The differential development of low and normal birth weight piglets? Effects of artificial rearing.

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I am fond of pigs.
Dogs look up to us.
Cats look down on us.
Pigs treat us as equals.

Winston Churchill

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LIST OF ABBREVIATIONS

ACN	acetonitrile
ADG	average daily gain
AEC	aminoethylcarbazol
AMP	adenosinemonophosphate
ApN	aminopeptidase N
BSA	bovine serum albumin
BTC	betacellulin
CGRP	calcitonin gene related peptide
CMP	cytidinemonophosphate
CWF	colostral whey fraction
D	day
DE	digestible energy
DNA	deoxyribonucleic acid
DTT	dithiothreitol
DPPIV	dipeptidylpeptidase IV
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
FA	formic acid
FFA	free fatty acids
FGF	fibroblast growth factor
GALT	gut-associated lymphoid tissue
GLP	glucagon-like peptide
GMP	guanosinemonophosphate
GnRH	gonadotropin-releasing hormone
GSH	glutathione
GSHPx	glutathione peroxidase
GSSG	oxidised glutathione
h^2	heritability

HB	heparin-binding
HE	hematoxylin-eosin
HPLC	high-performance liquid chromatography
IFN-γ	interferon-γ
IGF	insulin-like growth factor
IGF-1R	insulin-like growth factor-1 receptor
IGFBP	insulin-like growth factor-binding proteins
IL	interleukin
IMP	inosinemonophosphate
IP	intraperitoneal
IUGR	intrauterine growth restriction
LBW	low birth weight
LH	luteinizing hormone
LPS	lipopolysaccharide
MALDI	matrix assisted laser desorption ionization
MDGF	mammary derived growth factor
ME	metabolizable energy
MG	monoglycerides
MS	mass spectrometry
mRNA	messenger ribonucleicacid
MUFA	monounsaturated fatty acids
NBW	normal birth weight
NO	nitric oxide
NPN	non-protein nitrogen
OD	optical density
OS	oligosaccharides
PBS	phosphate-buffered saline
PDGF	platelet-derived growth factor

PUFA	polyunsaturated fatty acids
qRT-PCR	quantitative reverse transcription-polymerase chain reaction
RNA	ribonucleic acid
ROS	reactive oxygen species
S/N	signal to noise
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SGA	small for gestational age
SGLT1	sodium-dependent glucose cotransporter 1
SOD	superoxide dismutase
SP	substance P
TBS	tris(hydroxymethyl)aminomethane-buffered saline
TGF-α	transforming growth factor-α
TGF-β	transforming growth factor-β
TNF-α	tumor necrosis factor-α
TOF	time-of-flight
UMP	uridinemonophosphate
VIP	vasoactive intestinal peptide
W	week

GENERAL BACKGROUND AND STUDY OBJECTIVES

GENERAL BACKGROUND

During the last decades, the number of live-born piglets per litter increased tremendously through genetic selection and management techniques (1-3). Animal breeders believe that litter sizes will continue to increase in the near future. However, increasing litter size may be detrimental to piglet survival, because as litter size increased, so has piglet mortality (1). Levels of pre-weaning live-born mortality in European pig production are approximately 14% (1-4). The main reasons for the relatively (given the improved husbandry and managerial aspects) high pre-weaning mortality in modern pork production are the sows limiting lactating capacities and increased variability in birth weight. These are relevant modern traits in pig production that negatively affect the total number of weaned piglets per year.

In one French study (2008), 24.2% of all litters had over 14 piglets per litter and 36.7% of the litters had over 16 piglets per litter (5). This implies that the number of live born piglets often exceeds the normal teat number of 12 to 14 in Western pig breeds (1). The term supernumerary is referred to those piglets in excess of the number of teats (6). Moreover, milk yield in modern sows is insufficient to achieve the maximum growth potential of these larger litters, especially in the second half of the suckling period (7, 8).

Increased litter sizes results in a high variability in piglets' birth weight and consequently a higher proportion of low birth weight (LBW) piglets. The percentage of LBW piglets (<1 kg at birth) increases from 7% in case of 11 piglets per litter towards 23% in case of >15 piglets per litter (2).

The presence of supernumerary and LBW piglets can lead to increased aggression within a litter, reduced milk intake and growth of in particular the LBW piglets (9). These negative effects are a major cause of economic losses in pig production and have a negative impact on animal health and welfare

(1). As a result pig breeders need to implement management practices in order to raise these supernumerary and LBW piglets. According to a recent survey, cross-fostering and supplementary feeding is applied in almost every pig farm in Flanders (10). Additionally, 55% of the pig farmers performed euthanasia of disadvantaged piglets, 44% used foster sows and 19% performed split nursing. Moreover, 31% of the pig farmers apply split-weaning techniques (the practice whereby part of the litter is removed from the sow before weaning the rest of the litter). Split-weaned piglets are transferred to either nursery rooms/brooders or a growing-finishing facility. An understanding of the interventions that are practiced and their impact on growth and development of piglets is missing but nevertheless necessary for a scientifically based rearing strategy of supernumerary or LBW piglets. Therefore, the research presented in this thesis focuses on the effects of feeding artificially reared piglets on formulated milk and its impact on intestinal development, intermediary metabolism and performance.

OBJECTIVES AND OUTLINE OF THE THESIS

In the studies presented in this thesis, the differential development of LBW piglets compared to normal birth weight (NBW) littermates is investigated, in order to accommodate their nutritional needs (Fig.1). The **specific objectives** are:

1. To evaluate whether body composition and muscle lipid and glycogen contents are affected by age and birth weight.
2. To evaluate whether different rearing conditions influence piglets' growth, structural and functional gut maturation and the insulin-like growth factor-1 (IGF-1) system.
3. To evaluate whether supplementing a bovine colostrum fraction affects the small intestinal morphology and barrier function.

A literature review is presented in **chapter 1**, which consists of three major parts. In the first part of the literature review, interventions to prevent and rear LBW piglets are described because an understanding of these interventions is necessary for a scientifically based rearing strategy. One of the described interventions is artificially rearing piglets on milk replacer, which will be investigated in chapter 3. In order to facilitate the optimization of milk replacers used in practice, a species comparison between bovine and porcine milk composition is made in the second part of the literature review. The last part of chapter 1 covers the small intestinal histology, digestive and absorptive function because the latter are crucial elements in driving overall postnatal development, growth and health.

In order to adequately raise LBW piglets, either via artificially rearing systems or by naturally suckling, elucidation of the biological consequences of growth retardation is valuable. Therefore, the impact of birth weight on body composition and muscle energy stores in piglets was defined and presented in **chapter 2**.

In this respect, the influence of artificial rearing of LBW and NBW piglets was described in **chapter 3**. The experiment described in **chapter 3A**, assessed the effects of artificial rearing on small intestinal morphology and digestion capacity. **Chapter 3B** describes the IGF-1 serum levels and IGF-1 receptor expression in formula-fed piglets, because IGF-1 is one of the main regulators of prenatal and postnatal growth.

Additionally, **Chapter 4** describes the effects of supplementing formula-fed piglets with a bovine colostrum fraction on the small intestinal morphology and barrier function.

Finally, the obtained results are discussed in **Chapter 5** and new guidelines for rearing LBW piglets are proposed in conjunction with future research perspectives.

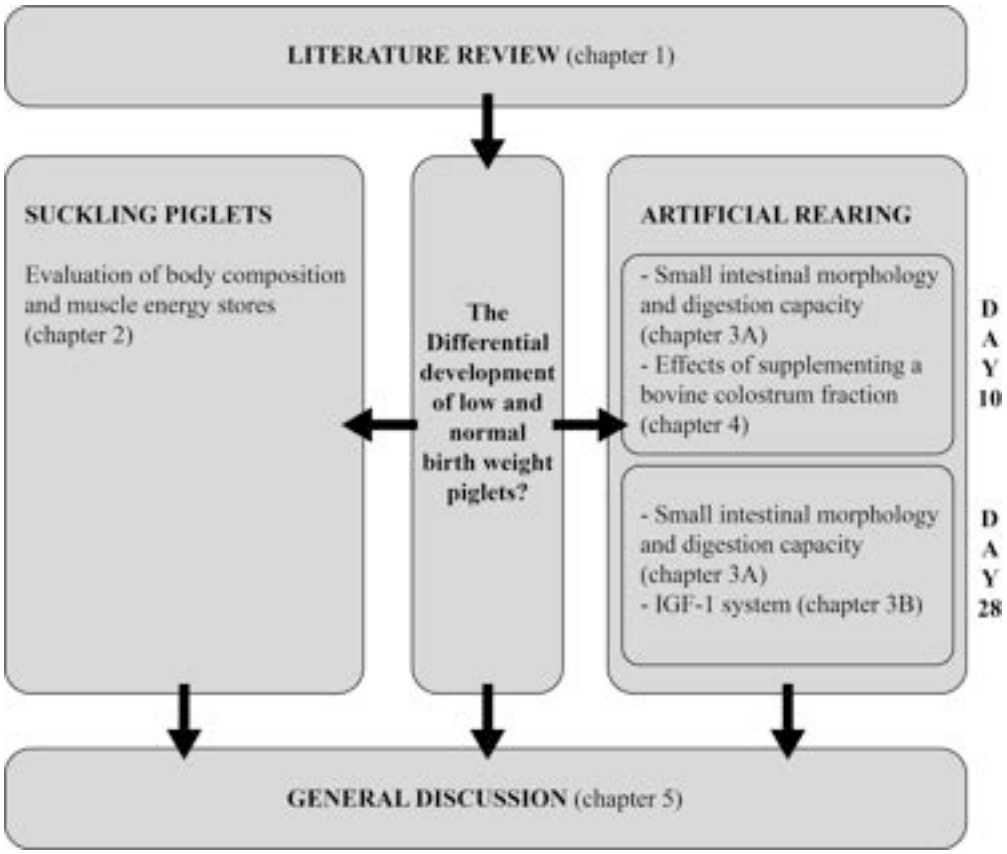

Fig 1. Outline of the thesis.

CHAPTER 1
LITERATURE REVIEW

The following chapter contains material, partly adapted, from:

M. De Vos, L. Che, V. Huygelen, S. Willemen, J. Michiels, S. Van Cruchten, C. Van Ginneken. Nutritional interventions to prevent and rear low birth weight piglets, Journal of Animal Physiology and Nutrition (2013), DOI: 10.1111/jpn.12133

M. De Vos, E. Weyn, C. Van Ginneken. Presence of VIP, CGRP, SP neuropeptides and PAF-AH enzyme in porcine colostrum. Livestock Science 134 (2010), 225-227

1. THE PRESENCE OF LOW BIRTH WEIGHT PIGLETS IN PIG PRODUCTION: POSSIBLE SOLUTIONS

1.1. Introduction

Generally, a newborn whose birth weight lies below the 10th percentile for its age is classified as small for gestational age (SGA) (11). Within the group of SGA newborns, a number of foetuses have not met their genetically determined potential size due to a process of intrauterine growth restriction (IUGR) (12, 13). Thus, the latter group excludes foetuses and newborns that are constitutionally small for their age. Additionally, the term low birth weight (LBW) is used for newborns having a birth weight below the 10th percentile of the mean birth weight of the litter (14). This term is also used for those having a birth weight less than the mean birth weight minus up to 2 times the standard deviation but also for those weighing less than 1 kg (piglets) or less than 2.5 kg (humans) at birth (15). In comparison with other livestock animals, pigs exhibit the highest number of naturally occurring LBW (15), which is probably associated with IUGR (Fig. 1.1). IUGR piglets can be identified as early as d 30 of gestation and IUGR is mainly caused by placental insufficiency (16). The prevalence of IUGR piglets increases in highly prolific sows, i.e. high ovulation rates, due to uterine crowding (17). Before d 35 of gestation, porcine embryos are uniformly distributed over the uterine horns (18). After d 35 of gestation, however, uterine capacity becomes the major limiting factor for foetal survival and growth and uterine crowding will reduce foetal size/weight (17). The high prevalence of LBW piglets may affect the profitability in pig production since these piglets exhibit high neonatal mortality rates and poor growth performances (19-21).

Figure 1.1 LBW piglet on the left hand side and a normal littermate at 3 days of age.

In addition, these LBW piglets have been associated with lower carcass and meat quality at slaughter age, though conflicting results exist (22-24). In this chapter an overview is given of the interventions that can be undertaken to influence piglets' birth weight and litter homogeneity (sow level) and to increase piglets' neonatal survival and growth (piglet level).

1.2. Maternal strategies to prevent the presence of LBW piglets

Due to genetic selection for hyper-prolificacy, the number of piglets per litter has increased over the last decades (2). Additionally, this selection increased within-litter birth weight variation, which is a major constraint for postnatal survival (20, 25, 26). Within-litter birth weight variation increased from 15% to 24% when litter size varied from less then 10 piglets to more than 15 piglets (26). The percentage of piglets below 1 kg at birth increased from 7% in case of 11 piglets per litter towards 23% in case of > 15 piglets per litter (2) (Fig. 1.2). Thus, the economic benefit of larger litters is partially outweighed by the fact that LBW piglets suffer from increased mortality and morbidity (27). Even if they survive, they show impaired growth performances (9, 20, 28). Besides less profit for the farmer, these increased

mortality and morbidity rates have important welfare and ethical consequences (21).

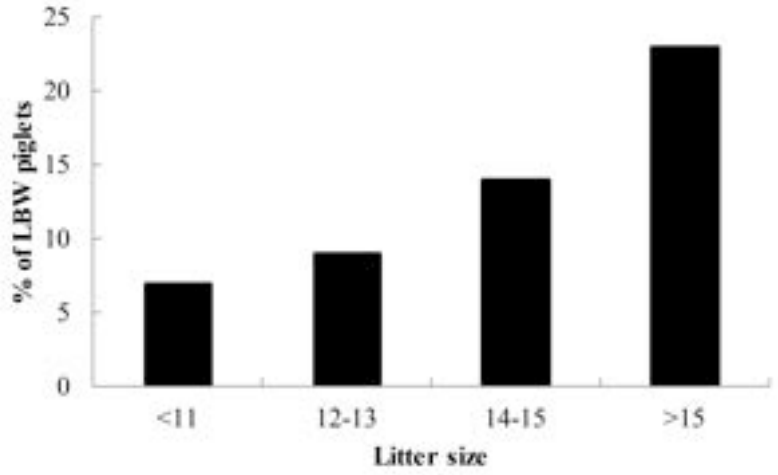

Figure 1.2 Effect of litter size on the % of LBW piglets (<1kg at birth) (2).

Several possible approaches, including genetic, nutritional and management strategies, have been proposed in order to prevent and rear LBW piglets. These are described below.

1.2.1. Genetic selection strategies to support good growth

Genetic selection strategies are of high importance because they can result, in the long term, in permanent changes in phenotypic characteristics. Different breeding strategies have been proposed in order to obtain a positive impact on both litter size and piglet survival, i.e. selection for increased gestation lengths (heritability (h^2) = 0.3) (29), selection for higher birth weights (h^2 = 0.04 - 0.36) (30-33), selection for more homogeneous litters (h^2 = 0.08) (34) and selection for good maternal behaviour (h^2 = 0.06) (35). All of them have advantages and shortcomings. Because of the positive correlation between

gestation length and birth weight (29), the small piglets would probably benefit from 2 extra days in the uterus. However, selection for increased gestation lengths might result in more stillborn piglets (29).

Phenotypically, individual birth weight is indeed closely related to piglet survival. In contrast, there are little genetic relations, which does not make it a recommendable selection strategy. Furthermore, higher birth weights might result in sow-related problems during gestation and parturition. Moreover, Meishan piglets, although very small at birth (i.e. 56% smaller than Dutch breeds), have an exceptional high viability (36, 37). This suggests that there is a role for genetic factors in piglet viability, which is not related to birth weight. The low pre-weaning mortality rates in Meishan piglets have previously been attributed to a more vascularized placenta (38, 39), higher teat number (40), high milk fat content (41), high activity of the hypothalamic-pituitary-adrenal axis (42) and differences in hepatic fatty acids (43).

The high mortality rates that are related to the within-litter-variation stimulated researchers to test whether maternal genetic variance exist for this trait. Damgaard et al. demonstrated that the positive relation between pre-weaning mortality and within-litter variation is partly genetic. However, the potential for genetic improvement by selective breeding seems limited (34).

Another selection strategy is to select for good maternal behaviour (44). As an example, Baxter et al. showed that genetic selection for high piglet survival dam lines, resulted in gilts that displayed less crushing behaviour (45). In addition, it seems possible to select for more functional teats (46). However, according to Nielsen et al. selection for more teats will not affect nursing capacity. The latter study described recently that the number of nursed piglets is heritable with an heritability around 0.07 (47).

Over the last decades, selection for piglet vitality (i.e. piglet's quality to stay alive through birth, suckling and weaning) has become increasingly important. Leenhouwers et al. indeed showed that piglet viability is heritable and positively correlated with circulating cortisol levels (48).
Professional breeding strategies will contribute to a solution to the problem related to large litters, but progress takes years before research is translated to results at the level of the farm. This implies that the short-term solution lies within nutritional and management interventions.

1.2.2. Maternal nutrient provision influences piglets' birth weight and performance

The feed requirements of gestating sows are generally subdivided into early (d 1 - 28), middle (d 29 - 84) and late (d 85 - 115) gestation. In the past, it was generally accepted that high levels of feed intake following mating results in increased embryo mortality due to a decrease in plasma progesterone levels (49). However, data in literature are conflicting (50-52) resulting in debate on the ideal feed intake levels during early gestation. Gilts are generally fed 2.0 kg/d, whereas sows 2.2 to 2.8 kg/d (dependent on their body condition and diet density) (53, 54). During mid gestation, feed intake should be increased by 0.15 to 0.20 kg/d in order to meet the energy requirements for maintenance and maternal body weight gain. During late gestation, where the focus is on foetal growth and mammary gland development, feed intake is generally increased by 0.3 to 0.5 kg/d.

Several researchers evaluated the effect of feeding gestating gilts and sows an increased amount of feed (55-58). In a study of Cromwell et al. individual piglet weights were increased (1.48 vs. 1.44 kg) after feeding 1.36 kg extra

feed/d from d 90 to farrowing (55). This effect was observed in both gilts and sows, which is in contrast to a more recent study in which 0.91 kg extra feed/ d was offered during the same period (56). In the latter, individual piglets' birth weight was increased in gilts (1.51 vs.1.42 kg) but decreased in sows (1.43 kg vs.1.54 kg). In addition, other studies did not find any effect of different feeding levels during gestation on piglets' birth weights. In these studies, different quantities of feed were tested during different periods of gestation (57-59). According to Martineau and Badouard the hyper-prolific sow is characterized by the lack of early embryonic death with overfeeding and a positive effect of overfeeding during the last weeks of gestation (2).

Several authors investigated the effect of low energy intake. Piglets birth weights were reduced when sows were fed 2.2 Mcal DE/d instead of 8.0 Mcal DE/d throughout gestation (60). In contrast, the majority of authors reported no effect of low energy supply on piglets' birth weight indicating that dams are able to mobilize maternal nutrients to support foetal development. Bee investigated different energy intake levels (1.4 and 2.4 Mcal DE/kg) during early gestation, which had no effect on average birth weight (61). Similar results were obtained by Lawlor et al. who tested different dietary digestible energy levels (7 to 14 Mcal DE/d) during different gestation phases (62).

According to some studies, supplementing sows with additional energy (5% to 10% extra fat) did not affect the offspring's birth weight (63, 64). However, the type and timing of fat supplementation during gestation may affect birth weights and litter performance.

Particularly medium-chain fatty acids incorporated in the sows' diet during late gestation (100 g/kg) have shown to improve the survival of LBW piglets during the postnatal period (65, 66), which could be related to a prenatal

effect (64). Long-chain polyunsaturated fatty acids (PUFA) including n-3 and n-6 PUFA are important in the development of the brain and other physiological functions. Researchers have incorporated different sources of these fatty acids (e.g. fish oil, flax seed oil) into the sow's diet and investigated their transfer and effects on the progeny. Supplementing sows' with fish oil (16.5 to 100 g/kg) has led to lighter piglets at birth (14, 67, 68). However, it reduced pre-weaning mortality rates and increased postnatal piglet growth, mainly due to a lower number of crushed piglets and an increased suckling behaviour (67, 68). Feeding flax seed oil (35 to 100 g/kg) to sows in late-gestation had no effect on piglets' birth weights (69). Laws et al. demonstrated that monounsaturated fatty acids (MUFA) (18:1 n-9) supplementation during the first half of gestation reduced the incidence of LBW piglets likely due to enhanced placental growth (70). Again, type and timing of fat supplementation appeared crucial in understanding the effects on the growing foetuses. Nevertheless, the main effect of feeding additional lipids during gestation is increasing sow's milk yield and quality, resulting in improved LBW piglet's survival rates (67, 71-73).

More important for foetal growth than energy source is the availability of proteins during gestation. Several protein-free (74) or low protein diets (ranging from 0.5% to 8.5%) (75-79) were given to gestating dams resulting in reduced piglets birth weights and impaired growth rates. Additionally, unphysiologically high dietary protein concentrations (30% vs. 12%) during gestation lowered piglet birth weight as well (77, 80). It is well known that for monogastric animals the functional value of dietary proteins is mainly dependent on its amino acid composition. During the course of gestation, the total amount of required amino acids changes as well as the ratio between the amino acids. It has previously been described that foetal growth occurs mainly during late gestation. Foetal protein gain is 0.25 g/d before d 69 of

gestation and increases to 4.63 g/d after d 69 of gestation (81). The recommended dietary amino acids ratios in gestation diets were constant in the previous *Nutrient Requirements of Swine* (82). However, the requirements in late gestation are higher than in early gestation (83). Therefore, the changing requirements have been addressed in a model provided in the 2012 edition (84). In addition, the ratio between the important amino acids changes throughout gestation. Sows in early gestation (d 0 - d 60) require high amounts of threonine, whereas sows in late gestation (d 60 - d 114) require high amounts of arginine and leucine for foetal and mammary growth (85). Feeding pregnant sows a diet with amino acid profile adjusted by stages of gestation resulted in increased litter uniformity with 4% compared to feeding a control diet (85).

Besides an ideally balanced amino acid diet, functional amino acids can be added into the sow's diet in order to stimulate foetal growth and development. Arginine and its precursor glutamine are key amino acids in controlling foetal growth (86). Several researchers demonstrated that dietary supplementation with L-arginine positively affected the progeny (87-89). Mateo et al. showed that 1% L-arginine supplemented to corn-soybean based diets from d 30 until parturition increased the number of live-born piglets with 23% and the total litter weight with 28% (87). Wu et al. showed that 0.83% dietary supplementation from d 14 to d 28 or from d 30 until parturition increased litter birth weights and number of live-born piglets (88). Ramaekers et al. found that 25 g/d of arginine from d 14 to d 28 increased litter size with 0.8 extra piglet per litter (89). Supplemental arginine can indeed enhance placental angiogenesis through the arginine-NO pathway. In that context, Hazeleger et al. showed that vascularization of the placenta was greater in gilts offered 40 g/d additional arginine from d 16 to d 28 of gestation (90).

Wu et al. supplemented 1% L-glutamine to the diet of gilts between 90 and 114 days of gestation. This increased the average birth weight, thereby reducing the number of IUGR piglets by 39% (86). L-glutamine serves as a precursor for arginine and is involved in multiple metabolic pathways including serving as an energy substrate for dividing cells. Little is known on the effects of L-glutamine on placental growth and development.

Besides amino acids and lipids, other substances involved in reproductive performance were added into the gestational diet. Since L-carnitine affects key enzymes involved in protein and lipid metabolism, researchers incorporated different amounts into the sow's diet, which resulted in piglets with increased birth weights (91-93). Musser et al. supplemented sows with 100 mg/d L-carnitine from d 5 to d 112 of gestation, which resulted in 2.7% heavier piglets (94). Eder et al. supplemented 125 mg/d during the entire gestation period, which resulted in piglets with 7% higher birth weights than control groups (92). Ramanau et al. supplemented sows with 25 and 50 mg/kg diet in tablet form until day 21 of gestation and from day 111 to 114 of gestation and incorporated L-carnitine in the feed from d 21 to d 111, so that sows were fed over the entire gestation period with L-carnitine (93). This resulted in 5.7% and 7.9% heavier piglets, respectively. Thus, one can conclude that it is best to supplement L-carnitine during the entire gestation period in order to achieve balanced litter weights. The increased birth weights observed after L-carnitine supplementation may be caused by elevated plasma IGF-1 levels during gestation. This in turn may enhance placenta development and intrauterine foetal nutrition (95). In addition, piglets of sows supplemented with L-carnitine, gain more weight during the suckling period due to longer suckling bouts (96). The increased suckling behavior is probably caused by a prenatal effect because L-carnitine supplementation to sows stimulates foetal muscle fibre development (94).

1.2.3. Management strategies

Optimizing maternal management to reduce stress during gestation may help reduce problems that are associated with large litter sizes. Although different studies observed that maternal stress resulted in lower birth weights (97, 98), in other studies birth weight remained unchanged (99, 100) or even increased (101). In these studies different types, timings and severities of maternal stress were tested. The conflicting results suggest a complex relationship between stress and birth weight.

Ambient temperatures in the farrowing houses as used in most Western European countries should ideally be set between 18 and 23°C. Higher ambient temperatures result in a lower feed intake and decreased litter birth weight (102). Additionally, sow's milk production is lowered, resulting in reduced postnatal piglet weight gain (103). From 1 January 2013 onwards, group-housing of pregnant sows in the European Union is mandatory. Sows should be kept in groups starting from 4 weeks after mating, until 1 week before the expected farrowing date (104). These interventions should stimulate social behaviour, but unfortunately studies indicate more aggressive behaviour resulting in more leg and reproductive problems (105-107). In addition, transferring pregnant sows to the farrowing crate may lead to increased stress at parturition. Whether group-housing will have impact on piglets' birth weights remains unclear and needs investigation. On the one hand, group housing may negatively affect piglet birth weight due to an expected increased stress level (97, 98). On the other hand, the physical exercise that is observed under group-housing conditions may help to improve the delivery of oxygen and substrates to the placenta and could thereby be beneficial for foetal growth (108).

1.3. Strategies to enhance survival and growth of LBW piglets during the nursing period

1.3.1. Feeding strategies

Colostrum is rich in nutrients and energy, providing the necessary elements for metabolism and heat production. In addition, colostrum contains immunoglobulins supplying piglets with passive immunity. Adequate colostrum ingestion (>200 g per piglet during the first 24 h after birth) is crucial for neonatal survival and especially for LBW piglets (109). Moreover, the lack of brown adipose tissue (110) renders colostrum the main energy source during the perinatal period.

LBW piglets are often less vital, making them less able to move towards the udder and compete (for both milk and space) with heavier littermates (21, 111, 112). Therefore, these LBW piglets are less efficient in gaining access to and ingesting sufficient amounts of colostrum and milk (113). Moreover, colostrum and milk intake is limited by the sows' productive capacity, resulting in an increased risk of insufficient milk intake in case of large litter sizes.

Studies investigating the influence of sow nutrition on colostrum yield are scarce. Decaluwé et al. reported that colostrum yield is not correlated to feed intake between d 111 of gestation and parturition or during the first 3 days of lactation (114). However, in a more recent study, they observed that sows receiving 4.5 kg/d instead of 1.5 kg/d (control group) between d 108 of gestation and d 3 of lactation, tended to have a greater total colostrum yield (4.0 kg vs. 3.5 kg) (115). Colostrum production was increased with 20% by feeding fermented potato protein during the last week of gestation (116).

Feeding the sow pectin residues instead of potato protein results in higher colostrum yield (117). Glucose is the main precursor for lactose synthesis in the mammary gland. Because lactose is the main osmotic determinant of milk (see 2.5.1), glucose uptake drives milk yield.

Modern sows give birth to large litters of piglets with high growth potential. Since milk production of these sows is not sufficient to accommodate the piglets' needs (7), nutritional interventions are recommended in particular for the survival and postnatal growth of LBW piglets. In Flanders, 85% of all pig farmers supplement their piglets during the suckling phase (10). Numerous commercial booster preparations or colostrum substitutes for neonates are available and widely used. These supplements are given shortly after birth, either to all live-born piglets (16%) or to the weakest piglets within the litter (18%) (10). They may contain supplemental energy (fat, lactose), immunoglobulins (mainly bovine derived), growth factors, etc. However, the efficacy of these supplements remains equivocal because detailed and peer-reviewed studies are scarce. Several authors supplemented neonatal piglets with medium-chain triglycerides without any positive effect on their survival (118-120). However, Casellas et al. demonstrated that oral supplementation of small piglets (< 1250 g) (1.95 g each 24h during the first 3 days of life) with an emulsion of medium and-long-chain triglycerides reduced their death hazard by 1.9 fold in comparison to unsupplemented littermates (121). Heo et al. demonstrated the favourable and accelerated oxidation of medium-chain compared to long-chain fatty acids in pig neonates thereby improving their energy intake (122).

Additionally milk-based creep diets and liquid milk replacers can be offered to the suckling piglet. However, consumption of creep diets before weaning seems to be highly variable (ranging from 24 to 690 g per piglet from d 11 to weaning at d 28) (66, 123, 124). The main purpose of supplementing creep

feed diets, besides the provision of extra nutrients, is to adapt the gastrointestinal tract to solid feed and plant-based ingredients via inducing the development of e.g. enzymes necessary to digest the post-weaning diets.
In Flanders, 54% of the farmers supplement their piglets during the suckling period with a milk replacer (10). In view of the increasing number of LBW piglets, researchers and feed companies are making efforts to optimize the formulation of milk replacers. The commercially available milk replacers generally use bovine milk products such as whey, lactose and skim milk, as main ingredients. A typical milk replacer constitutes of 25% crude protein, 40 to 50 % lactose and 10 to 12 % lipids (on dry matter basis), with mixing rates that range between 125 and 250 g/l (125). Supplementing suckling piglets with liquid milk replacers provides the piglets with a boost, which leads to an increased growth rate and heavier mean weaning weights (126-129). However, this additional milk supply is not effective in increasing LBW piglets' survival rates or in reducing the final weight variation between piglets at slaughter age (126, 127). Supplemental milk does not increase piglet LBW survival because to majority of deaths occur in the first 3 d of life. During this period, the voluntary milk intake is minimal (130) and orally drenching LBW piglets with the supplemental milk seems necessary (131). In addition, several companies have designed automatic milk suppliers (Fig. 1.3). The main concept of such a milk supplier is a milk tank, a pipeline system with pump, and milk cups with piglet-activated nipples. These can be placed in the farrowing pen, in a pre-weaning compartment or in a separate nursing device. Because there is little or no scientific evidence that these systems are worth the investment, they are currently not commonly used in practice (10). As an example, in Flanders, only 3% of the pig farmers use automatic milk suppliers.

Figure 1.3 A commercial brooder with automatic milk supplier.

However, the value of these systems will become increasingly important because litter sizes, and thereby the number of supernumerary and underprivileged piglets, are still increasing (1). In order to adequately raise these piglets, a better understanding of the differential development of LBW piglets compared to normal birth weight littermates is necessary to accommodate their nutritional needs. As an example, recent research suggests that organs (e.g. small intestine and skeletal muscle) in LBW piglets might suffer from more oxidative stress (132, 133). Moreover, we showed lower plasma antioxidant capacity (ferric reducing ability and glutathione-peroxidase enzyme activity) for the LBW pigs in the post-weaning period (134). This knowledge opens opportunities to provide LBW piglets antioxidant nutrients.

1.3.2. Management strategies

To date, various management strategies have been undertaken to enhance survival and growth of LBW pigs. Supervision immediately after farrowing focuses on reducing heat losses, preventing piglets from being crushed by the sow and ensuring adequate colostrum intake (135-137). To prevent heat

losses, the creep area can be warmed by the use of electric heat lamps and a floor heating system.

In addition, management strategies can include split-nursing, split-weaning and cross-fostering. Split-nursing, defined as the removal of the heaviest piglets for a fixed period of time to allow colostrum/milk intake of the smallest piglets, successfully decreased the weight variation amongst litters (138). In case of split-weaning, the heaviest piglets are permanently removed from the sow to a nursery facility usually at 3 weeks of age, to allow the remaining lightest piglets uncompetitive suckling for one more week (139). This practice results in an increased and prolonged milk intake and catch up growth of the lightest piglets during lactation (140). Cross-fostering is practiced to create litters of equal weights, in order to reduce competition between littermates. Cross-fostering is applied by either reallocating the largest or the smallest piglets to other litters. However, no matter which fostering strategy is used, the number of piglets must not exceed the number of functional teats (141). Ideally, cross-fostering should be performed during the first 3 days after parturition, since these days are crucial for survival of the weak piglets and teat order establishment. English and Bampton found that cross-fostering litters reduces pre-weaning mortality with 40% (142). In a more recent study, similar results were obtained. Cecchinato et al. showed that cross-fostered piglets have a 40% greater probability of survival than piglets that stayed with their own dam (143). Two types of cross-fostering are encountered: limited cross-fostering strategies (during the early postnatal period) and repeated cross-fostering during the whole lactation period (144, 145). Focusing on LBW piglets, results conflict on the effect of cross-fostering (pooling similar weight pigs and limiting litter size) on weight gain and piglet survival (146-148). In some studies, a negative effect on weight gain and piglet survival is noted for less vital piglets (149, 150). In addition,

frequent mixing maintains a continuous cycle of pathogen transmission (151, 152) and is stressful for both piglets and sows (153).

