aspect would be to start the application of Gln immediately after birth, because most underweight piglets have a massive energy deficiency, which greatly reduces their chances of survival.

The resources needed for to execute this should not be neglected. In our study, the workload during farrowing, the selection of piglets at birth and generating data, made the additional supplementation at birth impossible.

5.1 Impacts of glutamine

5.1.1 Influence on gut development and absorption

In contrast to studies supplementing Gln to weaned piglets (Haynes et al. 2009; Wu et al. 2011b; He et al. 2019), the supplementation of Gln to suckling piglets in this study showed no impact on GI development and only minor effects on immune cells. An improved GI development, especially of the morphological characteristics, with regards to the energy delivered by Gln for enterocytes (Darcy-Vrillon et al. 1994; Wu et al. 1995c), was hypothesised. The knowledge of the absorption kinetics of Gln in the intestine of pigs is scarce (Windmueller 1982; Wu et al. 1996b), but similar to other AA it can be expected to be absorbed in the SI (Wernerman 2011). The similar AA patterns in digesta and tissue of the jejunum might confirm that the absorption and or metabolism of the supplemented AA occurred in more proximal gut segments, leading to only minor carry over effects of Gln supplementation in distal gut segments. Interestingly, the concentration of plasma Gln was higher compared to Ala pigs, and vice versa (Li et al. 2022). This demonstrates, that the supplemented AA were absorbed, most likely in more proximal SI segments. Additionally, it is known that free Gln is not as stable as a Gln-dipeptide (Fuerst 2001) , especially in an acidic milieu. Therefore, Gln might have not have been fully available in the SI. Nevertheless, it has been reported that in weaned piglets supplemented with 1% free Gln, that the concentration of Gln in the duodenal digesta increases 8-fold (Wu et al. 1996b). Additionally, it was recently shown that supplemental Gln and Ala increased concentrations in the stomach content and duodenum tissue of suckling piglets (Sciascia et al. 2022). If or how far the Gln transits into the jejunum is still unknown, however, intestinal transit time studies show that the passage rate of the SI ranges from 1.8-3.2 hours (depending on diet composition) (Henze et al. 2021), this is enough time for any supplemental AA to pass to the jejunal section sampled for this study. Frequency, duration, and way of supplementing Gln could also play a role for possible effects of Gln in the GIT. A constant supplementation with Gln would only be possible by supplementing Gln parenterally. Parenteral Gln supplementation did not show a benefit compared to enteral Gln supplementation in weaned healthy and endotoxemic pigs. A combination of both seemed to be promising approach. It is expected, that the majority of enterally supplemented Gln will be utilized by enterocytes and immune cells, with the rest being absorbed into the portal vein by the liver. This would then result in a small fraction of Gln in the systemic circulation. It has been shown in pigs, that enteral Gln supplementation has only minor effects on the systemic

circulation, while the use for intestinal metabolic needs seemed to be sufficient. Stavrou et al. (2018) observed that enteral supplementation of 92 mmol Gln to endotoxemic swine resulted in a portal Gln concentration of 0.4 mmol/L compared to parenteral supplementation, leading to a perfusion of the GIT through the mesenteric artery resulting in a portal Gln concentration of 2.5 mmol/L. Intravenous supplementation displayed a similar Gln supply for the GIT compared to endogenous supply. A combination of 92 mmol intravenous and 92 mmol enteral Gln led to the highest portal concentration of 2.9 mmol. In total, 7 times higher than the 0.4 mmol portal Gln fraction of solely enterally fed endotoxemic pigs (Stavrou et al. 2018). The duration of enteral Gln application in most of the studies with suckling or weaned piglets was two weeks or longer (Wu et al. 1996b; Haynes et al. 2009; Cabrera et al. 2013)

5.1.2 Influence on immune cell development and bacterial metabolites

We reported no differences in tissue and digesta AA of the jejunal tissue sampled in this study (Schregel et al. 2022). This supports the findings of similar SCFA concentrations, microbial composition, and only minor differences in biogenic amines concentration in the digesta of the colon. However, effects on $CD3^+$ intraepithelial lymphocytes (IEL) in the colon of Gln supplemented piglets might be affected by changes in microbial composition in more proximal parts of the GIT. The $CD3^+$ IEL protect the intestinal epithelium and are the first cells with immunological function, colonizing the intestinal tissue. It is assumed that their abundance is related to T-cell dependent pathways which can be affected by changes in microbial composition due to Gln supplementation (Wu et al. 2016). Another explanation might be that the higher Gln concentration in the circulation (Li et al. 2022) led to a proliferation of intestinal lymphocytes. It has been shown that the small as well as the large intestine can use Gln from the digesta or the bloodstream (Wernerman 2014). Looking at these findings, the increase of $CD3^+$ IEL in the colon might be also related to the fact that intestinal lymphocytes use Gln as an energy source and require Gln for proliferation (Dugan et al. 1994; Wu 1996). Moreover, changes in concentrations of biogenic amines by Gln supplementation are difficult to evaluate, because of the current limited knowledge of their biological effects especially in the early life period. In the digesta, biogenic amines are produced by bacterial AA decarboxylation. In the literature, the function of biogenic amines is described to be related to concentrations and host general health, by maintaining barrier function and improving immunity of the intestine.(Fan et al. 2017; Ma et al. 2021). Their effects on intestinal development in suckling piglets are not known. The minor effects of Gln supplementation on biogenic amines and microbial composition in suckling piglets in this study might depend on a very limited carry-over effect of

proximal metabolic products into more distal gut segments assuming that the supplemental AA are being absorbed in more proximal tissues.

