F4ac-Fimbrial-Binding Proteins in Porcine Milk And the Absorption of Colostral Proteins by Piglets

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ABSTRACT

F4 positive enterotoxigenic *Escherichia coli* (ETEC) is the most common pathogen causing neonatal diarrhea in piglets. The pathogenesis requires the attachment of ETEC to the intestinal brush border, mediated by F4 fimbria. Colostral anti-F4 antibodies and some non-immunoglobulin porcine skim milk proteins can bind F4 and prevent colonization and infection by F4-positive ETEC. Little is known, however, about the F4-binding ability of porcine milk fat globule membrane (MFGM) proteins. In addition, the knowledge of the absorption of porcine colostral proteins into the blood of neonatal piglets is limited, despite the well accepted concept that in neonatal piglets, protein absorption from the intestine is non-selective.

In this study, the ability of porcine MFGM proteins to bind purified F4ac (one of the three subtypes of F4 fimbriae) was investigated. Porcine MFGM proteins were first separated by 2D SDS-PAGE and subsequently identified by mass spectrometry. Overlay western Blot was then employed to demonstrate the interaction between porcine MFGM proteins and purified F4ac. Several proteins from porcine MFGM reacted with F4ac, and of these, lactadherin, butyrophilin, adipophilin, and acyl-CoA synthetase 3 reacted strongly. The biological function of these proteins *in vivo* was not investigated but it is possible that their interaction with F4ac positive ETEC interferes with bacterial attachment and colonization.

In order to investigate protein absorption by neonatal piglets after natural suckling, the protein profiles of the plasma of pre-suckling and 24 h post-suckling neonatal piglets were studied by 2D SDS-PAGE. Those plasma proteins that increased prominently after suckling were then identified by mass spectrometry. Only immunoglobulins were unequivocally determined to be absorbed, because they were absent before suckling and present in large

quantity in plasma 24 h after suckling. The absorption of other colostral proteins was either equivocal or not detectable by our detection methods. These results suggest that, unlike immunoglobulins, major non-immunoglobulin proteins in porcine colostrum may not be absorbed into systemic circulation in substantial amounts.

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1D	one-dimensional
2D	two-dimensional
ACS3	acyl-CoA synthetase 3
AIDA	adhesin involved in diffuse adherence
BSA	bovine serum albumin
CV	column volume
DAEC	diffusely adhering *E. coli*
DTT	dithiothreitol
E. coli	*Escherichia coli*
EAST1	enteroaggregative E. coli heat-stable enterotoxin
ED	edema disease
EGF	epidermal growth factor
EPEC	enteropathogenic *E. coli*
ER	endoplasmic reticulum
ETEC	enterotoxigenic *E. coli*
FABP	fatty acid binding protein
FAD	flavin adenine dinucleotide
HBGAs	H-2 histo-blood group antigens
Ig	immunoglobulin
IGF-I	insulin-like growth factor I
IPG	immobilized pH gradient
LC MS/MS	liquid chromatography mass spectrometry/mass spectrometry

LDL	low density lipoprotein
LF	lactoferrin
LPS	lipopolysaccharide
LT	heat labile enterotoxin
MFGM	milk fat globule membrane
MS	mass spectrometry
MUC1	mucin 1
MUC15	mucin 15
MW	molecular weight
NeuGc-Gm3	N-glycolylneuraminyl-lactosyl-ceramide
NeuGc-SPG	N-glycolylsialoparagloboside
PAGE	polyacrylamide gel electrophoresis
PAS	periodic acid-Schiff
PfEMP-1	*P. falciparum*-erythrocyte-membrane-protein-1
pI	isoelectric point
PMF	Peptide mass fingerprinting
PVDF	polyvinylidene fluoride
PWD	post-weaning diarrhea
SDS	sodium dodecyl sulfate
ST	heat-stable enterotoxin
TIR	translocated intimin receptor
XDH/XO	xanthine dehydrogenase/oxidase

1. Literature review

1.1. Introduction

It is well known that colostrum and milk provide tremendous immune protection for neonates against local intestinal and systemic infectious diseases. Farm animals, including pigs, are born hypogammaglobulinemic (low concentration of immunoglobulin in the blood) because the epitheliochorial placenta prevents transfer of large proteins from dam to fetus during gestation. After suckling, a large amount of maternal immunoglobulins is absorbed from the gut of the neonates into the bloodstream, resulting in neonatal protection against pathogens. The importance of this passive transfer of immunoglobulins is well characterized and indisputable, but the ability to absorb immunoglobulins decreases sharply after 24 h of age [1]. There is, however, an increasing body of evidence demonstrating that non-immunoglobulin colostral and milk proteins are also involved in neonatal protection against disease. Failure of early proper colostral ingestion by neonates results in increased neonatal morbidity and mortality.

Escherichia coli (*E. coli*) causes a variety of intestinal and systemic diseases in neonatal and postweaning pigs and it is one of the most important pathogens in the swine industry. Neonatal diarrhea, which causes significant direct and indirect economic losses in the modern swine industry, is most commonly caused by enterotoxigenic *E. coli* (ETEC). Fimbrial adhesins, exotoxins and endotoxin are the major virulence factors of ETEC. Fimbrial adhesion of ETEC to the intestine is the necessary first step of infection leading to neonatal diarrhea. Anti-fimbrial antibodies in colostrum and milk prevent attachment of ETEC to the intestinal mucosa and protect piglets against diarrhea. In addition, various

non-immunoglobulin proteins in porcine milk have been reported to provide antibacterial defenses *in vitro*. Several proteins isolated from porcine skim milk fractions have been shown to bind either F4-fimbriae or endotoxin of *E. coli* [2, 3] or to kill bacteria *in vitro* [4]. Investigation of the potential defense role of the fat fraction of porcine milk has been limited. In addition, little is known about potential absorption of non-immunoglobulin defense proteins into the systemic circulation of neonates. Accordingly, the aims of studies presented in this thesis were to investigate the presence of F4-binding proteins in milk fat globule membrane (MFGM) isolated from the fat fraction of porcine milk and to determine the absorption of non-immunoglobulin proteins from milk by neonatal piglets.

1.2. Porcine milk proteins

1.2.1. Fractions of milk

By differential centrifugation, milk can be divided into four main fractions, namely milk fat, skim milk, whey milk and caseins. Milk fat is separated from the whole milk by low speed centrifugation. Skim milk refers to the non-fat fraction of milk. After ultracentrifugation, caseins can be pelleted from the clear supernatant termed whey milk. One should note, however, that the separation of these fractions by centrifugation procedures is not 100% efficient and cross contamination of components among these fractions cannot be completely eliminated.

1.2.2. Milk fat globule membrane proteins

In milk, fat appears as droplets which are called milk fat globules. These globules consist of a central triglyceride core enclosed by the milk fat globule membrane (MFGM) which has three layers. The innermost layer is derived from the endoplasmic reticulum (ER) of mammary glandular epithelium, and the outer double layers are formed from apical

membranes of the mammary glandular epithelial cells. The proteins associated with the MFGM are called MFGM proteins. Eight major and many minor proteins have been identified in human, murine and bovine MFGM [5, 6].

1.2.2.1. Major MFGM proteins

1.2.2.1.1. Mucin 1

Mucin 1 (MUC1) is a highly glycosylated large protein (molecular weight [MW] ranges from 160 kDa to > 200 kDa), and is present in ruminant, mouse and human MFGM as well as in many tissues (e.g. salivary gland, kidney, esophageal epithelium and stomach) [7, 8]. MUC1 is a filamentous type I membrane protein which has a single transmembrane anchor, an N-terminal exoplasmic domain and a short C-terminal cytoplasmic tail [6]. The amino acid sequence of the exoplasmic domain has a repetitive character [6]. Although structurally a membrane protein, MUC1 is not tightly bound to the MFGM, since cooling and stirring are sufficient to cause it to release [9]; accordingly, MUC1 is found in both MFGM and skim milk [10].

MUC1 is present in the mucosal secretion that forms a physical barrier protecting against the mucosal invasion of pathogens [8]. The carbohydrate structural moiety of MUC1 interacts with various pathogens *in vitro*; thus, a potential *in vivo* defense role has been hypothesized [10]. Mucin present in human MFGM inhibited binding of S-fimbriated *E. coli* to buccal epithelial cells [11]. Bovine MUC1 interfered with murine rotaviral infectivity both *in vitro* and *in vivo* [12]. Human MUC1 was demonstrated to aggregate poxvirus and inhibit its activity [13] and to inhibit infection of human T lymphoblastoid cells by HIV-1 [14].

1.2.2.1.2. Xanthine dehydrogenase/oxidase

Xanthine dehydrogenase/oxidase (XDH/XO) is a homodimer with MW ~ 300 kDa [6]. Each monomer has four domains: a molybdopterin cofactor, two Fe_2/S_2 clusters and one flavin adenine dinucleotide (FAD) [15]. XDH/XO is present in a variety of tissues including lactational mammary glandular epithelium and MFGM [16].

The enzymatic function of XDH/XO has been well studied [15]. XDH/XO catalyzes the oxidation of purines to uric acid by addition of oxygen from H_2O [6]; however, various inactive forms of the enzyme have been found, the functions of which are not clear [17, 18]. Two inactive forms (desulfo and demolybdo) of the enzyme have been reported and XDH/XO purified from milk is often inactive [6, 19, 20]. Although it has been suggested that the inactive XDH/XO can be activated in the gut [21], it is not clear whether the main function of XDH/XO in milk is as an enzyme. Alternatively, XDH/XO has been suspected to play an important role during the secretion of milk fat, facilitated by formation of a complex with butyrophilin and adipophilin [6, 22, 23]. This heterotrimer model of milk fat secretion has been previously well documented and accepted even though a recent study suggested that butyrophilin has a more dominant role than the other two proteins [24]. Reactive oxygen species generated by XDH/XO may also act as antibacterial components [6, 25, 26].

1.2.2.1.3. Mucin 15

Mucin 15 (MUC15) from MFGM was previously poorly characterized. This protein is highly glycosylated and stains well with Periodic acid-Schiff (PAS) stain. Several MFGM proteins stain positive with PAS and initially the name PAS III was used for this protein. Recently, PAS III from bovine milk has been characterized and renamed MUC15 [27]. Carbohydrates represent ~65% of the total molecular weight of bovine MUC15 [28]. Based

on PAGE migration, its molecular weight is 95 kDa to over 100 kDa [6]. There is no antigenic cross reactivity between PAS III and MUC1 [6]. The primary amino acid sequence shows that MUC15 is a type I membrane protein. It has an N-terminal exoplasmic domain which is rich in serine, threonine, and proline, a transmembrane domain and a C-terminal cytoplasmic tail. The same study showed that the MUC15 gene is expressed in a wide range of human and bovine parenchymal organs, lymphoid tissues, reproductive organs and on various mucosal surfaces [27, 29-31]. The function of MUC15 is not known.

1.2.2.1.4. CD36

CD36 is a highly glycosylated integral protein of the MFGM and is also present on monocytes, platelets, microvascular endothelial cells, adipocytes and many other tissues [6, 32]. Its structure is characterized by the presence of one transmembrane domain at both N-terminus and C-terminus and an exoplasmic hydrophobic region [33].

The functions of CD36, which acts as an adhesive protein and binds to a large number of ligands, have been reviewed extensively [32, 33]. Briefly, CD36 is a multifunctional protein involved, *inter alia*, in coagulation, apoptosis and atherosclerosis. It binds to collagen and contributes to platelet activation and aggregation. CD36 acts as a receptor for thrombospondin which can induce apoptosis via activated FAS ligand and associated caspase cascades. In addition, CD36 acts as a scavenger receptor and binds to apoptotic cells facilitating their phagocytosis [34]. CD36 on the surface of macrophages mediates the intake of oxidized low density lipoproteins (LDL) which are associated with formation of the foam cells characteristic of atherosclerosis [32, 35, 36]. This binding capacity to LDL [37] has recently become a focus of very active research. CD36 expressed on vascular endothelial cells and monocytes binds to *Plasmodium falciparum* infected red blood cells via *P. falciparum*-erythrocyte-membrane-protein-1 (PfEMP-1), assisting the localization of the

organism in peripheral tissues and the progression of infection [38, 39].

Although the function of CD36 in milk and the mammary gland is not clear, it has been hypothesized that CD36 acts as a thrombospondin receptor or functions as a transporter of long-chain fatty acids [6]. Recent data showed that CD36 and fatty acid binding protein (FABP) function in concert to increase the transport of fatty acid in heart [40], and perhaps CD36 and FABP have the same function in the milk, since FABP is also one of the major MFGM proteins [6]. Another possibility is that CD36 together with lactadherin, another major MFGM protein, facilitates the clearance of apoptotic cells in the mammary gland [34].

1.2.2.1.5. Butyrophilin

Butyrophilin is the most abundant protein on bovine MFGM, comprising over 20% of the total MFGM proteins [41, 42]. Butyrophilin, named from the Greek roots “butyros” (butter) and “philos” (loving), has an affinity for butter fat [43]. Butyrophilin is a type I membrane glycoprotein [44] considered to be an integral protein of the MFGM because it resists extraction by chaotropic agents and nonionic detergents [43]. Reducing agents (e.g. 2-mercaptoethanol) are needed to solubilize butyrophilin [45], which suggests that a disulfide bond stabilizes butyrophilin on the MFGM.

Butyrophilin belongs to the immunoglobulin superfamily based on its two immunoglobulin-like N-terminal folds (IgI and IgC1) [43]. A 27 hydrophobic amino acid segment of butyrophilin serves as the transmembrane domain. The C-terminal cytoplasmic segment is a highly conserved region known as B30.2 and is suspected to be the binding domain for xanthine dehydrogenase/oxidase [6].

Butyrophilin has a regulatory function in milk fat secretion from mammary epithelial cells. Secretion of milk lipid in mice with disruption or elimination of the butyrophilin gene is severely compromised in comparison to wild-type animals. As a result of butyrophilin

gene ablation, unstable and extremely large lipid droplets (up to 7 times larger than the diameter of wild-type) lacking MFGM accumulate in the mammary luminal spaces. The concentration of skim-milk proteins is not affected. Interestingly, probably due to the inability to get sufficient milk, approximately half the pups suckling dams lacking the butyrophilin gene died within the first 20 days, and weaning weights of the surviving pups were 60-80% of those suckling wild-type mice [46].

The concept that milk fat secretion is mediated by a complex of butyrophilin, XDH/XO and adipophilin is well accepted [47-49]. A recent study based on freeze-fracture immunocytochemistry suggests, however, that butyrophilin self-aggregates and alone controls the milk fat secretion [24].

It is not clear whether butyrophilin has any additional functions, even though it belongs to the immunoglobulin superfamily which contains a variety of adhesive proteins involved in important biological functions [50]. Several butyrophilin-like proteins have either potential receptor functions [51, 52] or T-cell regulatory functions [53, 54], but the biological importance of these newly identified activities has not been defined.

1.2.2.1.6. Lactadherin

Lactadherin, a 46 kDa peripheral glycoprotein, is a major protein on MFGM. There is no transmembrane hydrophobic sequence in its primary structure [6]. Amino acid sequencing revealed that lactadherin has N-terminal epidermal growth factor (EGF)-like domain(s) which contains an RGD cell adhesion sequence and C-terminal tandem repeats similar to C1 and C2 domains of coagulation factors V and VIII [6]. There is a putative amphipathic phospholipid-binding α-helix structure that may serve as a binding site to the membrane. Lactadherin is also found in several other cells, e.g. sperm and macrophages [6].

Multiple functions of lactadherin have been reported. The RGD motif in the EGF-like domain may mediate binding to integrin and this has been confirmed for bovine lactadherin [55]. Lactadherin is involved in cellular adhesion [56], neovascularization [57] and clearance of apoptotic cells [47, 58]. Lactadherin (aka p47) is also expressed on the membrane of sperm where it may be involved in adhesion during fertilization [59]. Purified porcine lactadherin is able to bind glycoprotein ZP on the zona pellucida of oocytes [60].

