

HUMAN MILK OLIGOSACCHARIDE SUPPLEMENTATION DID NOT AFFECT GUT
DEVELOPMENT IN THE HEALTHY NEONATAL PIGLET

BY

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THESIS

Submitted in partial fulfillment of the requirements
for the degree of Master of Science in Nutritional Sciences
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2017

Urbana, Illinois

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Abstract

Background: Intrauterine growth restricted (IUGR) is a category of infants that are born underweight for their gestational age due to nutrient deficiency in the womb. They are at a higher risk for a variety of health issues later in life, which can be exacerbated if the infant is not breast-fed. Breast milk is superior to infant formula in multiple ways, one of the advantages being the human milk oligosaccharide (HMO) content. HMO is beneficial for the immune health of the baby and cultivating a healthy gut microbiota population. It also has the ability to modulate gut function, but its effects in the gut of the healthy IUGR piglet are largely unknown.

Objective: To investigate the effect of supplementation of HMOs in piglet formula on gut development in IUGR piglets.

Methods: Appropriate for gestational age (AGA) and IUGR sex- and litter-paired piglets were weaned and taken on postnatal day (PD) 1. A subset of piglets was euthanized immediately for tissue sample collection and others were assigned to control or experimental diet. The control group was given standard piglet milk replacer, while the test group was given the same milk replacer with 0.351g per kg of body weight of 2'-fucosyllactose (2'FL). Gastric and intestinal samples were harvested on PD1, PD14, and PD28 and analyzed with immunohistochemistry. Villus and crypt lengths were measured in the small intestinal sections, and mucosal thickness was measured in the large intestinal sections. Chromogranin A, serotonin and somatostatin densities were quantified in the stomach and small intestine.

Results: Body weight gain was not affected by diet. AGA birth weight piglets had higher absolute weight gain while IUGR piglets had higher proportional weight gain. 2'-FL was not found to improve villi or crypt length in any segment of the small intestine nor mucosal height in any part of the large

intestine. Chromogranin A abundance in the stomach was not enhanced by 2'FL either. IUGR status was also found to have very few significant effects on the intestinal development of the piglets.

Conclusion: Overall, we found few differences in the AGA and IUGR piglets and the groups fed supplemented and control formula, suggesting that 2'-FL has little effect on gut health in piglets in terms of morphological growth and enteroendocrine cell development. Because so few differences were found between AGA and IUGR piglets, it is concluded that not all IUGR piglets have a disadvantage in terms of gut development. It is difficult to definitively conclude whether 2'-FL potentially may have had a positive effect on gut development because there seemed to be lack of an impairment in the IUGR cohort.

Acknowledgments

I would like to acknowledge my advisor, Dr. Rodney Johnson, and the rest of my committee, Dr. Hannah Holscher and Dr. Gregory Freund, for their input and contribution of advice towards my thesis. I appreciated their continued support throughout the process.

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Chapter 1: Introduction

Intrauterine growth restriction (IUGR) affects an estimated 8% of births in the United States and an even higher proportion in developing countries [1]. IUGR neonates experience higher rates of infant mortality as well as preterm birth or birth defects. As children, they are at risk for increased insulin resistance and higher triglyceride levels [2]. Some are growth-delayed in terms of stature, but neurodevelopment is a concern as well. The majority undergo catch-up growth, which additionally puts them at risk for greater abdominal adipose accumulation and metabolic syndrome [3]. It is widely hypothesized that the restriction of nutrients during pregnancy programs the fetus to adapt to a low-nutrient environment, rerouting the metabolic system to prepare for scarcity. Many of these changes are in the gut, where nutrient absorption occurs. It is important to characterize the differential development that IUGR children may undergo, in order to identify the best remedies to improve their metabolic destinies.

Breastfeeding may alleviate many of the challenges that IUGR infants face. It provides extensive immune protection and is rich in nutrients [4, 5]. Unfortunately, many infants are not able to receive breastmilk and must subsist on formula milk. Formula milk is heavily regulated and has improved drastically over the past decades, but still has deficiencies in comparison to breastmilk. The resplendent variety of human milk oligosaccharides (HMO) is one of the largest differences between breastmilk and formula. Each mother produces a unique array of these indigestible sugars, which play multiple roles in immune protection while at the same time providing substrate for beneficial gut microbiota colonies in the baby's colon [6]. They may also contribute to the development of the gut, as they are capable of modulating mRNA expression [7]. One HMO in particular has been recognized for both its abundance and functional capabilities, 2'-fucosyllactose. It has already been incorporated into many infant formulas, so it is pertinent to investigate the full range of its activities.

Piglets are an accepted and exceptional model for human neonates, especially in terms of the gut. We initiated our studies to further investigate the effects of IUGR on gut development in the first month of life and to evaluate whether HMO supplementation has any beneficial or detrimental effect on this process. We hypothesized that HMO supplementation would have positive effects for the IUGR piglets.

Chapter 2: Literature Review

2.1 Appropriate for Gestational Age and Intrauterine Growth Restricted Infants

2.1.1 Definition

There are different methods to classify neonates with body weights which do not meet the criteria of appropriate for gestational age (AGA). One way is to use absolute weight, another way is by using percentiles, and a third method is to take growth potential into account. Low birth weight (LBW), very low birth weight (VLBW) and extremely low birth weight (ELBW) are labels applied to infants born at under 2.5 kg, 1.5 kg and 1 kg respectively [8]. SGA, or small for gestational age, and intrauterine growth restriction (IUGR) are often used interchangeably. There is one distinction between the two terms. SGA can be applied all infants below the 10th percentile of birth weights. However, IUGR is more correctly applied to neonates which are (1) born SGA by weight and (2) stunted in growth and development by a pathologic restriction due to adverse circumstances while in the womb [9]. Based on this, the number of newborns which are classified as IUGR may be overestimated as some babies are simply constitutively small. In addition, to correctly diagnose as IUGR and identify infants that have not reached their growth potential, one must presuppose that each infant's individual growth potential can be accurately calculated [10].

IUGR babies can be placed along a continuum of symmetrical and asymmetrical growth retardation. The neonates classified as asymmetric have AGA head circumference but are accompanied by SGA weight and length measurements. This is attributed to inadequate oxygen or substrate supply starting in the third trimester [11]. Infants in this category present with generally smaller cells but with sparing of brain and skeletal growth [12]. Asymmetrical growth retardation is typically more common, accounting for about 70% of incidences [13]. Symmetrical growth retardation begins early in the pregnancy, and may be due to genetic disease, infection, or presence of toxins. Because cells are still dividing at this stage of pregnancy, reduced cell numbers are observed in certain

tissues and persist until birth, independent of substrate supply [14]. Symmetrical growth retardation is associated with higher rates of perinatal morbidity [13].

IUGR can be caused by maternal, fetal, or placental factors, which can further be divided into environmental or genetic categories. Pregnancy-associated hypertension and smoking are quantitatively the most important maternal factors, reducing birth weight by 10% and 5% (for each pack per day) respectively [12, 15]. Poor maternal diet and other drug use are both obvious factors. Less intuitively, overnutrition leading to obesity may also increase IUGR rates due to metabolic derangements as well as creating an inflammatory intrauterine environment [16]. Women who are at an age extreme (especially for women younger than and older than 40 [17, 18]), live at high altitudes, have short interpregnancy intervals, or present with other conditions such as diabetes or were SGA at birth themselves, have also been associated with higher rates of IUGR [19]. Fetal infections are related to less than 10% of IUGR cases, whereas almost 40% of infants with chromosomal abnormalities are IUGR [20]. Because the placenta is responsible for regulation of nutrition to the fetus as well as providing its surrounding environment, placental dysfunction is highly correlated with fetal growth restriction in addition to other serious problems for the infant [21].

2.1.2 Risk factors for IUGR/SGA infants

Prior to the era of modern medicine, the main risk for infants born SGA was drastically reduced ability for survival. In addition, many cases of stillbirth can be linked to IUGR [22]. In the United States, the risk of infant mortality for SGA infants is around 5% compared to about 0.2% for NBW infants [23]. Though still a problem, the concern over long-term health implications that are associated with IUGR has increased along with growing awareness of potential risks. Poor growth *in utero* puts individuals at an increased risk for developing a host of closely related conditions and diseases both in childhood and later in life, such as type 2 diabetes mellitus, obesity, hypertension and metabolic syndrome [2, 3, 24]. It is widely hypothesized that deficiencies in fetal nutrition may

result in developmental adaptations, causing metabolic derangement [25]. This may predispose the offspring to metabolic, endocrine, and cardiovascular, possibly due to epigenetic changes, which has been demonstrated with endocrine disrupters [26].