5.1.3 Effect on microbiota and reasons for little effects in suckling piglets

To date, the investigation of Gln and Glu catabolism by microbiota of the porcine SI has only been conducted *in vitro*, and shows that jejunal and ileal bacteria appear to use 22 to 40% of dietary Gln, as indicated by the incorporation of 2 mmol/L ^{14}C – labeled Gln into proteins (Dai et al. 2010). The catabolism of AA by bacteria is not yet sufficiently described, but it has been shown that the metabolism of AA is dependent on the microbial community and differs between gut segments (Dai et al. 2010; Dai et al. 2012b). Different utilization patterns of lysine, leucine, valine, ornithine and serine by jejunal and ileal mixed microbiota have been observed by adding different concentrations of Gln (Dai et al. 2013). We could not observe an effect of Gln on AA concentrations in digesta or tissue of the distal jejunum, indicated by similar concentrations of the different AA (Schregel et al. 2022). Effects on the microbiota of the colon digesta and on bacterial genera with an abundance below 1% were observed. Bacteria belonging to the *Clostridium* and P*eptostreptococcus* family, known for their extensive role in large intestine AA metabolism (Davila et al. 2013), were not greatly affected by Gln supplementation in our study. This had no obvious effect on the metabolism of AA into SCFA, which was reported in a previous study (Dai et al. 2011). It should be mentioned that all Gln and Ala supplementation groups in this study had the possibility to constantly suckle the dam. Milk Gln and Glu have been determined in the first 3 weeks of lactation. Concentrations of free Gln were 0.02-0.05 g/l milk, protein-bound Gln 11.0 – 7.24 g/l milk, free Glu 0.02-0.06 g/l milk and protein-bound Glu 13.8-5.54 g/l milk, were quite high in sow milk (Wu et al. 2011b). In total, the sum of Gln and Glu provided up to 20% of the total protein-bound AA in sow colostrum and milk (Wu et al. 2011b). To date the level of Gln and Glu in practical diets for weaned piglets is not analyzed, but it can be assumed from analyses measuring the content of total AA in different feed ingredients, that diets for weaned piglets have a similar or even higher content of total (free and protein bound) Gln plus Glu than sow milk. Animal and plant ingredients have a Glu plus Gln content, which makes up to 10 – 32% of total protein (Li et al. 2011). It is known that the high abundance of Gln in sow milk is an important factor for the growth and maturation of the piglet SI (Wu et al. 1998). Glutamine is converted to Glu and ammonia in the mitochondria of enterocytes by the phosphate-dependent GLS first (Blachier et al. 1999). Glutamate can then either be metabolized inside the mitochondria or exported to the cytosol. There, the transamination to alpha-ketoglutarate takes places, before it is used inside the mitochondria in the Krebs cycle (Duee et al. 1995). However, it has been shown, that if Gln and Glu are available for isolated pig enterocytes at the same time, the mitochondrial GLS

activity can be inhibited by Glu (Blachier et al. 1999), leading to a production of other functional AA like aspartate (Blachier et al. 1999), Ala, proline (Wu et al. 2011a), ornithine (Blachier et al. 1992) and citrulline (Wu et al. 1994b). Next to the high abundance of AA in sow milk, supporting the conversion into other important AA in the pig intestine, it should be mentioned, that sow milk includes a wide range of other bioactive factors, such as caseins, whey proteins, proteins associated with milk fat membranes, immunoglobulins, hormones, growth factors, enzymes and others (Theil and Hurley 2016). There is a natural drop in protein content of sow milk from approximately 7% at day two of lactation to reach a plateau of 5% during the rest of lactation (Hansen et al. 2012). This is related to the higher concentration of immunoglobulins and bioactive factors in colostrum compared to mature milk. The milk protein content is usually not affected by dietary treatment, because even in cases of malnutrition the sow mobilizes body reserves to maintain milk production and protein content (Theil and Hurley 2016). Therefore, possible beneficial effects of Gln supplementation might be diminished by an adequate milk intake. Not to forget the fact that the treatment of the control group supplemented with an isonitrogenous amount of Ala might have minimised differences as well. Alanine is a nitrogenous product of the intestinal catabolism of Gln (Windmueller 1982) and is regarded as an energy substrate on its own, as transamination of Ala produces pyruvate, which can be used as an energy source, yielding 13.7 – 18.3 kJ/g metabolizable energy depending on the concentration of urine nitrogen (May and Hill 1990). In addition, it must be mentioned that it has been shown that Gln may have beneficial effects under stressful conditions. This has been shown in pigs during infection and weaning studies (Wu et al. 1996b; Haynes et al. 2009; Stavrou et al. 2018; He et al. 2019). It also has to be considered that piglets in our study had a sufficient milk intake (Li et al. 2022), which can be different on the farm, with bigger litters, where LBW piglets get displaced from the teats by bigger littermates (Huting et al. 2021). A Gln supplementation might also have beneficial effects in these cases resulting in starvation of LBW suckling piglets followed by local gut inflammation, but this has not been studied before. Furthermore, studies in endotoxemia and sepsis models in other species have described the depletion of Gln results in impaired body homeostasis and the onset of disease (Kao et al. 2013; De Oliveira et al. 2014). Similar pathways can be expected in weaned piglets, who have a reduced feed intake in the first weeks and the Gln rich sow milk is not available for them anymore (Wu et al. 1996b). For this reason, it can be argued that selecting LBW piglets or the supplementation 3 x times daily mimic a stressful event. But as stated before, as long as the piglets survive the first day and have free access to Gln rich sow milk this might not have the same effect as after weaning or during an infection. It was shown that “drenching” is not a stressful event for piglets (Van Tichelen et al. 2022), as long as it is performed in the farrowing pen, like it was done in our study. A question which should be discussed is: are suckling piglets capable of using the surplus supplemental Gln in addition to the Gln taken up by sow milk? In the literature it is described

that the enzymatic activity for metabolizing Gln in the gut of suckling is less developed than in weaned piglets (Madej et al. 1999). Although beneficial effects of supplemental Gln to weaned piglets have been described, over supplementation to suckling piglets has also been reported. For example, by supplementing 1 g Gln/kg BW two times daily, weight gain was reduced by 19% in 7 – 21-d old suckling piglets (Haynes et al. 2009). Regarding these points, a sufficient dosage of Gln for the supplementation to suckling piglets still needs to be determined and it might be, that there is no need for a supplementation, as long as suckling piglets are healthy and have access to adequate amounts of milk.