Our understanding of the functions of lactadherin in colostrum and milk (including in MFGM) is limited. Lactadherin binds to apoptotic cells and facilitates their phagocytosis during postlactational mammary involution [47]. Human, but not bovine lactadherin, possesses anti-rotaviral activity *in vitro* [61] and is associated with protection of human infants against rotaviral infection [62]. Porcine lactadherin has binding affinity for F4ac-fimbriae and decreases binding of F4ac *E. coli* to intestinal villi *ex vivo* [3].

1.2.2.1.7. Adipocyte differentiation-related protein (Adipophilin)

This MFGM protein has been overlooked for a long time because its molecular weight is similar to that of lactadherin (46 kDa) [6]; thus, when subjected to one-dimensional (1D) SDS-PAGE, adipophilin and lactadherin are not separated. Using two-dimensional (2D) SDS-PAGE, however, they are visualized as two different proteins with similar molecular weights but different isoelectric points [6]. Initially, this protein was thought to be specifically expressed in adipocytes and termed adipocyte differentiation-related protein [63]; however, later it was demonstrated that it is also expressed in a variety of cultured cell and in tissues such as lactating mammary epithelium, steroid-producing adrenal cortical cells, Sertoli and Leydig cells in the testis, and in lipid laden hepatocytes in the cirrhotic liver of alcoholics [64]. Now, the name adipophilin is being more preferably used for this protein [6, 64, 65].

The amino acid sequence of adipophilin is similar to those of perilipin and TIP47; accordingly, these three proteins are now called "PAT proteins" (perilipin, adipophilin and TIP47) [6]. PAT proteins are all associated with lipid droplets [66, 67]. Since PAT proteins do not possess hydrophobic amino acids, the interaction between these proteins and lipid droplets has not been clear [63, 68]. More recently, however, an adipophilin fragment between 189 and 205 amino acids has been suggested to have an α-helical conformation proposed to interact with lipid droplets. [65].

The function of adipophilin still requires clarification. The association of adipophilin with cytoplasmic lipid droplets suggests that it is involved in lipid storage and metabolism [69]. Adipophilin enhances *in vitro* uptake of long-chain fatty acids [70]. It may be involved in lipid biogenesis, since it was recently found that lipid droplets are cupped, but not enclosed, by the endoplasmic reticulum at an adipophilin-rich site [71]. An association between the expression of adipophilin and development of artherosclerosis has also been demonstrated [72]. In mammary epithelium, complexing of adipophilin with butyrophilin and xanthine dehydrogenase/oxidase may facilitate lipid secretion in milk [47-49].

1.2.2.1.8. Fatty acid binding protein

Fatty acid binding protein (FABP) of MFGM appears as a ~13 kDa protein band in SDS-PAGE [6]. It was initially called mammary-derived growth inhibitor based on its ability to inhibit growth of mammary carcinoma cells [73] and later found to have a similar amino acid sequence to heart FABP in rats [74]. Subsequently it was determined that FABP in MFGM consists of a mixture of heart type and adipocyte type FABP [75], with the heart type predominating [6]. The characteristic of the structure of FABP is the presence of β-barrel composed of 10 antiparallel β-strands [76], which create a cavity lined by both hydrophobic and polar amino acids forming the binding ligands for fatty acids [76].

The function of milk FABP is not yet clear. It may transport intracellular fatty acids, control lipid metabolism and increase cytoplasmic lipid droplets [6]. FABP inhibits growth and stimulates differentiation of mammary epithelium [6], but any growth regulatory function has not been determined for the intestinal epithelium of neonates. Using affinity chromatography heart FABP was identified as one of the F4ac-fimbrial-binding proteins in porcine skim milk [3]. More studies are needed to determine if FABP is involved in innate immunity.

1.2.2.2. MFGM proteomics

The protein components of MFGM have recently attracted attention from various research groups working on different species even though the comparative knowledge about MFGM protein profiles is still limited. To date, MFGM proteins have been characterized only in human, mouse and bovine milk [5, 26, 77-79]. Using various experimental approaches, 107 proteins (derived from 39 genes) have been identified in human MFGM [78], 120 proteins in bovine MFGM [79] and 29 in mouse MFGM [5], but interestingly, only 15 of the bovine proteins were the same as those from human and mouse MFGM [79]. Accordingly, these results may indicate that differences exist in MFGM proteomes among various mammalian species; however, it is also possible that these differences were due to different experimental methodology employed. MFGM proteins can be grouped, based on function, into proteins involved in membrane trafficking, cell signaling, fat transport/metabolism, protein synthesis/folding, immune proteins and residual milk proteins. Also interesting to note is that the expression and concentration of several bovine MFGM proteins changes during the course of lactation. Twenty six proteins, including several major MFGM proteins, are up-regulated whereas 19 proteins, including some lipoproteins, are down-regulated [49, 80].

1.2.3. Immune functions of porcine skim milk proteins

The physico-chemical composition of porcine milk proteins has been comprehensively reviewed [81]. This section briefly summarizes some skim milk proteins in porcine milk that have immune functions. Immune components in the milk have been previously reviewed by different groups [4, 82, 83].

1.2.3.1. Immunoglobulins

Immunoglobulins are a well known immune component in colostrum and milk, providing an unequivocal protective activity against pathogens. This protection is considered to be specific, i.e., specific maternal antibody binds to specific antigen after the dam has been exposed or vaccinated with this antigen.

1.2.3.2. Caseins

A specific characteristic of porcine milk is the high concentration of caseins [81]. Caseins can be subdivided into α-casein, β-casein, κ-casein and casein micelles. Casein is a source of amino acids for the neonates; in addition, it has been increasingly reported to have potential immune functions. Antimicrobial activities have been demonstrated for many casein-derived peptides from human [84], bovine [85-87] and rabbit milk [88]. Semi-purified porcine casocidin-I has growth inhibitory activity against a wide spectrum of porcine pathogens [4]. Alpha-S_1 casein and β-casein have binding affinity to F4 and lipopolysaccharide (LPS) isolated from *E. coli* [2, 3]. Further investigations are needed to determine if various caseins or their fragments are important defense components *in vivo*.

1.2.3.3. Lactoferrin

Lactoferrin is an iron-binding protein present in milk, other body fluids and neutrophils [89]. The antimicrobial activity of human and bovine lactoferrin and its peptides has been

well documented [90-92]. Bovine lactoferrin protects germ-free piglets from endotoxic shock [93]. Porcine lactoferrin has binding affinity for LPS and pepsin-digested porcine lactoferrin inhibits growth of a wide spectrum of porcine bacterial pathogens [2].

Several other proteins in porcine skim milk exhibit binding affinity for LPS and F4ac in addition to those mentioned above; however, their biological relevance *in vivo* has not been determined [2, 3].

1.3. Pathogenic *E. coli* in pigs

Escherichia coli (*E. coli*) is a gram-negative, rod-like bacterium and is one of the major bacterial components of the normal intestinal flora in many species; however, some *E. coli* strains are pathogenic and can cause intestinal and/or extra-intestinal diseases in humans and animals. Pathogenic *E. coli* can cause a variety of diseases in pigs, including neonatal and post-weaning diarrhea, septicemia, enterotoxemia/edema disease, mastitis and urinary tract infection. This section is limited to a review of *E. coli*-associated porcine diarrhea and septicemia, which are the most important *E. coli*-associated diseases in pigs.

1.3.1. Diarrheagenic *E. coli*

Intestinal *E. coli* infection has been intensively studied and several diarrheagenic pathotypes of *E. coli* have been described in humans and animals. In pigs, enterotoxigenic *E. coli* (ETEC) is the most common type and causes neonatal and post-weaning diarrhea (PWD) [94]. Enteropathogenic *E. coli* (EPEC) is also associated with PWD.

1.3.1.1. Enterotoxigenic *E. coli* (ETEC)

Neonatal diarrhea causes significant economic loss in the pig industry, with ETEC being one of the most important pathogens. Affected piglets are usually under one week old and suffer varying degrees of watery diarrhea, dehydration and metabolic acidosis [95]. The

first step of bacterial colonization is the adhesin-mediated attachment to the intestinal epithelium [94]. Adhesins are important virulence factors of ETEC. ETEC strains that cause neonatal diarrhea may express F4, F5, F6 and F41 fimbrial adhesins, with F4-positive (F4+) strains being most common [95]. Adhesin involved in diffuse adherence (AIDA), a recently identified non-fimbrial adhesin, is also expressed in some porcine ETEC [96]. After attachment, ETEC multiply and excrete enterotoxins (LT, STa, STb, EAST1) which cause disturbed ion and water exchange by different molecular mechanisms, inducing secretory diarrhea in the absence of morphological changes of the intestinal epithelium [96, 97].

ETEC can also cause diarrhea in post-weaning pigs, usually in the first week after weaning. PWD is usually not as profuse as neonatal diarrhea. The feces are less watery and animals demonstrate a loss of weight gain [98]. Some ETEC strains can cause both neonatal diarrhea and PWD, e.g. some F4+ strains; however, other ETEC strains, such as F18+ *E. coli*, cause disease only in the post-weaning age group [95]. Some strains that typically cause edema disease are also associated with PWD and these two disease entities can be concurrent in one individual [95]. Adhesins involved in post-weaning ETEC diarrhea include F4, F18 and AIDA-I [95, 97]. That different strains of ETEC can cause disease in different age groups of pigs is probably due to age-related changes in expression of receptors in intestinal brush border and mucus [99, 100].

1.3.1.2. Enteropathogenic *E. coli* (EPEC)

Porcine EPEC strains typically cause PWD. EPEC do not express fimbrial adhesins [97]; instead, due to a non-fimbrial adhesin called intimin, they cause attaching and effacing lesions of the brush border of the intestinal epithelial cells [101]. The mechanism of diarrhea caused by EPEC is not entirely understood. Morphological changes of the

intestinal mucosal surface which cause malabsorption may be one explanation; however, other mechanisms may also be involved [97].

1.3.2. *E. coli* septicemia and endotoxemia

Extraintestinal *E. coli* strains can pass through the intestinal epithelium, enter the blood stream and cause septicemia with the release of LPS (aka endotoxin). Occasionally, LPS from ETEC may be absorbed from the intestine into the circulation, resulting in endotoxemia [96]. LPS triggers activation of macrophages and may induce an uncontrolled cascade of cytokine secretion resulting in shock and death [102].

1.3.3. Fimbrial adhesins

Fimbriae are rod-like protein structures on the surface of bacteria. ETEC typically use fimbriae to attach to the intestinal epithelial cells and this attachment is thought to be the first step of colonization. In pigs, ETEC may express F4, F5, F6, F18 or F41 fimbrial adhesins. ETEC fimbriae are composed of multiple repeating major subunits and several different minor subunits. Fimbriae bind to receptors on the brush border of the intestinal epithelial cell or to receptors in intestinal mucus. The binding between fimbriae and receptors is based on a lectin-glycan interaction, namely, a carbohydrate-binding domain of a protein binds to a carbohydrate moiety of a receptor. Accordingly, fimbrial receptors are most commonly glycoproteins or glycolipids.

1.3.3.1. F4 fimbriae

F4+ *E. coli* is the most common ETEC strain in pigs, causing both neonatal and post-weaning diarrhea [95]. F4 fimbriae are long filamentous surface appendages. F4 is divided into 3 subtypes, namely, F4ab, F4ac and F4ad, based on differences in primary amino acid sequences of the major FaeG subunit [99, 103]. The antigenically conserved segment

is “a”, while “b”, “c” and “d” refer to the variable antigenic segments. F4ac-positive ETEC is the most prevalent strain of porcine ETEC [104].

In addition to the major FaeG subunit, there are also several minor subunits, namely, FaeC, FaeD, FaeE, FaeF, FaeH, FaeI and probably FaeJ [99]. However, the major FaeG subunit, consisting of a ~ 27.5 kDa protein, is considered to possess both structural and adhesive functions [97]. F4 fimbriae have multiple exposed binding sites that recognize and bind to corresponding receptors [97].

Based on genetically determined differences in the ability of porcine intestines to bind the fimbrial subtypes F4ab, F4ac and F4ad *E. coli*, 6 different phenotypes (A-F) of pigs have been identified [105, 106] (Table 1.1). Phenotype A pigs bind all three F4 subtypes; phenotype B binds F4ab and F4ac; phenotype C binds F4ab and F4ad; phenotype D binds F4ad; phenotype E lacks binding and phenotype F binds only F4ab. It should be noted that phenotype C is poorly characterized and there is some concern that the binding of F4ab to this phenotype is artifactual, thus, phenotype C may be the same as phenotype D [107].

Intestinal receptors for F4 fimbriae are either glycoproteins or glycolipids [99, 107]. Van den Broeck and co-workers reviewed in detail the previous efforts to characterize F4 intestinal receptors [99]. The molecular weights of intestinal brush border receptors for different F4 subtypes are summarized in Table 1.2. A 4-receptor model (receptor *bcd*, *bc*, *b* and *d*) was established to explain the differences in susceptibility of various host phenotypes to the 3 subtypes of F4 *E. coli* [99] (Table 1.1). The *bcd* receptor has affinity for all 3 F4 fimbria subtypes; *bc* for F4ab and F4ac; *b* for F4ab and *d* for F4ad. Thus, phenotype A pigs have both *bcd* and *bc* receptors; phenotype B animals the *bc* receptor, phenotype C and D the *d* receptor (considering phenotype C may be the same as phenotype D); phenotype E lacks receptors for any F4 fimbriae; and phenotype F pigs have the *b* receptor [99]. Further, within the receptors summarized in Table 1.2, the 210 and 240 kDa proteins may represent

the *bc* receptor, the 74 kDa protein the *b* receptor and the set of 45-70 kDa proteins the *bcd* receptor [99]. The *d* receptor is likely a neutral glycosphingolipid [99]. In addition, F4ab and F4ac receptors have also been demonstrated in intestinal mucus (Table 1.2). F4ad mucus receptor(s) have not yet been investigated.

β-galactose residues are thought to be a basic carbohydrate structure recognized and bound by the carbohydrate-binding domain on F4 fimbriae. Galβ1-3GalNAc and Fucα1-2Galβ1-3/4GlcNAc have been proposed to be the main functional structures of F4-receptors [97].

Table 1.1 F4 intestinal brush border receptor model

Phenotypes	Receptors	Binding ability to F4 subtypes
A	*bcd*	ab, ac and ad
	bc	ab and ac
B	*bc*	ab and ac
C (same as D?)	*d*	ab (artifactual?) and ad
D	*d*	ad
E	No receptors	No binding
F	*b*	ab

Table 1.2 Molecular weights of intestinal F4 receptors

F4 subtypes	Brush border receptors (kDa)	Mucus receptors (kDa)
ab	16, 40-70, 74, 210 and 240	25, 30, 40-42, 60
ac	40-70, 210 and 240	25, 30, 60
ad	45-75	N/A

N/A: Not available

1.3.3.2. F5 fimbriae

F5+ ETEC infect pigs, cattle and sheep. F5 is a long fibrillar protein, the major 18.5

kDa subunit (FanC) of which serves as the adhesin [108, 109]. Since F5 mainly attaches to intestine of preweaning pigs, it most frequently causes diarrhea in this age group [96]. The gangliosides N-glycolylneuraminyl-lactosyl-ceramide (NeuGc-Gm3) and N-glycolylsialoparagloboside (NeuGc-SPG) serve as receptors for F5 fimbriae [109].

1.3.3.3. F6 fimbriae

F6 fimbriae are short, pili-like structures identified only on porcine ETEC. F6+ *E. coli* mainly cause disease in neonatal pigs. The F6 fimbria consists of a major repeating subunit (FasA) and several minor subunits, one of which, FasG, acts as the adhesin. This was the first recognition that a minor ETEC fimbrial subunit serves as adhesin [110]. The brush border receptors of F6 have not yet been definitely characterized but are either glycoprotein or glycolipid in nature [111]. Interestingly, that the susceptibility of piglets to F6-positive ETEC declines with increasing age is associated with an increased concentration of mucus receptors rather than a decrease in density of receptors in brush border [100]. Thus, it has been thought that the increased mucus receptors in older pigs bind to F6-positive ETEC and facilitate the clearance of the ETEC by intestinal peristalsis [100].