1.4. Conclusion

Selection for increased litter size is part of every day practice in pig production. However, this results in an increased number of LBW piglets. Thus, this selection for larger litters should be accompanied by selection for increased piglet survival and growth (1). Additional to a genetic selection (long term measure), approaches in preventing LBW need to be established as acute measures. These latter measures aim to optimize the intrauterine environment via supplementing the sow during gestation. Feeding sows additional lipids during gestation (dependent on type and timing of fat supplementation) reduces the incidence of LBW piglets and improves their survival rates. In addition, feeding pregnant sows a diet with balanced amino acid profile according to stage of gestation increases litter uniformity. Moreover, functional amino acids can be added into the sow diets, for example L-glutamine and L-carnitine positively affects piglets' birth weights. Moreover, at present it is an important ethical challenge to raise LBW piglets (1). Besides optimizing the intrauterine environment, both feeding and management strategies should focus on keeping LBW piglets alive and make sure they gain satisfactory weight. Feeding strategies include making sure that these piglets ingest sufficient colostrum and milk (e.g. via split-nursing, split-weaning and cross-fostering), giving LBW piglets a booster preparation (e.g. an emulsion of medium and long chain fatty acids), and supplementing these piglets with milk replacers and milk based creep diets.

2. BOVINE MILK AS THE MAIN INGREDIENT OF PORCINE MILK FORMULAS: A SPECIES COMPARISON

2.1. Introduction

The sow's milk production is insufficient to nurse larger litters, in particular in the later suckling period (7, 8). Therefore, farmers seek solutions to improve survival of the piglets and obtain satisfactory growth performances. According to a recent survey, at least half of the pig farmers in Flanders, Belgium, supplement their piglets during the suckling period with a milk replacer (10). However, the majority of farmers believe that the composition of milk replacers is inferior to sow milk. Sow milk is 'the golden standard' for piglet nutrition and the composition of milk replacers is based upon its gross composition. However, the commercially available milk replacers generally use bovine milk products such as whey, lactose and skim milk, as main ingredients.

2.2. Gross composition of colostrum and milk

Colostrum is the first milk secreted after parturition and is besides its nutritional components predominantly rich in immunoglobulins, anti-microbial peptides and bioactive factors. It is particularly important for the nutrition, growth and development of newborns and is involved in immunomodulation. The composition of milk changes throughout the suckling period (Table 1.1). The transition of colostrum to mature milk usually involves a decrease in total solids and protein and an increase in lactose. In addition, the composition of colostrum and milk is highly variable and depends on breed, diet, body condition, health status etc. (154).

Table 1.1 Gross composition (%) of porcine and bovine milk (155-160).

	SOW		COW	
	Colostrum	Milk	Colostrum	Milk
Total solids	17 - 26	17 - 19	15 - 28	10 - 20
Crude protein	6.4 - 16	5.1 - 6.4	5.4 - 16	2.9 - 9.0
Crude fat	4.8 - 5.6	5.3 - 6.5	3.5 - 8.0	3.5 - 6.0
Lactose	3.1 - 4.6	4.8 - 5.9	1.0 - 4.4	2.0 - 5.0
Crude ash	0.7 - 0.8	0.8 - 0.9	0.1 - 1.2	0.6 - 1.0

2.3. Proteins

The proteins in colostrum and milk are grouped according to their solubility in either water-soluble or whey proteins or water-insoluble proteins or caseins. Whey typically denotes the liquid part of milk that remains after milk coagulating and curd removal. Whey is a by-product of cheese manufacturing and generally contains a variety of bioactive factors (proteins, vitamins, minerals, etc.). In contrast, the casein-fraction is mainly a nutrient source (for other functions, see 2.3.1).

Porcine milk is generally high in whey proteins, which differs considerably from bovine milk that is high in caseins (Table 1.2) (161).

Table 1.2 Casein and whey protein (% of total N) in porcine and bovine milk (155, 160).

	SOW		COW	
	Colostrum	Milk	Colostrum	Milk
Casein	8.90 - 28.2	28.2 - 48.3	-	78.3 - 80.9
Whey protein	66.6 - 91.1	43.6 - 66.6	-	15.3 - 17.2

In both pigs and cows, colostrum and milk are mainly composed of the amino acids glutamic acid, proline and leucine (Table 1.3). In both animal species, the relative percentage of glutamic acid, proline, methionine, isoleucine and lysine increases from colostrum to later milk. On the contrary, the relative percentage of threonine, serine, glycine, alanine and cysteine decreases from colostrum to later milk. Mainly glycine and cysteine are higher in porcine milk compared to bovine milk.

Table 1.3 Individual amino acid composition (g/100g total amino acids) of porcine and bovine milk (160, 162, 163).

	SOW		COW	
	Colostrum	Milk	Colostrum	Milk
Alanine	4.4	2.8 - 3.6	3.9	3.1 - 3.2
Arginine	4.7 - 6.1	4.4 - 5.6	4.1	3.2 - 3.4
Aspartic acid	7.8 - 8.1	7.8 - 7.9	8.1	3.3 - 7.0
Cysteine	1.8 - 2.6	1.5 - 1.6	2.4	0.7 - 0.9
Glutamic acid	17 - 18	21 - 22	16.6	12 - 21
Glycine	3.1 - 3.5	2.3 - 3.2	3.1	1.7 - 1.8
Histidine	2.1- 2.5	2.3 - 2.4	2.2	2.4 - 2.6
Isoleucine	2.4 - 3.6	2.9 - 4.0	3.7	4.7 - 5.5
Leucine	9.6 - 9.9	8.9 - 9.8	8.6	9.1 - 9.9
Lysine	6.3 - 6.7	7.0 - 7.9	7.4	7.7 - 8.6
Methionine	1.7 - 1.9	1.6 - 2.2	2.1	2.6 - 2.7
Phenylalanine	4.2 - 4.4	3.8 - 4.3	4.2	4.6 - 5.0
Proline	9.1 - 9.2	11 - 12	8.3	9.3 - 10
Serine	6.1 - 6.5	5.1 - 5.4	8.0	5.6 - 5.9
Threonine	5.2 - 5.8	3.7 - 4.1	6.1	4.1 - 4.2
Tryptophan	1.6	1.6	-	1.4
Tyrosine	4.0- 4.7	3.9	5.0	4.7 - 5.2
Valine	5.0- 5.9	3.8- 4.6	6.1	5.2 - 6.1

2.3.1. Caseins

Caseins include several subtypes like α, β, κ and γ casein and serve as a source of essential amino acids. In addition, caseins prevent the adhesion of bacteria and viruses to the gut (164), have immunomodulatory effects (165) and bind enterotoxins and lipopolysaccharides (166). During digestion, caseinphosphopeptides are formed, which are calcium carriers and assist in its absorption (167). Furthermore, these peptides alter the proliferation of lymphocytes, stimulate antibody-formation and contain anti-microbial activities (168). As presented in Table 1.2, the amount of caseins increases with lactation.

2.3.2. Whey proteins

The whey protein fraction of milk contains four main proteins (immunoglobulins, β-lactoglobulin, α-lactalbumin and serum albumin).

2.3.2.1. Immunoglobulins and the complement system

Immunoglobulins and the complement system are the major antimicrobial components of colostrum. Colostrum collected at parturition contains approximately 16% proteins, from which 80% are immunoglobulins (155, 169). Three classes of immunoglobulins are present in colostrum and milk: IgG (subclasses IgG1 and IgG2), IgM, and IgA. In both porcine and bovine colostrum, IgG is the predominant immunoglobulin (Fig. 1.4). In porcine milk, IgG concentration rapidly declines during the first day of lactation and IgA becomes the most abundant immunoglobulin in milk at the third day of lactation. In contrast, in bovine milk IgG is the main immunoglobulin throughout the entire lactation period (Fig. 1.4) (170).

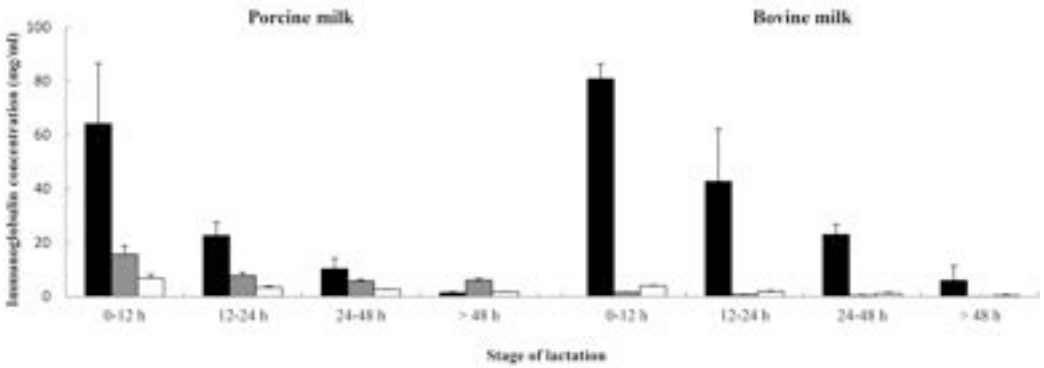

Figure 1.4 Changes in immunoglobulin concentration from colostrum to milk. Black bars represent IgG levels, grey bars IgA levels and white bars IgM levels (155, 171).

The complement system is composed of approximately 20 different proteins. Activation of this system leads to a cascade reaction resulting in antimicrobial activities. The complement system can be activated via antigen-antibody complexes (classical pathway), via carbohydrates (lectin pathway) or via components of microbial cell surfaces (alternative pathway) (169). Components of the complement system can be found in colostrum and milk (169).

2.3.2.2. Beta-lactoglobulin

Beta-lactoglobulin (18.3 kDa) is the most abundant whey protein in milk. Bovine milk contains 1.3 to 3.2 g/l β-lactoglobulin (172, 173), whereas in porcine milk levels between 3.2 and 8.0 g/l have been described (174). The physiological function of β-lactoglobulin is unclear.

Besides a valuable source of amino acids and bioactive peptides it binds to several molecules (e.g. fatty acids and retinol) and probably serves as a transport molecule (172, 175).

2.3.2.3. Alpha-lactalbumin

Alpha-lactalbumin is a 14 kDa protein that has an excellent nutritive value because it is high in lysine, cysteine and tryptophan. Moreover, it is involved in the lactose synthesis, functions as a calcium-carrier, has anti-carcinogenic and immunomodulating properties (172, 173). Bovine milk contains approximately 1.2 g/l α-lactalbumin (173) and similar values (1.2 - 3.0 g/l) have been reported in porcine milk (174).

2.3.2.4. Serum albumin

Serum albumin is a 66 kDa protein that is derived from the mammary circulatory system by passive diffusion. Besides a source of amino acids, it has anti-carcinogenic activities (176). Bovine milk contains 0.4 g/l serum albumin (172), whereas in porcine secretes 0.3 to 1.3 g/l serum albumin was detected (174).

2.4. Lipids

The main function of lipids in milk is to provide a source of energy to the neonate. Triglycerides, the main component of milk fat, are composed of fatty acids of different lengths (4 - 24 C-atoms) and saturation. Triglycerides are broken down into free fatty acids (FFA) and monoglycerides (MG), which contain anti-viral, anti-bacterial and anti-protozoa activities (177). Besides triglycerides, colostrum also contains di- and monoglycerides, phospholipids, glycolipids, free fatty acids and cholesterol (ester) (178).

The amount and constitution of fat is influenced by several factors: the dietary regimen, condition, breed, genetic factors, etc. In addition, the lipid content is affected by the stage of lactation. During the first 3 days of lactation, fat contents gradually increase in porcine colostrum/milk. Afterwards, milk fat contents decrease (155, 160). Similarly, in cows, the fat content is highest postpartum and gradually decreases as lactation proceeds (179, 180). Species differences have been described regarding the milk fat content. Generally, porcine milk contains more lipids in comparison with bovine milk (181). Compared with bovine milk, porcine milk lacks short chain fatty acids and is rich in oleic acid (C 18:1) and linoleic acid (C 18:2) (Table 1.4). Although fat content is different between colostrum and milk, the fatty acid profile is quite similar (Table 1.4).

Table 1.4 Fatty acid composition in porcine and bovine milk, expressed as the relative percentage of total fatty acids (%) (160, 182-184).

	SOW		**COW**	
	Colostrum	Milk	Colostrum	Milk
C 4:0	0	<0.09	3.89	3.3 - 3.84
C 6:0	0	<0.09	1.61	1.6 - 2.28
C 8:0	0	<0.03	0.76	1.3 - 1.69
C 10:0	0	<0.01	1.60	3.0 - 3.36
C 11:0	-	-	-	0.21
C 12:0	0	<0.02	2.08	3.1 - 3.83
C 14:0	3.18 - 3.20	3.06 - 4.02	9.15	11.2 - 14.2
C 14:1	0.01 - 0.03	<0.03	0.65	0.49
C 15:0	0.02 - 0.03	<0.02	0.61	1.03 - 1.3
C 15:1	<0.01	<0.02	-	0.08
C 16:0	31.1 - 33.3	27.6 - 37.0	38.4	32.2 - 42.7
C 16:1	5.47 - 5.56	5.32 - 10.5	2.41	1.53 - 3.7
C 17:0	0.05 - 0.09	0.06 -0.09	0.69	0.18
C 17:1	0.13 - 0.14	0.12 -0.17	-	0.08
C 18:0	6.31 - 6.41	5.93 - 6.36	7.9	5.7 - 11.1
C 18:1	37.5 - 39.5	32.3 - 40.3	23.0	16.7 - 21.7
C 18:2	12.6 - 12.7	8.4 -14.5	2.75	1.6 - 2.41
C 18:3	0.74 - 0.83	0.91 - 1.11	0.83	0.25 - 1.8
C 20:0	-	-	0.10	0.11
C 20:1	-	-	-	0.03
C 20:2	-	-	-	0.04
C 20:3	0.09 - 0.11	0.11 - 0.15	0.11	0.02
C 20:4	0.42 -0.46	0.48 -0.87	0.14	-
C 21:0	-	-	-	0.01
C 22:0	0.01 - 0.02	0.01	-	0.12
C 23:0	-	-	-	0.03
C 24:0	-	-	-	0.02

2.5. Carbohydrates

2.5.1. Lactose

The main carbohydrate in mammalian milk is the disaccharide lactose (342 Da). It is synthesized in the epithelial cells of the mammary gland and secreted in the alveolar lumen by exocytosis. Because lactose is not able to migrate through cell membranes, it is the primary osmotic component of milk and considered to be the main determinant of milk yield (185). Porcine colostrum contains 3.1 to 4.6 % lactose, whereas in mature milk 4.8 to 5.9 % lactose can be found (155). Bovine colostrum contains lactose levels between 1.0 and 4.4 % whereas in later milk 2.0 to 5.0 % lactose was measured (156-159) (Table 1.1).

2.5.2. Oligosaccharides and glycoconjugates

Oligosaccharides (OS) are carbohydrates containing three to ten sugar molecules. The monomers of which OS are composed are D-glucose, D-galactose, L-fucose, N-acetylglucosamine, N-acetyl neuraminic acid and N-glycolylneuraminic acid which are covalently linked through glycosidic bounds to make OS (186). The OS can be divided in two groups: a neutral and an acidic group. Neutral OS do not contain any charged groups, whereas acidic OS contain one or more negatively charged carbohydrate residues. Bovine and porcine milk contain only 1 to 2 g/l oligosaccharides (187) and concentrations of OS even decrease during the course of lactation (180). The majority of OS are resistant to digestion and absorption (180), implicating that they are present in milk for reasons other than nutrition. Consequently, undigested OS can affect the activity or growth of certain bacteria species in

favour of the host, and therefore these OS – e.g. galactosyl-lactose – are often called prebiotics (188). OS in milk structurally resemble pathogen binding sites coating the intestinal epithelial cell and therefore serve as soluble binding sites for pathogens; e.g. 3'-N-glycolylneuraminyl-lactose prevents the adhesion of *E. coli* to the piglets' intestinal wall (187).
Glycoconjugates are carbohydrate chains covalently attached to proteins (glycoproteins) or lipids (glycolipids). Both OS and glycoconjugates protect neonates against infection by preventing the adhesion of pathogens to the intestinal wall (189).

2.6. Vitamins

Vitamins can be grouped into fat- and water-soluble vitamins. The fat-soluble vitamins contain vitamin A, D, E and K. The water-soluble vitamins include the vitamin B and C-complex. The concentrations of vitamins in porcine and bovine milk are presented in Table 1.5. Generally, vitamin concentrations are higher in colostrum than in milk. However, their concentration is influenced by seasonal changes, liver deposition and the maternal diet (185).

Table 1.5 Concentration of vitamins (mg/l) in sow and cow colostrum and milk (157, 160, 185).

	SOW		COW	
	Colostrum	Milk	Colostrum	Milk
Fat-soluble vitamins				
Vitamin A	1.66	0.95 - 1.88	5.05	0.36
Vitamin D	0.02	<0.01	-	<0.01
Vitamin E	3.80	2.61 - 3.85	3.01	1.17
Vitamin K	0.10	0.09 - 0.10	-	0.03
Water-soluble				
Vitamin B1	-	0.7	0.90	-
Vitamin B2	-	2.8	4.55	-
Vitamin B3	-	7.4	0.34	-
Vitamin B5	-	4.6	-	-
Vitamin B6	-	-	0.04	-
Vitamin B12	-	0.02	0.60	-
Vitamin C	70.5	46.6 - 59.6	-	15.8
Vitamin H	-	0.01	-	-
Vitamin M	-	<0.01	-	-

2.7. Minerals

The mineral fraction of milk (8 - 9 g/l) is composed of both anions and cations, which can be grouped into a diffusible fraction and a non-diffusible fraction. The majority of the non-diffusible salts are bound to casein micelles and play a crucial role in their stability (190). Generally, the richer the milk is in proteins, the higher the calcium and phosphorus contents (191). The concentration of minerals is presented in Table 1.6. In cows, the highest mineral concentration can be found in colostrum and levels decrease during lactation (192). In pigs, calcium, phosphorus, and iron increase with the onset of lactation, whereas the concentration of potassium, sodium, zinc and copper decrease.

Table 1.6 Minerals concentrations (mg/l) in sow and cow colostrum and milk (157, 160, 191, 193, 194).

	SOW		COW	
	Colostrum	Milk	Colostrum	Milk
Calcium	707 - 1022	1192 - 2024	1210 - 8852	1074 - 2236
Phosphor	1032 - 1125	1121 - 1555	1845 - 8852	967 - 1022
Potassium	964 - 1133	770 - 938	1013 - 5676	1035 - 1874
Sodium	706 - 826	406 - 678	340 - 3057	278 - 870
Magnesium	80 - 94	100 - 114	237 - 1442	71.5 - 159
Iron	1.75 - 2.46	2.06 - 2.78	1.75 - 18.0	0.19 - 1.00
Zinc	12.77 - 16.17	5.77 - 9.06	11.33 - 86.52	2.30 - 6.60
Sulfur	-	26.78 - 47.38	916 - 4268	-
Copper	3.4 - 3.9	1.2 - 3.8	0.13 - 0.66	0.04 - 0.16
Manganese	0.06 - 0.11	0.06 - 0.10	0 - 0.36	0.03
Selenium	-	-	-	<0.03
Chloride	-	-	-	795 - 1243

2.8. Growth factors

Growth factors are proteins or polypeptides that are able to stimulate/inhibit growth and differentiation of a variety of cell types. The concentration is highest in colostrum and declines as lactation proceeds. In addition, the growth factor levels in colostrum and milk are highly species dependent (195). The most abundant growth factors in colostrum and milk are insulin-like growth factor-1 (IGF-1) and -2 (IGF-2), epidermal growth factor (EGF), fibroblast growth factor-1 (FGF-1) and -2 (FGF-2) and platelet-derived growth factor (PDGF).

2.8.1. Insulin-like growth factor

The insulin-like family is composed of IGF-1, IGF-2, insulin and relaxin. Two types of IGFs have been identified: IGF-1 (somatomedin, 7.6 kDa) and IGF-2 (7.5 kDa) (Table 1.7). Moreover, six IGF-binding proteins (IGFBP-1 to IGFBP-6) have been identified in milk (196, 197). These IGFBP are transport proteins for circulating IGFs. They regulate their bioavailability and modulate the interaction between IGFs and their receptors. In colostrum, most IGFs are present in a free form, whereas in milk the majority of IGFs are bound to IGFBP. As a result the bioavailability of IGF in colostrum is much higher in comparison with milk (198). IGF exert both metabolic and mitogenic effects at the level of the small intestine (199-201).

2.8.2. Epidermal growth factor family

The epidermal growth factor family includes epidermal growth factor (6 kDa), heparin-binding EGF (HB-EGF), amphiregulin, epireguline, transforming growth factor-α (TGF-α) and betacellulin (BTC). EGF is a heat and acid-stable peptide (202) that stimulates the proliferation and differentiation of several cell types and suppresses gastric acid secretion (203, 204) (Table 1.7). In addition, other regulatory peptides (e.g. HB-EGF, amphiregulin and TGF-α) belong to the epidermal growth factor family, some of which have not yet been detected in porcine and bovine milk (198, 205, 206). BTC (32 kDa), an important stimulator of wound healing and bone resorption, has been detected in bovine milk secretes (207), but not in porcine milk.

2.8.3. Other growth factors

Besides growth factors belonging to the IGF and EGF system, other growth factors have been identified in colostrum and milk.

Transforming growth factor-β (TGF-β) is a 25 kDa peptide from which 2 isomers have been identified (TGF-β1 and -β2) in bovine and porcine milk (Table 1.7). The majority of TGF-β is secreted in latent form, thus it requires activation by acids to become biologically active. The functionality of TGF-β is unclear, most likely it is involved in tissue repair, osteogenesis and immunoregulation (208).

Additionally, a number of growth factors has been identified in bovine milk, although not yet in porcine milk. FGF-2 (16.4 kDa) is an important stimulator of cell proliferation and differentiation (Table 1.7). This growth factor is involved in angiogenesis and stimulates wound healing and haematopoiesis. PDGF (30 kDa) has been found in bovine milk, although quantitative data are lacking. It plays a role in embryonic development; it is involved in the formation of kidneys, blood vessels, lungs and the central nervous system. Furthermore, it is involved in wound healing, angiogenesis and haematopoiesis (209). Mammary derived growth factor (MDGF; 62 kDa) stimulates the growth of the mammary glands and improves collagen production. Two bovine MDGF-types have been identified in bovine milk: b-MDGF-1 (30 kDa) and b-MDFG-2 (50 - 150 kDa) (198). In addition, a colostrum-specific mitogenic factor (bovine colostral growth factor (35 kDa)) has been found in bovine colostrum (198).

Table 1.7 Growth factor levels in porcine and bovine milk (195, 196, 198, 209-213).

	SOW		COW	
	Colostrum	Milk	Colostrum	Milk
IGF-1 (ng/ml)	70 - 350	4 - 27	49 - 2000	2 - 150
IGF-2 (ng/ml)	165 - 291	11 - 50	150 - 600	2 - 107
EGF (ng/ml)	5 - 1500	150 - 250	4 - 324	2 - 155
TGF- β1 (μg/ml)	12 - 51	1.5 - 2.4	12.4 - 42.6	0.8 - 4.3
TGF- β2 (μg/ml)	13 - 27	3.7 - 4.2	150 - 1150	38
FGF-2 (ng/ml)	-	-	-	0.5 - 1

2.9. Cytokines

Colostrum and milk contain a variety of cytokines such as interleukin (IL)-1β, IL-6, IL-8, IL-10, interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α). These are involved in immunomodulation, the induction of inflammatory response, the regulation of haematopoiesis and in wound healing. Moreover, they influence the growth and differentiation of a variety of cells (214). Cytokine concentrations in colostrum are higher than those in mature milk. Much higher levels were described in bovine compared to porcine milk (Table 1.8).

Table 1.8 Cytokine levels (ng/ml) in porcine and bovine milk (215, 216).

	SOW		COW	
	Colostrum	Milk	Colostrum	Milk
IL-1β	-	-	545 - 1143	0.49 - 239
IL-4	2 - 12	< 6	-	-
Il-6	0.5 - 2	< 1	46 - 77	0.06 - 14.1
IL-10	< 0.2	< 0.2	-	-
IL-12	0.2 - 1	< 0.4	-	-
IFN-γ	< 2	< 2	202 - 320	0.21 -71
TNF-α	< 0.2	< 0.2	510 - 1343	2.25 - 304

2.10. Nucleotides

Nucleotides, nucleosides and nucleobases are part of the non-protein nitrogen (NPN) fraction of colostrum and milk. Nucleosides are composed of a base (purine or pyrimidine), which is linked to a sugar molecule (ribose or deoxyribose). If nucleosides are being phosphorylated, nucleotides are formed. Nucleotides are the precursors of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). The important nucleotides in colostrum and milk are adenosinemonophosphate (AMP), cytidinemonophosphate (CMP), inosinemonophosphate (IMP), guanosinemonophosphate (GMP) and uridinemonophosphate (UMP) and concentrations are presented in Table 1.9. Nucleotide levels are at maximum within a day postpartum, decreasing concentrations are found in later milk (217). Nucleotides are involved in several biochemical processes e.g. immunomodulation, induction of apoptosis of malignant cells and growth stimulation (218). It is speculated that nucleotides are involved in iron absorption in the gut (219) and in lipid metabolism (220). The concentrations of 5' - AMP and 5' - CMP are comparable between both species. However, 5' - GMP and 5' - UMP are higher in pigs than in cows. The concentration of 5' - IMP has not yet been reported in other species than the pig.

Table 1.9 Nucleotide concentration (nmol/ml) in porcine and bovine milk (217, 218, 221).

	SOW		COW	
	Colostrum	Milk	Colostrum	Milk
5' - AMP	40 - 113	30 - 128	40 - 62	20 - 42
5' - CMP	15 - 71	9.8 - 71	32 - 53	19 - 47
5' - GMP	54 - 147	60 - 140	-	8.3
5' - UMP	3056 - 5556	1040 - 2631	186 - 390	<31.2
5' - IMP	11 - 18	4 - 26	-	-

2.11. Polyamines

Polyamines in milk include spermine, spermidine and putrescine. These substances regulate DNA, RNA and protein synthesis and are essential components for cell growth and differentiation. Moreover, they are involved in carcinogenesis and tumour growth (222). Polyamine levels in porcine and bovine milk are presented in Table 1.10. Spermine and putrescine in porcine milk are higher compared to bovine milk, whereas spermidine levels are similar (223).

Table 1.10 Polyamines concentration (μmol/l) in porcine and bovine milk (223-225).

	SOW		COW	
	Colostrum	Milk	Colostrum	Milk
Spermine	4.34 - 10.26	4.28 - 21.6	5 - 20	0.03 - 0.08
Spermidine	3.76 - 4.54	2.05 - 9.01	1.07 - 7.56	0.06 - 6
Putrescine	1.21 - 1.52	1.53 - 1.84	-	0.06 - 0.37

2.12. Hormones

The majority of hormones present in milk secretes are derived from the sexual glands, the adrenal glands, the hypothalamus and pituitary gland (Table 1.11). These hormones originate from the dams' blood flow and are secreted into the milk. In addition, the mammary gland is able to produce hormones itself. These substances are mainly involved in the growth and maturation of the gastrointestinal tract and immune function (226, 227).

Table 1.11 Hormone levels in porcine and bovine milk (227-241).

	SOW		COW	
	Colostrum	Milk	Colostrum	Milk
Gonadal hormones (pg/ml)				
Estrogens	500 - 2000	-	1000	5 - 10
Progesterone	0.01 - 0.04	-	-	2 - 20
Androgens	-	-	-	0 - 50
Adrenal gland hormones (ng/ml)				
Glucocorticoids	-	-	-	0 - 50
Androstenedione	-	-	-	3
Hypothalamic hormones (ng/ml)				
Gonadotropin-releasing hormone	-	-	-	0.5 - 3
Luteinizing-hormone-releasing hormone	-	-	-	0.5 - 3
Thyrotropin-releasing hormone	-	-	-	0 - 0.5
Somatostatin	-	-	-	10 - 30
Pituitary hormones (ng/ml)				
Prolactin	-	28 - 38	-	5 - 200
Somatotropin	-	-	-	0 - 1
Thyrotropin	-	-	-	-
Other hormones				
Insulin (ng/ml)	-	-	-	5 - 40
Calcitonin (ng/ml)	-	-	-	700
Triiodothyronin (ng/ml)	-	-	-	0.65 - 1.3
Thyroxin (ng/ml)	-	-	-	1.55
Bombesin (ng/ml)	0.91 - 1.20	0.91 - 1.20	-	0.25 - 450
Erythropoietin	-	-	-	-
Melatonin (pg/ml)	-	-	-	5 - 25
Leptin (ng/ml)	30 - 46	-	13.9	4 - 8
Relaxin (ng/ml)	9 - 19	< 2	-	1.47 - 4.77
Ghrelin (ng/ml)	5.7 - 8.0	5.7 - 8.0	2.60 - 3.04	-
Motilin (ng/ml)	-	-	166 - 293	19 - 52
Neurotensin (pg/ml)	193 - 339	66 - 112	-	-
Vasoactive intestinal peptide (pg/ml)	27- 56	-	-	-
Calcitonin gene related peptide (pg/ml)	242 - 466	-	-	-
Substance P (pg/ml)	108- 171	-	-	-

2.13. Antioxidants

Mammalian cells produce, under natural conditions, reactive oxygen species (ROS). If high amounts of ROS are formed, they cause damage to lipids, proteins and nucleic acids, a phenomenon called ‘oxidative stress’ (242). Protection against oxidative stress is possible via both enzymatic and non-enzymatic anti-oxidants. However, the anti-oxidative protection-mechanism is not yet fully developed during the first days after birth. Moreover, the extra-uterine environment is rich in oxygen, allowing the formation of ROS.
Milk contains many antioxidant factors, which are able to reduce the formation of ROS, scavenge radicals, and function as enzymes for the formation of non-enzymatic antioxidants (243). However, colostrum itself can be a source of ROS. ROS in colostrum are formed by phagocytes, which form superoxides in order to kill bacteria. Moreover, xanthineoxidase and lactoperoxidase in colostrum are sources of ROS as well, because hydrogen peroxide (244) and nitric oxide (245) can be generated, respectively.

2.13.1. Lactoperoxidase and lactoferrin

Lactoperoxidase is a 78 kDa glycoprotein capable of converting hydrogen peroxide into water. Via this reaction a toxic intermediary oxidation product is formed that inhibits bacterial metabolism (246). Lactoperoxidase levels of 11 to 45 mg/l and 13 to 30 mg/l have been detected in bovine colostrum and milk, respectively (247). Sow milk contains higher lactoperoxidase levels compared to cow milk (248).
Lactoferrin (80 kDa) is a glycoprotein with a high binding capacity to iron. Due to this capacity, competition for iron occurs with enteric bacteria, which explains its bactericidal activity. Moreover, lactoferrin is able to bind to the

bacterial cell wall, causing cell damage and/or cell death (249). In addition, lactoferrin functions as a non-enzymatic antioxidant by decreasing the conversion of hydrogen peroxide into hydroxyl radicals (243). Up to 1.6 mg/ml lactoferrin has been detected in porcine colostrum, whereas in later milk concentration declines towards 0.4 mg/ml (250). In bovine colostrum lactoferrin levels decline from 1.5 - 5 mg/ml in colostrum to 0.1 mg/ml in mature milk (213). Lactoferrin resists luminal degradation in the gastrointestinal tract and protects the gastrointestinal tracts of suckling piglets against infection. In addition, lactoferrin facilitates iron absorption along the small intestine, via a receptor-mediated mechanism (251).

2.13.2. Catalase, superoxide dismutase and glutathione peroxidase

Catalase (240 kDa), an enzyme that catalyses the decomposition of hydrogen peroxide, has been purified from bovine milk (243). It is present in both the cream and skim milk fraction.

Superoxide dismutase (SOD, 31 - 33 kDa) catalyses the reaction of superoxide anions into hydrogen peroxide. SOD activity has been detected in bovine skim milk and is known to vary between cows and breeds (243).

Glutathione peroxidase (GSHPx) reduces hydrogen peroxide to water. Glutathione peroxidase contains a seleno group, which is oxidised by the peroxide and reduced by glutathione (GSH), resulting in conversion of GSH into oxidised glutathione (GSSG).

GSPHx activity levels of 12 to 32 U/ml have been detected in cow’s milk but not in porcine milk (252).

2.13.3. (pro) vitamins

Vitamin C may function as an antioxidant by electron transfer. However, under certain metabolic conditions, vitamin C may act as a pro-oxidant as well. Vitamin E is composed of 8 vitamers, of which α-tocopherol is the main lipid-soluble antioxidant that acts as a radical scavenger. In addition, Vitamin A and its precursor β-carotene may act as oxygen scavengers (253).

