Defining the Porcine Colostral Proteome: Changes in the Array of Proteins from Colostrum to Mature Milk

by

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Abstract

The importance of colostrum (first milk) for immunological and nutritional support of newborn mammals is well established. Many bioactive peptides are present in milk at higher concentrations than in maternal circulation. Transmission of such factors from mother to offspring as a specific consequence of nursing is characterized as lactocrine communication. Because milk-borne, lactocrine-acting factors affect patterns of gene expression in neonatal somatic tissues, including the reproductive tract and heart, it is important to understand the biochemical nature of colostrum/milk. Data for relaxin, a prototypical lactocrine-acting factor in porcine colostrum, indicate that transmission of such factors is significant prior to gut closure in the neonatal pig. Additionally, amino acid sequences encoding potentially bioactive peptides are encrypted within porcine milk proteins, raising the possibility that proteolytic cleavage in the gut could release such factors into circulation after consumption. The array of proteins/peptides constituting the porcine colostral proteome has not been defined. Objectives of this study were to employ two-dimensional gel electrophoresis (2DE) and image analysis to: (1) define the porcine colostral proteome on lactation day (LD) 0; and (2) determine if and how this proteome changed from LD 0 to LD 6. Colostrum (LD 0) and milk (LD 6) samples were obtained from six lactating sows. Protein was extracted from individual samples

and total protein concentrations were determined. Extracted proteins were prepared for and subjected to both standard SDS-PAGE (10% total monomer) and 2DE. For 2DE, first dimension separations were carried out using pH 3-10 immobilized pH gradient strips followed by SDS-PAGE using gradient polyacrylamide gels (10-20% total monomer). Individual samples were run on duplicate 2DE gels and stained with Sypro RUBY. Digital images of individual gels were obtained and analyzed using a Typhoon 9400 digital scanner and PDQuest 2-D Analysis Software. Total protein concentrations for colostrum (LD 0) and milk (LD 6) were 8.5 mg/ml and 8.3 mg/ml. Standard SDS-PAGE analyses revealed distinct differences in the distribution of protein bands between LD 0 and LD 6. PDQuest analyses identified consistent qualitative and quantitative differences between colostrum from LD 0 and milk from LD 6. Systematic analyses of the primary amino acid sequences of porcine milk proteins using the BIOPEP program and related database (www.uwm.edu.pl/biochemia/index_en.php) revealed that potentially bioactive peptides are encrypted within porcine milk proteins. Thus, newborn pigs that nurse obtain a complex mixture of proteins and peptides from birth that changes with time during a period of neonatal life recognized to be critical for female reproductive tract development. Gilts deprived of colostrum for two days from birth exhibit altered gene expression patterns essential for normal development of female reproductive tract tissues. The presence of encrypted peptides in porcine colostrum increases the complexity of the porcine milk proteome. These studies provide a framework for future efforts to be aimed at identification of

colostral proteins and peptides that affect lactocrine programming of neonatal development.

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List of Abbreviations

AA	Arachidonic Acid
ACE	Angiotensin-Converting Enzyme
BAP	Bioactive Peptides
BSA	Bovine Serum Albumin
CCP	Casein Phosphopeptide
DHA	Docohexaenoic Acid
ESR1	Estrogen Receptor-α
EV	Estradiol Valerate
FITC-D	Fluorescub Isothiocyanate Labeled Dextran
HMG-CoA	Hydroxylmethylglutaryl Coenzyme A
HSA	Human Serum Albumin
IgA	Immunoglobulin alpha chain
IgG	Immunoglobulin gamma chain
IGF	Insulin-like Growth Factor
IGFBP	Insulin-like Growth Factor Binding Protein
LCPUFA	Long-Chain Polyunsaturated Fatty Acid
MbFs	Milk-borne Factors
miRNA	micro-RNA
MMP	Matrix Metalloprotease

Mya	Million years ago
PND	Post-Natal Day
RLX	Relaxin
RXFP-1	Relaxin Receptor
SSP	Standard Spot Numbers

CHAPTER 1
INTRODUCTION

Lactation is a ubiquitous and critical part of the mammalian reproductive strategy. Milk produced by the mammary gland is generally rich in fats, proteins, water and other nutrients critical for the survival of the newborn [1]. These milk-borne factors are not only nutritionally beneficial; they can also modulate numerous aspects of neonatal development [2, 3]. Additionally, the development of agriculture has enabled humans to consume milk throughout their life span. Thus it becomes necessary to understand the characteristics of lactation and milk, as they are essential parts of the mammalian reproductive strategy and human nutrition. Much of the current research involving milk has focused on the immunological and developmental effects it may have on the neonate, including transfer of antibodies for passive immunity, modulation of gastrointestinal (GI) tract function and other effects on systemic tissues. Neonatal pigs are immunologically naive and their survival rate increases if they are able to nurse and receive critical antibodies to defend against pathogens [4]. Colostrum, or first milk, is particularly high in immunoglobulins and other bioactive proteins produced by the maternal system when the neonatal small intestine is capable of transferring intact macromolecules into circulation. Data for the prototypical milk-borne peptide hormone relaxin indicate that such bioactive factors can affect gene expression in the neonatal porcine uterus, cervix, and other tissues via

a lactocrine mechanism soon after birth [5]. Delay or disruption of this mechanism leads to altered patterns of gene expression with potential for long lasting consequences. Additionally, bioactive peptides encrypted within the primary sequence of bovine milk proteins have been identified and can affect a variety of organ systems once released into circulation [2]. Milk is also a vector for environmental endocrine disruptors entering neonatal circulation, suggesting that lactocrine signaling can also be affected by environmental conditions [6]. The conserved nature of milk and mammary genes suggests that lactocrine signaling is a conserved mechanism that remains incompletely understood. Research described in this thesis was designed to investigate the nature of the porcine colostral proteome and to determine if potentially bioactive peptides are encrypted within porcine milk proteins.

CHAPTER 2
LITERATURE REVIEW

Lactation is a hallmark of mammalian reproductive physiology. Carl Linnaeus referred directly to the mammary gland in the naming of the class '*Mammalia'* as the dominant characteristic by which mammals can be identified despite other major characteristics such as thermoregulation and hair growth [7]. Considerable progress has been made in understanding the physiological and biochemical mechanisms required to support lactation. Following is a review of mammary gland structure and function and a description of the effects of nursing on neonatal development.

1. EVOLUTION OF LACTATION

The pathways leading to the establishment of modern lactation systems are uncertain due to the lack of direct fossil evidence for mammary gland development and reliable living counterparts of early mammals. However, a combination of fossil and molecular evidence indicates that mammals first appeared toward the end of the Triassic period approximately 220 Mya [8] and complex lactation systems were already established in the last common ancestor of all modern mammals in the late Triassic period [9]. This implies that lactation evolved in mammalian precursors of the therapsid and cyanodont lineages concurrently with other hallmarks of mammalian physiology such as endothermy and fur growth. Modern mammals evolved to have unique reproductive strategies that are reflected in their approach to

lactation regarding milk composition and lactation cycles [7, 9]. Nevertheless, genomic analyses revealed the close relationship of mammary and lactation genes between even the most phylogenetically distant mammals, some of which have origins dating as far back as 240 Mya [9, 10]

1.1 ORIGINS OF LACTATION, MAMMARY GLANDS, AND SUCKLING

Lactation involves the coordination of complex physiological, ecological, morphological, and biochemical factors [7]. Modern mammalian neonates are instinctually driven to nurse soon after birth. The mammary gland most likely evolved alongside other hallmarks of mammalian physiology in the therapsid, cyanodont, and mammaliforme lineages of the Triassic period [11]. Histological similarities between mammary glands and skin glands led to several hypotheses postulating that apocrine and sebaceous glands were the precursors to mammary gland development [9, 11]. Blackburn and colleagues suggested the predominant theory that mammary gland formation predated full differentiation of cutaneous glands and may have evolved as a “neomorphic mosaic” involving both cutaneous and apocrine glands [11]. This combination of skin glands may have formed the first primitive mammary gland able to secrete small amounts of organic substances similar to its precursors. Another theory points to evidence that the mammary gland may have evolved from an association between apocrine glands and hair follicles, the remnants of which can be seen in some modern day mammals [9]. In monotremes the mammary gland lacks a nipple and opens directly into the areola in association with a hair follicle [12]. This

relationship is also observed in the ontogeny of the mammary gland of marsupials, where a transient association is observed between the mammary duct and hair follicles. Direct association between mammary gland formation and hair follicles has not been detected in eutherian mammals, but there is active repression of hair follicle formation in the region of the mammary anlagen [13]. Though the exact mechanism of mammary gland evolution is unclear, it likely formed through cooption of existing structures and related biochemical pathways. This theory would explain the heavily conserved nature of milk and mammary genes, reviewed in the next section.

Numerous scenarios have been offered to explain the origins of lactation and suckling behavior. One of the earliest to propose a mechanism for the origin of lactation was Charles Darwin, who proposed that brood pouches used by certain fish to nourish their young might be the intermediate form of a mammary gland and lactation [14]. However, it is now clear that the mammary gland did not evolve from a piscine brood pouch format. Prevention of egg desiccation has been proposed as a theory, but observations on monotreme lactation produced conflicting evidence on whether mammary secretions are necessary to protect eggs from desiccation [7, 15]. Others proposed that apocrine secretions helped egg survivability through thermoregulation by evaporative cooling and heat transfer [16]. Graves and Duval [17] suggested that pheromones of reptilian-like mammals millions of years ago may have had a role in attracting offspring to the mother in order to enhance survival and that these or other secretions could have evolved to have a nutritional component. The

presence of antimicrobial peptides and other immuno-enhancing proteins in milk led some to hypothesize that early lacteal secretions were for the immunological benefit of the neonate [11], similar to amphibian secretion of antimicrobial and antitumor peptides on their skin [18]. Antimicrobial factors may have incorporated lysozyme, α-lactalbumin, and other iron binding peptides that are nearly universal in the milk of modern day mammals [19]. These factors were shown to predate the emergence of lactose as the primary disaccharide in milk, since α-lactalbumin is one of the functional subunits of lactose synthase [19]. Thus lactose production in proto-mammals could only have begun once high levels of α-lactalbumin were present in lacteal secretions. Though the origins of lactation are unclear, it is clear that the first mammal had a complex lactation system and differentiated mammary glands approximately 220 Mya [7].

1.2 CONSERVATION OF MILK AND MAMMARY GENES

The development of gene sequencing technology allowed for comparison of milk and mammary genes from various species [10]. Analysis of bovine milk genes revealed that milk genes coding for immunoglobulin, casein, fibrinogen, and milk fat membrane proteins (MFMPs) tend to be clustered with mammary genes [10]. Co-expressed genes and those that are more phylogenetically conserved are found in paired or triplet clusters across the genome, indicating that they are evolutionarily related [20]. Furthermore, a higher number of bovine milk protein orthologs were found in other mammals than would

normally be expected compared to gene orthologs not involved in lactation. Most of the highly conserved proteins were found in the milk fat membrane proteome, which generally does not contribute more than 5% of total protein in milk [10, 21]. Milk protein gene loss is minimal compared to the rest of the genome, but there are considerable gene copy variations that may contribute to the diversity of milk composition across species [10].

Caseins, a class of major milk proteins, are universally expressed in mammalian milk. Pigs express four different casein genes, including three calcium sensitive caseins and one κ-like casein to aid in the solubilization of other caseins [22]. The ubiquity of casein suggests that the ancestral precursor to casein existed before mammalian radiation over 300 Mya. The predominant theory on the evolution of caseins suggests that it developed alongside other ancient antimicrobial milk proteins such a lactoferrin, lysozyme, and immunoglobulins as a method for protecting the neonate against microbial threats [11]. An evolutionary relationship between caseins was found in the casein gene cluster region, where other genes involved in mineral homeostasis and host defense are also found. These genes are expressed in both mammary and salivary glands, which share developmental and morphological properties [23]. Genomic sequencing of casein genes from various eutherian and non-eutherian mammals revealed a high degree of divergence in coding [10, 22]. Caseins are the most divergent milk-borne proteins with an average pairwise percent identity (calculated from the human casein orthologue against 7 mammalian species) of <54% [10]. Nevertheless, the conserved function of

casein genes across mammals suggests that coding differences in their amino acid sequences does not impair their nutritional and immunological functions. Further analysis of the casein gene clusters showed that the organization and orientation of these genes is similar in placental mammals [22].

2. LACTATION FUNCTIONS

2.1 ONTOGENY OF THE MAMMARY GLAND

Porcine mammary glands undergo a coordinated morphological progression very similar to human mammary gland development [24]. Mammary glands arise from two lateral lines of thickened epidermal tissue known as the mammary ridge on the developing embryo [25]. Cells of the mammary ridge begin to push inwards forming the primary mammary bud. As the embryo develops, the primary mammary bud lengthens and branches out away from the epithelium. Towards the end of gestation these branches canalize to form the lactiferous ducts, which eventually empty to the exterior of the mammary gland [25]. The mammary gland differentiates further during puberty under the influence of estrogen, progesterone, prolactin, and growth hormone [25]. These hormones are responsible for the increased radial growth and branching of the mammary ducts in this period. The basic unit of the mammary gland is the sphere shaped alveolus, composed of a central lumen surrounded by a single layer of secretory epithelial cells and another layer of myoepithelial cells and capillaries. Alveolar formation at the terminal portion of each duct is

upregulated by progesterone produced during the luteal phase of estrous cycles. Complete mammary gland development takes place in the last trimester of pregnancy, specifically between the 75th and 90th day of gestation in gilts [26], characterized by a decrease in alveolar epithelial tissue combined with a proportional reduction in adipose and connective tissues.

2.2 LACTOGENESIS AND REGULATION OF MILK SECRETION

Towards the end of pregnancy, alveoli are separated into lobes that eventually empty into the mammary ducts [25]. The secretory epithelial cells are positioned such that the apical end is positioned next to the lumen and the basal end is separated from blood and lymph by the basement membrane [1]. These cells synthesize milk with components entering from the blood through the basement membrane, packaged by the Golgi apparatus, and excreted via exocytosis through apical membrane into the lumen. Prolactin secretion from the anterior pituitary plays a major role in the onset and maintenance of lactation in the sow [1]. Prolactin concentration in the blood of pregnant sows increases from ~25 ng/ml three days before parturition up to ~ 150 ng/ml on the day of farrowing [27]. When prolactin secretion is inhibited by bromocryptine during late pregnancy, plasma prolactin concentrations fall below 2 ng/ml and the onset of lactogenesis is delayed [28]. Lactogenesis is characterized by two phases in the pig. The first stage is the gradual accumulation of colostrum between the 90th and 105th day of gestation [26]. The second stage is characterized by copious milk secretion associated with

increasing lactose synthesis by the mammary gland. This results in an increased lactose concentration in milk and maternal blood which peaks during the hours prior to parturition [29].

Milk ejection or 'milk letdown' is primarily a neuroendocrine reflex involving both sensory neurons in the teats as wells as nontactile stimulation such as the sight and sound of the neonate. Teat stimulation causes a nerve impulse to travel from the nipple to the hypothalamus where it stimulates oxytocin synthesizing neurons of the paraventricular and supraoptic nuclei. Oxytocin is released into the bloodstream where it can bind to oxytocin receptors on the surface of myoepithelial cells surrounding the alveoli. These cells contract and force the milk stored in the lumen of the alveoli into the larger ducts closer to the teat where a suckling piglet has access [1, 25].

2.3. ABSORPTION OF INTACT MACROMOLECULES

The ability of neonatal intestinal cells to absorb whole macromolecules and transport them intact across the epithelium into circulation is a unique characteristic of intestinal development in farm animals, including the neonatal pig [30]. Though the exact time the porcine gut is open to macromolecule absorption varies, intestinal closure begins 6-12 hours after colostrum ingestion and progresses to completion by 24-36 hours[25, 30]. Such transfer of macromolecules facilitates the uptake of proteins such as immunoglobulins, growth factors, and bioactive compounds found in milk necessary for proper neonatal development. Transport involves enterocytes and M cells involved in

non-receptor passage of intact macromolecules [31]. More broadly, the transfer process can follow two pathways: 1) specific receptor mediated transocytosis and 2) nonspecific transocytosis mediated by nonselective vesicular transport [31]. Non-specific macromolecule absorption is significant for the first two days in neonatal ungulates, where immunoglobulins compete with other proteins for absorption. This mechanism is facilitated by decreased proteolytic degradation due to the presence of colostral protease inhibitors as well as low pancreatic and intestinal enzyme activities [31, 32]. Gut closure occurs when the intestinal epithelium matures in conjunction with increased proteolytic degradation within the intestinal lumen.

There are several factors influencing the length of time that the porcine intestinal epithelium remains open to macromolecule absorption. Until recently, the predominant view was that intestinal uptake capacity is due primarily to an immature 'leaky gut' that allows transport of all molecules [33]. More recent evidence suggests that signals inducing gut closure involve colostral and systemic factors that influence the rate of maturation of intestinal epithelium [34]. Additionally, diet and conditions of parturition have been inplicated as modulators of gut closure. Multiple studies showed that artificial rearing of newborn pigs with hormone-free milk replacer decreased capacity for whole macromolecule absorption [34, 35]. A study conducted by Jensen and colleagues [35] showed that neonatal pigs have a reduced capicity to absorb bovine and human serum albumin and IgG if they are fed milk replacer or bovine colostrum [35]. Piglets maintained on mik replacer also exhibited delayed gut closure.

Thus, milk replacer is adequate to induce normal intestinal growth [31, 36]. Collectively, data support the idea that there are factors in colostrum necessary to support normal patterns of macromolecule absorption across the neonatal gut.

Several systemic factors have been implicated in regulation of gut closure. Cortisol was shown to stimulate macromolecule transport across the newborn small intestine in farm animals [34]. Interestingly the neonatal porcine ileum expresses high numbers of glucocorticoid receptors during the suckling period that decrease to normal adult levels during weaning [37]. The presence of glucocorticoid receptors in the immature small intestine may have regulatory effects on intestinal transport of ions, amino acids, carbohydrates, lipids, and proteins [31]. Additionally, intestinal closure at 18 hours after birth is associated with an increase in serum immunoreactive insulin levels, an effect absent in fasted piglets. Exogenous insulin injections decreased bovine serum albumin (BSA) and fluorescein isothiocyanate labeled dextran 70,000 absorption at 12 after birth [38]. Since insulin is produced in response to nursing, colostrum may be at least partly responsible for initiating events leading to gut closure.

3. COMPOSITION OF PORCINE COLOSTRUM/MILK

The first studies on porcine milk composition were published in 1865 by von Gohren [3]. Porcine milk is a complex fluid composed of numerous constituents including proteins, carbohydrates, lipids, steroids, vitamins, minerals, nitrogen compounds, and cells [1]. Characteristics of mammalian milk, depend on the stage of lactation from which is it is obtained. Production of colostrum, or first milk, occurs during the periparturient period and ceases between 24 and 48 hours after parturition in sows [3, 25]. Mature milk is synthesized thereafter and production continues until weaning. Porcine colostrum differs in protein, carbohydrate, and lipid composition, though the most significant difference occurs in total protein and protein composition [1, 3, 39].

3.1 PROTEINS

Total protein content varies between breeds, but porcine colostrum can contain up to three times more proteins than mature milk [40]. Total protein declines rapidly after parturition, with protein content decreasing by 50% in the first 12 hours after the first piglet is born [3]. Major proteins in colostrum and milk are classified as either caseins or whey proteins, depending on their physical and chemical properties. Caseins make up about 8.8% of nitrogen-containing compounds in colostrum, but increase to 47.3% of the nitrogen-containing compounds in mature milk. Whey proteins make up 91.0 % of

nitrogen-containing compounds in colostrum, and about 52.6 % of these compounds in mature milk [3]. Caseins, such as α-, β-, γ-, and κ- variants are suspended in milk in the form of micelles. They are an important source of amino acids and calcium for suckling piglets, though they also have other bioactivities. Additionally, bioactive peptides encoded within these (and other) milk proteins have the potential to influence the function of the GI tract and other organ systems [1, 22]. Whey proteins include serum albumin, α-lactalbumin, β-lactoglobulin, immunoglobulins, growth factors and many other peptides with variable bioactivities [1]. Of the proteins found in colostrum, more than 90% are immunoglobulins that provide passive immunity for newborn piglets. Immunoglobulin concentrations decrease in mature milk, though secretory IgA levels remain high relative to other body fluids [3].

3.1.1 ANTI-INFECTION AGENTS IN PORCINE MILK

Porcine colostrum and milk contain a large number of defense factors including immunoglobulins, lactoferrin, lysozymes, lactoperoxidases, and leukocytes. These factors are crucial for developing the passive immunity in porcine neonates. Neonatal vitality has a positive correlation with the degree of passive immunization and circulating anti-infection agents. The most important of these is IgG, which constitutes a key element in host defense against pathogens [34]. The porcine fetus is well protected from antigens by the placenta. However, the neonatal porcine immune system is immature at birth, offering little resistance to pathogens. Unlike humans, pigs do not transfer

immunoglobulins through the placenta prior to parturition, thus the piglet is dependent on intestinal absorption of immunoglobulins and other immune-enhancing factors that exist in colostrum for their passive immunity [30].