1.3.3.4. F18 fimbriae

F18ac is associated with PWD worldwide [95]. F18 fimbria consists of the two antigenically different variants F18ab and F18ac [98]. The major subunit of F18 is FedA, but again the adhesin is located on one of the minor subunits, FedF [112]. The expression of the F18 receptor has been shown to increase with age [113]. Although the nature of F18 receptor is not fully understood to date, it is suspected that H-2 histo-blood group antigens (HBGAs) and A-2 HBGAs may be involved in the binding of F18+ *E. coli* to the host [113].

1.3.3.5. F41 fimbriae

F41 fimbriae are filamentous structures, composed of major subunits with a molecular weight of 29.5 kDa [114, 115]. Although F41 is mostly co-expressed with F5 fimbriae, F41 alone was shown to be able to mediate ETEC adherence to intestinal epithelium [96]. Characterization of the F41 receptor(s) is lacking.

1.3.4. Non-fimbrial adhesins

Several non-fimbrial adhesins have been discovered in recent years and adhesin involved in diffuse adherence (AIDA) and intimin have attracted the most interest in veterinary medicine.

1.3.4.1. AIDA-I

AIDA-I was first identified in human diffusely adhering *E. coli* (DAEC) isolated from infantile diarrhea [116]. Later, the prevalence of AIDA-I positive *E. coli* was found to be higher in porcine than in human diarrhea cases [117, 118]. AIDA-I isolated from porcine *E. coli* had a similar molecular weight (100 kDa) and relatively high amino acid homology (78-87%) with the AIDA-I adhesin expressed by human AIDA-I positive *E. coli* strain 2787 [119]. IgG Fc binding protein (~120 kDa) was identified as the most likely candidate to serve as a potential receptor in intestinal mucus for AIDA-I adhesin [119, 120].

1.3.4.2. Intimin

Porcine EPEC typically do not express fimbrial adhesins [96]. They attach intimately to the enterocytes via a non-fimbrial adhesin called intimin, which is a 94 kDa outer membrane protein [121]. Intimin binds to a translocated intimin receptor (TIR) which is expressed by EPEC and then transferred to the host enterocytes [101]. After attachment to

the brush border, EPEC causes attaching and effacing lesions, characterized by loss of microvilli and formation of a pedestal-like extrusion on the enterocyte at the site of adherence. Ultrastructurally, a dense plaque of cytoskeletal filaments is often seen in the pedestal-like structure [97].

1.4. Protein absorption by the porcine intestine

1.4.1. Non-selective protein absorption by the intestine of neonatal piglets

It is now accepted as dogma that the intestine of the neonatal piglet can non-selectively absorb proteins and macromolecules into the bloodstream. The absorption of proteins from gut to blood (so-called "transcytosis") is thought to consists of three steps: 1) endocytosis, i.e., the uptake of macromolecules by the enterocyte; 2) intracellular processing of the molecules; and 3) exocytosis, i.e., the release of the macromolecules into the blood [122].

Intestinal absorption of a variety of proteins and macro- and micromolecules has been investigated in pigs. These includes bovine IgG [123], porcine IgG [124, 125], dextrans of different molecular weights [123, 126, 127], bovine serum albumin [123, 126, 127] and egg proteins [128], and all these were able to be transferred from neonatal (24 h) intestines into blood stream. This leads to the accepted concept that protein absorption by neonatal pigs is a non-selective process [122, 129]. In addition, it has been shown that the efficiency of absorption negatively correlates with molecular size [127].

This non-selective absorption of macromolecules drops significantly at about 24 h after birth [123, 127]. The cessation of macromolecular transfer after this time frame is referred to as gut closure. The mechanism of gut closure is not well understood. The fetal type of porcine enterocytes are able to endocytose macromolecules non-selectively from intestinal lumen [122]. At 6 days postnatally, mature enterocytes, which have much lower endocytotic activity, start to replace fetal enterocytes. This replacement is complete at about

6 weeks after birth [123, 130]. Therefore, during the first 6 days of life, the neonatal porcine enterocytes maintain the ability for non-selective endocytosis. Since gut closure happens at about 24-36h, prior to the enterocyte replacement, the cessation of absorption must be explained by other mechanisms.

Little is known about the intracellular processing of proteins in relationship to gut closure. Since intracellular proteolytic activity of capthepsin B and D was found to be similar in newborn and 6-day old piglets, Ekstrom hypothesized that gut closure is not due to increased intracellular degradation of the endocytosed proteins, but is most likely related to a decrease in exocytosis [123]. This hypothesis has yet to be tested.

Three aspects of intestinal protein absorption after gut closure need to be mentioned: 1) micromolecules (molecular weight less than a few kDa) can be absorbed independent of gut closure [127]; 2) a small amount of macromolecules can be transferred to blood after gut closure [127]; and 3) certain proteins can be selectively absorbed from intestinal lumen into blood by a receptor mediated process termed specific protein absorption.

1.4.2. Selective intestinal protein absorption in piglets

As mentioned above, the absorption of macromolecules, including immunoglobulin, by neonatal piglets has been thought to be primarily non-selective; however, an increasing body of evidence indicates that in addition to the non-selective absorption of proteins through "leaky" epithelium of neonatal intestine, there is also receptor-mediated absorption of some proteins such as lactoferrin and insulin-like growth factor-I, and also of immunoglobulin.

1.4.2.1. Immunoglobulin

The absorption of colostral immunoglobulin by newborn pigs is well documented and has been long regarded as non-selective endocytosis. The results of the recent studies, however, suggest that the immunoglobulin absorption may also involve selective mechanisms:

first, the intestinal transfer of immunoglobulin exceeds that of other macromolecules [131]; further, the efficiency of absorption of porcine IgG by neonatal piglets is higher than that of bovine IgG [132]. Thus, Sangild suggested that the endocytosis of immunoglobulins by neonatal enterocytes is a highly specific process [1]. In enterocytes of rodents, an immunoglobulin Fc receptor has been identified and is responsible for the transport of immunoglobulin [133, 134]. Recently, the immunoglobulin Fc receptor has also been identified and characterized in pigs [135]. The same study showed that 4-week-old pigs are still able to absorb bovine IgG, despite that gut closure occurs much earlier, which made the possibility of non-selective mechanism unlikely. It is possible that non-selective and selective intestinal absorption of immunoglobulins occur together in pre-closure piglets.

1.4.2.2. Lactoferrin (LF)

Lactoferrin is a major ion-binding protein in porcine milk and its antimicrobial activities have been well documented. The fact that oral administration of bovine lactoferrin protects pigs from endotoxic shock indicates that lactoferrin can be absorbed into the blood stream [93]. The absorption of bovine lactoferrin by neonatal pigs has been investigated by Harada and his research group [124, 136-138], who demonstrated that bovine lactoferrin was absorbed from the gut into blood. Enterohepatic recycling of lactoferrin as well as transfer of lactoferrin from plasma to cerebrospinal fluid were also shown [124, 137]. Further, absorption of bovine lactoferrin was demonstrated in growing pigs (10-12 weeks old) [139].

The observation that growing pigs can absorb lactoferrin after gut closure points to a selective mechanism of absorption. Indeed, the presence of lactoferrin receptors on enterocytes of several species has been reported and extensively reviewed [140, 141]. The initial investigation of porcine intestinal lactoferrin receptor by Gislason confirmed the presence of lactoferrin receptors on porcine enterocytes, the density of which was

age-independent [142, 143]. In 2007, the cDNA of the porcine homologue of human lactoferrin receptor was cloned. The molecular weight of the receptor protein was 35 kDa under reducing conditions and ~135 kDa under non-reducing conditions, suggesting that the porcine lactoferrin receptor is a tetramer [144]. The physiological role of this receptor needs further investigation.

1.4.2.3. Insulin-like growth factor I (IGF-I)

Insulin-like growth factor I (IGF-I), a 7.5 kDa protein present in porcine colostrum and milk [145], stimulates cellular proliferation and differentiation. The absorption of IGF-I by neonatal piglets is independent of gut closure [146], and an IGF-I receptor in porcine small intestine has been identified [147], strongly suggesting that IGF-I absorption is receptor-mediated.

1.5. Plasma protein profiles in pigs

The plasma protein profiles in neonatal pigs are poorly characterized. Several studies have investigated the developmental changes of major porcine plasma proteins, such as albumin, α-fetoprotein, α_1-antitrypsin, α_1-acid glycoprotein, fetuin, transferrin and immunoglobulin, in porcine fetuses and neonates [148-150]. Recently, the serum and plasma protein profiles of 4 months old pigs have been studied [151]. Thirty-nine proteins were identified in that study, including most of those previously reported as major proteins in neonatal porcine plasma, except α-fetoprotein [151]. The concentration of α-fetoprotein has been reported to drop below detectable level at one month of age [148]. Other studies investigating porcine plasma proteins have focused on characterization of the acute phase protein changes, e.g., C-reactive protein, haptoglobin and pig major acute phase protein [148, 152]. Otherwise there is little information on changes of plasma protein profiles in piglets pre- and post- suckling to determine which proteins are absorbed or up-regulated after

suckling.

2. Rationale, hypotheses and objectives

2.1. Experiment 1

2.1.1. Rationale 1

It has been demonstrated that porcine milk fat globule membrane (MFGM) is a binding target of F4 *E. coli* [153], and that porcine milk MFGM is able to inhibit the binding of F4 fimbriae to porcine intestinal brush border membrane [154]. Many proteins have been identified on MFGM from human, murine and bovine milk. A previous study in our laboratory have demonstrated that two major MFGM proteins in porcine milk, namely, porcine lactadherin and heart fatty acid binding protein, interact with F4ac-fimbria [3]. The potential interaction of the other MFGM proteins with F4 fimbria has not been investigated.

2.1.2. Hypothesis 1

There are several milk fat globule membrane proteins that bind F4ac-fimbria.

2.1.3. Objective 1

To visualize milk fat globule membrane proteins that can interact with F4ac-fimbria by 2D Overlay Western blot and to identify them by LCMS/MS.

2.2. Experiment 2

2.2.1. Rationale 2

Many non-immunoglobulin proteins with potential defense function have been identified in porcine milk [2-4]. One of these proteins, namely, porcine lactoferrin can be absorbed

into the circulation to function systemically and protect against septicemia [124, 137-139]. Neonatal pigs are assumed to non-selectively absorb many proteins and macromolecules from the gut into the blood stream, suggesting that many colostral proteins can be absorbed before 24~36h of life. However, there are limited studies demonstrating unequivocally the identity of porcine milk proteins that can be absorbed by neonatal piglets.

2.2.2. Hypothesis 2

Numerous porcine colostral proteins can be absorbed from the intestine of neonatal piglets into the blood during the first 24 h of life.

2.2.3. Objective 2

To identify absorbable non-immunoglobulin porcine colostral proteins by comparison of plasma protein profiles of neonatal piglets before and after suckling.

3. F4ac-fimbrial-binding proteins on porcine milk fat globule membrane (MFGM)

3.1. Introduction

F4-positive enterotoxigenic *E. coli* (ETEC) is the most commonly encountered diarrheagenic pathogen in neonatal piglets in the modern swine industry. The attachment of ETEC fimbriae to specific glycoprotein and glycolipid receptors on the intestinal mucosa is a necessary initial step in the pathogenesis of enterotoxigenic colibacillosis. Prevention of bacterial attachment to the intestinal mucosa is the most effective defense against ETEC. Accordingly, colostral anti-F4 antibodies induced by previous exposure or vaccination of the sows effectively prevent neonatal diarrhea caused by F4-positive ETEC. Many studies have demonstrated additionally that colostrum and milk contain non-immunoglobulin components that decrease or prevent attachment of pathogenic bacteria *in vitro* [3, 11, 154]. Atroshi et al. demonstrated that F4-positive *E. coli* bind to sow milk fat globule membranes (MFGM) [153] and Choi et al. reported that MFGM inhibited binding of the F4 fimbria to the intestinal brush border [154]. Neither of these studies, however, identified the specific individual components of porcine MFGM involved in the binding of F4.

Milk fat droplets are surrounded by a membrane which originates from the apical cell membrane of the mammary glandular epithelium [23]. This membrane is called MFGM and it contains several major and minor proteins [6, 26, 77-79, 155, 156]. MFGM proteomic profiles have been defined for human [78], bovine [79] and murine [5] milk. Several MFGM proteins in milk were reported to be associated with host defense. Both lactadherin and mucin in human MFGM have anti-rotaviral activity [61, 62]. Human mucin inhibits adhesion of S-fimbriated *E. coli* to buccal epithelium [11], aggregates poxvirus [13] and

inhibits infection of human T lymphoblastoid cells with HIV-1 [14]. These *in vitro* studies suggest that MFGM proteins may play an important defensive role in the protection of mammalian neonates of various species from infectious diseases.

The porcine MFGM proteomic profile has not been defined and little is known about the function of porcine MFGM proteins. Previously, we employed affinity chromatography and isolated several F4-fimbrial-binding proteins from porcine skim milk [3], two of which, namely lactadherin and heart fatty acid binding protein, have been reported to be major MFGM proteins in several species [6]. We also demonstrated that lactadherin interfered with attachment of F4-positive ETEC to porcine small intestinal villi *ex vivo* [3]. Accordingly, it is possible that milk components that bind to F4-fimbriae may act as receptor analogues and interfere with the binding of F4-positive ETEC to the intestinal surface [11, 157, 158].

The purpose of this study was to investigate the ability of porcine MFGM proteins to interact with F4ac *in vitro* and to characterize protein profiles of porcine MFGM by liquid chromatography/mass spectrometry (LC MS/MS).

3.2. Materials and methods

3.2.1. Isolation of porcine milk fat globule membrane (MFGM)

Porcine milk was obtained from healthy sows 21 d after farrowing (Prairie Swine Centre Inc., Saskatoon, SK, Canada) in a previous study [3]: after subcutaneous administration of 2 ml of oxytocin (MTC Pharmaceuticals, Cambridge, ON, Canada), the milk was expressed manually and kept at 4°C during transportation to the laboratory. The milk fat was obtained by centrifugation at 3600 x *g* for 15 minutes (min) at 4°C, the skim milk was removed and the fat fraction was pooled and stored at –70°C. MFGM was isolated from porcine milk fat according to a previously published protocol [26, 159]. Briefly, the milk fat was washed

three times with PBS (1.6 mM NaH_2PO_4, 9.4 mM Na_2HPO_4, 154 mM NaCl, pH=7.2) and centrifuged at 2000 x *g* for 15 min at 20ºC. The supernatant creamy layer was collected, resuspended in PBS, and subjected to intensive sonication (6 x 60 sec with cooling intervals on ice) to break and release MFGM which was harvested by ultracentrifugation at 100,000 x *g* for 90 min at 4ºC. The MFGM pellet was resuspended in double-distilled (dd)H_2O and the protein concentration of MFGM proteins was adjusted to 5 mg/ml ddH_2O, based on the volumes obtained, using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) with a bovine serum albumin (BSA) standard.

3.2.2. Purification of F4 fimbriae

F4ac was prepared in a previous study and kindly provided by Dr. Shahriar [2]. F4ac were isolated from F4ac-positive ETEC according to a published method [160] and further purified by gel filtration chromatography using a Superdex 75 column (Amersham Biosciences, Uppsala, Sweden) as described previously [2]. The purity of F4ac was determined to be about 95% by semi-quantitative densitometry [2].

3.2.3. Electrophoresis

3.2.3.1. One-dimensional SDS-PAGE

One-dimensional (1D) sodium dodecyl sulfate (SDS) polyacrylamide gel (12%, discontinuous) electrophoresis (PAGE) was performed under reduced conditions using glycine buffer system according to the instructions of the PAGE equipment manufacturer (Mini-PROTEAN® 3 Cell, Bio-Rad Laboratories, Mississauga, ON, Canada).

3.2.3.2. Two-dimensional SDS-PAGE

Isolated MFGM proteins were subjected to isoelectric focusing in linear immobilized pH gradient strips (IPG) (Immobiline Dry Strip, pH 3–10, 13 cm, GE Healthcare Bio-Sciences

AB, Uppsala, Sweden) using an isoelectric focusing system (IPGphor; GE Healthcare Bio-Sciences AB, Uppsala, Sweden) [2], and subsequently separated based on MW using a large electrophoretic system (PROTEAN® II xi Cell, Bio-Rad Laboratories, Mississauga, ON, Canada) as previously described [161]. Approximately 500 µg protein was loaded per gel for colloidal Coomassie G-250 stain [162] and 25 µg for gels used for 2D Overlay Western Blot.