5.2 Impact of Birthweight

5.2.1 Influence on morphological characteristics

Only minor effects of BiW were detected on parameters investigated in both studies comparing LBW and NBW. As mentioned, it could be related to the relatively high BiW (0.8 – 1.2 kg) of LBW piglets selected in this study. Nevertheless, these piglets are defined as LBW piglets according to the definition of (Cooper 1975). In general, the BiW range of LBW piglets in different studies varies (0.59 kg - < 1.0 kg) (Xu et al. 1994b; Morise et al. 2008; Huygelen et al. 2015; Chen et al. 2021b). Morphological or growth characteristics and gene expression differences have mostly been reported when using very low BiW piglets (Xu et al. 1994b) or LBW piglets with a BiW around 0.8 kg (Chen et al. 2021b). In contrast, Wang et al. (2016) and Wiyaporn et al. (2013) have reported no differences in morphological characteristics and gene expression of jejunum between LBW and NBW piglets. In addition, no major differences in the morphological development of the colon between piglets influenced by IUGR and NBW suckling piglets at 5 days of age or earlier, have been observed (D'inca et al. 2010a; D'inca et al. 2010c). To date, these are the only studies comparing morphometric development of the GIT between LBW and NBW GIT, indicating a paucity of information in this field. The selection of LBW piglets in the different studies, indicates the difficultly in keeping them alive. The study by Feldpausch et al. (2019) demonstrates that nearly 100% of piglets with a BiW of 0.5-0.6 kg die before weaning. Therefore, if the LBW piglets survive the first days of life and take up sow milk it might be that existing differences in morphological characteristics, tissue proliferation and gene expression diminish, as reviewed by Everaert et al. (2017). Supporting this statement, different studies (Xu et al. 1994b; Wang et al. 2008; Chen et al. 2021b) found differences in gene expression and morphologic characteristics of jejunum between LBW and NBW piglets right at birth but not later during the suckling period. However, other studies could not detect differences in morphological development of the gut and gene expression of jejunum

between LBW and NBW piglets (Wiyaporn et al. 2013; Huygelen et al. 2015; Thongsong et al. 2019), thus supporting the findings of our study. Nevertheless, it has to be noticed that the studies with and without differences in morphological development and gene expression of jejunum used pigs with different genetic (Wiyaporn et al. 2013; Huygelen et al. 2015; Thongsong et al. 2019; Chen et al. 2021b), which could have an influence on the results.

5.2.2 Effects on immune cells

The differences observed in $CD3^+$ IEL and $CD3^+$ lamina propria lymphocytes of colonic tissue but not in jejunal tissue between LBW and NBW piglets is not clear. One explanation could be that the jejunum adapts faster after birth, whereas the development of the colon is slower than that of the SI (Mccance 1974).The jejunum is very permeable right after birth and has been shown to host more IEL than the large intestine. The greater surface area of the SI, created by the villi and only having a relatively liquid mucus layer, whereas the colon has no villi and more firm mucus layers, may lead to higher numbers of IEL and faster immune development (Wiarda et al. 2020). Another reason may be that the SI is exposed more to luminal contents than the large intestine, leading to less IEL in the large intestine. Also, the large intestine as the fermentation chamber is dependent on a symbiotic relationship with the microbes. Tolerating the beneficial bacteria, but still regulating them, might result in a lower number of IEL in the colon (Wiarda et al. 2020). The higher number of $CD3^+$ cells in jejunum might lead to no detectable differences in $CD3^+$ IEL in our study. However it is reported that LBW piglets (1.12 kg) have a higher amount of $CD4^+CD8^+$ T-cells and dendritic cells in blood and mesenteric lymph nodes and a lower number of blood cytotoxic T – cells at 16 days of age than NBW piglets (1.81 kg) (Verso et al. 2018). The flow cytometric investigation of these data is not comparable with the quantitative immunohistomorphometric method we used in our study. To the best of our knowledge there is no published studies investigating colonic immunological cell development at this early time point of life. Another reason could be the fact that we compared all LBW piglets supplemented with Gln or Ala against all NBW piglets in the study investigating the colon, because of little effects of supplementation and BiW. A higher number of investigated piglets might have led to a difference here. Feed intake differences between LBW and NBW piglets might also play a role in developing an immune response. Sow milk is highly digestible, leading to only small amounts of minimally- or un-digested substances reaching the colon (Lærke and Hedemann 2012), which might also influence the immunological development of the colon. Normal birthweight pigs are more vital and therefore it can be expected that they take up more spilled feed and sow´s faeces (Sansom and Gleed

1981). Thus, the intake of a small amounts of solid feed might lead to an altered colonic immune cell development.

5.2.3 Influence on biogenic amines

A finding of our study, regarding the effect of Gln in the colon, which might support the immunological development is, that we measured a higher concentration of total biogenic amines, cadaverine and tyramine in colon digesta of NBW suckling piglets. Biogenic amines are bacterial metabolites, produced by decarboxylation of AA. Cadaverine is mainly derived from lysine and source of the monoamine tyramine is mainly exogenous trough the ingestion of feed which is rich in tyrosine degrading bacteria like *Enterococcus* and *Lactobacillus* spp.*,* leading to the accumulation of tyramine (Fan et al. 2017). Cadaverine is expected to regulate an adaptive response to acidic conditions using in vitro cell cultures by modulating outer membrane permeability (Samartzidou and Delcour 1999). Changes in low abundant bacteria with an abundance below 1 % might also explain why differences in SCFA concentration in digesta of the colon were not seen between LBW and NBW piglets. Only bacteria with a relative high abundance contribute in the production of bacterial metabolites to a bigger extent.

