MATERNAL INGESTION OF GLUTAMINE AND GLUTAMATE DURING SOW PREGNANCY AND LACTATION: LIPID PROFILE ANALYSIS OF MILK AND NEONATAL ADIPOSE TISSUES

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ABSTRACT OF THE THESIS

Maternal Ingestion of Glutamine and Glutamate During Sow Pregnancy and Lactation: Lipid Profile Analysis of Milk and Neonatal Adipose Tissues

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Glutamine and glutamate are the most abundant amino acids found in milk and are thought to be important for rapid growth of neonatal tissues. Although glutamine and glutamate metabolism in sows during pregnancy and lactation have been explored, the impact of glutamine and glutamate supplementation on lipids in milk and neonatal tissues are less well understood. The research goals of this project were to better understand the impact of glutamine and glutamate supplementation during gestation and lactation in pigs, and how it affects the lipid composition of colostrum, milk, and neonatal adipose tissue. This master's thesis is comprised of two chapters, which will examine the influences that supplemental glutamine and glutamate have on the sow and the neonate. The significance of this work as it relates to porcine neonatal adipose tissue composition will be explained along with the results of our findings.

DEDICATION

To My Parents, Grandparents, and My Brother Michael

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CHAPTER 1

Literature Review

A. Purpose and scope of literature review

The objective of this research is to better understand the impact of glutamine supplementation during gestation and lactation in pigs and how it affects the lipid composition of colostrum, milk, and neonatal adipose tissue. Glutamine and glutamate are the most abundant protein-bound and free alpha amino acids found in milk (Wu and Knabe 1994, Sarwar *et al.* 1998, Haynes *et al.* 2009, Wu *et al.* 2011) and are thought to be important for rapid growth of neonatal tissues (Manso Filho *et al.* 2008, Manso *et al.* 2012). Although the role of glutamine metabolism in sows during pregnancy and lactation has been explored, the impact of glutamine supplementation on lipids in milk and neonatal tissues is less well understood. Thus, the purpose here is to review the literature on the influence that supplemental glutamine has on the sow and the neonate. Additionally, studies indicating glutamine as an important bioactive factor during pregnancy and lactation will be described. The effects of glutamine on neonatal tissue development will also be reviewed. Finally, the significance of this work as it relates to porcine neonatal adipose tissue composition will be explained and the working hypothesis and objectives will be presented.

B. Maternal programming of neonatal development

The epigenome can be described as the complex relationship between genes and environmental factors that ultimately affect the expressed phenotype (Waddington 1942). Epigenetics is the study of changes in gene expression that do not arise from alterations to the DNA sequence (Goldberg *et al.* 2007). Developmental plasticity is defined as the idea that an organism's genome adapts to environmental cues during distinct developmental phases that alter the resulting phenotype (Hochberg *et al.* 2011). The

ability of developing tissues to respond to conditions in the beginning stages of life that ultimately alter tissue structure and function is defined as programming (Langley-Evans 2007). This maternal programming of offspring tissues establishes a developmental trajectory that determines the future state of a particular tissue phenotype (Burggren 1999). An example of developmental programming comes from several studies using rodents. For instance, rat dams fed a restricted diet during gestation gave birth to offspring with low birth weights compared to offspring from dams fed a non restricted diet (Jones and Friedman 1982). When the low birth weight offspring were introduced to a non-restricted diet after birth, they became obese compared to offspring from mothers fed a non-restricted diet during gestation (Jones and Friedman 1982, Vickers *et al.* 2000). In another study, pregnant mice were fed a low protein diet thus subjecting their fetuses to malnutrition *in utero* (Ozanne and Hales 2004). Postnatally, these offspring were then nursed from control-fed mice, resulting in a shorter life expectancy compared to control mice (Ozanne and Hales 2004). These authors hypothesized that the shorter life expectancy was due to over eating that compensated for reduced growth *in utero* which ultimately resulted in obesity in adulthood (Ozanne and Hales 2004). Together, these findings suggest that maternal programming due to alterations in nutrition during fetal and neonatal development stages can alter metabolism and tissue function both after birth and into adulthood.

C. Composition of colostrum and milk

Lactation is a characteristic of all mammals (Peaker 2002). Milk is synthesized in the mammary gland, provides nutrition, and acts as an energy source for tissue growth

and development of nursing neonates (Blackburn and Babayan 1989, Peaker 2002). The initial secretion from the mammary gland is termed colostrum, or first milk. Not only is the composition of colostrum and milk different, but it is also species-specific. Milk is a dynamic fluid that changes composition throughout lactation (Jenness 1974). In horses and goats, colostrum is higher in fat compared to mature milk (Csapo *et al.* 1995, Sanchez-Macias *et al.* 2013) while bovine colostrum is higher in protein and lower in fat compared to mature milk (Tsioulpas *et al.* 2007). In kangaroos, lactation lasts 200 d and the composition of milk is lower in fat and protein and higher in carbohydrates compared with composition of the milk in the beginning stages of lactation (Trott *et al.* 2003). In the pig, colostrum differs from mature milk in that it has higher concentrations of immunoglobulins, proteins, and carbohydrates while it is lower in fat and lactose (Klobasa *et al.* 1987). In the pig, the transition from colostrum to mature milk occurs by approximately two days after birth and is characterized by higher fat and lactose and lower protein and immunoglobulins (Klobasa *et al.* 1987, Ogawa *et al.* 2014).

In general, colostrum functions in providing newborn animals with passive immunity and nutrition (Rooke and Bland 2002). The first 48 h after birth is important because nutrient demands increase, not only for the growth of the neonate, but for the start of milk synthesis in the mammary gland (Grummer *et al.* 1995). Colostrum is also important for gut development (van Barneveld and Dunshea 2011). It is unknown whether colostrum functions in programming neonatal tissue development and, therefore, this is the focus on research in our laboratory.

Relaxin (RLX) is present in porcine colostrum and milk (Yan *et al.* 2006). Yan and colleagues (2006) demonstrated that milk-borne RLX was detectable at high

concentrations during the first 24 h of lactation and declined by postnatal day (PND) 14. RLX was only detectable in the serum of gilts that nursed after birth on PND 1, but was undetectable in the circulation of newborns and in those fed a RLX-free milk replacer for 12 hours after birth (Frankshun *et al.* 2010). Therefore, our laboratory coined the term lactocrine and defined the lactocrine hypothesis to describe a mechanism in which milk-borne bioactive factors (MbFs), such as RLX, enter intact into the circulation of nursing young after birth and can affect developmental programming of neonatal tissues (Yan *et al.* 2006, Bartol *et al.* 2008). Recent evidence from our laboratory suggests that MbFs present in colostrum support neonatal development of male (Rahman *et al.* 2014) and female reproductive tissues (Miller *et al.* 2013), however, whether MbFs support non-reproductive tissue development and the identity of the component(s) in colostrum/milk that cause these effects are unknown.

D. Glutamine as a bioactive factor in colostrum

Colostrum contains many MbFs including proteins, peptides, and steroids that have a role in affecting development of neonatal tissues (Grosvenor and Mena 1992, Donovan and Odle 1994, Rodriguez-Palmero *et al.* 1999). Glutamine is the most prevalent protein-bound and free alpha amino acid in milk of most species including humans, baboons, horses, cows and pigs (Wu and Knabe 1994, Sarwar *et al.* 1998, Haynes *et al.* 2009, Wu *et al.* 2011). In pigs, the concentration of glutamine in milk is reported to increase from 0.1 mM glutamine in colostrum at LD 1 to 4.0 mM glutamine in milk collected at LD 28 (Wu and Knabe 1994).

Although glutamine is considered a non essential amino acid, it functions as an energy source for certain cells as well as plays a key role in cell regulating pathways.

Furthermore, through the glutaminase reaction, glutamine serves as a precursor for glutamate in the cell (Newsholme *et al.* 2003). Intracellularly glutamate is the most abundant amino acid (2-20mM), while glutamine is the most abundant extracellular amino acid 0.7 mM (Newsholme *et al.* 2003). Additionally, glutamine and glutamate are necessary for protein synthesis within many organs such as the liver, intestine, immune cells, and kidneys (Haussinger and Gerok 1983, Haussinger 1989, Reeds *et al.* 1996, Reeds *et al.* 1997, Schuldt *et al.* 1999).

The amount of free glutamine in the plasma is species-dependent and ranges from 0.5-1.0 mM (Rhoads and Wu 2009). The concentration of free glutamine is critical since many tissues depend on this source for glutamine metabolism (Rhoads and Wu 2009). Evidence to suggest that glutamine is important in regulating neonatal porcine tissue development originates from a study by (Wu and Knabe 1995). In this study, the relationship between glutamine and arginine was explored. Since the amino acid arginine, deficient in colostrum and milk, is required for neonatal growth, glutamine is important because it can be converted to arginine. Wu and Knabe (1995) found that enterocytes from 0 to 2 day old pigs synthesized arginine from glutamine via citrulline at three-fold greater amounts than enterocytes from seven day old pigs. Since glutamine is abundant in colostrum and milk, (Wu 1998) suggested milk as the source of glutamine for the neonate. Thus, data suggest that studies designed to test the role of glutamine as an important MbF in regulating neonatal porcine tissue development are warranted.