3.2.4. Overlay Western Blot

Overlay Western Blot was used to investigate the interaction between the isolated MFGM proteins and purified F4-fimbriae. The isolated MFGM proteins separated by 1D and 2D SDS-PAGE were electro-blotted on to polyvinylidene fluoride (PVDF) membranes using a small (for 1D PAGE) or a large (for 2D PAGE) electrophoretic transfer cell apparatus (Bio-Rad Laboratories, Mississauga, ON, Canada) and Overlay Western Blot detection of F4-binding proteins was performed as described previously [120]. Briefly, 25 µg of MFGM proteins were separated by 2D SDS-PAGE and blotted to the PVDF membrane which was subjected sequentially to the following procedures: blocking of any remaining protein binding sites with blocking buffer (2% non-fat dry milk and 0.1% Tween-20 in PBS) at 4ºC overnight; incubation with 2 µg/ml purified F4ac in blocking buffer with gentle shaking for 90 min at room temperature; three 15-min washes with PBS-Tween-20 (PBST) buffer (0.1% Tween-20 in PBS); incubation with primary rabbit anti-F4 polyclonal serum (Dr. J. M. Fairbrother, Faculté de Médecine Vétérinaire, Université de Montréal, Saint-Hyacinthe, QC, Canada) in blocking buffer (1:2000) for 90 min at room temperature; three 15 min washes in PBST; incubation with secondary goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma Chemical Co, St Louis, MO, USA) in blocking buffer (1:20,000) for 90 min at room temperature; three 15-min washes in PBST, and exposure to a substrate solution (Immun-Star

Substrate, Bio-Rad Laboratories, Mississauga, ON, Canada). Chemiluminescent signals demonstrating F4ac-binding proteins was detected by exposure of the PVDF membrane to an X-ray film (Eastman Kodak Company, Rochester, NY, USA) which was developed in an automated X-ray developer (Kodak, M35AX-OMAT processor, NY, USA).

One-dimensional Overlay Western Blot was used initially in a pilot study to determine if any MFGM proteins interact with F4ac, and to demonstrate the presence of interaction with porcine lactadherin used as a positive control and the absence of interaction with BSA used as a negative control. In addition, omission of incubation with F4ac for both 1D and 2D Overlay Western Blots was employed to demonstrate any non-specific binding between primary or secondary antibodies to blotted MFGM proteins. We also carried out both 1D and 2D Overlay Western Blot experiments using protein-free blocking buffer (Pierce, Rockford, IL, USA) instead of skim milk to rule out potential interaction between F4ac and skim milk proteins in blocking buffer, and we obtained similar results.

3.2.5. Protein identification

Porcine MFGM protein spots in 2D SDS-PAGE gels were excised manually by pipet tips, transferred into 0.5 ml Eppendorf tubes and submitted for liquid chromatography mass spectrometry/mass spectrometry (LC MS/MS) identification, performed by the National Research Council's Plant Biotechnology Institute (Saskatoon, SK, Canada). The spots were placed in a 96-well microtiter plate and were digested in gel using a MassPREP station robotic protein handling system Waters/Micromass (Milford, MA, USA) following the standard digestion protocol. The digested samples were evaporated to dryness, then dissolved in 15 µl of 1% aqueous trifluoroacetic acid, of which 3 µl was injected on to a NanoAcquity UPLC (Waters, Milford, MA, USA) interfaced to a Q-Tof Ultima Global hybrid tandem mass spectrometer fitted with a Z-spray nanoelectrospray ion source

(Waters/Micromass, Manchester, UK). The digested peptide sample was loaded onto a C18 trapping column (Symmetry 180 μm X 20 mm; Waters, Milford, MA, USA) and washed for 3 min using solvent A (0.1% formic acid in H_2O) at a flow rate of 15 μL/min. The trapped peptides were eluted onto a C18 analytical column (1.7 μm BEH130 C18 100 μm X 100 mm; Waters, Milford, MA, USA). Separations were performed using a linear solvent gradient of 10:95% to 45:55% (solvent A:solvent B [0.1% formic acid in acetonitrile]) over 45 min. The composition was then changed to 20:80% (A:B) and held for 10 min to flush the column before re-equilibrating for 7 min at 100:0% (A:B). Mass calibration of the Q-TOF instrument was performed using a product ion spectrum of Glu-fibrinopeptide B acquired over the m/z range 50 to 1900. LC MS/MS analysis was carried out using data-dependent acquisition, during which peptide precursor ions were detected by scanning from m/z 400 to 1900 in TOF MS mode. Multiply-charged ions (2+, 3+, or 4+) rising above predetermined threshold intensity are automatically selected for TOF MS/MS analysis, by directing these ions into the collision cell where they fragment using low energy CID by collisions with argon, and varying the collision energy by charge state recognition, product ion spectra are acquired over the m/z range 50 to 1900. LC MS/MS data was processed using Mascot Distiller (ver. 2.1.1.0, Matrixscience) and searched against the NCBInr or Swiss-Prot databases using MASCOT (Matrix Science Inc., Boston, MA). Searches were performed using carbamidomethylation of cysteine as a fixed modification and oxidation of methionine as a variable modification, allowing for one missed cleavage during trypsin digestion. In this study, those proteins with at least 2 matched peptides are considered to be successfully identified (personal communication with Doug Olson [National Research Council's Plant Biotechnology Institute, Saskatoon, SK, Canada]). In the search results, a protein score and a score threshold is given to each protein. Protein scores greater than the threshold are considered to be significant. In some cases, more than one protein is identified from the

same spot. These proteins will all be listed and the one that has the highest score is called "first hit".

3.3. Results

Isolated porcine MFGM proteins were separated by 1D and 2D SDS-PAGE and visualized by Coomassie stains (Figure 3.1 and 3.2A, respectively). The major protein bands/spots observed in Coomassie stained 2D gel were submitted for LC MS/MS identification (Table 3.1) and in total, 17 distinct porcine MFGM proteins were identified (Table 3.2). Purified F4ac fimbriae (Figure 3.3) were used in 1D and 2D Overlay Western Blot for detection of interacting MFGM proteins (Figure 3.1 and 3.2B). Four bands (~ 66kDa, 47kDa, 44kDa and 31kDa) and several spots were detected by 1D and 2D Overlay Western Blot, respectively (Figure 3.1 and 3.2B). In the 1D experiment, porcine lactadherin, used as a positive control, was positive; while BSA, used as a negative control, was negative (Figure 3.1). By omission of F4ac, both the 1D and 2D assays showed no positive signals (Figure 3.1 and 3.2C). Comparative matching of MW and isoelectric points (pI) of the detected F4ac-fimbrial-binding spots with the previously identified MFGM proteins in Coomassie stained 2D gels (Figure 3.2A and B) revealed that lactadherin, butyrophilin, adipophilin, and acyl-CoA synthetase 3 gave strong F4ac-fimbrial-binding signals *in vitro*.

3.4. Discussion

Using 2D SDS-PAGE protein separation and LC MS/MS identification, 17 proteins were identified in porcine MFGM. Lactadherin, butyrophilin, adipophilin, and acyl-CoA synthetase 3 were demonstrated and identified by Overlay Western Blot and LC MS/MS, respectively, to interact with F4ac purified from clinically relevant F4 positive enterotoxigenic *E. coli*.

MFGM proteins are comprised of 8 major proteins according to previous reports in

several species [5, 6, 26, 79], namely, xanthine dehydrogenase, butyrophilin, lactadherin, adipophilin and fatty acid binding protein, mucin 1, mucin 15 (previous known as PAS III [27]) and CD36. Consistent with the previous reports [5, 26, 78, 156], only the first five of these 8 could be separated by 2D SDS-PAGE and visualized; whereas, the last three (i.e. mucin 1, mucin 15 and CD36) were not visualized and identified in this study. Accordingly, the overall two-dimensional electrophoretic pattern of porcine MFGM in this study is similar to previously reported human [78], bovine [26] and murine [5] MFGM patterns. In addition, butyrophilin, adipophilin and lactadherin were also identified or co-identified from multiple minor spots in our 2D SDS-PAGE gel (Table 3.2), but these minor spots have smaller MW than those of butyrophilin, adipophilin and lactadherin. These findings are unexpected; the LC MS/MS data is however convincing and accordingly, we suspect that these minor spots may be, or may contain, fragments of the native (parent) proteins. These fragments may not be present naturally in the porcine MFGM; instead, they may be generated during sample processing (i.e. sample degradation).

By Overlay Western Blot technique, several spots were demonstrated to interact with F4ac. In the 1D experiment, F4ac interacted with the positive control, porcine lactadherin, while it did not react with the negative control, BSA. Further, when F4ac was omitted, both 1D and 2D experiments demonstrated no positive signals. Taking all together, we believed that Overlay Western Blot in this study reliably demonstrated the *in vitro* interactions between F4ac and porcine MFGM proteins.

The interaction of lactadherin with F4ac-fimbriae, detected by Overlay Western Blot in this study, agrees with our previous study in which lactadherin was isolated from porcine skim milk by F4ac-fimbrial affinity chromatography and demonstrated to inhibit the attachment of F4ac positive *E. coli* to intestinal villi *ex vivo* [3]. In addition to lactadherin, butyrophilin, adipophilin and acyl-CoA synthetase 3 all were demonstrated in this study to

give strong F4ac-fimrial-binding signals (Figure 3.2B).

Adipophilin, previously called adipose differentiation-related protein, has a similar MW (47 kDa) and pI (~ 6-7) to lactadherin, and was thus overlooked for a long time [6] due to the similar electrophoretic migration of these two proteins. Adipophilin is located on the surface of lipid droplets and it is thought to be involved in lipid storage [22, 69]. Based on a crude comparison with previously published 2D SDS-PAGE images of human [78], murine [5] and bovine [26] MFGM proteins, it seems that adipophilin is more abundant in porcine MFGM.

The 31 kDa positive band in 1D Overlay Western Blot is composed of several spots in the 2D Overlay Western Blot. One (Figure 3.2B: MW ~ 31 kDa, pI ~ 5.1) of the strongest positive signals was identified as acyl-CoA synthetase 3 (ACS3). ACS3 is related to lipid metabolism and is highly expressed in the brain [163]. Several ACSs, including ACS3, have recently been identified in bovine MFGM recently [80]. Even though ACS3 has a relatively low abundance compared to the other major porcine MFGM proteins (Figure 3.2A), it generated a strong detection signal with F4ac fimbria in Overlay Western Blot (Figure 3.2B), indicating potential high affinity of porcine ACS3 to F4ac fimbria. Two other strong positive signals (Figure 3.2B: MW ~ 28 kDa, pI ~ 7.4; and MW ~ 28 kDa, pI ~ 8) in the same MW area were not detected in Coomassie-stained 2D SDS-PAGE (Figure 3.2A), thus were not possible to be indentified in this study; however, these proteins may be biologically important.

A positive band at ~ 66 kDa in 1D and 2D Overlay Western Blot has similar electrophoretic migration pattern as the monomer of butyrophilin (65-67 kDa) [5, 6, 164]; however, we failed to identify this protein by LC MS/MS. A protein band, identified by LC MS/MS as butyrophilin, located in high molecular weight region of 2D SDS-PAGE reacted strongly with F4ac fimbria (Figure 3.2B). An unexplained aggregation of butyrophilin has

been previously reported in 2D but not 1D SDS-PAGE [164] and was observed also in this study. Butyrophilin is the major structural and most abundant protein on bovine MFGM [6, 43]. Butyrophilin is a member of the immunoglobulin (Ig) superfamily, a broad family which is characterized by the presence of Ig-like folds and includes various proteins that serve as receptors and immune regulators [165]. A recent study demonstrated that the receptor for the subgroup C avian sarcoma and leukosis viruses was similar to mammalian butyrophilin [52]. Nevertheless, the most commonly reported function of butyrophilin is the control of milk fat secretion [6, 22, 24, 47], and little is known about any potential role as a receptor in mammalian species.

It should be noted that in addition to those spots that interacted strongly with F4ac fimbriae, several spots reacted weakly with F4ac. One of these is fatty acid binding protein (spot group 36, Figure 3.2A), and this agrees with the previously reported affinity of fatty acid binding protein to F4ac fimbria [3].

Binding interaction between porcine MFGM and F4 (K88) positive *E. coli* [153] and inhibition of *E. coli* attachment to porcine small intestinal brush border [154] were previously demonstrated. In this study, interaction between F4ac fimbria and lactadherin, butyrophilin, adipophilin, and acyl-CoA synthetase 3 was demonstrated. It is possible that these proteins serve as receptor analogues for F4ac fimbria and may prevent attachment of *E. coli* to intestinal epithelium *ex vivo* as demonstrated previously [3, 154].

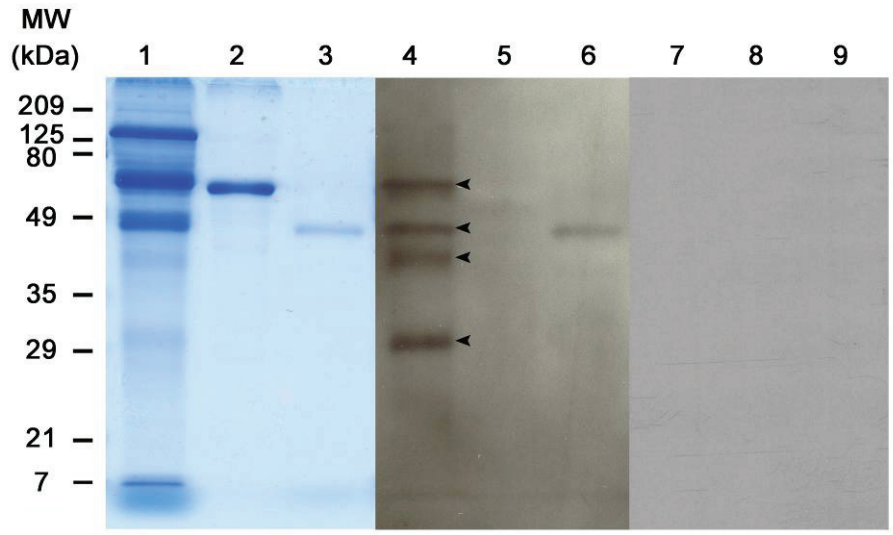

Figure 3.1. One dimensional (1D) SDS-PAGE (lanes 1-3) and 1D Overlay Western Blot using purified F4ac as a probe (lanes 4-6). Porcine MFGM proteins (lane 1), bovine serum albumin (BSA) (lane 2) and purified porcine lactadherin (lane 3) were subjected to electrophoresis and stained by Coomassie. The proteins were transferred to PDVF membrane and probed with purified F4ac. Using a chemiluminescent method, 4 positive bands (arrow heads, from top to bottom, ~ 66kDa, 47kDa, 44kDa and 31kDa) from porcine MFGM (lane 4) were detected. The negative control, BSA, (lane 5) did not react, while porcine lactadherin used as a positive control (lane 6) reacted with F4ac fimbriae. When F4ac was omitted, none of the samples showed positive signals (7: porcine MFGM; 8: BSA; 9: porcine lactadherin)