5.2.4 Influence of birthweight on microbial development

Previously it has been described, that the microbiota of the colon and the faeces of LBW (0.88 kg) suckling piglets differs from the microbiota of NBW (1.43 kg) suckling piglets (Li et al. 2018; Li et al. 2019). Studies comparing the microbiota of LBW and NBW suckling piglets in the colon are scarce. To the best of our knowledge the study of (Li et al. 2019) is the only one comparing the colonic microbiota of LBW and NBW piglets, like it was done in our study. We could neither find the same pattern of microbial changes as (Li et al. 2019) in colon nor as (Li et al. 2018; Gaukroger et al. 2020) in faeces of LBW compared to NBW piglets. But interestingly in these studies the majority of changes in microbial abundance between LBW and NBW suckling piglets was observed in the low abundant bacterial families. The different observations might be also partly explained by the different feeding regime between our study and the previous studies. In the study by (Li et al. 2018; Li et al. 2019) piglets were offered a solid starter feed from 3 to 5 days after birth, which might be partly responsible for different microbial abundances in the colon digesta. Nevertheless, one main finding on phylum level is similar, which reveals an association of a certain microbial pattern of LBW or NBW. Low birthweight piglets seem to have a lower abundance of *Firmicutes* and a relatively higher abundance of *Bacteroidetes* (Li et al. 2018; Li et al. 2019). With regards to humans this finding is in line with

literature, because it is reported that the *Firmicutes* to *Bacteroidetes* ratio is higher in obese and lower in lean infants (Bervoets et al. 2013). Since the *Firmicutes* to *Bacteroidetes* ratio is also higher in obese minipigs (Pedersen et al. 2013), a lower abundance of *Firmicutes* and a higher abundance of *Bacteroidetes* might be associated with a lower BW of piglets . Other than Li et al. (2019) in ileum content of LBW the observation of higher abundances of *Alistipes* in colon content of LBW piglets in our study might be closer to what is reported in literature, describing an association of LBW and fat accumulation in the body of pigs (Morise et al. 2008). The genus *Alistipes* seems to involved in the lipid metabolism (Radka et al. 2020), and has been shown to have beneficial effects in humans (Parker et al. 2020). Together with observations of a higher back fat mass and intramuscular fat accumulation (Tang et al. 2020), also a higher lean body mass in pigs (Chen et al. 2021a), was associated with the bacterial genus *Alistipes*. All in all, the influence of BiW on the microbiota of the colon needs further research.

5.3 Impact of Age

5.3.1 Morphological characteristics, immune cells and goblet cells affected by age

In the literature a similar rapid morphological development of the jejunum (Hampson 1986; Adeola and King 2006) and the colon (Qi et al. 2021) of suckling piglets as observed in our study is described. An increasing villus height and crypt depth in jejunum and increasing crypt depth in colon are indicators of an expanding mucosal absorptive surface. The increasing crypt depth, as an indicator of crypt cell proliferation, which starts at the bottom of the crypts, leads to deeper crypts and elongation of villi in the SI (Hampson 1986). Nevertheless, contrary to our study it is also reported that the villus height in jejunum decreases during the first two weeks of life in suckling piglets (Van Ginneken et al. 2002; Huygelen et al. 2015). Different feed, microbial development and gut segments (Umesaki et al. 1995; Van Ginneken et al. 2002; Al Masri et al. 2015) could explain the different observations. In the colon it is also known, that development of the microbiota influences crypt depth. For example, germfree mice had a lower crypt depth than in colonized mice (Umesaki et al. 1995). A decrease in the abundance of goblet cells in crypts of the jejunum and the colon during the first two weeks of life in suckling piglets was observed in our studies. Since goblet cells produce mucins which act as diffusion barrier and also have protective function against microorganisms (Pelaseyed et al. 2014), it could be, that their abundance is changed due to a changing bacterial composition in digesta or an immune system maturation (Forder et al. 2007). Moreover, it was shown, that a balance between major abundant microorganisms and *Bacteroides* ssp influence mucus production and led to reduced numbers of neutral and mixed mucins (Wrzosek et al. 2013). In colonic

tissue the same decrease in the number of goblet cells was described in suckling piglets from 0 to 7 days of age (Arnaud et al. 2020). Even though no change in $CD3^+$ IEL was observed in the jejunum in our study, the increase of $CD3^+$ IEL and lamina propria $CD3^+$ cells in colon of our study and the appearance of IgA positive cells might indicate the maturation of the immune system. However, it is not totally clear which number of $CD3^+$ cells is the healthy status, but it is known that a suitable density of $CD3^+$ IEL's supports epithelial barrier function (Van Kaer and Olivares-Villagómez 2018). No identification of IgA positive B-cells in the lamina propria of 5 day old piglets, but at 12 days of age, mostly in the lamina propria of jejunum and colon, is in line with the literature (Rothkotter et al. 1991). IgA positive B-cells are play a part in the innate immune response and it is known that they are affected by age (Rothkotter et al. 1991), composition of the microbiota (Collinder et al. 2002) and diet (Hedemann et al. 2006).