2.14. Milk cells

Colostrum and milk contain a variety of cell types, which include macrophages, neutrophils, lymphocytes, eosinophils and epithelial cells (Table 1.12). The cell number declines as lactation proceeds. However, the number of epithelial cells, which originate from abrasion of the epithelial lining of the mammary gland, increases as lactation proceeds. The physiological functions of milk cells are not well understood. Macrophages and neutrophils are capable of phagocytosis. Moreover, macrophages secrete IgA and produce lysozyme, lactoferrin and complement components (254, 255). Lymphocytes are grouped into B- and T-lymphocytes. Lymphocytes present in colostrum of the piglets' own mother are able to migrate through the intestinal wall and serve as immunomodulators (256). Milk leucocytes are probably involved in both local protection of the gastrointestinal tract and systemic immune protection at least in the early neonatal period.

Table 1.12 Composition of milk cells (%) in porcine and bovine milk (214, 257, 258).

	SOW		COW	
%	Colostrum	Milk	Colostrum	Milk
Neutrophils	63 - 72	41 - 45	-	-
Macrophages	1.3 - 25	8.6 - 16	35	80
Lymphocytes	6.5 - 27	0.0 - 23	4	16
Eosinophils	0 - 0.7	0 - 0.4	-	-
Epithelial cells	0.4 - 35	6.1 - 89	0	2
Polymorphonuclear cells	45 - 72	12 - 55	62	3

2.15. Hydrolytic enzymes and protease inhibitors

Several hydrolytic enzymes have been reported to be present in mammary secretions. Lipolytic (241, 259-261) and proteolytic enzymes (262) play a role in the digestion of milk and the release of bioactive components. However, extensive proteolyic hydrolysis is unlikely because protease inhibitors are also present (263). The activity of protease inhibitors is especially high in colostrum (264), which are involved in macromolecule absorption in the intestine (265). In addition, protease inhibitors may be involved in the protection of luminal digestion of milk-borne growth factors (266).

2.16. Conclusion

In general, it is difficult to compare the described levels in porcine and bovine milk, due to a number of variables such as time of milk collection, sample handling and method of analysis. Despite this, it may be concluded that porcine milk has a number of unique features compared to milk replacers. Its composition changes throughout lactation and thereby is, unlike milk replacers, a dynamic food that possibly reflects the need of piglets at different stages during postnatal development. In addition, there are several differences between porcine milk and bovine milk. The former contains more proteins (especially rich in whey proteins) and fat (especially rich in oleic acid and linoleic acid) than bovine milk. Moreover, porcine milk provides the piglet not only with nutrients. It is also a carrier of numerous bioactive compounds (e.g. rich in EGF, spermine, 5' - GMP), which influence the piglets' postnatal metabolism and development. These features should be considered when formulating the milk replacers for artificial rearing of piglets.

3. THE SMALL INTESTINE

3.1. Introduction

One of the main functions of the gastrointestinal tract, and more specifically the small intestine, is the digestion of ingested feed and absorption of nutrients. In addition, the gastrointestinal tract functions as a barrier to prevent the entrance of pathogenic microorganisms, toxins and allergens. At birth, nutrient supply changes from the parental route via the umbilical cord and foetal swallowing of amniotic fluid towards enteral nutrition by suckling the sow. At weaning, the young piglet is being separated from the dam, which includes the change from suckling to non-suckling, a change from liquid to solid feed and a change in composition of the diet. In addition, the piglets are being exposed to several other stressors: a new environment, mixing with strangers, etc.

In addition to the ontogenic maturation, the function and health of the small intestine is influenced by the intake and the composition of the ingested feed, which can be investigated by several methods including intestinal mass, functional morphology, digestion and absorptive capacity and barrier function.

3.2. Anatomy and histology

The small intestine, starting at the pylorus and ending at the ileocecal junction, consists of the duodenum, jejunum and ileum (267) (Fig. 1.5). The duodenum, the first part of the small intestine, forms a U-shaped loop and can be anatomically divided into four parts: pars cranialis, pars descendens, pars transversa and pars ascendens. In the duodenum, digesta from the

stomach are mixed with bile (via the ductus choledochus) and pancreatic juice (via the ductus pancreaticus accessorius). They end at the level of the papilla duodeni major and papilla duodeni minor, respectively. The pars ascendens of the duodenum is connected with the colon descendens via the plica duodenocolica, forming the anatomical hallmark to distinguish the duodenum from the jejunum. The jejunum, being the longest part of the small intestine, forms numerous loops in the right half of the abdominal cavity. The ileum, the short terminal part of the small intestine, forms the connection with the colon ascendens and is attached to the caecum via the plica ileo-caecalis.

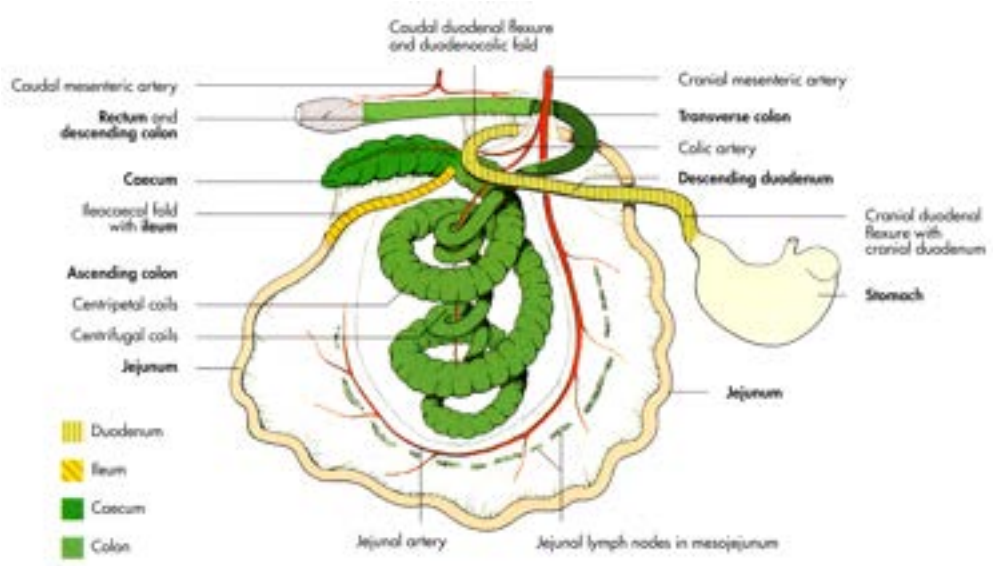

Figure 1.5 A schematic presentation of the gastrointestinal tract of the pig (268).

The small intestinal wall consists of four layers: tunica mucosa, tela submucosa, tunica muscularis and tunica serosa (269) (Fig. 1.6). The surface of the tunica mucosa reflects the area available for absorption. This absorptive area is amplified by (1) the plicae circulares (valves of Kerkring), (2) the intestinal villi, (3) the intestinal glands and (4) the microvilli on the apical surface of the intestinal cells (270). The plicae circulares are permanent folds of the tunica mucosa and tela submucosa, whereas intestinal villi are projections of the tunica mucosa. At the base of the intestinal villi, intestinal crypts of Lieberkühn can be found, which contain the epithelial stem cells. These stem cells differentiate and migrate from the crypts to the tip of the villi and are shed into the lumen (271). In the small intestine, there is a continuous cell renewal of the enterocytes, which takes 3 to 4 days. The epithelial cells lining the villi consist of various cell types: enterocytes, entero-endocrine cells and goblet cells. Underneath this epithelial layer lies the lamina propria, which contains mainly lymphatic tissue as scattered lymphocytes or aggregated as Peyer's patches. Moreover, the lamina propria contains the capillary network and nerve fibres surrounded by connective tissue. Each villus contains an arteriole, which drains into a venule at the base of the villus, thereby facilitating absorption. In addition, lymphatic capillaries are found in order to drain the products of fat digestion (see 3.2.3) (Fig. 1.7). Underneath the lamina propria, two layers of smooth muscle cells (an inner circular and outer longitudinal) form the lamina muscularis mucosae. The activity of these muscle cells causes the motility of the villi, increasing the contact with the luminal digesta.

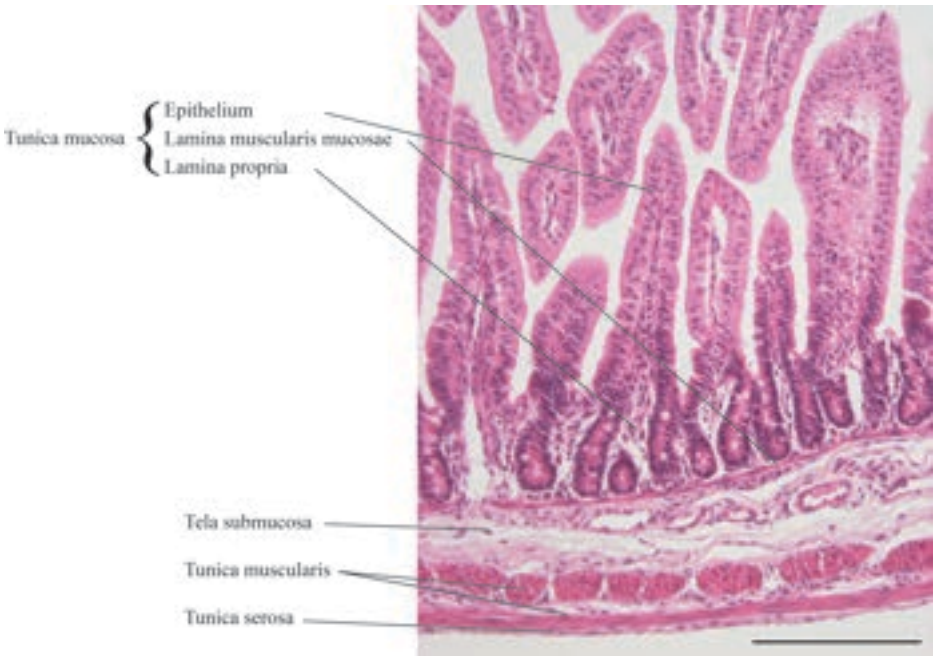

Figure 1.6 Microscopic structure of the small intestinal wall of a suckling piglet. Haematoxylin-eosin stained section, scale bar = 200 µm.

The submucous layer (tela submucosa) consists of blood and lymphatic vessels, lymph follicles and nerve plexuses (inner and outer submucous plexuses) imbedded in loose connective tissue (272). In the duodenum and first part of the jejunum, glands - so called Brunner's glands - can be found in the tela submucosa (269). These glands secrete an alkaline content (pH 8.1 - 9.3) in the intestinal crypts in order to protect the duodenal mucosa from the acidic digesta coming from the stomach. Additionally, by increasing the pH of the intestinal contents, the pancreatic enzyme activity is optimized. The tunica muscularis consists of an inner circular and an outer longitudinal muscle layer. In between these two layers, a myenteric nerve plexus (Auerbach's plexus) is interposed (272). The serosal layer (tunica serosa), being the outer layer of the small intestine, consists of connective tissue. The mesentery connects the small intestine with the dorsal body wall and contains blood vessels, nerves and lymph nodes (267).

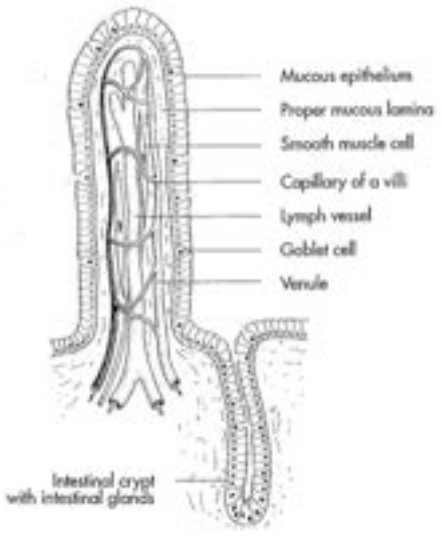

Figure 1.7 A schematic presentation of the intestinal villi and crypts (268).

Because piglets are born agammaglobulinemic, maternal antibodies have to be transmitted postnatally via the colostrum (214). Selective IgG transmission is mediated by Fc receptors which have been identified on the intestinal cells in neonatal piglets (273). In addition, non-selective uptake of these large molecules is performed via the apical canalicular system (274). From the second trimester of gestation, vacuolated foetal-type enterocytes are observed throughout the small intestine (Fig. 1.8). These cells contain cytoplasmatic vacuoles and have the ability to non-selectively transport intact proteins from the intestinal lumen into the circulation (transport vacuoles) or to digest ingested proteins inside the cell (digestive vacuoles). Enterocytes containing transport vacuoles are present along the small intestine and play a crucial role in macromolecular uptake via an apical canalicular system. This type of enterocytes disappears 2 to 3 days after birth, a phenomenon called "gut closure" (275). After closure of transmission, digestive vacuoles can still

be observed in the ileal absorptive cell. This vacuolated cell-type gradually disappears from the jejunum towards the ileum. The gradual replacement of vacuolated foetal-type enterocytes with adult-type enterocytes occurs with ontogenic maturation and takes approximately 18 days, often referred to as "ileal closure" (274).

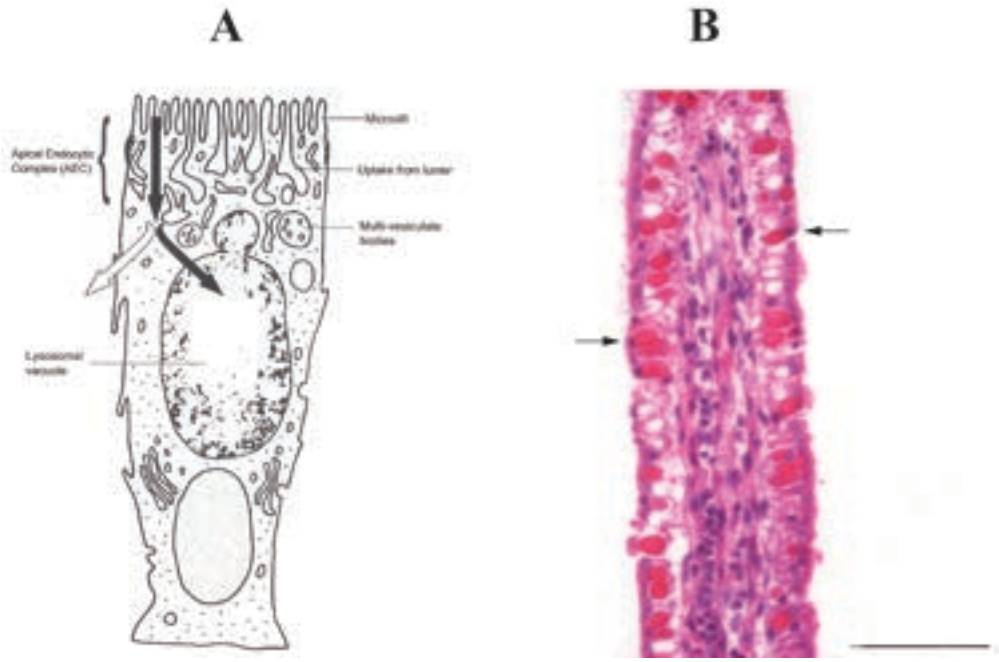

Figure 1.8 (A) Schematic presentation of a vacuolated cell (276). Ingested substances enter the cell into the apical endocytic complex. Transport is either via the lysosomes (dark arrow) or across the cell via the lateral intercellular space (open arrow) (B) Presence of vacuolated enterocytes (arrows) in the intestinal villi of a 1 d old suckling piglet. Haematoxylin-eosin stained section, scale bar = 100 µm.

Adult-type enterocytes lining the intestinal villi are columnar cells containing an oval nucleus at the basal region. The surface of the enterocyte is covered by microvilli facing the intestinal luminal content, enlarging the apical surface area. This apical membrane is often called the brush border membrane or striated border and is the active site for nutrient digestion and absorption. The glycocalyx, a complex surface coat consisting of glycoproteins, covers the microvillus membrane. Neighbouring enterocytes are joined by junctional complexes that are comprised of the zonula occludens (tight junction), zonula adherens (belt desmosome) and macula adherens (spot desmosome) in order to prevent the transmission of pathogens, toxins and other antigens through the intercellular space.

3.3. Digestion and absorption of nutrients in the small intestine

The small intestine plays a crucial role in the final digestion and absorption of nutrients. Enzymes in the lumen of the intestinal tract are derived from the salivary glands, the gastric glands and the pancreas. The end-products of luminal digestion are hydrolysed further into absorbable nutrients by enzymes located in the enterocytes. The major groups of digestive enzymes within the enterocytes are the disaccharidases and peptidases (277) (Fig. 1.9).

3.3.1. Digestion and absorption of carbohydrates

In the lumen, amylose and amylopectin are degraded by α-amylase (derived from the salivary gland and pancreas) into disaccharides, which are further hydrolysed by enzymes on the epithelial cell to monosaccharides. The latter are absorbed along the small intestine through paracellular transport and/or predominantly transcellular transport. The enterocytes of the pig small

intestine produce lactase, trehalase, the maltase-glucoamylase complex, and the sucrase-isomaltase complex (277).

Ingested lactose is hydrolysed by lactase to glucose and galactose (277). In addition to lactose, lactase can also hydrolyse cellobiose. Trehalase hydrolyses trehalose into two glucose molecules. Sucrase forms an enzyme complex with isomaltase, the sucrase-isomaltase complex. Sucrase breaks sucrose into glucose and fructose. The isomaltase-part, together with maltase from the maltase-glucoamylase complex, hydrolyses maltose and maltotriose to glucose. The glucoamylase enzyme degrades, in turn, amylose and amylopectin (277).

Monosaccharides (glucose, galactose and fructose) can be absorbed via either passive or active transport through the intestinal wall (278). Passive transportation is possible via both the paracellular and transcellular pathway. However, the paracellular glucose pathway has not yet been investigated in pigs. For transcellular transport, the presence of specific transporters has been identified. In the small intestine of pigs, the sodium-independent fructose transporter GLUT5 has been detected for the facilitated absorption of fructose (279). For glucose and galactose transport, against their concentration-gradient, the sodium-dependent glucose cotransporter (SGLT1) has been identified at the apical side of the enterocytes (280). Basolateral export of monosaccharides, by facilitated diffusion, is mediated by the GLUT2 transporter (281). Additionally, glucose can be transported into the circulation by exocytosis (282).

3.3.2. Digestion and absorption of proteins

Proteins in food are degraded by pepsin from the gastric glands and proteases from the pancreas. The degradation products consist primarily of peptides while little amino acids are formed. Peptides containing four or more amino acids, are extracellularly hydrolysed by enzymes located in the brush border. Smaller peptides are either hydrolysed by peptidases (at the level of the brush border/intracellular) or are being directly absorbed and transported into the circulation (277).

A large number of peptidases are found in the epithelial cells of the small intestine (277). Besides enterokinase and endopeptidases, other peptidases have been identified participating in the final stages of protein digestion. The major peptidases located in the brush border are aminopeptidase A, aminopeptidase N and dipeptidylpeptidase IV (277). Aminopeptidase A hydrolyses mainly oligopeptides with aspartic acid and glutamic acid in the N-terminal position. Aminopeptidase N mainly cleaves amino acids with neutral or basic amino acids at the N-terminus of oligopeptides. It is the most abundant brush border enzyme in pigs. The activity of dipeptidylpeptidase IV is complementary with aminopeptidase N; preferentially peptides with proline at the N-terminus position are cleaved.

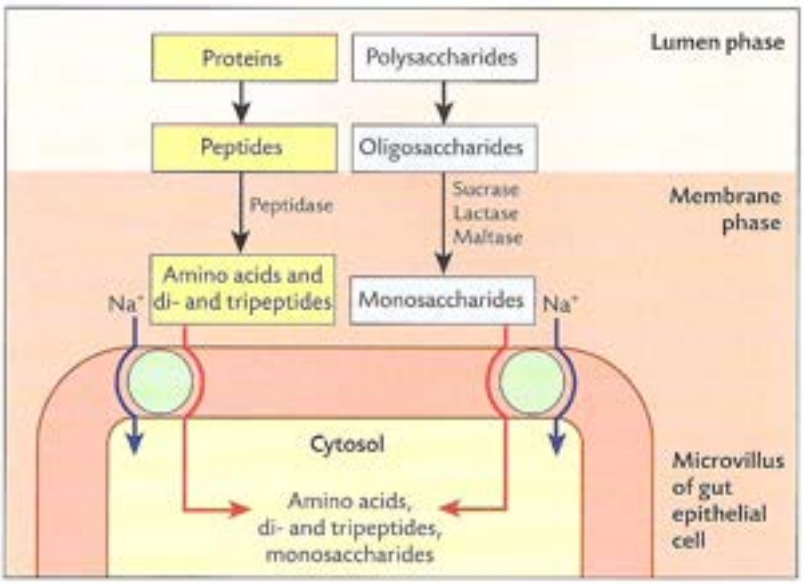

Figure 1.9 Schematic overview on the luminal and membranous breakdown of carbohydrates and proteins (283).

Amino acids can either be transported via the paracellular or transcellular (passive and carrier-mediated) pathway. Via the active transport system, using sodium-dependent transporters, amino acid transport is possible against the concentration gradient. Several amino acid transport systems have been identified for the transportation of anionic, cationic, neutral and imino acids in the small intestine (284). Besides amino acids, peptides are being absorbed in the intestinal tract of pigs. Nosworthy and coworkers recently described the peptide transport protein (PepT1) expression in the intestinal tract of pigs and demonstrated that short chain peptides are being absorbed throughout the small intestine (285). Intact peptides entering the enterocyte are hydrolysed to free amino acids, consequently no intact peptide can be observed in the portal blood of pigs (286).

3.3.3. Digestion and absorption of fat

Dietary fat is primarily composed of triglycerides and some phospholipids, sterols and sterol esters. Triglycerides are digested into free fatty acids and monoglycerides by lipolytic enzymes (lipases). When triglycerides come into contact with bile acids, fat is emulsified (reducing the size of fat droplets) and micelles (water-soluble aggregates) are formed. These micelles function as transporters for fat degradation products allowing these to diffuse across the cell membrane into the enterocyte. Coming in contact with the epithelial cells, micelles release monoglycerides and free fatty acids, which are transformed into triglycerides in the enterocyte. These triglycerides are coalesced together with cholesterol and phospholipids to lipoproteins called chylomicrons. These chylomicrons are accumulated as vesicles and transported via exocytosis into the lymphatic vessels (283). Phospholipids and sterols are hydrolysed into non-esterified fatty acids, lysophospholipids and cholesterol by pancreatic phospholipase and cholesterol esterase, respectively. Short-chain fatty acids pass directly into the portal blood, without being esterified (287). Fat absorption occurs along the entire length of the small intestine but is most intense at the end of the duodenum and proximal two thirds of the jejunum.

3.3.4. Water and electrolyte absorption

The small intestine absorbs a large amount of fluid and electrolytes, which are both of dietary and endogenous origin. Water in the small intestine is mainly derived from the secretions of the gastrointestinal tract, liver and pancreas and needs to be reabsorbed by the enterocytes and returned to the systemic circulation. Water and electrolytes transverse the epithelium via the

transcellular or paracellular pathway in an isosmotic manner. The majority of fluid transport in the small intestine occurs via passive diffusion through the intercellular junctions between the epithelial cells. It is a consequence of osmotic and hydrostatic gradients set up by the sodium-potassium-pumps located at the basolateral membrane.

The uptake of electrolytes into the epithelial cells is regulated by two types of carrier-mediated transport mechanisms: a electronically neutral sodium chloride absorptive process and a sodium-substrate coupled process (288). The first mechanism exchanges sodium for hydrogen and chloride for bicarbonate. The second process links sodium to the uptake of substrates (e.g. glucose and amino acids) through the small intestine. Both transport mechanisms allow the uptake of substrates into the cell against their concentration gradients.

3.4. Effects of small intestinal growth and function

3.4.1. Distribution along the crypt-villus axis and along the small intestine

Small intestinal growth is not evenly distributed along the length of the intestine. The proximal part starts to develop earlier during suckling; as a result vacuolated enterocytes are restricted to the ileum after gut closure. The length of the villi usually increases from the duodenum to the mid jejunum and again decreases towards the ileum (289-291). Villi in duodenum and jejunum have a more regular shape (from finger to more tongue-like) in comparison with a more irregular shape in the ileum (289). Crypts are usually deeper in the proximal part compared to in the middle and distal part of the small intestine (292).

Studies confirmed the presence of lactase, sucrase, aminopeptidase N and dipeptidylpeptidase IV from the crypts to the villus in porcine enterocytes (293, 294), with increasing activities reaching the villus tip (295).
Lactase-activity is highest in the proximal part of the intestine and lowest in the distal part in newborn pigs (296, 297), while it is evenly distributed along the intestine at older ages (298). At birth, maltase and sucrase are evenly distributed along the small intestine. However, in suckling piglets both enzymes have a low activity in the distal part of the small intestine (299). In contrast, sucrase activities are high in the distal part of the small intestine in suckling piglets (297) and during the post-weaning period (277). Kelly et al. stated that maltase was equally distributed along the small intestine at 2 to 3 weeks of age (300). Peptidase activities are highest in the distal part of the small intestine in suckling and weaned piglets (277, 293).

3.4.2. Age related changes

Villus height and shape change with aging. At birth, the small intestinal mucosa is lined with finger-like villi (301). During the first days after birth, the length of the intestinal villi quickly increases from approximately 252 μm at birth to 533 μm at 3 days of age (301). After 3 days of age, the length of the villi decreases during the suckling period. Parallel with the villus shortening during suckling, villus diameter increases, resulting in leaf-like shaped villi (302).
Intestinal crypt depth in newborn piglets is lower compared to piglets that are 3 days of age, while crypt depth decreases when piglets become older (301). The weaning process, i.e. separation from the dam, change of housing conditions, mixing, change of feed is stressful to the piglets and induces physiological changes at the level of the small intestine. These changes can

be separated into two phases. The weaning transition is usually followed by a period of low feed intake. During the first 3 to 5 days after weaning, typically a reduction in villus height, decreased number of enterocytes, lower enterocyte maturity and reduced absorptive function is observed (302-304). However, in the second phase, the intestine responds to the luminal presence of nutrients by crypt deepening and cell proliferation. As a result, villus height increases and a change in shape from longitudinally flattened villi to tongue-like villi is seen (302). The length of the adaptation period is mainly determined by the age at weaning and the composition of the post-weaning diet.

Lactase-activity is present in the pig foetus (starting from 7 weeks of gestation) and increases towards birth (296). Feeding colostrum and milk rapidly induces an increase in lactase activity, which activity is highest during the first postnatal week. Lactase-activity starts to decline during the second and fifth week after birth and continues to decline towards weaning (305). Maltase activity, but not sucrase activity, can be detected in the foetal pig at 105 days of gestation (298). Both maltase and sucrase activities are, in contrast to lactase activity, low at birth (296). Increasing activities are seen starting from the second week after birth reaching a plateau at about 6 to 8 weeks (299). Peptidase activities are generally high at birth and decrease during suckling (306). A reduction of enzyme activities is seen in the early post-weaning period (303), whereas enzyme activities elevate following the recovery of villus heights after 1 to 2 weeks after weaning (307).

3.4.3. Birth weight related changes

In the immediate postnatal period, LBW piglets possess shorter small intestines with reduced wall thickness and villus height and a decreased density of the villi compared to normal-birth-weight piglets (133, 308-311). This implies a reduction in small intestinal surface for nutrient absorption. The reduction in mass and wall thickness of the gastrointestinal tissue is caused by a reduced cell population rather than cell volume (308). In addition, LBW changes the maturation pattern of intestinal digestive enzymes with an altered digestive capacity as a result. Xu et al. demonstrated a decreased total mucosal lactase activity in the small intestine of LBW piglets at birth compared to normal control animals as did Che et al. with regard to sucrase at 2 days after birth (311).

Apparently these alterations do not persist until later stages of the suckling period because earlier studies from Wiyaporn et al. and results from our own lab suggested that the small intestine of LBW piglets develops normally (291, 312). In agreement, D'Inca et al. showed that in LBW piglets of 0 to 5 days of age, the mucosal brush border enzyme activities were not significantly reduced (313).

3.4.4. Feeding related changes

3.4.4.1. Dietary effects

In case of deprivation of colostrum intake or starvation, gut closure can be delayed and uptake of proteins prolonged (314, 315). Besides the fact that colostrum intake induces gut closure, it is also believed to play an essential role in maintaining gastrointestinal growth. Feeding colostrum increases intestinal weight, absorptive area and brush border enzyme activities (316, 317). These findings may partly be the result from the presence of colostrum-borne growth factors. Comparing animals fed different diets, showed that colostrum and milk contain substances capable of stimulating tissue growth and of inducing functional changes in the small intestine (317-320).

The effects of feeding piglets formulated milk on the small intestinal morphology and enzyme activities are contradictory. Feeding neonatal piglets for six hours with milk replacer instead of porcine colostrum decreased total lactase, maltase and peptidase activities (321). Feeding neonatal pigs with milk replacer instead of sows' milk during the first week of artificial rearing slows down intestinal development. Formula-fed piglets had lower mitotic index, higher apoptotic index and slower replacement of foetal-type with adult-type enterocytes (322, 323). Wolinski et al. even showed decreased villus lengths and crypt depths in this group of piglets, whereas Biernat et al. could not find alterations in these parameters. Surprisingly, formula-feeding enhances the peptidase activities (322). In contrast, feeding piglets from day 7 until day 28 after birth on formulated milk results in higher villi and deeper crypts, whereas few effects on enzyme activities were noticed (324).

3.4.4.2. Effect of milk-borne components

Several studies have demonstrated that specific nutrients alter the morphological and functional capacity of the intestine. Recently, there is an increasing interest in the physiological functions of bioactive substances (growth factors, hormones, nucleo(s)tides, etc) present in colostrum and milk, and this opens opportunities to the use of these substances as feed supplements (325).

Various studies have proven a beneficial effect of orally given IGF-1 (200 - 3500 µg/kg/d), which stimulates small intestinal growth and maturation (200, 201, 326, 327). Supplementing IGF-1 to neonatal formula-fed piglets (500 - 20 000 µg/l), increased crypt cell proliferation (199), villus heights (200, 201) and elevated intestinal brush border enzyme activities (201). Exogenously administered epidermal growth factor (EGF) (60 - 263 µg/kg/d) increased intestinal brush border enzyme activities (328) and has a healing effect on intestinal tissue damage (329). Experiments carried out in sheep or rabbit models of IUGR, indicate a beneficial effect on growth in general as well as at the level of the intestinal tract when amniotic IGF-1 (330, 331) or EGF (332, 333) supplements were given. The latter experiments illustrate the importance of IGF-1 and EGF in modulating fetal growth and were conducted since circulating concentrations of both growth factors are reduced in human IUGR foetuses (334). The effects of postnatal (oral) supplementation of IGF-1 and/or EGF in case of porcine IUGR or LBW remain to be investigated. Nevertheless systemic administration of IGF-1 (4 µg/h from d 3 to d 10 of age) via an osmotic minipump to IUGR piglets restores growth to normal levels (335).

Since leptin seems to be a crucial factor in foetal development (336), Wolinski et al. added leptin (2 - 10 µg/kg) to the diet of formula-fed neonatal

piglets during the first 7 days of life. This has led to longer small intestines, but resulted in some undesirable effects such as reduced villus heights and reduced brush border enzyme activities (322). Additionally, no beneficial effect on weight gain was noticed. In contrast, recent studies showed a beneficial effect on growth of postnatal leptin administration to IUGR rat pups (2.5 mg/kg/d) (337) and to IUGR piglets (0.5 mg/kg/d) (338).

For many years, infant formulas are supplemented with nucleotides (339). In addition, studies in rats showed a beneficial effect of these compounds on gut growth and maturation (340). In piglets, nucleotide supplementation has only been investigated during the weaning period (341) with higher mitosis to apoptosis ratios and a decrease in villus atrophy after weaning as result (342, 343). Arginine is the first limiting amino acid in sow's milk and milk replacers and the endogenous synthesis is insufficient to cover the requirements of suckling piglets. For example the arginine provision from sow milk is ≤1 g/d whereas arginine requirements for growth and metabolic function of a 7-d old piglet are ≥2.7 g/d (344). Studies investigating supplementation of this amino acid in the IUGR lamb (345) and piglet (346) showed accelerated growth. A deficiency in L-glutamine, a precursor of arginine, is a crucial factor contributing to IUGR in pigs. IUGR piglets, which were supplemented with oral L-glutamine (1 g/kg/d) between day 0 and day 21 of age, showed accelerated growth (167 ± 4 g/d vs. 144 ± 3 g/d) and reduced pre-weaning mortality (21.8% vs. 42.3%) (86).

Since additional milk supply is still not effective in reducing neonatal survival rates or in reducing weaning or slaughter weight variation (126, 127), is seems clear to us that the formulation of milk replacers needs optimization. Studies have shown that the addition of 500 to 20 000 µg/l IGF-1 and 500 to 1000 µg/l EGF to milk replacer positively affects intestinal growth (199-201, 329, 347). Moreover, 0.2 to 0.4% arginine on the basis of

milk replacer powder enhances average daily gain (ADG) by 28 and 66% and body weight by 15 and 32% compared with control piglets (346).