Permeability of the gut after birth is an important factor that can limit the delivery of MbFs, such as glutamine, to enter into circulation. In the neonate, intestinal cells take up macromolecules by endocytosis for transport into circulation (Sangild 2003). The term

"gut closure" refers to the termination of the neonatal gut's ability to absorb macromolecules and its timing is species specific (Pacha 2000). In pigs, gut closure occurs within the first few days after birth (Sangild 2003). A study by Lecce and Morgan demonstrated that nursing pigs were able to absorb the macromolecule, polyvinvlpyrrolidone, up to 36 h after birth (Lecce and Morgan 1962). In another study, by Lin and associates, protein digestibility of porcine colostrum by neonatal pigs was examined and at day 3, 98.8% of glutamine and glutamate from the colostrum was digested in the gut (Lin *et al.* 2009). Thus, the first days after birth are crucial for the neonate to absorb molecules from colostrum into the gut including glutamine and glutamate.

E. Metabolism of glutamine/glutamate during lactation

Late in gestation, the sow is in a mild catabolic state given that muscle protein is lost to meet the demanding needs of the growing fetus. In a study conducted by Clowes and associates (2003) body size and body protein loss during lactation was determined by evaluating sows that were either a standard or high body mass at parturition and lost either a moderate or high level of protein in lactation. Sows with a standard body mass at parturition and a high level of protein loss during lactation exhibited a decrease in lactation performance and follicular development compared to the other treatment groups (Clowes *et al.* 2003). Additionally evidence to suggest that glutamine metabolism is linked to a catabolic state during lactation comes from a study conducted in the horse. In the mare, like the sow, changes in glutamine metabolism also result in a mild catabolic state during lactation (Manso Filho *et al.* 2008). These authors suggested the catabolic

state during lactation in horses was due to the loss of lean body mass, since muscle protein is being used to provide amino acids for milk synthesis during lactation.

Since muscle glutamine is being catabolized during gestation and lactation, other molecular sources are used by the tissues to create novel glutamine (Manso Filho *et al.* 2008). For instance, the lactating sow requires a high amount of branched-chain amino acids (BCAA) to support milk production (Kim *et al.* 2009). Researchers found that BCAA in the lactating mammary gland (76 g/d) are much greater than the amount found in milk (46 g/d) (Trottier *et al.* 1997). Furthermore, protein expression of glutamine synthetase, the enzyme responsible for glutamine synthesis from BCAA, was measured by way of immunoblotting in mammary gland tissue collected from sows on lactation day 28 (Li *et al.* 2009). Since results revealed that glutamine synthetase protein expression was high, authors concluded that the BCAA are catabolized in order to produce novel glutamine within the mammary tissue. Thus, to prevent depletion of other substrates, including BCAA, and produce novel glutamine, supplementation of this amino acid may be necessary during gestation and lactation.

F. Effects of AminoGut supplementation during gestation and lactation

Data collected by Wu and co-authors suggest that glutamine can serve as an energy substrate for the developing tissues of the porcine fetus during gestation (Wu 2010). For instance, sows fed a restricted protein diet during gestation (2 kg/d protein) exhibited protein deficiency and limited fetal development compared to sows fed a control diet (Kim *et al.* 2009). Whether supplementation with glutamine could remedy this was determined using AnimoGut (Ajinomoto; Sao Paulo, Brazil), a low-cost feed-grade glutamine and glutamate supplement for swine and poultry diets produced by

Ajinomoto Co. Inc (Wu *et al.* 2011). Indeed, research conducted by Manso and colleagues in 2012 demonstrated that AminoGut supplemented to gilts 30 d prior to expected parturition through LD 21 prevented a decrease in glutamine in skeletal muscle and increased the presence of glutamine in milk (Manso *et al.* 2012). These results suggest that supplementing sow diets with 1.5% AminoGut is beneficial to the mother because the glutamine in the skeletal muscle is being preserved, however the effects of increased lipid content in the milk produced by the sow on the nursing neonate is not clear.

In an unpublished study by this same group (Santos de Aquino et al), sows were either fed a control diet or a diet supplemented with 1.5% AminoGut seven days prior to parturition and up to day 21 of lactation. They reported that the fat content of colostrum from sows supplemented with AminoGut increased by 60 % to 7.38% fat in AminoGut fed sows compared to the control group (4.64% fat). However, the fat content of the milk from sows fed AminoGut was similar to sows on the control diet at LD 7. By LD 21 the fat content of milk from control sows was 5.0% whereas in AminoGut-fed sows milk fat was 6.64% .These results suggest that AminoGut supplementation can increase the lipid content in colostrum and milk.

G. Effects of glutamine on small intestine development

Glutamine was initially considered a nonessential amino acid however; Windmueller and Spaeth (1978) showed the importance of glutamine as metabolic fuel in the small intestine as a source of energy. This was accomplished by quantifying the uptake of circulating metabolites including glutamine, glucose, acetoacetate, and 3-hydroxybutyrate, and by measuring those metabolites found in blood samples from rat

jejunal segments of the small intestine (Windmueller and Spaeth 1977). The results of this experiment included a 30 % net flux of circulating glutamine in the tissue suggesting that glutamine can serve as an intestinal metabolic fuel. Furthermore, glutamine signals intestinal cells to rapidly grow via mitogen-activated protein kinases pathway to increase survival rates and prevent apoptosis in the intestine (Rhoads *et al.* 2000). Glutamine is also used to treat chronic diseases such as inflammatory bowel disease and diarrhea (Wang *et al.* 2009), which is especially important considering that pigs are susceptible to developing scours (neonatal diarrhea) shortly after birth (Tzipori *et al.* 1985). Whether supplemental glutamine can improve intestinal function in young pigs is of interest in the swine industry. A study conducted by Cabrera and colleagues (2013) determined pre- and post-weaning growth performance and intestinal health of pigs that were fed creep feed supplemented with AminoGut compared to pigs fed creep feed alone. Results suggested that compared to the control diet, supplementation with AminoGut (1) improved feed conversions (feed/gain) within the first three weeks post weaning; (2) improved pre and post weaning growth performance and intestinal health of pigs; and (3) reduced atrophy in villi found in the small intestine of the neonatal pig (Cabrera *et al.* 2013). This study supports the use of glutamine supplementation in support of intestinal health of pigs. However, whether the benefits of glutamine supplementation in pigs extends to include other tissues, remains to be determined.

H. Adipose tissue: Types, depots and effects of glutamine

Adipose tissue consists of two distinct types: brown and white fat depots (Saely *et al.* 2010). Brown adipose tissue regulates thermogenesis and is located in newborns and in mammals that hybernate (Cannon and Nedergaard 2004). White adipose tissue is a

storage site for triglycerides (TG) and cholesterol (Montoye *et al.* 1966) and also functions as an endocrine organ (Ahima and Flier 2000). Adipose tissue is present in different locations in the body including, but not limited to, subcutaneous and visceral fat depots (Shen *et al.* 2003). While subcutaneous fat is found underneath the skin, visceral adipose tissue is found associated with digestive organs. Within these depots, morphological and functional differences are found. Morphologically, subcutaneous adipose tissue contains higher numbers of preadipocytes when compared to visceral adipose tissue (Tchkonia *et al.* 2005). Functionally, triglyceride storage and lipolysis are greater in visceral fat when compared with subcutaneous adipose tissue (Engfeldt and Arner 1988, Wajchenberg *et al.* 2002). Visceral adipose tissue also has a higher rate of apoptosis compared with the subcutaneous adipose depot (Sethi and Hotamisligil 1999, Wajchenberg *et al.* 2002). Researchers have also reported differential gene expression in subcutaneous versus visceral adipose tissues. (Gesta *et al.* 2006) reported depot-specific differences in gene expression in subcutaneous and visceral adipose tissues in mice and humans. Thus, whether maternal ingestion of a glutamine supplement during gestation and lactation alters the lipid profile of neonatal adipose tissues or has differential effects on visceral or subcutaneous fat depots remains to be determined.