There are three types of immunoglobulins in porcine colostrum; IgG, IgA, and IgM [1, 39]. The predominant immunoglobulin in colostrum, IgG is absorbed into the neonatal circulation where it provides passive protection against immune challenges. Some mammals exhibit intestinal selectivity to IgG because of the similar concentrations of IgG in colostrum and within the plasma of piglets allowed to nurse. [34]. The intestinal Fc receptor in the rodent was implicated in regulating IgG absorption in the suckling rat, however this receptor has yet to be identified in the neonatal pig [34, 41]. By the third day of lactation IgA becomes the predominant immunoglobulin in colostrum/milk. It is found in the form of secretory IgA in milk, which combines IgA with a glycoprotein. Secretory IgA functions primarily in the intestinal lumen where it blocks adhesion of pathogens and toxins to the epithelial surface. The largest immunoglobulin, IgM is detectable in low concentrations in milk throughout lactation. It is thought to serve a function similar to that of IgA in protection of the intestinal lumen [42].

Lactoferrin is an iron binding glycoprotein expressed in most biological fluids and is an important part of the immune system. Milk-borne lactoferrin, identified in 1960, is synthesized by mammary epithelial cells [43]. Lactoferrin concentrations are higher in porcine colostrum than in milk by four weeks after parturition [44]. Lactoferrin was shown to protect the body against bacteria, viruses, fungi, inflammation and even cancer [43]. This milk protein functions by

binding free iron, thereby competing with pathogens to limit their growth. It also binds directly to and destabilizes bacterial and fungal membranes and activates the host immune cells through nuclear activation pathways [43, 45, 46]. Lactoferrin contains an abundance of bioactive peptides within its primary sequence that may have higher antimicrobial capabilities than the parent protein [47]. These bioactive peptides are released and activated after proteolytic degradation.

Lysozyme, one of the most intensively studied antibacterial milk proteins, is also found in mucus, tears, saliva, and cytoplasmic granules [44]. Found ubiquitously, it has a close structural relationship with α-lactalbumin [10]. Lysozyme functions by binding to peptidoglycans in bacterial cell walls and hydrolyzing the glycosidic bond that connects *N*-acetylnuramic acid with the fourth carbon atom of N-acetylglucosamine [48]. It is stable in acid and trypsin solutions and is considered an important part of GI defense in suckling neonates. However, its role in the pig has yet to be determined as porcine milk-borne lysozyme has not been identified[1]. Lactoperoxidase is another common bacteriostatic factor found in milk, tears, and saliva. It catalyzes the oxidation of certain molecules with hydrogen peroxide to generate reactive products with a wide range of antimicrobial activity [49, 50]. Lactoperoxidase activity in porcine milk was detected in both colostrum and milk 36 hours after parturition[49]. Though the importance of this enzyme in neonatal porcine GI defense is unclear, lactoperoxidase is important in protecting the newborn piglet against reactive

oxygen species created during parturition and subsequent adaptation to living outside the womb [50].

3.1.2 GROWTH FACTORS AND PEPTIDE HORMONES

Growth factors are a heterogeneous group of proteins that promote cellular growth, differentiation, and may act in a similar manner to classic endocrine hormones in circulation [51]. The ability of porcine milk and, to a greater extent, colostrum to stimulate gastrointestinal DNA and protein synthesis in the neonate is recognized. Colostrum-fed piglets have greater protein synthesis in liver, kidney, spleen and skeletal muscle compared to those fed mature milk. This indicates that colostral factors affect the growth of systemic tissues [52]. Porcine colostrum and milk contains epidermal growth factor (EGF), insulin, insulin-like growth factor I (IGF-I), insulin-like growth factor II (IGF-II), IGF binding proteins, transforming growth factor-α (TFG-α), transforming growth factor-β 1 and 2 (TGF-B1 & B2), relaxin, growth hormone, prolactin, and numerous other peptide growth factors [51, 53]. Colostrum contains a greater concentration of these growth factors compared to mature milk and was shown to play an important role in support of growth and development of the neonatal piglet [53].

The EGF family of growth factors includes EGF, TGF-α, and TGF-β as the three major proteins having a role in development of neonatal mammals [54]. EGF is a single chain polypeptide of approximately 6 kDa. Porcine colostrum was reported to contain up to 1500 ng/ml of EGF, with concentrations declining

to 150-250 ng/ml nine days after parturition [1, 53]. Additionally, EGF receptors were identified in the villi and crypts of the neonatal porcine small intestine [53]. Data for mice suggest that EGF has a role in the regulation of GI tract development. The influence of EGF on porcine systemic tissues has yet to be defined. Data for the mouse indicate that EGF conveys important regulatory signals affecting development of the liver, pancreas and pulmonary system [54]. TGF-β is a dimeric protein of 25 kDa. Three isoforms were identified in porcine colostrum and milk, with concentrations in colostrum being higher than those observed for milk [1]. Recent studies in mice suggest that TGF-β is also involved in intestinal, hepatic, cardiac, and pulmonary system development[1, 54]. However, the physiological function of milk-borne TGF-β is not well understood in pigs. TGF-α is found in low levels in both porcine colostrum and milk and can stimulate mitotic events through the EGF receptor [51]. The most likely role for TGF-α in milk is as a local regulator for GI function and repair [51].

The insulin-like family of peptides is composed of four members (insulin, relaxin, IGF-I, and IGF-II). These peptides are very closely related, with the IGFs sharing 70% amino acid homology to proinsulin and 40% to insulin [1, 51]. The primary structures of these proteins are heavily conserved across species; IGF-I and IGF-II are identical in humans, pigs, and cattle [1]. Porcine colostrum contains all members in high concentration with comparatively reduced levels found in mature milk [1, 51, 53]. Insulin, IGF-I, and IGF-II receptors were identified in the small intestine. Though relaxin is primarily known for its role in

cervical softening prior to parturition, it also promotes mammary gland development and is involved in uterine morphogenesis in the pig [5, 51, 55].

Milk-borne insulin is absorbed from the neonatal GI tract in a biologically active form. Oral administration of pharmacological levels of insulin to the suckling rat and pig results in hypoglycemia, indicating that insulin is absorbed intact and retains its biological activity [51]. However, studies in pigs showed that insulin can also survive in the lumen of the GI tract and subsequently act on receptors there [56]. Newborn piglets bottle-fed formula containing 60 unit/l of insulin had higher levels of brush border enzyme activities than those fed formula alone [57]. Svendsen and colleagues [38] determined that milk-borne insulin may be a player in the timing of gut closure of neonatal piglets, as those piglets given exogenous insulin had a 70% reduction of macromolecular transport. They hypothesized that insulin changes enterocyte basement membrane proteoglycan synthesis, which enhances gut closure.

IGF-I and IGF II are similar peptides with a molecular weight of approximately 7.5 kDa [1]. They exist in milk in association with specific high molecular weight binding proteins called IGF binding proteins (IGFBPs). IGFBPs modulate the ability of IGFs to interact with target tissues and provide the IGFs with protection from proteolytic degradation[53]. Porcine colostrum can contain 500-fold higher levels of IGF-I and IGF-II compared to mature milk. [58]. IGFs and IGFBPs are very stable in acidic environments. Also, type I and II receptors were identified in both mucosal and serosal surfaces of the small intestine where they stimulate GI cell proliferation. There is relatively low absorption of ingested

IGF-I or Long-R^3-IGF-I in piglets [53, 58], though several investigators dispute this assessment [56, 59]. A study conducted by Xu *et al* [59] indicated that labeled IGF-I administered via orogastric tube represents 20% of serum IGF-I in newborn piglets and 10% in three day old piglets, implying that IGF-I absorption can occur after gut closure.

Relaxin (RLX) is a 6 kDa peptide hormone similar in structure to insulin and has been detected in human, canine, rat, bovine, and porcine milk [51, 55]. The concentration of RLX in milk over time is species specific. Milk-borne human RLX concentrations are low early in lactation, increasing as milk matures [60]. Conversely, RLX concentrations in porcine colostrum are high but drop after the second day of lactation [61]. The role of RLX and other milk-borne bioactive factors (MbFs) on neonatal development has only recently been explored in the pig. Ingested RLX is absorbed into the neonatal circulation of dogs and pigs where it can affect downstream targets [61, 62]. Evidence for the pig shows that the neonatal uterus is RLX receptor (RXFP1) positive at birth and that RLX is important for the proper development of the porcine female reproductive tract (FRT) [5, 63]. A more expansive review of porcine MbFs and their effects on mammalian neonates is presented below.

3.2 CARBOHYDRATES & LIPIDS

Several reviews described the relative nutrient composition of porcine colostrum and milk [1, 3, 64]. Colostrum has a high concentration of total solids including proteins and a low concentration of carbohydrates, lipids, and ash. The

transition from colostrum to milk is marked by a decline in total protein (15.7-6.4%) with a simultaneous increase in lactose (3.1-5.5%) and fat (5.0-13.0%) [3]. Lactose is the predominant carbohydrate in porcine colostrum and milk [1]. It is produced by mammary epithelial cells and secreted actively into the alveolar lumen. Lactose is the major carbohydrate in milk and is one of the factors that determines milk volume [64]. Lactase readily hydrolyzes lactose in the neonatal porcine small intestine. Additionally, milk contains smaller quantities of nucleotide sugars, glycolipids, glycoproteins, oligosaccharides, and monosaccharides [1]. Some milk-borne oligosaccharides have protective effects against pathogenic bacteria in the intestinal lumen while promoting the growth of beneficial bacteria.

Porcine milk fat is mainly composed of triglycerides, though there are smaller quantities of phospholipids, glycolipids, cholesterols, fat-soluble vitamins, and free fatty acids [1]. The newborn piglet does not metabolize free fatty acids very well on its own because tissue and liver concentrations of carnitine are low. Carnitine is a compound that is responsible for transferring free fatty acids into the mitochondrial membrane, thus playing an important role in fat metabolism. However, Kerner *et al* [65] showed that colostrum contains a high concentration (370 nmol/ml) of carnitine while serum levels of the compound increase dramatically after two days of suckling. They concluded that colostrum is the primary source for carnitine, suggesting lactocrine regulation of neonatal fat metabolism.

3.3 microRNAs

The first microRNA (miRNA) was discovered in 1993 in nematode worms [66]. The recent development of deep sequencing technologies accelerated the number of miRNAs discovered in multiple species. These small, bioactive RNA molecules display regulatory functions including cell differentiation, developmental timing, apoptosis, cell proliferation, metabolism, transposon silencing, and immunity [66]. miRNAs are 20-30 nucleotides in length that target mRNA, thus they are post-transcriptional regulators. They function as guide molecules for mRNA by binding to untranslated 3' region of target RNAs, which typically leads to regression and exonucleic mRNA decay. Other types of regulation such as transcriptional activation and heterochromatin formation may also be important [66]. Like most other types of RNA, miRNAs are transcribed by RNA polymerase II, though a smaller subclass of miRNAs are transcribed exclusively by RNA polymerase III [67]. Although individual miRNAs repress their targets only moderately, miRNAs can have broad effects because each can have multiple targets [66].

A recent study identified miRNAs in human breast milk [68]. The highest concentrations of miRNAs were found between days 4 and 240 of lactation with most relating to immune function. *In vitro* observations of milk-borne miRNAs indicate that these molecules are resistant to RNases , may be encapsulated in microvesicles and are resistant to harsh conditions [68]. This suggests that they can survive in the neonatal intestinal lumen and could be absorbed into circulation, affecting downstream targets. miRNAs in humans were shown to

increase T-cell numbers and B-cell differentiation. miRNAs may also serve as a vehicle for transferring genetic material from mother to offspring. It was estimated that 1.3 X 10^7 copies/liter/day of mR181a, an immunomodulating miRNA, are transferred from mother to infant [68]. There were also hundreds of miRNAs found in breast milk with undetermined function. While the jury is still out on this new class of potential MbFs, evidence of the presence of miRNAs in milk opens the possibility that milk-borne miRNAs may have significant impact on the growth and development of the neonate.

3.4 STEROID HORMONES

Several different steroid hormones are present in the milk and colostrum of a wide variety of mammals. Bovine milk contains testosterone, progesterone, cortisol, and several different types of estrogens including 17-β estradiol [69]. All were shown to consist of 58-92% conjugated (inactive) forms, which render them more easily water soluble for excretion. Conjugated steroids can become activated upon exposure to bacterial sulfatases or glucouronidases in the GI tract [53]. Data for steroids in porcine milk is less abundant. Farmer *et al* [70] reported that porcine colostrum contains almost 15 ng/ml of estrone, decreasing to ~2 ng/ml by 30 hours after parturition. Since non-conjugated steroids can pass through biological membranes, it is reasonable to assume that estrogens in milk arise from the ovary. However, a recent study showed that ovariectomized sows can secrete 17-β estradiol into circulation from their mammary glands [71]. It has not been determined whether 17-β estradiol produced by the

porcine mammary gland is secreted into milk, though the diffusible nature of steroids makes this scenario likely.

The roles of milk-borne estrogens have yet to be fully understood. A positive correlation exists between estrogen levels in neonatal circulation and survival [70]. Estrogen receptor (ER) expression and activation in the neonatal porcine uterus is required for adenogenesis and uterine maturation [72]. Administration of 17-β estradiol-valerate (EV) to neonatal gilts promoted uterine gland genesis by postnatal day (PND) 14 while administration of the specific antiestrogen ICI 182,780 retarded uterine wall development and inhibited gland formation [72]. Uterine ER is activated by MbFs [55], a mechanism that will be reviewed in the next section. Nevertheless, uterotrophic effects of EV on the neonatal uterus were ultimately detrimental to uterine development and functional uterine capacity. Adult, primiparous gilts exposed to EV for two weeks from birth exhibited abnormal responses to signals associated with the periattachment phase of early pregnancy and displayed: 1) reduced uterine fluid protein content; 2) abnormal uterine growth response to early pregnancy; and 3) altered endometrial gene expression patterns compared to unexposed controls [73, 74]. In humans, estrogenic endocrine disruptors found in the environment can be detrimental to the health and development of the infant, and evidence indicates that these disruptors can enter neonatal circulation by ingestion of compromised milk from the mother [75]

3.4.1 ENDOCRINE DISRUPTORS

In recent years there has been considerable interest on environmental endocrine disruptors (EDs) and their effects on humans, domestic animals, and wildlife [75]. The Environmental Protection Agency defines EDs as "exogenous agents that interfere with the production, release, transport, metabolism, binding, action, or elimination of natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental processes" [6]. These compounds are diverse in their structure and can be found in water, soil, air, food, household products, and packing material [75]. Common endocrine disruptors include bisphenol A (BPA) and other phthalates, paraben compounds, polychlorinated biphenols, pesticides, herbicides, heavy metals, and many others [6]. Particular attention has been paid to estrogenic EDs that mimic the effects of endogenous estrogen once they are in circulation. Biologically active xenoestrogens can freely cross cellular plasma membranes and target cytoplasmic estrogen receptors, ERα (ESR1) and ERβ (ESR2), where they can induce transcription [75]. Exposure to these xenoestrogens may have significant consequences in reproductive growth and sexual differentiation.

Milk is well recognized as a potential sink for toxic substances and endocrine disruptors [75]. Adipose tissue is a reservoir for lipophilic EDs such as xenoestrogens and fat deposits in breast tissue were used to monitor ED levels in peripheral adipose tissue during pregnancy and lactation. Not surprisingly, multiple xenoestrogenic compounds are found in milk since these compounds are able to diffuse into the alveolar lumen from circulation [75].

Zearalenone (ZEA), an estrogenic mycotoxin produced predominantly by fungi in the genus *Fusarium,* is the most common ED affecting swine [76]. Fungi producing ZEA contaminate maize and other cereals in the field, but it can also grow after harvesting if the cereals are not handled correctly. Pigs can absorb 80-85% of an oral ZEA dose and are very efficient in internalizing ZEA from the GI tract [77]. Once ingested, intestinal epithelial cells degrade ZEA into its metabolites α-zearalenol (α-ZEA) and β-zearalenol (β-ZEA). Malekinejad *et al* [69] showed that pigs produce mainly α-ZEA upon oral ingestion of ZEA. Reduced forms of ZEA display increased estrogenic activity compared to the parent compound, since they can bind to both ESR1 and ESR2 competitively [75]. ZEA and its metabolites were reported in porcine urine, plasma, and milk, though concentrations in porcine milk were much lower when compared to intrauterine concentrations of ZEA [78, 79]. Therefore, signs of hyperestrogenism associated with ZEA exposure in the neonatal pig were mainly attributed to prenatal rather than postnatal exposure through milk.

Pigs and sheep are very sensitive to the reproductive and developmental effects of ZEA. It reduces embryonic survival, fetal weight, and is known to decrease luteinizing hormone (LH) and progesterone secretion as well as altering uterine morphology [76]. Chen *et al* [80] reported that prenatal and postnatal ZEA exposure affected uterine gene expression in the neonatal pig [80]. Pregnant sows were fed 1500 μg ZEA/kg of feed/day for 14 days prior to parturition and 21 days afterwards. Gilts were cross-fostered to obtain four groups; unexposed controls, prenatal exposure, postnatal exposure, and

continuous exposure. Results indicated that continuous ZEA exposure decreased *RXFP1, RXFP2, ER-α, Wnt7a,* and *Hoxa10* uterine transcripts while postnatal exposure only decreased *RXFP1* transcripts in the piglets[80]. Thus, neonatal exposure to estrogen or estrogen-like compounds can alter the uterine developmental program [5, 81].

4. ENCRYPTED BIOACTIVE PEPTIDES

Milk exhibits a wide range of biological activities that can influence neonatal digestion, metabolism, immunity, and development. Most of this activity is due to the hormones, proteins and peptides synthesized by the mother, deposited in colostrum/milk and consumed by the newborn. However, it is possible that some bioactive peptides communicated via a lactocrine mechanism from mother to offspring in milk have latent activity, becoming active only after proteolytic digestion of the parent protein while in the neonatal GI tract or circulation [82]. Peptides in this category are likely to be 'encrypted' within the primary amino acid sequence of parent proteins. Such encrypted peptides can be multifunctional, meaning that specific peptide sequences have two or more biological activities with their expression depending on degree of proteolysis [82, 83]. Logically, the two biggest contributors to milk proteins, caseins and whey proteins, would be expected to contain the highest number of encrypted, potentially bioactive peptides.

Proteolytic activation of encrypted peptides can be achieved through two

mechanisms. Most of the known milk-borne bioactive peptides were identified by hydrolyzing the parent protein with pancreatic enzymes such as trypsin, chymotrypsin, carboxypeptidases, and aminopeptidases [83]. Encrypted peptides have also been released using pepsin, thermolysin, and proline-specific peptidases [84]. However, the neonatal pig does not have high concentrations of proteolytic enzymes due to the immaturity of the GI tract and pancreas. This suggests the likelihood of encrypted peptide release in the GI tract to be low [35]. However, naturally occurring bacteria in the small intestine can produce bioactive peptides from milk-borne proteins. Yamamoto *et al* [85] found that *Lactobacillus helveticus* produces a serine-type proteinase that can bind to β-casein and produce several casokinins [85]. Casokinins are ACE-inhibitory peptides derived from α- and β-caseins [2, 85]. Many different bacterial proteinases with specificities to the different caseins found in milk are known. Not surprisingly, specific cleavage cites to bacterial proteinases have been identified in αS1-casein, αS2-casein, β-casein, and κ-caseins [86].

Milk-borne encrypted peptides identified in bovine and human milk can be divided into four groups, including those affecting gastrointestinal function, modulation of postprandial metabolism, antimicrobial defense, and immunoregulation [2]. These peptides can interact with target receptors in the intestinal lumen or may be absorbed and potentially reach target sites via the circulation [2]. Among the most abundant of milk peptides with latent activity are the opioid peptides and their antagonists, both mainly affecting GI function in the neonate. Opioid agonists are called casomorphins and exorphins for their

ability to bind to opioid receptors. These peptides are encrypted within αS1-casein, β-casein, and most of the major whey proteins [87]. In contrast, opioid antagonists, known as casoxins and lactoferroxins, are derived from κ-casein and lactoferrin respectively [84]. Casomorphins/exorphins bind to opioid receptors and decrease intestinal mobility while increasing amino acid and electrolyte uptake in the calf, while casoxins/lactoferroxins have the opposite effect [84, 87]. Presence of bioactive peptides encrypted within milk proteins has enormous implications concerning the extent of influence that milk-borne bioactive peptides have on intestinal and systemic health. Colostrum has higher concentrations of αs1-casein and β-casein compared to κ-casein and lactoferrin [87], implying that the number of opioid agonist peptides out numbers opioid antagonists . This may be important, as the decrease in intestinal mobility combined with higher amino acid uptake that such signaling might effect would give more time for colostral peptides and growth factors to influence GI development and get absorbed into circulation. Opioid agonists can influence postprandial metabolism in canines by stimulating the secretion of insulin and somatostatin once inside circulation [88, 89]. Antimicrobial bioactive peptides can be found within whey proteins casein micelles [47]. The most common is lactoferricin, found after hydrolyzing lactoferrin with pepsin, though others were identified after hydrolysis with different GI enzymes. Lactoferricin has activity against a broad spectrum of Gram-positive and negative bacteria. [47]. Antimicrobial peptide fragments can also come from all the different caseins, though αS1-casein is the primary source [90]. Immunomodulatory peptides can

enhance the immune response by stimulating lymphocyte proliferation and interleukin production [91].