The knowledge that milk-borne substances positively affect the gastrointestinal development offers opportunities to supplement milk replacers with these factors to assure the growth and maturation of the gastrointestinal tract, in particular for the LBW piglet.

3.4 Conclusion

In this chapter, the literature pertinent to the morphological and functional development of the porcine small intestine has been reviewed. From recent studies it appears that, besides ontogenic maturation, the small intestinal function can be influenced by environmental inputs such as dietary change and weaning. Based on the literature cited, one might state that knowledge on the small intestinal development is gradually being unravelled, but the picture is far from complete.

CHAPTER 2
EVALUATION OF BODY COMPOSITION AND MUSCLE ENERGY STORES IN PIGLETS

Adapted from: **M. De Vos**, V. Huygelen, M. Hesta, S. A. Willemen, E. Fransen, C. Casteleyn, S. Van Cruchten, C. Van Ginneken. Birth weight has no influence on chemical body composition and muscle energy stores in suckling piglets, Animal Production Science, in revision

1. ABSTRACT

Economic losses in pig production are high due to neonatal mortality and poor postnatal growth performances predominantly of LBW piglets. To explore underlying mechanisms, we describe in this paper the effects of age and birth weight on body composition and muscle energy stores. Different parameters were assessed in pairs of low birth weight (LBW, n = 32) and normal birth weight (NBW, n = 32) piglets, at day 0 (n = 16), day 3 (n = 16), day 10 (n = 16) and day 28 (n = 16) of age. In total 6 piglets (3 LBW and 3 NBW) per age group were euthanized for chemical total body composition analysis. The semimembranosus muscles of 10 additional piglets (5 LBW and 5 NBW) per age group were sampled for the analysis of muscle lipid and glycogen contents. For none of the tested parameters differences related to birth weight were observed ($P > 0.05$). With increasing age, water percentage decreased, whereas fat and protein percentages increased in both LBW and NBW piglets ($P < 0.01$). Body ash content remained constant during growth ($P > 0.05$). Muscle glycogen contents decreased with increasing age for both types of piglets ($P < 0.05$), whereas no age effects could be observed for muscle lipid deposition ($P > 0.05$). In conclusion, the age of the suckling piglet has a major impact on its body composition and muscle energy stores but its birth weight unexpectedly has no influence.

2. INTRODUCTION

By using hyper-prolific sow breeds, litter sizes have increased tremendously during the last decades. This increase has been associated with higher within-litter birth weight variation and concomitantly a high number of low birth weight piglets (< 1.0 kg at birth) (2). The occurrence of LBW piglets is a major threat to pig production resulting in economic losses for two reasons. First of all, these piglets are characterized by increased mortality and morbidity (27). In addition, surviving LBW piglets show reduced zootechnical performances in the postnatal period (28).

In neonatal piglets, energy is stored as glycogen in muscle and liver and as lipids in adipose tissue but the total amount of body lipids in newborn piglets is low and ranges from 1 to 2% of body mass (348). During the last decades, selection for leaner meat resulted in piglets with smaller organs and consequently less glycogen deposition (349).

Crushing is the most common cause of death during the suckling period and is part of a hypothermia and starvation complex (350, 351). Chilled hungry piglets are more likely to be in risky areas at the udder. Because neonatal piglets have limited capacity to oxidize protein, mobilization of glycogen and fat is essential for survival immediately after birth (352). Additionally, it has previously been shown that insufficient energy supply from degraded glycogen or ingested colostrum and milk are (as part of the hypothermia and starvation complex) important causes of death before weaning (71).

Reports on LBW piglets' body compositions at birth are contradictory (59, 353, 354). At slaughter age, various studies suggest that LBW piglets have gained more fat (22, 61) that is also present within muscles (355).

The effect of birth weight on body energy stores in suckling piglets has been poorly studied. However, knowledge on the biological consequences of intrauterine growth restriction is required in order to adequately raise LBW piglets.

Therefore, chemical total body composition and intramuscular lipid and glycogen deposition of piglets were evaluated in relation to their birth weights and at various ages during the suckling period.

3. MATERIAL AND METHODS

3.1. Animal selection

Institutional and national guidelines for the care and use of animals were followed and all experimental procedures involving animals were approved by the Ethical Committee of Animal Experimentation, University of Antwerp, Belgium. A total of 64 crossbred piglets (Piétrain x (Finnish Yorkshire x stress negative Belgian Landrace)) were selected from 2nd to 4th parity sows, which had equal litter sizes of 12 piglets. From 32 litters, pairs of LBW (0.96 ± 0.06 kg at birth) and NBW (1.49 ± 0.12 kg at birth) piglets were selected at birth and euthanized at day 0 (n = 16), whereas the other piglets remained suckling the sow until day 3 (n = 16), day 10 (n = 16) or day 28 (n = 16). Piglets with a birth weight below 1 kg were considered to be LBW, whereas those with a birth weight close to the mean of the litter were considered to be NBW. Each age-group consisted of 16 gender-matched piglets, i.e. 2 littermate piglets (LBW and NBW) from 8 different litters. Of each age-group 10 piglets, i.e. 5 littermate LBW and NBW piglets were sacrificed for organ and tissue sampling. All intestinal organs were emptied and rinsed before weighing. Of each age-group 6 piglets, i.e. 3 littermate

LBW and NBW piglets were euthanized for chemical body composition analysis. Euthanasia was performed by intraperitoneal (IP) injection of an overdose sodium pentobarbital (200 mg/kg, Kela Laboratoria, Hoogstraten, Belgium) followed by exsanguination.

3.2. Histochemistry

Samples were taken from the pork ham (*m. semimembranosus*), snap frozen in liquid nitrogen and stored at -80°C. Cryostat sections (10 µm) were air-dried for 30 min and fixated in formaldehyde-calcium for 30 min. Slides were stained in Oil Red O solution for 20 min. Stock solution was prepared by adding 500 mg Oil Red O dye to 100 ml isopropanol (98%). Afterwards the sections were dedifferentiated in 60% isopropanol for 10 min and washed in distilled water. The lipid deposition was determined using image analysis (phase analysis, Olympus BX 61, analySIS Pro®, Aartselaar, Belgium). Lipid deposition was estimated by calculating the area (mm^2) that showed lipid deposition per area of muscle tissue (i.e. section area) (using 20x objective).

3.3. Glycogen contents

To hydrolyse glycogen to glucose, 25 mg of muscle samples were heated with 1 ml of 1 *M* HCl at 100°C for 2 h in sealed glass tubes. Subsequently, the contents were centrifuged at 2500 *g* for 20 min at 4°C. Glucose was measured spectrophotometrically at 450 nm (Tecan sunrise, Tecan Group Ltd., Männedorf, Switzerland). Muscle glycogen was expressed as µmol of glucose residues per g of wet muscle weight (356).

3.4. Chemical composition

After euthanasia, 6 piglets (i.e. 3 littermate LBW and NBW piglets) per age group were frozen in aluminium containers for chemical total body composition analysis. Carcasses were autoclaved for 24 h and mixed. Representative samples of each animal were taken and lyophilized. Chemical total body composition was determined according to the Association of Official Analytical Chemists methods (357). Crude ash, crude fat and crude protein contents were determined by ashing, Soxhlet extraction with petroleum ether and the Kjeldahl method, respectively. The obtained data were corrected for moisture gain or loss during autoclaving.

3.5. Statistical analysis

A linear mixed model was fitted to model the data. The starting model included birth weight, age, gender and their pairwise interactions as fixed effects. To account for the dependence between observations within the same litter and animal, random effect terms for litter and animal, nested within litter, were added to the model. The starting model was simplified using stepwise backward modelling, testing the significance of the fixed effect terms using an F-test with Kenward-Roger correction for the number of degrees of freedom. In case the effect of age was significant, a post-hoc test was performed with a Tukey correction for multiple testing. The modelling was performed using the statistical package R, version 2.13.1, and add-on packages lme4, pbkrtest and multcomp. In case data were non-normal, calculations were performed on log-transformed data. Results represented in tables and figures represent the non-transformed data.

4. RESULTS

The effects of age and birth weight on body and organ weights are presented in Tables 2.1 and 2.2. Because of the differences in final body weights, both the absolute weight of the internal organs (Table 2.1) and the weight relatively to the piglets' final body weights (Table 2.2) are expressed. With aging, body weights and absolute organ weights increased ($P < 0.01$). LBW piglets had lower body weights and absolute organ weights compared to NBW piglets ($P \leq 0.05$) (Table 2.1). Age had an influence on relative caecum, kidney, pancreas and spleen weights ($P < 0.01$). Being born small resulted in relatively heavier stomachs, small intestines and livers ($P < 0.05$) (Table 2.2).

Table 2.1 Body weights and absolute organ weights of the different age groups of piglets (day 0, day 3, day 10, day 28) [1,2].

		DAY 0	DAY 3	DAY 10	DAY 28		*P*-value	
		Weight (g)				SEM	Birth weight	Age
Body weight (g)	LBW	841[a]	1008[a]	2466[b]	5308[c]	252	< 0.01	< 0.01
	NBW	1740[a]	1734[a]	3860[b]	8210[c]	253		
Stomach weight (g)	LBW	5.43[a]	6.65[a]	22.1[b]	36.1[c]	1.48	< 0.01	< 0.01
	NBW	9.77[a]	11.9[a]	15.7[b]	48.3[c]	2.49		
Small intestinal weight (g)	LBW	33.5[a]	39.4[a]	88.4[b]	196[c]	9.76	< 0.01	< 0.01
	NBW	64.9[a]	69.1[a]	138[b]	269[c]	11.6		
Colon weight (g)	LBW	7.40[a]	11.6[a]	15.6[a]	61.4[b]	7.39	< 0.01	< 0.01
	NBW	15.1[a]	19.5[a]	26.5[a]	78.1[b]	7.61		
Caecum weight (g)	LBW	0.80[a]	0.64[a]	2.23[b]	11.2[c]	0.32	0.05	< 0.01
	NBW	1.48[a]	1.42[a]	3.52[b]	13.2[c]	1.2		
Liver weight (g)	LBW	21.9[a]	28.8[a]	69.8[b]	148[c]	8.26	< 0.01	< 0.01
	NBW	43.1[a]	55.8[a]	103[b]	211[c]	7.3		
Kidney weight (g)	LBW	4.49[a]	4.43[a]	8.60[b]	17.2[c]	0.81	< 0.01	< 0.01
	NBW	8.43[a]	8.28[a]	14.5[b]	23.4[c]	1.11		
Pancreas weight (g)	LBW	1.15[a]	2.03[a]	3.97[b]	7.13[c]	0.71	< 0.01	< 0.01
	NBW	2.27[a]	4.03[a]	6.47[b]	10.5[c]	0.54		
Spleen weight (g)	LBW	1.03[a]	1.37[a]	5.74[b]	15.3[c]	1.4	< 0.01	< 0.01
	NBW	2.24[a]	2.95[a]	8.87[b]	23.9[c]	1.13		

[a-c] Within birth weight group, means not sharing a common superscript letter differ significantly ($P < 0.05$).

[1] LBW = low birth weight; NBW = normal birth weight.

[2] Values are presented as means and pooled SEM (n = 5/group).

Table 2.2 Relative organ weights of the different age groups of piglets (day 0, day 3, day 10, day 28) [1,2].

		DAY 0	DAY 3	DAY 10	DAY 28		*P*-value	
			% of body weight			SEM	Birth weight	Age
Stomach weight (g/kg)	LBW	6.58	6.94	6.69	6.87	0.34	< 0.01	0.96
	NBW	4.41	6.63	5.66	5.88	0.67		
Small intestinal weight (g/kg)	LBW	39.8	37.7	38.1	37.7	2.95	0.04	0.73
	NBW	37.3	35.8	36.2	33.0	2.31		
Colon weight (g/kg)	LBW	8.56	10.9	6.60	12.8	1.75	0.44	0.47
	NBW	8.68	10.5	7.40	9.44	1.13		
Caecum weight (g/kg)	LBW	0.92^{a}	0.62^{a}	0.95^{a}	2.14^{b}	0.16	0.42	< 0.01
	NBW	0.87^{a}	0.75^{a}	0.98^{a}	1.57^{b}	0.13		
Liver weight (g/kg)	LBW	26.2	30.4	28.8	27.9	1.84	0.02	0.15
	NBW	24.9	29.0	26.4	25.9	1.06		
Kidney weight (g/kg)	LBW	5.32^{a}	4.29^{b}	3.50^{b}	3.28^{c}	0.29	0.28	< 0.01
	NBW	4.91	3.93	3.92	2.86	0.23		
Pancreas weight (g/kg)	LBW	1.42^{a}	1.66^{b}	1.79^{ab}	1.37^{ac}	0.25	0.61	< 0.01
	NBW	1.35^{a}	2.12^{b}	1.77^{ab}	1.29^{ac}	0.11		
Spleen weight (g/kg)	LBW	1.30^{a}	1.36^{a}	2.23^{b}	2.82^{c}	0.17	0.40	< 0.01
	NBW	1.25^{a}	1.54^{a}	2.47^{b}	2.93^{c}	0.23		

[a-c] Within birth weight group, means not sharing a common superscript letter differ significantly ($P < 0.05$).

[1] LBW = low birth weight; NBW = normal birth weight.

[2] Values are presented as means and pooled SEM (n = 5/group).

For none of the tested chemical body composition parameters, birth weight differences could be observed ($P > 0.05$). As expected, dry matter, protein and fat percentages increased with aging ($P < 0.01$) (Table 2.3). In contrast to the previous parameters, ash percentage did not differ significantly ($P > 0.05$) (Table 2.3). In each age and birth weight group, lipid droplets were located within (intracellular) and between muscle fibres (extracellular) (Fig. 2.1). The mixed model analysis indicated that neither birth weight, nor age ($P > 0.05$) had a significant effect on the number of lipid droplets (data not shown).

Table 2.3 Chemical body composition of the different age groups of piglets (day 0, day 3, day 10, day 28) [1,2].

		DAY 0	DAY 3	DAY 10	DAY 28		*P*-value	
		Content (%)				SEM	Birth weight	Age
Dry matter	LBW	20.93[a]	22.41[a]	27.82[b]	30.30[b]	1.53	0.14	<0.001
	NBW	18.35[a]	22.56[a]	26.33[b]	27.77[b]	1.37		
Protein	LBW	13.43[a]	13.86[ab]	15.30[ab]	16.56[b]	0.54	0.18	< 0.001
	NBW	13.36[a]	14.37[ab]	14.34[ab]	14.76[b]	0.40		
Fat	LBW	2.96[a]	4.69[a]	8.89[b]	10.34[b]	1.38	0.56	0.02
	NBW	1.98[a]	4.01[a]	8.72[b]	9.73[b]	1.62		
Ash	LBW	3.33	3.25	2.92	3.28	0.16	0.54	0.73
	NBW	2.81	3.34	3.12	3.11	0.28		

[a-c] Within birth weight group, means not sharing a common superscript letter differ significantly ($P < 0.05$).

[1] LBW = low birth weight; NBW = normal birth weight.

[2] Values are presented as means and pooled SEM (n = 5/group).

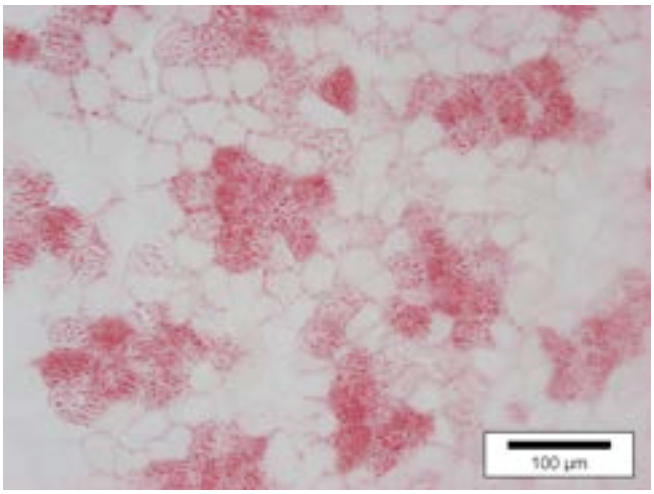

Figure 2.1 Oil red O stained semimembranosus sections from a NBW piglet of 10 days of age.

There was a significant interaction between birth weight and age regarding muscle glycogen content ($P < 0.05$). Qualitatively, the graph shows that glycogen content decreased with increasing age in both the LBW and NBW group, but the differences between age groups are more pronounced in the NBW piglets (Fig. 2.2). In the LBW group, 28 d-old piglets had significantly lower values compared to 0 and 3 d-old piglets ($P < 0.01$). Moreover, 10 d-old piglets had decreased glycogen values compared to 0 d-old piglets ($P = 0.05$). In the NBW group, significant differences between all groups were present ($P < 0.01$), except between 0 and 3 d-old piglets. For none of the performed analyses gender-dependent differences were observed ($P > 0.05$).

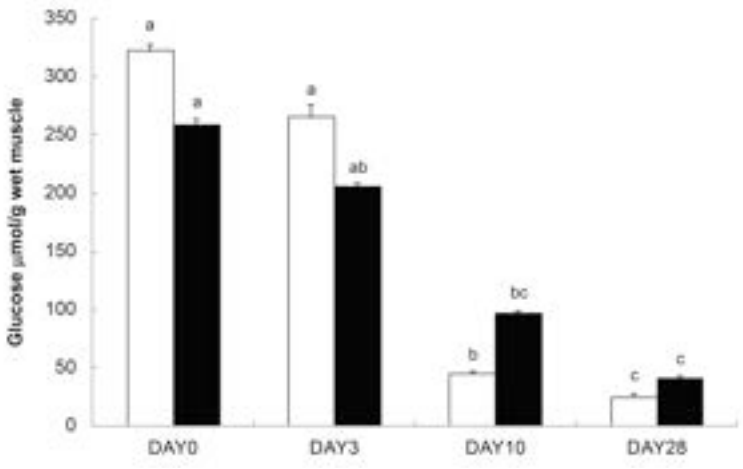

Figure 2.2 Muscle glycogen contents expressed as µmol glucose residues per g wet muscle of the different age groups of piglets (day 0, day 3, day 10, day 28). White bars represent NBW piglets and black bars represent LBW piglets. Values represent means and SEM, n = 5/group. Within birth weight group, means not sharing a common superscript letter differ significantly ($P < 0.05$).

5. DISCUSSION

Piglets are, in contrast to human neonates, born with low total body fat contents. Because sow milk contains high amounts of fat, the neonatal piglet transfers much of the milk-fat to its body lipid stores (358). In our study, in which piglets were sampled during the suckling period, body weights, absolute organ weights, dry matter, fat and protein percentages increased. Ash content remained constant during growth. These age-related observations correspond to previously reported data (359, 360).

In the present study, body and absolute organ weights were lower in LBW piglets. As reported previously, the relative weights of some organs were higher in LBW piglets (311, 313, 361). This could be explained by the thrifty phenotype hypothesis and its associated mechanisms (362). This hypothesis states that poor nutrition in early life initiates a sparing response by selective

preservation of key organs e.g. the brain (362). In this respect, the present study showed that poor foetal nutrition significantly influenced the weights of some metabolically active organs such as the gastrointestinal tract and as such also the liver in piglets. When nutrition becomes more abundant in the postnatal compared to the prenatal period, the prenatal changes in the foetus may lead to a nutritional mismatch between energy intake, storage and expenditure. As such the development of the adipocyte is affected in utero, with increasing capacity to form new fat cells in adipose depots or to store more lipids in existing adipocytes during postnatal development when nutrition was impaired in early - prenatal - development (363). However, birth weight-related differences for the chemical body composition analyses were not observed, and more specifically no difference in fat percentages were noticed. This agrees with previous reports in which piglets of different birth weight categories (0.5 - 1.86 kg) were examined at birth and 10 d of age (353, 354). However, a more recent study showed that neonatal LBW piglets (< 1.20 kg) contained less fat and dry matter compared to piglets with middle and heavy birth weight (59). In contrast to our study, the latter excluded piglets with a birth weight below 800 g. However, one should keep in mind that outcomes may differ when comparing pigs that are slaughtered at the same age or at the same weight. In addition, differences amongst studies may be attributed to the genetic dam lines used. At slaughter, some studies showed that carcasses of LBW piglets contain more adipose tissue (22, 59, 61). It has repeatedly been described that LBW piglets and infants show compensatory growth, i.e. accelerated growth, when being fed *ad libitum* after a period of nutritional stress (e. g. intrauterine growth restriction) (364-366). This catch-up growth has been associated with increased adult adiposity (367). The same effect is seen in LBW infants who frequently develop obesity in later life due to postnatal overnutrition concomitant with a

reduction of energy expenditure (365). Thus, both animal and human observations support the concept of 'foetal programming' as the origin of obesity (368, 369). Additionally, when intrauterine nutritional conditions are suboptimal, metabolic and endocrine shifts in the foetus result in altered postnatal metabolism (370, 371). In our study, LBW piglets did not became fatter than NBW piglets, possibly because in suckling piglets the feed intake is largely controlled by the sow and competition among littermates. This implies that the increased fatness that is observed in LBW piglets at slaughter age is not initiated during the suckling period but during the post-weaning period.

Similar as the total body fat content, no birth weight effects could be observed regarding intramuscular fat in the semimembranosus muscle. In contrast, Karunaratne et al. reported higher intramuscular fat contents in the semitendinosus muscles of the smallest foetuses compared to their larger littermates (372). This suggests a prenatal development of adipocytes at the expense of muscle fibres. Indeed, it has been described several times that the total number muscle fibers (especially secondary) is reduced in LBW piglets compared to NBW littermates (28, 57, 373). Apparently, study results depend on the muscle monitored. When slaughter age is reached, intramuscular fat has been reported to be unchanged in the longissimus and rhomboideus muscles, whereas intramuscular fat increased in the semitendinosus muscles of LBW piglets compared to heavy neonatal piglets (22, 28). The higher amount of intramuscular lipids in LBW piglets at slaughter age is most likely due to the fact that LBW piglets attain their maximum muscle fibre growth potential earlier and therefore use feed energy for fat deposition.

The muscle glycogen values measured in our study correspond to previously reported data (356, 374, 375). In our study, the glycogen contents decreased with increasing age implicating that glycogen is mobilised for

thermoregulation during the suckling period (110) and for physical activity in order to reach the udder (376). Moreover, we observed that the differences in glycogen content were more pronounced in the NBW group. Muscle glycogen contents were not affected by piglets' birth weights, which is in accordance with a previous study that examined piglets shortly after birth (375). According to a previous study the rate of glycogen mobilization is similar in piglets of different birth weights until 20 - 36 h of age (377), which is in agreement with our study. After 3 d of age we observed that the glycogen concentrations were depleted faster in NBW piglets, suggesting that glycogen mobilization is easier in case piglets are heavier.

Although in literature no dietary effects were observed on muscle glycogen concentration (375, 377), previous studies described that neonatal survival, and in particular survival of LBW piglets, may be improved by enlarging the energy pools at birth by supplementing the sow with medium-chain triglycerides during gestation (65). Nissen and Oksbjerg recently reported that LBW piglets do not have a lower requirement for dietary protein. Further studies are needed to elucidate the effect of dietary changes, e.g. feeding piglets on formulated milk, on body composition and muscle energy stores (378).

In conclusion, piglets deposit fat soon after birth, with increasing contents with aging. Intramuscular fat contents in the m. semimembranosus did not change with age nor birth weight, whereas intramuscular glycogen stores are rapidly depleted after birth in both LBW and NBW piglets.

CHAPTER 3
ARTIFICIAL REARING OF PIGLETS

CHAPTER 3A

ARTIFICIAL REARING OF PIGLETS: EFFECTS ON SMALL INTESTINAL MORPHOLOGY AND DIGESTION CAPACITY

Adapted from: **M. De Vos**, V. Huygelen, S. Willemen, E. Fransen, C. Casteleyn, S. Van Cruchten, J. Michiels, C. Van Ginneken. Artificial rearing of piglets: effects on small intestinal morphology and digestion capacity, Livestock Science 159 (2014) 165-173

1. ABSTRACT

The use of hyper-prolific sows results in large litters but also leads to an increasing number of supernumerary and underprivileged (e.g. low birth weight (LBW)) piglets. The effects of artificial rearing on the growth, small intestinal morphology and digestion capacity of these piglets remain unclear. Therefore, the aim of this study was to assess the effect of sow-feeding versus formula-feeding on piglets' structural and functional gut maturation. To this purpose, pairs of LBW and normal birth weight (NBW) piglets (n = 40) were allocated to four treatment groups. Groups 1 and 2 contained piglets that suckled until either d 10 or 28 of age, respectively. Groups 3 and 4 contained animals that suckled until 3 d of age and were then formula-fed until either d 10 or 28. During d 3 to 10, formula-fed piglets showed reduced average daily gain (ADG; -112 g/d) and lactase activities (-4.50 U/g tissue) compared to suckling piglets ($P < 0.01$). In contrast, animals that were formula-fed until d 28 had a comparable ADG compared to sow-fed pigs. In addition, formula-fed piglets had a greater absorptive area ($P < 0.01$; +59.1 μm^2), maltase and sucrase activities ($P < 0.05$; +0.97 and +0.23 U/g tissue) and deeper crypts ($P < 0.03$; +42.5 μm) compared to suckling piglets. In general, the differences between LBW and NBW piglets were scarce. These results suggest that the combination of *ad libitum* access to formulated milk and an increased capacity to absorb nutrients makes artificial rearing a good alternative to raise supernumerary and/or LBW piglets.

2. INTRODUCTION

The hyper-prolificacy of modern hybrid sows has resulted in a high number of piglets per litter (2, 379), often even higher than the number of available functional teats. Unfortunately, these larger litters are characterized by high within-litter birth weight variation and consequently greater mortality and lower growth rates of undersized piglets (9, 59). Additionally, the sow milk yield is insufficient to achieve the maximum growth potential of these larger litters (7). Thus, in contrast to the initial goal of increasing sows prolificacy, increasing litter size can negatively affect profitability. Therefore, farmers seek solutions to assure piglet survival and optimize their growth. According to a recent survey, cross-fostering and supplementing piglets is practiced in almost every (Belgian) pig farm (10). Additionally, 56% of the pig farmers euthanize the weakest piglets and 31% of the pig farmers apply split-weaning, amongst which artificial rearing. These data illustrate the negative consequences of hyper-prolificacy and their implications on animal welfare and health. Moreover, an understanding of the interventions that are practiced and their impact on growth and development of piglets is missing. This is, however, a prerequisite for a scientifically based rearing strategy of supernumerary or LBW piglets. Up to now, a limited number of studies on artificial rearing have been conducted (322, 380, 381), which makes it difficult to assess the effect of artificial rearing on the growth and health of piglets. Nevertheless, the value of these rearing systems will become increasingly important because litter sizes, and consequently the number of supernumerary piglets, are increasing.

Therefore, the objective of our study was to investigate the growth performance and the structural and functional characteristics of the small intestine, in artificially reared versus suckling piglets of different weight categories (LBW vs. NBW) and at various points in time (d 10 and d 28 of age).

3. MATERIAL AND METHODS

3.1. Animal experiments

A total of 20 crossbred (Piétrain x (Finnish Yorkshire x stress negative Belgian Landrace)) pairs of NBW (1.48 ± 0.11 kg at birth) and LBW piglets (0.87 ± 0.04 kg at birth) were selected from 10 litters at a local farm. All piglets (n = 40), i.e. 20 gender matched pairs of LBW and NBW piglets were allotted to 4 groups. Sow-fed piglets remained at the farm and were either euthanized at d 10 of age (n = 10) or d 28 of age (n = 10). The other piglets were separated from the sow at d 3 of age and were subsequently artificially reared using a commercial milk formula until d 10 (n = 10) or d 28 (n = 10). The formula-feeding was started at d 3 after birth to allow piglets to ingest sufficient amounts of colostrum. These piglets were penned in a commercial brooder where they were provided with *ad libitum* access to formulated milk and water via a nipple system. The formula used in this study was a complete milk replacer for young piglets, allowing rearing piglets in absence of the sow. The commercial diet was composed of skimmed milk powder, whey powder, coconut fat, palm oil and wheat concentrate and nutritional additives such as amino acids, minerals and vitamins (calculated analysis: 28 g crude protein, 23 g crude fat, 56 g lactose per l and calculated ME 548 kcal/l).

Piglets were group-housed without separating littermates to avoid additional stress and to enable natural competition between light and heavier piglets. The ambient temperature was set at 28 to 30°C and gradually reduced to 22°C at the age of d 28. The pens were provided with a heat lamp (250 W) during the first wk of artificial rearing to create a temperature of 30°C inside the brooder. The entire feeding system (milk tank, pipeline system and nipples) was cleaned and disinfected twice weekly (Cid Lines N.V., Ieper, Belgium). The first time-point for evaluating the effect of BW and diet was set at d 10 after birth because at that point of time sows milk production starts to become a limiting factor for piglets' growth (7). A second time-point was chosen to observe piglets at the end of a normal suckling period of 4 wk. In both housing settings, piglets had free access to water and creep feed. Institutional and national guidelines for the care and use of animals were followed and all experiments involving animals were approved by the Ethical Committee of Animal Experimentation, University of Antwerp, Belgium.

3.2. Milk intake

Milk intake was estimated with the weigh-suckle-weigh technique (382). To this purpose, piglets were isolated from the sow or the automatic brooder one h before suckling (fasting period). Piglets were weighed individually at the end of the fasting period and admitted to the sow or brooder. When suckling was completed, piglets were weighed again and fasted prior to next measurements. Six suckle cycles were recorded with suckling intervals of 75 min. Milk intake was estimated at 3 time points before euthanasia at 28 d of age (d 5, d 9 and d 16). Daily milk intake (l/piglet/d) was calculated as the average milk intake per suckling, multiplied by 60 min/h and by 24 h/d and divided by 75 min per suckling. Because sow milk and milk formula were

different in energy content, the gross energy intake of each piglet (kcal/piglet/d) was calculated by multiplying daily milk intake (l/piglet/d) by the energy content of the ingested feed (kcal/l).

3.3. Sample collection

At d 10 or 28, piglets were weighed and euthanized with an overdose of sodium pentobarbital (200 mg/kg, IP) followed by exsanguination. The intestinal organs were emptied of their contents, rinsed, weighed and the lengths of small and large intestines were determined. The small intestine was divided into three equally long segments (proximal, middle and distal) and from the middle of each segment tissue samples were taken. Samples were either snap frozen in liquid nitrogen and stored at -80°C or fixated in 4% paraformaldehyde solution for 2 h (pH 7.4) at room temperature. After fixation, samples were rinsed with phosphate-buffered saline solution (pH 7.4, PBS) for 24 h and further processed for paraffin embedding.

3.4. Histology

Transverse paraffin sections (5 µm) were stained with hematoxylin-eosin (HE) and processed for morphometric measurements (the height and width of the intestinal villi, the depth of the crypts and the thickness of tela submucosa and the tunica muscularis). Measurements were performed for each tissue block in 30 longitudinally cut villi and adjacent crypts (Olympus BX 61, analySIS Pro®, Aartselaar, Belgium).

Assuming that villi resemble a cylindrical shape (301), villus surface area was calculated using the following equation:

$$\text{Villus surface area} = 2\pi \times \left(\frac{\text{villus width}}{2}\right) \times \text{villus height}$$

Villi are considered as approaching a cylindrical shape (301). Accounting for the variable villus shape and position in which each villus is sectioned, the mid-villus width was used in this equation.

3.5. Bacterial screening

Additional sections of the small intestine were cut in order to perform a gram staining and an immunohistochemical lipopolysaccharide (LPS)-staining. Adherent gram-positive bacteria to the small intestinal mucosa were visualized by the gram-staining. In order to visualize gram-negative bacteria, an immunohistochemical staining for LPS-containing organisms was applied. Paraffin sections were deparaffinised, rehydrated and subjected to antigen retrieval with TRIS-EDTA (pH 9, DakoCytomation, Glostrup, Denmark). Non-specific staining was blocked by incubation in 3% H_2O_2 and 10% normal serum. Subsequently, sections were incubated with anti-LPS (Anti-LPS, Acris antibodies, Hiddenhausen, Germany). Immune complexes were visualised following application with a commercial preconjugated enzyme complex (Envision, DakoCytomation) and a staining substrate (3,3'-diaminobenzidinetetrahydrochloride or 3-Amino, 9-ethyl-carbazole). Between incubations, sections were washed in Tris-buffered saline. All dilutions were made in 0.01% Tris-buffered saline (pH 7.4) with 0.3% Triton-X100, except for H_2O_2 where Triton-X100 was omitted.

Intestinal sections were considered colonized when at least one cluster of either gram-positive bacteria (gram-staining) or gram-negative (LPS-staining) was seen in the lower 2/3 of the intervillous space while scanning the entire circumference of three tissue sections (383).