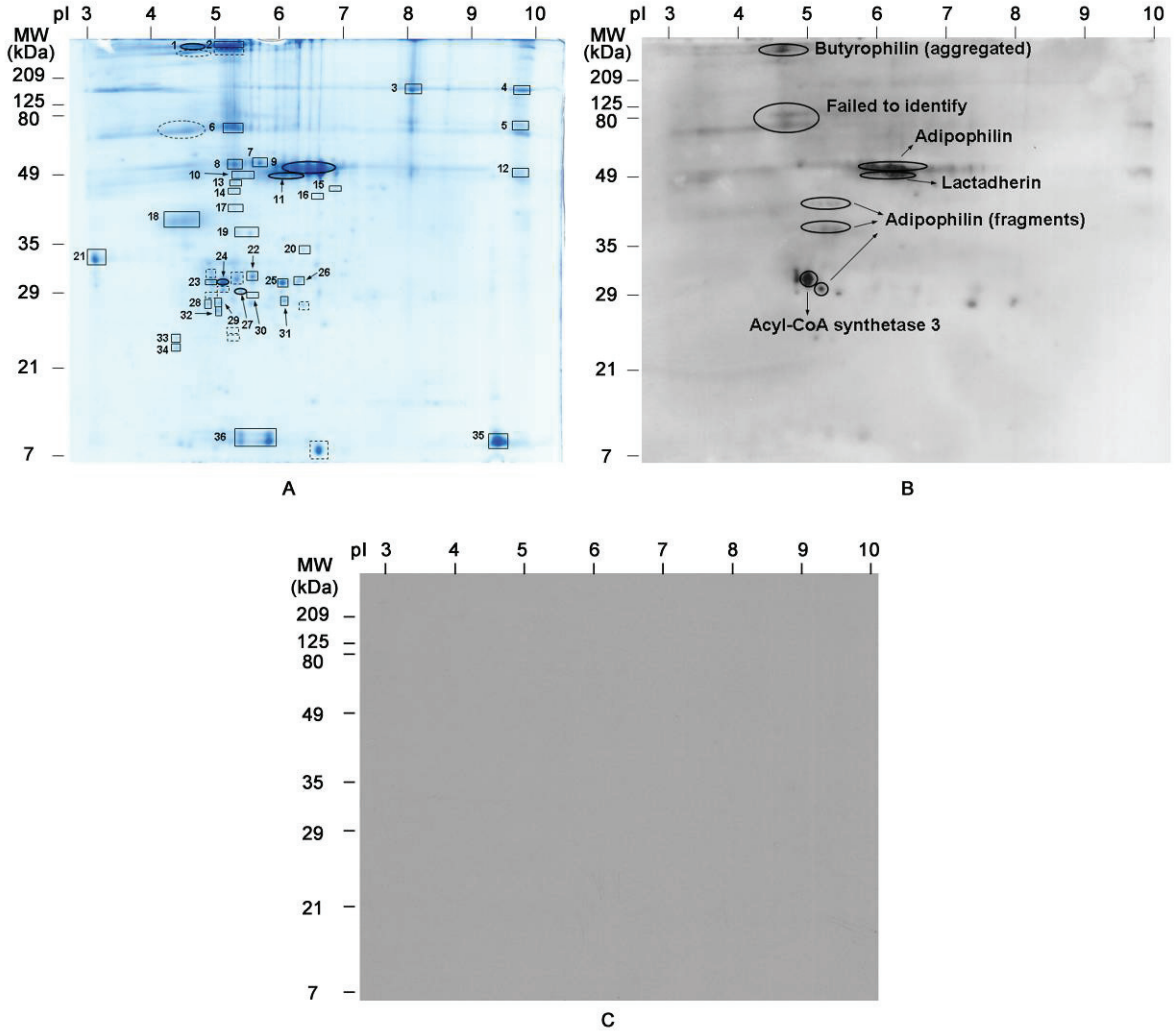

Figure 3.2. Coomassie-stained two dimensional (2D) SDS-PAGE (A) and 2D Overlay Western Blot (B) of porcine MFGM proteins using purified F4ac as a probe. Major F4ac-fimbrial-binding spots (closed ovals) and other protein spots (closed squares) are indicated on the SDS-PAGE (A). Several spots (dashed ovals and squares) were visible by Coomassie stain, but failed to be identified by LC MS/MS. Some of these spots reacted with F4ac (dashed ovals) while others (dashed squares) did not. When F4ac was omitted, none of the MFGM proteins showed positive signals (C). The numbers correspond to those in Table 3.1 and Table 3.2.

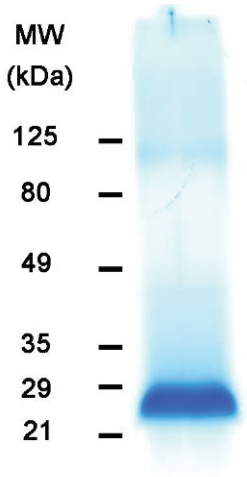

Figure 3.3. Coomassie-stained 1D SDS-PAGE of purified F4ac fimbria. A prominent band at ~ 27 kDa is demonstrated.

Table 3.1. Identification of porcine milk fat globule membrane (MFGM) proteins by LC MS/MS

ID#	Identities of matched proteins	Predicted MW (kDa)	Total MASCOT protein score*	Score threshold	# of matched peptides	Estimated MW (kDa)	Estimated pI
1[a]	Butyrophilin	59.9	102	52	2	> 200	4.5~4.8
2	Xanthine dehydrogenase	148.9	101	43	2	>200	5.1~5.4
	Butyrophilin	14.3	74		2		
3	Xanthine dehydrogenase	148.9	230	52	6	150	8.1
4	Xanthine dehydrogenase	148.9	512	52	13	150	10
5	Acyl-CoA synthetase 3	81.3	354	43	6	72	10
6	Heat shock protein 70 kDa	71	219	52	5	70	5.1~5.4
7	Adipophilin	50.6	766	52	13	52	5.7
	Lactadherin	49	334		6		
8	Lactadherin	46.7	548	43	11	50	5.3
	Adipophilin	50.6	519		10		
9[a]	Adipophilin	50.6	1347	52	36	50	6~6.8
	Lactadherin	46.7	441		8		
10	Lactadherin	49	363	52	7	49	5.2~5.5
	Mannose 6 phosphate receptor binding protein 1	48	337		6		
11[a]	Lactadherin	49	862	52	18	49	5.8~6.4
	Adipophilin	50.1	395		7		
12	Adipophilin	50.1	589	52	9	49	10
	Lactadherin	49	387		7		
13	Beta-actin	42	377	52	6	47	5.4
	Lactadherin	46.7	97		2		
14	Beta-actin	42.1	294	52	5	45	5.4
15	Adipophilin	50.6	425	43	6	45	6.9
16	Adipophilin	50.6	346	52	7	44	6.7
17	Adipophilin	50.6	325	42	5	42	5.4
	Gamma-soluble NSF attachment protein	35.0	232		5		
	Lactadherin	46.7	85		2		
18	Alpha S2 casein	27.7	106	52	2	40	4.2~4.6
19	Adipophilin	50.6	226	43	5	37	5.4 and 5.5
	G protein beta subunit	38.0	159		4		
	Lactadherin	46.7	149		3		
20	Adipophilin	50.6	384	42	7	34	6.3
	Lactadherin	46.7	169		3		

ID#	Identities of matched proteins	Predicted MW (kDa)	Total MASCOT protein score*	Score threshold	# of matched peptides	Estimated MW (kDa)	Estimated pI
21	Kappa casein	21.2	92	52	2	33	3
22	Acyl-CoA synthetase 3	81.3	141	52	2	31.5	5.6
	Butyrophilin	60	91		2		
23	Acyl-CoA synthetase 3	81.3	100	43	2	31	5
	Butyrophilin	14.3	87		4		
24[a]	Acyl-CoA synthetase 3	81.4	237	52	6	31	5.1
	Butyrophilin	60	188		5		
	Adipophilin	50.6	144		3		
25	Adipophilin	50.6	162	43	3	31	6.1
26	Adipophilin	50.6	194	42	3	31	6.3
27[a]	Adipophilin	50.6	65	43	2	29	5.4
28	Butyrophilin	60	102	52	2	28	5
29	Apolipoprotein A-I	18.8	129	43	2	28	5.1
	Butyrophilin	59.9	73		2		
30	Adipophilin	50.6	111	43	2	28	5.7
31	Alpha(s1) casein	24.2	81	43	2	28	6
32	Butyrophilin	60	102	52	2	27	5.1
	Adipophilin	50.1	95		2		
33	Chain A, Structure Of Porcine Beta-Lactoglobulin	18.1	124	43	3	24	4.2
34	Chain A, Structure Of Porcine Beta-Lactoglobulin	18.1	187	43	3	23	4.2
35	Serum amyloid A2	14.6	81	43	3	11	9.4
36	Fatty acid binding protein	13.3	597	52	17	11	5.3 and 5.8
	Adipophilin	50.6	149		3		

a: Spots gave strong F4ac-fimbrial-binding signals
MW: Molecular weight
pI: Isoelectric point
LC MS/MS: Liquid chromatography/Mass spectrometry
* Protein scores are derived from ions scores (not included in this table) as a non-probabilistic basis for ranking protein hits and are provided in the MASCOT search results. Ions score is -10*Log (P), where P is the probability that the observed match is a random event. Individual ions scores > threshold indicate identity or extensive homology ($p<0.05$).

Table 3.2 Distribution of porcine milk fat globule membrane (MFGM) proteins in 2D SDS-PAGE

Proteins	**Identified with highest scores (first hit) from this/these spots**	**Identified with 2nd or 3rd highest scores (not first hit) from this/these spots**
Xanthine dehydrogenase	2, 3, 4	None
Butyrophilin	1[a], 28,32	2, 22, 23, 24[a], 29
Adipophilin	7, 9[a], 12, 15, 16, 17, 19, 20, 25, 26, 27[a], 30	8, 11[a], 24, 32, 36
Lactadherin	8, 10, 11[a],	7, 9[a], 12, 13, 17, 19, 20
Fatty acid binding protein	36	None
Actin	13, 14	None
Acyl-CoA synthetase 3	5, 22, 23, 24[a]	None
Heat shock protein 70 kDa	6	None
Mannose 6 phosphate receptor binding protein 1	None	10
Gamma-soluble NSF attachment protein	None	17
G protein beta subunit	None	19
Alpha (S1) casein	31	None
Alpha (S2) casein	18	None
Kappa Casein	21	None
Apolipoprotein A-I	29	None
Lactoglobulin	33, 34	None
Serum amyloid A	35	None

a: Spots gave strong F4ac-fimbrial-binding signals

Mammalian neonates are protected against infectious diseases by a myriad of protective substances present in ingested colostrum and milk. Neonatal local and systemic protection by colostral and milk immunoglobulins is well characterized and indisputable. In addition, some other substances in porcine colostrum/milk have been demonstrated to have potential protective effects (e.g. LPS-binding proteins, F4-fimbrial-binding proteins and bactericidal peptides). However, only few (e.g. lactoferrin) have been reported to be absorbed by piglets from the gut into blood and to potentially exhibit protection against systemic diseases. Since protein absorption by neonatal piglets has been assumed to be non-selective, it is reasonable to hypothesize that other non-immunoglobulin colostral proteins can be absorbed and are involved in systemic protection of neonates. Accordingly, experiments presented in the following chapter were designed to compare plasma protein profiles of piglets before and after suckling to characterize absorption of colostral proteins by piglets.

4. Plasma protein profiles of neonatal pigs before and after suckling

4.1. Introduction

The epitheliochorial placentation of pigs and several other domestic animals prevents the transfer of maternal plasma macromolecules from dam to fetus *in utero*. Thus, piglets are born hypogammaglobulinemic and maternal immunoglobulins are absorbed from the intestine into the systemic circulation from colostrum ingested within the first 24 to 36 hours after birth. The importance of absorption of colostral immunoglobulins for neonatal protection against disease is well characterized and considered indisputable [166].

In addition to immunoglobulins, colostrum contains a myriad of substances potentially involved in various aspects of neonatal development and immune defense [2, 61, 93]; however, neonatal absorption of intact colostral components other than immunoglobulins is poorly characterized. Reports have documented the ability of neonatal piglets to absorb a variety of substances from the gastrointestinal tract to the systemic circulation within 24 to 36 hours after birth, e.g., bovine IgG [123], porcine IgG [124, 125], dextrans of different molecular weights [123, 126, 127], bovine serum albumin [123, 126, 127], egg proteins [128], lactoferrin [137] and insulin-like growth factor I [146]. It has also been shown that the degree of absorption negatively correlates with molecular size [127]. Accordingly, early post-natal protein absorption by neonatal piglets was inferred to be non-selective [122]. The magnitude of the non-selective absorption of macromolecules drops significantly at about 24 h after birth [123, 127]. This sudden decrease and cessation of macromolecular transfer is referred to as gut closure. While the mechanism of gut closure is not well understood, it is assumed that after gut closure only limited selective absorption of macromolecules takes

place in the gastrointestinal tract.

Based on the concept that proteins can be non-selectively absorbed by neonatal piglets before gut closure, and the fact that neonatal digestive enzymes have low activity [167], it may be reasonably assumed that many intact proteins are absorbed from ingested colostrum into the blood stream by neonatal piglets; however, unequivocal supporting experimental evidence is limited. Harada et al. used two-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (2D SDS-PAGE) to investigate pre- and post-suckling plasma protein profile changes in neonatal piglets and reported that 2D SDS-PAGE of plasma from post-suckling piglets demonstrated ~ 30 more protein spots than plasma from pre-suckling piglets; however, the identity of these proteins was not determined [124]. Based on the well characterized absorption of colostral immunoglobulins, it is clear that a substantial portion of the protein spots identified by Harada et al. in post-suckling plasma correspond to immunoglobulins. It is possible, however, that the increase in protein spots in post-suckling plasma is also in part due to absorption of non-immunoglobulin colostral proteins and/or up-regulation of plasma protein synthesis induced by suckling.

The objectives of this study were to characterize the major proteins in porcine neonatal plasma and to determine the changes that occur in plasma protein profiles, including those of minor proteins, in piglets after natural suckling.

4.2. Materials and methods

4.2.1. Experimental design

To determine the changes of plasma protein profiles before and after natural suckling of neonatal piglets, the plasma of 9 piglets from 3 sows (3 piglets/sow) was studied by 2D SDS-PAGE. Blood samples were collected from the anterior vena cava into tubes containing sodium citrate right after the birth and again at 24 hours after suckling the dam.

The whole plasma and the plasma with immunoglobulins removed, termed immunoglobulin-removed plasma in this study, were subjected to 2D SDS-PAGE. Electrophoretic patterns of paired plasma samples pre- and post- suckling, with and without immunoglobulin removal were compared. Prominently increased proteins after suckling were defined in this study as protein spots that were absent before and present after suckling, or protein spots with low concentration before suckling and obvious increased concentration after suckling, determined by visual comparison of staining intensity. The proteins that increased prominently after suckling and the major plasma proteins were identified by mass spectrometry (MS) and liquid chromatography mass spectrometry/mass spectrometry (LC MS/MS).

4.2.2. Separation of plasma

Blood samples (~ 1.5-2 ml) were collected into 4 ml tubes containing 0.25 ml of buffered 0.105 M sodium citrate solution as an anti-coagulant, and then stored on ice for less than 8 hours before separation of plasma. Plasma samples were obtained by centrifugation of the whole blood at 2000 x g at 4ºC for 15 minutes and stored at -20ºC.

4.2.3. Removal of immunoglobulins

In order to increase the visibility of minor plasma proteins on SDS-PAGE, affinity chromatography was employed to remove immunoglobulins from the plasma. Approximately 7 ml of protein-A sepharose medium was packed in the column according to manufacturer's instruction (GE Healthcare Bio-Science AB, Uppsala, Sweden). Plasma (100 µl) was diluted with double distilled (dd) H_2O to a final volume of 1 ml and filtered through a 0.45 µm syringe filter (Whatman Inc, Clifton, NJ, USA). The column was equilibrated with 3 column volumes (CV) of buffer A (PBS, 1.6 mM NaH_2PO_4, 9.4 mM Na_2HPO_4, 154 mM NaCl, pH=7.2) at a speed of 1 ml/minute. Next the diluted plasma

samples were injected by a sample loop at a speed of 0.5 ml/minute. The unbound proteins were washed out by 3 CV of buffer A at a speed of 0.5 ml/minute and collected. This washed out fraction is the immunoglobulin-removed plasma and contained plasma proteins with majority of the immunoglobulins removed. Immunoglobulins were eluted by 3 CV of buffer B (0.1 M glycine, pH 3), separated by 1D and 2D SDS-PAGE and visualized by colloidal Coomassie G-250 (Appendix A). The whole process was performed in AKTA Purifier 10 (Amersham Biosciences, Uppsala, Sweden) and controlled by software UNICORN 5.11 (GE Healthcare Bio-Science AB, Uppsala, Sweden). The immunoglobulin-removed plasma samples were dialysed against distilled H_2O for 72 hours, frozen at -20ºC and subsequently lyophilized in Free Zone Plus freeze dry system (Labconco Corp., Kansas City, MO, USA). The lyophilized proteins were resuspended in ddH_2O and adjusted to the final concentration of 3 μg/μl determined by Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) using bovine serum albumin (BSA) as a standard.