The mechanism by which these peptides exert their effects is undefined, though it was hypothesized that they act through opiate receptors found on the surface of lymphocytes and macrophages [2]. Although direct studies aimed at identifying encrypted peptides in porcine milk are lacking, the idea that encrypted peptides affect the nursing piglet is believable considering the conserved nature of milk and mammary genes.

Some milk-borne proteins contain angiotensin I-converting enzyme (ACE) inhibitory peptides within their sequence [2, 83, 84]. ACE is predominantly found in membrane bound vascular endothelial cells, neuroepithelial cells, and solubilized in the blood. Its main function is to cleave the C-terminal dipeptide from angiotensin I to ultimately form angiotensin II, a powerful vasoconstrictor [92]. ACE inhibitory peptides in milk are called casokinins and lactokinins depending on whether the parent proteins are α/β-caseins or α-lactalbumin/β-lactoglobulin respectively [2]. These peptides lower blood pressure by competing with angiotensin I for the active site on the enzyme. Over 50 different ACE inhibitory peptides were identified *in vitro* by hydrolysis of milk proteins using proteases and bacterial fermentation [92]. However, some of the larger peptides have no effect *in vivo*, most likely because they are hydrolyzed to smaller inactive fragments in the intestine before they are able to pass into circulation.

Mineral binding properties of casein are well described. The high

concentration of calcium in milk has long been credited to the presence of phosphorylated serine and glutamyl residue clusters in casein that are able to bind other minerals present in milk such as zinc and iron [2]. Tryptic digestion of caseins yields caseinphosphopeptides (CCPs) containing clusters of phosporylated serine and glutamyl residues, thus retaining the mineral binding capabilities of the intact casein molecule [2, 90]. Studies showed that CCPs in milk can increase the availability of calcium, iron, magnesium and zinc to the neonate [93, 94]. Though milk-borne zinc concentrations are rarely deficient in porcine milk [1], zinc levels in breast milk can vary greatly and low concentrations of milk-borne zinc in humans can lead to a severe zinc deficiency in nursing infants which has behavioral, neurological, and immunological consequences [95]. However, there are conflicting reports on whether or not increasing the availability of minerals using CCPs leads to an increase in passive mineral absorption, particularly calcium [90].

5. COLOSTRUM/MILK AS A CONDUIT FOR DEVELOPMENTAL SIGNALS

The previous section focused on describing some of the most important components of colostrum and milk. Colostrum is known to contain significant amounts of immunoglobulins and other anti-infection agents that protect against pathogens in the neonatal GI tract and enhance passive immunity within circulation [4]. Colostrum and milk are both rich in growth factors that function as mediators of neonatal development. The EGF and IGF families of proteins in

milk affect multiple aspects of neonatal development, from GI maturation to reproductive tract development [51, 53]. Bioactive peptides encrypted in milk proteins ~2-20 residues are also important as they have latent activity in the GI tract and circulation following hydrolysis of the parent protein. These small peptides can modulate neonatal immunity, blood pressure, GI development, and systemic targets [90]. Milk-borne steroids have also been described in the milk of several organisms, including the pig, though their role has yet to be fully determined [53]. The diversity of growth factors and hormones in colostrum/milk requires that the role of this maternal lacteal secretion be extended beyond nutritional and immunological borders to include consideration of colostrum/milk as a conduit for lactocrine transmission of developmental signals from mother to offspring [96].

The term *lactocrine* was coined to describe a mechanism by which milk-borne factors are delivered from mother to offspring as a specific consequence of nursing [5]. In pigs, lactocrine-acting factors are present predominantly in colostrum synthesized within the first two days of lactation, a timing that coincides with the period of gut closure to macromolecule absorption. Studies reviewed in the next section were designed to determine how colostrum consumption during this period affects development of the female reproductive tract (FRT), male reproductive tract, and other tissues in the neonatal pig [55, 61, 63, 97-99].

Studies of marsupial species support the idea that lactocrine regulation of development is not unique to the pig [100]. Additionally, mammotroph

differentiation in the rodent is dependent on milk-borne factors shortly after birth [96]. In rats, colostrum consumption in the first two days is necessary to induce the appearance of prolactin-releasing cells within the anterior pituitary gland and delays development of mammotrophs in the neonate. Milk-deprived rats had altered mammotroph function as adults compared to their siblings allowed to nurse normally [96]. This suggests that there are milk-borne factors in rat colostrum necessary for proper differentiation and long-term function of mammotrophs. Finally, the World Health Organization recommends that mothers nurse their infants for at least six months after birth, citing multiple studies describing the short- and long-term benefits of breastfeeding [101]. The fact that all newborn mammals consume milk containing bioactive proteins and peptides combined with the ability of the neonatal gut to absorb these molecules intact makes the lactocrine hypothesis [5] for maternal programming of mammalian neonatal development compelling.

5.1 LACTOCRINE REGULATION OF PORCINE FRT DEVELOPMENT

In the pig, FRT development begins prenatally and is completed postnatally [81, 102]. Throughout this period, there are systematic cellular interactions involving epithelial and stromal cells of the FRT, that support both structural and functional patterning of the oviduct, uterus, cervix, and vagina [102, 103]. One of the most important events during this period is uterine adenogenesis, or budding and differentiation of glandular epithelium from luminal epithelium. Genesis of uterine endometrial glands in the neonatal pig requires both the expression and activation of

uterine ESR1 [102] . Development of the neonatal porcine uterus is both estrogen sensitive and ESR1 dependent [74]. Patterns of expression and activation of the uterine ESR1 system can define the developmental program and determine the developmental trajectory porcine FRT tissues, including the uterus [5].

Using relaxin as a prototypical milk-borne morphoregulatory factor, a series of experiments was conducted to determine the extent to which lactocrine mechanisms might support development of the neonatal porcine uterus. Bioactive prorelaxin was detected in porcine milk, with highest concentrations found in colostrum at birth [99]. Immunoreactive RLX was only detectable in serum of neonatal piglets allowed to nurse [63], indicating lactocrine transmission of this bioactive morphoregulatory peptide. The neonatal uterus is RLX receptor (RXFP1) -positive and ESR1-negative at birth [5]. Administration of exogenous RLX upregulated uterine ESR1 expression and had trophic effects on uterine growth similar to estrogen that could be inhibited by co-administration of the ESR1 antagonist ICI 182,780 [55, 63, 104]. Evidence supporting lactocrine transmission of RLX to the neonatal circulation [55] indicated that the window for delivery of such MbFs is open for approximately 48 h from birth. This is approximately the same time when the gut closes and mammary secretions characteristic of colostrum cease [55]. It is important to note that, as reviewed above, RLX is only one of many potentially lactocrine-active factors.

Piglets maintained in a lactocrine null state (deprived of colostrum) for the first 48 hours from birth displayed dramatically altered uterine expression patterns for ESR1, vascular endothelial growth factor (VEGFA) and matrix metalloproteinase 9

(MMP9) on PND 2 as compared to nursed controls [105]. While no effects on patterns of growth as reflected by body weight were noted, uterine ESR1, VEGFA and MMP9 protein levels were uniformly below assay sensitivity in lactocrine null gilts at PND 2. Results provided the first, unequivocal evidence of a requirement for lactocrine support of gene expression events necessary to establish an optimal uterine developmental program [105]. Similar effects of lactocrine signaling were also seen in the cervix [106], male reproductive tissues [97] and the heart [98]. Generally, the fact that trophic effects of exogenous RLX were more pronounced in nursing gilts than in lactocrine null gilts indicated that factors other than RLX in colostrum/milk cooperate to support RLX-dependent effects [98, 105].

5.2 EVIDENCE FOR LACTOCRINE REGULATION OF MARSUPIAL DEVELOPMENT

Given the conserved nature of milk and mammary genes (see above) it is reasonable to theorize that lactocrine regulation of development occurs across a wide range of mammalian species. Direct studies on lactocrine signaling in mammals excluding the pig are scarce. However, studies focusing on marsupial lactation strategies provide evidence that lactocrine signaling supports development in the metatherians. The tammar wallaby is a member of the kangaroo family indigenous to Australia. As a marsupial their lactation strategy differs from that of eutherian mammals in that they have long lactation periods relative to gestation, where the inverse relationship is true for eutherians [100].

In contrast to eutherian mammals that display one major change in milk composition, the transition from colostrum to mature milk [107], marsupial (wallaby) lactation involves multiple changes in milk-protein composition which occur through a series of lactational phases matched to the extrauterine development of offspring. *Phase 1* is the preparatory phase beginning 26 days before parturition; *Phase II* begins when, following birth, the neonate climbs into the pouch and attaches to a teat secreting *Phase II* milk for ~200 days. *Phase II* includes two sub-phases: *Phase IIA* – when milk contains high levels of α-lactalbumin, β-lactoglobulin, α-casein, and β-casein; and *Phase IIB* – involving concurrent changes in whey protein composition - when pouch young (PY) cease to be permanently attached to the teat [108]. *Phase III* occurs as the PY begin consumption of herbage. This phase of wallaby lactation is characterized by high milk volume coupled with an increase in milk protein and fat content[108]. Key stages of PY development are correlated with changes in lactation patterns of the mother [109]. Joss *et al* [109] found that all eight milk samples of specific time points tested between lactation day (LD) 0 and LD 250 contained unique proteins. Proteins that changed in abundance at least 3-fold between time points consisted of up to 80% of the total proteins within that stage [109]. Clearly, given the extreme, altricial state of development of metatherian PY at the time they emerge from the uterus, lactocrine signaling is central to developmental success in these species.

Kwek *et al* [100] provided further evidence that lactocrine regulation of development functions the tammar wallaby. They determined that disruption of the lactational/developmental relationship between mother and joey results in altered

patterns of gene expression in the forestomach [100]. In that study, PY at 120 days of age were cross-fostered to mothers at 170 days of lactation for 60 days. Thus, the developmental age of the joey and lactation stage of the mother was asynchronous. Joeys around 120 days of age exhibit significant changes in different regions of their stomach and the effect of later stage milk on fore-stomach maturation was unknown. They found that gross morphology and cell proliferation were not altered, however there was increased apoptosis and decreased parietal cell numbers in the fore-stomach of fostered joeys at 180 days of age [100]. Parietal cells are normally abundant in 180 day-old joey fore-stomachs but disappear by day 230. Not surprisingly, the parietal cell marker ATP4A was reduced in the forestomach of the fostered group. Other gastric glandular cell markers, such as GHRL and GKN2, were also downregulated. These observations suggested that milk consumed by the fostered PY from a later stage in lactation contained factors that retarded development of gastric glandular cell types of the forestomach and increased related apoptosis [100]. Moreover, fostered PY were over twice as heavy and had a head length 11 mm longer than non-fostered PY [100]. While the advanced rate of growth could be attributed to higher protein and lipid content of later stage milk consumed by the fostered group, it is also possible that the different proteins or other factors consumed by the fostered joeys affected their development.

A similar study by Menzies *et al* [110] showed how cross-fostering between wallaby species can alter growth and development of the fostered group [110]. In this case tammar joeys were fostered to parma wallaby mothers and parma joeys were fostered to tammar wallaby mothers at the same stage of lactation and development.

Parma and tammar wallabies were chosen for their similarities in size and lactation length [110]. Joeys were fostered at 15 days and 30 days and timing of developmental milestones were recorded. Fostered tammar joeys grew at similar rates to the controls and there was no change in the timing of developmental milestones such as appearance of hair, pigmentation, and opening of the eyes. In contrast, parma joeys did not survive when fostered at 15 days and struggled when fostered at 30 days of age. The surviving joeys had retarded growth rates and delayed appearance of developmental milestones. The difference in size between fostered joeys is not dependent on nutritional factors because fostered tammar joeys grew at a similar rate to control tammar and parma joeys, demonstrating that the nutritional milk components of each species are similar. It has long been known that an intrinsic maternal program unaffected by suckling patterns of the neonate regulates changes in expression of tammar milk proteins [111]. The authors suggested that differences in key milk-proteins consumed by fostered parma joeys produced an immature endocrine growth axis and retarded development. They pointed to early lactation protein (ELP), whey acidic protein (WAP) and IGF-1 as possible candidates affecting development of the parma endocrine growth axis [110]. Concentrations of these candidate proteins peak earlier in parma lactation compared to tammar lactation, suggesting that growth defects observed in fostered parmas were caussed by delayed consumption of ELP and WAP [110]. The fact that no parma joeys fostered before PND 30 survived supports this theory, as the concentration of these proteins in tammar milk would have increased enough to support the needs of fostered parma joeys. Authors concluded that maternal milk influenced aspects of joey development

and that altering the period when each joey receives critical milk components can have profound developmental consequences [110]. While mechanistic details remain to be delineated, developmental differences observed between these groups makes a convincing argument that maternal factors in wallaby milk may be affecting gene expression events critical to the success of neonatal development in this metatherian species.

6. BREASTFEEDING AND LONG-TERM EFFECTS

Short-term benefits of breastfeeding are clear. Numerous studies show that nursed infants have reduced risk of mortality from infectious diseases and decreased morbidity from GI and allergic ailments [112]. Long-term benefits of breastfeeding are less clear and more difficult to describe considering the limitations inherent in human studies. However, in a review published by the World Health Organization several correlations between breastfed children and long-term health such as risks for hypertension, high cholesterol, obesity, type II diabetes, and intelligence were described [101]. There is still controversy regarding the effects of breastfeeding beyond infancy since numerous studies suggest opposite conclusions (101) Nevertheless, infant formula typically does not contain the variety of milk-borne factors that the infant would ingest with breast milk. Meta-analytical studies and observations of the impact of imposition of a lactocrine null state on human development are imperfect. However, such studies may provide some insight into the role of lactocrine signaling in postanatal development of our species.

In humans, neonatal diet may influence risk of hypertension and heart disease in adulthood [113]. On average, the risk of hypertension in adults that were breastfed as infants was lower than those that were not [101]. The leading theory explaining this finding involves the presence of long-chain polyunsaturated fatty acids (LCPUFA) in breast milk that are not found in most brands of milk formula [101]. These fatty acids are incorporated into tissue membrane systems, including neural membranes and vascular epithelia [114]. Supplementation of formula with LCPUFAs results in lower blood pressure by age 6 compared to those fed with standard formula and comparable to infants that were breastfed [115]. After correcting for confounding factors and publication bias, meta-analyses of several studies concluded that both systolic and diastolic blood pressure are reduced in adults breastfed as infants [101]. Higher LCPUFA concentrations in breast milk were suggested as the reason for higher performance on intelligence tests applied to breastfed infants compared to infants that were fed formula. The major lipid components of neural membranes are docosahexaenoic acid (DHA) and arachidonic acid (AA), both of which are necessary for cortical and retinal brain development [101, 116]. DHA and AA levels decrease soon after parturition, but was also shown that DHA and AA levels are higher in breastfed infants, suggesting that the milk-borne LCPUFAs are replenishing DHA and AA levels. However, breastfeeding also enhances bonding between mother and child, which may contribute to intellectual development [117]. These factors are impossible to separate in observational studies and the extent to which breastfed infants are cognitively superior to those fed formula exclusively remains unclear [118].

Breastfeeding was associated with lower cholesterol levels in adults [101]. This may be due to the ability of milk to downregulate hepatic hydroxylmethylglutaryl coenzyme (HMG-CoA), as observed in piglets nursed normally compared to those fed with milk replacer (101) Similarly, piglets fed a high cholesterol diet had lower cholesterol levels as adults [119]. HMG-CoA is the rate limiting enzyme in the conversion of acetate to cholesterol [101]. Breast milk has significantly higher cholesterol compared to standard formulas, suggesting that high cholesterol levels have a long-term programming effect on cholesterol synthesis [101]. In contrast to the analysis of hypertension, protective effects of milk against high cholesterol were observed exclusively in adults (101)

Risks of obesity and type 2 diabetes in adults were also linked to breastfeeding [101]. A possible explanation is that formula fed infants express higher levels of insulin, motilin, enteroglucagon, neurotensin, and pancreatic polypeptide compared to breastfed counterparts [120]. Higher insulin results in increased fat deposition and adipocyte numbers, leading to a higher risk for obesity and insulin resistance in the future [101]. Studies (101) showed that breastfeeding has a small protective effect against obesity and development of type 2 diabetes, though the issue still remains controversial.

7. SUMMARY AND IMPLICATIONS

The studies discussed in this literature review conveyed the importance of colostrum and milk in enhancing the health and development of the neonate. Milk is a

complex fluid consisting of a vast array of substances suited for the survival of the neonate. The fact that all mammals nurse their young, the highly conserved nature of milk and mammary genes, and the changes observed in milk composition across several different species during developmentally critical periods suggests the value of milk to the neonate exceeds pure nutritional and immunological needs. Data for the pig demonstrate the effects of nursing within the first 48 hours of life on development of FRT and other somatic tissues in both male and female neonates.

The first objective of research described here was to determine if and how the array of proteins/peptides that define the porcine colostral proteome changes in the transition from colostrum to milk by determining qualitative and quantitative differences between protein profiles characteristic of colostrum at PND 0 with milk obtained at PND 6 using two-dimensional gel electrophoresis. The second objective was to confirm the hypothesis, as established for other species, that potentially bioactive peptides are encrypted within porcine milk proteins. Results will serve as a reference point for future studies aimed at identifying novel lactocrine acting factors in the porcine colostral proteome that may be contributing to FRT programming.

CHAPTER 3
DEFINING THE PORCINE COLOSTRAL PROTEOME

ABSTRACT

Colostrum (first milk) provides nutritional and immunological support for newborn mammals. In addition, colostrum contains a variety of bioactive peptides and growth factors, some of which are present in higher concentrations than in maternal circulation. Milk-borne, lactocrine acting factors affect patterns of gene expression in neonatal somatic tissues such as the reproductive tract and heart. Data for relaxin, a prototypical lactocrine-acting factor in porcine colostrum, indicate that its transmission of such factors from mother to offspring is significant prior to gut closure in the neonate. Additionally, amino acid sequences encoding potentially bioactive peptides may be encrypted within porcine milk proteins. These encrypted peptides become activated after proteolytic cleavage in the intestinal lumen or within the circulation. Thus it is important to understand the biochemical nature of colostrum/milk. The array of proteins/peptides constituting the porcine colostral proteome has not been defined. Objectives of this study were to employ two-dimensional gel electrophoresis (2DE) and image analysis to: (1) define the porcine colostral proteome on lactation day (LD) 0; (2) determine if and how this proteome changes from LD 0 to LD 6; and to (3) determine if potentially bioactive peptides are encrypted within porcine milk proteins. Colostrum (LD 0) and milk (LD 6) samples

were obtained from six lactating sows. Protein was extracted from individual samples and total protein concentrations were determined. For 2DE, first dimension separations were carried out using pH 3-10 immobilized pH gradient strips followed by SDS-PAGE using gradient polyacrylamide gels (10-20% total monomer). Individual samples were run on duplicate 2DE gels and stained with Sypro RUBY. Digital images of individual gels were obtained and analyzed using a Typhoon 9400 digital scanner and PDQuest 2-D Analysis Software. Total protein concentrations for colostrum (LD 0) and milk (LD 6) were 8.5 mg/ml and 8.3 mg/ml. PDQuest analysis identified 304 spots on 2DE gels that defined the colostral/milk proteome. Of these, 25 were unique to LD 0 and 15 were unique to LD 6. Differences in relative spot intensity between groups were also identified. There were 158 spots common to LD 0 and LD 6 that changed quantitatively at least 2-fold between days. Of these 158 spots, 105 increased in abundance while 53 decreased in abundance from LD 0 to LD 6. Data indicate that the colostral proteome (LD 0) is distinct from that identified for milk (LD 6). A large number of encrypted peptides were found within the sequences of common porcine milk-borne proteins. Thus, newborn pigs that nurse obtain a complex mixture of proteins and peptides from birth, which changes with time during a critical developmental period. This idea is supported by the fact that gilts deprived of colostrum for only two days from birth exhibit altered gene expression patterns associated with development of reproductive tract tissues and which persist when colostrum-deprived neonates are returned to milk feeding immediately thereafter. Though this study is largely predictive, it serves as a starting point in identifying

potential bioactive peptides in colostrum that act in a lactocrine faction and are necessary for the normal programming of the neonate.

INTRODUCTION

Milk is the main source of nutrients for the neonatal pig early in development. [3]. Colostrum is produced exclusively during the late stages of pregnancy and early lactation. It is high in protein and is classically known for boosting passive immunity in the neonate [1]. Fifty to eighty percent of the crude protein in porcine colostrum consists of immunoglobulins that are necessary in protecting the piglet from infection [4, 121]. However, colostrum also contains a large number of bioactive compounds, including growth factors, hydrolytic enzymes, hormones, and anti-infection agents that have local and systemic targets [1, 121]. Additionally, many milk proteins contain potentially bioactive peptides encrypted within their primary sequences that may become active after proteolysis.