3.6. Enzyme assays

Frozen samples were homogenized using a 1% Triton X-100 solution. Enzymatic activities of aminopeptidase A, aminopeptidase N and dipeptidylpeptidase IV were kinetically measured using Glu-p-nitroaniline (Sigma-Aldrich, St. Louis, USA), L-alanine 4-nitro-anilide hydrochloride (Sigma-Aldrich, St. Louis, USA) and H-glycyl-prolyl-p-nitroaniline p-tosylate (Bachem AG, Bubendorf, Switzerland) as substrates, respectively. Lactase, maltase and sucrase activities were determined with lactose (Sigma-Aldrich, St. Louis, USA), maltose (Sigma-Aldrich, St. Louis, USA) and sucrose (Sigma-Aldrich, St. Louis, USA) as substrates, respectively. Enzyme activities are expressed as units per gram wet tissue. One unit of activity is defined as the amount of enzyme that hydrolyses 1 μmol substrate per min.

3.7. Statistical analyses

The effect of diet, birth weight, gender, age and intestinal region on the different parameters was studied by fitting linear mixed models. We accounted for the dependence between observations within the same litter and (if applicable) within the same individual by including random effects for litter and for individual, nested within litter. Diet, birth weight, age and (if applicable) region or gender were included as fixed affects, as well as their pairwise interactions.

The fixed effect model was simplified in a stepwise backward way. Significance of the fixed effects was tested by performing an F-test between the larger model and the reduced model, adjusting the number of degrees of freedom using the Kenwardroger method. In case the final model included a significant effect of region, a post-hoc test was performed to test which of the categories has a different mean outcome, with a Tukey correction for multiple testing. All statistical calculations were performed in the software package R version 2.13.1. Mixed models were fit using the lme4 package. The F-test with Kenwardroger correction was performed using the package pbkrtest, and the post-hoc test with Tukey correction was carried out as implemented in the multcomp package.

4. RESULTS

4.1. Growth and milk intake

Overall, NBW piglets had an ADG of 69.7 g/d higher compared to LBW piglets ($P < 0.01$). There was an interaction between diet and age. Between d 3 and d 10, the ADG of sow-fed piglets was on average 112.0 g/d higher compared to the formula-fed piglets ($P < 0.01$) (Table 3.1). Between d 3 and d 28, on the other hand, there was no significant difference in ADG between sow-fed and formula-fed piglets ($P = 0.09$ - on average, sow-fed pigs had an ADG of 57 g/d lower than formula-fed piglets) (Table 3.1).

In this study, creep feed consumption started when the piglets were 10 d of age. Although not measured quantitatively, we observed that the intake of creep feed was generally negligible. Additionally, individual creep feed consumption did not appear to be influenced by birth weight or housing system. Furthermore, no significant effect of birth weight could be observed

for the absolute or relative energy intake (P > 0.22). Absolute energy intake from milk (kcal/piglet/d) showed a significant interaction between diet and age ($P < 0.01$). In the formula-fed group, there was a significant effect of age on energy intake ($P < 0.01$), with a linear increase of intake with age (d 5 < d 9 < d 16). No significant increase was observed in the sow-fed group ($P = 0.07$). When taking the body weight of the piglets into account, the relative energy intake (kcal/kg BW/d) showed a significant main effect of the ingested feed. Sow-fed piglets had significantly reduced relative energy intake ($P = 0.01$; mean difference = 57.9 kcal/kg BW/d), compared to formula-fed piglets. There were no significant interactions, so this difference was observed regardless of age or weight.

Table 3.1 Birth weight, body weight and growth of 10-d old and 28-d old formula-fed and sow-fed LBW and NBW piglets [1,2].

	FOR		SOW		
	LBW	NBW	LBW	NBW	SEM
D 10					
Birth weight (kg)	0.87[a]	1.48[b]	0.87[a]	1.48[b]	0.09
Body weight (kg)	1.80[a*]	2.74[b*]	2.40[a]	3.77[b]	0.18
Average daily gain (g/d)	90.3[a*]	115[b*]	168[a]	261[b]	23.4
D 28					
Birth weight (kg)	0.87[a]	1.48[b]	0.87[a]	1.48[b]	0.09
Body weight (kg)	6.22[a]	9.67[b]	5.31[a]	8.21[b]	0.45
Average daily gain (g/d)	228[a]	302[b]	163[a]	251[b]	24.4

[a,b] Within feeding group, means not sharing a common superscript letter differ significantly ($P < 0.05$). An asterisk refers to a significant difference from the SOW-group ($P < 0.05$).

[1] FOR = formula-fed piglets; SOW= sow-fed piglets; LBW = low birth weight piglets; NBW = normal birth weight piglets.

[2] Values are presented as means and pooled SEM (n = 5).

4.2. Internal organ characteristics

When analysing the final body weights the interaction between birth weight and age ($P < 0.01$) and between diet and age ($P < 0.01$) needed to be taken into account; meaning that the differences between LBW and NBW, and between sow- and formula- feeding, are not the same in the d 10 and d 28 group. Splitting the data into a d 10 and a d 28 group showed that LBW piglets were lighter than NBW piglets ($P < 0.01$) and 10 d-old formula-fed piglets weighed less than sow-fed piglets ($P < 0.01$). At 28 d of age, NBW piglets were still heavier than LBW piglets ($P < 0.01$), whereas there was no longer a significant difference between the diets ($P = 0.07$) (Table 3.1). Because of the differences in final body weights, the anatomy of the internal organs is being expressed relatively to the piglets' final body weights.

Both the small intestine and colon were relatively longer for LBW piglets compared to NBW piglets ($P < 0.01$). The relative weights of the stomach ($P < 0.01$), small intestine ($P < 0.01$) and caecum ($P = 0.04$) were greater for LBW piglets compared with those of the NBW piglets (Table 3.2). As for the small intestinal and colon length and the stomach weight there was a significant interaction between age and diet ($P < 0.01$). At 10 d of age, the lengths of both the small intestine and colon, and the stomach weights were greater in the formula-fed group compared to the sow-fed group ($P < 0.01$). At 28 d of age, there was no longer a significant difference between the two groups ($P > 0.11$) (Table 3.2).

Table 3.2 Organ to body weight ratios from 10-d old and 28-d old formula-fed and sow-fed LBW and NBW piglets [1,2].

	FOR		SOW		
	LBW	NBW	LBW	NBW	SEM
D 10					
Stomach weight (g/kg)	9.05[a*]	7.65[b*]	6.49[a]	5.89[b]	0.64
Small intestinal weight (g/kg)	40.4[a]	33.7[b]	36.9[a]	36.7[b]	3.56
Small intestinal length (cm/kg)	290[a*]	212[b*]	222[a]	169[b]	14.80
Colon weight (g/kg)	13.5	16.3	6.55	7.02	1.74
Colon length (cm/kg)	49.4[a*]	36.1[b*]	41.1[a]	26.4[b]	2.31
Caecum weight (g/kg)	1.20[a]	1.03[b]	0.93[a]	0.93[b]	0.17
D 28					
Stomach weight (g/kg)	6.37[a]	5.23[b]	6.87[a]	5.88[b]	0.37
Small intestinal weight (g/kg)	53.0[a]	42.1[b]	37.7[a]	33.0[b]	3.02
Small intestinal length (cm/kg)	154[a]	117[b]	164[a]	116[b]	14.3
Colon weight (g/kg)	12.3	12.2	11.8	9.44	2.25
Colon length (cm/kg)	23.9[a]	17.1[b]	23.6[a]	17.0[b]	2.13
Caecum weight (g/kg)	1.72[a]	1.44[b]	2.14[a]	1.57[b]	0.21

[a,b] Within feeding group, means not sharing a common superscript letter differ significantly ($P < 0.05$). An asterisk refers to a significant difference from the SOW-group ($P < 0.05$).

[1] FOR = formula-fed piglets; SOW= sow-fed piglets; LBW = low birth weight piglets; NBW = normal birth weight piglets.

[2] Values are presented as means and pooled SEM (n = 5).

4.3. Small intestinal structural characteristics

Villus height did not differ between LBW and NBW piglets ($P = 0.43$) (Table 3.3). There was a significant interaction between diet and age ($P = 0.02$) and between region and age ($P < 0.01$) for the height of the intestinal villi, meaning that the effect of diet and region differs between d 10 and d 28. At 10 d of age, there were no significant effects of diet or region ($P > 0.08$). At 28 d of age, no significant effect of the diet was observed ($P = 0.40$), but there was a difference between the regions with mean villus height decreasing from proximal towards the distal part of the intestine ($P < 0.01$).

In addition to villus height, villus width did not differ between the two birth weight categories ($P = 0.36$). However, a significant interaction was observed between diet and age ($P = 0.02$). At 10 d of age, no effect of the diet was observed ($P = 0.71$), whereas at 28 d of age, formula-feeding led to wider villi ($P < 0.01$) in comparison with sow-feeding (Table 3.3).

For the calculated villus area, no effect on piglets' birth weight could be noticed ($P = 0.14$) but we observed a significant interaction between diet and age ($P < 0.01$). At 10 d of age, no significant differences in villus area were observed for diet ($P = 0.12$). However, at 28 d of age, formula-feeding resulted in greater villus area compared to sow-feeding ($P < 0.01$).

For the crypt depths, a significant interaction was observed between diet and weight ($P = 0.02$) and between age and weight ($P = 0.04$). This means that the difference in crypt depth between sow- and formula-feeding differs between LBW and NBW piglets and that the difference in crypt depth between d 10 and 28 differs between LBW and NBW piglets. For the LBW piglets, formula-fed animals had significantly deeper crypts compared to sow-fed piglets ($P < 0.01$); for the NBW piglets the difference between formula- and sow-feeding was smaller but still significant ($P = 0.03$) (Table

3.3). In LBW piglets, the difference in crypt depth between d 10 and d 28 is significant ($P < 0.01$), with 28-d old piglets having deeper crypts than 10-d old piglets. In NBW piglets, there is no longer a significant difference ($P = 0.06$) (Table 3.3).

There was a significant interaction for the tela submucosa thickness between diet and weight ($P = 0.02$) and between age and weight ($P < 0.01$). The effect of the diet does not reach significance in the separate weight groups ($P > 0.08$). In the LBW group, pigs of 28 d have a significantly thicker tela submucosa compared to pigs of 10 d ($P = 0.03$). In the NBW group, there was no significant age effect ($P = 0.07$). Only a main effect of age was observed for the tunica muscularis thickness ($P = 0.02$), with a larger value at 28 d of age compared to 10 d of age.

Table 3.3 Histology of the small intestine from 10-d old and 28-d old formula-fed and sow-fed LBW and NBW piglets [1-3].

	FOR		SOW		
	LBW	NBW	LBW	NBW	SEM
D 10					
Villus height (μm)	361.89	419.54	487.31	490.75	7.27
Villus width (μm)	87.96	87.18	83.38	89.06	1.05
Villus area (μm^2)	98.88	117.91	118.96	135.07	8.30
Crypt depth (μm)	118.85*	119.67*	74.03	78.36	1.57
Tela submucosa (μm)	64.99	102.63	70.45	73.75	2.02
Tunica muscularis (μm)	75.65	76.96	91.34	94.19	2.35
D 28					
Villus height (μm)	457.41	523.89	487.31	490.75	6.64
Villus width (μm)	113.85*	118.46*	89.85	91.92	1.68
Villus area (μm)	167.37*	193.15*	129.62	117.52	10.88
Crypt depth (μm)	147.48*	125.23*	104.86	112.94	1.79
Tela submucosa (μm)	84.23	75.26	81.92	73.08	2.90
Tunica muscularis (μm)	119.33	105.50	103.95	94.04	3.15

[1] An asterisk refers to a significant difference from the SOW-group ($P < 0.05$).

[2] FOR = formula-fed piglets; SOW= sow-fed piglets; LBW = low birth weight piglets; NBW = normal birth weight piglets.

[3] Values are presented as means and pooled SEM (n = 5).

4.4. Bacterial screening

During formula-feeding, either until 10 or 28 days of age, the intestine became predominantly colonized with adherent gram-negative bacteria compared to sow-feeding ($P = 0.02$). Subsequent formula-feeding until 28 d of age, increased the number of samples with gram-positive adherent bacteria as well ($P = 0.03$) (Fig. 3.1).

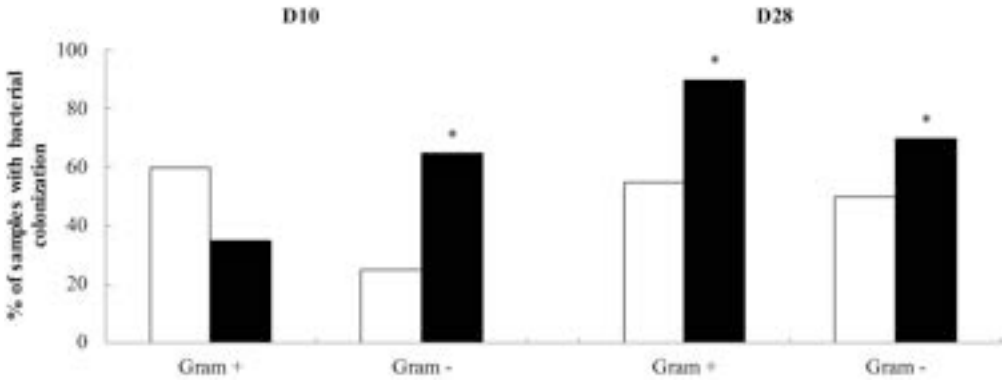

Figure 3.1 Adherent bacterial colonization. The observed fraction of intestinal samples colonized with adherent gram-positive bacteria and gram-negative bacteria is shown according to age and diet. White bars represent sow-fed piglets and black bars represent formula-fed piglets. An asterisk refers to a significant difference from the SOW-group, $P < 0.05$.

4.5. Small intestinal functional characteristics

For aminopeptidase A, no significant effect of weight ($P = 0.53$), diet ($P = 0.10$) or age ($P = 0.06$) could be observed, whereas a significant effect of region was noticed ($P < 0.01$), with the distal region significantly higher than the mid ($P < 0.01$) and almost significantly higher from the proximal part of the small intestine ($P = 0.06$) (Fig. 3.2A). For aminopeptidase N (ApN), a significant interaction was observed between diet and region ($P = 0.03$). Splitting the data into the separate regions, no difference in ApN activities could be observed between the 2 feeding groups ($P > 0.17$). For the sow-fed piglets, ApN activity differs between all regions of the small intestine ($P < 0.01$), with increasing activities from the proximal towards the distal part of the small intestine (Fig. 3.2B). For the formula-fed piglets, the effect of region was not significant ($P = 0.06$). A significant interaction was observed between region and diet ($P < 0.01$) for the dipeptidylpeptidase IV (DPPIV) activities. Sow-fed piglets had higher DPPIV activities compared to formula-fed piglets in the middle ($P = 0.01$) and distal part ($P < 0.01$) of the small intestine, whereas no significant difference could be observed in the proximal part ($P = 0.30$) (Fig. 3.2C). In sow-fed piglets, DPPIV differs between all 3 regions of the small intestine ($P = 0.02$, distal > middle > proximal) (Fig. 3.2C). In formula-fed piglets, no significant difference was observed between the 3 regions ($P = 0.61$).

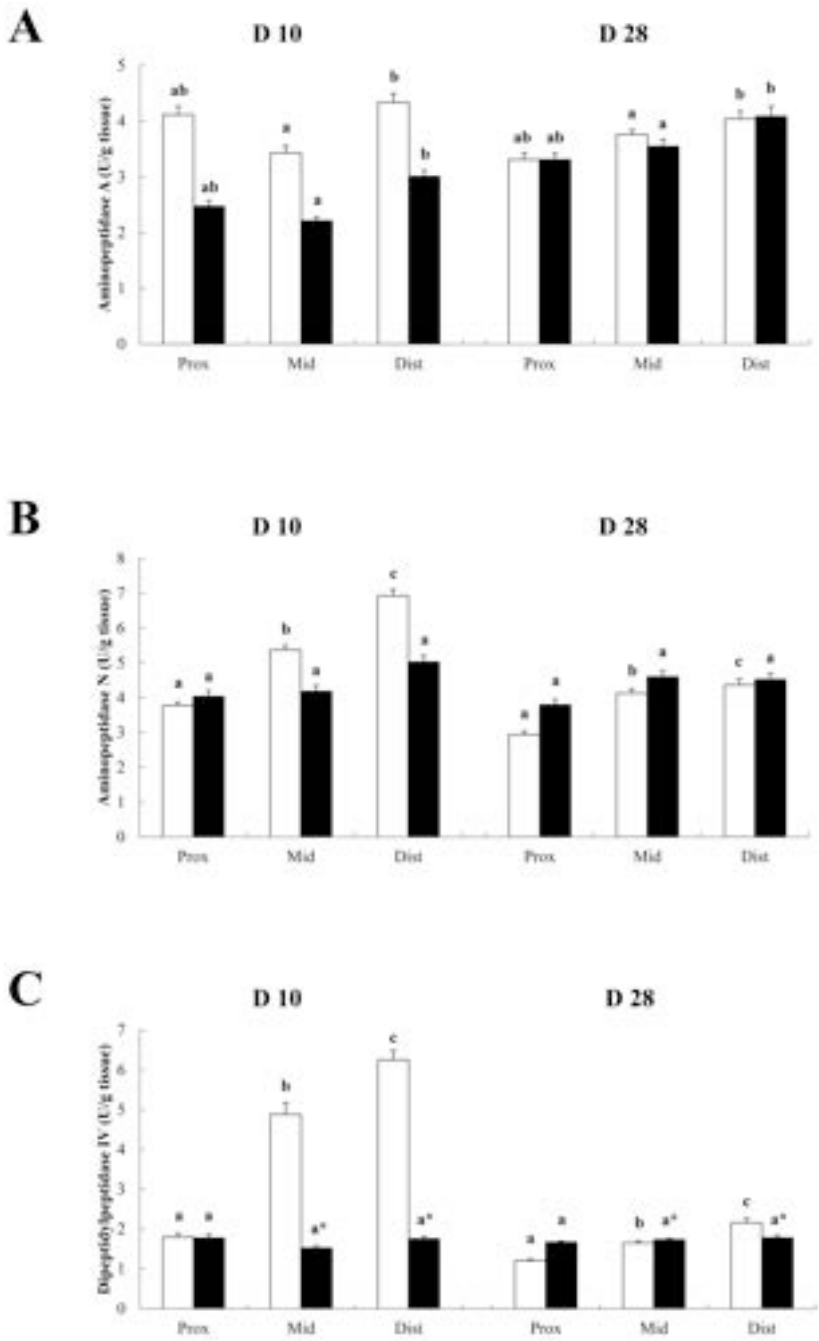

Figure 3.2 Small intestinal peptidase activities (U/g tissue) from 10-d old and 28-d old formula-fed and sow-fed LBW and NBW piglets (A, aminopeptidase A; B, aminopeptidase N; C, dipeptidylpeptidase IV). White bars represent LBW and NBW sow-fed piglets and black bars represent LBW and NBW formula-fed piglets. Values represent means and SEM, n = 5. Means not sharing a common superscript letter differ significantly, $P < 0.05$. An asterisk refers to a significant difference from the SOW-group, $P < 0.05$.

Lactase activities showed a significant interaction between diet and age (P = 0.01), and between region and age (P = 0.03) At 10 d of age, the effect of the diet was significant (P = 0.01), with sow-fed piglets showing a higher lactase activity compared to formula-fed piglets (Fig. 3.3A), whereas no significant difference between regions was observed (P = 0.86). At 28 d of age, lactase differed significantly between regions (P < 0.01), with all small intestinal regions differing significantly from each other (proximal > middle > distal) (Fig. 3.3A). For the maltase activities we observed significant interactions between diet and weight (P = 0.02), between diet and age (P < 0.01) and between region and age (P = 0.03). The effect of weight depends on the ingested feed: in the sow-fed group, no significant difference between LBW and NBW piglets could be observed (P = 0.13), whereas in the formula-fed group, NBW piglets showed significantly increased values of maltase compared to the LBW piglets (P < 0.01). The effect of the diet depends on the combination of age and birth weight. At 10 d of age, there was no significant difference in maltase activity between sow-fed and formula-fed (P > 0.29) piglets. At 28 d of age, sow-fed piglets had a significantly lower maltase activity compared to formula-fed piglets (P < 0.01) (Fig. 3.3B). At 10 d of age, the proximal part has significant higher values compared to the other regions (P < 0.01) (Fig. 3.3B). At 28 d of age, the distal part shows significantly lowered values (P = 0.01) compared to the proximal and middle part (Fig. 3.3B).

For the sucrase activities we observed significant interactions between diet and weight ($P = 0.01$), between diet and age ($P < 0.01$) and between region and age ($P < 0.01$). There was no difference in sucrase activity between LBW and NBW in case of sow-feeding ($P = 0.34$), but in the formula-fed piglets, NBW piglets had significantly greater sucrase activities compared to LBW piglets ($P < 0.01$). At 10 d of age, sow-fed LBW piglets had significantly higher sucrase activities compared to formula-fed piglets ($P = 0.03$) (Fig. 3.3C); whereas for the NBW piglets this difference was not significant ($P = 0.15$). At 28 d of age, no significant difference could be observed for the LBW piglets, whereas in case NBW piglets were formula-fed sucrase activities were elevated ($P = 0.02$) (Fig. 3.3C). At 10 d of age, there was no significant effect of region ($P = 0.07$), whereas at 28 d of age, all 3 regions differed significantly ($P < 0.01$) (Fig. 3.3C).

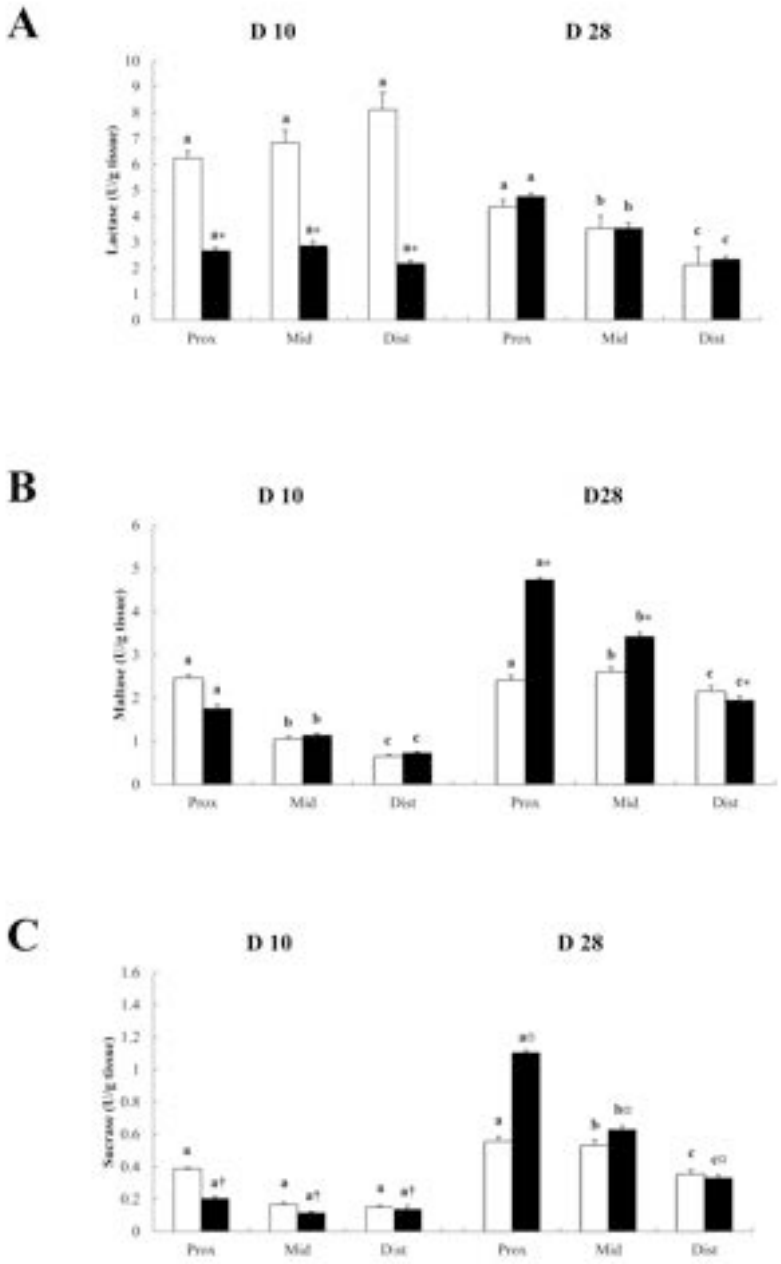

Figure 3.3 Small intestinal disaccharidase activities (U/g tissue) from 10-d old and 28-d old formula-fed and sow-fed LBW and NBW piglets (A, lactase; B, maltase; C, sucrase). White bars represent sow-fed LBW and NBW piglets and black bars represent LBW and NBW formula-fed piglets. Values represent means and SEM, n = 5. Means not sharing a common superscript letter differ significantly, $P < 0.05$. An asterisk refers to a significant difference from the SOW-group, $P < 0.05$. The symbol "†" refers to a significant difference from the SOW-group, but only for the LBW piglets, $P < 0.05$. The symbol "□ "refers to a significant difference from the SOW-group, but only for the NBW piglets, $P < 0.05$.

5. DISCUSSION

A major problem in pig production is the presence of supernumerary and underprivileged piglets. Therefore, there is a need for alternative systems to raise those piglets the sow cannot nourish. Many feed companies have designed commercial brooders but negligible scientific evidence exist that these systems give rise to heavy and healthy piglets, which implicates that they are currently rarely used in practice (10).

It has been well described that sow's milk contains bioactive compounds (e.g. growth factors, cytokines, hormones), which stimulate and regulate the process of small intestinal growth and maturation (384). Additionally, it has previously been reported that short-term formula-feeding alters the structural and functional maturation of the small intestine due to a lack of these 'milk-borne' bioactive compounds (322, 323, 385, 386). However, reports on long-term effects of formula-feeding are scarce. Therefore, the present study investigated the short-term and long-term consequences of artificially rearing piglets on formulated milk in a brooder. In addition to their growth, the morphological characteristics and digestive capacity of the small intestine were analysed, because the latter are crucial elements in driving postnatal development, growth and health (387).

In our study, feeding milk formula for one wk inhibited the growth of the piglets by 112 g/d, resulting in reduced body weights at 10 d of age (on average -0.818 kg). These results correspond with an earlier report by Wolinski et al. in which the short-term effects of formula-feeding were examined (322). In contrast to the latter, formula-feeding in our study did not inhibit the growth of the gut since it resulted in a relatively heavier stomach (on average +2.16 g/kg) and longer gastrointestinal tract (on average +63.6 cm/kg). Although in our study the postnatal intestinal structural growth

between the feeding groups was similar, it was clear that formula-feeding slows down intestinal functional maturation, increasing the risk of gut malfunctioning in both LBW and NBW piglets.

In our study, the delay in the functional maturation of the small intestine was shown by decreased lactase (-4.50 U/g tissue), dipeptidylpeptidase IV (middle and distal part of the small intestine; -3.35 and -4.50 U/g tissue) and sucrase activities (LBW piglets; -0.283 U/g tissue). It has previously been described that formula-feeding results in different brush border enzyme activities in both pigs and rats (322, 388-390) which may result from direct diet-dependent effects on the developing enterocytes (391).

The reduced functional capacity of the small intestine that was observed in the present study at 10 d of age contributes to the reduced ability to absorb water and optimally utilize the nutrients that are offered via the formulated milk. This contributed to a reduced body weight of 10-d old formula-fed piglets compared with sow-fed piglets.

Additionally, the long-term effects (compared to the neonatal period) of artificial rearing on formulated milk were assessed at 28 d of age. Although ADG (-46.7 g/d) and final body weights (-2.20 kg) of LBW piglets were still inferior to their NBW littermates, formula-fed piglets had similar growth performances and final body weights compared to suckling animals. In previous studies in which piglets were artificially reared shortly after birth (starting at d 1 - 3), piglets achieved growth rates that even exceeded those of suckling piglets (7, 392, 393). In contrast, Huysman et al. found reduced growth performances of piglets reared with an electronic mother milk applicator during the same period (394). Bacterial screening of the small intestines displayed that artificially-reared piglets had an altered bacterial colonization pattern. However, it is essential to bear in mind that we only

evaluated the gut flora species adhering to the mucosal lining of the intestine and that the different bacterial genera were not evaluated. Overall, very few birth weight-related differences were observed with regard to small intestinal morphology and physiology. In general, the effect of the ingested diet was similar for both types of piglets, except for the sucrase and maltase activities. The greater area for nutrient absorption (+59.1 μm^2) combined with deeper crypts (+42.5 μm) observed in NBW and LBW piglets of the formula-fed group in our study until 28 d of age is in agreement with a report of Boudry and colleagues (324). Deeper crypts when being formula-fed were also described in studies with human infants and rat pups (395-398). Deeper crypts suggest an increase in crypt cell population, which may result in expansion of the intestinal surface of the small intestine, reflecting the enlarged villus area observed in our study. Maltase (+0.97 U/g tissue) and sucrase (+0.23 U/g tissue) activities more rapidly increased in formula-fed piglets compared to suckling animals. A similar disaccharidase maturation pattern was observed in other studies with rats (395-397) and guinea-pigs (399) and is probably caused by the presence of plant-based stimulatory components in formulated milk. In our study, NBW formula-fed piglets had even higher values compared to LBW formula-fed piglets (maltase, +0.85 U/g tissue and sucrase, +0.17 U/g tissue), indicating a slower maturation in the latter group of piglets.

Based on the results obtained in our study in which the pre-weaning period was covered, we believe that it is also worth investigating what the effect on growth performances and intestinal development would be after the weaning transition on solid feed. Additionally, further investigations on the effects of artificially rearing piglets on welfare and behavioural changes are warranted. Although not directly comparable, researchers reported increased restlessness, increased vocalisations and more aggression in case of early

weaning. In addition, early-weaned piglets performed more belly-nosing behaviour and spent more time under the heating lamp (400, 401). Widowski et al. showed that a feeding device that accommodates both sucking and massage could reduce piglet-directed behaviour (402). This implies that the use of the cup system with piglet-activated nipples may facilitate social housing of artificially reared piglets and increase their welfare when compared to the classic method of early weaning on solid feed.

In conclusion, artificial rearing of 3 d-old piglets resulted in a reduction in body weight and delayed functional maturation of the small intestine during the first week of formula-feeding. Despite the short-term differences encountered, long-term formula feeding resulted in similar growth performances and improved gut growth and functional maturation. Very little differences were observed between LBW and NBW piglets. The results of our study illustrate that piglets of all birth weight categories can be housed within these systems. Moreover, artificially reared piglets benefit from the *ad libitum* availability of formulated milk by an increased absorptive intestinal capacity (+59.1 μm^2). Additionally, due to the latter and their elevated sucrase (+0.23 U/g tissue) and maltase (+0.97 U/g tissue) activities, formula-fed piglets will probably better adapt to the shift towards solid feed.

CHAPTER 3B

INCREASED IGF-1 SERUM LEVELS AND DISCORDANT PROTEIN AND mRNA IGF-1 RECEPTOR EXPRESSION IN THE SMALL INTESTINE OF FORMULA-FED PIGLETS

Adapted from: **M. De Vos**, L. Che, V. Huygelen, S. Willemen, C. Casteleyn, S. Van Cruchten, C. Van Ginneken. Increased IGF-1 serum levels and discordant protein and mRNA IGF-1 receptor expression in the small intestine of formula-fed piglets. Livestock Science 154 (2013) 224-228

1. ABSTRACT

The present investigation addresses the question whether feeding piglets on formulated milk alters the insulin-like growth factor-1 (IGF-1) system differently in normal versus low birth weight piglets. In this study, both types of piglets were either sow-fed until 28 days of age or until 3 days of age and subsequently formula-fed until day 28. Formula-fed piglets had higher serum IGF-1 levels compared to suckled piglets, whereas low birth weight piglets had lower IGF-1 levels in comparison with normal birth weight piglets. In contrast, the mRNA expression of IGF-1 receptor (IGF-1R) in the small intestine showed lower expression in formula-fed piglets versus suckled piglets. However, the opposite effect was observed for IGF-1R protein abundance. Moreover, birth weight did not affect IGF-1R mRNA or protein abundance. In conclusion, formula-fed piglets have higher IGF-1 serum levels and altered gene and protein expression of IGF-1R in the small intestine, irrespective of birth weight.

2. INTRODUCTION

Insulin-like growth factor-1 (IGF-1) is one of the main regulators of prenatal and postnatal growth. Piglets that are born with low birth weights, have suffered from prenatal undernutrition. These piglets have decreased small intestinal mRNA IGF-1 expression (309) and lower plasma IGF-1 levels during the first 3 days after birth (403). During the first week after parturition, the serum IGF-1 levels of the piglets increase (404), showing a positive correlation with their body weight gain. Sows' colostrum and milk are known to contain various growth factors, including IGF-1, whereas this growth factor is undetectable in milk replacer (405). Orally administrated

IGF-1 improves the development of the gastrointestinal tract (406). Because little IGF-1 is absorbed in the intestine, it is unlikely that milk-borne IGF-1 contributes to increasing circulating IGF-1 levels (403). But, the presence and location of IGF-1 receptors in the gastrointestinal tract of foetal, neonatal and weaned piglets (407) indicate that IGF-1 may act via both the luminal and parenteral route. Additionally, human studies showed that endogenous IGF-1 production is influenced by the infant's diet (408).