In studies comparing children who were born SGA to those born AGA, SGA children had increased prevalence of the factors that define metabolic syndrome. At 12 months, insulin resistance is already correlated with LBW [27]. At two years of age, VLBW children have higher glucose levels than AGA children, while SGA children at that age have higher triglyceride levels [2]. In a study examining overweight children of ages ranging from 4-16, the SGA group had increased metabolic risk factors compared to the AGA group, including increased hypertension, triglycerides, and impaired glucose tolerance [28]. These results may be related to compensatory or “catch-up” growth for SGA infants, which is defined as a period of accelerated growth up to two years after birth and occurs in approximately 85% of SGAs. The catch-up weight gain is accompanied by higher rates of abdominal adipose accumulation and insulin resistance seen already at age 4 [24].

Health problems have been documented to persist into adulthood. Increased fat accumulation accompanied by greater increase in waist circumference from the age of 22 to 30 was observed in SGA adults compared to AGA adults [29]. If LBW individuals do become obese, they seem to suffer more health consequences from obesity than do NBW obese persons [30]. Blood pressure in a cohort of adults 46-54 years of age was found to be higher in individuals with a low birth weight and high placental weight; enlargement of the placenta is thought to be an adaptive response to undernutrition in pregnancy [31]. A number of studies show a relationship linking low birth weight and higher incidence of impaired glucose tolerance in the elderly [32]. Low birth weight has also found to be correlated with higher risk of type 2 diabetes and cardiovascular events [33, 34].

2.1.3 The piglet as a model for the human infant

The neonatal piglet has been identified as perhaps the best model for the human infant in terms of the gastrointestinal tract for several reasons. Since pigs eat an omnivorous diet just as humans do, they have much of the same digestive anatomy, physiology and biochemistry. They produce most of the same digestion enzymes in similar proportions [35]. Digestion transit rates are comparable, though the pig has greater ability to utilize fibrous material [36]. The rat is an oft-used experimental species, but as an altricial species it has only a partially developed gastrointestinal tract at birth, rendering it an unreliable model for the human neonatal gut.

Although there are a variety of methods that can be used to increase the rate of IUGR piglet births [37], it often occurs spontaneously in the large litters, which are frequently seen with the style of livestock farming in the United States. Large litter sizes are associated with lower mean birth weight and higher incidence of IUGR due to placental deficiencies [38]. Therefore, it is easy to obtain a naturally-farrowed piglet to use as a model for the human IUGR infant.

2.1.4 Development of the fetal gastrointestinal tract

The gastrointestinal tract (GIT) is initially formed from the endoderm and mesoderm in week 3 of fetal life [39]. Distinct organs such as the pancreas, liver and esophagus can be observed in utero at week 4 [40]. The stomach grows steadily from week 13 to 39 [41]. The intestine undergoes linear growth until week 20, at which point its maturation begins to accelerate [42]. During the last 15 weeks of pregnancy, the intestine doubles its length [42]. By the time of birth, the intestine has reached 275 cm in length [42]. It continues to elongate through the 10th year of childhood, and finishes growing by 20 years of age [42]. An adult human has a total intestinal length of 7.5 m [43]. Villi begin to form in the small intestine as well as the large intestine at week 8 [41]. After week 28, the villi rapidly disappear from the large intestine [44]. Gastrointestinal hormones such as insulin and glucagon begin appearing at week 8, as do somatostatin and serotonin [45, 46]. By 12 weeks in

utero, the majority of the enteroendocrine cell types are present [47]. Crypts begin to form around the 12th week as well [47]. By week 24, the fetus is capable of nearly all absorptive and digestive functions [48].

Swallowing can be detected at 11 weeks in utero, while sucking motions appear at week 18 [49]. The fetus begins consuming a few milliliters of amniotic fluid per day and progresses to around 450 mL/day by the end of the third trimester [40]. Gastroanal motility progresses to a more mature stage by 34 weeks [50]; preterm infants often have feeding difficulties due to low motility rates [48]. At birth, term infants are able to feed but do not have the same volume and range of digestive activity as adults.

2.1.5 Effects of IUGR on the gut

In humans, little is definitively known about the effect of IUGR on neonatal gut maturation. Preterm infants and LBW infants generally have less developed GITs [51]. Yet it is of great consequence because the gastrointestinal tract is responsible not only for defending against the threat of foreign microorganisms, but also for extracting nutrients from the diet; both are crucial objectives for the neonate. IUGR and preterm infants are at higher risk of contracting necrotizing enterocolitis, probably in large part due to the relative immaturity of their guts [52].

Many studies of the impact of IUGR in piglet gut development have been conducted. In the IUGR piglet, organ weights such as that of the pancreas are usually significantly smaller but proportionally so in relation to body weight [53]. Due to reduced villi height and quantity and crypt depth along with reduced cell number and lower secretion of digestive juices in IUGR compared to NBW piglets, luminal digestive and absorptive capabilities may be reduced [37, 54]. Stomach wall thickness is decreased which may bear impact on the robustness of the organ [55]. Enlargement of gastric pits has also been observed in the IUGR piglet, again suggesting reduced wall protection [53].

Many hormones important for growth and development are seen to have altered concentration levels in IUGR neonates, including insulin-like growth factor I, cortisol, leptin and growth hormone [56, 57]. A reduction of glucagon-like peptide 2 (GLP 2), which is responsible for stimulating gastrointestinal tract development, has also been observed [58]. In pig studies, it was shown in particular that GLP 2 improved barrier function and increased villi and crypt size [59]. Levels of GLP 2 were low in IUGR piglets in the weeks after birth, but increased above the levels of NBW piglets in the months afterwards, possibly indicating catch-up growth [60].

2.2 Potential Benefits of Human Milk Oligosaccharides

2.2.1 Definition and characterization of human milk oligosaccharides

Breast-feeding is recognized as having beneficial effects over formula-feeding. Infants nourished by breast milk experience a significantly lower risk of mortality [61]. In the long-term, breastfeeding is associated with lower rates of obesity, diabetes and higher IQ, although there may be confounding social factors [62]. One of the advantages that breast milk has over formula is the presence of a large variety of indigestible glycans, termed human milk oligosaccharides (HMOs). HMOs are reported to have a range of activities in the neonate, from improving immune activity to modulating the microflora population to changing developmental aspects of the gut [4, 6, 63, 64].

Human milk oligosaccharides are the third most abundant bioactive found in human breast milk at concentrations ranging from 12-14 g/L, with even greater concentrations during colostrum [65]. Over 200 distinct structures have been identified [66]. They are more complex than other strictly linear dietary oligosaccharides such as fructo-oligosaccharides or galacto-oligosaccharides. HMOs can be classified by whether they are fucosylated, sialylated, or have neither modification. In human breast milk, about 50% are both unfucosylated and unsialylated, and there is a higher concentration of fucosylated HMOs compared to sialylated HMOs, though the absolute amounts vary depending on secretor or Lewis blood group status [67].

The overall concentration and collection of HMOs varies immensely not only from mother to mother, but also over time in a single individual as the lactation period continues. Even the number of different HMOs present has been shown to vary from as few as 33 up to 124 in a single sample [66]. The specific collection of HMOs present depends partly on the mother's Lewis blood group and secretor gene status, which determine production capability of specific transferases, affecting fucosylation sites [68, 69]. This may reflect global variations in prevalence of perinatal infection, as different profiles are more protective against certain pathogens [70, 71].

Of note, 2'-fucosyllactose (2'-FL) is the oligosaccharide which is found in the largest quantity in most human breast milk samples, at an average concentration of 2.38 g/L [72]. The high concentration of 2'-FL suggests that it has a critical role for the neonate. About 81-85% of women can produce it; those of non-secretor status are not able to synthesize it [72, 73]. Infants with non-secretor mothers, and therefore receiving no 2'-FL, have been shown to experience higher incidences of diarrhea [74]. Mice that were supplemented with 2'-FL after an intestinal resection demonstrated improved weight gain, positive morphological developments in the gut, and more efficient energy metabolism [75].

In other mammals, the concentrations of milk oligosaccharides (MOs) are lower than in humans, and display far less diversity [76]. Primate milk is richer compared to the domesticated mammals, but evidence shows that the MO structures are not as large or complex as in humans [77]. In porcine milk, at least 60 distinct MOs have been identified [78]. As with most other mammals, sialylated structures are more common than fucosylated oligosaccharides. However, the oligosaccharides of porcine milk has a more similar profile to human milk than other domesticated mammalian milks, including bovine milk [76]. One of the reasons for this is the presence of 2'-FL and other fucosylated structures throughout all stages of lactation, though in comparatively lower quantities [78].

2.2.2 HMOs and the gut

Approximately 97% of HMOs pass through the intestine unaltered [71]. Though they cannot be digested, they perform several crucial roles in the infant gut.

They may serve as antimicrobial agents either by preventing the adhesion of microorganisms to the intestinal mucosa via competitive binding or by serving as decoy ligands for pathogens [79]. The large variety of HMOs suggest an ability to block many different pathogens [80]. Some HMOs may also modulate the expression of cell surface glycans, diminishing the ability of certain pathogens to attach [81]. Various studies have found that the concentration of HMOs, especially 2'-FL and other fucosylated milk oligosaccharides, is inversely proportional to diarrhea incidence in infants [74, 82]. Furthermore, HMOs have also been proven in *in vitro* setting to be capable of producing both inhibitory and stimulatory effects on cell proliferation and differentiation, which could be another potential role for it in the gut [83].