Effects of milk-borne factors on the health and development of the neonatal gastrointestinal (GI) tract are well documented [32]. The porcine GI tract develops rapidly during the perinatal period after colostrum consumption and intestinal villi are temporarily damaged after milk withdrawal [57]. Newborn piglets fed colostrum for 24 hours had greater intestinal villous height and crypt depth compared to those that were fed mature milk or milk replacer as well as greater internalization of transforming growth factor-β receptors of the intestinal epithelium [39]. Bioactive factors can also affect systemic targets before the porcine GI tract closes to absorption of whole macromolecules at 24-48 hours after birth [32] This process is facilitated by

protease inhibitors present in colostrum as well as the undeveloped production of gastric acids and pancreatic enzymes [32, 96]. Collectively this arrangement provides an effective method for milk-borne bioactive factors (Mbfs) to enter systemic circulation.

Evidence suggests that colostrum serves as conduit for developmental signals required for the normal growth of the neonatal mammal. Prolactin secreting cells of the anterior pituitary in the rat proliferate quickly immediately after birth [96]. A study where neonatal rats were immediately fostered to mothers at day 4 of lactation showed that fostered pups had a decreased percentage of prolactin-secreting cells compared to nursed controls [122]. Similar studies done with tammar wallabies where fostered joeys nursing from mothers at 60 day advanced lactation phase exhibited altered patterns of gastrointestinal and somatic growth [100]. Analysis of wallaby milk revealed that changes in milk proteins throughout lactation correlated with the changing needs of the joey during development [109]. Using a porcine model the term 'lactocrine' was coined to describe a mechanism through which milk-borne bioactive factors enter circulation as a specific consequence of nursing [5]. The prototypical milk-borne bioactive factor relaxin (RLX) is present in porcine colostrum and only detectable in neonatal circulation if piglets are allowed to nurse [55, 61]. Subsequent studies showed that maternally driven relaxin signaling is required for estrogen receptor-α (ESR1) and vascular endothelial growth factor (VEGFA) expression in the porcine uterus by postnatal day 2. The lactocrine null state describes neonates deprived of colostrum and instead fed a hormone free milk replacer. Piglets maintained in this state for 48h from birth exhibit abnormal,

developmentally critical gene expression patterns the heart and both male/female reproductive tract tissues. [97, 98].

Relaxin is only one of many Mbfs present in porcine milk with potential to affect somatic development in the neonate. However, the milk proteome is poorly characterized in pigs and indeed in most mammals. In this study two dimensional polyacrylamide gene electrophoresis (2DE), image analysis and bioinformatic methods were used to (1) define the porcine colostral proteome on lactation day (LD) 0; (2) determine if and how this proteome changes from LD 0 to LD 6; and to (3) determine if potentially bioactive peptides are encrypted within porcine milk proteins.

MATERIALS AND METHODS

PORCINE MILK COLLECTION AND WHEY PROTEIN EXTRACTION

Purebred Yorkshire sows were milked manually immediately after parturition (lactation day 0) for colostrum and on lactation day 6 for mature milk (n = 6 sows/day). Teats were washed with warm water and massaged to stimulate milk letdown for both days. Composite samples were obtained on ice from multiple teats from each sow and frozen at -4°C. To isolate the whey protein fraction individual milk samples were diluted 1:1 with ethylenediamine tetra-acetic acid (EDTA; pH 7.0) and centrifuged twice for 15 minutes at 4000 x g at 4°C [61, 123]. Excess fat was removed with a spatula after each centrifugation. All samples were stored at -20°C.

SAMPLE PREPARATION AND DETERMINATION OF TOTAL PROTEIN

Samples were not pooled. Individual samples were prepared for 2DE. Total protein concentration was determined with the RCDC assay kit (Bio-Rad, Hercules, CA) using bovine serum albumin (BSA) as a standard. For each sample, 500µg of protein was subjected to third level sequential extraction (Bio-Rad) and further processed using the ReadyPrep 2-D Clean-Up Kit (Bio-Rad) to improve spot quality. Protein pellets were rehydrated with 50µl of ReadyPrep 2-D Starter Kit Rehydration/Sample Buffer (Bio-Rad).

ISOELECTRIC FOCUSING (IEF) AND 2DE

For each sample, 75µg of protein were loaded unto 11cm pH 3-10 ReadyStrip IPG Strips (Bio-Rad) following the protocol from the manufacturer. Strips were loaded on to a Protean IEF Cell (Bio-Rad) and focused for 20 hours per the protocol established by Boehmer *et al* in 2008: 500V for 1h, 1000V for 1h, 2000V for 2h, 4000V for 4h, 8000V for 12h[124]. Focused IPG strips were equilibrated in Buffer I (6 *M* Urea, 2% SDS, 0.375 *M* Tris-HCl pH 8.8, 20% Glycerol, and 2% wt/vol dithiotrheitol) for 30 minutes and Buffer II (6 *M* Urea, 2% SDS, 0.375 *M* Tris-HCl pH 8.8, 20% Glycerol, and 2.5% wt/vol iodoacetamide) for an additional 30 minutes (Bio-Rad). In every case, strips representing each animal and lactation day were run together to avoid procedural confounding.

Equilibrated IPG strips were run in duplicate in the second dimension on 10-20% Tris-HCl polyacrylamide gels (Bio-Rad) in 1X Tris-glycine-SDS running buffer at 200V for 1 hour with a Criterion Dodeca Cell (Bio-Rad). Since 12 second dimension gels can be run simultaneously on this device, care was taken to insure that

samples representing each animal and lactation day were run together to avoid procedural confounding.

Second dimension gels were removed from casings and fixed in 40% methanol, 10 % acetic acid solution for 1 hour and stained with SYPRO Ruby overnight. 2D gels were destained with 10% methanol, 7% acetic acid solution for 2 hours. Each gel was scanned using a Typhoon 9410 Variable Mode Imager (GE Healthcare Bio-Sciences).

2D GEL IMAGE ANALYSIS

Digital images of each gel were analyzed using PDQuest Advanced 2-D Analysis software version 8.0.1 (Bio-Rad). Gel images were set up such that the pI range (3-10) was oriented left-to-right and the molecular weight range (M_r x 10^{-3}, 250-to-10) was oriented top-to-bottom. All images were cropped to standardize their size and minimize false spots detected around gel edges. Individual spots were detected using the automatic spot detection wizard along with manual modifications to spot detection parameters. The speckle filter was turned to a sensitivity of 200 and images were corrected for horizontal and vertical streaks. Gel images were matched automatically to create a 'master 'gel, or a digital image combining all spots identified in each raw image for a given animal and day. Three master gels were created; LD 0 master, LD 6 master and the combined master which included data from all gels in both groups. Matched spots were inspected visually using the Spot Review Tool to verify accurate spot matching and identification. Some spots were matched or unmatched manually based on visual inspection. The Multi-Channel

Viewer was used to manually overlay gel images stained digitally with different colors (pesudocolored) to aid in manual spot matching. Analysis sets were then created to determine qualitative and quantitative differences between groups, which were identified using PDQuest software.

SPOT IDENTIFICATION

Spots indicative of proteins and peptides separated by 2DE that changed qualitatively and up to two-fold quantitatively from LD 0 to LD 6 were identified preliminarily using the ExPASy TagIdent tool (http://expasy. org/tools/tagident. html). To do this, estimated molecular weight and isoelectric point values were obtained for each targeted spot. These values were entered into the ExPASy TagIdent database obtain a preliminary identification. The error ranges for molecular weight and isoelectric point were set at 10% and pH 0.2 respectively. The organism name was specified to *Sus scrofa* whenever possible. If no matches were found, a general search for mammalian proteins in the approximated pI x molecular weight range was conducted. Molecular weight and pI values with multiple matches and no consensus between matches were labeled 'No Consensus" in Tables 1 and 2.

ENCRYPTED PEPTIDE IDENTIFICATION

Primary amino acid sequences of common porcine milk proteins were obtained from the NCBI protein database (http://www.ncbi.nlm.nih.gov/protein/) and each was analyzed using the BIOPEP database (http://www.uwm.edu.pl/ biochemia/index _en.php). A correlation was obtained between the size of the protein

and the incidence of unique encrypted peptides occurring three times or > 4 times per sequence using SAS analytics software version 9.1 (SAS Institute, Cary, NC). In silico analyses were performed to determine projected effects of proteolytic enzyme action on targeted porcine milk proteins. The enzymes chosen for these studies included pepsin, trypsin, chymotrypsin, elastase I, and elastase II. These enzymes were selected as they are known to be expressed by or to be present in the porcine GI tract during early postnatal development (35).

RESULTS

Relative qualitative and quantitative changes in the colostral/milk proteome observed between LD 0 and LD 6 of lactation are illustrated in Figure 1. Proteins and peptides are separated by pI (3-10, left to right) and molecular weight (250-10 x 10^{-3}, top to bottom). In this example, illustrating results for a single animal, details within outset boxes (Figure 1, A, B and C) show how the same areas in LD 0 and LD 6 gels differ in both spot number and relative spot density between days as assessed visually.

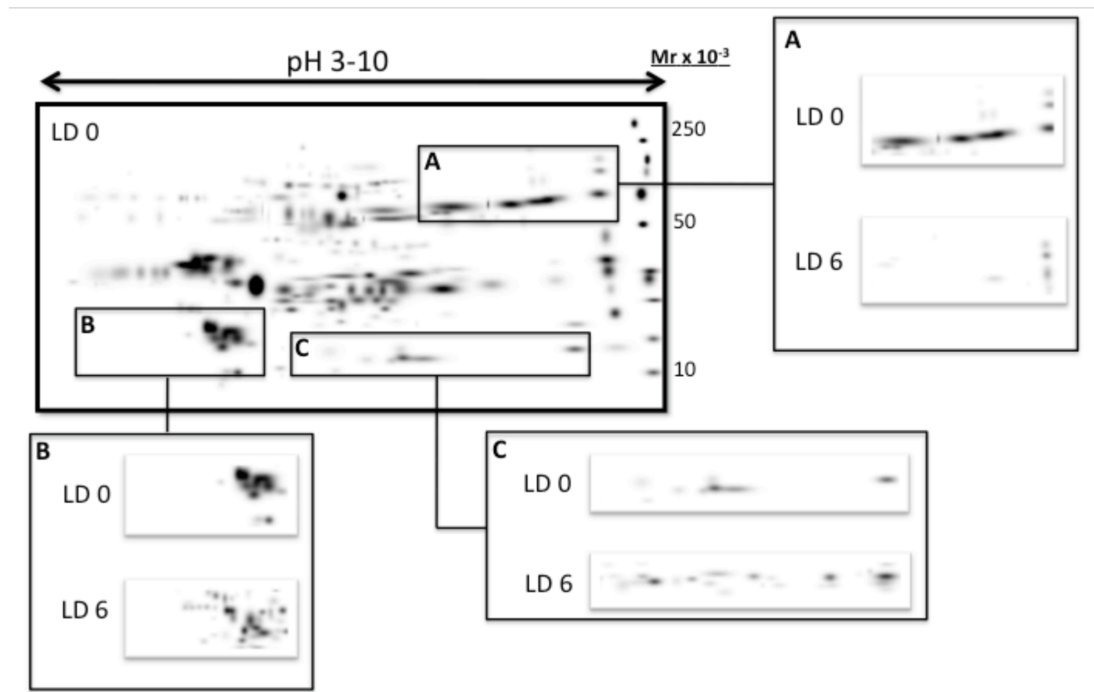

Figure 1: **Visual comparison of 2DE master images of porcine colostrum/milk at LD0 and LD 6 for representative sow.** Molecular weight range (250-10 X 10^{-3}) and pI (3-10) markers are shown. Boxes A, B, and C show areas where quantitative and qualitative changes were observed in the porcine milk proteome between LD 0 and LD 6.

Figure 2 depicts master gels for LD 0 (top) and LD 6 (bottom). Qualitative and quantitative differences in the proteomes are shown. Figure 3 is the master image including all components from both LD 0 and LD 6. Differences between the images in Figures 2 and 3 can be explained by the higher number of gels that were matched for Figure 3. Digital matching procedures resulted in fewer spots matched at 70% in all gels.

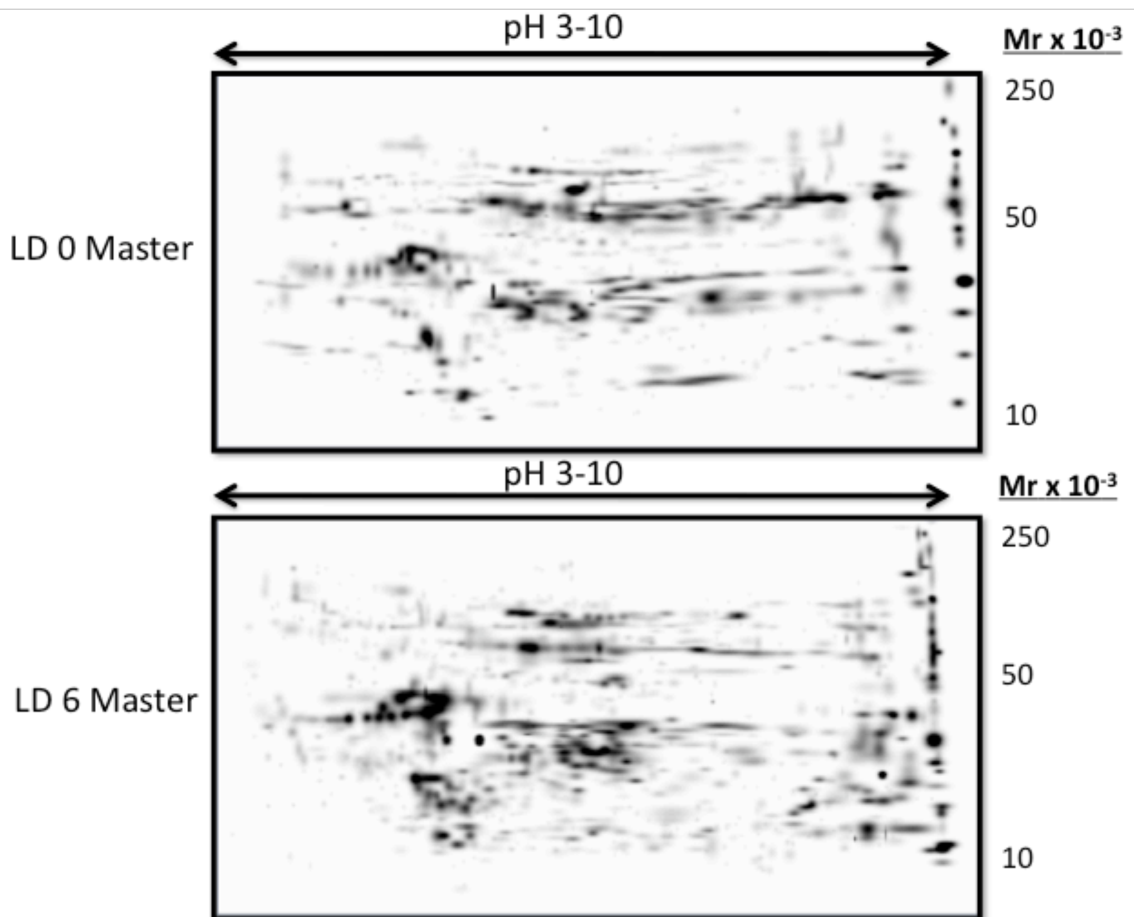

Figure 2: **Comparison of 2DE master images for colostrum at LD 0 and milk at LD 6 combining milk samples from all sows on respective days.** Molecular weight range (250-10 X 10^{-3}) and pI (3-10) markers are shown.

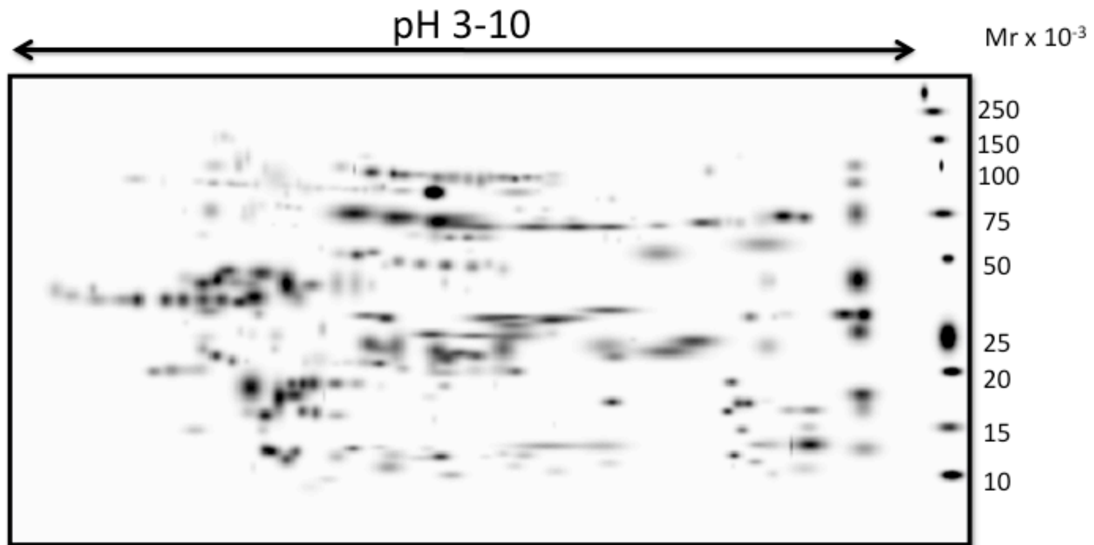

Figure 3: **Overall master image showing consensus spots from all gels in both groups (LD 0 and LD 6).** PDQuest identified 304 common spots representative of the porcine milk proteome at LD0 and LD 6. Molecular weight range (250-10 X 10^{-3}) and pI (3-10) markers are shown.

Figures 4 and 5 are the master gel images showing qualitative and two-fold or greater quantitative changes between LD 0 and LD 6.

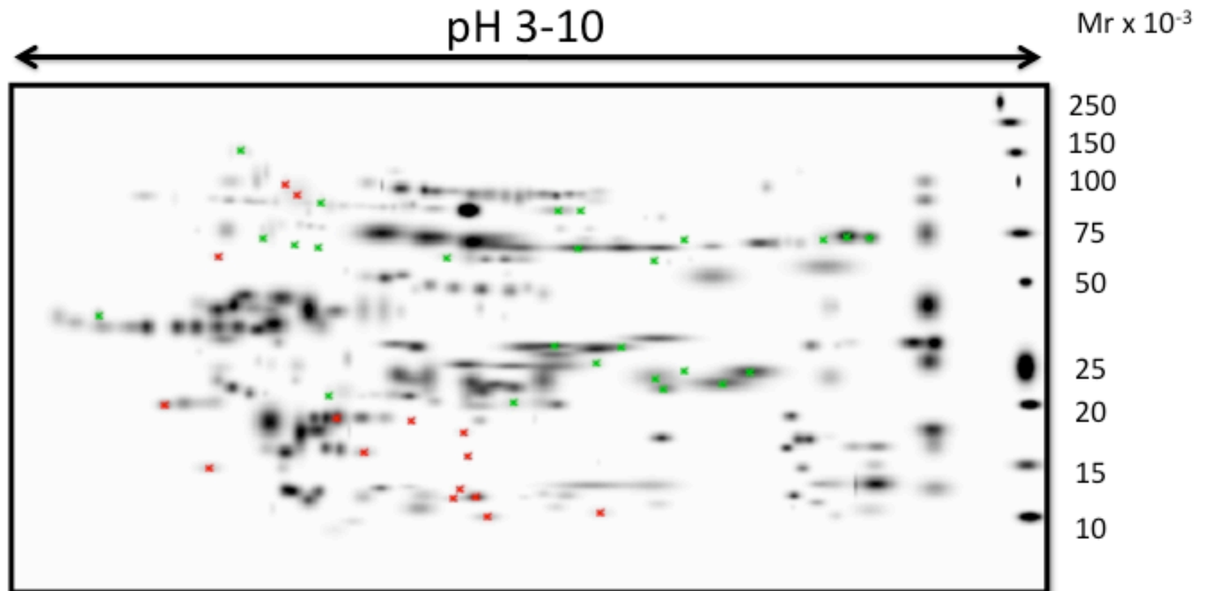

Figure 4: **Master image with colored overlays showing qualitative changes between LD 0 and LD 6.** Molecular weight range (250-10 X 10^{-3}) and pI (3-10) markers are shown. Green X's represent the 25 spots unique to LD 0 while Red X's represent the 15 spots unique to LD 6. These spots are described in Tables 1 and 2.