In this study, the hypothesis that birth weight and nutrition alter the insulin-like growth factor-1 system in neonatal piglets was tested by measuring the serum IGF-1 and IGFBP-3 levels, the small intestinal IGF-1R expression, the liver IGF-1 and IGF-1R levels in piglets of two birth weight categories (low birth weight (LBW) versus normal birth weight (NBW)), which were either sow-fed or formula-fed from 3 d of age until 28 d of age.

3. MATERIAL AND METHODS

3.1. Animal experiment and tissue collection

Institutional and national guidelines for the care and use of animals were followed and all experimental procedures involving animals were approved by the Ethical Committee of Animal Experimentation, University of Antwerp, Belgium. Gender-matched pairs of LBW piglets (0.87 ± 0.04 kg) and NBW piglets (1.48 ± 0.11 kg) were selected at a local farm. A birth weight less than 1 kg defined LBW piglets, whereas NBW piglets had weights close to the mean litter weight. All piglets (n = 20), i.e. ten gender matched pairs of LBW and NBW piglets were assigned to 2 dietary treatments. One group of piglets (n = 10), containing an equal number of LBW and NBW animals, remained suckling until 28 d of age. Another group

of piglets (n = 10) were formula-fed in a commercial brooder starting at 3 d of age to ensure the intake of sow colostrum. The formula used in this study was a complete milk replacer for young piglets, allowing rearing piglets in absence of the sow. The commercial diet was composed of skimmed milk powder, whey powder, coconut fat, palm oil and wheat concentrate and nutritional additives such as amino acids, minerals and vitamins (calculated analysis: 28 g crude protein, 23 g crude fat, 56 g lactose per l and calculated ME 548 kcal/l). Piglets had free access to the milk replacer and water. The intake of creep feed was negligible. At 4 weeks of age LBW and NBW piglets were weighed and killed by severing the carotid arteries under deep barbiturate anaesthesia (sodium pentobarbital, 200 mg/kg, IP). Blood samples were collected in tubes without anti-coagulant, centrifuged at 2000 g for 10 min and the serum was frozen at -20°C until analysis. Considering IGF-1R is highly expressed in the distal part of the small intestine (409), ileal tissues were collected. These were either snap frozen in liquid nitrogen and stored at -80°C until gene expression analysis and enzyme-linked immunosorbent assay (ELISA) or fixed in formalin pending immunohistochemical staining. Additionally, liver samples were collected, snap frozen and stored at -80°C.

3.2. Enzyme-linked immunosorbent assays

IGF-1 and IGFBP-3 levels were measured with an IGF-1 (Mediagnost Inc, Rentlingen, Germany) and IGFBP-3 ELISA kit (Cusabio Biotech, Wuhan, China), respectively, according to the manufacturer's protocol. Serum of all piglets, porcine colostrum and milk replacer were assayed. Porcine colostrum samples of four sows were collected at parturition and defatted at 11.000 g for 5 min. The aqueous fraction was used for analysis.

Porcine ELISA kits (Cusabio Biotech, Wuhan, China) were used to measure IGF-1 and IGF-1R levels in tissue homogenates. Small intestinal and liver samples (100 mg) were homogenized in 1 ml Phosphate Buffered Saline (PBS) and stored overnight at -20°C. Two freeze-thaw cycles were performed in order to break the cell membranes. Afterwards, homogenates were centrifuged at 5000 g for 5 min at 4°C. The supernatants were immediately assayed according to the manufacturer's protocol.

3.3. Total RNA isolation and IGF-1R gene expression

Total RNA was isolated from the small intestine samples by using TRIzol® (Invitrogen, Merelbeke, Belgium). The RNA quality was verified by both agarose gel (1%) electrophoresis and spectrometry (A260/A280, Beckman DU-800, Beckman Instruments, Fullerton, CA, USA). The mRNA IGF-1R level was analysed using quantitative reverse transcription-polymerase chain reaction (qRT-PCR) (ABI 7900HT, Applied Biosystems, Beijing, China). qRT-PCR was performed in duplicate to amplify the target gene and the reference gene, and performed by using one step SYBR® PrimeScript™ RT-PCR kit II (Takara Bio Inc., Shiga, Japan). Briefly, the reaction mixture (10 μL) contained 5.6 μL of freshly premixed one step SYBR® Green RT-PCR Master mix and PrimeScript™ Enzyme Mix, 0.8 μmol/l of the primer pair, and ~100 ng of RNA template. The procedures followed the manufacturer's instructions. The PCR consisted of 1 cycle of 42°C for 5 min, 1 cycle of 95°C for 10 s, 40 cycles of 95°C for 5 s and 60°C for 34 s, followed by the dissociation step at 95°C for 15 s, 60°C for 60 s, and 95°C for 15 s. To confirm the specific amplification, melt curve analysis was performed. Relative mRNA abundance was determined using the Δ cycle threshold (ΔCt) method as outlined in the protocol of Applied Biosystems. In brief, a ΔCt

value was the Ct difference between the target gene and the reference gene ($\Delta Ct = Ct^{target} - Ct^{reference}$). The ΔΔCt values of all the samples were then calculated by subtracting the average ΔCt of the group of piglets that was sow-fed (control group). The ΔΔCt values were converted to fold differences by raising 2 to the power –ΔΔCt ($2^{-\Delta\Delta Ct}$). Primers for the *IGF-1R* gene and reference gene, *β-actin* (ACTB), were as follows forward 5' – CCA GGC CAA AAC GAC ATA TGA – 3', reverse 5' – CAA CAG AA CGG CCA CTG GTA – 3' for IGF-1R (67 bp); forward 5' – GGC GCC CAG CAC GAT – 3', reverse 5' – CCG ATC CAC ACG GAG TAC TTG – 3' for β-actin (66 bp) and designed using Primer Express 3.0 (Applied Biosystems).

3.4. IGF-1R immunohistochemical staining

The presence and distribution of IGF-1R were demonstrated on paraffin sections of the small intestine as described previously (134, 407). In short, sections were incubated in 1% H_20_2 in methanol for 30 min to block for non-specific staining. Afterwards, sections were incubated with rabbit polyclonal IGF-1R anti-serum, 1/100 diluted in PBS, (Santacruz Biotechnology, California, US) overnight at 4°C. Following rinsing in PBS, sections were incubated with Envision anti-rabbit (DakoCytomation) for 30 min at room temperature. In order to visualize the IGF-1R immunocomplexes, aminoethylcarbazol (AEC) (DakoCytomation) were applied to the sections. IGF-1R scores were based on the proportion and intensity of immunohistochemical staining of smooth muscle cells (tunica muscularis, lamina muscularis mucosae and tunica media of submucosal arterioles) and tunica mucosa (enterocytes) of the small intestinal samples. Staining was semi-quantitatively scored (0 = no staining, 1 = weak staining, 2 = moderate

staining, 3 = strong staining) according to the proportion and intensity of staining.

3.5. Statistics

Data were subjected to statistical analysis by Analysis of Variance (General linear model procedures) using SPSS 16.0 (SPSS Inc., Chicago, IL, USA). This model included the effect of birth weight (LBW versus NBW) and diet (sows' milk versus formula) and their interactions. Pearson correlation tests were used to detect correlations between body weights and serum IGF-1 levels. IGF-1R scores were analysed using a Chi-square test. P values < 0.05 were considered significant.

4. RESULTS

Formula-fed piglets were heavier (P = 0.036) (7.94 ± 2.14 kg (formula-fed) vs. 6.76 ± 1.75 kg (sow-fed)) and had higher serum IGF-1 levels (P = 0.002) at 28 d of age compared to suckled piglets (Fig. 3.4).

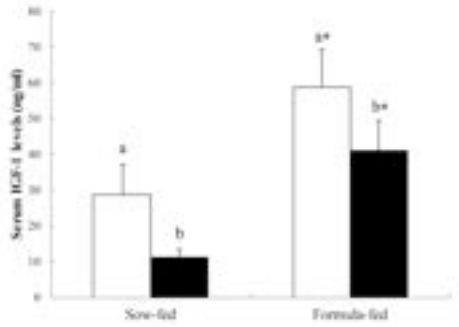

Figure 3.4 Serum IGF-1 levels (ng/ml) in NBW (white bar) and LBW (black bar) piglets, which were either sow-fed or formula-fed. Values are means, with their SEM (n = 5) represented by vertical bars. Within feeding group, means not sharing a common superscript letter differ significantly (P < 0.05). An asterisk refers to a significant difference from the SOW-group.

Additionally, immunohistochemical staining for IGF-1R in the small intestine showed significant higher scores in the smooth muscle cells (P = 0.043) and tunica mucosa (P = 0.008) in formula-fed piglets (Table 3.4). In accordance, IGF-1R levels in small intestinal tissue homogenates detected by ELISA, showed higher IGF-1R levels in case piglets were formula-fed (P < 0.001) (Fig. 3.5). In contrast, IGF-1R mRNA expression in the small intestine was lower in formula-fed compared to suckled piglets (P = 0.027) (Fig. 3.6). IGF-1 and IGF-1R levels in liver homogenates did not differ between the tested diets (P > 0.375; data not shown). For none of the tested parameters, significant interactions could be observed between birth weight and diet.

Table 3.4 Effect of the way of feeding on the IGF-1R staining scores in smooth muscle cells and epithelial cells of the tunica mucosa [1].

	Sow-fed	Formula-fed	*P*-value
Smooth muscle cells (%)			0.043
Score 0	13.3	0.0	
Score 1	50.0	29.6	
Score 2	33.3	63.0	
Score 3	3.3	7.4	
Tunica mucosa (%)			0.008
Score 0	33.3	3.7	
Score 1	43.3	44.4	
Score 2	23.3	51.9	
Score 3	0.0	0.0	

[1] Score 0 = no staining, score 1 = weak staining, 2 = moderate staining, 3 = strong staining.

At 28 d of age, piglets' body weights were lower in LBW piglets (P < 0.001) (5.69 ± 1.10 kg (LBW) vs. 8.94 ± 1.30 (NBW)). Final body weights were positively correlated with serum IGF-1 levels (R^2 = 0.71; P < 0.001). This implies that lower IGF-1 levels were present in LBW piglets (P = 0.045)

(Fig. 3.4). No differences were noticed regarding the small intestinal IGF-1R immunohistochemical scores (P = 0.182), IGF-1R levels in small intestinal (Fig. 3.5) and liver homogenates (P = 0.121) and small intestinal gene expression (P = 0.815) (Fig. 3.6) between both body weight categories.

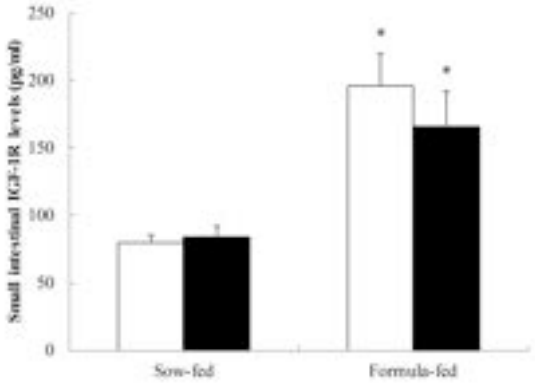

Figure 3.5 Small intestinal IGF-1R levels (pg/ml) in NBW (white bar) and LBW (black bar) piglets, which were either sow-fed or formula-fed. Values are means, with their SEM (n = 5) represented by vertical bars. Within feeding group, means not sharing a common superscript letter differ significantly (P < 0.05). An asterisk refers to a significant difference from the sow-fed group.

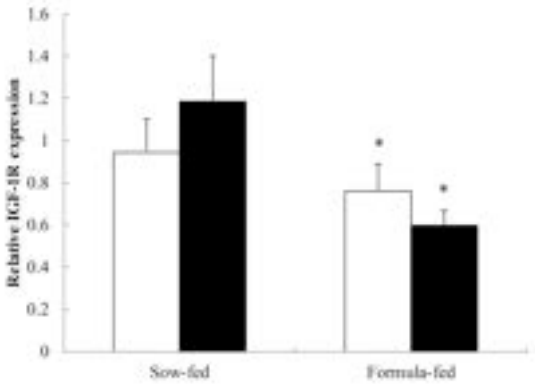

Figure 3.6 Relative expression of small intestinal IGF-1R in NBW (grey bar) and LBW (white bar) piglets that were either sow-fed or formula-fed. Values are means, with their SE (n = 5) represented by vertical bars. Within feeding group, means not sharing a common superscript letter differ significantly (P < 0.05). An asterisk refers to a significant difference from the sow-fed group.

IGF-1 was not detected in the milk formula used in this experiment, whereas in porcine colostrum high levels were measured (154 ± 28 ng/ml). Although IGFBP-3 is the most abundant binding protein, in none of the serum samples this binding protein could be detected. Porcine colostrum contained IGFBP-3 levels ranging from undetectable (<78 ng/ml) to 89 ng/ml, whereas in the milk formula it was undetectable.

5. DISCUSSION

The objective of the present study was to examine whether feeding piglets on formulated milk alters the insulin-like growth factor system differently in normal versus low birth weight piglets. In this study, formula-fed piglets had higher serum IGF-1 levels in comparison to suckled piglets. This finding is consistent with observations from human studies in which higher IGF-1 levels were observed in formula-fed infants compared to breast-fed infants (410). Because no IGF-1 could be detected in milk formula, and absorption of IGF-1 via the gut is considered to be negligible, we assumed that the rise in serum levels was due to an increased endogenous IGF-1 production in the liver. Surprisingly, liver IGF-1 levels did not differ between the two tested diets. However, it has previously been described that high protein intake, stimulates IGF-1 secretion and thereby results in elevated serum IGF-1 levels (408). Calculated protein intake was indeed higher in the formula-fed group (based upon daily milk intake and protein content of the diets). Moreover, the higher IGF-1 levels may be ascribed to the increased body weight gain in formula-fed subjects (411). All serum IGFBP-3 levels were below detection limit (< 78 ng/ml). The effect of these binding proteins on the circulating IGF-1 levels will probably be minor, for both tested diets and birth weight categories (412, 413). In sow-fed piglets, the gene expression of IGF-1R in

the small intestine was up-regulated, whereas IGF-1R protein was down-regulated compared to formula-fed piglets. It is likely that the increased IGF-1R mRNA expression is compensating for the decreased circulating IGF-1 levels in sow-fed piglets (414). However, excessive secretion of IGF-1 may desensitize IGF receptors, as suggested by Petersen et al. who observed that increasing glucagon-like peptide-2 (GLP-2) negatively regulates GLP-2 receptor expression (415). Furthermore, enteral nutrition itself may regulate IGF-1R activity, as studies in pigs (416, 417) have already suggested.

At 28 d of age, serum IGF-1 levels were reduced in LBW piglets. Previous studies in swine have indeed described lower circulating IGF-1 levels for LBW piglets not only during the pre-weaning period (335) but also in the post-weaning period (134) and at slaughter age (28). These reduced IGF-1 levels might be the result of alterations in the IGF-1 axis maturation or the inadequate nutritional status of LBW piglets, and are involved in the regulation of postnatal growth.

In this study, no birth weight related differences in small intestinal IGF-1R expression could be observed. This is in accordance with an earlier study in which 21 d-old intrauterine growth restricted piglets showed no difference in mRNA expression of IGF-1R in *m. longissimus*, liver and kidney (413). However, our data contrast with a study in which weaned piglets expressed lower protein IGF-1R abundance in the small intestine of low birth weight piglets (134). The discrepancy among these findings demonstrates that IGF-1R expression has a differential response to birth weight depending on the organs and age groups that are examined.

In conclusion, formula-fed piglets had higher serum IGF-1 levels and discordant IGF-1R expression in the small intestine irrespective of birth weight. Moreover, regardless of the enteral nutrition type, LBW piglets showed lower circulating IGF-1 concentrations.

CHAPTER 4
EFFECTS OF SUPPLEMENTING FORMULA-FED PIGLETS WITH A BOVINE COLOSTRUM FRACTION

Adapted from: **M. De Vos**, V. Huygelen, G. Van Raemdonck, S. Willemen, E. Fransen, X. Van Ostade, C. Casteleyn, S. Van Cruchten, C. Van Ginneken. Supplementing formula-fed piglets with a low molecular weight fraction of bovine colostrum whey results in an improved intestinal barrier. Journal of Animal Science, accepted for publication

1. ABSTRACT

In this study, we hypothesized that a low molecular weight fraction of colostral whey could affect the morphology and barrier function of the small intestine. Therefore, the addition of colostral whey was investigated via both *in vitro* and *in vivo* trials. Our *in vitro* data showed that a low molecular weight fraction of colostral whey stimulated the proliferation of IPEC-J2 cells ($P = 0.019$), tended to increase cell migration of IPEC-J2 cells ($P = 0.082$) and increased monolayer integrity ($P = 0.021$). In the *in vivo* study, 30 three-day-old piglets (normal or low birth weight) were suckled (n = 5) or artificially fed with milk formula (n = 5), or artificially fed with milk formula with a low molecular weight fraction of colostral whey (n = 5) until 10 d of age. The small intestine was sampled for histology (haematoxylin and eosin stain; anti-KI67 immunohistochemistry) and enzyme activities (aminopeptidase A, aminopeptidase N, dipeptidylpeptidase IV, lactase, maltase, and sucrase). In addition, intestinal permeability was evaluated via a dual sugar absorption test and via the measurement of occludin abundance. Artificially feeding of piglets reduced final body weight ($P < 0.001$), villus height ($P < 0.001$), lactase ($P < 0.001$) and dipeptidylpeptidase IV activities ($P < 0.07$), whereas crypt depth ($P < 0.001$) was increased. No difference was observed with regard to the permeability measurements when comparing artificially fed with naturally suckling piglets. Supplementing piglets with the colostral whey fraction did not affect body weights, enzyme activities, or the outcome of the dual sugar absorption test. On the contrary, the small intestines of supplemented piglets had even shorter villi ($P = 0.001$) than unsupplemented piglets and contained more occludin ($P = 0.002$).

In conclusion, at 10 d of age no differences regarding intestinal morphology and permeability measurements were observed between the two birth weight categories. In both weight categories, the colostral whey fraction affected the morphology of the small intestine but did not improve the growth performances or the in vivo permeability. These findings should be acknowledged when developing formulated milk for neonatal animals with the aim of improving the performance of low birth weight piglets.

2. INTRODUCTION

Interest in the physiological functions of bioactive substances present in colostrum and milk has increased in recent years (418). Bovine colostrum has been used as an alternative for foetal bovine serum to support growth in rat (419, 420), human (421, 422) and swine intestinal cells (423). Additionally, various studies have confirmed that orally fed given bioactive substances such as insulin like growth factor-1 (200, 201), epidermal growth factor (329), leptin (322), ghrelin (424), insulin (425) and arginine (346) affect small intestinal growth and maturation of piglets. The positive effect of milk-born substances could be particularly important for smaller piglets because several studies reported that intra-uterine growth restriction prevented the development and maturation of the small intestine, hampering nutrient utilization and barrier function (133, 199, 309, 310).

Most of these milk-derived bioactive factors are small proteins or polypeptides, which are found in the whey fraction of colostrum and/or mature milk (172). Surprisingly, these milk-born factors are poorly studied when they are administered simultaneously or in their natural matrix.

The hypothesis tested in the current study was that supplementing formula-fed piglets with a low-molecular-weight fraction of colostrum, stimulates small intestinal growth, improves intestinal barrier function and digestive enzyme activities, and, thus, positively affects body weight gain and the functional maturation of the gut.

3. MATERIAL AND METHODS

Institutional and national guidelines for the care and use of animals were followed and all experimental procedures involving animals were approved by the Ethical Committee of Animal Experimentation, University of Antwerp, Belgium.

3.1. Preparation of the colostral whey fraction

Bovine colostrum powder (Biofiber - Damino A/S, Gesten, Denmark) was reconstituted to 10% (wt/vol) in saline (NaCl, 0.9%) and centrifuged (10,000 x g for 30 min at 4°C) to separate the cream from the skim milk. Colostral whey was prepared by adding rennin (R 7751; Sigma-Aldrich, Steinheim, Germany) to milk and removing the curds by centrifugation (3000 x g for 30 min at 4°C). The resulting whey was microfiltrated (0.2 μm pore filter; Schleicher and Schuell, Dassel, Germany). Clear whey was further ultrafiltrated using a ultrafiltration concentrator with filter device of 50 kDa (Vivacell 250; Sartorius Stedim, Vilvoorde, Belgium). The colostral whey filtrates (CWF), and not residues, were used for all further experiments.

3.2. Analysis of the colostral whey fraction – determination of the protein content

The protein content of the colostral whey fraction was determined (Pierce BCA Protein Assay Kit; Thermo Scientific, Rockford, IL) according to the manufacturer's instructions. Before digestion, 100 µg of the ultrafiltrated sample was precipitated overnight at -20°C, by adding 6 volumes of ice-cold acetone. After centrifugation the supernatant was removed and the protein pellet was resuspended in 50 mM Tris-HCl/6 M urea/5 mM DTT/10% beta-mercaptoethanol (25 µl/100 µg protein) at a pH of 8.7. For the denaturation and reduction process, all samples were incubated at 65°C for 1 h. Subsequently, proteins were diluted in 50 mM Tris-HCl/1 mM $CaCl_2$ (75 µl/100 µg protein) and then alkylated by adding 200 mM iodoacetamide (10 µl/100 µg protein) for 1 h at room temperature. Finally, proteomics-grade modified trypsin (Roche, Mannheim, Germany) was added at a 30:1 protein-to-enzyme ratio. After incubation at 37°C for 18 h the digestion was stopped by freezing the fractions at -80°C.

Peptides were separated based on hydrophobicity by using a reverse phase C18 column on a micro-capillary HPLC system (Agilent 1100 series; Agilent Technologies, Waldbronn, Germany). Ten micrograms of peptides were injected on a Zorbax 300SB-C18 guard column (0.3 x 5 mm; particle size 3.5 µm; Agilent Technologies) serially connected with a Zorbax 300SB-C18 analytical column (0.3 x 150 mm; particle size 3.5 µm; Agilent Technologies). Solvent A contained 0.1% formic acid (FA) in water, while solvent B contained 0.1% FA in 90% acetonitrile (ACN). Then, ACN gradient was applied using the capillary pump with a constant flow rate at 6 µl/min: 5 to 55% B in 56.7 min, increase quickly to 90% B over 3.3 min, persistent 90% B for 5 min, 85% B for 5 min, and back to equilibrating

conditions of 3% B. Starting from min 5 till min 51.7 of the chromatographic run, 350 spots (800 nl/spot) were spotted (Opti-TOF MALDI-Target; Applied Biosystems, Inc., Foster City, CA). Afterwards, each spot was covered with matrix (2 mg/ml α-cyanohydroxy cinnamic acid in 70% ACN; internal calibrant: 63 pmol/ml human [Glu^1]-fibrinopeptide B) using an external syringe pump at a flow rate of 6µl/min (800 nl/spot).

Spotted fractions were analysed using a matrix assisted laser desorption/ionization (MALDI; AB4800 Proteomics Analyser; Applied Biosystems, Inc.). The MALDI-ToF MS-analysis (reflectron mode; laser intensity, 3200; 25 x 20 laser shots per spot; mass-range 800-3000 Da) was performed first, after which precursors with a signal-to-noise (S/N) ratio above or equal to 100 were selected. The MALDI-ToF/ToF MS/MS-analysis was performed on these selected precursors, and a maximum of 50 unique precursors per spot were selected for fragmentation, beginning with the precursors with the lowest S/N-ratio. These precursors were ionized (laser intensity: 4200; 25 x 20 laser shots per spot) and fragmented in a collision cell (1kV collision) with air.

Acquired MS/MS spectra were screened against the mammalian Swiss-Prot database (Version: 57.1) using the MASCOT search engine (Matrix Science; Version 2.1.03) with *Bos Taurus* being the only criterion. Carbamidomethylation of cysteines was listed as fixed modification, and oxidation of methionine was listed as a variable modification. A maximum of 2 missed cleavages of trypsin was tolerated. The mass tolerance was set to 200 ppm for the precursors and 0.2 Da for the fragment ions. The MudPIT scoring algorithm of MASCOT was used. Scaffold (Version Scaffold_3_00_03; Proteome Software Inc., Portland, OR) was used to validate MS/MS-based peptide and protein identifications. Protein probabilities were assigned by the Protein Prophet algorithm.

3.3. Cell culture experiments

IPEC-J2 cells (passage 55 - 65), were cultured in 50% Dulbecco's Modified Eagle Medium (DMEM) - 50% (v/v) F12 medium (Gibco, Life Technologies, Merelbeke, Belgium) containing 5% (v/v) foetal calf serum, 1% (v/v) insulin/transferrin/Na-selenite media supplement (Gibco, Life Technologies, Merelbeke, Belgium), 100 units penicillin/ml and 100 μg streptomycin/ml (penicillin/streptomycin, Gibco, Life Technologies, Merelbeke, Belgium). The cells were maintained in serial passage in 75 cm^2 flasks at 37°C in a 5% CO_2 atmosphere.

The effect of the colostral whey fraction on the viability of IPEC-J2 cells was measured using a 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (CellTiter96 Non-radioactive Cell Proliferation Assay, Promega, Madison, WI, USA). IPEC-J2 cells were placed in 100 μl culture medium (for composition details see above) at a concentration of 5 x 10^3 cells/well in a 96-well plate. After 24 h, medium and non-adhering cells were removed. Stimulation of cell growth was assessed by the addition of various concentrations of CWF (1%, 5%, 10%) to basal medium for 24 h. The basal medium consisted of 50% DMEM and 50% (v/v) F12 medium. Subsequently, 15 μl MTT solution was added to the wells, which was reduced to formazan by living cells and absorbance was measured at 570 nm (Tecan sunrise, Tecan Group Ltd., Männedorf, Switzerland). Relative activity (RA) of cell growth was determined using the following equation:

RA percentage = $(\frac{OD+}{OD-} - 1)$ x100

In this formula OD = optical density with (+) or without (-) CWF

We studied the involvement of CWF in cell migration as a model of wound repair. IPEC-J2 cells were grown to confluence in six well plates. Subsequently, the monolayers were wounded by scraping a pipet tip across the well. Cells were cultured in basal medium in the presence of various doses CWF (1%, 5%, 10%) for 3 h. The distance of cell movement from the edges of the original wound was measured at fixed time-points after the addition of CWF (0 h, 3 h). At least 3 measurements were taken per time point and per well.

TEER measurements were used to evaluate the permeability of the cell monolayer and its barrier function (426). IPEC-J2 cells were seeded at a concentration of 2.5 - 4.0 x 10^4 cells/ cm^2 on 4.2 cm^2 filters in 6 well culture plates. Cells were cultured for at least 7 days and medium was changed every other day. The integrity of each cell monolayer was confirmed by measuring the TEER using a Millicell electrical cell resistance meter (Millipore Corporation, Bedford, MA, USA). CWF was added to the upper side of the filter at concentrations of 1%, 5% and 10% to basal medium.
TEER measurements were performed before and 24h after incubation with CWF. Results are expressed using the following equation:

$$TEER\ (\%\ of\ initial\ value) = \left(\left(\frac{value\ after\ incubation}{value\ before\ incubation}\right) - 1\right) x\ 100\%$$

3.4. Animal experiment

Crossbred piglets ((Piétrain x (Finnish Yorkshire x stress negative Belgian Landrace)) were obtained from a commercial farm. From 15 litters, pairs of low birth weight (LBW; 0.96 ± 0.06 kg at birth) and normal birth weight (NBW; 1.49 ± 0.12 kg at birth) piglets were selected at birth and allotted to 3

experimental treatments at 3 d of age. Piglets with a birth weight (BW) below 1 kg were considered to be LBW, whereas these with a BW close to the mean of the litter were considered to be NBW. Each experimental group consisted of 10 sex-matched piglets, i.e., 2 littermate piglets (LBW and NBW) from 5 different litters. The LBW and NBW piglets remained with the sow and suckled until d 10 (SOW; n = 5), weaned at 3 d of age and fed formula until d 10 (FOR; n = 5), or weaned at 3 d of age and fed formula with CWF supplement (SUP; n = 5). After transporting from the farm, piglets of the FOR and SUP group were group-housed, without separating littermates, in a commercial brooder. All formula fed (FOR and SUP) piglets were *ad libitum* fed using a semi-automatic milk dispenser. All piglets learned to use the feeding system within 6 h. The formulated milk used in this study was a complete milk replacer for young piglets, allowing rearing piglets in absence of the sow. The commercial milk replacer was composed of skimmed milk powder, whey powder, coconut fat, palm oil and wheat concentrate and nutritional additives such as amino acids, minerals and vitamins (calculated analysis: 28 g crude protein, 23 g crude fat, 56 g lactose per l and calculated ME 548 kcal/l). Supplemented piglets received the CWF once a day via gavage at a dose of 10 ml/kg BW. This dose was chosen as it was calculated to be 1 to 10% of the piglets' daily milk intake. Piglets in all experimental groups had free access to water, while no solid feed was available.

3.5. Permeability measurements and tissue collection

To measure *in vivo* gut permeability, the piglets were dosed intra-gastrically with 0.75 g lactulose/kg BW and 0.3 g mannitol/kg BW (Sigma-Aldrich, Steinheim, Germany) 4h before euthanasia (427-429). Urine was collected by cystocentesis at the time of euthanasia. Concentrations of lactulose and

mannitol in urine were measured as a percentage of urine recovery using an enzymatic spectrophotometric method (430, 431). Piglets were euthanized by the intraperitoneal injection of an overdose sodium pentobarbital (200 mg/kg) followed by exsanguination.

3.6. Tissue collection

After euthanasia, the intestinal tract was flushed and rinsed and internal organs were weighed. The lengths of small and large intestines were recorded. The entire small intestine was equally divided into a proximal, middle, and distal part. Samples were taken from each small intestinal segment. One set of samples was immediately frozen in liquid nitrogen and stored at -80°C. Another set was immersed for 2 h in 4% paraformaldehyde solution at room temperature (0.1 M, pH 7.4).

3.7. Morphometric analysis and enzyme activities

After fixation, samples were rinsed with phosphate-buffered saline solution (pH 7.4, PBS) for 24 h and routinely processed to paraffin blocks. Transverse paraffin sections (5 μm) were conventionally stained with haematoxylin-eosin (HE). The thickness of the tunica muscularis and tela submucosa, the length and width of the intestinal villi, crypt depth and the percentage of crypt fission were measured at a magnification of 100x. Villus length and crypt depth were measured, for each tissue block, in 30 well-orientated villi and crypts (Olympus BX 61, analySIS Pro, Olympus Belgium, Aartselaar, Belgium). Crypt fission is expressed as the percentage of crypts having a split or fissure in their base. The percentage of crypt fission was determined in at least 100 crypts in a total of 10 systematically at random sections (Olympus

BX 41; Olympus Belgium). Enzyme activities (aminopeptidase A, aminopeptidase N, dipeptidylpeptidase IV, lactase, maltase and sucrase) of homogenized small intestinal samples were measured according to De Vos *et al* (432). Enzyme activity levels were expressed as units per gram wet tissue.

3.8. Immunohistochemical staining

After antigen retrieval with citrate (pH 6, DakoCytomation, Glostrup, Denmark) for the detection of mitotic cells via anti-KI67 protein (433) or with proteinase K (DakoCytomation) for the detection of occludin, paraffin sections (5 μm) were incubated with 3% H_2O_2 and 20% normal goat serum. Subsequently, sections were incubated overnight at 4°C with monoclonal mouse anti-human Ki67 antibody (1/25, DakoCytomation) diluted in 50 mM Tris(hydroxymethyl)aminomethane-buffered saline solution enriched with 1% bovine serum albumin (TBS/BSA) or with polyclonal rabbit anti-occludin (1/250; Invitrogen, Camarillo, CA) diluted in TBS enriched with 0.3% Triton-X-100 (Sigma, St Louis, MO). After washing in 50 mM TBS (pH 7.4), sections were incubated with biotinylated secondary goat anti-mouse antibody (for anti-KI67 protein) or with biotinylated goat-anti-rabbit antibody (for occludin) (1/200 diluted in TBS/BSA; DakoCytomation) for 30 min at room temperature. Following rinsing with TBS, sections were incubated for 30 min with streptavidin-conjugated horseradish peroxidase (1/200 diluted in TBS/BSA, DakoCytomation) at room temperature. After rinsing with TBS and demineralized water, proliferating cells and the tight junction protein occludin were visualized with 3,3'-diaminobenzidinetetrahydrochloride and tissues were counterstained with haematoxylin. The number of immunoreactive cells for KI67 was expressed as a percentage of the total number of nuclei per crypt. At least 30 well-

orientated crypts were counted in the proximal, middle and distal part of the small intestine of each animal.