HMOs have been proven through *in vitro* studies to influence the infant immune system, generally promoting anti-inflammatory activity. 2'-FL specifically was found to decrease levels of membrane-bound CD14 mRNA, undermining LPS-induced inflammation [7]. Both fucosylated and sialylated HMOs cause decreased levels of pro-inflammatory cytokines such as IL-12, IFN- γ , IL-8 and TNF- α [84, 85]. They also appear to help drive the infant immune system from its birth phenotype, the type-2 pathway of humoral immunity, towards a more mature, balanced immune profile [86]. At the present, few *in vivo* studies with HMOs have been conducted.

2'FL has several direct effects on the gut in the murine model: supplementation of 2'-FL was shown to decrease necrotizing enterocolitis, a condition affecting many preterm infants, by increasing expression of endothelial nitric oxide synthase [87]. In another experiment, 2'-FL and 3'-FL were also able to diminish motor contractility of the colon in mice, suggesting therapeutic benefits for gut disorders [88]. In terms of immune function, oral supplementation of 2'-FL (as well as 6'-

sialyllactose) was found to attenuate food allergy symptoms and decrease anaphylaxis response in mice [89].

2.3 Enteroendocrine Cells

2.3.1 Introduction

The enteroendocrine cells (EECs) together, by cell number and peptides produced, comprise the largest endocrine organ in the body [90]. Unlike other endocrine organs, the cells are not grouped together but rather spread throughout the gastrointestinal tract and pancreas, representing approximately 1% of all the epithelial cells in stomach and intestine. The hormones they secrete are primarily responsible for the regulation of digestion, gut motility, appetite, and metabolism. There are at least 16 distinct types of EECs which secrete various hormones [91]. The cells are classified by their main product or products and the structure of the secretory granules. Due to various methods of gene transcription and translation modification methods, over 100 different peptides can be produced in the gut. Based on the method of release, these can act as blood-borne hormones, local growth factors, or neurotransmitters [92].

EECs can be divided into open- or closed-type cells. Most EECs are open cells and have microvilli extending into the lumen. They are able to directly sense and respond to luminal contents [91]. Closed cells such as enterochromaffin-like cells and the pancreatic islet cells respond indirectly through neural and humoral pathways [93]. EECs may contain one of two vesicle types, large dense-core vesicles (LDCVs) or synaptic-like microvesicles (SLMVs). LDCVs are a typical endocrine vesicle found in most EECs, with the release of its contents triggered by Ca^{2+} [94]. SLMV intestinal cells exocytose their contents upon membrane depolarization, similar to neuronal postsynaptic vesicles [95].

As with other cells of the gastrointestinal system, EECs are derived from the endodermal epithelium [96]. The notch signaling pathway control basic helix-loop-helix transcription factors and mediates

differentiation of the EECs, preventing them from developing next to each other [97]. Unlike other endocrine cells, EECs have high plasticity due to their short life span of 4 to 6 days [98].

Environmental factors can influence the quantity of EECs, correspondingly altering overall effect [99]. They have been shown to respond to changes in diet [100].

Given the immaturity of the gut at birth, the plasticity of the gastrointestinal tract, and the key role of the EECs in nutrient extraction, the EEC population could be affected by the IUGR of neonatal piglets. Past studies have shown mixed results depending on the specific cell type. Willemen et al. showed that serotonin cell density was unchanged in IUGR vs AGA piglets at day 0 in the proximal small intestine, but also that ghrelin-expressing cells in the stomach were increased in AGA compared to IUGR piglets [101, 102]. Multiple groups have shown that morphology of the gut changes over time, so it is probable that cell population evolves as well [60, 103].

2.3.2 Chromogranin A

Chromogranin A (CgA) is a member of the granin family, which is a group of acidic soluble proteins which are expressed by endocrine, neuroendocrine and neuronal cells. Its name is derived from the adrenal gland, where it was originally discovered. CgA is stored in large dense cell vesicles (LDCV) and co-released with other peptide hormones [104]. Cleavages of CgA itself can produce at least 11 biologically active peptides [105]. Chromogranin A is present in all cells of the enteroendocrine system and therefore is a useful marker for identifying EECs [94]. It is also present in circulation.

CgA regulates and stabilizes the formation and packaging of peptides within the secretory granule [106]. It is able to bind to calcium with high capacity but low affinity, utilizing it to facilitate the condensation of solutes inside the LDCV [107]. CgA also serves as an important prohormone, with its bioactive fragments exerting a wide variety of effects including antimicrobial activity, modulation of cell adhesion, influence on the regulation of glucose and blood pressure [108, 109].

CgA has been used as a marker for disease. In human patients with irritable bowel syndrome (IBS), the concentration of CgA-secreting cells in the stomach as assessed by gastroscopy were found to be significantly reduced compared to healthy controls, but increased towards normal levels after strict dietary guidelines were imposed [110]. CgA can be useful in identifying neuroendocrine tumors (NET) using histopathology due to increased posttranslational modification in NET cells; normal NE cells express fewer of these CgA fragments [111]. Elevated CgA in circulation is associated with many disease states including gastritis, impaired kidney function, heart disease, and arthritis [112].

2.3.3 Serotonin

Serotonin is an ubiquitous substance involved in countless biological processes, especially well-known for its effects in the brain. Nearly all human behaviors are regulated in some way by serotonin, including mood, appetite, and sexuality [113]. Apart from its function as a neurotransmitter, it is active in many other organs of the body, playing roles as a growth factor and hormone [114]. This is reflected by the presence of at least 15 different identified serotonin receptors throughout the body [115]. In the gut, it is found in enterochromaffin (EC) cells, the most common type of EEC in the gastrointestinal tract. EC cells are located all throughout the intestinal tract, but in humans are seen most abundantly in the duodenum [116]. Approximately 95% of the body's serotonin is localized in the gastrointestinal tract. A flood of serotonin is released from the ECs after food is physically sensed in the intestine [117], and then quickly inactivated by reuptake through the serotonin reuptake transporter (SERT)[118]. This prevents desensitization of serotonin receptors and possible deleterious or disruptive effects on distant tissues [118, 119].

In the gut, serotonin regulates motility, enzyme secretion, and intestinal development. Upon mechanical stimulation of the mucosa, release of serotonin is capable of initiating the peristaltic reflex [120]. Its secretion is crucial in the stimulation of intestinal transit as well as pancreatic secretion [121-123]. In addition, growth in the intestinal mucosa has been demonstrated to be

directly linked to serotonin using serotonin reuptake transporter knockout mice (SERTKO). As SERT-mediated reuptake usually operates with high affinity, its deletion greatly augments and prolongs the effects of serotonin. SERTKO mice not only had greater mucosal growth, but also experienced increased proliferation of mucosal cells [124].

Serotonin has been identified as an enteric source of intestinal inflammation, with EC cell hyperplasia responding to T lymphocyte activation [125-127]. SERTKO mice presented with higher measures of inflammation in the colon [125]; SERT function may be decreased by circulating cytokines [128]. Interestingly, serotonin originating from neuronal cells has been shown to alleviate intestinal inflammation [114]. Trinitrobenzene sulfonic acid (TNBS)-induced ileitis in mice caused an increase in both EC cells and a decrease in SERT. In humans, serotonin positive cell populations are increased in Crohn's ileitis and ulcerative colitis [129, 130]. Conversely, constipation-predominant irritable bowel syndrome has been associated with lower EC cell counts, characteristically accompanied by increased colonic transit rate [131].

2.3.4 Somatostatin

Somatostatin is the main antisecretory hormone in the gastrointestinal tract, decreasing the release of digestive enzymes including itself [132]. It is produced in D enteroendocrine cells, which are found throughout the gastrointestinal tract, with scatter numbers in the ileum and colon [133]. There are two forms, somatostatin-14 and somatostatin-28. The stomach and duodenum contain predominantly somatostatin-14, while the quantity of somatostatin-28 increases further down the gastrointestinal tract [133]. The release of the two types of somatostatin is stimulated by the ingestion of food [134] as well as by insulin-induced hypoglycemia [135].

Somatostatin acts by binding to high-affinity plasma membrane receptors found in pituitary cells, pancreatic cells, and adipocytes [136]. Upon binding, somatostatin inhibits calcium-dependent processes [137]. One effect somatostatin produces is suppression of motility along the

gastrointestinal tract [138, 139]. It inhibits the secretion of GI and pancreatic enzymes as well as growth hormone and thyroid-stimulating hormone [140]. It has also been found to have antiproliferative effects on cells [141].

In mice, as with 5HT-immunoreactive cells, D cells were shown to be increased in TNBS-induced ileitis [142]. This may have occurred in response to the increase in other enteroendocrine cells. In human patients with gastritis, somatostatin-producing D cells were shown to be significantly lower in the stomach [143]. Patients with inflammatory bowel diseases presented with fewer D cells in the colon [144]. Correspondingly, concentration of somatostatin by weight was also found to be lower in the colon of patients with IBD [145]. Considering its role, the decrease in somatostatin could be contributing to the pathogenesis of inflammatory gastrointestinal disorders.