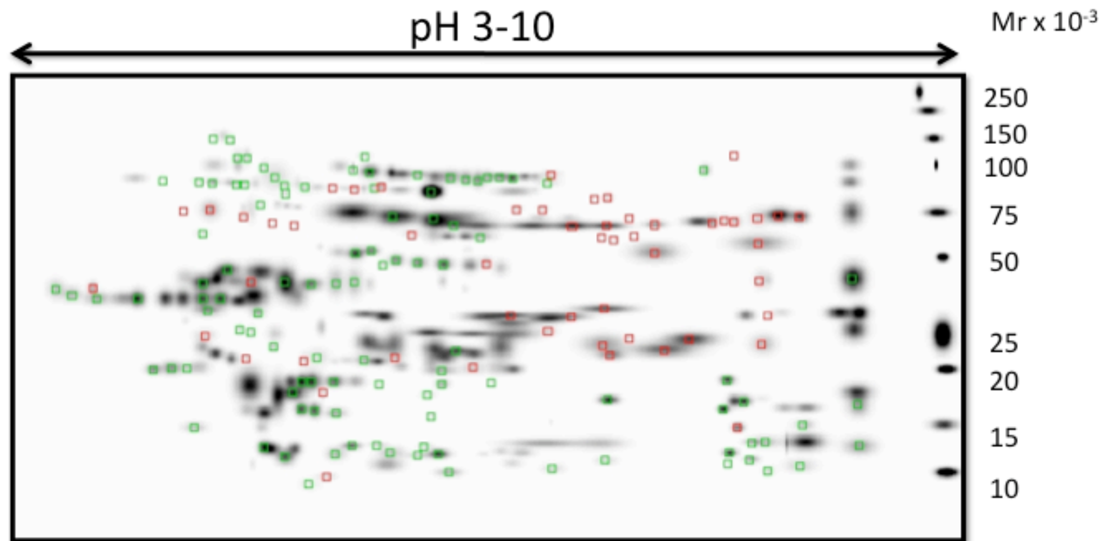

Figure 5: **Master image with colored overlays showing quantitative changes between LD 0 and LD 6.** Molecular weight range (250-10 X 10^{-3}) and pI (3-10) markers are shown. Red squares highlight the 53 spots that decrease at least two-fold from LD0 while green squares show the 105 spots that increase two-fold from LD 0.

With spot identification parameters established using both automated and visual inspection protocols, PDQuest identified 304 spots defined here to represent the LD 0-6 porcine milk proteome. Twenty-five of these spots were unique to LD 0 and 15 were unique to LD 6. There were 158 spots common to LD 0 and LD 6 that changed quantitatively at least 2-fold between days. Of these 158 spots, 105 increased in relative abundance while 53 decreased in relative abundance from LD 0 to LD 6. Figure 6 shows the location of spots and Standard Spot Numbers (SSP) at each location on the master gel image. The SSP numbers were used as a reference for identification of proteins/peptides. Results of preliminary TagIdent identification of spots unique to LD 0 and LD 6 are listed in Tables 1 and 2.

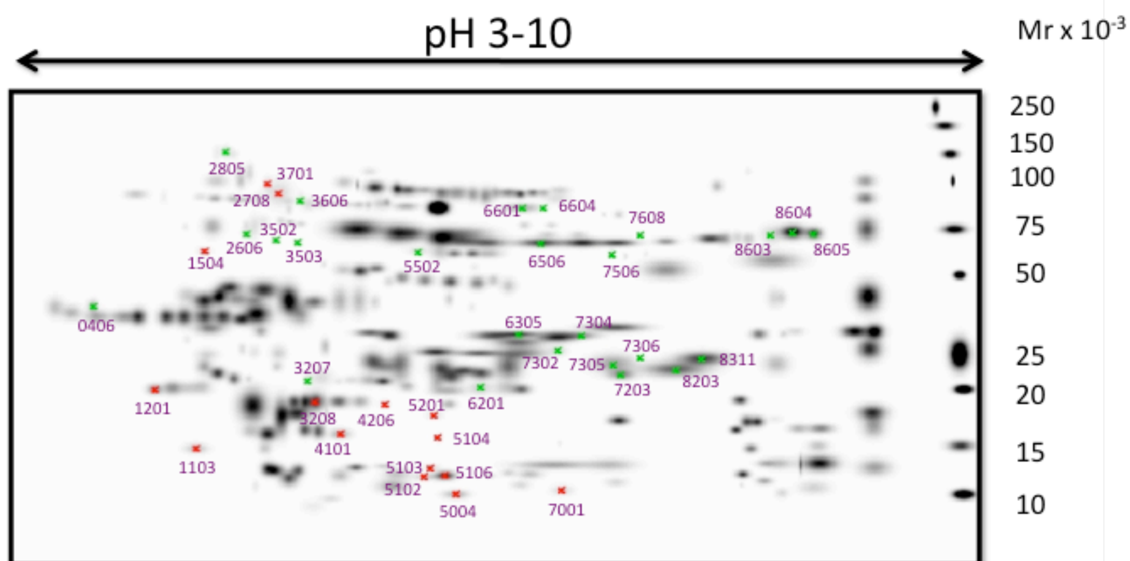

Figure 6: **Master image with qualitative overlays and additional SSP numbers in purple.** SSP numbers arbitrarily identify the identification of all spots in the master image. Additional information on SSP identified spots and can be tracked to Tables 1 and 2. Molecular weight range (250-10 X 10^{-3}) and pI (3-10) markers are shown.

SSP	Mr x 10^{-3}	pI	TagIdent ID	Protein Name
SSP 0406	44.16	3.30	Unknown	No Matches
SSP 2606	71.02	4.49	RSCA1_Pig	Regulatory Solute Carrier Protein
SSP 2805	121.26	4.32	Unknown	No Matches
SSP 3207	25.46	4.96	FA12_Pig	Coagulation Factor XII
SSP 3502	68.00	4.71	GHR_Pig	Growth Hormone Receptor
SSP 3505	67.34	4.88	C1S_Pig	Complement C1S Subcomponent
SSP 3606	88.15	4.91	HS90A_Pig	Heat Shock Protein 90
SSP 5502	63.11	5.95	HS71L_Pig	Heat Shock Protein 71
SSP 6201	24.13	6.52	APOM_Pig	Apolipoprotein M
SSP 6305	36.00	6.87	FCN1_Pig	Ficolin 1
SSP 6506	66.50	7.06	PLMN_Pig	Plasminogen
SSP 6601	83.84	6.90	TRFE_Pig	Serotransferrin
SSP 6604	84.24	7.08	PLMN_Pig	Plasminogen
SSP 7203	25.86	7.82	Unknown	No Consensus
SSP 7302	31.89	7.22	IBP2_Pig	Insulin-like Growth Factor BP 2
SSP 7304	35.60	7.43	TGFI_mouse	Transforming Growth Factor 1
SSP 7305	29.92	7.77	Unknown	No Matches
SSP 7306	30.18	7.97	Unknown	No Consensus
SSP 7506	62.08	7.71	FIBA_Bovin	Fibrinogen Alpha Chain
SSP 7608	70.60	7.96	Unknown	No Consensus
SSP 8203	70.22	9.15	Unknown	No Consensus
SSP 8311	29.82	8.52	Unknown	No Consensus
SSP 8603	70.22	9.15	Unknown	No Consensus
SSP 0604	71.39	9.34	Unknown	No Consensus
SSP 8605	70.93	9.53	Unknown	No Consensus

Table 1: **Preliminary identification of spots unique to LD0 using ExPASy TagIdent tool.** SSP numbers can be tracked to their location on the image in Figure 6. The table shows TagIdent IDs and protein names based on molecular weight and pI.

SSP	Mr	pI	TagIdent ID	Protein Name
SSP 1103	15.40	4.10	THRB_Pig	Prothrombin
SSP 1201	23.78	3.78	Unknown	no matches
SSP 1504	63.31	4.16	Unknown	no matches
SSP 2708	98.53	4.65	Unknown	Too many matches
SSP 3208	21.69	5.03	CATB_Pig	Cathepsin B Heavy Chain
SSP 3701	92.68	4.73	ENPL_Pig	Endoplasmin
SSP 4101	17.14	5.25	GKN3_Pig	Gastrokine 3
SSP 4206	21.30	5.65	APOR_Pig	Apolipoprotein R
SSP 5004	10.93	6.30	S10AA_Pig	Protein S100-A11
SSP 5102	12.46	6.01	TGFB3_Pig	Transforming Growth factor Beta 3
SSP 5103	13.28	6.07	TGFB3_Pig	Transforming Growth factor Beta 3
SSP 5104	16.64	6.13	SODC_Pig	Superoxide Dismutase (Cn, Zn)
SSP 5106	12.55	6.20	TGFB3_Pig	Transforming Growth factor Beta 3
SSP 5201	19.65	6.10	FRIH_Pig	Ferritin Heavy Chain
SSP 7001	11.19	7.25	B2MG	Beta Microglubulin

Table 2: **Preliminary identification of spots unique to LD 6 using ExPASy TagIdent tool.** SSP numbers can be tracked to their location on the image in Figure 6. The table shows TagIdent IDs and protein names based on molecular weight and pI.

Figure 7 illustrates the regression line ($p < 0.0001$) with incidence of unique peptides occurring three times per sequence as the dependent variable. Figure 8 illustrates the regression line ($p < 0.0001$) with incidence of unique peptides occurring > 4 times per sequence.

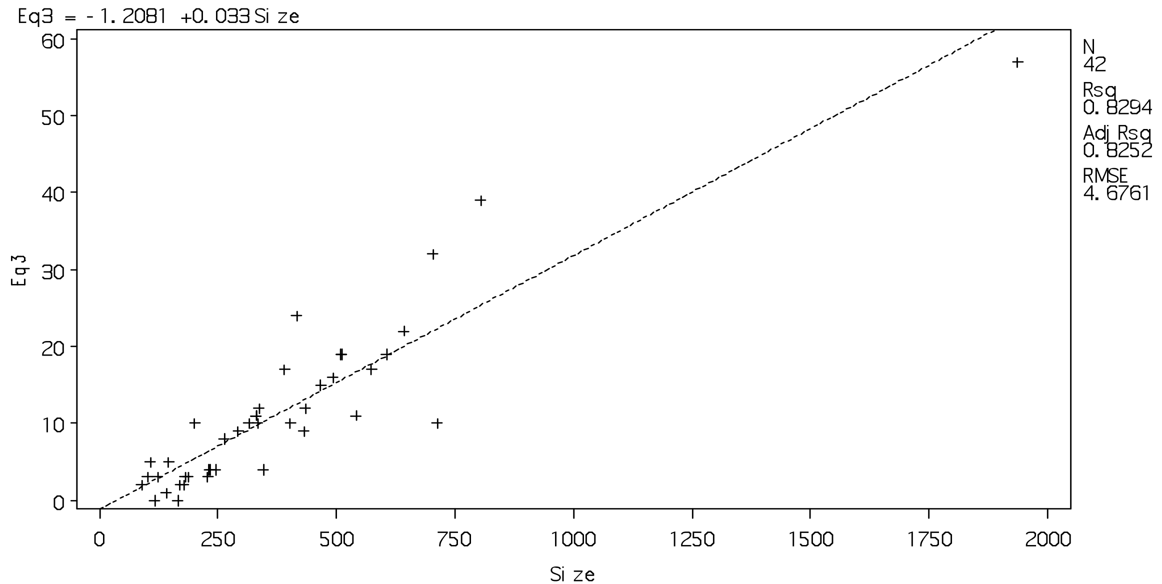

Figure 7: **Comparison of parent protein size (by number of amino acids) and incidence of unique encrypted peptides occurring exactly three times within one parent protein**. The equation of the line appears above the graph. Number of proteins (N), R^2, Adjusted R^2, and root-mean square error (RMSE) is shown to the right of the graph.

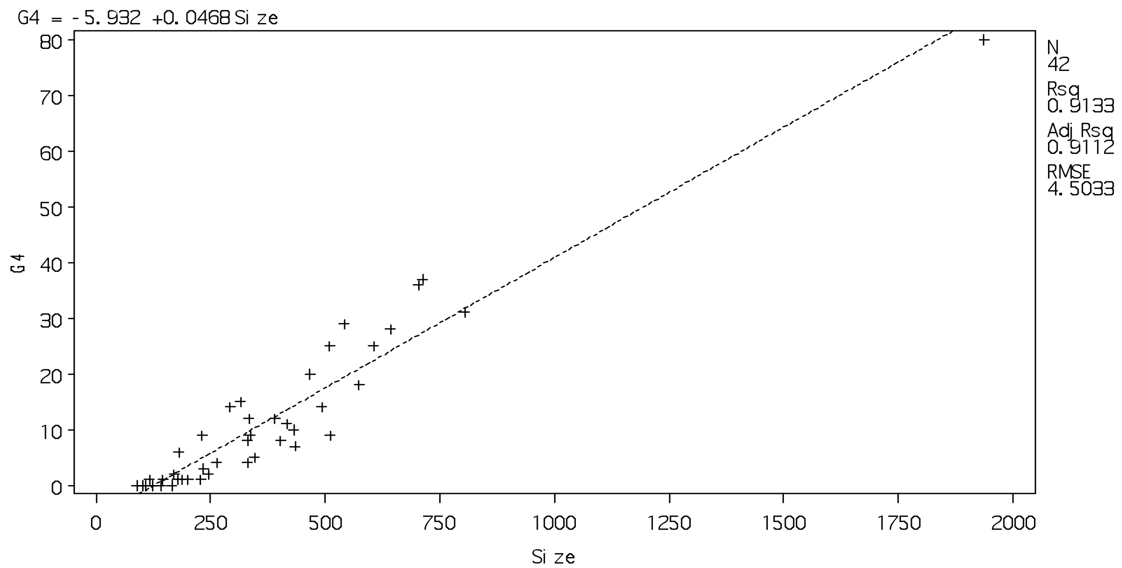

Figure 8: **Comparison of parent protein size (by number of amino acids) and incidence of unique encrypted peptides occurring greater than 4 times within one parent protein**. The equation of the line appears above the graph. Number of proteins (N), R^2, Adjusted R^2, and root-mean square error (RMSE) is shown to the right of the graph.

Not surprisingly, the number of potentially bioactive peptides likely to be encrypted repeatedly (three or more times) within parent proteins was determined to be related positively to the size, as defined by number of amino acids, of each parent molecule (Figures 7 and 8).

BIOPEP analysis of common milk-borne proteins revealed the presence of potentially bioactive encrypted peptides (BAPs) in all proteins tested (Table 3). Many of these encrypted peptides occurred over 100 times in a single parent molecule. Tables 4 and 5 show projected effects of proteolytic enzyme action on porcine milk proteins as reflected by the number of potential BAPs that could be excised. As expected, the decrease in number of potential BAPs identified through this *in silico* procedure likely reflects the limited number of enzyme-specific excision sites in the parent molecule.

Milk Proteins	# AAs	Potential BAP	BAP Milk Org	Occur ≥3
Actin	246	89	8	6
Amylase	511	146	8	28
Apolipoprotein A-1	264	90	6	14
Ceruloplasmin	335	109	8	22
Creatine Kinase	102	46	1	3
EGF Precursor	574	125	11	35
Endoplasmin	804	128	11	70
Haptoglobin	347	103	4	9
Heat Shock 27 kDA	124	50	3	3
Heat Shock 70 kDa	643	134	10	50
IgA	338	99	8	21
IgD	541	126	8	40
IGF-1	171	75	6	4
IGF-2	181	65	10	9
IGFBP-II	316	113	4	25
IGFBP-III	293	111	5	23
IgG	467	133	11	35
IgM	403	115	6	18
Insulin	108	56	4	5
Lactadherin	431	134	11	19
Lactate Dehydrogenase	332	99	3	19
Lactoperoxidase	712	127	11	47
Leptin	167	60	6	0
Lipoprotein Lipase	492	150	9	30
Lymphocyte Cytosolic Protein	510	137	11	44
Lysozyme	146	49	2	6
Myosin-4	1937	204	22	137
Phosphoglycerate Kinase	417	112	5	35
Pregnane X Receptor	330	114	6	15
Prolactin	229	75	5	4
Serum Albumin	607	140	12	44
Serum Amyloid A	89	47	1	2
TGFα	391	132	9	29
TGFβ2	434	121	12	19
α-lactalbumin	141	51	7	1
α-lactoferrin	703	155	8	68
αs1-Casein	201	105	8	11
αs2-Casein	235	8	11	7
β-Casein	232	107	8	13
β-lactoglobulin	178	56	5	3
β2-Microglobulin	118	42	4	1
κ-Casein	188	82	16	4

Table 3: **BIOPEP analysis of common porcine milk proteins and the potential number of bioactive encrypted peptides that can be found within one sequence of the parent protein.** The first two columns describe the protein names and their size based on amino acid number. The third column shows the number of bioactive peptides (BAPs) occurring in one protein. The fourth and fifth columns show the number of BAPs that occur exactly three times or greater than four times respectively in one protein.

Milk Proteins	Pepsin	Trypsin	Chymotrypsin	Elastase I	Elastase II
Actin	1	3	3	11	2
Amylase	0	2	2	3	0
Apolipoprotein A-1	1	3	1	9	2
Ceruloplasmin	2	2	5	25	3
Creatine Kinase	4	8	13	10	9
EGF Precursor	1	3	3	18	1
Endoplasmin	8	5	11	22	9
Haptoglobin	2	4	3	21	3
Heat Shock 27 kDA	1	0	1	3	1
Heat Shock 70 kDa	3	4	5	40	3
IgA	3	0	4	14	3
IgD	6	2	9	50	6
IGF	3	2	1	8	3
IGF-2	3	3	3	9	1
IGFBP-II	3	0	0	6	1
IGFBP-III	2	0	2	3	0
IgG	5	1	7	31	5
IgM	0	1	2	12	0
Insulin	3	1	1	10	3
Lactadherin	5	3	4	13	6
Lactate Dehydrogenase	3	8	4	10	5
Lactoperoxidase	5	5	9	23	5
Leptin	2	0	3	13	2
Lipoprotein Lipase	5	6	8	21	9
Lymphocyte Cytosolic Protein	7	5	3	15	2
Lysozyme	0	0	0	2	0
Myosin-4	17	22	12	76	11
Phosphoglycerate Kinase	2	5	2	12	4
Pregnane X Receptor	2	2	5	14	0
Prolactin	2	0	4	5	2
Serum Albumin	2	5	4	12	2
Serum Amyloid A	0	1	0	8	0
TGFα	8	0	13	24	9
TGFβ2	6	1	12	14	6
α-lactalbumin	0	1	1	4	1
A-lactoferrin	7	4	12	31	9
αs1-Casein	2	1	3	6	3
αs2-Casein	1	2	2	3	2
β-Casein	3	1	3	9	4
β-lactoglobulin	1	1	1	11	1
β2-Microglobulin	4	3	3	6	4
κ-Casein	0	3	3	3	0

Table 4: **BIOPEP analysis showing the potential number of bioactive peptides excised using common proteolytic enzymes.** The first column names the porcine milk proteins and the subsequent columns list the number of bioactive peptides excised using pepsin, trypsin, chymotrypsin, elastase I, and elastase II respectively.

Milk Proteins	Pepsin + Trypsin + Chymotrypsin	Elastase I + Elastase II
Actin	8	11
Amylase	24	42
Apolipoprotein A-1	8	16
Ceruloplasmin	17	28
Creatine Kinase	3	2
EGF Precursor	9	26
Endoplasmin	34	36
Haptoglobin	24	34
Heat Shock 27 kDA	3	2
Heat Shock 70 kDa	14	47
IgA	12	15
IgD	12	50
IGF	5	12
IGF-2	10	9
IGFBP-II	9	13
IGFBP-III	8	10
IgG	11	32
IgM	8	12
Insulin	3	9
Lactadherin	4	11
Lactate Dehydrogenase	4	18
Lactoperoxidase	29	36
Leptin	3	13
Lipoprotein Lipase	13	39
Lymphocyte Cytosolic Protein	15	18
Lysozyme	3	4
Myosin-4	39	19
Phosphoglycerate Kinase	20	21
Pregnane X Receptor	10	21
Prolactin	4	7
Serum Albumin	20	23
Serum Amyloid A	3	9
TGFα	10	28
TGFβ2	14	19
α-lactalbumin	2	7
α-lactoferrin	27	35
αs1-Casein	3	9
αs2-Casein	8	5
β-Casein	6	12

Table 5: **BIOPEP analysis showing the potential number of bioactive peptides excised using combinations of common proteolytic enzymes**. The first column names the porcine milk proteins and the subsequent columns list the number of bioactive peptides excised using combinations of pepsin, trypsin, chymotrypsin, elastase I, and elastase II respectively.

DISCUSSION

Previous studies on proteins found in porcine milk focused on those necessary to maximize the health of the piglet. Transfer of milk-borne antibodies from mother to offspring is particularly important in pigs because the epitheliochorial nature of the placenta prevents transfer of immunoglobulins into neonatal circulation [4]. This, combined with an undeveloped immune system in neonatal pigs at birth, makes ingestion of colostrum crucial in the short period where the neonatal gut is open to absorption of whole macromolecules. Nevertheless, as can be inferred from studies of pigs [5] and metatherians [100, 108], lactocrine communication of bioactive factors from mother to offspring is likely to represent a conserved mechanism for maternal regulation of extrauterine development of mammalian neonates.

Present results are consistent with observations indicating considerable changes in porcine milk quality during the transition from colostrum to mature milk [1, 3]. As expected, both qualitative and quantitative changes were documented for the array of proteins found on LD 0 as compared to that identified on LD 6. Of the 304 total spots defined as porcine colostral and milk proteomes, 25 were unique to colostrum and 15 were unique to mature milk (Figure 4). Quantitative changes were also observed; 53 spots decreased at least 2-fold while 105 spots increased at least 2-fold in abundance between days (Figure 5). Interestingly, nearly half of the spots downregulated at least 2-fold from LD 0 were those determined to be unique to colostrum (Figure 6, Table 1). Fairly liberal PDQuest settings were used to establish conditions for identification of these spots by their pI and molecular weight coordinates using TagIdent. This may explain why this approach to identification of

peptides and proteins in colostrum and milk proved to be less than accurate and of minimal objective value. Many of the spots matched using TagIdent had potentially hundreds of candidate protein identities matched to a specific pI and molecular weight range. As a consequence, no consensus sequence or related peptide/protein identify could be defined. However, some systematic patterns of change in colostral/milk proteomic patterns were observed between days.