4.2.4. Two dimensional SDS-PAGE

The whole plasma samples (25 μl) were subjected to isoelectric focusing in linear immobilized pH gradient strips (IPG) (Immobiline Dry Strip, pH 3–10, 13 cm, GE Healthcare Bio-Sciences AB, Uppsala, Sweden) using an isoelectric focusing system (IPGphor; GE Healthcare Bio-Sciences AB, Uppsala, Sweden) [2] and subsequently separated based on molecular weight using a large electrophoretic system (PROTEAN® II xi Cell, Bio-Rad Laboratories, Mississauga, ON, Canada) as previously described [161]. Proteins of whole plasma separated by 2D SDS-PAGE (9 pairs of gels, 3 pairs per litter, 18 gels in total) were visualized by Coomassie R-250 stain.

Resuspended immunoglobulin-removed plasma samples (16.7 ml) containing 3 μg/μl

protein (~ 50 μg) were subjected to 2D SDS-PAGE (9 pairs of gels, 3 pairs per litter, 18 gels in total) and visualized by silver stain (data not presented). To obtain sufficient amount of proteins suitable for LC MS/MS identification, equal amounts (120 μg) of immunoglobulin-removed plasma from each of the three littermates, before and after suckling, respectively, were pooled and lyophilized. The pooled samples were prepared for each litter. Subsequently, 360 μg of pooled proteins from the pre- and post-suckling immunoglobulin-removed plasma samples were subjected to 2D SDS-PAGE (3 pairs of gels, 1 pair per litter, 6 gels in total) and visualized by colloidal Coomassie G-250 stain [162]. The silver-stained pattern of the 18 individual 2D SDS-PAGE gels of immunoglobulin-removed plasma samples pre- and post-suckling were very similar to the 6 colloidal Coomassie G-250 stained gels of pooled littermate immunoglobulin-removed plasma protein samples before or after suckling.

The detection limit of Coomassie R-250 is ~ 50 ng according to the manufacturer (Bio-Rad Laboratories, Hercules, CA, USA), that of colloidal Coomassie G-250 stain was reported to be ~ 1 ng protein [162] and that of silver stain is ~ 0.1 ng [168]. (See Appendix B for sensitivities of detection in our laboratory)

4.2.5. Protein identification

Proteins increased prominently after suckling and the major proteins in neonatal porcine plasma were excised manually from the Coomassie stained gels, transferred to 0.5 ml Eppendorf tubes and submitted for identification by mass spectrometry (MS) or liquid chromatography mass spectrometry/mass spectrometry (LC MS/MS) performed by the National Research Council's Plant Biotechnology Institute (Saskatoon, SK, Canada). All samples were first subjected to MS analysis. Samples that failed to be identified by MS were then subjected to LC MS/MS. Briefly, selected spots were placed in a 96 well

microtiter plate and digested using a MassPREP station robotic protein handling system Waters/Micromass (Milford, MA, USA), following the standard digestion protocol.

For MS, Peptide Mass Fingerprinting (PMF) analysis of the digested peptide samples was carried out on an Applied Biosystems Voyager DE-STR (Foster City, CA, USA) equipped with a nitrogen laser operated at 337 nm, 3 ns pulse. The instrument was operated in positive ion reflection mode acquiring from m/z 700-m/z 3000. The samples were analyzed as follows: to the MALDI plate, 0.75 µl of CHCA matrix (5 mg/ml in 75% acetonitrile, 0.1% trifluoroacetic acid with 10 mM ammonium phosphate) and 0.75 µl of the protein digest was added, mixed on the plate and air dried under a gentle stream of warm air. The MALDI plates had previously been mass accuracy optimized with the OptiPlate software in the Voyager 5.1 software (Applied Biosystems). PMF analysis of the digested proteins was done in the Automatic Control Mode with recalibrations every 4 samples using a mixture of Angiotensin 1 (M+H 1296.6853), ACTH 1-17 (M+H 2093.0867), and ACTH 18-39 (M+H 2465.1989), and when presented, the spectra were internally calibrated with the autolytic fragment from trypsin (MH+ 842.5100 m/z). 200 laser shots were averaged, and then smoothed, background corrected and converted to the mono-isotopic values with Data Explorer (Applied Biosystems). The resulting peak lists were submitted to MASCOT for database searching. Proteins that had at least 5 matched peptides with significant protein scores were considered to be successfully identified in this study (personal communication with Doug Olson [National Research Council's Plant Biotechnology Institute, Saskatoon, SK, Canada]).

For LC MS/MS, the digested samples were evaporated to dryness, then dissolved in 15 µl of 1% aqueous trifluoroacetic acid, of which 3 µl was injected on to a NanoAcquity UPLC (Waters, Milford, MA, USA) interfaced to a Q-TOF Ultima Global hybrid tandem mass spectrometer fitted with a Z-spray nano-electrospray ion source (Waters/Micromass,

Manchester, UK). The peptide digested sample was loaded on to a C18 trapping column (Symmetry 180 μm X 20 mm; Waters, Milford, MA, USA) and washed for 3 minutes using solvent A (0.1% formic acid in H_2O) at a flow rate of 15 μL/min. The trapped peptides were eluted on to a C18 analytical column (1.7 μm BEH130 C18 100 μm X 100 mm; Waters, Milford, MA, USA). Separations were performed using a linear solvent gradient of 10%:95% to 45%:55% (solvent A:solvent B [0.1% formic acid in acetonitrile]) over 45 min. The composition was then changed to 20%:80% (A:B) and held for 10 min to flush the column before re-equilibrating for 7 min at 100%:0% (A:B). Mass calibration of the Q-TOF instrument was performed using a product ion spectrum of Glu-fibrinopeptide B acquired over the m/z range 50 to 1900. LC MS/MS analysis was carried out using data dependent acquisition, during which peptide precursor ions were detected by scanning from m/z 400 to 1900 in TOF MS mode. Multiply charged ions (2+, 3+, or 4+) rising above predetermined threshold intensity are automatically selected for TOF MS/MS analysis, by directing these ions into the collision cell where they fragment using low energy CID by collisions with argon, and by varying the collision energy by charge state recognition, product ion spectra are acquired over the m/z range 50 to 1900. LC MS/MS data was processed using Mascot Distiller (ver. 2.1.1.0, Matrixscience) and searched against the NCBInr or Swiss-Prot databases using MASCOT (Matrix Science Inc., Boston, MA, USA). Searches were performed using carbamidomethylation of cysteine as a fixed modification and oxidation of methionine as a variable modification, allowing for one missed cleavage during trypsin digestion. Proteins that had at least 2 matched peptides with significant scores were considered to be identified in this study (personal communication with Doug Olson [National Research Council's Plant Biotechnology Institute, Saskatoon, SK, Canada]).

4.3. Results

The 2D electrophoretic pattern of post-suckling plasma (Figure 4.1B) is comparable with that of 4-month porcine plasma [151]. The changes in 2D SDS-PAGE protein profiles of post-suckling, compared to pre-suckling, whole plasma samples, were dominated by the presence of protein spots located in ~50 and 25 kDa region, which represent high and low molecular weight chains of colostral immunoglobulins absorbed after suckling (Figure 4.1B). Two additional protein spots increased prominently after suckling (Figure 4.1B, spots a and b). Spot a (MW=25 kDa, pI=4) was identified as the immunoglobulin J chain (i.e. the joining molecule in IgA and IgM) and spot b (MW=40 kDa, pI=5.3) as apolipoprotein A-IV.

In plasma samples that were subjected to protein-A affinity chromatography to remove immunoglobulins and increase the detection limit, the concentration of immunoglobulins was considerably decreased but not eliminated from post-suckling plasma (Figure 4.2B). Comparison of two-dimensional electrophoretic pattern of immunoglobulin-removed plasma revealed 4 additional protein spots (or groups of spots) that were increased prominently after suckling (Figure 4.2B). They are spot group 7 (MW=47 kDa, pI=5.6~6.5), spot 8 (MW=45 kDa, pI =4.8), spot group 9 (MW=45 kDa, pI =5.2~6) and spot 10 (MW=17 kDa, pI =6.6). All four spots contained fragments of albumin, while one of these, spot 8, contained both albumin and haptoglobin (Table 4.1).

The major proteins in neonatal porcine plasma identified by MS or LCMS/MS are listed in Table 4.1.

4.4. Discussion

In this study, only the concentrations of immunoglobulins, apolipoprotein A-IV and albumin were increased in neonatal porcine plasma after suckling as detected by comparison of Coomassie stained two-dimensional electrophoretic patterns of plasma samples obtained

from neonatal piglets before and after suckling.

The absorption of colostral immunoglobulins by neonatal piglets has been well characterized previously and was expected in this study. However, we were surprised by the fact that other colostral proteins (e.g. caseins, lactoglobulin and lactalbumin) were not absorbed in detectable amounts together with immunoglobulins and that only a few non-immunoglobulin proteins (i.e. albumin, haptoglobin and apolipoprotein A-VI) had increased moderately in concentration in plasma from piglets after suckling. It has been reported that bovine serum albumin can be absorbed by neonatal pigs [126, 127]. Albumin is one of the major porcine colostral proteins [81], thus it is possible that at least some of the demonstrated fragments of the albumin in the post-suckling porcine plasma are absorbed from colostrum. Nevertheless, we cannot rule out increased hepatic synthesis of albumin stimulated by colostral ingestion as the source. There is currently no report of the presence of haptoglobin in porcine milk. In a study of acute phase proteins in bovine milk in an experimental model of subclinical mastitis caused by *Staphylococcus aureus*, haptoglobin was present in milk from infected cows, but not in milk from control cows [169]. The increase of haptoglobin after suckling in this study is thus not likely to be the result of protein absorption, but may be due to increase hepatic synthesis. The increase in apolipoprotein A-IV in plasma of piglets after suckling was considered to be a consequence of increased hepatic production, because the synthesis of apolipoprotein A-IV was reported to be up-regulated by lipid absorption [170], and because its concentration is not detectable in Coomassie-stained 2D SDS-PAGE gels of porcine colostrum (data not presented).

Data generated in this study do not seem to support the previous assumption that protein absorption by neonatal piglets within 24-36 h after birth is a non-selective process [122]. Colostral digestion is limited during the first 24 h due to the low proteolytic activity of digestive enzymes in the gastrointestinal tract of the neonates [167] and due to the presence

of protease inhibitors in porcine colostrum protecting the degradation of colostral proteins in the neonatal intestines [81]. Accordingly, in this study, if substantial non-selective absorption was taking place in neonatal intestines, the other major colostral proteins would have been absorbed together with immunoglobulins and detected in plasma of neonatal piglets after suckling. Alternative possibilities are that colostral proteins were non-selectively taken up by enterocytes and non-immunoglobulin proteins were subsequently degraded before being transferred into systemic circulation; or possibly, once absorbed into the portal circulation, non-immunoglobulin proteins were removed and degraded by hepatocytes, thus were not detected in this study.

The detection limit of our method needs to be taken into account in this study. Coomassie R-250 stain (Bio-Rad Laboratories, Mississauga, ON) that we used for the whole plasma has a detection capability of ~50 ng of protein (50 ng protein in 25 µl plasma equals to a concentration of 2 mg/L) listed in the manufacturer's catalogue (Bio-Rad, Life Science Research Product Catalog & Price List, Canada, 2008/09, p.162). Interpreting conservatively, i.e. allowing for a 10 fold increase (i.e. 500 ng) in the minimal amount of protein detectable by Coomassie R-250 stain, then our study would not detect those proteins with a concentration in plasma of less than 20 mg/L. Accordingly, non-specific absorption of some non-immunoglobulin colostral proteins may exist but the amount of proteins absorbed by neonates may have been below the detection limit in this study.

The major proteins (i.e. alpha-1 acid glycoprotein, alpha-2-HS-glycoprotein, alpha-fetoprotein, serotransferrin, albumin and immunoglobulin) in neonatal porcine plasma composed over 90% of the total proteins as shown in this study (Figure 4.1). Removal of some of these major proteins can improve the visualization of minor plasma proteins. Accordingly, we employed Protein-A affinity chromatography to remove immunoglobulins, which represent a substantial proportion of the proteins in plasma from the piglets after

suckling. It is interesting that the protein-A column did not remove all the immunoglobulins (Figure 4.2 B). The reason for this phenomenon was not definitely determined, but may be due to the different affinities of protein-A to the immunoglobulins from different species [171] or some non-identified technical insufficiencies; however, after applied to the column, a substantial amount of immunoglobulins was removed from the plasma, which resulted in the demonstration of more minor spots than it did in the whole plasma (Figure 4.2). Further, a more sensitive colloidal Coomassie G-250 stain [162] was used for 2D SDS-PAGE of immunoglobulin-removed plasma. Nevertheless, only several additional protein spots were detected (Figure 4.2B), all of which had peptides matching albumin, but with lower MW than albumin. Thus, these spots may be fragments of albumin that were generated either *in vivo* after absorption or *in vitro* during sample processing. It should be noted that, spot 8 (Figure 4.2B) was identified to contain both albumin fragments and haptoglobin by LC MS/MS (Table 4.1). Although this spot was at similar location to that of haptoglobin β chain in 4-month old porcine serum [151], based on significant LC MS/MS data, it is likely that albumin fragments and haptoglobin co-exist in this same spot. As discussed above, the increase of haptoglobin may be due to post-suckling hepatic synthesis, but not absorption.

Our results indicate that only 4 proteins, namely immunoglobulin, albumin, haptoglobin and apolipoprotein A-IV, are prominently increased in post-suckling plasma. This seems to contradict previous findings reported by Harada *et al* [124] that ~ 30 more protein spots were found in post-suckling porcine plasma than in pre-suckling plasma. Accordingly, we also compared the numbers of protein spots in whole plasma samples before and after suckling, determined by automatic count using a 2D analysis software (ImageMaster^{TM} 2D Platinum Version 5.00 (1.1.2), GE Healthcare Bio-Sciences AB, Uppsala, Sweden). On average, ~ 50 more protein spots were present in post-suckling whole plasma compared to pre-suckling plasma (data not shown), which agrees with the result of Harada *et al* [124]; however, the

great majority of these spots were located in the region of immunoglobulin heavy and light chains. Accordingly, as indicated before, immunoglobulins are the most prevalent proteins absorbed from colostrum by neonatal piglets and the absorption of other colostral proteins is very limited.

In conclusion, based on comparison of Coomassie-stained 2D SDS-PAGE gels of pre-suckling and post-suckling neonatal porcine plasma, immunoglobulins were the only proteins unequivocally demonstrated to be absorbed from colostrum. The absorption of other colostral proteins was either equivocal or not detectable by the methods employed in this study. These results suggest that, unlike immunoglobulins, other major proteins in porcine colostrum may not be absorbed into systemic circulation and utilized in their intact forms in substantial amounts.

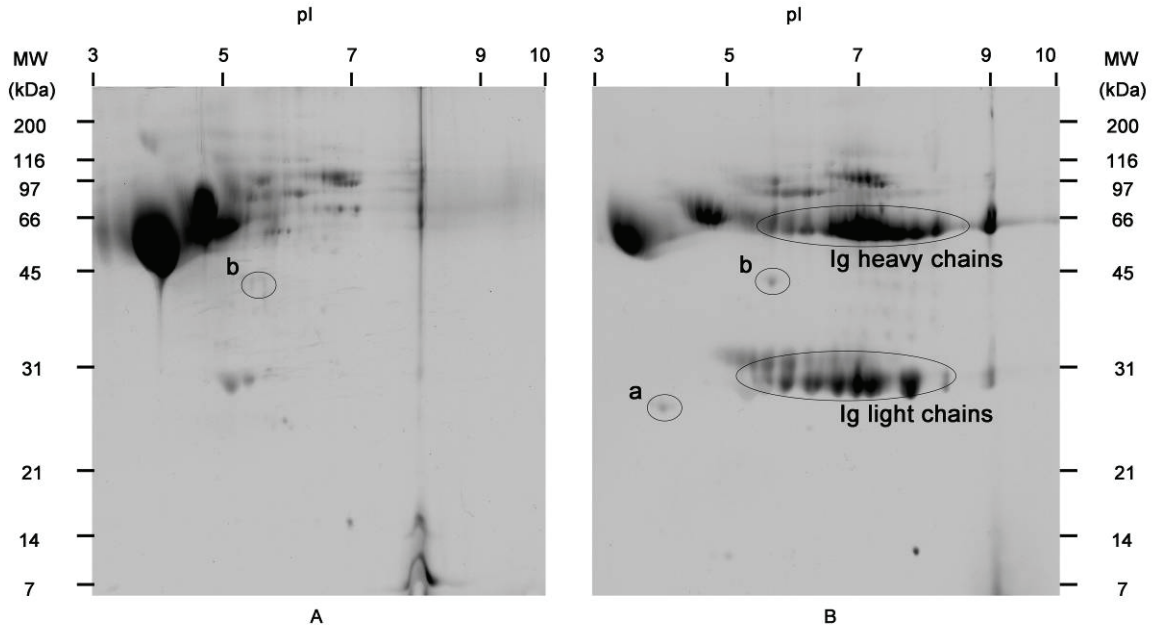

Figure 4.1. Representative images of Coomassie-stained 2D SDS-PAGE of whole pre-suckling (A) and 24 h post-suckling (B) plasma. Immunoglobulins are present in large amount after suckling. Two additional spots (spot a and b) are prominent after suckling.