Chapter 3: Materials and Methods

3.1 Animals, Housing and Feeding

Naturally-farrowed sex-matched littermate pairs of AGA and IUGR piglets (AGA, 1.2-2.0 kg; IUGR, 0.5-0.9 kg) from the University of Illinois swine herd were used. They remained with their dam and littermates 24-48 h after birth to obtain colostrum and then were weaned and transferred to the biomedical animal facility. Once received, each piglet was given iron dextran (1 mL, Butler Schein Animal Health, Dublin, OH) and Gentamicin (1 mL, Agri Laboratories, Ltd., St. Joseph, MO). Each piglet was housed individually in a cage fitted with flooring designed for neonatal animals and provided with a toy (plastic Jingle Ball™, Bio-Serv, Frenchtown, NJ, USA) and a blanket, which was regularly replaced. Cages were arranged in blocks of 6 slots (0.75 m L×0.58 m W×0.47 m H) separated by plexiglass dividers as described in previous studies [146]. The room was maintained at 27°C, with heat lamps installed on each cage for supplementary warmth.

Several AGA and SGA littermates were randomly selected and euthanized at postnatal day (PD) 0 for tissue sample collection (n=7 per group). Other AGA and SGA piglets were assigned to either a control or HMO study group designated for euthanasia at PD14 (n=8 per group) or PD28, (n=9 per group). Fresh sow-milk replacer was reconstituted each morning with tap water to a concentration of 206 g/L (Advance Liqui-Wean, Milk Specialties Co., Dundee, IL, USA). For the HMO group, the milk was supplemented with 1.17 g/L of HMO derived from bacterial synthesis which was provided by Abbott Nutrition. Piglets were fed 5 times per day out of bowls at 3h intervals beginning at 10:00 AM. A total of 300 mL of milk per kg of body weight was provided each day. No additional water was administered. The room was maintained on a 12h light/dark schedule with the lights coming on at 8:00 AM in the morning.

On the day of sacrifice, piglets were anesthetized with a Telazol:ketamine:xylazine drug cocktail (50 mg of tiletamine plus 50 mg of zolazepam reconstituted with 2.5 mL ketamine (100 g/L) and 2.5 mL

xylazine (100 g/L; Fort Dodge Animal Health, Fort Dodge, IA) before euthanasia via intracardial injection with sodium pentobarbital (1 mL; Fatal Plus, Vortech Pharmaceuticals, Dearborn, MI). All animal care and experimental procedures were in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals and approved by the University of Illinois at Urbana-Champaign Institutional Animal Care and Use Committee as part of protocol 14170.

Immediately after sacrifice, the stomach and segments of the duodenum, jejunum, ileum, ascending colon, descending colon and rectum were dissected as follows. The stomach was removed and emptied, splayed with a butterfly cut, and affixed to cardboard with the first portion of the duodenum still attached. A block of tissue ~1.5 cm in length was taken from each part of the intestinal tract. The suspensory muscle of duodenum connects the junction of the duodenum and the jejunum to the connective tissue of the abdomen, but this landmark was not used to identify the jejunum in our study, From the small intestine, a medial jejunal piece was selected along with an ileal segment from the distal portion of the ileum. The ascending colon segment was taken from the proximal colon immediately behind the cecocolic junction, and a descending colon section was removed from the distal portion of the colon. The rectum was obtained from the anal cavity. Each part was then rinsed and placed in a jar of 10% zinc formalin for 48h before being embedded in paraffin and sectioned at 5 μ m to mount on glass slides.

3.2 Immunohistochemistry

Histological sections were dewaxed and rehydrated using xylene and ethanol. Each section was incubated overnight with a primary antibody and 5% bovine serum albumin. The primary antibodies used were: chromogranin A (CgA, Immunostart 20086, Abcam ab80787), serotonin (SER, Abcam Ab16007), and somatostatin (SOM, Dako a0566). The primary antibodies were detected using an adapted secondary biotinylated antibody and avidin–biotin–peroxidase complex (ABC) system (Vector Laboratories, Burlingame, CA, USA) and diaminobenzidine DAB (Zymed, San Francisco, CA, USA) as chromogens. Aqueous hematoxylin was applied for counterstaining.

3.3 Morphological Analysis

Slides were digitized using the NanoZoomer-XL (Hamamatsu Photonics, Bridgewater, NJ). The density of enteroendocrine cells in the gastric mucosa was measured by selecting two random ~1mm² areas of the mucosa per gastric section (fundus, cardia, and pylorus) and then counting the cells. Cell density was expressed as the number of cells/mm² of the epithelium. Quantification of EECs in the small intestine was done by measuring two 0.4 to 0.6 mm lengths of mucosa base in each section (duodenum, jejunum and ileum) and counting the positive cells in the associated area. Cell density here was expressed as the number cells/mm. Quantification in the gastric mucosa was done using AxioVision (Carl Zeiss Microscopy GmbH, Jena, Germany), while quantification in the small intestine was done in the Nanozoomer viewer (NDP.view2; Hamamatsu Photonics, Bridgewater, NJ) by researchers blinded to the experimental treatments.

All morphological measurements were made using the Nanozoomer viewer. Mucosal height in the large intestine (ascending colon, descending colon, and rectum) was measured to be from the bottom of the submucosa to the top of the mucosa. Villi height was measured by picking three intact, representative villi for each small intestinal section. Crypt depth was measured from three crypts that were associated with intact villi. These measurements were made on slides stained with either CgA, serotonin, or somatostatin.

3.4 Statistical Analysis

Statistical analyses were performed using SAS 9.4 (SAS Institute, Cary, NC) and GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA. Two-way (diet and prenatal growth status) analyses of variance were used in order to identify differences between groups. Three-way analyses of variance were also utilized to take timepoint into account. Results were considered statistically significant when $p < 0.05$. Values are expressed as mean \pm SEM.

Chapter 4: Results

4.1 Measurements

4.1.1 Body Weight Gain (Table 1)

Average birth weight for PD1 AGA piglets was 1.38 kg compared to 0.81 kg for IUGR piglets. The magnitude of the PD14 body weight gain was greater in AGA piglets ($p=0.0001$), but proportional body weight gain did not differ significantly between groups ($p=0.957$). When using single pairwise comparisons, the IUGR HMO group had higher percentage weight gain than the AGA control group. In PD28 piglets, absolute body weight gain was higher in AGA piglets ($p=0.0001$) but proportional body weight gain was higher in IUGR piglets ($p=0.001$). Using pairwise examination, higher proportional body weight gain was only significant in HMO IUGR compared to both AGA groups. Though there were no differences in body weight gain due to overall effect of diet, HMO IUGR piglets at D28 had a higher body weight percentage gain compared to AGA piglets while this attribute was not observed in the control IUGR group.

Table 1 Body weight gain

Treatment	Body weight		Weight Gain	% Weight Gain
	PD1	Final		
PD14	<i>kg</i>		<i>kg</i>	%
AGA Control	1.41 ± 0.031	2.95 ± 0.13	1.53 ± 0.12 ^{A,b}	108 ± 7.6 ^e
AGA HMO	1.40 ± 0.027	3.05 ± 0.14	1.65 ± 0.085 ^{C,D}	119 ± 8.5
IUGR Control	0.77 ± 0.028	1.71 ± 0.072	0.94 ± 0.12 ^{A,C}	121 ± 12
IUGR HMO	0.84 ± 0.026	1.95 ± 0.11	1.12 ± 0.089 ^{b,D}	134 ± 7.9 ^e
PD28				
AGA Control	1.43 ± 0.033	6.69 ± 0.21	5.25 ± 0.20 ^{F,G}	365 ± 15 ^J
AGA HMO	1.47 ± 0.049	6.95 ± 0.34	5.48 ± 0.29 ^{H,I}	373 ± 10 ^K
IUGR Control	0.83 ± 0.025	4.34 ± 0.26	3.51 ± 0.25 ^{F,H}	423 ± 27
IUGR HMO	0.81 ± 0.039	4.59 ± 0.37	3.78 ± 0.34 ^{G,I}	469 ± 27 ^{J,K}

Table 1. Values with the same letters were different at the statistical level mentioned. Statistical level: b,e $p<0.05$; A, C, D, F, G, H, I, J, K $p<0.01$

4.1.2 Morphological Development (Table 2, Figure 1)

In order to evaluate the effect of birth weight and diet on the development of digestive structures, villus length and crypt depth were measured in the small intestine, and mucosal height was measured in the large intestine.