Spots unique to colostrum and those downregulated at least two-fold from LD 0 were observed, in general, to have higher molecular weights and pI estimates compared to those spots defned to be unique to LD 6 milk and those upregulated two-fold from LD 0. (Figures 4 and 5) Variation in proteomic profiles was also seen between individual sows of the same experimental group within lactation day. This could be explained by the natural variation that exists between individual dams. However, evidence that milk protein composition varies depending on mammary gland location was recently reported. Wu and colleagues [125] used 2DE procedures to identify proteomic differences in porcine colostrum and LD 14 milk from anterior and posterior mammary glands [125]. In the present study, care was taken to collect milk from multiple teats in different locations. Therefore, variation of the kind described by Wu et al [125] should have been masked. Careful visual comparisons of proteomic profiles between the present study and images published by Wu et al [125] revealed a number of similarities, including the location of several proteins identified in their study by mass spectrometry. A high degree of consensus was observed upon visual comparison of gel images presented by Wu et al [125] with LD 0 gel images generated in this study. Interestingly, some spots identified by Wu *et al*

[125] using mass spectroscopy were also identified here using TagIdent. For example, albumin (SSP 5502 in appendix Table 6) was found in the same area in both of these studies. Nevertheless, positive identification of these peptides and proteins will require more sophisticated analysis such as mass spectrometry.

A hidden component of the porcine colostral/milk proteome, not visible in 2DE images, is the presence of potentially bioactive peptides encrypted within larger, parent proteins. These peptides are generally a few amino acids in length and require enzymatic release from their milk-protein precursors [2]. Meisel and colleagues [2, 83] studied the activities of encrypted peptides in bovine milk. This group determined that such encrypted peptides can be divided into four general categories including peptides that affect gastrointestinal function, those that modulate postprandial metabolism or those that function in antimicrobial defense or immunoregulation [2, 83]. Potentially bioactive peptides in similar categories were identified in porcine milk proteins.

Though the porcine neonatal GI tract is immature, some proteolytic enzymes are present and active early in development. Although chymosin is the predominant enzyme in the stomach at birth, it has weak proteolytic activity [126]. Trypsin, chymotrypsin, pepsin and pancreatic elastases are present in variable levels in the young pig [126, 127]. Analysis of the primary sequences of common porcine milk proteins using BIOPEP, a database for identification of encrypted bioactive peptides, revealed the presence of potentially thousands of bioactive peptides present within milk-borne proteins (Table 3) and hundreds that may be released as a result of enzyme action (Table 4 and Table 5).

Results reported here not only illustrate the number of potentially bioactive peptides that can be identified within porcine milk proteins, they also emphasize the extreme complexity of the potential lactocrine signaling domain after degradation of MbFs. The positive correlation identified between the size of parent milk proteins and the incidence of specific encrypted peptides occurring three or more times per parent molecule (Figure 7 and 8) was expected. These relationships reinforce the idea that molar quantities of potentially bioactive peptides encrypted in colostral/milk proteins could be available to be unleashed in the neonate and affect development and growth. This dimension of lactocrine signaling remains to be explored systematically.

Changes occurring in the porcine milk proteome may reflect changing needs of the neonatal piglet in a manner similar to that described for lactationally dependent tammar wallaby development [100]. Genomic studies analyzing the relationship between milk and mammary genes from marsupials, monotremes, and placental mammals revealed that milk and mammary genes are more likely to be present in all mammals and are highly conserved across Mammalia [10]. These relationships are likely to reflect the importance of such factors in the mammalian reproductive continuum. The array of potentially bioactive peptides consumed by the neonate changes with time, suggesting a critical window for lactocrine programming of neonatal development. Future studies will focus on identifying colostral peptides and determining their role as mediators of lactocrine programming in the neonate.

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Appendix A.

TagIdent Identification of spots downregulated at least two-fold from LD 0

SSP	MR x 10^{-3}	pI	TagIdent ID	Protein Name
406	44.16	3.3	Unknown	No Matches
1305	30.78	4.18	Unknown	No Consensus
1603	73.95	4.01	Unknown	No Matches
1604	74.47	4.22	CAD1_PIG	Cadherin 1
2203	25.89	4.5	Unknown	No Consensus
2406	45.88	4.54	Unknown	No Consensus
2606	71.02	4.49	RSCA1_PIG	Regulatory Solute Carrier Protein
3005	10.54	5.17	ICTL_PIG	Cathelin
3207	25.46	4.96	FA12_PIG	Coagulation Factor XII
3210	19.94	5.13	A1AG1_PIG	Alpha 1 Acid Glycoprotein
3502	68	4.71	GHR_PIG	Growth Hormone Receptor
3505	67.34	4.88	C1S_PIG	Complement C1S Subcomponent
3611	86.1	5.23	CAD5_PIG	Cadherin 5
4208	26.14	5.8	Unknown	No Consensus
4603	85.14	5.42	Unknown	No Consensus
4606	86.49	5.68	Unknown	No Consensus
5502	63.11	5.95	ALBU_PIG	Serum Albumin
6201	24.13	6.52	APOM_PIG	Apolipoprotein M
6305	36	6.87	Unknown	No Consensus
6403	52.04	6.64	Unknown	No Consensus
6603	74.51	6.92	Unknown	No Consensus
7302	31.89	7.22	IBP2_PIG	Insulin-like Growth Factor BP 2
7304	35.6	7.43	Unknown	No Consensus
7305	28.65	7.72	Unknown	No Consensus
7306	30.18	7.97	Unknown	No Consensus
7307	37.82	7.74	Unknown	No Consensus
7505	66.64	7.44	TPA_PIG	Tissue Type Plaminogen Activator
7506	62.08	7.71	FIBA_PIG	Fibrinogen Alpha Chain
7507	67	7.76	FIBA_PIG	Fibrinogen Alpha Chain
7508	61.08	7.82	G6PI_PIG	Glucose 6 Phosphate Isomerase
7510	62.32	8.01	G6PI_PIG	Glucose 6 Phosphate Isomerase
7512	55.94	8.2	Unknown	No Consensus
7602	74.55	7.16	Unknown	No Consensus
7605	80.29	7.64	Unknown	No Consensus
7606	80.49	7.77	Unknown	No Consensus
7608	70.6	7.96	Unknown	No Consensus
7703	93.8	7.24	PLMN_PIG	Plasminogen
8106	15.31	8.96	Unknown	No Consensus
8308	28.91	9.19	Unknown	No Consensus
8309	35.97	9.25	LEG4_PIG	Galcetin4
8404	46.58	9.17	Unknown	No Consensus

SSP	MR x 10^{-3}	pI	TagIdent ID	Protein Name
8501	67.38	8.2	Unknown	No Consensus
8504	68.44	8.73	SC5AA_PIG	Sodium/glucose cotransporter 5
8505	69.07	8.84	FA5_PIG	Coagulation Factor V light chain
8506	68.75	8.94	FA5_PIG	Coagulation Factor V light chain
8603	70.22	9.15	Unknown	No Consensus
8706	106.41	8.93	TLR9_PIG	Toll Like Receptor 9
8707	108.22	9.15	Unknown	No Consensus
8709	67.2	9.2	Unknown	No Consensus
8711	90.7	8.78	Unknown	No Consensus
8717	45.6	9.2	Unknown	No Consensus
8720	97.9	9.1	Unknown	No Consensus

Table 6: Preliminary spot identification of 53 spots downregulated at least 2-fold from LD 0. For clarity, SSP numbers are not tracked to a table. Molecular weight and isoelectric point for each spot are shown. Tagident ID and preliminary protein identification are also listed.

Appendix B.

Tagident ID of spots upregulated at least two-fold from LD 0

SSP	Mr x 10^{-3}	pI	TagIdent ID	Protein Name
403	43.9	3	Unknown	no matches
404	41.94	3.13	Unknown	no matches
407	40.95	3.33	PEPA_PIG	Pepsin A Precursor
409	40.86	3.65	PEPC_ PIG	Gastricsin
1103	15.4	4.1	THRB_PIG	Prothrombin
1201	23.78	3.78	Unknown	no matches
1203	23.95	3.91	Unknown	no matches
1205	23.97	4.04	Unknown	No Consensus
1306	37.26	4.2	CHYM_PIG	Chymosin (rennin)
1407	40.69	4.16	Unknown	No Consensus
1408	45.62	4.16	D4N5N1_PIG	Calreticulin
1504	63.31	4.16	Unknown	No Consensus
1701	90.14	3.85	Unknown	No Consensus
1703	89.75	4.13	Unknown	No Consensus
2108	13.19	4.65	LALBA_PIG	Alpha-Lactalbumin
2301	32.03	4.46	Unknown	No Consensus
2303	31.55	4.54	Unknown	No Consensus
2305	36.59	4.6	Unknown	No Consensus
2402	40.84	4.3	Unknown	No Consensus
2403	49.79	4.36	D4N5N1_PIG	Calreticulin
2604	88.44	4.42	Unknown	No Consensus
2608	88.32	4.5	Unknown	No Consensus
2611	76.85	4.62	Unknown	No Consensus
2701	89.16	4.24	Unknown	No Consensus
2705	104.75	4.44	TSP4	Thrombospondin 4
2707	105.36	4.51	TSP4	Thrombospondin 4
2708	98.53	4.65	Unknown	No Consensus
2801	119.83	4.25	Unknown	No Consensus
2806	118.85	4.38	Unknown	No Consensus
3001	12.34	4.81	Unknown	No Consensus
3004	10	5	Unknown	No Consensus
3103	17.56	4.94	LACB_PIG	Beta Lactoglobulin
3104	17.41	5.06	LACB_PIG	Beta Lactoglobulin
3105	12.51	5.24	LALBA_PIG	Alpha-Lactalbumin
3204	19.95	4.87	LACB_PIG	Beta Lactoglobulin
3206	21.71	4.95	CASA1_PIG	Alpha S1 Casein
3208	21.69	5.03	CATB_PIG	Cathepsin B Heavy Chain
3209	26.01	5.08	APOA1_PIG	Apolipoprotein A-1
3211	21.75	5.23	CASB_PIG	Beta Casein
3301	28.22	4.72	Unknown	no matches
3401	45.93	4.81	Unknown	no matches
3403	45.21	5.02	Unknown	no matches
3404	45.68	5.25	Unknown	no matches

SSP	MR x 10^{-3}	pI	TagIdent ID	Protein Name
3602	87.35	4.81	Unknown	no matches
3603	82.97	4.82	Unknown	no matches
3609	86.38	4.97	Unknown	no matches
3701	92.68	4.73	ENPL_PIG	Endoplasmin
4102	13.33	5.4	CASA2_PIG	Alpha S2 Casein
4103	13.4	5.63	CASA2_PIG	Alpha S2 Casein
4104	12.64	5.76	CYTA1_PIG	Cystatin A-1
4203	25.48	5.51	CASA1_PIG	Alpha S1 Casein
4206	21.3	5.65	APOR_PIG	Apolipoprotein R
4402	45.91	5.42	Unknown	no matches
4406	51.67	5.69	CLUS_PIG	Clusterin
4504	55.69	5.43	Unknown	no matches
4505	56.75	5.57	Unknown	no matches
4507	53.02	5.81	Unknown	no matches
4605	86.02	5.61	Unknown	no matches
4607	71.17	5.77	Unknown	no matches
4703	96.89	5.41	Unknown	no matches
4704	106.25	5.52	Unknown	no matches
4705	95.6	5.57	Unknown	no matches
5004	10.93	6.3	S10AA_PIG	Protein S100-A10
5102	12.46	6.01	TGFB3_PIG	Transforming Growth factor Beta 3
5103	13.28	6.07	Unknown	no matches
5104	16.64	6.13	SODC_PIG	Superoxide Dismutase (Cn, Zn)
5106	12.55	6.2	TGFB3_PIG	Transforming Growth factor Beta 3
5201	19.65	6.1	FRIH_PIG	Ferritin Heavy Chain
5204	23.62	6.23	CASK_PIG	Kappa Casein
5205	21.23	6.23	CASK_PIG	Kappa Casein
5207	27.43	6.37	Unknown	no matches
5402	52.34	6.01	Unknown	no matches
5405	51.73	6.24	Unknown	no matches
5507	67.26	6.35	Unknown	no matches
5604	70.64	6.16	Unknown	no matches
5606	83.76	6.14	Unknown	no matches
5701	93.61	6.01	Unknown	no matches
5705	92.07	6.31	Unknown	no matches
5708	91.17	6.45	Unknown	no matches
6203	21.46	6.69	Unknown	no matches
6502	62.01	6.59	Unknown	No Consensus
6701	90.5	6.57	Unknown	No Consensus
6703	92.45	6.65	Unknown	No Consensus
6704	92.12	6.77	Unknown	No Consensus
6705	91.91	6.88	Unknown	No Consensus
7001	11.19	7.25	B2MG	Beta Microglubulin
7002	11.95	7.75	B2MG	Beta Microglubulin
7105	18.95	7.77	IL10_PIG	Interleukin 10
7702	88.8	7.21	Unknown	No Consensus
8006	11.7	8.87	IL13_PIG	Interleukin 13
8008	12.02	9.08	Unknown	No Consensus

SSP	MR x 10^{-3}	pI	TagIdent ID	Protein Name
8010	11.04	9.24	ELAF_PIG	Elafin
8102	17.54	8.83	Unknown	No Consensus
8104	12.7	8.89	AQN3_PIG	Carbohydrate Binding Protein
8107	18.65	9.02	TNFB_PIG	Lymphotoxin Alpha
8110	13.79	9.22	IL4_PIG	Interleukin 4
8201	21.98	8.87	SAMP_PIG	Serum Amyloid P Component
8704	96.86	8.66	Unknown	No Consensus
9001	11.48	9.54	ANFB_PIG	Natriuretic Peptides B
9104	15.62	9.57	Unknown	No Consensus

Table 7: Preliminary spot identification of 105 spots downregulated at least 2-fold from LD 0. For clarity, SSP numbers are not tracked to a table. Molecular weight and isoelectric point for each spot are shown. Tagident ID and preliminary protein identification are also listed

Appendix C.

Milk Collection

Materials:

Latex gloves

15 ml Fisherbrand centrifuge tubes

Rescue decks

Cheese-cloth

Procedure:

1. Sows were milked manually following the birth of the first piglet and milked again 6 days later.
2. Teats were milked directly into centrifuge tubes, with the cheese-cloth serving as a filter for foreign particles.
3. Piglets were temporarily separated from the mother for 1 hour in order to collect a significant volume of milk at LD 6. After 1 hour, 2-3 piglets were reintroduced to induce milk let down. This process was repeated several times in order to obtain a significant volume of milk.
4. Samples were put on ice and stored at -4.0°C

Considerations:

1. Teats were cleaned with warm water before collection.
2. Milk was collected from as many teats as physically feasible from each sow.

3. Bloody or injured teats were not included in the study.

Appendix D

Milk Preparation: Lipid Removal and Protein Solubilization

Materials:

2.0 ml Eppendorf centrifuge tubes

0.2 M EDTA pH 7.0

10 M NaOH

Stir bars

pH sensor (Symphony)

Centrifuge (Fisher Scientific Marathon 26KM)

Procedure:

1. Create 0.2 M EDTA

37.22g EDTA in 500 ml distiller water. Add stir bar to bottle.

2. Use pH sensor and 10 M NaOH to adjust pH of stock EDTA to 8.0 to solubilize the EDTA. Add sequential drops of HCl to reduce pH to 7.0.
3. Add 0.8 ml EDTA to 0.8 ml thawed and vortexed milk samples
4. Centrifuge for 15 minutes at 6000 rpms.
5. Remove whey fraction with 1.0 ml pipette and transfer to new centrifuge tube.
6. Centrifuge for 15 minutes at 6000 rpms.
7. Remove whey fraction from any remaining lipids and transfer to new centrifuge tube.
8. Store at -20°C.

Appendix E

RCDC Protein Assay

Materials:

Prepared milk/EDTA samples

RCDC Protein Assay Kit (Bio-Rad)

BSA standard

0.1 M TRIS

1.5 ml centrifuge tubes (Eppendorf)

Vortex Genie Mixer

96 well plates (Bio-Rad)

Densitometer (Molecular Devices)

Centrifuge (Fisher Scientific Marathon 26KM)

Procedure:

1. Create stock BSA solution. 1.25 mg/ml
2. Create 1-5 serial dilution for standards using 0.1 M TRIS as buffer. Make enough volume for triplicate wells.
3. Create 100X and 200X dilutions of all milk samples using 0.1 M TRIS.

   5µl sample / 495µl TRIS for 100X 200µl 100X / 200 µl TRIS for 200X
4. Add 25µl samples and standards to labeled 1.5 ml centrifuge tubes.

5. Add 125µl of RC Reagent I to each tube. Incubate for 1 minute.
6. Add 125µl of DC Reagent II to each tube. Centrifuge at 15,000 g for 4 minutes.
7. Discard supernatant. Remove remaining supernatant with a quick spin in the centrifuge and a gel loading pipette tip.
8. Create Reagent A' by adding 60.96µl of Reagent S and 2987µl of Reagent A.
9. Add 127µl of Reagent A' to each sample/standard. Vortex. Incubate at room temperature for 5 minutes.
10. Add 1ml of Reagent B to each sample/standard. Vortex.
11. Add 300µl of sample/standard to each well. Each sample is done in triplicate.
12. Incubate at room temperature for 15 minutes.
13. Insert well plate in densitometer for analysis.

Appendix F.

Sample Preparation: Sequential Extraction 3

Materials:

Sequential Extraction 3 Kit (Bio-Rad)

Tributyl Phosphine (TBP)

PMSF

Vortex Genie Mixer

Centrifuge (Fisher Scientific Marathon 26KM)

Procedure:

1. Mix a 1:100 ratio of TBP and Reagent 3 to create extraction buffer in the hood. Vortex
2. Calculate 500µg of protein in a volume based on the RCDC protein assay and transfer to 1.5ml centrifuge tube.
3. Add 200µl of extraction buffer to each sample.
4. Add 8µl of PMSF to each sample. Incubate at room temperature for 15 minutes.
5. Vortex for 5 minutes. Centrifuge at 10,000 g for 5 minutes.
6. Recover supernatant and proceed to 2D Clean-Up Kit protocol.

Appendix G.

Sample Preparation: 2D Clean-Up Kit

Materials:

Vortex Genie Mixer

ReadyPrep 2D Clean-Up Kit (Bio-Rad)

Centrifuge (Eppendorf 5417R)

ReadyPrep 2-D Starter Kit Rehydration/Sample Buffer (Bio-Rad)

Procedure:

1. Add 300µl of Precipitating Agent I to each sample. Incubate on ice for 15 minutes.
2. Add 300µl of Precipitating Agent II to each sample. Vortex.
3. Centrifuge at 15,000 g for 5 minutes.
4. Discard supernatant.
5. Add 40µl of Wash Reagent I. Centrifuge at 15,000 g for 5 minutes
6. Remove supernatant and transfer to new microfuge tube.
7. Add 25µl of nanodrop water to each sample. Vortex for 30 seconds.
8. Add 1ml of Wash Reagent II to each sample. Vortex for 1 minute.
9. Add 5µl of Wash Additive to each sample. Vortex.
10. Incubate at -20°C for 30 minutes. Every 10 minutes vortex for 30 seconds.

11. Centrifuge at 15,000 g for 5 minutes. Discard supernatant. Air-dry tubes for 5 minutes.
12. Re-suspend each sample with 50μl Rehydration Buffer.
13. Store at -20°C.

Appendix H.

Isoelectric Focusing

Materials:

Protean IEF cell (Bio-Rad)

ReadyStrip IPG Strip 11cm pH 3-10 (Bio-Rad)

ReadyPrep 2-D Starter Kit Rehydration/Sample Buffer (Bio-Rad)

Loading Trays (Bio-Rad)

Mineral Oil (Bio-Rad)

Paper Wicks

Procedure:

1. Thaw IPG strips and prepared samples on ice.
2. Transfer 75µg of protein by volume from samples and add enough rehydration buffer to total 185µl. Vortex.
3. Transfer the 185µl of sample onto their respective loading trays evenly.
4. Remove plastic backing from 11cm IPG strips and load gel side down onto loading wells, taking care that there are no bubbles under the sample. Incubate for 1h at room temperature.
5. Cover each strip with mineral oil and incubate at room temperature overnight.
6. Cover IEF cell tray electrodes with paper wicks weighted down with nanodrop water.

7. Load IPG strips gel side up onto IEF cell tray and cover strips with mineral oil.
8. Load tray onto IEF cell focus strips for 20 hours per the following protocol: 500V for 1h, 1000V for 1h, 2000V for 2h, 4000V for 4h, 8000V for 12h.
9. Remove tray from IEF cell and transfer IPG strips gel side up to clean loading tray. Cover with plastic wrap and freeze immediately at -80°C.

Appendix I.