3.9. Protein extraction and western blot

Protein levels of occludin were measured by Western immunoblotting. Frozen tissues were homogenized in lysis buffer (50 mM Tris, 150 mM NaCl, 1% (wt/vol) Nonidet P40, 0.5% (wt/vol) deoxycholate, protease inhibitor tablet (Roche Diagnostics GmbH, Mannheim, Germany)) and subjected to 10 - 15% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Biorad Labs, Hercules, CA) under reducing conditions. Proteins were electrophoretically transferred to nitrocellulose membranes. Non-specific protein binding was blocked with 5% (wt/vol) non-fat dry milk in Tris-buffered saline with Tween 20. Membranes were incubated with a rabbit polyclonal antibody to occludin (Invitrogen, Merelbeke, Belgium; 1/1000). After washing, blots were incubated with an anti-rabbit secondary antibody conjugated to biotin (DakoCytomation; 1/1000). After incubation with avidin/biotinylated horseradish peroxidase (DakoCytomation) detection of bound antibodies was performed using a chemiluminescence system (Super Signal West Femto; Thermo Scientific) according to the manufacturer's instructions. A normalized OD was obtained by dividing the protein density with the density of the loading control β-actin (Sigma-Aldrich, Steinheim, Germany). Band intensities were quantified by densitometry using GeneSnap and GeneTools software (Syngene, Cambridge, UK).

3.10. Statistical analysis

Each *in vitro* experiment was run in duplicate and repeated three times. *In vitro* data were subjected to the Kruskal-Wallis test followed by the Mann-Whitney U test.

The effects of the ingested feed and BW on the different anatomical, physiological, and enzymatic characteristics were assessed by fitting linear mixed models. The BW, feed, and (if applicable) region were included as fixed effects, as well as the 2-way interactions between them. The dependence between observations within the same litter and (if applicable) within the same individual was taken into account by including random intercept and random slope terms into the regression model. The fixed effect model was simplified in a stepwise backward way.

Significance of the fixed effects was tested by performing an F-test between the larger model and the reduced model, adjusting the number of degrees of freedom using the Kenwardroger method. In case the final model included a significant effect of region or feed, a post-hoc test was performed to test which of the categories had a difference with a Tukey correction for multiple comparisons. All statistical calculations were performed in the software package R version 2.13.1. Mixed models were fit using the Ime4 package. The F-test with Kenwardroger correction was performed using the package pbkrtest, and the post-hoc test with Tukey correction was carried out as implemented in the multcomp package.

4. RESULTS

4.1. Composition of the colostral whey fraction

The protein content of the colostral whey fraction was 285 µg/ml. Plotting the MS/MS spectra of this fraction (<50 kDa) against the mammalian Swiss Prot Database with *Bos Taurus* as organism being the only criterion revealed with a high probability (over 80%) that this fraction contained: α-S1 casein, α-S2 casein, β-lactoglobulin, β-casein, β-2-microglobulin, κ-casein, fibrinogen α chain, complement C3, α-lactalbumin, serum amyloid A, polymeric immunoglobulin receptor, cathelicidin-1, cathelicidin-2, fibroblast growth factor-binding protein 1, haemoglobin subunit α and zinc finger protein 746.

4.2. In vitro assessments

The proliferative effect of CWF as determined using the MTT assay is shown in table 4.1. The relative activity of cell growth was significantly increased after the addition of the various doses CWF (P = 0.019). This suggested a role for CWF in regenerative capacity, which tended to increase cell migration 3 h after injury (Table 4.2) (P = 0.082).

We observed a drop in resistance after the replacement of culture medium by basal medium. This drop in resistance was reduced after the addition of CWF (P = 0.021), suggesting CWF increased monolayer integrity.

Table 4.1 Relative activity of cell growth after the addition of various concentrations (1%, 5%, 10%) CWF to IPEC-J2 cells [1].

Variable (%)	Basal medium	CWF				P-value
		1%	5%	10%		
	Mean	Mean	Mean	Mean	SEM	
Relative activity of cell growth	-	9.2	17.9*	45.3*	5.23	0.019
Regenerative capacity	50.94	65.11	70.67	80.61	3.93	0.082
Resistance drop	51.10	42.58	24.07*	13.45*	4.71	0.021

[1]An asterisk refers to a significant difference from the basal medium ($P < 0.05$).

4.3. Piglets' growth and anatomy of the gastrointestinal tract

Because average daily weight gain was lower in artificially-fed piglets compared to those suckled the sow, they had lower final body weights ($P < 0.001$; Table 4.2). Consequently, gross morphometrical data were expressed relative to the final body weights. Birth weight and feed-related differences were found with regard to the relative size of the digestive organs. Artificially reared piglets had heavier stomachs ($P = 0.02$) and colons ($P < 0.001$), whereas spleen weight ($P = 0.009$) was decreased compared to the SOW treatment. Caecum weight ($P = 0.01$) was greater for piglets on the SUP treatment compared to the other treatments. Small intestinal ($P < 0.001$) and colon lengths ($P < 0.001$) were greater for artificially fed piglets compared to those suckled the sow. Moreover, SUP piglets had a longer colon compared to FOR piglets ($P < 0.01$). The LBW piglets showed heavier stomach weights ($P = 0.01$), whereas the small intestine and colon ($P < 0.001$) were longer compared to NBW piglets (Table 4.2).

Table 4.2 Body weight and relative size and weight of digestive organs of piglets [1,2].

	NBW			LBW				P-value for F-test		
	SOW	FOR	SUP	SOW	FOR	SUP	SEM	BW	Feed	I
Birth weight (kg)	1.49	1.49	1.49	0.96	0.96	0.96	0.09	<0.001	-	
Body weight (kg)	3.77[a]	2.74[b]	2.63[b]	2.40[a]	1.79[b]	1.74[b]	0.19	<0.001	<0.001	0.11
Stomach weight (g/kg)	5.89[a]	7.64[b]	7.60[b]	6.49[a]	9.05[b]	8.68[b]	0.63	0.01	<0.001	0.66
Small intestinal weight (g/kg)	36.72	33.70	44.15	36.97	40.36	43.00	3.23	0.22	0.13	0.05
Small intestinal length (cm/kg)	169.9[a]	212.3[b]	251.2[b]	222.1[a]	288.9[b]	309.7[b]	16.4	<0.001	<0.001	0.56
Colon weight (g/kg)	7.02[a]	9.79[b]	11.72[b]	6.55[a]	10.81[b]	13.14[b]	2.32	0.73	<0.001	0.12
Colon length (cm/kg)	26.41[a]	36.06[b]	46.48[c]	41.07[a]	49.40[b]	59.53[c]	2.95	<0.001	<0.001	0.93
Caecum weight (g/kg)	0.93[a]	1.03[a]	1.69[b]	0.93[a]	1.20[a]	1.68[b]	0.16	0.33	0.003	0.23
Liver weight (g/kg)	27.41	28.25	28.65	29.54	27.80	29.43	1.35	0.39	0.70	0.61
Pancreas weight (g/kg)	1.71	2.78	2.31	1.72	2.10	2.44	0.38	0.45	0.09	0.33
Spleen weight (g/kg)	2.34[a]	1.71[b]	1.69[b]	2.35[a]	1.88[b]	1.84[b]	0.18	0.45	0.003	0.89

[a-c] Within normal or low birth weight, means not sharing the same superscript letter are different ($P<0.05$).

[1] SOW = remained with the sow and suckled until d 10, FOR = weaned at 3 d of age and fed formula until d 10, SUP = weaned at 3 d of age and fed formula with colostral whey fraction until d 10, and I = birth weight x feed interaction.

[2] Values are presented as means and pooled SEM (n = 5/treatment).

4.4. Structural and functional characteristics of the small intestine

The microscopic analysis of the small intestine mainly displayed feed-related differences.

Artificial feeding was associated with a marked decrease in villus height ($P = 0.001$) and increase in crypt depth ($P < 0.001$) compared to sow-feeding (Table 4.3). Piglets on the SUP treatment had shorter villi ($P = 0.001$) compared to piglets on the FOR treatment. In addition, the percentage of crypt fission was reduced ($P < 0.001$) (Table 4.4). There was a birth weight x feed interaction in the tela submucosa layer ($P < 0.001$) (Table 4.4). Fitting separate models for LBW and NBW piglets showed that in the LBW group, there was an effect of feed, with the SUP treatment having a greater mean

value compared to the 2 other treatments. No effects of dietary treatment were observed in the NBW group. No birth weight, feed or region-related differences were observed for the villus width, tunica muscularis thickness, and the KI67 proliferating index in the small intestine. The KI67 immunoreactive proliferating epithelial cells were predominantly located in the crypt region (Fig. 4.1).

Table 4.3 Small intestinal morphological characteristics and proliferation index in piglets [1,2].

	NBW			LBW				P-value for F-test		
	SOW	FOR	SUP	SOW	FOR	SUP	SEM	BW	Feed	I
Crypt depth (μm)	78.3[a]	119.67[b]	135.47[b]	74.07[a]	118.85[b]	136.20[b]	1.57	0.39	<0.001	0.80
Crypt fission (%)	4.42[a]	5.08[a]	2.54[b]	5.02[a]	4.13[a]	2.23[b]	0.52	0.71	<0.001	0.42
Villus height (μm)	490.8[a]	419.5[b]	309.0[c]	487.3[a]	361.9[b]	253.9[c]	6.65	0.03	<0.001	0.39
Villus width (μm)	89.06	85.04	89.27	83.38	87.96	85.85	1.09	0.09	0.82	0.36
Tela submucosa (μm)	73.75	102.63	92.83	70.45[a]	64.99[a]	82.10[b]	2.72	-	-	<0.001
Tunica muscularis (μm)	94.19	76.96	81.90	91.34	75.65	68.41	2.31	0.13	0.11	0.27
Proliferation index (%)	42.55	43.66	47.33	37.87	44.85	46.57	3.90	0.56	0.16	0.73

[a-c] Within normal and low birth weight, means not sharing the same superscript letter are different ($P < 0.05$).

[1] SOW = remained with the sow and suckled until d 10, FOR = weaned at 3 d of age and fed formula until d 10, SUP = weaned at 3 d of age and fed formula with colostral whey fraction until d 10, and I = birth weight x feed interaction.

[2] Values are presented as means and pooled SEM (n = 5/treatment). No main and interaction effects of region.

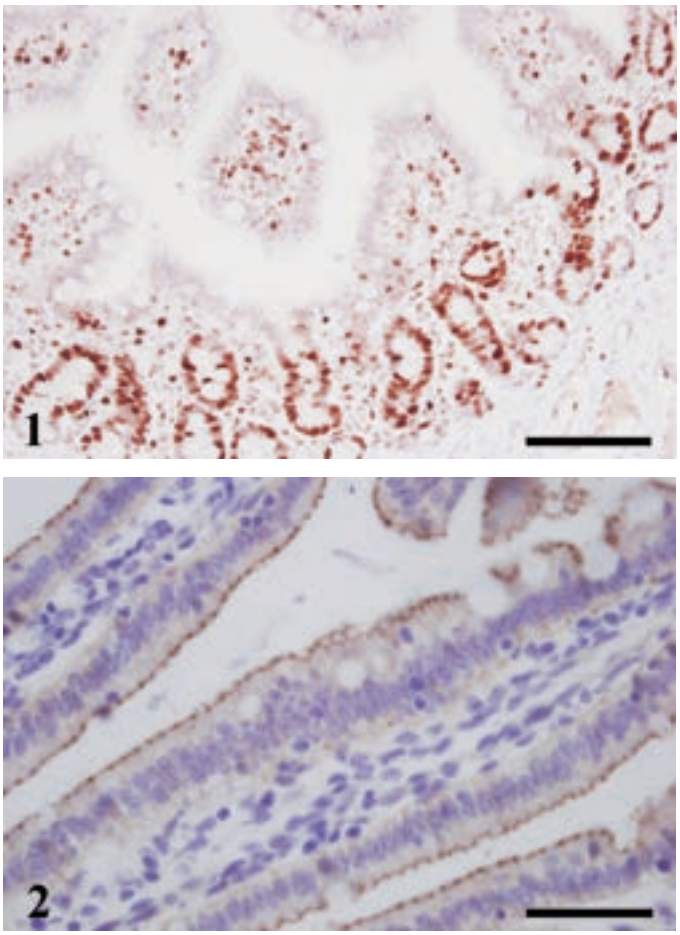

Figure 4.1 (1) The KI67 proliferating cells (red-brown) are visible in the lamina propria and are predominantly seen in the crypts of the distal small intestine of a piglet of 10-d old that suckled the sow. Scale bar = 100 µm. (2) Occludin-immunoreactivity (red) is located at the apicolateral side of the enterocytes of the (distal) small intestinal villi and crypts of a piglet of 10-d old that suckled the sow. Scale bar = 50 µm.

The small intestinal enzyme activities mainly displayed region-related differences, except for lactase (Fig. 4.2A). Sucrase had greater ($P < 0.01$) activities in the proximal compared to the middle and distal part of the small intestine (Fig. 4.2B) whereas maltase activities decreased from proximal towards distal part of the small intestine ($P < 0.01$; Fig. 4.2C). For aminopeptidase A, an effect of region was observed, with the distal part of the small intestine having a significantly greater ($P < 0.01$) activity compared to the middle part (Fig. 4.3A), with the difference between the proximal and distal small intestine showing a trend ($P = 0.09$). Aminopeptidase N activity increased ($P = 0.01$) towards the distal part of the small intestine (Fig. 4.3B). An interaction was observed between region and feed for dipeptidylpeptidase IV ($P < 0.001$, Fig. 4.3C). Splitting the data into proximal, middle, and distal regions showed that in the proximal region, there was no difference in mean among 3 treatments. In the middle and distal part of the small intestine, suckling the sow led to a greater value than the 2 other treatments. Additionally, lactase activity was greater in piglets suckled the sow compared to artificially-fed piglets ($P < 0.01$; Fig. 4.2A).

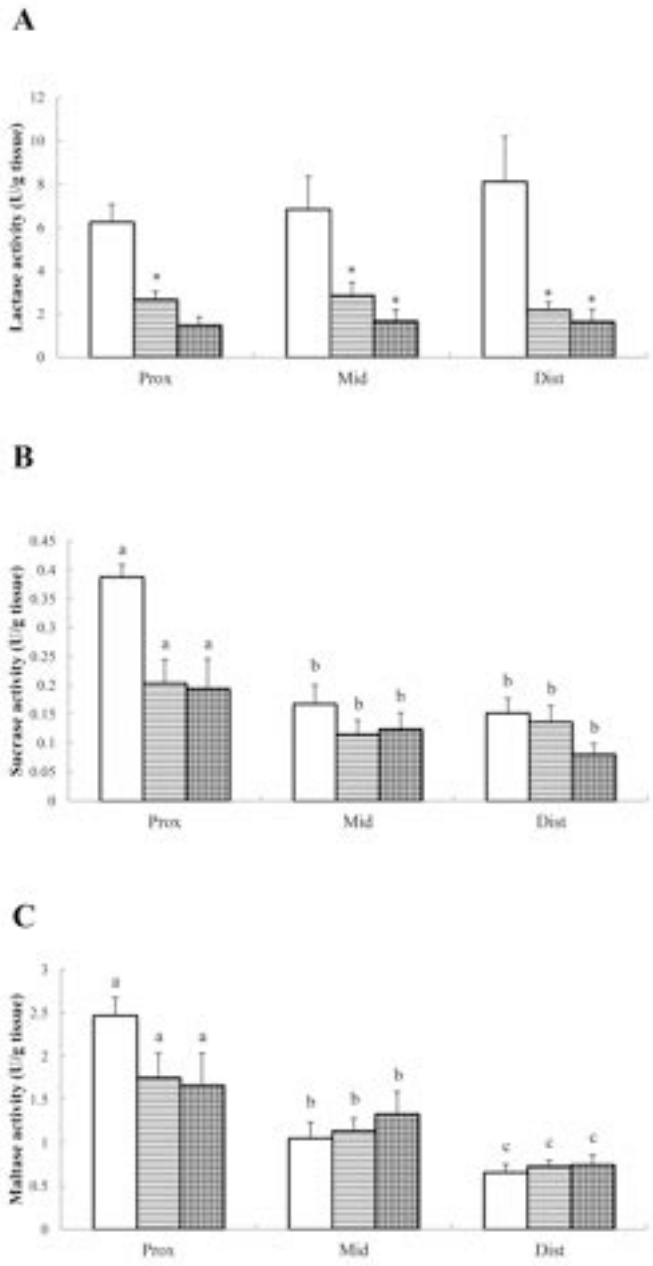

Figure 4.2 Small intestinal disaccharidase (lactase (A), sucrase (B), maltase (C)), of SOW, FOR and SUP piglets, according to small intestinal region. White bars represent sow-fed piglets, striped bars formula-fed piglets and hatched bars represent supplemented piglets. Values represent means and SEM, n = 5/treatment. Within feeding group, means not sharing a common superscript letter differ significantly. An asterisk refers to a difference from the SOW-treatment ($P < 0.05$).

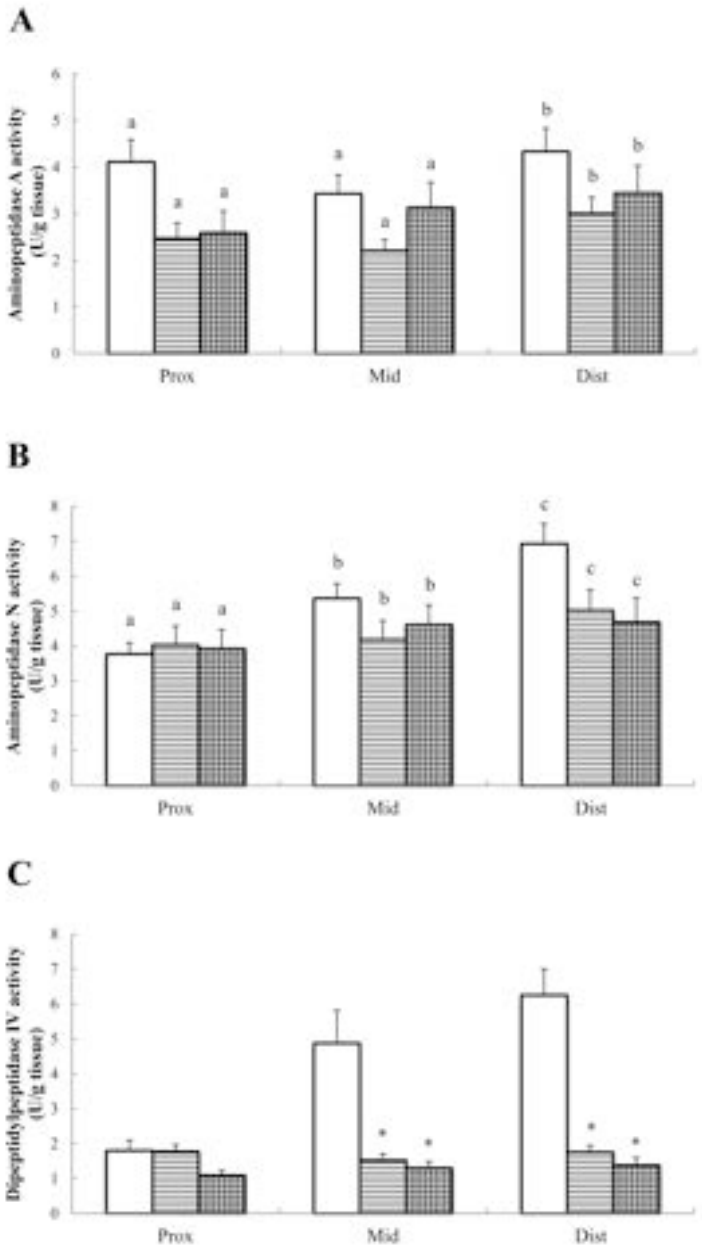

Figure 4.3 Small intestinal peptidase activities (aminopeptidase A (A), aminopeptidase N (B) and dipeptidylpeptidase IV (C)) of SOW, FOR and SUP piglets, according to small intestinal region. White bars represent sow-fed piglets, striped bars formula-fed piglets and hatched bars represent supplemented piglets. Values represent means and SEM, n = 5/treatment. Within feeding group, means not sharing a common superscript letter differ significantly. An asterisk refers to a difference from the SOW-treatment ($P < 0.05$).

4.5. Intestinal Permeability

No statistical differences between the birth weights or among feed treatments were observed for the urinary lactulose-to-mannitol ratio (Table 4.4). For lactulose, no effects of feed and birth weight were found. On the contrary, the greatest levels of mannitol were recovered in the urine of SOW piglets ($P < 0.05$). An effect of feed treatment was found for the occludin expression ($P < 0.05$), with the SUP diet having a greater value compared to the other 2 dietary treatments (Table 4.4). The correlation between the lactulose levels and occludin expression was weak (Spearmann correlation, -0.34) and no significant association between the two variables could be observed ($P = 0.22$). The immunohistochemical staining against occludin revealed that the protein was located at the apico-lateral side of the enterocytes (Fig. 4.1).

Table 4.4 Lactulose/mannitol in urine and occludin expression ratio in piglets[1,2].

	NBW			LBW				P-value for F-test		
	SOW	FOR	SUP	SOW	FOR	SUP	SEM	BW	Feed	I
Occludin/β-actin	0.19[a]	1.64[a]	12.62[b]	0.18[a]	0.79[a]	18.53[b]	3.14	0.42	<0.001	0.59
Lactulose (mmol/l)	27.95	5.06	2.68	24.84	3.81	7.42	9.83	0.45	0.15	0.27
Mannitol (mmol/l)	69.57[a]	6.80[b]	ND[b]	52.64[a]	55.18[b]	5.15[b]	16.6	0.48	<0.001	0.08
Lactulose/mannitol	0.48	0.77	ND	0.39	0.15	0.80	0.23	0.20	0.78	0.15

[a-b] Within normal and low birth weight, means not sharing the same superscript letter are different ($P < 0.05$).

[1] SOW = remained with the sow and suckled until d 10, FOR = weaned at 3 d of age and fed formula until d 10, SUP = weaned at 3 d of age and fed formula with colostral whey fraction until d 10, and I = birth weight x feed interaction.

[2] Values are presented as means and pooled SEM (n = 5/treatment).

5. DISCUSSION

The ingestion of colostrum is believed to play an essential role in gut growth and development via the direct and indirect effects of various bioactive components on the gut epithelium (384, 434). Both *in vitro* (419, 421, 423) and *in vivo* studies (318, 419) have shown that colostrum contributes to cell proliferation and differentiation and mediates several changes in the gut during the postnatal period. Various milk-born factors stimulate the proliferative capacity of the intestinal epithelium, which is visible as crypt hyperplasia or crypt fission or both (435-438), and is a prerequisite for increasing the area for nutrient absorption. Although the value of single milk-born substances on piglets' intestinal growth and maturation has well been established (199, 200, 322, 328, 341, 424), only a few studies investigated the value of a combination of these components in their natural matrix.

In this study, it was demonstrated that early weaning on a milk replacer resulted in shorter villi, reduced lactase activities, and increased crypt depths. Similar changes in small intestinal architecture are described at weaning on solid feed (303, 304, 439), irrespective of the weaning age. In a previous study, no variation in villus lengths or crypt depths were observed between 7-d old formula-fed and suckling piglets (323); but in contrast to our study, those piglets were bottle-fed. Earlier studies unanimously reported the weaning process to affect, irrespective of the age at weaning, brush border enzymes activities (440). Usually, a reduction of enzyme activities is observed in the early post-weaning period (441, 442), whereas elevated enzyme activities are observed following the recovery of villus heights 1 to 2 wk after weaning (443). In a previous study, Thymann and coworkers suggested that formula-feeding resulted in insufficient lactase activity, which is in accordance with our study (389). Similar as in our study, at 10 d of age,

body weights of artificially-fed piglets were lower compared to sow-reared pigs (322). The reduced absorptive capacity (shorter villi and lesser mannitol absorption) and reduced functional capacity (lesser lactase activity) in the small intestine of artificially-reared piglets contributes to their reduced body weights. No differences (organ weights, intestinal morphology, enzyme activities, and permeability) between both birth weight categories could be detected. Boudry and coworkers made a similar observation (324). The finding that LBW piglets responded similarly to artificial rearing as NBW piglets, illustrates that a more in depth analysis of the composition of the milk formula is needed in order to find out which factor improves the growth performance of the LBW piglets. In this respect, our study attempted to investigate the effect of a fraction of colostral whey (bovine) that promoted enterocyte proliferation *in vitro* and improved the barrier functioning of the intestine (own observations).

The protein analysis of the < 50 kDa fraction of colostral whey corresponds to earlier descriptions of the bovine whey proteome (444-447). Proteins with a documented effect on intestinal health (e.g., α-lactalbumin and cathelicidin) (444) were present in the fraction that was supplemented to the piglets.

However, supplementing CWF did not affect the body weights at d 10. Nevertheless, the addition of CWF induced changes in gut architecture. Namely, CWF decreased villus length and crypt fission percentage. Park et al. stated that crypt fission rate is decreased by epidermal growth factor (437), a growth factor that was however absent or present below detectable levels in our fraction. No effect was observed on the proliferation index, which differs from our *in vitro* trials that showed a positive effect of CWF on the proliferation of IPEC-J2 cells. Previous studies have confirmed that the addition of colostrum (419, 421, 448-450) or whey (451, 452) to cell cultures results in increased cell proliferation. Moreover, no changes in brush border

enzyme activities were observed in the supplemented and unsupplemented piglets. Treatment with CWF resulted in mannitol levels below the detection limit (NBW piglets) or very low levels (LBW piglets). This is an indication for decreased transcellular transport, an observation that can be explained by the reduced absorptive area and related to the shortening of the villi. Although lactulose levels did not differ between the feeding groups, occludin expression was increased in the supplemented group. Barrier function is maintained by, amongst others, tight junctions, in which occludin is an important integral membrane protein (453). The epithelial barrier forms the first line of defense within the intestinal lumen and prevents the passage of pathogens, toxins and other antigens. In our *in vitro* study, the addition of CWF reduced the drop in resistance that was observed after the addition of basal medium. Likewise, in our *in vivo* study, occludin expression was increased in piglets that had received the colostral whey fraction. In an earlier study, it was demonstrated that bovine colostrum protects swine intestinal cells against increased membrane permeability caused by enterotoxigenic *Escherichia coli* (423).

Previous studies have shown that several proteins, which were identified in our fraction, maintain epithelial barrier integrity in both cell cultures and *in vivo* experiments (454-457). Besides proteins, individual amino acids may have a specific role in maintaining intestinal barrier function (458, 459). The role of L-glutamine in reducing intestinal permeability has been thoroughly described (460). L-glutamine maintains epithelial barrier integrity in cell cultures (461) and *in vivo* (462, 463). Additionally, other amino acids such as arginine (464), citrulline (465) and threonine (466) improve gut barrier integrity.

The formation of tight junctions is not only influenced by alimentary components but also by intestinal microbiota and inflammatory processes

(461, 467). As a result, our findings need to be corroborated by further experiments, in which these parameters are taken into account.

In conclusion, at the age of 10 d, no morphological and functional differences could be observed between the NBW and LBW piglets. Moreover, the supplementation of a low molecular weight fraction of bovine colostral whey to piglets fed on a milk replacer did not stimulate weight gain, digestive enzyme activities, or *in vivo* permeability, but resulted in structural changes of the small intestine. These findings should be acknowledged when developing formulated milk with the aim of improving the survival and performance of especially piglets with low birth weight.

CHAPTER 5

GENERAL DISCUSSION AND FUTURE PROSPECTS

1. BRIEF OVERVIEW OF THE STUDY RESULTS

Chapter 2 Evaluation of body composition and muscle energy stores in piglets

- The chemical total body composition, intramuscular glycogen and lipid stores of LBW piglets were not different from NBW piglets.
- Body dry matter, fat and protein content increased with aging.
- Body ash content and intramuscular lipid stores remained constant during growth.
- Muscle glycogen contents decreased with increasing age.

Chapter 3A Artificial rearing of piglets: effects on small intestinal morphology and digestion capacity

- The morphology of the small intestine of LBW piglets was not different from NBW piglets.
- The small intestinal functional capacity of LBW piglets showed minor differences from NBW piglets.
- During d 3 to d 10, formula-fed piglets showed reduced average daily gain and lactase activity compared to suckling piglets.
- During d 3 to d 28, formula-fed piglets had a comparable average daily gain, greater absorptive area, maltase and sucrase activities, deeper crypts compared to suckling piglets.

Chapter 3B Increased IGF-1 serum levels and discordant protein and mRNA IGF-1 receptor expression in the small intestine of formula-fed piglets.

- LBW piglets had lower IGF-1 levels in comparison with NBW piglets.
- Birth weight did not affect IGF-1R mRNA or protein abundance.
- Formula-fed piglets had higher serum IGF-1 levels compared to suckled piglets.
- mRNA expression of the IGF-1 receptor in the small intestine showed lower expression in formula-fed versus suckled piglets.
- Protein expression of the IGF-1 receptor in the small intestine showed higher expression in formula-fed versus suckled piglets.

Chapter 4 Effects of supplementing formula-fed piglets with a bovine colostrum fraction

- A colostral whey fraction stimulated IPEC-J2 cell proliferation and monolayer integrity.
- Supplementing piglets with a colostral whey fraction did not affect body weights, enzyme activities or the dual absorption test.
- Supplementing piglets with a colostral whey fraction resulted in villus shortening and elevated occludin expression.

2. ARTIFICIAL REARING OF PIGLETS

2.1. General observations of feeding piglets on formulated milk

Under natural conditions, weaning is a gradual process in which the sow reduces the nursing frequency. In free-living conditions the weaning process is completed by the age of 13 to 17 weeks (468). However, in commercial farms, weaning piglets implicates an abrupt withdrawal of mother care and milk supply, a sudden shift towards solid feed and mixing with strangers. In the EU, it is forbidden to wean piglets before 4 weeks of age unless advantages for the health of the dam or the piglets justify earlier weaning. However, piglets may be weaned up to 7 days earlier if they are moved to a specialized, cleaned and disinfected housing (469). Thus piglets are routinely weaned at 21 to 28 days of age. Currently, pig production - especially in US - is moving towards reducing the weaning age to 10 to 14 days and even earlier. The reasons for weaning at this early age are related to increasing the yearly production by rebreeding the sow as soon as possible. In addition, the concepts of medicated early weaning and segregated early weaning are used to eradicate diseases (e.g. PRRSV) or to produce a healthier breeding stock.

In Flanders, 31% of the pig farmers apply split-weaning, i.e. the practice whereby part of the litter is removed from the sow before weaning the rest of the litter, usually before 3 weeks of age (10). In case of split-weaning, piglets are permanently removed from the dam to either a nursing or a growing-finishing facility. Nursery facilities are generally temperature-controlled facilities that have pens with perforated floors (470). These contain specialized feeders in which warm liquid feed is supplied to the piglets. In addition, brooders have been designed that are placed on top of the farrowing

crate. When piglets are moved to a growing-finishing facility either a liquid or solid feed can be offered via different types of feeders.

Typically, the heaviest piglets are split-weaned in order to avoid additional stress to the smallest piglets. However, the former piglets usually have a more severe growth check and reduced average daily gain compared with smaller piglets (471, own observations).

In this thesis, we noticed few disadvantages of rearing 3-d old piglets in brooders. As described in chapter 3A, feeding piglets in a brooder on formulated milk for one wk inhibits the growth of the piglets, resulting in reduced body weights at 10 d of age. In contrast, artificially reared piglets had a comparable ADG compared to sow-fed pigs at 28 days of age. Although LBW piglets had generally a lower ADG (70 g/d) compared to NBW piglets we believe that piglets of all birth weight categories can be housed within the artificial rearing systems.

Therefore, we evaluated the effects of artificial rearing in a field trial. In this field trial, the conventional system used on the farm was compared with our experimental setting used in chapter 3A. In the conventional system, the heaviest piglets were split-weaned at 10 d of age and raised on milk replacer A (addendum Table 1) in round feeders (addendum Fig. 1). In the experimental system, LBW piglets were split-weaned at 4 d of age and raised on milk replacer B (addendum Table 1) provided via rescue cups (addendum Fig. 2).

Although we are aware of the fact that this practical set-up had important confounding factors, in general we may conclude that the results of this field trial were very disappointing (mortality in the split-weaned group of the experimental system was 90%) due to a combination of factors. The first problem was the starting temperature which was not warm enough to house 4-d old split-weaned piglets. The second problem was the water quality used

for the reconstitution of the milk formula, which was severely contaminated (addendum Table 2). A third problem was that the milk replacer used was not stable in solution and protein flocculation and fat separation occurred when applying it in the cup system. Combining the results of Chapter 3A and our own observations in practice led to some recommendations when artificially rearing piglets. In general, our results illustrate that when piglets are removed from the dam at 3 d of age, a high degree of care and management, controlled environmental and hygiene conditions and specialized diets are required. Undoubtedly, piglets should receive colostrum from the sow in order to obtain passive immunity (472). According to our experience, piglets can be small but should be healthy and vital in order to be withdrawn from their mother. Additionally, both environmental and hygiene conditions should be totally under control. Artificially reared piglets should be housed warm enough. The following temperature ranges are recommended: during the first week 30 to 32°C; during the second week: 28 to 30°C; during the third week 26 to 28°C and during the forth week: 24 to 26°C (473). A key factor in successfully using liquid nutrition, including artificial rearing of piglets is sanitation. Apart from overall hygiene, feeders must be rinsed and cleaned daily. Mixing milk replacers with water should be done thoroughly and manufacturer's dosages and guidelines should be followed precisely. Some milk replacers should be mixed with warm water, but they may never be mixed with hot water as nutrient degradation may occur. In addition, the water used for mixing must be of drinking quality and not chemically or bacteriologically contaminated. The success of the rearing system strongly depends on the physical characteristics of milk replacers used. The ideal milk replacer should be fresh with a typical milk smell. In addition, it should be soluble, stable in solution, with minimal protein flocculation and fat separation. Although some milk replacers remain stable for 2 or 3 days, it is recommended to daily prepare a fresh solution.