At PD1, there were no differences between the AGA and IUGR groups in terms of villus length, crypt depth or mucosal thickness. Average duodenal villus length was 515.4 μm in AGA piglets, and 665.9 μm in IUGR piglets ($p=0.181$). Average jejunal villus length was 888.7 μm in AGA piglets and 799.1 μm in IUGR piglets ($p=0.140$). Average ileal villus length was 751.6 μm in AGA piglets and 583.0 μm in IUGR piglets ($p=0.407$). Overall, the average villus length was 716.9 μm for AGA piglets and 693.1 in IUGR piglets ($p=0.539$). The average crypt lengths at PD1 similarly showed no significant differences. The overall crypt length was 138.5 μm in AGA piglets and 132.1 μm in IUGR piglets ($p=0.430$). The average mucosal thickness was 297.8 μm in the ascending colon, 277.7 μm in the descending colon, and 268.9 in the rectum.

At PD14, AGA control piglets had longer villus lengths than HMO AGA ($p=0.028$) and IUGR ($p=0.048$) piglets in the jejunum. In the duodenum, AGA control piglets had an average villus length of 675.4 μm , AGA HMO had average villus length of 634.3, and IUGR control and HMO were 729.4 μm and 731.2 μm respectively. In the ileum, it ranged from 583.7 μm to 760.3 μm . AGA piglets had significantly greater ileal crypt depth ($p=0.047$) measured at 186.9 μm compared to 164.7 μm in IUGR piglets. No significant differences were found in mucosal thickness. The average mucosal thickness was measured at 271.4 μm in the ascending colon, 316.1 μm in the descending colon, and 329.4 in the rectum of the PD14 piglets.

At PD28, there were no apparent main effects of size or diet in the intestinal morphology. In the small intestine, average duodenal villi length was 694.8 μm , 738.4 in the jejunum, and 547.9 μm in the ileum. The average crypt depth was 298.1 μm in the duodenum, 200.6 μm in the jejunum, and

208.9 μm in the ileum. In the large intestine, average mucosal thickness was 338.7 μm in the ascending colon, 361.2 μm in the descending colon, and 420 μm in the rectum.

Among the HMO piglets but not control piglets, there was greater ileal crypt depth in PD28 compared to PD14. However, only PD28 control piglets had significantly deeper jejunal crypts than their PD14 counterparts. In the duodenum, crypt depth was significantly different between all three time points but without a diet or size effect.

Crypt depth increased consistently from PD0 to PD28, with significant increase in thickness in both AGA and IUGR piglets at PD14 compared to PD28. Villi length did not always differ between timepoints, but had an overall tendency to decrease from PD0 to PD28. Mucosal thickness in the colon, as with crypt depth, tended to increase from PD0 to PD28.

Table 2 Morphology

Treatment	Villus Height			Crypt Depth			Mucosal Height		
	Duodenum	Jejunum	Ileum	Duodenum	Jejunum	Ileum	Asc. Colon	Desc. Colon	Rectum
PD1					μm				
AGA	515 ± 47	889 ± 68	752 ± 93	177 ± 12	118 ± 5.6	117 ± 9.1	318 ± 13	275 ± 16	266 ± 14
IUGR	666 ± 73	799 ± 101	583 ± 41	152 ± 9.8	137 ± 12	98 ± 3.3	274 ± 21	280 ± 14	272 ± 12
PD14									
AGA Control	675 ± 56	876 ± 111 ^{a,b}	681 ± 47	237 ± 17	171 ± 12	195 ± 12*	291 ± 21	342 ± 18	342 ± 17
AGA HMO	634 ± 46	591 ± 39 ^a	584 ± 45	232 ± 9.7	175 ± 14	179 ± 11*	265 ± 11	305 ± 19	313 ± 21
IUGR Control	729 ± 68	646 ± 73	760 ± 85	210 ± 8.5	150 ± 7.9	165 ± 9.2*	265 ± 8.9	323 ± 10	344 ± 16
IUGR HMO	731 ± 65	607 ± 54 ^b	618 ± 42	241 ± 7.9	154 ± 9.3	165 ± 9.6*	267 ± 4.7	296 ± 13	321 ± 11
PD28									
AGA Control	688 ± 46	767 ± 105	552 ± 47	291 ± 18	208 ± 18	197 ± 12	332 ± 15	354 ± 13	423 ± 20
AGA HMO	697 ± 18	751 ± 79	480 ± 49	336 ± 30	195 ± 14	232 ± 17	339 ± 18	357 ± 16	429 ± 28
IUGR Control	674 ± 34	633 ± 42	580 ± 32	284 ± 25	204 ± 17	208 ± 11	356 ± 17	398 ± 25	431 ± 23
IUGR HMO	684 ± 52	813 ± 106	583 ± 42	294 ± 23	196 ± 15	198 ± 8.9	328 ± 11	346 ± 19	399 ± 30

Table 2. Values with the same letters were different at $p < 0.05$.

* = there was a main effect of size at $p < 0.05$

4.2 Enteroendocrine System (Figures 2 and 3)

Distribution and quantity of cells varied from PD1 to PD28. This was true for each experimental group and for each of the markers studied. In the small intestine, enteroendocrine cells were most highly concentrated in the duodenum, while in the stomach, the pylorus area contained the most densely concentrated cells.

4.2.1 Chromogranin A

Chromogranin A is present in nearly all enteroendocrine cells. At PD1, there were no significant differences between the AGA and IUGR piglets in terms of CgA expression in the stomach or the small intestine. The average density in the duodenum was 87.0 cells/mm for AGA piglets and 76.0 cells/mm for IUGR piglets ($p=0.433$). At PD14, there was a main effect of size but not diet. AGA piglets presented with more CgA positive cells in the duodenum ($p=0.043$) and the jejunum ($p=0.017$) with an average density of 192.0 cells/mm and 102.7 cells/mm respectively compared to 149.9 cells/mm and 70.5 cells/mm in IUGR piglets (*see figure 2*). Also in PD14 piglets, there was a significant interaction ($p=0.001$) in the pylorus section of the stomach (*see figure 3*). IUGR control piglets had higher levels of CgA, 526.4 cells/mm², than IUGR HMO piglets, 414.8 cells/mm². However, AGA HMO piglets had higher levels at 543.8 cells/mm² compared to the control AGA group with 414.1 cells/mm². There were no significant differences due to diet or size at PD28.

As found previously, CgA cell density in the duodenum was significantly greater at PD14 and PD28 compared to PD1 ($p<0.001$), 164.9 cells/mm at PD14 and PD28 combined and 82.1 cells/mm at PD1. The jejunum contained similar levels of CgA across timepoints with an average of 76.3 cells/mm. In the ileum, levels of CgA were lowest at PD28 with 64.0 cells/mm and 79.1 cells/mm at PD14. In the stomach, levels of CgA tended to be lower at PD1 in the fundus and pylorus at 364.4 cells/mm² and 357.2 cells/mm² respectively. For PD14 and PD28 combined, the density was 457.4 cells/mm² 392.5 cells/mm² in the fundus and pylorus. In the cardia, no differences were measured between the timepoints. The average concentration was 204 cells/mm².

4.2.2 Serotonin

The number of serotonin positive cells did not differ at PD1 between AGA and IUGR piglets. At PD14, control diet piglets had greater serotonin expression in the cardia of the stomach ($p=0.032$) with 58.0 cells/mm² compared to 42.0 cells/mm² in HMO piglets (*see figure 3*). At PD28, no significant differences in serotonin density due to size or diet were detected.

In the duodenum, serotonin staining was much more concentrated at PD28 at 79.4 cells/mm as compared to PD1 with 46.8 cells/mm. In the jejunum, there was no discernible difference in positive cell density at the various timepoints. The overall average was 46.5 serotonin cells/mm of jejunal mucosa. In the ileum, the positive expression of serotonin was highest at PD1, 52.3 cells/mm while it was an average of 37.8 cells/mm at PD14 and PD28 timepoints. As with CgA, serotonin in the cardia was similar across the timepoints, an average of 53.8 cells/mm. Both in the fundus and pylorus, serotonin expression was highest at PD1, at 130.2 cells/mm² and 188.5 cells/mm² respectively, whereas at PD14 and PD28, the combined average for PD14 and PD28 was 80.1 cells/mm² in the fundus and 78.9 cells/mm² in the pylorus. Otherwise, there were no significant differences in gastric serotonin density between PD14 and PD28.

From PD14 to PD28 in the cardia, there was an interaction by size ($p=0.0457$). IUGR piglets had an increased average number of serotonin positive cells from PD14 to PD28, while AGA piglets did not have significant difference in serotonin-positive cells between PD14 to PD28.

4.2.3 Somatostatin

At PD14, control IUGR piglets expressed more somatostatin in the jejunum compared to the other three treatment groups ($p=0.008$), 64.4 cells/mm compared to 36.0 cells/mm (*see figure 2*). No significant differences due to diet or size were observed at PD28.

In the duodenum, PD28 somatostatin levels were higher than in the PD1 cohort, with 96.4 cells/mm as compared to 58.0 cells/mm. In the jejunum, there were no significant differences but average levels decreased slightly from PD1 to PD28 from 49.8 cells/mm to 35.8 cells/mm² at PD28.