Equilibration and 2D-SDS-PAGE

Materials:

SDS

Urea

Tris

Glycerol

Acetic Acid

Methanol

Iodoacetamide (Bio-Rad)

Dithiothreitol (DTT) (Bio-Rad)

Focused IPG strips (Bio-Rad)

Bromophenol Blue Agarose (Bio-Rad)

Precision Plus Unstained Protein Standard (Bio-Rad)

Loading Trays (Bio-Rad)

Orbital Shaker (Stovall)

Power Pack 300 (Bio-Rad)

Criterion Dodeca Cell (Bio-Rad)

Water Circulator / Cooler (Brinkmann)

Criterion Tris-HCl Gels 10-20% 11cm

Sypro Ruby Protein Gel Stain

Buffers:

1. 60ml Equilibration Buffer I.

   6M Urea, 0.375M Tris pH 8.8, 2% SDS, 20% Glycerol, 2% (w/v) DTT

2. 60ml Equilibration Buffer II

   6M Urea, 0.375M Tris pH 8.8, 2% SDS, 20% Glycerol, 2.5% (w/v) Iodoacetamide

3. 10X and 1X tank buffers. To make 1L of 10X tank buffer:

   30g Tris, 144g Glycine, 10g SDS, bring to volume using distilled water.

   6L 1X tank buffer: Mix 600ml 10X tank buffer with 5.4L distilled water.

4. Fixative solution. 40% Methanol, 10 % acetic acid

5. De-stain solution. 10% Methanol, 7% Acetic acid

Procedure:

1. Thaw focused IPG strips at room temperature until gel is pliable and translucent.

2. Cover each strip with 4ml of Equilibration Buffer I. Place tray in orbital shaker and incubate for 30 minutes.

3. Carefully decant buffer I from tray. Cover each strip with 4ml of Equilibration Buffer II. Place in orbital shaker and incubate for an additional 30 minutes.

4. Melt the agarose overlay during the incubation period.

5. Prepare the SDS-PAGE gels by removing combs and blotting away excess water from the IPG well.
6. Fill dodeca tank with approximately 5L of 1X tank buffer and connect to water circulator. Set temperature to 20°C. Place 2 stir bars at the bottom of the tank
7. Mount the tank on stir plate.
8. Remove IPG strips from buffer II and dip strips briefly in 1X tank buffer.
9. Lay strips gel side up on back casing of SDS-PAGE gel directly above the IPG well.
10. Use pipette to place overlay agarose solution into the IPG well of the gel.
11. Gently push IPG strip into the well using forceps or a spatula.
12. Place SDS-PAGE gels in slots of the dodeca tank.
13. Load 10µl of protein standard in standard well of the gel.
14. Fill reservoirs with 1X tank buffer.
15. Connect dodeca to powerpack and begin electrophoresis with running conditions of 200V for approximately 1 hour or until bromopehnol blue line is 1cm from bottom of the gel.
16. Remove gels from casing after electrophoresis is complete. Place gels in 100 ml of fixative solution and incubate for 1 hour on orbital shaker.
17. Remove fixative and add 60 ml Sypro RUBY stain to each gel. Cover containers in aluminum foil and incubate overnight on orbital shaker.
18. Remove stain and add 100 ml of destain solution to each gel. Incubate for 2 hours on orbital shaker. Minimize light exposure to gels.

19. Remove destain and wash gels in distilled water before imaging. Minimize light exposure to gels.

Appendix J.

2-D Gel Imaging

Materials:

Typhoon 9410 Variable Mode Imager

PC with ImageQuant version 5.2

Squirt bottle with distilled water

Procedure:

1. Open Typhoon Scanner Control on the desktop.
2. Change acquisition mode to fluorescence.
3. Click Set-Up button → Emission Filter → Sypro RUBY
4. Set up scan size to an H-6 grid
5. Open the scanner and carefully place a single gel on the bottom left corner. Use water to remove any air bubbles that appear under the gel.
6. Close the scanner and press the scan button on the PC. Save and name the gel image when prompted. Repeat for all gels.
7. Open ImageQuant on the desktop.
8. Open all gels from ImageQuant. Click Save As → File Type → TIFF file. Save.

Appendix K.

Gel Analysis: PDQuest

Materials:

2-D gel images

PC with PDQuest Advanced 2-D Analysis Software version 8.0.1

Microsoft Excel

Cropping Images:

1) Open PDQuest software and close the experiment wizard that appears.
2) Click File →Open → open collected 2-D gel images.
3) Click the ‘Advanced Crop Tools’ tab → Crop → select area of interest of gel. Crop an area that includes the molecular weight markers but avoids all four edges of the gel.
4) Select the ‘Crop and Save’ button that appears. Name and save the new gel images.
5) Click the ‘Save Crop Settings’ button under the ‘Advanced Crop Tools’ tab. Name the settings.
6) Click ‘Load Saved Cropped Settings’ under the ‘Advanced Crop Tools’ tab for each subsequent gel. Repeat step 4 for every gel. The crop size and gel size will be the same for each gel.

New Experiment Wizard

1) Click ‘New Experiment’ in PDQuest Analysis Quick Guide.
2) Name the experiment and click ‘Next’.
3) Click the ‘Add’ button and select all the images cropped using PDQuest. Click ‘Next’.
4) Group replicate gel images. LD 0 and LD 6 gels were grouped separately.
5) Select the ‘Use Spot Detection Wizard” when prompted. Select ‘Warp Gel Images Before Matching’ under Matching Options.

Spot Detection Parameters Wizard

1) Click on the ‘Advanced’ tab on the top right to open up all the options.
2) Zoom in on an area of the gel with a low spot concentration. Select the ‘Click on a faint spot’ button and find the faintest spot on the gel.
3) Follow the same steps for the ‘Click on a small button if different from the faint spot’ button and ‘Box the largest spot cluster’. The casein clusters around pI of 5.0 MW of 30 kD was chosen as the largest spot cluster.
4) Under ‘Test Settings’ check all three options. Change ‘Sensitivity’ to 8.00, ‘Size Scale’ to 5. Leave the ‘Min Peak’ unchanged.
5) Under ‘Optional Controls’:
    a) Streaks: Check ‘Vertical’ and ‘Horizontal’
    b) Background: Do not change settings
    c) Smoothing: Do not change settings
    d) Speckles: Check ‘ Apply speckle filter’. Change ‘Sensitivity’ to 200

6) Click the 'Proceed' button. Click 'Next' until software begins to count the spots for each gel. Do not change any of the standard settings. When analysis is completed, select 'Open Spot Review Tool' when prompted.

Spot Review:

1) This tool is used to make sure that the spots identified by the software are real and accurately matched to each other. Each histogram numbers each spot and represents the relative abundance of each matched spot per gel.
2) Open the 'Edit Spot Tools' tab.
3) Click on each histogram and inspect the spot matching accuracy in each gel.
4) If the spot is false use 'Cancel spot at cursor' under the 'Edit Spot Tools' tab and cancel the false spot in each gel.
5) If the software identifies a large saturated spot as multiple spots, click on 'Combine spots in a box' button under the 'Edit Spot Tools' tab and draw a square around the spots to combine them into a single spot.
6) Repeat steps 4 and 5 for every histogram to improve the accuracy of the matching software.
7) Make sure to cancel all the spots associated with the MW markers.

Creating Analysis Set for Qualitative Changes:

1) Click on 'Analysis' and select 'Analysis Set Manager'.
2) Click on 'Create' and select 'Qualitative'.
3) Name the analysis set (On LD 0, Off LD 6).

4) Check the ‘Repl. Gels’ button under ‘Compare’ and select LD 0 for ‘A’ and LD 6 for ‘B’. Check ‘Estimate missing spots’ button and ‘ Estimate saturated spots’ button under ‘Options’.
5) Spot count should appear on the lower left hand corner. Click on the ‘Save’ button.
6) Repeat steps 1-5 to analyze for spots unique to LD 6. For step 4 choose LD 6 for ‘A’ and LD 0 for ‘B’.

Creating Analysis Set for Quantitative Changes:

1) Click on ‘Analysis’ and select ‘Analysis Set Manager’.
2) Click on ‘Create’ and select ‘Quantitative’.
3) Name the analysis set (2-Fold Change from LD 0 to LD 6)
4) Check the ‘Repl. Gels’ button under ‘Compare’ and select LD 0 for ‘A’ and LD 6 for ‘B’. Check ‘Estimate missing spots’, ‘ Estimate saturated spots’, and ‘Include qualitative changes’ under ‘Options’.
5) Check ‘Outside limits’ under ‘Method’
6) Spot count should appear on the lower left hand corner. Click on the ‘Save’ button to accept changes.
7) To see spots that increase in expression, select ‘Upper limit’ under ‘Method’. To see spots that decrease in expression, select ‘Lower limit’ under ‘Method’.

Determining MW and pI:

1) Click on the 'Mr, pI Standard Tools' tab and select 'Enter Mr, pI data for spot'.
2) Estimate the MW and pI for 5-7 spots on various locations of the gel, include areas around the 4 corners as well.
3) After estimating 5-7 spots, the software will be able to plot a MW and pI grid over the gel and will be able to estimate the MW and pI for the remaining spots.

Exporting Spot Data and Gel Images

1) Click on 'File' → Export → Export (Text) Experiment.
2) Specify the analysis set to export and leave default settings unchanged.
3) Specify analysis set under 'Spots to export'. Check 'Clipboard' under 'Export to'. Click on the 'done' button
4) Open Microsoft Excel, Edit→ Paste
5) To export gel images click on 'File' → Export → Export JPEG image. Click on the image to be exported.
6) Click 'Export'. Image will be saved on the desktop.

Appendix L.

Spot Identification Using Bioinformatic Methods

Materials:

Spot MW and pI data

ExPASy Proteomics Server (http://ca.expasy.org/tools/tagident.html)

Procedure:

1) Obtain the MW and pI data for each spot. For the maximum and minimum pI range, enter the actual pI given by PDQuest $\pm$ 0.01 respectively.
2) Enter the MW in the specified box. Reduce the MW range to 10% error.
3) Check the box with ‘Check for protein sequences with cysteines in reduced form (-SH)’
4) Under ‘Organism name or classification’ input *Sus scrofa*.
5) Scroll down the screen and click on the ‘Start Tagident’ button.
6) If minimal or no results appear, input *mammalia* under ‘ Organism name or classification’.

Appendix M.

Encrypted Peptide Identification

Materials:

BIOPEP database (http://www.uwm.edu.pl/biochemia/index_en.php)

NCBI protein database (http://www.ncbi.nlm.nih.gov/protein/)

List of porcine colostral /milk proteins

Finding Primary Amino Acid Sequences of Porcine Milk Proteins:

1) Access the NCBI protein database and type the name of the target protein in the 'Search' box.
2) Click on the protein name. Under 'Display Settings' select 'FASTA' and click 'Apply'.
3) Copy and paste the sequence to a text document for future reference.

Determining Potential Biological Activity of Encrypted Peptides:

1) Access the BIOPEP database. Select the 'BIOPEP' tab and click on 'Bioactive Peptides'.
2) Select the 'Analysis' tab and click on 'Profiles of Potential Biological Activity'. Select the 'For Your Sequence' button.
3) Copy and paste the selected amino acid sequence onto the search box. Click 'Report' on the far right of the page.

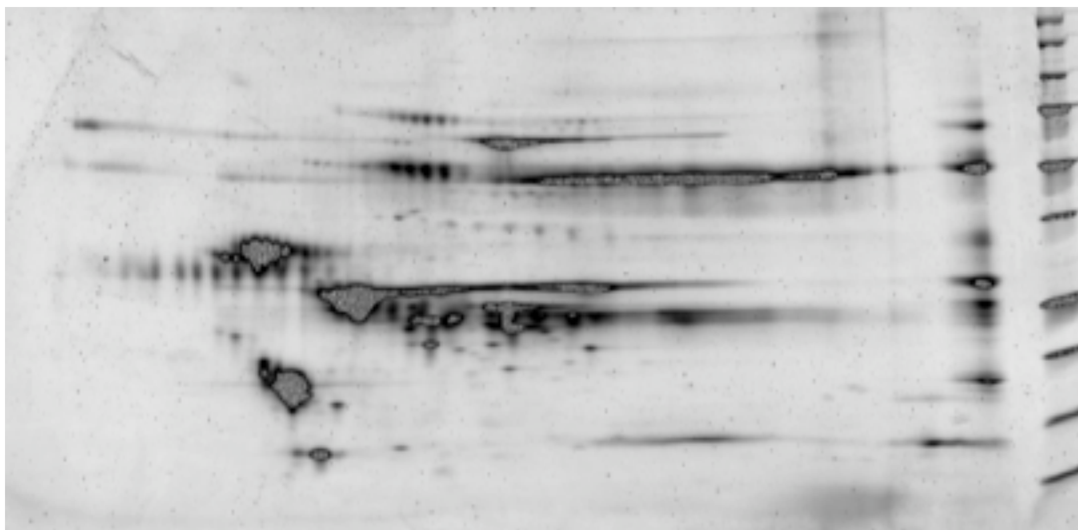
4) The website will display all potential bioactive peptides for the inputted sequence.

Determining Possible Encrypted Peptides After Enzymatic Cleavage:

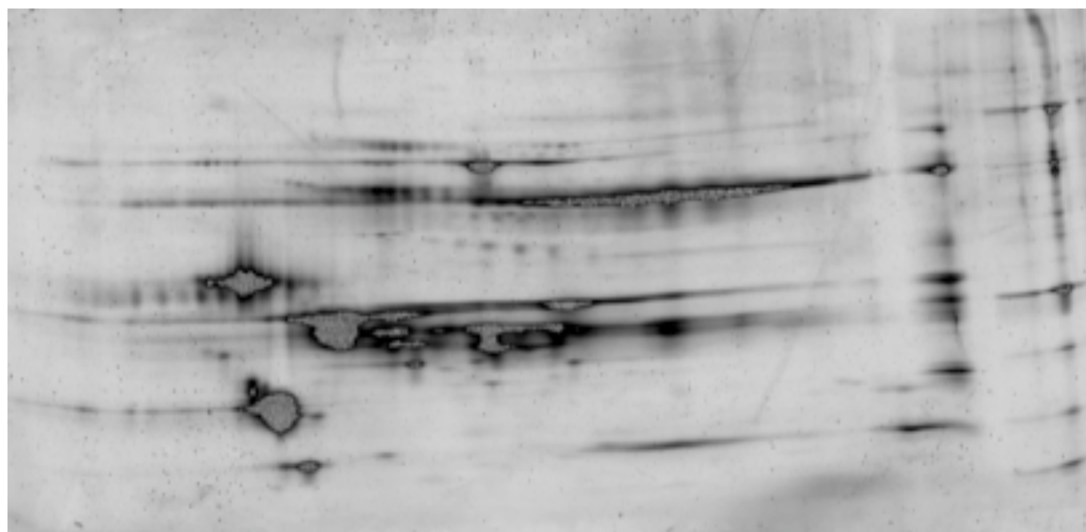
1) Access the BIOPEP database. Select the ‘BIOPEP’ tab and click on ‘Bioactive Peptides’.
2) Select the ‘Analysis’ tab and click on ‘Enzymes Action’. Select the ‘For Your Sequence’ button.
3) Copy and paste the selected amino acid sequence onto the search box. Under ‘Select enzymes’ choose the enzymes required for analysis. Click on the ‘Report of enzyme action’ button.
4) Under results, click on the ‘Search for active fragments’ button. The database will search the cleaved protein for bioactive peptides within the BIOPEP database.

Appendix N.

Raw 2D Gels



Sow 129 LD 0 Gel Run Date: 5/28/09



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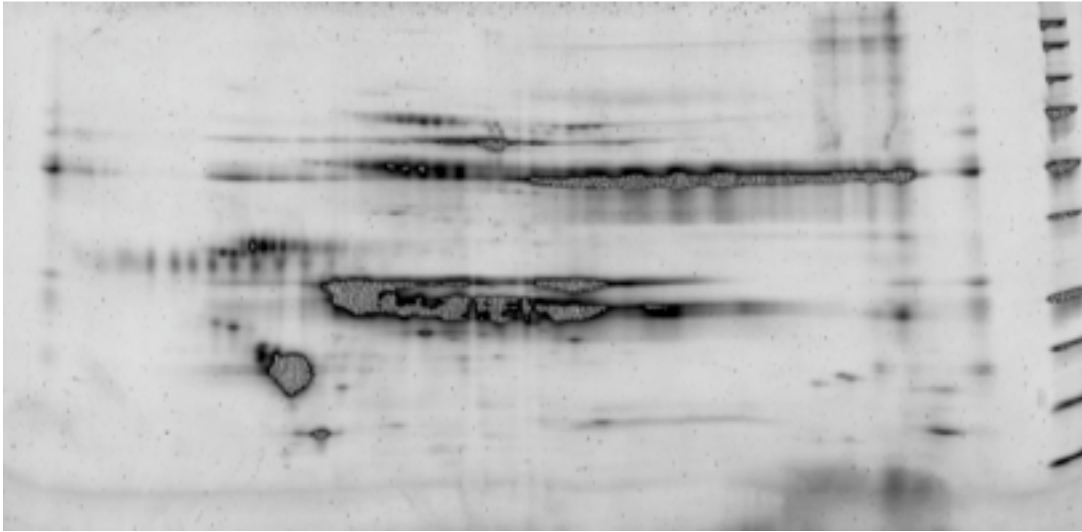

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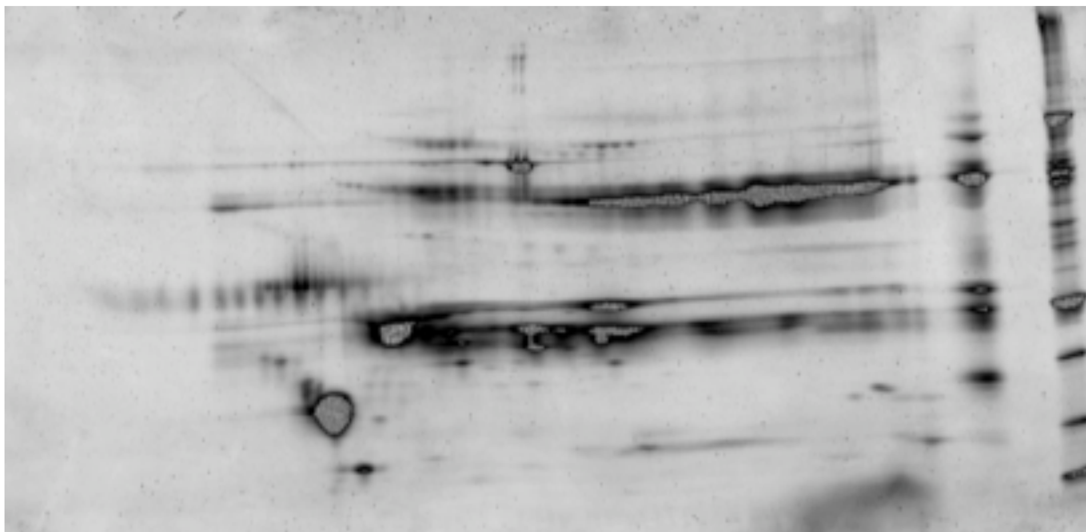

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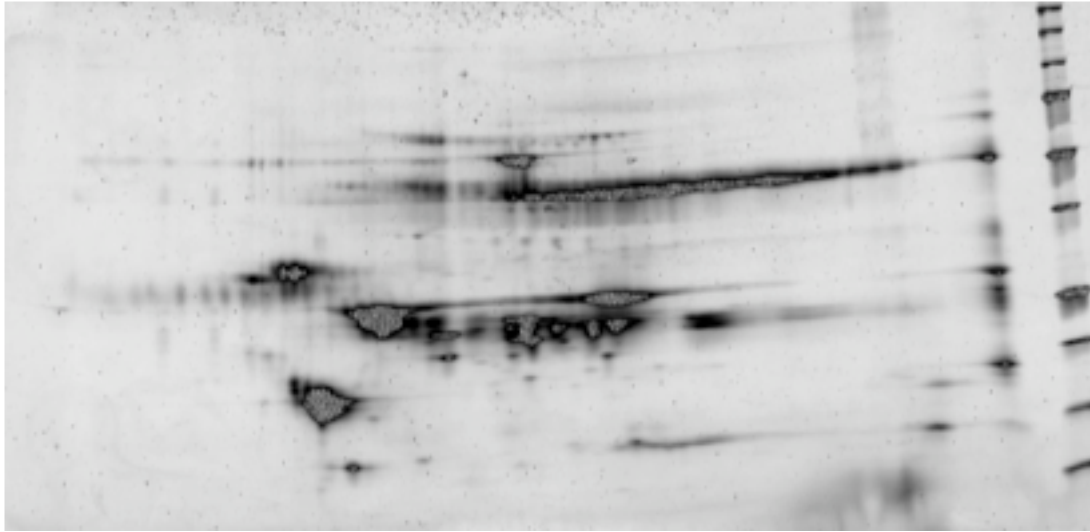