5.3.2 Effects on abundance of amino acids

Interestingly we found that certain jejunal free AA, protein bound AA and AA metabolites changed in concentration over time. The decrease in jejunal AA concentrations may be due to very young animal's having the highest AA requirements and that AA absorption capacity decreases later in life (Wang et al. 2018). The transport systems in the intestine seem to adapt to changes of dietary intake and AA requirements, depending also on the regulation of the ontogenic development (Buddington et al. 2001). But the overall knowledge about the whole process of AA absorption in the intestine of piglets during the suckling phase is still scarce. Here again the microbiota (Arnaud et al. 2020), structural and functional changes in cells or in absorption (Wang et al. 2018), could be responsible. We could also show a decrease in DNA concentration in the jejunal tissue in suckling piglets from 5 to 12 days of age. This is an indicator of a lower cell number per mg of jejunal tissue and goes in line with the findings of lower protein, RNA, free AA and protein-bound AA. However, this was observed for the first time, and is possibly indicating a rise in apoptosis, after enhanced mitosis and reduced apoptosis at birth (Godlewski et al. 2005).

5.3.3 Alterations in bacterial metabolites

In this study, only the microbiota of the colon were investigated, assumptions and correlation to results possibly affected by microbial changes cannot be made for the jejunum. Changes in biogenic amines in colonic digesta, which are products of AA decarboxylation by bacteria (Fan et al. 2017), could be related to an increasing richness of bacteria in colon digesta or exogenous intake of materials containing biogenic amines (Girdhar et al. 2006; Van Zyl et al. 2015). However the decrease in concentration of spermidine could also be linked to its similar function to spermine (He et al. 2015), which has been shown to regulate mucosal repair and

intestinal health in rats (Wang and Johnson 1992). But a decrease of spermidine could be also linked to the increase of putrescine which is a precursor of spermidine (Gupta et al. 2009). For the production of spermidine, the enzyme spermidine synthetase is necessary (Gupta et al. 2009), which could be reduced or not activated at this early point of life. Putrescine is known to be an additional source of energy for the intestine because it is also a precursor of succinate (Desury et al. 2002). Additionally it is reported that exogenous dietary putrescine protects the intestinal mucosa (Girdhar et al. 2006). In the colon digesta of suckling piglets the increase of tyramine can be also dependent on diets rich in tyramine or synthesis by bacteria in the GIT (Van Zyl et al. 2015). Nevertheless, next to the positive effects of biogenic amines the concentration dependent possibly toxicity should not be neglected. For example, in weaned piglets increased production of biogenic amines, by bacterial fermentation of precaecally undigestible protein, is leads to diarrhea (Kozak et al. 2015). The same mechanism could also explain the higher concentrations of biogenic amines in digesta of colon of our study. These concentrations did not lead clinical signs of diarrhea.

5.3.4 Influence on microbial development

The general development of the core microbiota, mainly *Firmicutes* and *Bacteroidetes*, in suckling piglets is also pointed out in other studies (Li et al. 2018; Li et al. 2019; Arnaud et al. 2020). Moreover, since the microbial mass in the intestine increases with age, it makes sense that there is an increase in bacterial diversity. We observed an age related increase in bacteria, which were less abundant in digesta of the colon at 5 days of age. The bacterial genus unknown *Muribaculaceae*, having no clear functional role other than the degradation of carbohydrates, were within the most abundant bacteria at 5 days and declined at 12 days of age. As the name of the bacterial genus suggests, most studies regarding this bacteria have been conducted in mice and rats (Lagkouvardos et al. 2019). *Muribaculaceae* spp. is considered to be the major bacterial family in the gut of healthy mice (Nagpal et al. 2018). Thus, it cannot be excluded, that environmental factors, like contamination in the stable and stable hygiene, lead to higher abundances of *Muribaculaceae.* Comparable to other studies, increasing abundances of bacteria belonging to the *Ruminococcaceae* family were determined (Li et al. 2018; Li et al. 2019), though. Whether there is a relationship to SCFA production due to fermentation of bioactive factors in sow's milk, or if other bacteria lead to the increase of *Ruminococcaceae,* needs further clarification. However, the knowledge so far is, that bacteria belonging to the *Ruminococcaceae* family ferment dietary fiber and build a dominant part of the colonic microbiota in pigs (Gaukroger et al. 2020). The ontogenetic development observed in the study of the jejunum and colon of suckling piglets, reveal a physiological gut development, which is in line with data from the literature. Nevertheless, it should be stated,

that the whole physiological development process in neonates is majorly dependent on sufficient and adequate quality milk intake, supplementation of other feeds and the environment (Everaert et al. 2017).

5.4 Options for future studies

Future *in vivo* studies investigating the effect of Gln supplementation in piglets are necessary and might concentrate more on the effect of a supplementation to underweight piglets right at birth or in stress conditions such as starvation or weaning. According to the literature, under stressful conditions the most promising effects were observed. Next to adding an isonitrogenous supplementation group with Ala, it should be considered to add an additional negative control group, to see if possible positive effects of Gln might be also diminished by using Ala as a control group. In general, the whole AA metabolism in neonates and the influence of sows milk, with its bioactive factors and other dietary and environmental factors, like the intake of faeces of sows and littermates, spilled feed, bedding material and general housing conditions, need further evaluation. Further *in vivo* and *ex vivo* experiments should consider the Gln effects in more proximal parts of the GIT, the influence of Gln on immunological development in proximal parts of the intestine using quantitative analyses such as flow cytometry, and the production and influence of biogenic amines for neonates as well. The dosage and the biochemical form of Gln, and the content of Gln in starter and weaner feed, best for supplementation should also be tested before starting new experiments. Moreover, the whole mechanism behind changes in bacterial abundances and their metabolites should be elucidated. This should also include minor abundant bacterial species as their effect is often neglected.

6 Conclusion

The results of this study revealed a minor impact of Gln supplementation and BiW on the measured jejunal and colonic traits in male suckling piglets, whereas most of the observed effects were age dependent. The findings suggest that Gln supplementation during the first two weeks of life may not be an appropriate way to stimulate the development of the jejunum and colon during the suckling period. However, reviewing the literature, Gln supplementation might be beneficial under more challenging conditions. Further research should focus on the influence of more proximal parts of the GIT, changes in microbial abundances and their metabolites in the digesta and mucosa of these proximal parts, starting with a Gln supplementation right at birth using LBW piglets. Alternatively, this should be done in weaning piglets, where already positive effects of Gln supplementation were reported.