There is evidence to indicate that glutamine may play a role in lipogenesis in a brown adipocyte cell line. Studies showed that the contribution of glutamine to lipogenic acetyl CoA was equal to glucose in wild type brown adipocytes (Yoo *et al.* 2004). This was done by evaluating flux of carbon sources during brown adipocyte differentiation. They suggested that glutamine acts as a precursor for lipogenic acetyl-CoA and occurs by reductive carboxylation in brown adipocytes. According to (Yoo *et al.* 2008) these results

were unexpected since glutamine is not generally thought to be a lipogenic precursor. Whether glutamine is lipogenic in white adipose tissue is unknown. Since neonatal pigs lack brown adipose tissue (Berg *et al.* 2006) studies to determine whether glutamine is lipogenic in porcine neonatal tissues is warranted.

I. Significance and Objectives

Glutamine and glutamate are the most abundant amino acids found in milk (Wu and Knabe 1994, Sarwar *et al.* 1998, Haynes *et al.* 2009, Wu *et al.* 2011) and studies suggest that gestation and lactation can cause a mild catabolic state depleting glutamine stores (Manso *et al.* 2012). Given the report by this group suggesting that supplementing sows with glutamine, by feeding AminoGut, increased the lipid content of colostrum or milk (Santos de Aquino et al., unpublished) we were interested in following up on these observations by studying effects of maternal glutamine supplementation on neonatal adipose tissues. Availability of excess lipids in colostrum and milk may alter the lipid profile of neonatal adipose tissue. This is important especially considering that newborn pigs do not have brown adipose tissue as a source of energy for maintaining body temperature (Berg *et al.* 2006). Thus, whether feeding sows a glutamine supplement (such as AminoGut) during gestation and lactation can alter the lipid profile of neonatal pigs remains to be determined.

Therefore, the working hypothesis of this thesis research states that supplementation with dietary glutamine during sow pregnancy and lactation will increase the lipid content and alter the lipid profile of colostrum, milk and adipose tissues of the offspring. Thus, the objective of this Master's project was to determine the effects of dietary AminoGut supplementation during gestation and lactation on the lipid content and

lipid profile of the following; 1) porcine colostrum and milk and; 2) visceral and subcutaneous adipose tissue depots of PND 14 gilts.

CHAPTER II

Maternal Ingestion of Glutamine and Glutamate During Sow Pregnancy and Lactation: Lipid Profile Analysis of Milk and Neonatal Adipose Tissues

ABSTRACT

Glutamine and glutamate are the most abundant amino acids found in milk. There is evidence in pigs that ingestion of AminoGut, a glutamine and glutamate supplement for swine, increases glutamine and lipid content of colostrum and milk. In this study, we tested the hypothesis that supplementation with dietary glutamine and glutamate during sow pregnancy and lactation will increase the lipid content and alter the lipid profile of colostrum, milk and adipose tissues of the offspring. Sows were randomly assigned at pregnancy day (PD) 84 (30 days prior to expected parturition) to be fed either 1) a commercial control diet (n=4) or 2) a commercial diet supplemented with 1.5% AminoGut (n=4) mixed into the rations. Milk at lactation day (LD) 0 and LD 7 was extracted via Folch method. AminoGut supplementation had no effect on total lipid, cholesterol, or triglyceride (TG) content of milk. However, the TG content of LD 7 milk was greater ($P<0.01$) than milk at LD 0. In objective 2, neonatal gilts (n=6/group) born from sows fed either the control diet or AminoGut (1.5%) supplemented diet, nursed *ad libitum* for 14 d. At postnatal day (PND) 14 visceral and subcutaneous adipose tissue were extracted via Folch method. Maternal ingestion of AminoGut increased TG content in neonatal subcutaneous adipose tissue compared to offspring from the control-fed group ($P < 0.01$). Overall, TG content of neonatal subcutaneous fat was greater ($P<0.01$) than TG in visceral fat depots. Cholesterol content of neonatal subcutaneous fat was 10-fold greater ($P < 0.01$) than visceral fat. Maternal ingestion of AminoGut increased ($P < 0.05$) cholesterol content in neonatal visceral fat compared to the control group. However, maternal glutamine supplementation did not affect lipid content, TG in visceral fat or cholesterol in subcutaneous fat. In conclusion, maternal glutamine supplementation during pregnancy and lactation did not increase total lipid content of colostrum or milk

but did result in a small, but significant, increase in triglyceride content of neonatal subcutaneous adipose tissue at PND 14. In addition, in the neonate depot-specific effects were observed in that cholesterol and triglycerides were higher in subcutaneous fat when compared to visceral fat, independent of maternal diet.

INTRODUCTION

Glutamine is a free amino acid that circulates in the body in most mammals that is considered to be metabolic fuel for dividing cells (Rhoads and Wu 2009) and is mainly stored in skeletal muscle (Curthoys and Watford 1995, Watford 2008). Skeletal muscle accounts for 40-45% body weight in pigs, which makes this site important for glutamine storage (Wu *et al.* 2006). Metabolic stress increases the demand for glutamine in tissues, depletes glutamine storage in skeletal muscle and requires increased glutamine synthesis (Tjader *et al.* 2007). Studies suggest that gestation and lactation can cause a mild catabolic state depleting glutamine in skeletal muscle of pigs (Clowes *et al.* 2003) and horses (Manso Filho *et al.* 2008, Manso et al. 2012). Branched-chain amino acids support glutamine production in the mammary gland in the process of milk synthesis (Kim *et al.* 2009). Glutamine is the most abundant amino acid found in milk (Wu and Knabe 1994, Sarwar *et al.* 1998, Haynes *et al.* 2009, Wu *et al.* 2011). There is evidence in pigs that ingestion of AminoGut (Ajinomoto Co., Inc; Sao Paulo, Brazil), a low-cost, feed-grade glutamine supplement for swine and poultry (Wu *et al.* 2011) increased milk glutamine levels (Manso *et al.* 2012) as well as the lipid content of colostrum and milk (Santos de Aquino et al., unpublished). However, the impact of maternal ingestion of AminoGut on lipids in neonatal porcine tissues is unknown.

The initial secretion from the mammary gland is termed colostrum, or first milk. Milk is a nutritious energy source for tissue growth and development of nursing neonates (Blackburn and Babayan 1989, Peaker 2002). In the pig, the transition from colostrum to mature milk occurs by approximately lactation day (LD) 2 and is characterized by higher fat and lactose and lower protein and immunoglobulins in milk when compared with

colostrum (Klobasa *et al.* 1987, Ogawa *et al.* 2014). How the lipid composition of colostrum and milk influences the lipid profile of neonatal tissues, including adipose tissue is not well understood.

Adipose tissue is present in different locations in the body including, but not limited to, subcutaneous and visceral fat depots (Shen *et al.* 2003). While subcutaneous fat is found underneath the skin, visceral adipose tissue is found associated with digestive organs. Within these depots, morphological and functional differences are found. Morphologically, subcutaneous adipose tissue contains higher numbers of preadipocytes when compared to visceral adipose tissue (Tchkonia *et al.* 2005). Functionally, triglyceride storage and lipolysis are greater in visceral fat when compared to subcutaneous adipose tissue (Engfeldt and Arner 1988, Wajchenberg *et al.* 2002). Visceral adipose tissue also has a higher rate of apoptosis compared to the subcutaneous adipose depot (Sethi and Hotamisligil 1999, Wajchenberg *et al.* 2002). Researchers have also reported differential gene expression in subcutaneous versus visceral adipose tissues. (Gesta *et al.* 2006) reported depot-specific differences in gene expression in subcutaneous and visceral adipose tissues in mice and humans. Thus, whether maternal ingestion of a glutamine supplement during gestation and lactation alters the lipid profile of neonatal adipose tissues or has differential effects on visceral or subcutaneous fat depots remains to be determined.

Thus, the objective of this study was to determine the effects of dietary AminoGut supplementation during sow gestation and lactation on the lipid profile of: 1) porcine colostrum and milk and; 2) visceral and subcutaneous adipose tissue depots of PND 14 gilts.

MATERIALS AND METHODS

Animals

Pregnant crossbred sows (*Sus scrofa domesticus*) from the Swine Unit of the New Jersey Agricultural Experiment Station, Rutgers University were housed in individual pens. After parturition, neonatal pigs were housed with their dams in pens equipped with heating lamps to make sure body temperatures were maintained in the neonates. Care was taken to ensure that treatments were balanced for potential effects of litter (n=7 litters) for the neonatal pig study. All procedures involving animals were reviewed and approved by the Rutgers Institutional Animal Care and Use Committee and conducted in accordance with the Guide for the Care and Use of Agricultural Animal in Agricultural Research and Teaching (1999; Federation of Animal Science Society, Savory, IL, USA).