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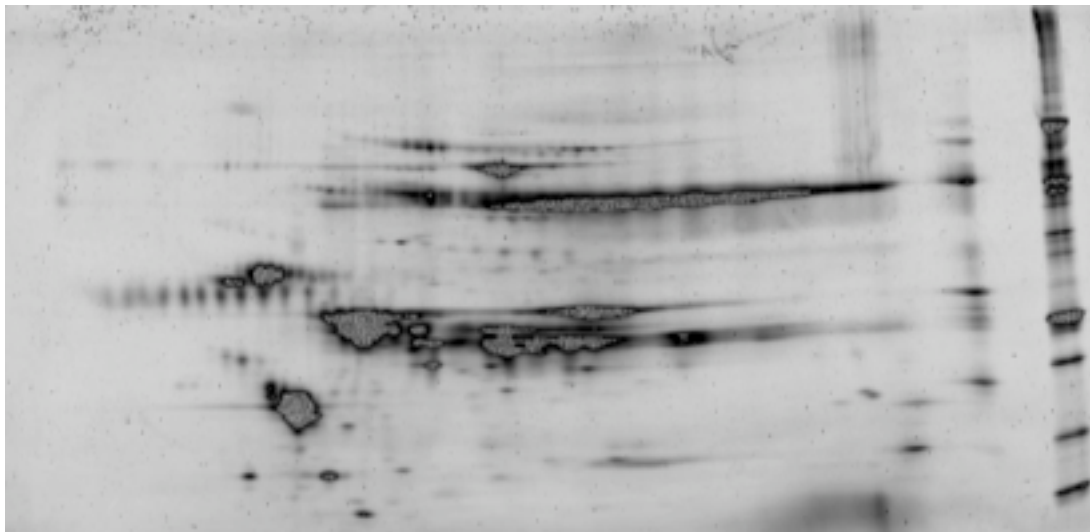

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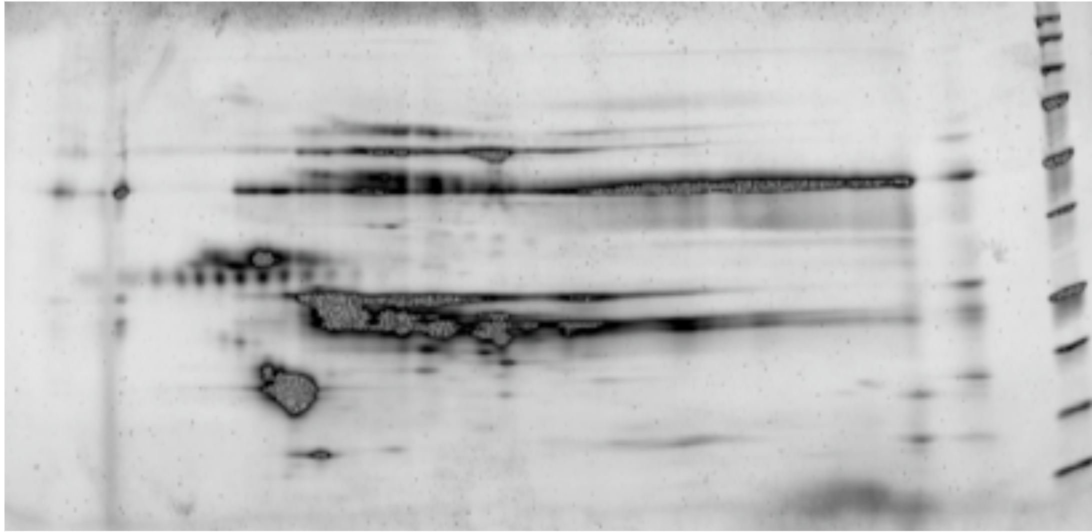

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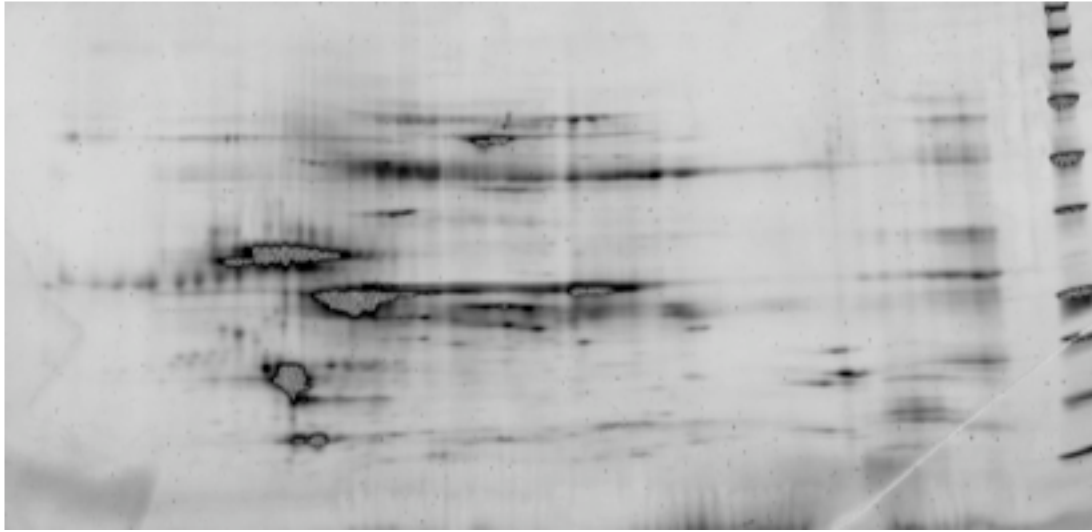

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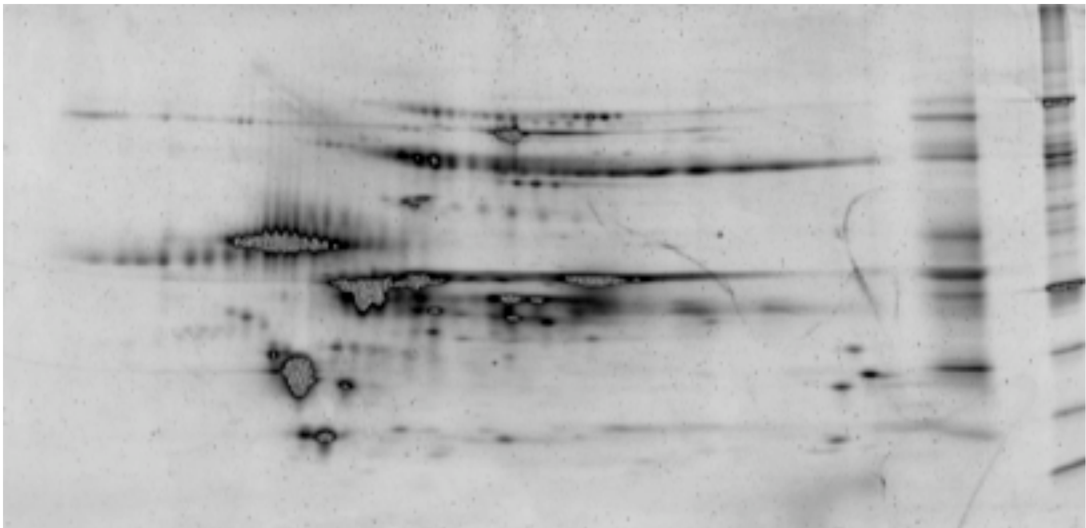

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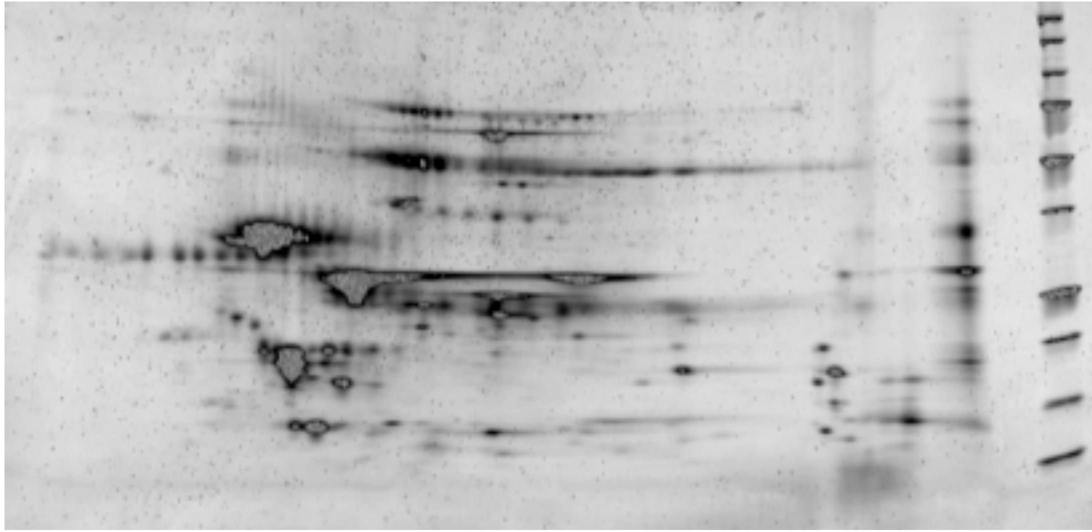

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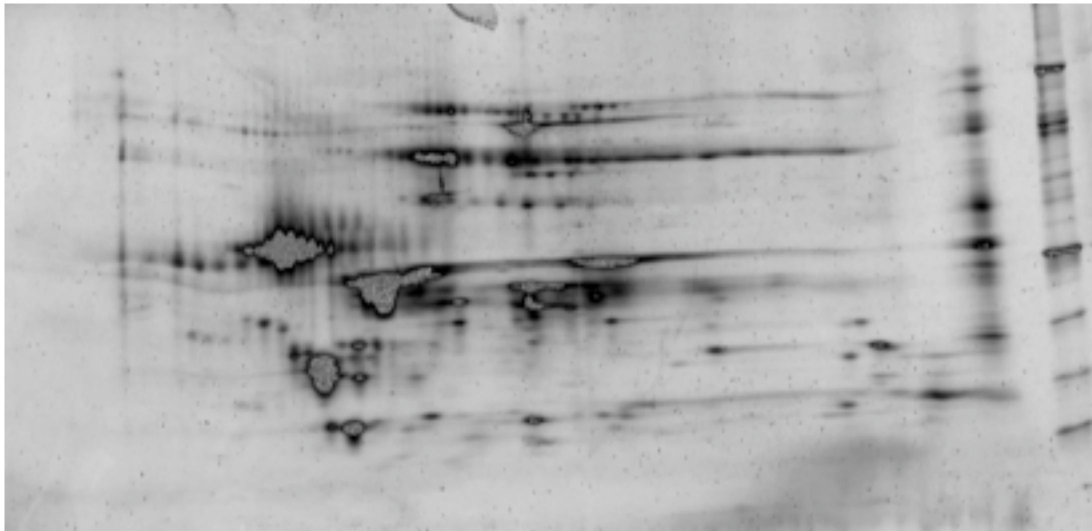

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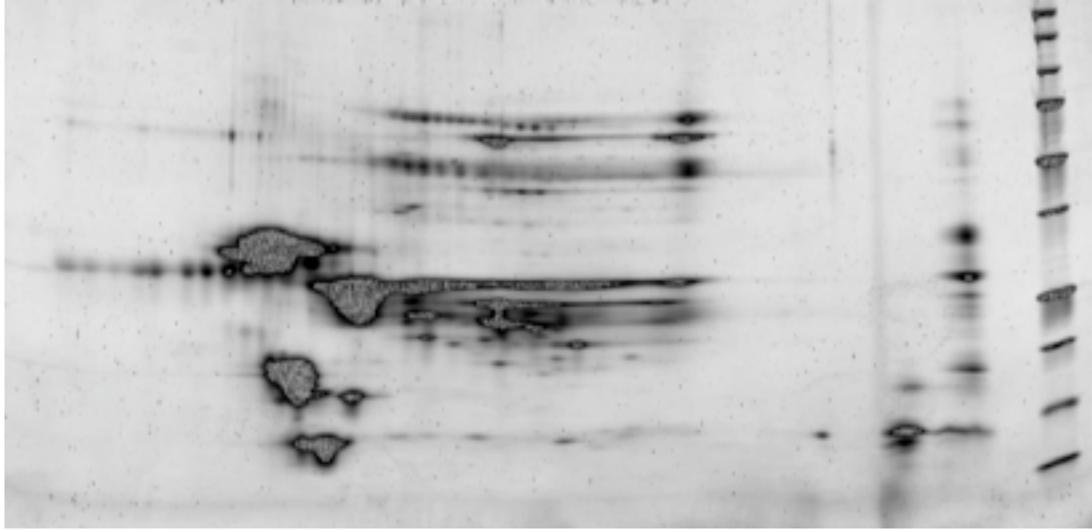

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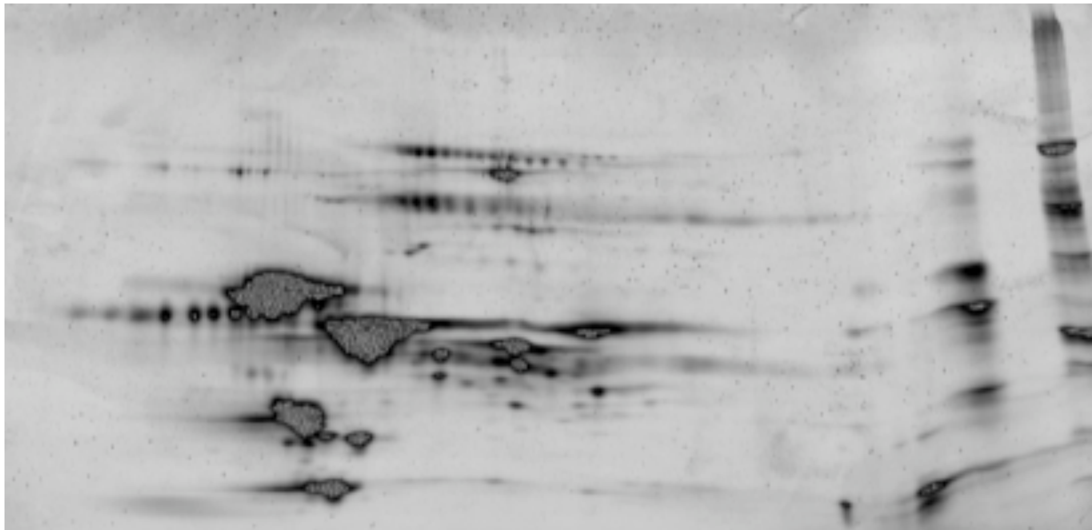

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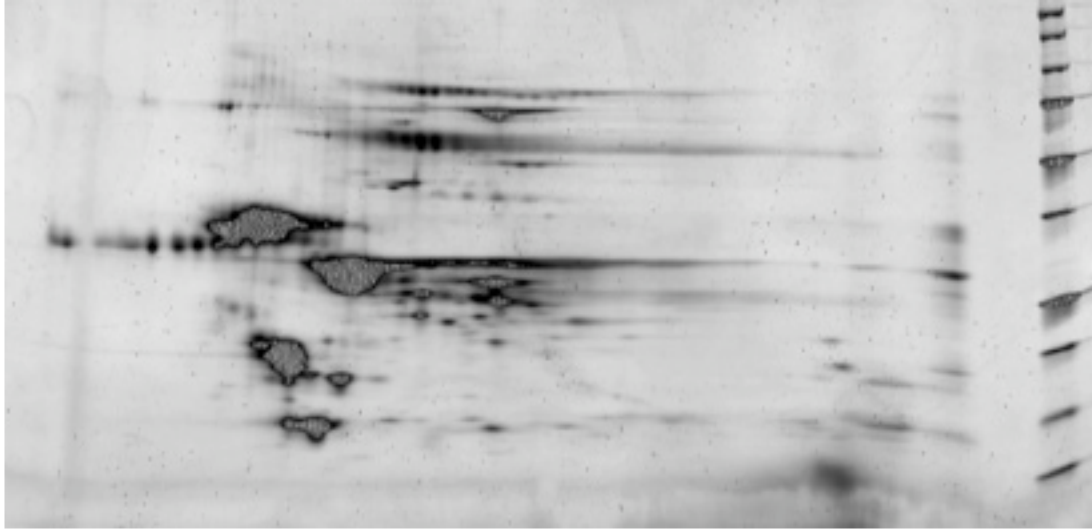

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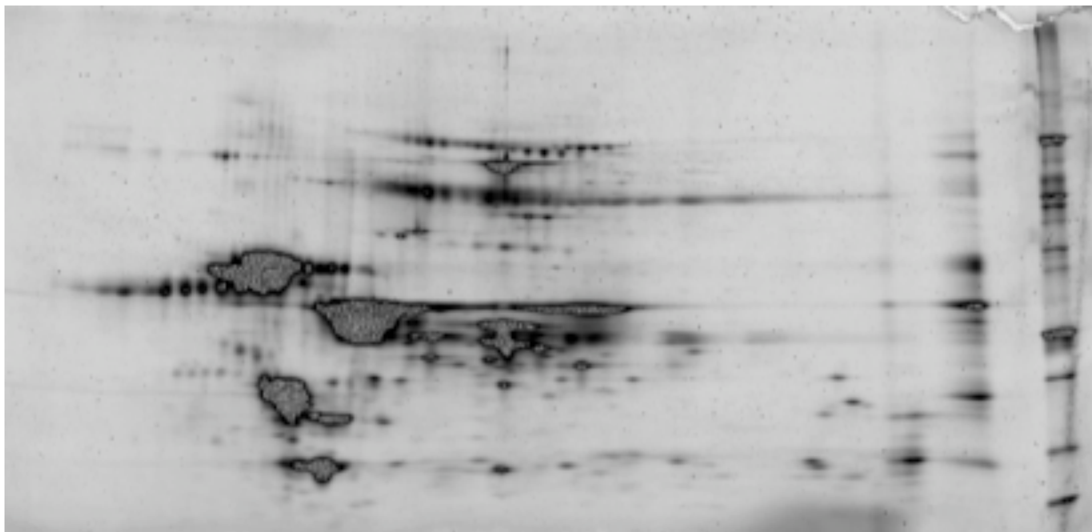

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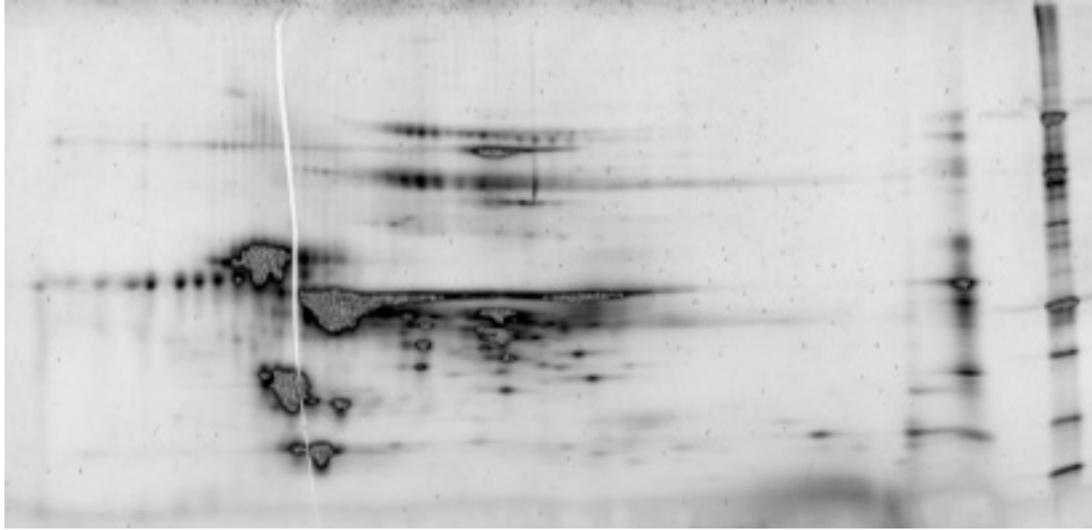

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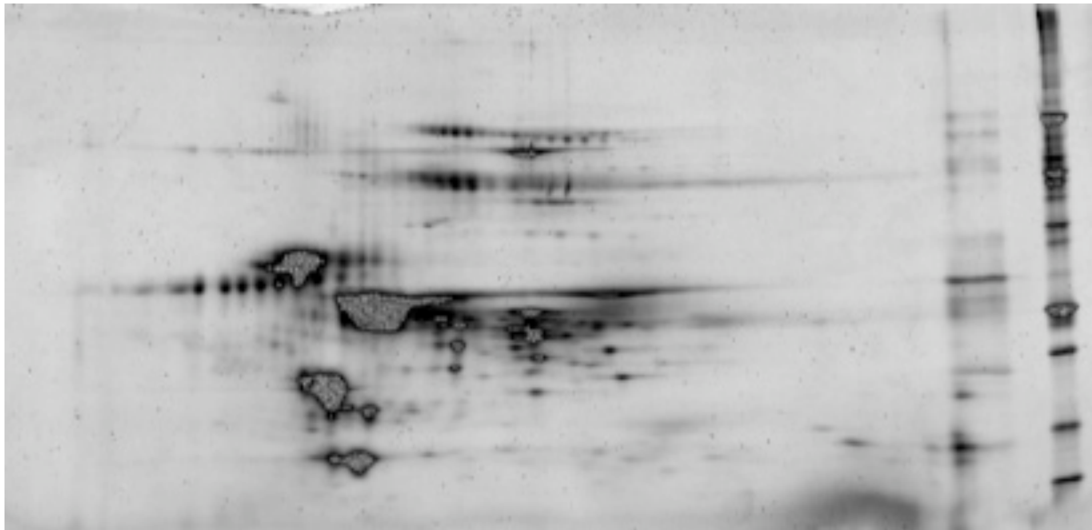

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