7 Summary

Summary of the Thesis

The impact of glutamine supplementation on morphological gut development, immune cells, tissue and digesta amino acid profiles, microbiota and bacterial metabolites in low birthweight and normal birthweight male suckling piglets

Due to the breeding of hyperprolific sows with large litters size, the number of low birthweight piglets (LBW) increased in the last decade. Low birthweight piglets show a higher preweaning mortality and reduced growth performance. The high mortality cannot be accepted regarding animal welfare and the reduced growth performance leads to economic losses. Promising results of glutamine (Gln) supplementation to piglets were observed at weaning and under stressful conditions, such as infections, leading to better gut development and growth. An important aspect of the non-essential amino acid Gln is, the function as an energy fuel for enterocytes and immune cells of the intestine.

The objective of this study was to investigate the impact of Gln supplementation on gut and immune cell development, amino acid absorption, the microbiota and their metabolites in LBW male suckling piglets.

To test the effect of Gln on male suckling piglets an animal trial was performed. LBW (0.8–1.2 kg) and normal birthweight (NBW) (1.4–1.8 kg) male littermates born to gilts were paired at birth. The piglets received an oral supplementation of either 1 g Gln or an isonitrogenous amount of alanine (Ala) (1.22 g/kg BW) until 12 days of age. In total four different groups were studied: LBW+Gln; NBW+ Gln; LBW+Ala; NBW+Ala (n = 12/age group). The piglets had free access to suckle the dam throughout the study. At 5 and 12 days of life (n = 96) piglets were euthanized. Tissue and digesta of the jejunum and colon were snap frozen in liquid nitrogen or fixed in formalin for subsequent analysis.

Interestingly the supplementation of Gln led to no differences in most of the investigated parameters in the jejunum. Glutamine supplementation had no effect on jejunal morphology, development, tissue and digesta amino acid profiles and mRNA abundance of genes involved in amino acid transport, metabolism, glutathione synthesis in LBW piglets compared Ala supplementation and birthweight (BiW) at 5 and 12 days of age. Only the jejunal tissue Gln concentration was higher in NBW piglets supplemented with Gln compared to Ala at 5 days of age. The BiW comparisons revealed no difference between LBW and NBW piglets. However,

age seemed to have the biggest effect, because morphological characteristics, immune cells, the ratio of jejunal RNA to DNA and the concentration DNA, protein and RNA, as well as amino acid profiles changed from 5 compared to 12 days in suckling piglets.

In the colon we observed similar effects. Concentrations of biogenic amines were lower and the number of $CD3^+$ intraepithelial lymphocytes (IEL) were higher in Gln compared to Ala supplemented piglets. Low birthweight of piglets had minor effects on the microbial composition and led to a lower concentration of some biogenic amines and IEL. In contrast to Gln supplementation and BiW the factor age had a major influence on histological and microbial characteristics in male suckling piglets.

In conclusion, the oral supplementation of Gln and the BiW of piglets had only minor effects on the observed parameters in the distal small intestine and the colon of suckling piglets, whereas age had a major impact. This indicates, that the intestinal development is mainly driven by ontogenetic factors during the suckling period of piglets.

8 Zusammenfassung

Zusammenfassung der Doktorarbeit

Die Auswirkungen einer Glutaminsupplementierung auf morphologische Entwicklung des Darms, Immunzellen, Aminosäureprofile von Gewebe und Digesta, die Mikrobiota und die bakteriellen Stoffwechselprodukte bei männlichen Saugferkeln mit niedrigem und normalem Geburtsgewicht

Aufgrund der Verfügbarkeit von fruchtbaren Sauen mit großen Würfen hat die Zahl der Ferkel mit niedrigem Geburtsgewicht (LBW) im letzten Jahrzehnt zugenommen. Ferkel mit niedrigem Geburtsgewicht weisen eine höhere Sterblichkeit vor dem Absetzen und ein geringeres Wachstum auf. Die hohe Sterblichkeit ist aus Sicht des Tierschutzes nicht akzeptabel, und die verringerte Wachstumsleistung führt zu wirtschaftlichen Verlusten. Es wurden vielversprechende Ergebnisse bei der Gabe von Glutamin (Gln) an Ferkel zum Zeitpunkt des Absetzens und unter Stressbedingungen, wie zum Beispiel bei Infektionen, beobachtet, die zu einer besseren Darmentwicklung und einem höheren Wachstum führten. Ein wichtiger Aspekt der nicht-essentiellen Aminosäure Glutamin ist, dass sie als Energieträger für Enterozyten und Immunzellen des Darms von Bedeutung ist.

Ziel dieser Studie war es, die Auswirkungen einer Gln-Supplementierung auf die Entwicklung des Darms und von Immunzellen, die Aminosäureabsorption und die intestinale Mikrobiota sowie deren Metaboliten bei männlichen LBW Saugferkeln zu untersuchen.

Um die Wirkung von Gln auf männliche Saugferkel zu testen, wurde ein Fütterungsversuch durchgeführt. Für den Versuch wurden männliche Wurfgeschwister mit LBW (0,8-1,2 kg) und normalem Geburtsgewicht (NBW) (1,4-1,8), die von Jungsauen geboren wurden, bei der Geburt gepaart. Die Ferkel erhielten bis zum Alter von 12 Tagen entweder 1 g Gln oder eine isonitrogene Menge an Alanin (Ala) (1,22 g/kg Körpergewicht) oral verabreicht. Insgesamt wurden vier verschiedene Gruppen untersucht: LBW+Gln; NBW+ Gln; LBW+Ala; NBW+Ala (*n* = 12/Altersgruppe). Die Ferkel hatten während der gesamten Studie freien Zugang zum Säugen des Muttertiers. Am 5. und 12. Lebenstag (n = 96) wurden die Ferkel zur Probenentnahme euthanasiert. Das Gewebe und die Digesta des Jejunums und des Kolons wurden für die anschließende Analyse in flüssigem Stickstoff eingefroren oder in Formalin fixiert.