In the ileal section of the small intestine, PD1 piglets again had a slightly denser concentration of positive somatostatin cells, 50.5 cells/mm compared to an average of 32.2 cells/mm in PD14 and PD28 combined. This was similar to observations of serotonin in the ileum. Somatostatin in the cardia at PD14 was lower than at PD28, 50.3 cells/mm² vs 74.5 cells/mm². In the fundus, somatostatin was significantly higher at PD28 with 141.5 cells/mm² compared to an average of 84.6 cells/mm² for PD1 and PD14 combined while in the pylorus, levels were lowest at PD1. PD1 somatostatin density in the pylorus was 122.6 cells/mm² and an average of 250.7 cells/mm² in PD14 and PD28 combined.

From PD14 to PD28 in the jejunum, there was an interaction by size ($p=0.006$). AGA piglets had an increased number of somatostatin positive cells from PD14 to PD28, while IUGR piglets saw a decrease in the number of somatostatin positive cells.

Chapter 5: Discussion

This present study found that neither birth weight nor supplementation with 2'-FL made a significant impact on gut development. Although a few differences were detected as mentioned above, there is overall far more evidence that indicates that the groups did not differ from each other. While 2'-FL supplementation did not confer any benefits based on the measurements that we chose, it likewise did not have any observable detrimental effect. It was surprising that there were so few significant differences between AGA and IUGR piglets at any of the timepoints, but there are many possible explanations for this finding which are discussed below.

Milk oligosaccharides are a component of breast milk that confer a range of benefits to the human neonate. They are best known for their effects on immunity and as substrate for the gut microbiota, but have many other conjectured roles. We hypothesized that supplementation of 2'-FL in sow milk replacer would improve the gut health and development of the IUGR piglet, but our results did not confirm this. Only one significant difference was attributed to the supplementation of HMO in terms of gut morphology or enteroendocrine cell quantity in the piglet. This finding occurred at the PD14 timepoint; serotonin expression in the cardia of the stomach was observed to be lower in piglets fed the HMO diet. As elevated serotonin is often accompanied by inflammation, HMO may be associated with lower inflammation levels in the piglets. This difference did not persist at PD28. HMOs are associated with low inflammation through several mechanisms. They lower inflammation associated with infection by competing with pathogens for epithelial binding sites and can also prevent excessive accumulation of leukocytes [147].

Some infants experience restricted growth in the womb due to insufficient nutrient supply from the placenta. They are born small for their gestational age because their body and other organs have received a reduced nutrient stream in order to spare the brain from deficiency. In addition, the lack of nutrients may also signal epigenetic changes. The resulting IUGR infants are often at a

disadvantage compared to their AGA counterparts due to this fetal programming. The IUGR piglet is often used as a model for the IUGR infant due to similarities in pathology. Gut development is one component where IUGRs may experience inadequacies. However, in this experiment, there were only several significant differences between AGA and IUGR piglets were detected in this experiment, and none at the PD1 timepoint. At PD14, higher chromogranin A levels were observed in the duodenum and jejunum of the AGA piglets. In addition, these AGA piglets also had slightly deeper crypts. These differences could allude to higher digestive capacity in AGA piglets, as proximal portion of the intestine is most active in digestive and absorptive capacity. None of these distinctions were observed at the PD28 time point.

Other groups have seen morphological differences in villi depth, crypt height and mucosal thickness between AGA and IUGR piglets at PD0 or PD1, but in this study we did not observe any. There are many possible reasons for this discrepancy. Some differences could arise from differences in study design. First of all, it is possible that the difference in birth weight was too small or that our IUGR piglets were too large. The breeds of the pigs or the conditions of the sow may also play a role. All of our piglets received colostrum, which may have evened the playing field between AGA and IUGR groups. In addition, the specific characteristics which were found to be significantly different varied somewhat sporadically from study to study, suggesting that true morphological differences can be difficult to detect.

Our AGA piglets at PD1 were 1.38 kg while the PD1 IUGR piglets were 0.81 kg, a difference of 0.57 kg. Overall in the study, AGA piglets had an average birth weight of 1.42 kg and IUGR piglets were 0.81 kg. Xu et al. used PD0 IUGR piglets with an average birth weight of 0.59 kg while the AGA piglets were 1.33 kg, a difference of 0.74 kg. Xu's piglets were also completely prevented from receiving any colostrum, which could have allowed our IUGR piglets to have an advantage [55]. The PD0 piglets of D'Inca et al. and Zhong et al. were also taken before receiving colostrum [148, 149]. Our study featured larger sample sizes than some other studies making similar measurements. At

our PD1 timepoint, there were 7 piglets per group, 8 for each treatment at PD14, and 9 in each treatment at PD28. Xu et al. had 5 piglets in each PD1 group, D’Inca et al. had 6 piglets per group, and in a previous study by our group, only 4 piglets per group were used at the PD1 timepoint.

Comparing the same measurements across different studies elicits a high level of variation, suggesting that the breed of pig, maternal sow diet, piglet environment or even subtle discrepancies in how the measurement is made may play a role. Xu et al. used Landrace and Large White crossbred sows. Alizadeh et al. used Landrace and Yorkshire crossbred sows [103]. Mickiewicz et al. used German Landrace gilts [60]. Using the jejunal villi as an example, the AGA jejunal villi length ranged from 690 to 980, while IUGR jejunal villi length ranged from 487 to 790 [55, 103, 149]. In comparison, our PD1 AGA and IUGR mixed lineage piglets were not unusual with jejunal villi lengths of 889 and 799 μm respectively.

Crypt and villus measurements of the distal ileum also varied between studies using piglets taken at PD0 or PD1. Piglets of Xu et al. had ileal villus lengths of around 600 μm for both AGA and IUGR, piglets of the D’Inca study had ileal villus lengths of 364 and 329 μm for AGA and IUGR, while Zhong’s were measured at 693 and 394. Our piglets had average ileal villus lengths of 752 and 583 μm for AGA and IUGR. Crypt depth in our piglets was 117 and 98 for AGA and IUGR, compared to 75 and 60 μm as observed by Xu et al., 53 vs 50 as reported by D’Inca et al, and 46 and 110 as seen by Zhong et al.

Not only are there large variations in the actual measurements, but the measurements producing a significant difference between AGA and IUGR at PD0 or PD1 differed from study to study. Che et al. had PD0 AGA and IUGR piglets which did not receive colostrum, but also did not display significant differences in villus length and crypt depth. The PD0 AGA and IUGR piglets of Xu et al. had significant differences in the villi and crypts of the jejunum and ileum, but not in the duodenum. The AGA and IUGR piglets of D’Inca et al. had differences in the ileal villi but not crypts. Similarly,

Mickiewicz et al. saw differences in the jejunal villi but not the crypts. Zhong et al. saw differences between their AGA and IUGR piglets in the villi of the duodenum, jejunum and ileum as well as in the crypts of the ileum.

The differences between studies in terms of morphological features persist after a few weeks. At this time period, differences become more comprehensible due to variable diet and environmental factors. PD26 AGA control piglets of Alizadeh et al. had an average jejunal villus length of 837 μm while the PD28 AGA control piglets of Mickiewicz et al. had an average jejunal villus length of 365 μm and a length of 422 μm for IUGR [60, 103]. Our PD28 AGA control piglets had an average jejunal villus length of 767 μm while IUGR control had an average length of 633 μm . In conclusion, considering the high degree of variability in results across studies, it is understandable that our experiment failed to detect differences between AGA and IUGR.

In terms of enteroendocrine cell density, there were again few significant differences between AGA and IUGR piglets at PD1. There is limited data available from other experiments, but in a study by Willemen et al. measuring serotonin levels in the gut, they similarly did not find any differences at PD1 between AGA and IUGR piglets in terms of chromaffin-like cell density or serum levels [101].

Past PD1, our experimental groups were statistically indistinguishable from each other in most respects based on the characteristics that were measured. Though few differences were observed between the treatment groups, distinctions in enteroendocrine cell development arose within each segment of the gastrointestinal tract. Cell staining always increased in density in the duodenum between PD1 and PD28, while the concentration tended to decrease from PD1 to D28 in the ileum. This could signify that enteroendocrine cell numbers stay constant in the ileum while the tissue size enlarges. Alternatively, this observation could indicate that ileal enteroendocrine cell numbers decline slightly over the first month of life. EEC cell numbers rapidly increase in the duodenum during the first month which is unsurprising as the duodenum is a critical site for the digestion and

absorption of nutrients. In the stomach, CgA and somatostatin cell concentrations tended to be higher at PD28 in the fundus and pylorus while the opposite was true for serotonin cells.