Objective 1 was conducted to evaluate the effects of dietary supplementation of sows with AminoGut during late gestation and early lactation on the lipid profile of colostrum and milk (Figure 1). Sows were randomly assigned at pregnancy day (PD) 84 (30 days prior to expected parturition) to be fed either 1) a commercial control diet (n=4) or 2) a commercial diet supplemented with 1.5% AminoGut (n=4) mixed into the rations. AminoGut (Ajinomoto do Brazil; Sao Paulo, Brazil) and contains a minimum of 10% L-Glutamine, and 10% L-Glutamate. During gestation, sows were fed 3 lbs per feeding twice a day and 6 lbs per feeding three times a day during lactation. These diets were fed through lactation day (LD) 7 and are in accordance with the NRC nutrient requirements for swine. Colostrum (LD 0; ~ 20 ml/sow) and milk (LD 7; ~5 ml/sow) were collected manually from each sow and frozen at -20ºC until subjected to triglyceride and cholesterol assays. To facilitate milk collection at LD 7, sows were treated with oxytocin

(20 USP intramuscularly, Butler Schein, Dublin, Ohio, USA). Lipid extracts from the colostrum and milk samples were also subjected to lipidomic analysis in collaboration with Dr. Anita Brinker and Nutritional Metabolomics Research Core Facility at Rutgers University.

In objective 2, the effects of maternal ingestion of AminoGut during late gestation and early lactation on the lipid profile of two neonatal adipose tissue depots, visceral and subcutaneous fat, were studied (Figure 2). Sows were randomly assigned at pregnancy day (PD) 84 (30 days prior to expected parturition) to be fed either 1) a commercial control diet or 2) a commercial diet supplemented with AminoGut (1.5%) mixed into rations through LD 14 as described above. Neonatal gilts (n=6/group) born from sows fed either control diet or AminoGut (1.5%) supplemented diet, nursed *ad libitum* for 14 d. At postnatal day (PND) 14 gilts were euthanized using Aerrane Vet Isoflurane (Butler Schein, Melville, NY, USA). Visceral adipose tissue from the duodenal mesenteric region and subcutaneous adipose tissue from the subscapular region were collected, frozen on dry ice and stored at -80ºC until subject to testing. All neonatal gilts used in these studies weighed 1.3 kg or greater at birth based on studies indicating that pigs with lower body weight are less likely to survive and have lower postnatal growth rates (Rehfeldt and Kuhn 2006).

Lipid Extractions for Lipidomics Analysis

Total lipids in colostrum/milk were extracted using the Folch method (Folch et al., 1957). Frozen colostrum or milk samples were thawed at 37ºC, mixed by vortexing (1 min) and 500 ul of the sample was inserted to a 15 ml glass tube. PBS (pH 7.4) was

added to the samples to bring the volume to 600 ul. Samples were vortexed again for one min and 50 ul of internal standard (Trinonanoin, 1,2-ditridecanoyl-sn-glycero-3-phosphocholine, 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine) was added. Samples were vortexed for a minute and then brought to the fume hood where 12 ml of 2:1 CHCl3:MeOH was added with a glass pipette and vortexed for a minute. Next 50 mM KCl (2.4 ml) was added, samples were stored at -80ºC for at least 1 h, and then centrifuged at 1200 rpm at 4ºC for 20 min. The lower chloroform phase was transferred to a 15 ml glass tube. The chloroform extract was dried in a Speed-Vac at medium heat setting. Isooctane:THF (500 ul; 9:1) was added to each tube with dried lipids, vortexed lightly so that solvent did not splash up to reach the cap, sonicated for 2 min and then centrifuged 2000 rpm x 10 min @ 4ºC to pellet any debris. Isooctane:THF was removed with a Pasteur pipette to a HPLC vial. The 15 ml tube was washed with an additional 500 ul of Isooctane:THF and added into the same HPLC vial and dried under nitrogen. This procedure was to prevent loss of lipids through the transferring process of glass tube to HPLC vial. The dried lipids were re-dissolved in 500 ul isooctane:THF and stored at -80 C.

Lipid Extractions for Total Cholesterol and Triglyceride Assays

Total lipids from colostrum/milk and adipose tissue samples were extracted using the Folch method (Folch *et al.* 1957) and modified as reported in (Shabrova *et al.* 2011). For colostrum and milk, frozen samples were thawed at 37ºC, vortexed and 0.5 ml samples were pipetted into 15 ml tubes along with 0.5 ml of cold phosphate-buffered saline (PBS; Invitrogen; 10 mM phosphate, 150 mM NaCl) pH 7.4. For adipose tissue analysis,

frozen tissue (40 mg) was homogenized using a Tekmar Tissumizer in 1 ml of cold phosphate buffered saline (PBS; Invitrogen; 10 mM phosphate, 150 mM NaCl) pH 7.4. The tissue homogenate (1 ml) was transferred to a 15 ml glass tube.

For both milk and adipose tissue samples, chloroform:methanol (3.75 ml of 2:1 CHCl3:MeOH) was added to the 15 ml tubes. Samples were vortexed for 1 min and centrifuged at 1800 rpm for 10 min. The lower chloroform phase was transferred to a 15 ml glass tube. Another 1 ml of CHCl3:MeOH was added to the original 15 ml tube vortexed for a minute and centrifuged at 1800 rpm for 10 min. The lower chloroform phase was transferred and combined with the 1st portion. The lipid extract dissolved in chloroform was dried down with nitrogen.

For the triglycerides assay, chloroform/2% Triton (500 ul) was added to each tube of dried lipids, vortexed lightly so that solvent did not splash up to reach the cap, sonicated for 2 min and then centrifuged 2000 rpm x 10 min @ 4ºC to pellet any debris. Chloroform/2% Triton was transferred with a Pasteur pipet to a 5 ml tube. The 15 ml tube was washed with an additional 500 ul of chloroform/2% Triton and added to the 5 ml tube. This was to prevent loss of lipids in the transfer process from the glass tubes. After drying under nitrogen, the lipid extract was re-dissolved in 500 ul of chloroform/2% Triton and stored at -80ºC. For the cholesterol assay, the same procedures were followed except isopropyl alcohol was used in place of chloroform/2% Triton. Triglycerides were measured using the L-Type Triglyceride M assay for each sample in triplicate following the manufacturer's protocol (Wako Chemicals, Richmond,VA, USA). Total cholesterol in each sample was measured in triplicate using the Cholesterol E assay following the manufacturer's protocol (Wako Chemicals, Richmond,VA, USA).

Total Lipid Weight

Total lipids in colostrum/milk and adipose tissue samples were extracted using the Folch method (Folch *et al.* 1957). Extracts dissolved in chloroform:methanol (2:1) as described above were added to pre-weighed dishes. Extracts were air-dried and total lipid weight was determined by calculating the difference between final and initial weight of the dishes. Total lipid weight was divided by amount of tissue sample used, multiplied by 100 to calculate the percentage of fat in each sample.

Validating Folch Extraction procedures

To confirm the accuracy of the lipid extraction procedures using the Folch method, a pilot study was conducted using commercial bovine milk samples at defined fat concentrations [0, 2, and 4% milk fat and heavy cream (38%); Stop and Shop, distributed by FOODHOLD USA Landover, MD, USA]. Samples were subjected to Folch extraction as described above and lipid weights were obtained. All four forms of milk were extracted in triplicate. The observed lipid weights were calculated as % fat (mean + SEM) and recorded for each group.

Statistical Analysis

Data were subjected to analyses of variance (ANOVA) using GLM procedures (SAS Cary, NC, USA) and are presented as least squares means (LSM) ± SEM. For milk samples, ANOVA with repeated measures considered variation due to main effects of treatment [AminoGut (AG) vs control (C)], day (LD 0 vs LD 7), as well as interactions as appropriate. For adipose tissue, analyses considered variation due to main effects of

treatment (AG vs. C), adipose tissue depot at PND 14 (subcutaneous vs. visceral), as well as interactions as appropriate.

RESULTS

Pilot study: Validating Folch extraction procedures

In this experiment commercial bovine milk samples, ranging from 0% to 38% fat, were used to validate the accuracy of the Folch method for lipid extraction (Folch *et al.* 1957, Shabrova *et al.* 2011). The percentage of fat found in commercial bovine milk products following Folch extraction is presented in Figure 3. Results confirmed that the observed % fat for each of the milk samples was similar to expected % fat validating the Folch extraction procedures.

Maternal ingestion of AminoGut: Analysis of porcine milk lipids during lactation

Maternal ingestion of AminoGut during pregnancy had no effect on total lipid content of milk at LD 0 as shown in Figure 4a (C; 8.06 ± 0.41 % vs AG; 8.42 ± 1.36 %). Likewise, the lipid content of milk collected at LD 7 from control (8.85 ± 0.74 %) and AG-fed pigs (9.06 ± 0.83 %) were similar. Also, overall the lipid content of milk at birth LD 0 (8.2 ± 0.89 %) was similar to the lipid content at LD 7 (9 ± 0.79 %).