Interessanterweise führte die Supplementierung von Gln zu keinen Unterschieden bei den meisten der untersuchten Parameter im Jejunum. Glutamin hatte keinen Einfluss auf die Morphologie des Jejunums, die Entwicklung der Immunzellen und die Aminosäureprofile im Gewebe und im Verdauungstrakt bei LBW Ferkeln im Alter von 5 und 12 Tagen. Dies war auch für die mRNA-Expression von Genen, die am Aminosäuretransport, dem Stoffwechsel und der Glutathion Synthese beteiligt sind zu beobachten. Nur die Gln-Konzentration im Gewebe des Jejunums war bei NBW-Ferkeln mit Gln-Supplementierung im Vergleich zu Ala im Alter von 5 Tagen höher. Es wurden keine Einflüsse des Geburtsgewichts auf die untersuchten Parameter nachgewiesen. Das Alter schien jedoch die größte Auswirkung zu haben, da sich die morphologische Entwicklung, die Immunzellen, das Verhältnis von jejunaler RNA zu DNA und die Konzentration von DNA, Protein und RNA sowie die Aminosäureprofile bei Saugferkeln im Alter von 5 Tagen im Vergleich zu 12 Tagen veränderten.

Im Dickdarm beobachteten wir ähnliche Auswirkungen. Die Konzentrationen biogener Amine waren niedriger und die Zahl der $CD3^+$ intraepithelialen Lymphozyten (IEL) bei Ferkeln nach Gabe von Gln im Vergleich zu Ferkeln, die Ala erhielten, höher. Ein niedriges Geburtsgewicht der Ferkel hatte geringfügige Auswirkungen auf die mikrobielle Zusammensetzung und führte zu einer geringeren Konzentration einiger biogener Amine und geringerer Anzahl an IEL. Im Gegensatz zur Gln-Supplementierung und zum Geburtsgewicht hatte der Faktor Alter einen größeren Einfluss auf die histologischen und mikrobiellen Merkmale bei männlichen Saugferkeln.

Zusammenfassend lässt sich sagen, dass die orale Supplementation von Gln und das Geburtsgewicht der Ferkel nur geringe Auswirkungen auf die beobachteten Parameter im distalen Dünndarm und im Dickdarm der Saugferkel hatten, während das Alter einen großen Einfluss spielte. Dies deutet darauf hin, dass die Entwicklung des Darms hauptsächlich durch ontogenetische Faktoren während der Säugezeit der Ferkel bestimmt wird.

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10 List of publications

10.1 Published and accepted papers (peer reviewed)

Schregel, J.; Schulze Holthausen, J.; Sciascia, Q. L.; Li, Z.; Görs, S.; Eggert, A.; Tuchscherer, A.; Zentek, J.; Metges, C. C. Effects of oral glutamine supplementation on jejunal morphology, development, and amino acid profiles in male low birth weight suckling piglets. PLOS ONE. **2022**, 17(4),
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Schulze Holthausen, J.; Schregel, J.; Sciascia, Q. L.; Li, Z.; Vahjen, W.; Metges, C. C.; Zentek, J.; Effects of Oral Glutamine Supplementation, Birthweight and Age on Colonic Morphology and Microbiome Development in Male Suckling Piglets. Microorganisms **2022**, 10(10), 1899
doi:10.3390/microorganisms10101899

10.2 Congress contributions

10.2.1 Oral presentations

Schulze Holthausen, J.; Sciascia, Q. L.; Schregel, J.; Andreotti, S.; Vahjen, W.; Metges, C.C.; Zentek, J. (2023):
Influence of oral glutamine supplementation on the abundance of gene expression and metabolic pathways in digesta of jejunum in neonatal piglets. 77.Tagung der Gesellschaft für Ernährungsphysiologie Göttingen – 07.03.-09.03.2023. In: Society of Nutrition Physiology - Gesellschaft für Ernährungsphysiologie; (Hrsg.) DLG-Verlag; 32, ISBN: 978-3-7690-4116-3

Saliu, E. M.; **Schulze Holthausen, J**.; Zentek, J. **(2022)**:
The impact of chemical and physical characteristics of two fibre sources on performance and nutrient digestibility in growing pigs. 7th EAAP International Symposium on Energy and Protein Metabolism and Nutrition (ISEP) Granada – 12.09.-15.09.2022. In: Animal; **13**(3), 309.

Schulze Holthausen, J.; Sciascia, Q. L.; Schregel, J.; Li, Z.; Andreotti, S.; Vahjen, W.; Metges, C. C.; Zentek, J. **(2022)**. Der Einfluss von Glutaminergänzung, Geburtsgewicht und Alter auf die Mikrobiota mit geringer Abundanz im Magen und Jejunum von Saugferkeln. DVG Kongress Berlin – 13.10 – 15.10.2022

Schulze Holthausen, J.; Sciascia, Q.; Schregel, J.; Metges, C.C; Zentek, J. **(2022)**: Influence of oral glutamine supplementation on bacterial metabolites in stomach and jejunum in neonatal piglets. 76. digitale Tagung der Gesellschaft für Ernährungsphysiologie – 08.03.-10.03.2022. In: Society of Nutrition Physiology - Gesellschaft für Ernährungsphysiologie; (Hrsg.) DLG-Verlag; 31 ,104. ISBN: 978-3-7690-4115-6