There are many possible explanations of why HMO supplementation did not appear to noticeably affect the neonatal piglets either way. First of all, our piglets were kept in a well-maintained, clean setting. In this non-stressful environment, it is possible that all of them were able to thrive equally. Many of the benefits of HMOs are related to immune function, which was not a challenge faced by any of the treatment groups. As mentioned before, they were allowed to receive colostrum after birth, conferring protective advantages. Next, the only HMO included was 2'-FL. In breast milk, as mentioned before, most mothers produce a wide variety of HMOs for their offspring. It may be that greater HMO diversity is essential towards conferring an advantage. In addition, although 2'-FL is consistently found among samples of porcine milk oligosaccharides, it is not the most abundant oligosaccharide. 2'-FL may not have as much relevance in the development of the neonatal piglet as in the human infant.

In the future, it would be useful to define a threshold IUGR piglet birth weight at which increased developmental problems occur. Perhaps only the extreme IUGR piglets truly suffer from digestive limitations in the gut. It would also be interesting to supplement with a larger variety of milk oligosaccharides, or include a few that are specifically crucial to piglets. To address the concern of “catch-up” growth and the later associated health consequences, adding a long-term component to the study would also be interesting both for pigs that are allowed to feed ad libitum and pigs that have access to HMOs. Further work is necessary to determine what roles milk oligosaccharides play in the development of the neonatal gastrointestinal tract, including impact on disaccharide activity as well as proliferation, differentiation and apoptosis activity of EECs.

Figure 1

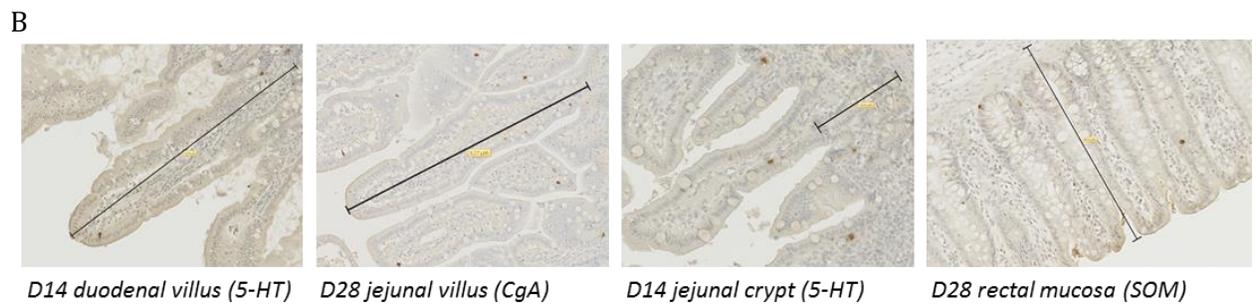
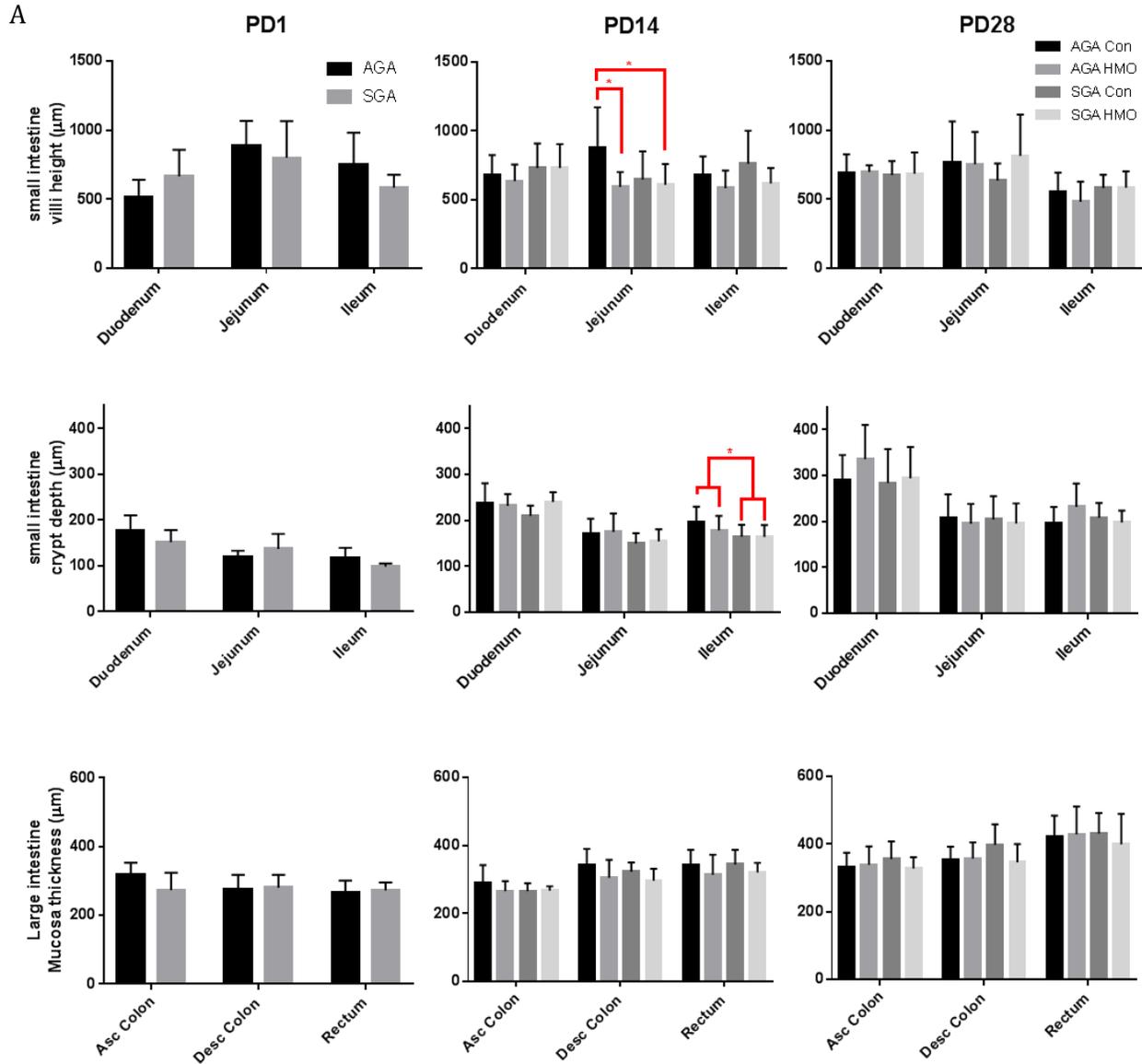


Figure 1. Graphs (A) compare the morphological measurements of the villi and crypts of the small intestine and the mucosa of the large intestine at PD1, PD14, and PD28. * $p=0.05$ Pictures (B) show measurements of the villus, crypt, and mucosa; 5-HT=serotonin, CgA=chromogranin A, SOM=somatostatin