Figure 4b illustrates that the triglyceride content of milk was similar in Control (6.3 ± 0.07 mg/dl) and AG-fed sows (5.4 ± 0.04 mg/dl) at LD 0. There were also no effects of maternal ingestion of AG on triglyceride (TG) content of milk at LD 7 (8.2 ± 0.02 mg/dl) when compared to controls (8.3 ± 0.02 mg/dl). However, overall the triglyceride content of milk at LD 7 (8.2 ± 0.02 mg/dl) was greater ($P<0.01$) than at LD 0 (5.9± .05 mg/dl).

Cholesterol content in porcine milk at LD 0 and LD 7 is represented in Figure 4c. Results showed that cholesterol content in porcine milk of controls and AG-fed sows was

similar at LD 0 (C; 6.6± .17 vs AG: 6.4 ± 0.07 mg/dl) and at LD 7 (C; 6.8 ± 0.67 vs AG; 6.6± 0.04 mg/dl). Overall, there were no differences in cholesterol content of milk at LD 0 (6.5± 0.13 mg/dl) or LD 7 (6.7 ± 0.35 mg/dl).

Maternal ingestion of AminoGut: No effects in body weight

Body weights of neonatal pigs at birth for both control (1.6 ± 0.12 kg) and AminoGut fed groups (1.7 ± 0.11 kg) were similar. Likewise, at PND14 AminoGut feeding did not increase body weights (4.6 ± 0.37 kg) compared to the control fed group (4.2 ± 0.43 kg).

Maternal ingestion of AminoGut: Analysis of neonatal porcine fat tissue depots

As illustrated in Figure 5a, there was no effect of maternal ingestion of AminoGut during pregnancy and lactation on the lipid content of neonatal visceral fat (C; 86 ± 1.48 % vs AG-fed; 87 ± 2.63 %) or subcutaneous fat (C; 85 ± 1.60 % vs AG-fed; 87 ± 3.19 %) depots at PND 14. Overall, the lipid content of neonatal visceral fat (86 ± 5 %) was similar to that of subcutaneous fat (86 ± 6 %) at PND 14.

Maternal ingestion of AminoGut increased TG content in neonatal subcutaneous adipose tissue (0.94 ± 0.02 mg/g) compared to offspring from the control-fed group (0.69 ± 0.05 mg/g; $P < 0.01$; Figure 5b). However, for neonatal visceral fat, there was no maternal diet effect on TG content at PND 14. Overall, TG content of neonatal subcutaneous fat (0.81 ± 0.15 mg/g) was greater ($P<0.01$) than TG in visceral fat depots (0.58 ± 0.07 mg/g) at PND 14.

Figure 5c illustrates that the cholesterol content of neonatal subcutaneous fat tissue (1.07 ± 0.01 mg/g) was 10-fold greater ($P < 0.01$) than visceral fat tissue (0.08 ±

0.01 mg/g) at PND 14. Maternal ingestion of AminoGut increased ($P < 0.05$) the cholesterol content in neonatal visceral fat (0.19 ± 0.02 mg/g) at PND 14 compared to the control group (0.05 ± 0.01 mg/g). However, for subcutaneous fat, cholesterol content was similar in neonates from both control (1.05 ± 0.03 mg/g) and AG-fed sows (1.09 ± 0.04 mg/g).

DISCUSSION

Glutamine is the most abundant free alpha amino acid found in milk (Wu and Knabe 1994, Sarwar *et al.* 1998, Haynes *et al.* 2009, Wu *et al.* 2011) and is thought to be important for neonatal intestinal health and growth. In pigs ingestion of a commercial glutamine supplement for swine and poultry (AminoGut) increased milk glutamine levels (Manso *et al.* 2012) as well as the lipid content of colostrum and milk (Santos de Aquino et al., unpublished). However, these studies were conducted at a commercial swine facility and were limited to non-invasive milk collection only. Although the role of glutamine metabolism in sows during pregnancy and lactation has been explored, the impact of glutamine supplementation on lipids in pigs' milk and neonatal tissues is less well understood. Thus, the purpose of this study was to evaluate the impact of glutamine supplementation during pregnancy and lactation on the lipid profile of colostrum, milk and of subcutaneous and visceral adipose tissues from the offspring.

To evaluate the accuracy of the Folch method for lipid extraction, a pilot study was conducted with commercial bovine milk samples. Results confirmed that the observed % fat for each of the milk samples was similar to expected % fat validating the Folch extraction procedures. Total lipid content in milk did not differ between colostrum, collected at birth, and milk collected at LD 7. Some authors have reported that fat content is higher in mature milk compared to colostrum in the pig (Braude *et al.* 1947, Klobasa *et al.* 1987). However, (Elliott *et al.* 1971) reported that for pregnant and lactating sows the fat content of colostrum varied from 4-16% while milk fat content was also variable ranging from 4- 9.4%. However, these authors also reported that the average concentration of fat in colostrum and milk was similar in agreement with the

findings for colostrum and milk fat reported here. In contrast to the study conducted by Santos de Aquino et al (unpublished), who supplemented sows with AminoGut for the last 7 days of pregnancy and through d 21 lactation, in the studies presented here there was no effect of 30 days of supplemental glutamine ingestion by sows during pregnancy on the lipid content in LD 0 milk compared to the non-supplemented controls. Similarly, there was no effect of maternal glutamine supplementation on milk fat content at LD 7, which was in agreement with the Santos de Aquino et al (unpublished) study.

Regarding cholesterol and triglyceride content of colostrum and milk, (Elliott *et al.* 1971) reported that cholesterol values in colostrum were about 40-50% higher when compared to mature milk at LD 7. In contrast, no differences in the cholesterol content of colostrum were detected in the present studies. However, LD 7 milk was higher in triglyceride content compared to colostrum at LD 0. Supplementing AminoGut in the sow's diet also had no effect on the triglyceride and cholesterol content of colostrum at LD 0 or milk at LD 7. Information on TG content of pig colostrum and milk was not reported by (Elliott *et al.* 1971) and the reasons for differences in milk cholesterol content between our study and the Elliott paper are unknown. This may be due to animal differences, timing of collection and/or methods of lipid analysis. For example, our studies were conducted during the summer of 2013 and the possibility of seasonal differences in pig milk composition should be considered. However, in a study on the effect of season on pig milk composition, (Braude *et al.* 1947) found that milk fat composition in winter and summer was similar.

Maternal ingestion of AminoGut during pregnancy and lactation did not affect lipid content in neonatal porcine adipose tissue depots (subcutaneous and visceral) at

PND 14. (Martin *et al.* 1994) reported that lipids and water account for over 90% of the composition of adipose tissue which agreed with our findings. However, maternal glutamine supplementation during pregnancy and lactation did increase the triglyceride content in the neonatal subcutaneous adipose tissue compared to offspring from the control fed sows. Triglyceride content did not differ between treatment groups in the visceral adipose tissue depot. Overall, triglyceride content was greater in neonatal subcutaneous adipose tissue compared to visceral adipose tissue. These results were unexpected, since triglyceride storage was higher in human visceral fat compared to subcutaneous fat depots (Engfeldt and Arner 1988). The observation that cholesterol content of neonatal subcutaneous fat tissue was 10 fold greater than visceral fat tissue at PND 14 was the biggest difference in fat composition found. The explanation for this substantial difference in cholesterol content of subcutaneous versus visceral adipose tissue is unclear. Cholesterol content was not affected by maternal glutamine supplementation in subcutaneous adipose tissue of the offspring but increased in visceral adipose tissue compared to the offspring from the control feed sows. Studies indicate that there are differences between subcutaneous and visceral adipose tissue depots. While subcutaneous fat is found underneath the skin, visceral adipose tissue is found associated with digestive organs. Morphologically, subcutaneous adipose tissue contains higher numbers of preadipocytes when compared to visceral adipose tissue (Tchkonia *et al.* 2005). Visceral adipose tissue also has a higher rate of apoptosis compared to the subcutaneous adipose depot (Sethi and Hotamisligil 1999, Wajchenberg *et al.* 2002). Researchers have also reported differential gene expression in subcutaneous versus

visceral adipose tissues. Gesta and associates (2006) reported depot-specific differences in gene expression in subcutaneous and visceral adipose tissues in mice and humans.

Though, glutamine and glutamate levels were not examined in the sow's milk or in the blood stream from the neonate, other studies report increased levels of glutamine and glutamate in milk at LD 0 and LD 7 when sows were supplemented with AminoGut prior to parturition and during lactation (Manso *et al.* 2012), Santos de Aquino Unpublished). A study conducted by Cabrera and colleagues (2013) determined pre- and post-weaning growth performance and intestinal health of pigs that were fed creep feed supplemented with AminoGut compared to pigs fed creep feed alone. Results suggested that compared to the control diet, supplementation with AminoGut (1) improved feed conversions (feed/gain) within the first three weeks post weaning; (2) improved pre and post weaning growth performance and intestinal health of pigs; and (3) reduced atrophy in villi found in the small intestine of the neonatal pig (Cabrera *et al.* 2013). This study supports the use of glutamine supplementation in support of intestinal health of pigs and suggests that the increase of glutamine in milk by supplementation may benefit the intestinal health of the neonate.