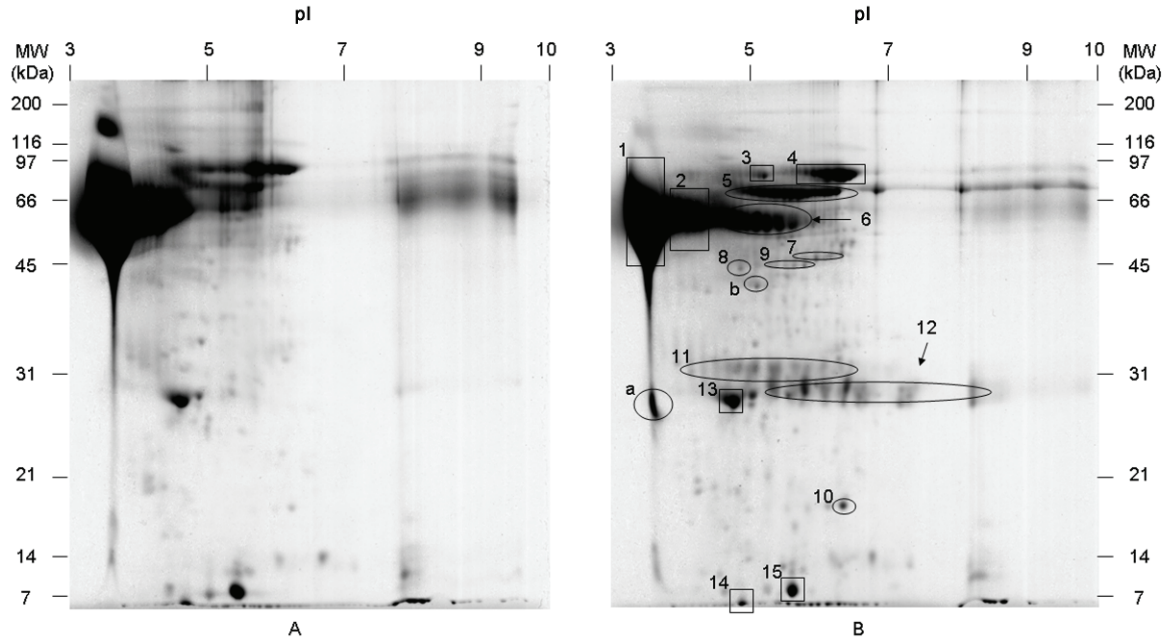

Figure 4.2. Representative images of Coomassie-stained 2-D SDS-PAGE of immunoglobulin-removed pre-suckling (A) and 24 h post-suckling (B) porcine plasma. Major plasma proteins (squares) and proteins increased prominently after suckling (ovals) are noted. Protein ID #s correspond to those in Table 4.1.

Table 4.1. Identification of neonatal porcine plasma proteins by mass spectrometry

ID#	Protein identity	Total protein score*	Score threshold	Numbers of matched peptides	Estimated MW	Estimated pI	Method of identification
A	Immunoglobulin J chain	65	32	2	25	4	LC MS/MS
B	Apolipoprotein A-IV	1462	38	52	40	5.3	LC MS/MS
1	Alpha-1 acid glycoprotein	100	71	13	60	3.8	MS
2	Alpha-2-HS-glycoprotein (fetuin)	435	47	12	60	4.2~4.8	LC MS/MS
3	Alpha-fetoprotein	264	71	31	90	5.2~5.6	MS
4	Serotransferrin	241	71	25	90	6~6.8	MS
5	Albumin	333	71	29	70	4.8~6.6	MS
6	Ig alpha chain C region (fragment)	121	71	15	55	4.8~6	MS
	Immunoglobulin alpha heavy chain C region	89		11			
7	Albumin	82	71	8	47	5.6~6.5	MS
8	Albumin	141	39	3	45	4.8	LC MS/MS
	Haptoglobin	138		4			
9	Albumin	192	71	17	45	5.2~6	MS
10	Serum albumin precursor	142	40	5	17	6.6	LC MS/MS
11	Ig lambda chain C region	97	80	6	33	4.2~6.8	MS
	Ig lambda chain V-C region PLC3 (fragment)	89		6			
12	Ig lambda chain V-C region PLC3 (fragment)	89	51	6	28	5~8.2	LC MS/MS
	Immunoglobulin kappa variable region	129		2			
13	Apolipoprotein A-I	441	49	8	27	4.8	MS
14	Alpha-1 acid glycoprotein	132	52	3	6.5	4.8	LC MS/MS
15	Failed to be identified	N/A	N/A	N/A	7	5.6	MS and LC MS/MS

MW: Molecular weight pI: Isoelectric point MS: Mass spectrometry LC MS/MS: Liquid chromatography Mass spectrometry/Mass spectrometry
N/A: Not applicable * In MS, protein score is -10*Log(P), where P is the probability that the observed match is a random event. Protein scores greater than the threshold are significant ($p<0.05$). In LC MS/MS, protein scores are derived from ions scores (not included in this table) as a non-probabilistic basis for ranking protein hits and are provided in the MASCOT search results. Ions score is -10*Log (P), where P is the probability that the observed match is a random event. Individual ions scores greater than the threshold indicate identity or extensive homology ($p<0.05$). .

5. General discussion

The aims of the work presented in this thesis were to investigate the ability of porcine MFGM proteins to interact with *E. coli* F4ac fimbriae and to evaluate the absorbability of porcine colostral proteins by neonatal piglets. Overlay Western Blot technique demonstrated that the following porcine MFGM proteins interacted relatively strongly with F4ac *in vitro*: butyrophilin, adipophilin, lactadherin and acyl-CoA synthetase 3. Comparison of the two-dimensional electrophoretic protein profiles of plasma obtained from neonatal piglets before and after suckling revealed that immunoglobulins are the only proteins unequivocally absorbed from colostrum by neonatal piglets.

Besides their nutritional value, colostrum and milk provide important immunity to the neonates in their early stage of lives. Immunoglobulins are commonly speculated to be the most important immune components and exert their protective function locally in the gastrointestinal tract and/or systemically after their absorption into the blood stream. After a dam has been exposed to or vaccinated with a specific pathogen, specific immunoglobulins against that pathogen are produced by the dam and secreted into colostrum and milk. After ingestion of these immunoglobulins, the neonates are protected against this specific pathogen. The importance of immunoglobulins in neonatal protection from infection is well characterized and indisputable [166].

Recent studies have shown increasingly that non-immunoglobulin proteins in colostrum and milk may be involved in host defense functions, e.g., human lactadherin can interact with rotavirus *in vitro* and it is associated with protection of human infants against rotaviral infection [61, 62]; casein- and lactoferrin-derived peptides also have bactericidal activities

[86, 92].

While the immune functions of colostral and milk non-immunoglobulin proteins in human and bovine milk have attracted active research, those of porcine colostral and milk proteins have yet to be explored. The pre-weaning period in modern pig industry has been shortened to improve production. However, this leads to a decrease of overall uptake of milk proteins, which potentially may cause health problems. Intensive research to characterize the innate immune functions of porcine milk proteins may provide aid for the further improvement of management in the pig industry. Previously in our laboratory, the porcine skim milk fraction was investigated for its ability to interact with virulence components of *E. coli*. Several proteins have been shown to be able to bind either F4ac or LPS of *E. coli in vitro* [2, 3]. Further, lactadherin was demonstrated to inhibit the attachment of F4ac *E. coli* to intestinal villi *ex vivo* [3]; and pepsin-digested lactoferrin to possesses anti-bacterial activity against several pathogenic bacteria in pigs [4].

These results suggest the potential immune importance of non-immunoglobulin proteins in porcine milk. Two F4ac-fimbrial-binding proteins (i.e. lactadherin and fatty acid binding protein) identified in porcine skim milk by Shahriar et al [3] are predominantly present on MFGM in milk. In addition, porcine MFGM is able to bind F4 *E. coli* and inhibit the attachment of F4 fimbria to the intestinal brush border [153, 154]; however, the components responsible for the anti-attachment activity have not been identified. Besides lactadherin and fatty acid binding protein, there are various other proteins on MFGM of human [78], bovine [79, 80] and rat [5] milk. Based on these data the following hypothesis was created: other F4ac-fimbrial-binding proteins exist on porcine MFGM, besides lactadherin and fatty acid binding protein.

Therefore, the interaction of porcine MFGM proteins with *E. coli* F4ac was tested in Chapter 3. Porcine MFGM proteins were first separated by 1D and 2D SDS-PAGE and then

subjected to 1D and 2D Overlay Western Blot in which purified F4ac was used as a probe.

In SDS-PAGE, reducing agents were added according to the instructions of PAGE equipment manufacturer (Bio-Rad Laboratories, Mississauga, ON, Canada) to break down disulfide bonds between and within proteins, so that the protein complexes and subunits could be separated. Xanthine dehydrogenase, butyrophilin and adipophilin can form a protein complex in native MFGM, thus, it is necessary to separate these proteins by the addition of reducing agents. In 1D SDS-PAGE, mercaptoethanol was used as reducing agent, and the migration patterns of porcine MFGM (Figure 3.1, lane 1) are comparable with the published bovine 1D gel, in which the complex mentioned above were separated [6]. In 2D SDS-PAGE, dithiothreitol (DTT) was used as reducing agent, and the monomer of xanthine dehydrogenase and adipophilin could be successfully identified by LC MS/MS. In addition, a band (Figure 3.2A, MW=66 kDa, pI=4~4.7) with similar migration pattern of butyrophilin [5, 6, 164] was observed. Although this band failed to be identified by LC MS/MS, it is likely to be the monomer of butyrophilin. All together, the amount of reducing agents added in SDS-PAGE in this experiment was able to separate at least partially the protein complex of xanthine dehydrogenase, butyrophilin, adipophilin, as well as other proteins. Nevertheless, given that butyrophilin was also present in high MW region (Figure 3.2A, greater than 200 kDa), the question could arise as to whether this represented unreduced protein aggregates. Similar phenomenon was observed in human MFGM [164] and the reason for this was not determined. It might be an artifact in 2D SDS-PAGE due to high concentration of some proteins in the sample [172] or may represent unreduced butyrophilin.

The reason that Overlay Western Blot, instead of affinity chromatography in which native samples are applied, was employed to test our hypothesis was to avoid the potential false positive results caused by protein complexes in native MFGM formed by disulfide

bonds, such as those among butyrophilin, adipophilin and xanthine dehydrogenase [6]. Had affinity chromatography been used in this study, and if one of the proteins in the complex mentioned above could bind to F4ac, the other two proteins, not necessarily themselves F4ac-fimbrial-dinding proteins, would also remain in the column and be eluted together with other real F4ac-fimbrial-binding proteins, thus produce false positive results. In this study, prior to Overlay Western Blot, reducing agent was added to the porcine MFGM (during SDS-PAGE) to break the disulfide bonds, thus allowing the separation of these three proteins. Our results demonstrated that xanthine dehydrogenase does not bind to F4ac, and the other two proteins in the complex, butyrophilin and adipophilin, bind to F4ac. By affinity chromatography, on the other hand, all three proteins of this complex would have been interpreted to be F4ac-fimbrial-binding proteins.

Several positive spots were demonstrated by Overlay Western Blot, the signals of which ranged from weak to very strong, and some proteins did not react with F4ac at all (e.g. bovine serum albumin, xanthine dehydrogenase and serum amyloid A), which can be considered as internal negative controls. Purified porcine lactadherin, which was demonstrated previously by affinity chromatography to bind F4ac, was used as a positive control in 1D Overlay Western Blot and showed strong reaction with F4ac. Initially Overlay Western Blot was performed with bovine skim milk used as blocking proteins. In order to eliminate possible interference of bovine skim milk proteins in this assay, the Overlay Western Blot was also carried out using a protein free blocker and essentially similar results were obtained. Further, when F4ac was omitted (omission control), none of the proteins gave positive signal, thus it was considered that the positive signals in Overlay Western Blot were given by the *in vitro* reaction between F4ac and porcine MFGM proteins.

Based on signal intensity demonstrated by Overlay Western Blot, butyrophilin, adipophilin, lactadherin and acyl-CoA synthetase 3 were the four major

F4ac-fimbrial-binding proteins. In addition, some fragments of adipophilin also reacted strongly with F4ac.

The results of Overlay Western Blot in Chapter 3 support the previously reported the affinity between lactadherin and F4ac [3]. The finding that butyrophilin, adipophilin and acyl-CoA synthetase 3 can bind F4ac is novel. Butyrophilin belongs to the immunoglobulin superfamily, which includes various adhesive proteins; however, the previous studies of butyrophilin were mainly focused on its association with milk fat secretion [6, 24]. Similarly, no anti-bacterial functions have been reported in adipophilin and acyl-CoA synthetase 3. The result of this study suggests these proteins may be potentially involved *in vivo* in host defense against pathogens. Further studies, e.g., an attachment inhibition assay, to evaluate the roles of each individual F4ac-fimbrial-binding protein are needed.

Interestingly, Overlay Western Blot revealed that FABP reacted weakly with F4ac. Affinity between F4ac and FABP was previously demonstrated in pepsin-digested porcine milk, but not in undigested milk [3]. These phenomena may be explained by the fact that the concentration of FABP is low in skim milk and affinity between intact FABP and F4ac is low, therefore, it was not detected by affinity chromatography in the previous study when non-digested skim milk were employed. On the other hand, concentration of FABP is high in MFGM [6], and therefore, it was detected by Overlay Western Blot in Chapter 3. It is possible that partial pepsin-digestion of FABP generates fragments that have a high affinity to F4ac, permitting the detection by F4ac-affinity chromatography in pepsin-digested skim milk [3].

Several defense proteins in porcine colostrum and milk have been identified and characterized. Some milk proteins, such as IgA, exert their host defense functions locally in the gastrointestinal tract by preventing attachment of pathogens to the intestinal surface. F4ac-binding proteins identified in Chapter 3 may function as F4ac receptor analogues and

also prevent F4-positive *E. coli* attachment to intestines. Some other proteins, for example IgG and lactoferrin [137], have been demonstrated to provide systemic protection after their absorption into the blood circulation [93].

The intestinal protein absorption by neonatal piglets within 24-36 h after birth has been well accepted to be non-selective [122] based on the previously demonstrated absorption of various substances (e.g. bovine serum albumin, dextran and egg proteins) [127, 128]. In addition, selective receptor-mediated absorption of immunoglobulin [135], lactoferrin [143] and insulin-like growth factor I (IGF-I) [147] by piglets has been also reported and the receptors for these proteins are already present at birth. Harada *et al* used 2D SDS-PAGE to investigate plasma protein profile changes of neonatal piglets after suckling and reported that in plasma from post-suckling piglets, ~ 30 more protein spots were present than in plasma from pre-suckling piglets [124]. However, the identity of these proteins was not determined. Accordingly, experiments in Chapter 4 were performed to identify the changes in plasma protein profiles of piglets after natural suckling.