Schulze Holthausen, J.; Sciascia, Q. L.; Schregel, J.; Li, Z.; Andreotti, S.; Vahjen, W.; Metges, C. C.; Zentek, J. **(2022)**. The effect of glutamine supplementation, birth weight and age on the dominant microbiota in the jejunum of suckling piglets. 26th ESVCN Congress Basel – 06.09.-08.09.2022. In: Proceedings of the European Society of Veterinary and Comparative Nutrition, 167. ISBN: 978-3-033-09422-2

Schulze Holthausen, J.; Sciascia, Q. L.; Schregel, J.; Metges, C.C.; Zentek, J. **(2021)**. Infuence of oral glutamine supplementation on piglet jejunal immunological parameters at the end of the suckling period. 25th ESVCN Congress - 09.09.-11.09.2021 digital, In: Proceedings of the European Society of Veterinary and Comparative Nutrition, 126. ISBN: 978-9-09-035058-5

Schulze Holthausen, J.; Sciascia, Q. L.; Schregel, J.; Li, Z.; Metges, C.C.; Zentek, J. **(2019)**: Effect of glutamine supplementation on intestinal development in preweaned low birthweight piglets. Berlin – 27.09.2019. In: 12. Doktorandensymposium & DRS Präsentationsseminar "Biomedical Sciences" Berlin: Mensch & Buch, 30. ISBN:

10.2.2 Poster presentations

Saliu, E.; **Schulze Holthausen, J.**; Zentek, J. **(2022)**. Chemical and physical characteristics of two fibre sources influence performance and nutrient digestibility in growing pigs. 26th ESVCN Congress Basel – 06.09.-08.09.2022. In: Proceedings of the European Society of Veterinary and Comparative Nutrition, 150. ISBN: 978-3-033-09422-2

Saliu, E.; **Schulze Holthausen, J.**; Zentek, J. **(2022)**. Sows' preference for fibre-rich feedstuffs and the impact of fibre particle size on feed intake duration, faecal consistency and short-chain fatty acids in faeces from sows. 26th ESVCN Congress Basel – 06.09.-08.09.2022. In: Proceedings of the European Society of Veterinary and Comparative Nutrition, 151. ISBN: 978-3-033-09422-2

Saliu, E.-M.; **Schulze Holthausen, J.**; Zentek, J. **(2022)**. Differences in feed intake duration, faecal consistency and short chain fatty acids in faeces from sows fed dried hemp plants (Cannabis spp.) either intact or grinded. 76. digitale Tagung der Gesellschaft für Ernährungsphysiologie – 08.03.-10.03.2022. In: Proceedings of the Society of Nutrition Physiology - Gesellschaft für Ernährungsphysiologie (Hrsg.) DLG-Verlag; 32 (2022), S. 143 ISBN: 978-3-7690-4115-6

Saliu, E.-M.; **Schulze Holthausen, J.**; Zentek, J. **(2022)**. Preference for fibre rich feedstuffs in a cafeteria trial with sows. 76. digitale Tagung der Gesellschaft für Ernährungsphysiologie – 08.03.-10.03.2022. In: Proceedings of the Society of Nutrition Physiology - Gesellschaft für Ernährungsphysiologie (Hrsg.) DLG-Verlag; 32 (2022), S. 142 ISBN: 978-3-7690-4115-6

Saliu, E.-M.; **Schulze Holthausen, J.**; Zentek, J. **(2022)**. Differences in faecal parameters from sows fed dried hemp plants or apple pomace intact or ground. 15th International Symposium on Digestive Physiology of Pigs, Rotterdam – 17.05.-20.05.2022. In: Animal; 13(2), S. 186–187

Saliu, E.; **Schulze Holthausen, J.**; Vahjen, W.; Zentek, J. **(2022).** The impact of feeding sows diets enriched with dried hemp plants or apple pomace either coarse or grinded on faecal parameters. Zero Zinc Summig 2022, Copenhagen – 22.06.-23.06.2022.

Schulze Holthausen, J.; Sciascia, Q. L.; Schregel, J.; Metges, C. C.; Zentek, J. **(2022)**, Bacterial metabolites in stomach and jejunum of neonatal piglets and the influence of oral glutamine supplementation on them. 15th International Symposium on Digestive Physiology of Pigs, Rotterdam – 17.05.-20.05.2022. In: Animal; 13(2), S. 197

Schulze Holthausen, J.; Sciascia, Q. L.; Schregel, J.; Li, Z.; Andreotti, S.; Vahjen, W.; Metges, C. C.; Zentek, J. **(2022)**, The influence of glutamine supplementation on microbial composition in the stomach and jejunum digesta of suckling piglets. 7th EAAP International Symposium on Energy and Protein Metabolism and Nutrition (ISEP), Granada – 12.09. 15.09.2022. In: Animal; 13(3), S. 399

Schulze Holthausen, J.; Sciascia, Q. L.; Schregel, J.; Li, Z.; Metges, C. C.; Zentek, J. **(2021).** Histomorphological development of the jejunum in low and normal birth weight piglets orally supplemented with glutamine during the suckling period. 75. digitale Tagung der Gesellschaft für Ernährungsphysiologie Berlin – 16.03.-18.03.2021. In: Proceedings of the

Society of Nutrition Physiology - Gesellschaft für Ernährungsphysiologie (Hrsg.) DLG-Verlag; 30 (2021), S. 63 ISBN: 978-3-7690-4114-9

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13 Conflict of Interest

The author declares no conflict of interest.

14 Selbstständigkeitserklärung

Hiermit erkläre ich Eides statt, die vorliegende Arbeit selbständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet zu haben. Die Arbeit ist in dieser Form noch keiner anderen Prüfungsbehörde vorgelegt worden.

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