Among the limitations of the current study was the relatively low sow numbers (n=4) in each treatment group. We were limited by the number of sows available for these experiments on our campus farm. In addition, two AminoGut-fed sows died during this study, one at pregnancy day 107 and the other at lactation day 7 and these sows were replaced with another two sows. Necropsy failed to reveal evidence that death was due to AminoGut supplementation. Glutamine itself is not toxic (Curi *et al.* 2005). Pigs typically consume 4% of their diet as glutamine and glutamate, therefore adding an additional

1.5% represents a minor increase and is seen in variation with foodstuffs from other sources (Watford 2008, Manso *et al.* 2012).

Also, we expected to see treatment differences, even with relatively low sow numbers, based on the report of a 60% increase in the lipid content of colostrum in glutamine supplemented sows (Santos de Aquino, unpublished). Colostrum and milk samples from control and AminoGut-fed sows were submitted to the Lipidomics Core Facility (Rutgers U) for lipidomic analysis that will provide additional information about the lipid profile in response to treatment however; those data are not yet available.

Maternal glutamine supplementation during pregnancy and lactation resulted in a small, but significant, increase in triglyceride content of neonatal subcutaneous adipose tissue at PND 14. However, overall the findings from these studies did not support the hypothesis that supplementation with dietary glutamine and glutamate during sow pregnancy and lactation will increase the lipid content and alter the lipid profile of colostrum, milk and in visceral adipose tissue of the offspring. In addition, in the neonate depot-specific effects were observed in that cholesterol and triglycerides were higher in subcutaneous fat when compared to visceral fat, independent of maternal diet. Future efforts should examine the lipid profile of other neonatal tissues (liver and small intestine) from the offspring of AminoGut supplemented sows. Whether the uptake of glutamine into neonatal tissues occurs following maternal glutamine supplementation should also be monitored. The possibility that adipose tissues from male offspring are affected differently from females should also be considered in future work.

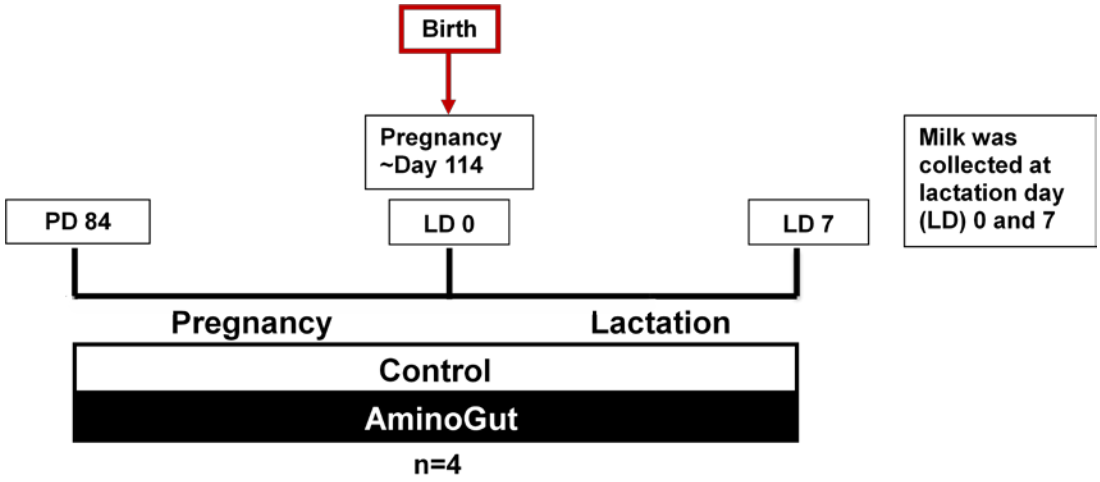

Figure 1. Experimental design – milk studies. Sows at pregnancy day (PD) 84 (30 d prior to expected parturition) were fed either 1) a commercial control diet (C) or 2) a commercial diet supplemented daily with AminoGut (AG; 1.5%) mixed into rations through lactation day (LD) 7. Milk at birth (LD 0) and at LD 7 was collected manually from each sow and frozen at -20°C until subject to testing (n=4/group). To facilitate milk collection at LD 7, sows were treated with oxytocin (20 USP intramuscularly).

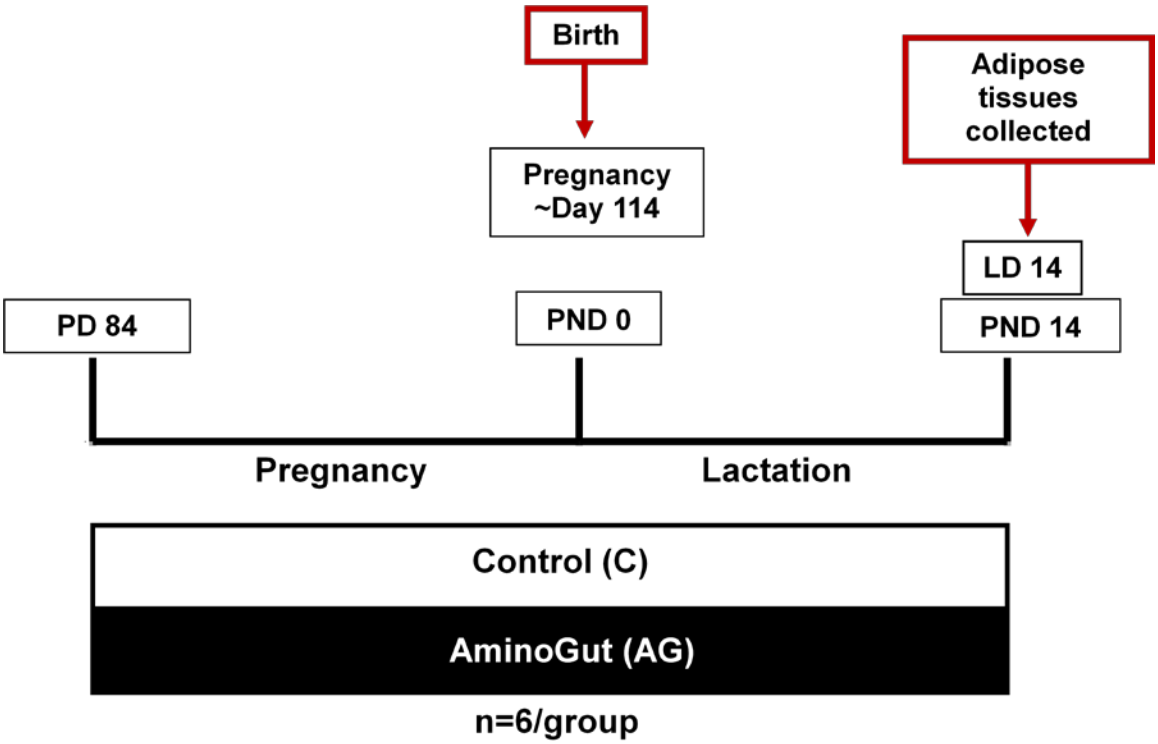

Figure 2. Experimental design – neonatal adipose tissue studies. Sows at pregnancy day (PD) 84 (30 d prior to expected parturition were fed either 1) a commercial control diet (C) or 2) a commercial diet supplemented daily with AminoGut (AG; 1.5%) mixed into rations through lactation day (LD) 14. Neonatal gilts (n=6/group), born from these sows fed either the control diet or AminoGut supplemented diet, nursed *ad libitum* for two weeks from birth [post natal day (PND) 0]. Adipose tissues (visceral fat and subcutaneous fat) were collected on PND 14.