Figure 2

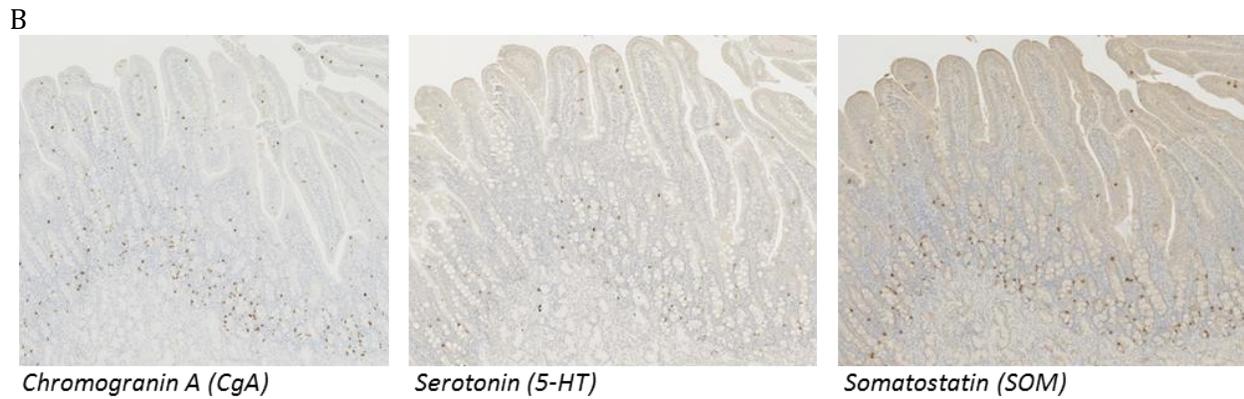
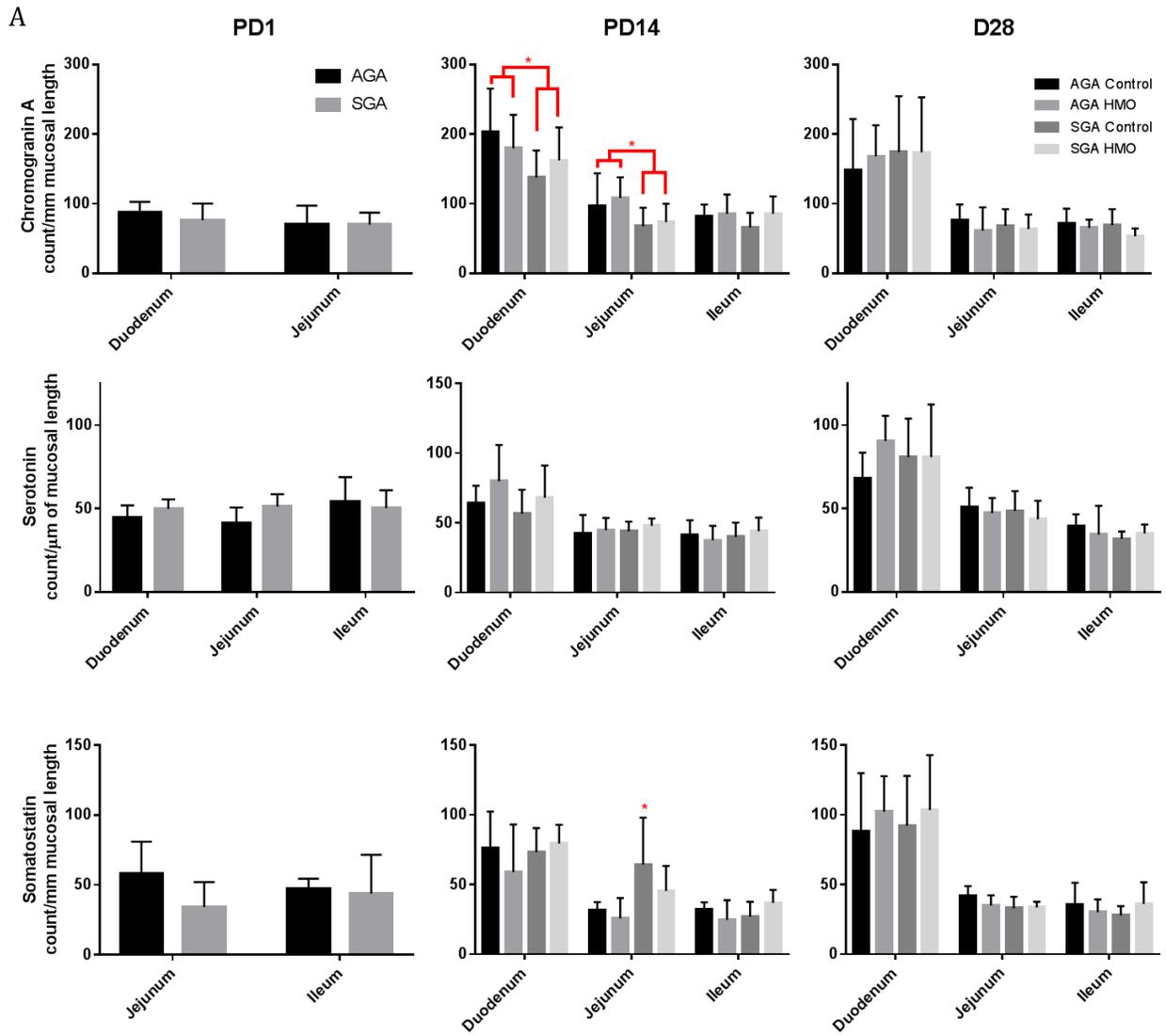


Figure 2. Graphs (A) compare CgA, serotonin and SOM concentration in the small intestine at PD1, PD14, and PD28. $\ast = p < 0.05$; Pictures (B) show positively stained cells in the D14 duodenum with different stains.

Figure 3

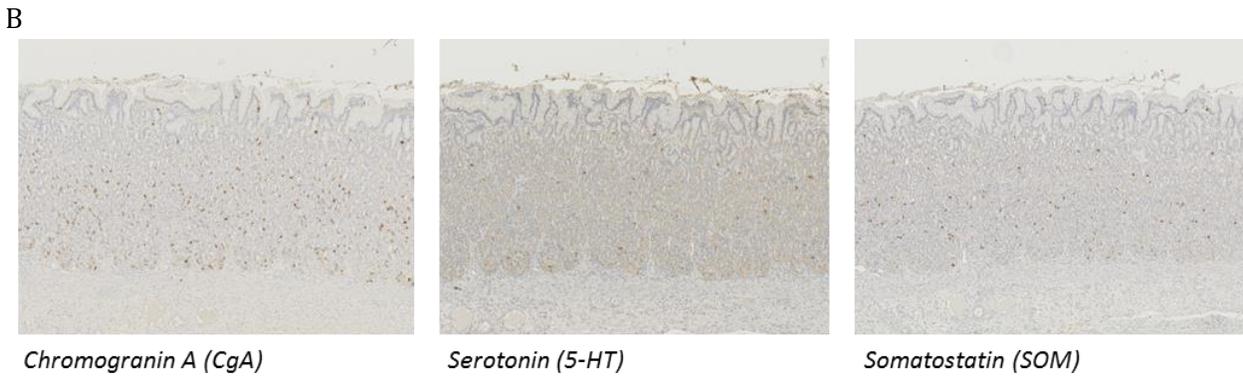
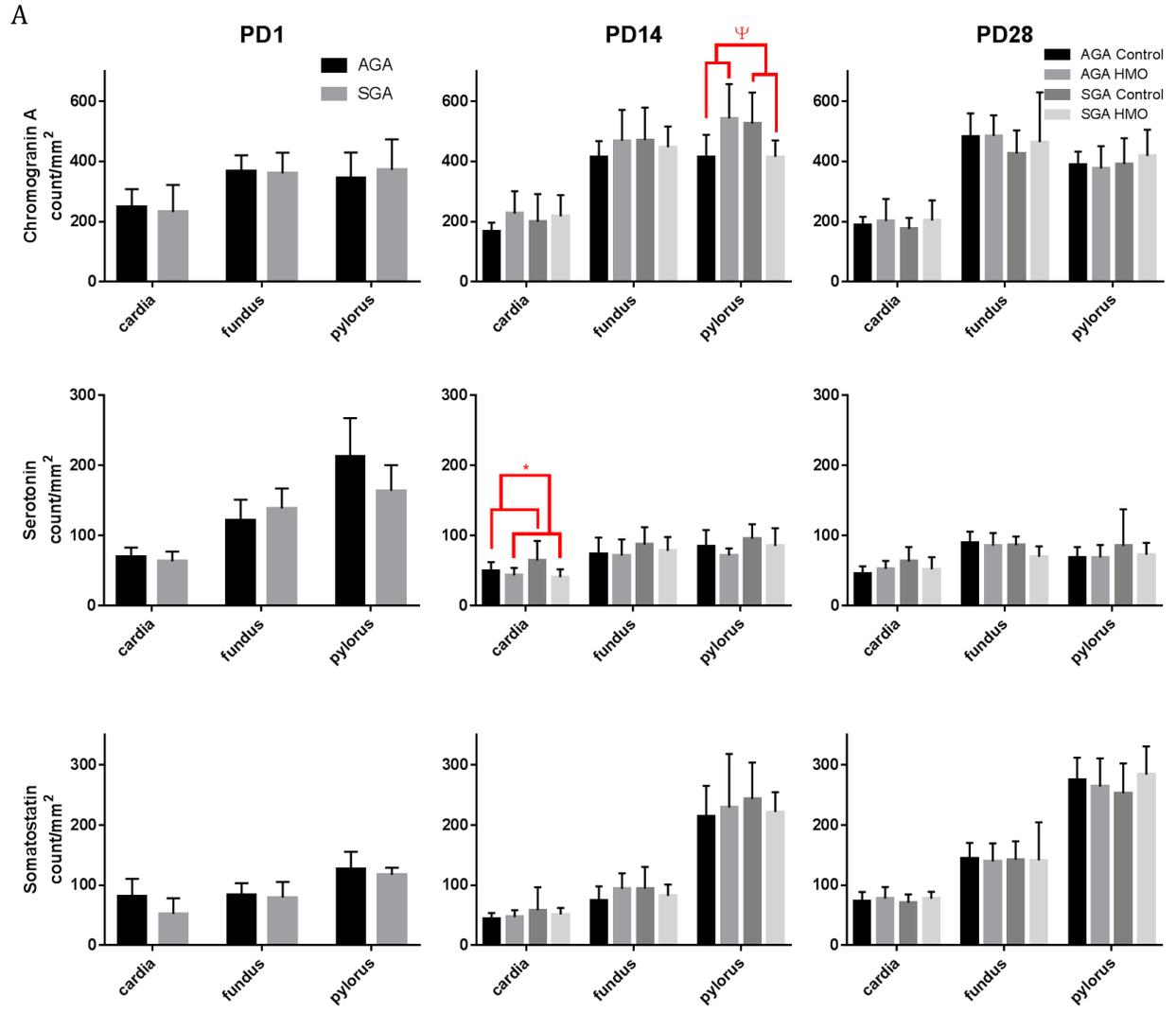


Figure 3. Graphs (A) compare CgA, serotonin and SOM concentration in the stomach at PD1, PD14, and PD28. *= $p < 0.05$, ψ =significant interaction; Pictures (B) show positively stained cells in the D28 fundus with different stains.

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