2.2. Small intestinal consequences of feeding piglets on formulated milk

Artificial rearing of 3-d old piglets led to some degree of gastrointestinal dysfunctioning during the first week. In our experiments, we could relate this dysfunctioning to a slower functional maturation of the gastrointestinal tract. The slower intestinal functional maturation was reflected by altered brush border enzyme activities and thereby a reduced capacity to optimally utilize the nutrients that were offered via the formulated milk. Similar short-term consequences of formula-feeding were observed in previous studies with piglets (322, 389, 391). Nevertheless, the experimental setting of our study differs substantially from the previously mentioned studies since in our study piglets were not individually housed and different birth weight categories were taken into account. Despite the short-term negative consequences, long-term formula-feeding resulted in improved gut growth and functional maturation. Artificially rearing of piglets until 28 d of age resulted in a greater area for nutrient absorption and an increase in crypt cell population, which was also shown by Boudry and coworkers (324). Additionally, maltase and sucrase activities increased more rapidly in formula-fed piglets compared to suckling animals. Furthermore, formula-fed piglets had inconsistent IGF-1R expression in the small intestine, namely lower IGF-1 mRNA expression and higher IGF-1 protein abundance, compared to sow-feeding. These results confirm that enteral nutrition (i.e. formula versus sow milk) indeed regulates both structural and functional gut maturation differently. Although it is known that formulated milk lacks ‘milk-borne’ bioactive compounds, in this thesis it was demonstrated that long-term formula feeding can have a trophic effect on the intestine and induces maturation of intestinal disaccharidases, as was previously shown in studies with human infants and rats (396, 398).

Apparently birth weight does not alter the feeding induced structural gut adaptation, since in our study, for none of the measured structural parameters of the small intestine differences could be observed between the two birth weight categories (324). In addition, no birth weight related differences in small intestinal IGF-1R expression could be observed. On the contrary, with regard to the functional maturation of the small intestine, the effect of birth weight did depend on the ingested feed. At 28 d of age, NBW formula-fed piglets had higher maltase and sucrase activities compared to LBW formula-fed piglets, indicating a slower maturation in the latter group of piglets. In conclusion, LBW piglets have only limited different responses on formulated milk compared to NBW piglets, suggesting that piglets of both weight categories can be successfully artificially reared.

2.3. Future prospects with regard to artificial rearing of piglets

2.3.1. How will the gut microflora be affected?

The gut flora consists of a diverse collection of microorganisms colonizing the healthy gastrointestinal tract of pigs. These microorganisms are involved in a variety of functions e.g. protection, metabolism and nutrition, by which the intestinal physiology and whole-body immunity is affected. In our study, small intestinal samples were analysed for the presence of basic gut flora species (Gram-positive vs. Gram-negative) adhering to the mucosal lining of the intestine. Artificial rearing of piglets generally resulted in a shift towards more Gram-negative adherent bacteria in the small intestine. In addition, when piglets are being fed on formulated milk until 28 days of age, more Gram-positive adherent bacteria are present than when being sow-fed. Unfortunately, we have not identified the different bacterial genera and did not include the intestinal lumen microbiota. Both would provide useful

information and ideally need further research. Generally, the microflora composition is analysed after isolating organisms from faecal or intestinal material. Although not directly comparable with the artificial rearing protocol applied in our study, weaning piglets on solid feed at 28 days of age results in a shift towards more Gram-negative bacteria in the large intestine of piglets. Jensen showed that weaning results in a decreased number of Gram-positive lactobacilli and an increase in the proportion of Gram-negative coliforms, in particularly *Escherichia coli* (474). However, effects on the composition of the microflora seem to depend on the age at which piglets are weaned (475). Poroyko et al. evaluated the microbial gene expression in caecal contents of 21-d old piglets. In this study, Gram-negative *Prevotella* was most abundant in mother-fed piglets, whereas Gram-negative *Bacteroides* were predominant in formula-fed piglets (476). In a study where the microbial composition of colonic contents of 9 and 17-d old piglets was tested, sow-reared piglets had predominantly Gram-positive *Bifidobacteria* whereas formula-fed piglets had more Gram-positive *Clostridium* and Gram-negative *Bacteroides* (477). In agreement with the different studies carried out in piglets, breast-fed infants had a microflora rich in Gram-positive *Bifidobacteria*, whereas in bottle-fed infants the microflora mainly contains Gram-negative *Coliforms* and *Bacteroides* (478-480).

2.3.2. How will the immune system respond?

Sows have an epitheliochorial placenta, which implies that maternal antibodies are not transferred from the sow to the foetal pig *in utero*. Consequently, neonatal piglets are born agammaglobulinemic and immunologically naïve. Therefore, piglets entirely depend on colostrum for maternal immunoglobulin intake. At birth, natural killer cell activity is low or absent (481), neutrophil numbers are low (482) and the gut-associated

lymphoid tissue (GALT) is poorly developed (483). Due to the postnatal development of the immune system, the early age when piglets were removed from the sow in our study (3 days after parturition) will most likely affect piglets' immune responses. In addition, artificial rearing results in environmental, nutritional and social changes, which will most likely provoke an acute stress response suppressing cell-mediated immunity (484, 485). Whether LBW piglets will respond immunologically different than NBW piglets on the changes following artificial rearing needs to be further elucidated. Especially since recent studies showed that artificially reared LBW piglets contain altered cytokine profiles in the serum and in the intestine compared to NBW piglets (486, 487).

Surprisingly, birth weight does not influence serum IgG concentration in suckling neonatal piglets (488, 489). Probably, LBW piglets have a greater capacity to absorb large molecules (490). Because 50% of the pre-weaning mortality occurs during the first 3 days of life (491), future research should focus on testing the effect of supplementing piglets with booster preparations and colostrum substitutes during this period. In particular, because of their high capacity to absorb immunoglobulins, LBW piglets would most likely benefit from extra immunoglobulin administration. Although scientific evidence on the efficacy of booster preparations and colostrum substitutes is scarce (492, 493), piglets are often supplemented shortly after birth. In Flanders for example, 16% of the farmers supplements all piglets at time of birth, whereas 18% of the farmers only supplement the smallest piglets (10).

2.3.3. What is the profitability of artificial rearing during the nursing period?

In recent years, pig production is under severe pressure because of the low pig prices and the high costs for e.g. feed and housing. Several Western breeds have more piglets than functional teats, implicating that there is a need for alternative systems to raise these supernumerary piglets. However, whether the farmer has a higher profit after motherless rearing piglets within the available systems remains unknown.

The producer of the commercial brooder used in our study (Rescue deck system©, Provimi, The Netherlands) calculated that the installation and maintenance costs of these systems are €1.67 per piglet per year. When all costs and profits of these systems are taken into account (amongst which the price of the milk replacer), a positive return on investment of 2.54% was calculated. In his master-thesis, Gruwier proposed an economic model in order to calculate the profitability of several rearing systems used during the nursing period (494). Within this theoretic model, keeping supernumerary piglets alive elevated the costs for feed, health, manure and labour. Because of the higher number of produced piglets and the greater weaning weight after milk-supplementation, the expectation would be that the farmers' profit increased. However, milk supplementation seemed to give rather negative results predominantly because of the high costs of the milk replacer. What the actual return on investment is of motherless rearing in practice needs to be investigated, but is expected to be somewhere between the calculations of both described studies.

3. EFFECTS OF SUPPLEMENTING FORMULA-FED PIGLETS WITH A BOVINE COLOSTRUM FRACTION

Hyper-prolific sows are pushing the limit of numbers reared per year to above 30 piglets. When litter size increases, total milk yield increases, but not in a proportional manner, implicating that with each additional piglet, milk intake per piglet decreases (495). Hence, liquid milk replacers are used in almost every swine farm in Europe. Today, the most common application of liquid milk replacers is supplementing piglets that are suckling the sow. For example, formulated milk is supplemented in 54% of the pig farms in Flanders. Total artificial rearing of piglets on a milk replacer is less frequently practiced (in Flanders 6.2% of the pig farms use brooders) (10).

Sows produce rather thick milk that is especially rich in fat. As a result, the ideal milk replacer should be a dense powder (496). Ideally, mixing rates should be at least 20%. However, such a powder is difficult to manufacture and market. Therefore, the majority of commercial milk replacers are manufactured to mix at 10 to 17.5%, implicating that after reconstitution, the milk replacer is lower in energy than sow milk. Recently, novel milk products have been designed which are to be mixed at 25 to 50%.

In addition to the nutrient content of milk replacers, the ingredient composition is equally important (497). The digestive system of piglets is developed to digest milk components. Therefore, the majority of milk replacers are composed of skim milk, whey, lactose etc. In addition, vitamins, minerals, lipids, taste and flavour enhancers are added. In some milk replacers, plant-based ingredients are added (e.g. soy protein), in order to lower the price or as emulsifiers (e.g. lecithin).

Because sow milk is rich in bioactive substances, some milk replacers are supplemented with functional ingredients such as immunoglobulins. In general these immunoglobulins can be derived from dried animal plasma,

bovine colostrum, or could be egg-derived (498). Of these products, preparations can be produced in order to give effective specific protection against different enteric diseases (e.g. *E. coli*) in piglets (499).

One of the main differences between the two milk replacers used for artificial rearing on the farm is that milk replacer A contains animal plasma (5%) whereas milk replacer B does not (addendum Table 1). Studies have proven that feed intake of young pigs can be stimulated by the inclusion of animal plasma (5 to 7%) in solid diets immediately after weaning (500-503). Moreover, the inclusion of animal plasma has beneficial effects on small intestinal barrier integrity and the occurrence of diarrhoea (504). Quigley and Wolfe incorporated spray dried plasma in calf milk replacer with positive effects on calf mortality and morbidity (505). Unfortunately, the effect of the inclusion of animal plasma in piglet milk replacer has not yet been investigated in scientific literature, which opens opportunities for further research on this subject. The mechanism responsible for the increased feed intake response upon plasma supplementation and its positive effect on health is mainly related to the immunoglobulin fraction (506). Additionally, Van Dijk et al. described that the effect of plasma is greater when performance in weanling piglets without plasma is low. This implies that healthy piglets that are housed in good facilities do not benefit much from plasma (507).

Besides plasma, bovine colostrum is a protein source rich in immunoglobulins. Although not directly comparable with our study, previous studies in weaned piglets described increased growth rates when consuming bovine colostrum during the post-weaning period (246, 501, 508, 509). In contrast, King et al. described no differences in growth rate but an improved intestinal morphology and immune status in weaned piglets consuming colostrum (510). In a study on artificial rearing 1 d-old piglets researchers replaced whey protein isolate in sow milk replacer with a bovine colostrum

protein isolate. This replacement resulted in positive effects on piglet growth rates, although most of the immunoglobulins in the colostrum isolate were removed (511). This indicates that other substances present in colostrum such as growth factors (e.g. IGF-1) are equally important on piglets' performance. Although the study of Van Barneveld and Dunshea resulted in increased gut growth, small intestinal morphological measurements were not performed (511).

In our study, in which formula-fed piglets were supplemented with a bovine colostrum fraction, supplementation did not stimulate weight gain or digestive enzyme activities, but resulted in structural changes of the small intestine with high barrier integrity as a result.

The intestinal epithelial barrier is anatomically composed of a single layer of enterocytes, joined by junctional complexes that comprise the zonula occludens, zonula adherens and macula adherens. On the one hand, the epithelial layer seals the intestinal tract, to form a robust barrier against the passage of luminal pathogens, toxins and other antigens. On the other hand, the barrier needs to be permeable to facilitate the uptake of nutrients. Weaning young animals, such as piglets, causes mucosal damage and alterations in tight junction integrity, which compromise the small intestinal barrier (512). Hence, weaning is associated with gastrointestinal disorders such as diarrhoea, and the impaired barrier increases the susceptibility to disease. It has been described several times that nutrition plays a crucial role in maintaining the intestinal barrier function. The experiments described in this thesis suggest that supplementing formula-fed piglets with a bovine colostrum fraction resulted in an increased barrier function at 10 d of age. This is an important finding and could perhaps explain why in the study of Van Barneveld and Dunshea there was no incidence of *E. coli* toxaemia in pigs fed the diet containing the colostrum isolate whereas half of the pigs consuming the diet containing whey protein concentrate exhibited symptoms

of *E. coli* toxaemia. These findings should be acknowledged when developing formulated milk for neonatal animals. In addition, it may be possible that barrier function is not only influenced by alimentary components but also by intestinal microbiota and inflammatory processes. Yet, further more focused research is necessary to elucidate this hypothesis.

4. OTHER CONSEQUENCES OF ARTIFICIAL REARING

Weaning the entire litter of sows before 14 days lactation results in increased weaning-to-first service interval, which negatively affects sow productivity (513-515). Weaning-to-first service interval is the time it takes for a weaned dam to return to heat and be bred. Previous studies showed that shortening the lactation period results in reduced post-weaning luteinizing hormone levels, the hormone that triggers ovulation (516, 517). However, early weaning the entire litter does not affect sows' ovulation rate (513, 518) or fertilization rate (519). The main detrimental effect of shortening the lactation period is a greater embryonic mortality (513, 518). Both the uterine environment and the developing embryo can be responsible for this increased embryonic mortality. By one week after farrowing, the involution of the uterus is almost completed and the renewal of the endometrial epithelium well progressed. Nevertheless, it takes at least three weeks before the uterus is completely recovered, both morphologically and histologically (520).

It has previously been described that maturation of ovarian follicles (521-523) and the oocyte (522, 524) is delayed with extended lactations and loss in body condition. Data on the effect of shortened lactations on follicle and oocyte maturation are scarce. Early weaning between 8 and 17 d of lactation (compared to 20 - 31 d in the control group) tended to reduce total follicles by approximately 2 (525), whereas early weaning at 14 d of lactation

(compared to 24 d in the control group) had no effect on ovulation or fertilization rate (519). Thus, the duration of lactation has been reported to influence post-weaning fertility of sows by different mechanisms. However, study results depend on what is considered as extended/shortened lactations and which parameters are tested.

Modern hyper-prolific sows nurse large number of piglets, which results in loss of body reserves during lactation. This can compromise follicle development and reproductive performance, a phenomenon that is often seen in primiparous sows. Indeed, Quesnel et al. showed that sows weight loss increased when nursing a large litter, which negatively affect follicle development (521).

Based on previous studies we could conclude that both shortening the lactation period and nursing large litters have negative effects on the reproductive cycle. Split-weaning, thereby reducing litter size, may improve the reproductive performance of sows in the subsequent parity. Stimulation of teats by the piglets suppresses luteinizing hormone (LH) secretion by inhibiting the gonadotropin-releasing hormone (GnRH) pulse, beginning at 2 to 3 days post partum (526). In case of split-weaning, suckling intensity will be decreased, which could implicate a decrease in the inhibition of LH secretion. This could results in shorter weaning-to-oestrus intervals (139). Matte et al. reviewed data on the effects of split-weaning piglets a few days before complete weaning on sows reproduction. In general, no effects on fertilization rate or litter size were noticed after split-weaning (527). However, whether split-weaning at a very early age has any effects on sow fertility needs to be investigated.

5. CONCLUSION

This thesis documents several findings with regard to the postnatal development of LBW piglets compared to NBW piglets. In our study, it was shown that birth weight had no influence on chemical body composition and muscle energy stores in suckling piglets. In addition, few morphological or functional differences could be seen between the normal and LBW piglets, indicating that the latter piglets develop normally.

Additionally, based on the results obtained in this thesis it can be concluded that artificial rearing is a good alternative to raise supernumerary and/or LBW piglets. However, the success of artificial rearing strongly depends on management, hygiene conditions and the milk replacer offered. Moreover, this study demonstrated that a bovine colostrum fraction maintains the small intestinal barrier integrity, which is an interesting finding to take into account in the development of formulated milk. While we harbour no illusions that the work represented here completes the lack of knowledge on artificial rearing, we do hope that it offers an important foundation on which others can build.

ADDENDUM

Figure 1 Time schedule of the conventional system in which heavy piglets were split-weaned at 10 d of age and raised on milk replacer A in round feeders.

Figure 2 Time schedule of the experimental system in which LBW piglets were split-weaned at 4 d of age and raised on milk replacer B in rescue cups.

Table 1 Composition of the experimental diets used in the field trial.

Composition	Milk replacer A[A]	Milk replacer B[B]
Protein (g/l)	39	39
Lipid (g/l)	35	32
Lactose (g/l)	80	85
ME (kcal/l)	797	768
Ingredients		
Vitamin A (IE/kg)	50000	55000
Vitamin D3 (IE/kg)	10000	5500
Vitamin E (mg/kg)	150	310
Vitamin C (mg/kg)	150	110
Calcium (%)	0.6	0.9
Phosphorus (%)	0.5	0.7
Lysine (%)	1.8	1.7
Methionine (%)	0.8	0.8
Sodium (%)	0.5	0.5
Fe (mg)	235	88
I (mg)	3.5	1.1
Cu (mg)	150	8.8
Mn (mg)	50	60
Zn (mg)	140	77
Se (mg)	0.4	0.3
Antioxidants BHT/BHA(mg)	31	38

[A] major ingredients in the milk replacer A were: whey powder, coconut fat, wheat concentrate, spray-dried plasma

[B] major ingredients in the milk replacer B were: skimmed milk powder, whey powder, palm oil, coconut fat, wheat concentrate

Table 2 Bacteriological and chemical analysis of the water used to reconstitute the milk formula powder.

	Result	Norm
Bacteriological analysis		
Coliforms (cfu/100 ml)	> 100	< 10000
E. Coli (cfu/100 ml)	> 100	< 100
Sulphite-reducing Clostridia (cfu/20ml)	6	< 1
Clostridium perfringens (cfu/100 ml)	> 100	< 1
Intestinal enterococci (cfu/100 ml)	> 100	< 1
Total aerobic plate count 22°C (cfu/ml)	96000	< 100000
Total anaerobic plate count 37°C (cfu/ml)	2000	< 100000
Chemical analysis		
Ammonium (mg/l)	0.69	< 2.0
Calcium (mg/l)	83.5	< 270
Chloride (mg/l)	40.7	< 250
Fluoride (mg/l)	0.16	< 1.5
Phosphate (mg/l)	1.4	< 5.0
Magnesium (mg/l)	9.2	< 50
Manganese (mg/l)	0.19	< 1.0
Nitrate (mg/l)	14.71	< 200
Nitrite (mg/l)	< 0.08	< 0.5
Sulphate (mg/l)	102.3	< 250
Total iron (mg/l)	1.89	< 2.5
Salinity (mg/l)	67.2	< 3000
Conductivity (mS/cm)	602	< 2100
pH	6.1	4-9
Total hardness (°D)	13.8	< 20

SUMMARY

The use of hyper-prolific sow breeds has resulted in increased litter sizes, with often more piglets than the number of available functional teats. Moreover, these larger litters are characterized by high within-litter birth weight variation, which is adversely correlated with piglet survival and growth rates of the undersized piglets. Additionally, the sow's milk yield is insufficient to achieve the maximum growth potential of these larger litters. Consequently, farmers seek solutions to assure piglet survival and optimize their growth. In **chapter 1** an overview is given of the interventions that can be undertaken to influence piglets' birth weight and to increase piglets' neonatal survival and growth. One of the described interventions is to artificially rear supernumerary or LBW piglets on formulated milk. The commercially available milk replacers generally use bovine milk products such as whey, lactose and skim milk, as main ingredients. In order to facilitate the optimization of milk replacers used in practice, a species comparison between bovine and porcine milk composition is made in this chapter. Chapter 1 also covers the small intestinal histology, digestive and absorptive function because these are crucial elements in driving overall postnatal development, growth and health. Furthermore, the function and health of the small intestine is influenced by the intake and the composition of the ingested feed.

The differential development of low and normal birth weight piglets and the impact of artificial rearing on the growth and development of piglets are incompletely documented but nevertheless necessary for a scientifically based rearing strategy of LBW piglets. Thus, we hypothesized (1) that body composition and muscle lipid and glycogen contents are affected by age and birth weight (2) that different rearing conditions influence piglets' growth, structural and functional gut maturation and the IGF-1 system (3) and, that

supplementing a bovine colostrum fraction affects the small intestinal morphology and barrier function.

Chapter 2 focused on the differential development of LBW piglets compared to NBW littermates. The effects of age and birth weight on body composition and muscle lipid and glycogen contents were assessed in pairs of LBW and NBW piglets, at day 0, day 3, day 10 and day 28 of age. In this chapter, we describe that the age of the suckling piglet has a major impact on its body composition and muscle energy stores but its birth weight unexpectedly has no influence. With increasing age, dry matter, fat and protein percentages increased in both LBW and NBW piglets. Body ash content remained constant during growth. Muscle glycogen contents decreased with increasing age for both types of piglets, whereas no age effects could be observed for muscle lipid deposition. Body composition and muscle energy stores were not affected by birth weight.

In the experiments presented in **chapter 3**, artificial rearing was evaluated.
In **chapter 3A**, the effect of artificial rearing on the growth, small intestinal morphology and digestion capacity of piglets was evaluated. In a short-term experiment, piglets were either sow-fed until 10 d of age, or sow-fed until 3 d of age and subsequently formula-fed until 10 d of age. In this experiment, formula-fed piglets showed reduced ADG and lactase activities compared to suckling piglets. In a long-term experiment, piglets were either sow-fed until 28 d of age, or sow-fed until 3 d of age and subsequently formula-fed until 28 d of age. In this experiment, piglets that were formula-fed until d 28 had a comparable ADG compared to sow-fed pigs. In addition, formula-fed piglets had a greater small intestinal mucosal absorptive area, maltase and sucrase activities and deeper intestinal crypts compared to suckling piglets. The

results obtained in chapter 3A suggest that the combination of *ad libitum* access to formulated milk and an increased capacity to absorb nutrients makes artificial rearing a good alternative to raise supernumerary and/or LBW piglets.

In **chapter 3B**, the question was addressed whether feeding piglets on formulated milk affects the IGF-1 system differently in NBW versus LBW piglets. In this experiment, both types of piglets were either sow-fed until 28 d of age, or sow-fed until 3 d of age and subsequently formula-fed until d 28. In this experiment, we observed that formula-fed piglets have higher IGF-1 serum levels and altered gene and protein expression of IGF-1R in the small intestine, irrespective of their birth weight. Thus, birth weight does not alter the feeding induced gut adaptation of the IGF-1 system.

The finding that LBW piglets responded similarly to artificial rearing as NBW piglets, illustrated that a more in depth analysis of the composition of the milk formula is needed in order to find out which factor improves the growth performance of the LBW piglets. Therefore, we tested the effect of a fraction of bovine colostral whey on the morphology and barrier function of the small intestine of 10-d old artificially fed piglets in **chapter 4**. The small intestine was sampled for histology and enzyme activities. In addition, intestinal permeability was evaluated via a dual sugar absorption test and via the measurement of occludin abundance. Supplementing piglets with the colostral whey fraction did not affect body weights, enzyme activities or the outcome of the dual sugar absorption test. On the contrary, the small intestines of supplemented piglets had even shorter villi than unsupplemented piglets and contained more occludin. In both weight categories the colostral whey fraction affected the morphology of the small intestine, which could be

acknowledged when developing formulated milk for neonatal animals with the aim of improving the performance of LBW piglets.

Finally, in **chapter 5**, the main results are summarized and discussed. From the results obtained in this thesis, the following conclusions can be drawn:

1. Piglets deposit fat soon after birth, with increasing contents with aging. Intramuscular glycogen stores are rapidly depleted after birth in both LBW and NBW piglets.

2. Artificial rearing is a good alternative to raise supernumerary and/or LBW piglets. Few morphological or functional differences could be seen between the normal and LBW piglets. However, the success of artificial rearing strongly depends on management, hygiene conditions and the milk replacer offered.

3. A bovine colostrum fraction maintains the small intestinal barrier integrity, which is an interesting finding to take into account in the development of formulated milk.

SAMENVATTING

Het inzetten van hoogproductieve zeugenlijnen heeft geleid tot grotere worpen, met vaak meer biggen dan het aantal beschikbare functionele spenen. Bovendien worden deze grotere nesten gekenmerkt door een grote variatie in geboortegewicht, wat een negatieve impact heeft op de overleving en groei van in hoofdzaak de lichte biggen. Daarenboven is de melkgift van de zeug onvoldoende opdat het maximale groeipotentieel van deze grotere nesten wordt bereikt. Bijgevolg zoeken varkenshouders oplossingen om de overleving van biggen te verzekeren en hun groei te optimaliseren. In **hoofdstuk 1** wordt een overzicht gegeven van de maatregelen die kunnen worden ondernomen om het geboortegewicht van biggen te doen toenemen en om de overleving en groei te verhogen. Een van de beschreven interventies is het kunstmatig opfokken van overtallige of lichte biggen met gebruik van kunstmelk. De commercieel verkrijgbare melkvervangers gebruiken doorgaans koemelkproducten zoals wei, lactose en magere melk als voornaamste ingrediënten. Om het optimaliseren van melkvervangers in de praktijk te vergemakkelijken wordt in dit hoofdstuk een species vergelijking gemaakt van runder- en varkensmelk. Hoofdstuk 1 omvat ook een beschrijving van dunne darm histologie, verterings- en absorptiefunctie. Dit zijn cruciale elementen in het regelen van de postnatale ontwikkeling, groei en gezondheid. Verder wordt de functie en de gezondheid van de dunne darm beïnvloed door de samenstelling van het opgenomen voer.

De impact van kunstmatige opfok op de groei en ontwikkeling van lichte en normale biggen is onvoldoende gedocumenteerd, maar niettemin is dit noodzakelijk om een wetenschappelijk onderbouwde opfokstrategie voor lichte biggen uit te denken. In deze thesis werden - om bijkomende informatie over kunstmatige opfok te structureren en te verzamelen - volgende hypotheses gepostuleerd: (1) dat de lichaamssamenstelling en de

intramusculaire vet- en glycogeengehaltes beïnvloed worden door leeftijd en geboortegewicht (2) dat verschillende opfokstrategieën de groei, de structurele en functionele darmmaturatie en het IGF-1 systeem van biggen beïnvloeden (3) en dat het supplementeren van een fractie uit rundercolostrum een effect heeft op de dunne darm morfologie en barrière functie.

In **hoofdstuk 2** werd nagegaan of lichte biggen zich anders ontwikkelen dan normale nestgenoten qua lichaamssamenstelling. De effecten van leeftijd en geboortegewicht op de lichaamssamenstelling en intramusculaire vet- en glycogeengehaltes werden beoordeeld in paren van lichte en normale biggen, op dag 0, dag 3, dag 10 en dag 28 na geboorte. In dit hoofdstuk werd aangetoond dat de leeftijd van de zogende big een belangrijke invloed heeft op de lichaamssamenstelling en intramusculaire energie voorraad, terwijl het geboortegewicht geen invloed uitoefent. Met toenemende leeftijd, stijgen de droge stof, vet- en eiwitpercentages in beide types biggen. Het mineralengehalte blijft constant tijdens de groei. De intramusculaire glycogeengehaltes nemen af met toenemende leeftijd voor beide types biggen, terwijl er geen leeftijdseffecten kunnen worden waargenomen voor de intramusculaire vetaanzet. Om te besluiten kunnen we stellen dat lichte biggen niet verschillen van hun normale nestgenoten wat betreft hun lichaamssamenstelling en intramusculaire energievoorraad.

In de experimenten beschreven in **hoofdstuk 3** werd kunstmatige opfok geëvalueerd.

In **hoofdstuk 3A**, werd het effect van kunstmatige opfok nagegaan op de groei, dunne darm morfologie en verteringscapaciteit van biggen. In een korte-termijn experiment, werden biggen ofwel gevoed door de zeug tot d 10, ofwel tot d 3 en vervolgens gevoed met kunstmelk tot de leeftijd van d 10. In dit experiment vertoonden biggen gevoed met kunstmelk verminderde dagelijkse groei en lagere lactase activiteiten in vergelijking met zogende biggen. In een lange-termijn experiment werden biggen ofwel door de zeug gevoed tot d 28, ofwel tot d 3 en vervolgens gevoed met kunstmelk tot de leeftijd van d 28. In dit experiment vertoonden biggen gevoed met kunstmelk een gelijkaardige dagelijkse groei als zogende biggen. Daarnaast beschikten de met kunstmelk-gevoede biggen over een groter absorptieoppervlak, hogere maltase en sucrase activiteiten en diepere intestinale crypten in vergelijking met zogende biggen. De verkregen resultaten in hoofdstuk 3A suggereren dat de combinatie van *ad libitum* toegang tot kunstmelk en een verhoogde capaciteit om voedingsstoffen op te nemen, van kunstmatige opfok een goed alternatief maakt om overtallige en/of lichte biggen op te kweken.

In **hoofdstuk 3B**, werd de onderzoeksvraag gesteld of het voeden van biggen met kunstmelk het IGF-1 systeem anders beïnvloed in lichte versus normale biggen. In dit experiment werden beide types biggen ofwel door de zeug gevoed tot d 28, ofwel tot d 3 en vervolgens gevoed met kunstmelk tot d 28. In dit experiment werd waargenomen dat kunstmatig opgefokte biggen hogere IGF-1 serum gehaltes vertoonden en een gewijzigd gen- en eiwitexpressie van de IGF-1R in de dunne darm, ongeacht hun geboortegewicht.

De bevinding dat lichte biggen vergelijkbaar reageerden op kunstmatige opfok als normale biggen, toont aan dat een meer diepgaande analyse van de samenstelling van de kunstmelk noodzakelijk is om na te gaan welke factor de groei van lichte biggen verbetert. Daarom werd **in hoofdstuk 4** het effect nagegaan van een laag moleculair gewicht fractie van rundercolostrum op de morfologie en barrièrefunctie van de dunne darm in 10 dagen oude biggen gevoed met kunstmelk. De dunne darm werd bemonsterd voor histologie en enzymactiviteiten. Bovendien werd de intestinale permeabiliteit geëvalueerd via een suikerabsorptietest en via de meting van occludine expressie. Supplementeren van biggen met de colostrale weifractie heeft geen invloed op het lichaamsgewicht, enzymactiviteiten of het resultaat van de suikerabsorptiestest. Integendeel, de dunne darm van gesupplementeerde biggen vertoonde zelfs kortere villi en bevatte meer occludine. In beide types biggen beïnvloedt de colostrale weifractie de morfologie van de dunne darm, wat in rekening kan worden gebracht bij het ontwikkelen van kunstmelk met als doel de prestaties van lichte biggen te verbeteren.

In **hoofdstuk 5** worden de belangrijkste bevindingen samengevat en besproken. Op basis van de resultaten behaald in deze thesis kunnen de volgende conclusies worden getrokken:

1. Biggen zetten kort na geboorte vet aan, met stijgende vetgehaltes bij ouder worden. De intramusculaire glycogeen voorraad wordt snel na geboorte aangesproken en dit zowel bij lichte als normale biggen.

2. Kunstmatige opfok is een goed alternatief om overtallige en lichte biggen op te kweken. Een beperkt aantal morfologische en functionele verschillen werden geobserveerd tussen normale en lichte biggen. Het succes van kunstmatige opfok is echter sterk afhankelijk van het algemeen management, hygiëne en de aangeboden kunstmelk.

3. Een fractie uit rundercolostrum houdt de dunne darmbarrière intact, wat een interessante bevinding is om rekening mee te houden bij het ontwikkelen van kunstmelk.

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DANKWOORD

Doctoreren is…

Doctoreren is… zoveel meer dan onderzoek doen en publiceren. Doctoreren is een lange weg afleggen, waarin je jezelf op zowel professioneel als persoonlijk vlak op zoveel gebieden tegenkomt en verrijkt. Soms een weg vol hindernissen, maar bovenal van zovele hoogtepunten.

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CURRICULUM VITAE

Personalia

Maartje De Vos, geboren te Gent op 26 juni 1984, behaalde in 2002 het diploma van secundair onderwijs (Latijn-Wetenschappen) aan het Don Boscocollege te Zwijnaarde. In 2008 studeerde ze met onderscheiding af aan de Universiteit Gent als dierenarts en behaalde in hetzelfde jaar het FELASA certificaat type C in de proefdierkunde aan de Universiteit Gent.

Sinds november 2008 verricht zij onderzoek aan het laboratorium voor toegepaste morfologie aan de faculteit FBD aan de Universiteit Antwerpen. Gedurende deze periode werkte Maartje aan dit doctoraal proefschrift, stond ze in voor de begeleiding van de practica anatomie en begeleide ze 5 studenten bij het uitvoeren van hun bachelor thesis, 3 studenten bij het uitvoeren van hun masterstage en 3 studenten bij het uitvoeren van hun masterproef. Daarnaast stond ze in voor de les en practica anatomie van de proefdieren aan Thomas More Kempen.

Maartje is auteur en co-auteur van verscheidene publicaties in peer-reviewed wetenschappelijke tijdschriften. Daarnaast verzorgde ze diverse voordrachten en postervoorstellingen op nationale en internationale symposia.

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