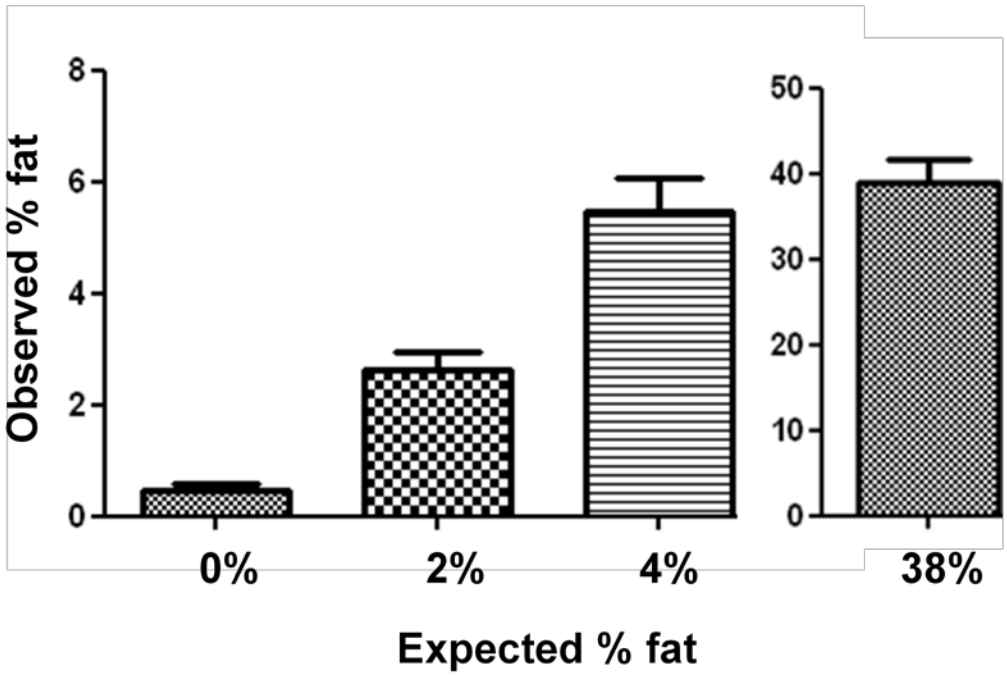

Figure 3. Pilot study - Validating the Folch Method using bovine milk as standards. Commercial bovine milk samples at expected fat concentrations of 0, 2, and 4% milk fat and heavy cream (38% fat) were subjected to Folch extraction. All four forms of milk were extracted in triplicate. Lipid weights were obtained and expressed as observed % fat (mean + SEM).

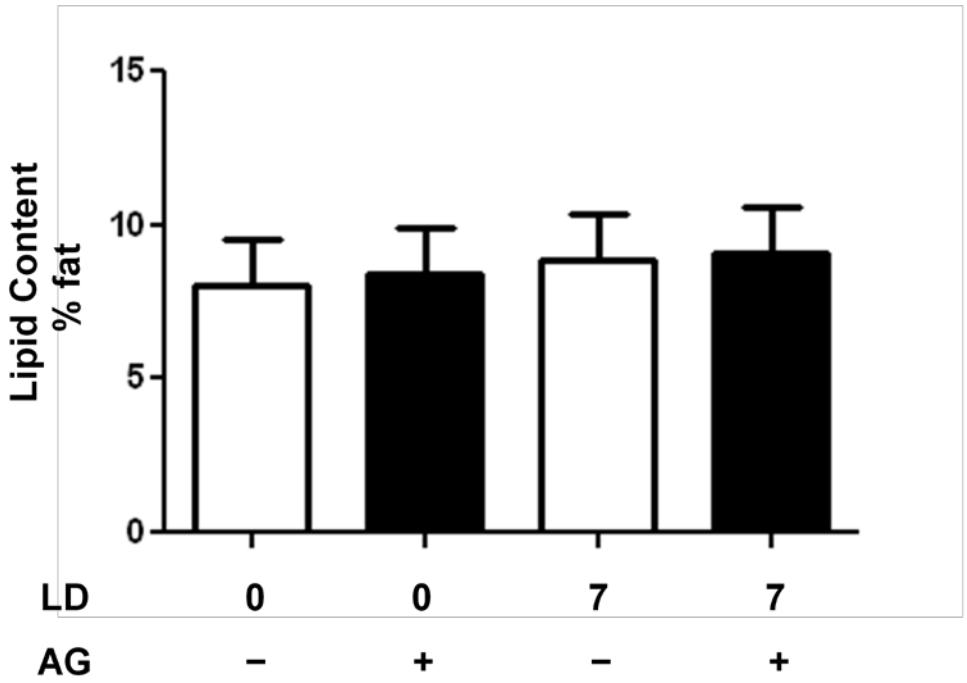

Figure 4a. Effects of maternal ingestion of AminoGut (AG) on lipid content (expressed as % fat) of porcine milk at LD 0 and 7. Results are expressed as LSM ± SEM**.** No differences between control-fed (open bars) and AG-fed (closed bars) sows.

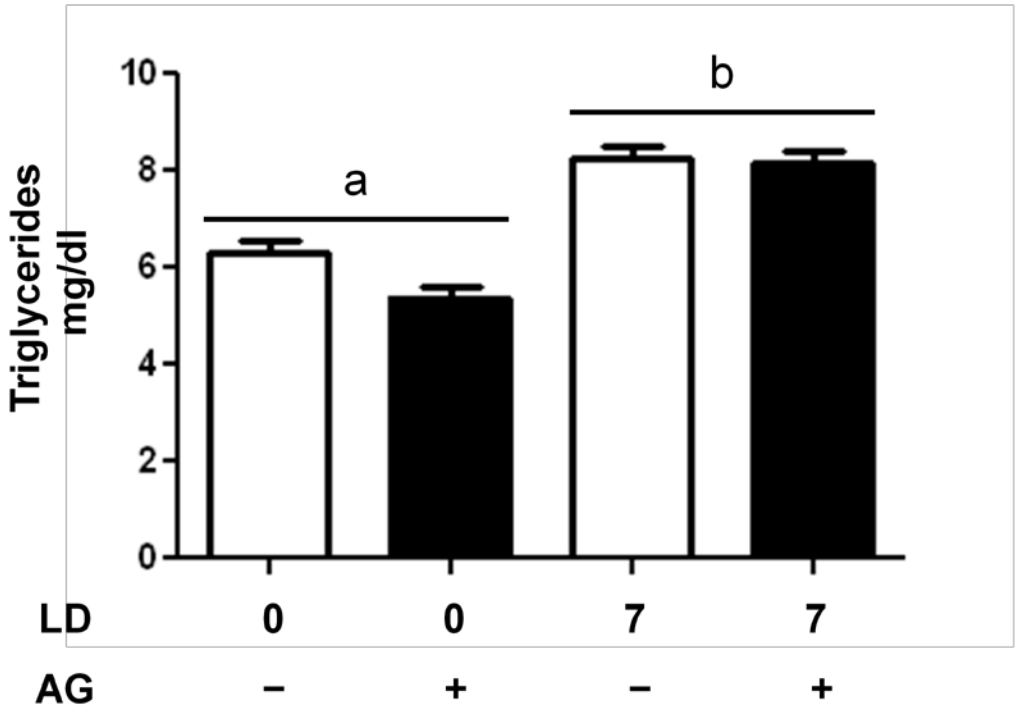

Figure 4b. Effects of maternal ingestion of AminoGut (AG) on triglyceride (TG) content of porcine milk at lactation day LD 0 and LD 7 from control-fed (open bars) and AG-fed (closed bars) sows. Results are expressed as LSM ± SEM**.** Different letters above horizontal lines indicate differences between LD 0 vs. LD 7 $P<0.01$.

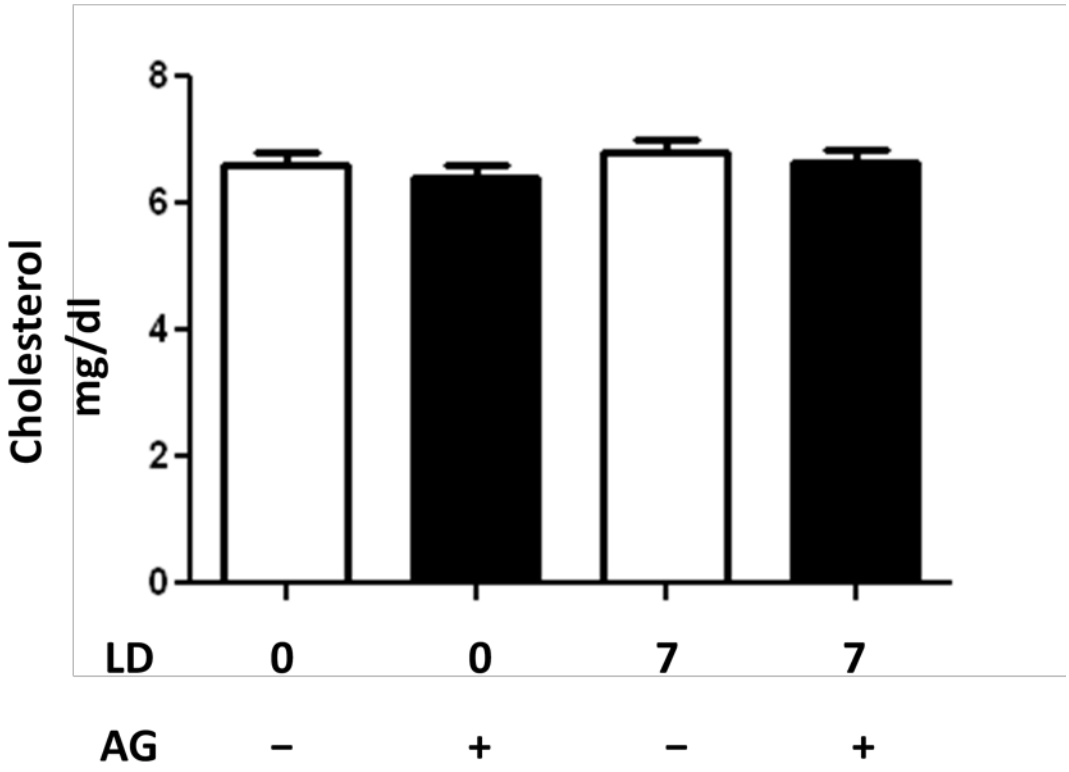

Figure 4c. Effects of maternal ingestion of AminoGut (AG) on cholesterol content of porcine milk at lactation day LD 0 and LD 7. Results are expressed as LSM ± SEM**.** No differences between control-fed (open bars) and AG-fed (closed bars) sows.

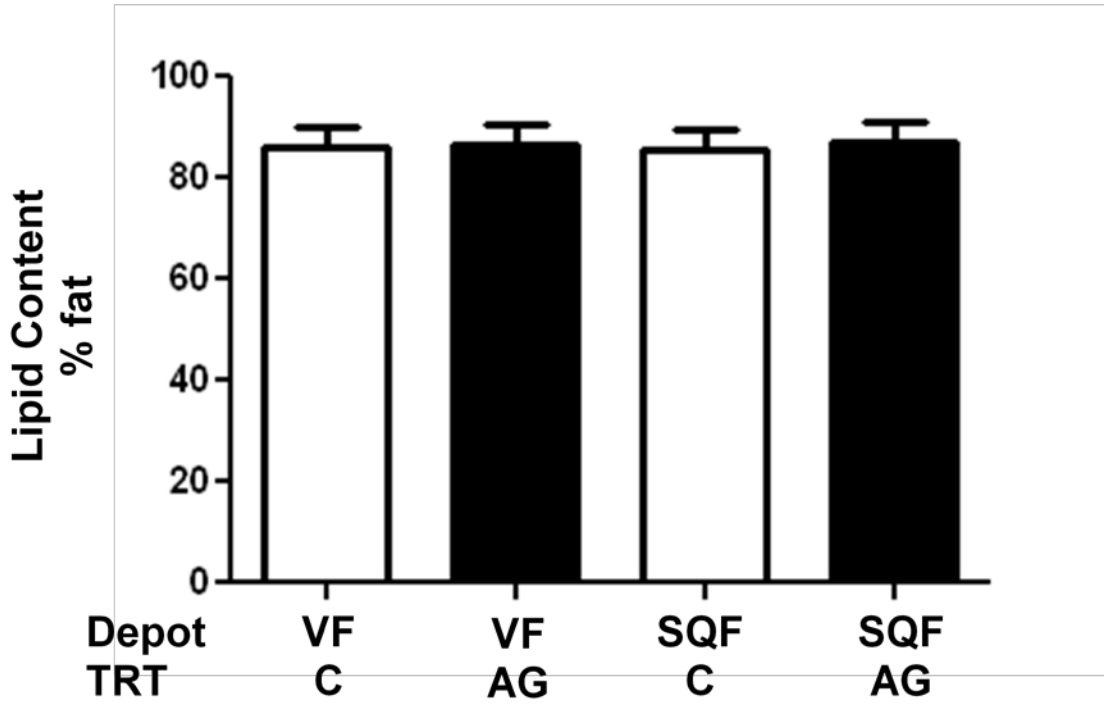

Figure 5a. Effects of maternal ingestion of AminoGut (AG) on lipid content (expressed as % fat) of neonatal porcine visceral fat (VF) and subcutaneous fat (SQF) at PND 14. Results are expressed as LSM ± SEM**.** No differences between neonates from control-fed (C; open bars) and AG-fed (closed bars) sows.

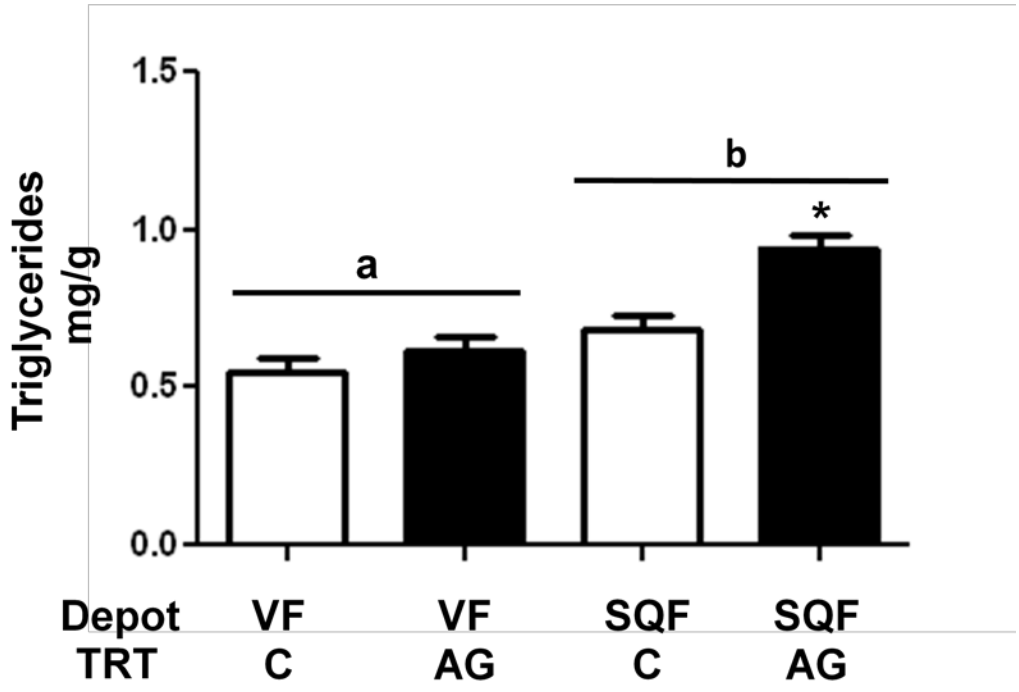

Figure 5b. Effects of maternal ingestion of AminoGut (AG) during late pregnancy and early lactation on triglyceride (TG) content of neonatal porcine visceral fat (VF) and subcutaneous fat (SQF) at PND 14. Results are expressed as LSM ± SEM**.** Different letters above horizontal lines indicate differences between fat depots (VF vs SQF) at $P<0.01$. Asterisk denotes treatment difference ($P<0.01$) between neonates from control-fed (C; open bars) and AG-fed (closed bars) sows.

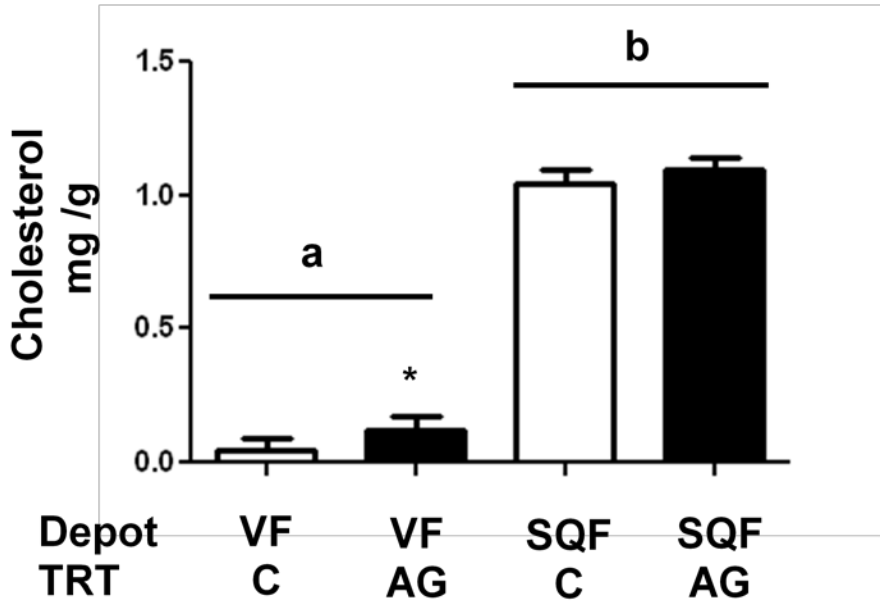

Figure 5c. Effects of maternal ingestion of AminoGut (AG) on cholesterol content of neonatal porcine visceral fat (VF) and subcutaneous fat (SQF) at PND 14. Results are expressed as LSM ± SEM**.** Different letters above horizontal lines indicate differences between fat depots (VF vs SQF) at $P<0.01$. Asterisk denotes treatment difference ($P<0.05$) between neonates from control-fed (C; open bars) and AG-fed (closed bars) sows.

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