Comparative 2D SDS-PAGE was chosen to grossly screen the changes of plasma protein profiles after suckling. Whole plasma was first separated by 2D SDS-PAGE and stained by Coomassie R-250, which can promisingly detect proteins with concentrations > 20 μg/ml as was discussed in Chapter 4. The 2D electrophoretic pattern of post-suckling plasma (Figure 4.1B) is comparable with that of 4-month porcine plasma [151]. To our surprise, only immunoglobulins were unequivocally demonstrated to be absorbed by piglets within 24 h after suckling by comparing the electrophoretic patterns of pre- and post-suckling whole plasma. A large amount of immunoglobulins was present in the plasma after suckling, but not in the pre-suckling plasma. Other major colostral proteins, such as casein, lactoglobulin and lactalbumin were not detectable in plasma after suckling.

In order to demonstrate more minor plasma proteins, protein-A affinity chromatography

was employed to remove the immunoglobulins from the plasma. After passing through the protein-A column, a substantial amount of immunoglobulins was removed from the plasma; however, it should be noted that the immunoglobulins were not totally removed. The reason of this phenomenon was not definitely determined. Possible explanations may include technical insufficiencies and the different affinities between protein-A and immunoglobulins from various species [171]. Additionally, a more sensitive stain, colloidal Coomassie G-250, which was reported to be able detect ~1 ng of protein [162], was used to visualize the proteins. The above approaches resulted in the visualization of more minor spots in this study (Figure 4.2B). Only 4 additional protein spots (or groups of spots) were demonstrated to increase after suckling (Figure 4.2B). These spots all contain fragments of albumin, which is one of the major proteins in porcine colostrum. The increase of albumin after suckling could be due to absorption, since it was reported that bovine albumin can be absorbed by neonatal piglets [126, 127]. The fragments of albumin observed in this study are not present in plasma of 4-month pigs [151]. Accordingly, fragments of albumin observed in this study may be a result of the absorption of the partial digested albumin from the colostrum into systemic circulation. On the other hand, increased post-suckling hepatic synthesis of albumin was also reported [150]. As no antiprotease was used in this study, it is also possible that the presence of albumin fragments in post-suckling plasma is an artifact due to degradation of the absorbed non-digested and/or increased synthesized albumin during sample collection and storage. Based on current experimental design, it is impossible to determine which possibility is a more likely explanation for the presence of albumin fragments in post-suckling plasma.

These results do not support the well accepted concept that protein absorption by neonatal piglets is a non-selective process. There are several major proteins in porcine colostrum: immunoglobulins (~126 mg/ml) [81], caseins (~14 mg/g) [173], albumin (~19

mg/ml) [174], and lactoglobulin (~7 mg/ml) [175]. If the absorption of intact colostral proteins is non-selective, all of these major colostral proteins should have been demonstrated in post-suckling porcine plasma; however, only immunoglobulins and albumin are increased in neonatal porcine plasma after suckling, while others are not detectable. Moreover, the post-suckling increase of plasma albumin is much less significant compared to that of immunoglobulins. There are five possible explanations for these findings: 1) non-immunoglobulin major colostral proteins are not absorbed; 2) non-immunoglobulin major colostral proteins can be absorbed, but the degree of absorption is very low, so the presence of these proteins in porcine plasma after suckling was not detectable by the methods employed; 3) non-immunoglobulin major colostral proteins are absorbed but are removed from the portal circulation by the liver; 4) the methods employed were not sensitive enough to detect the absorption of minor proteins; or, 5) Protein-A column may have removed minor portions of some plasma proteins together with immunoglobulins and produced false negative findings (See also Appendix A). The Coomassie R-250 stain (Bio-Rad Laboratories, Mississauga, ON, Canada) we used to visualize the 2D SDS-PAGE of whole plasma can detect as little as ~ 50 ng of protein (Bio-Rad, Life Science Research Product Catalog & Price List, Canada, 2008/09, p.162). Divided by the maximum sample load (25 µl) of the 2D SDS-PAGE equipment (PROTEAN® II xi Cell, Bio-Rad Laboratories, Mississauga, ON, Canada), Coomassie R-250 stain can detect a protein with a concentration of 2 µg/ml (50 ng protein in 25 µl plasma equals to a concentration of 2 µg/ml). Interpreting conservatively, i.e. allowing for a 10 fold increase (i.e. 500 ng) in the minimal amount of protein detectable by Coomassie R-250 stain, then our method can promisingly detect proteins with concentrations > 20 µg/ml in the whole plasma.. Based on this, our detection method should have been appropriate to detect the absorption of major colostral proteins into plasma, if their absorption is substantial and proportional to that of immunoglobulins. On the other hand,

proteins with low concentrations in colostrum and plasma (e.g. cytokines) cannot be detected by our methods, and their absorption was not determined in this study. This, however, does not imply that they are biologically not important. Thus, within the major colostral proteins, only immunoglobulins are absorbed in substantial amounts and utilized by the piglets in their intact forms.

Comparative 2D SDS-PAGE seems not to be a good method to investigate our objective in Chapter 4, which was to determine the changes in plasma protein profiles in piglets after natural suckling. This method cannot detect many minor proteins in the samples. Removal of major proteins in the samples and chromatographic fractioning of the samples are two common solutions for this problem. In one study, after removal of most of the major human plasma proteins by immunoaffinity chromatography and further chromatographic fractioning, minor proteins known to have concentrations < 10 ng/ml, such as interleukin-6, were able to be visualized by Coomassie stain [176]. This approach, however, is not suitable for comparative studies, because it is very difficult to accurately evaluate the original concentration of a protein after many chromatographic steps and the antibodies against many major porcine plasma proteins are not available. Quantitative comparative mass spectrometry is an alternative choice of achieving our objective. By this method, the developmental changes of bovine MFGM protein profiles have been successfully demonstrated [80]. In future studies, this approach can be used to further evaluate the changes in plasma protein profiles in piglets after natural suckling.

In conclusion, the studies present in this thesis demonstrated that there are several proteins on porcine MFGM can bind F4ac, and that within the major colostral proteins, immunoglobulins are the only proteins absorbed and utilized in large amount and in intact form by neonatal piglets.

This research has extended our knowledge of innate immune substances in porcine milk.

The F4ac-fimbrial-binding proteins identified in Chapter 3 may not be the main innate defense against pathogenic *E. coli,* but possibly contribute useful adjunct protection. Future confirmation and characterization of the protective role of each major F4ac-fimbrial-binding protein on porcine MFGM is needed. These proteins can be purified and tested individually for their ability to inhibit F4ac *E. coli* from binding to the porcine intestinal villi *ex vivo*, as was used to characterize the anti-attachment ability of lactadherin [3]. Further, the interactions between porcine MFGM proteins, including their fragments, and various bacterial and viral pathogens require more study. Overlay Western Blot can be used as a fast screening method to select potential MFGM and milk proteins that interact with pathogens. More intense research is needed to determine if non-immunoglobulin defense substances identified in porcine milk have potential to be used for future treatment and prevention of certain intestinal infections. Last but not least, since more and more defense proteins have been identified from porcine milk, research efforts should be made to investigate whether the decreased overall ingestion of these proteins associated with the early weaning time in modern swine industry leads to a change of disease prevalence in pigs.

6. References

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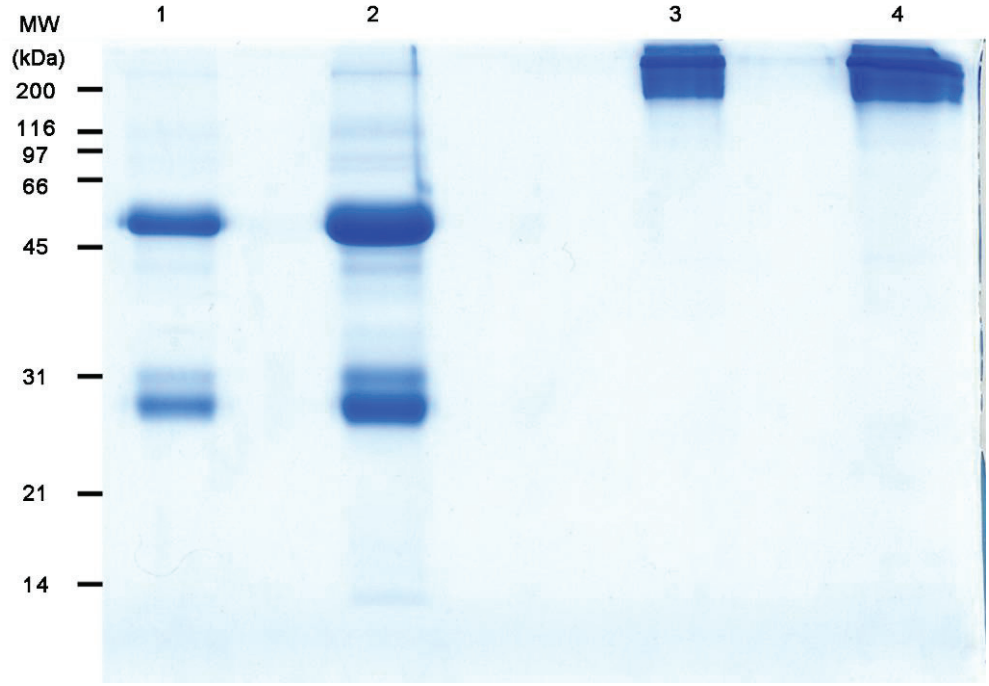
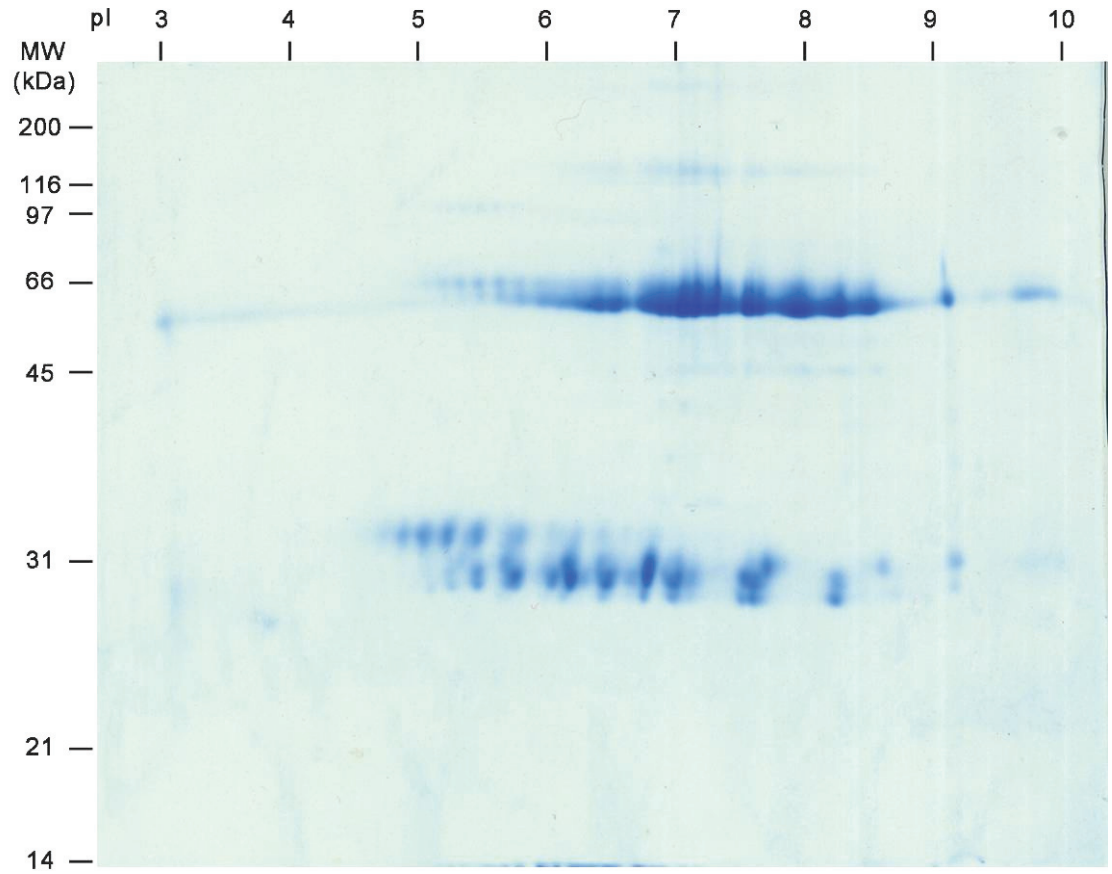
Appendix A

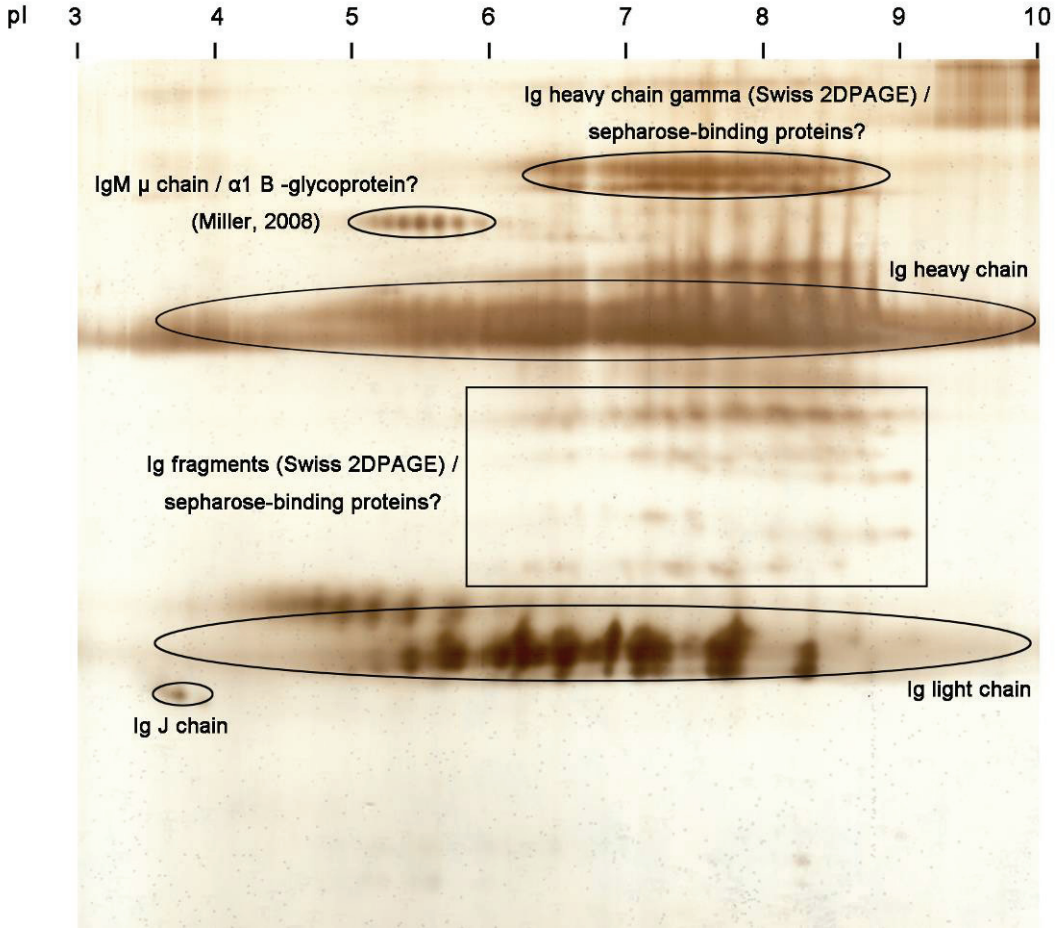
Proteins removed from 24 h post-suckling porcine plasma by protein-A affinity chromatography



Appendix A1. Colloidal Coomassie G-250 stained 1D SDS-PAGE of the proteins removed from 24 h post-suckling porcine plasma by protein-A affinity chromatography. Five µg (lane 1 and 3) and 10µg (lane 2 and 4) of the eluted proteins were separated under reduced (lane 1 and 2) and non-reduced (lane 3 and 4) conditions. By visual estimation, bands represent immunoglobulin heavy chain (~ 50 kDa) and light chain (~ 28-31 kDa) compose more than 95% of the total proteins in the elutant.



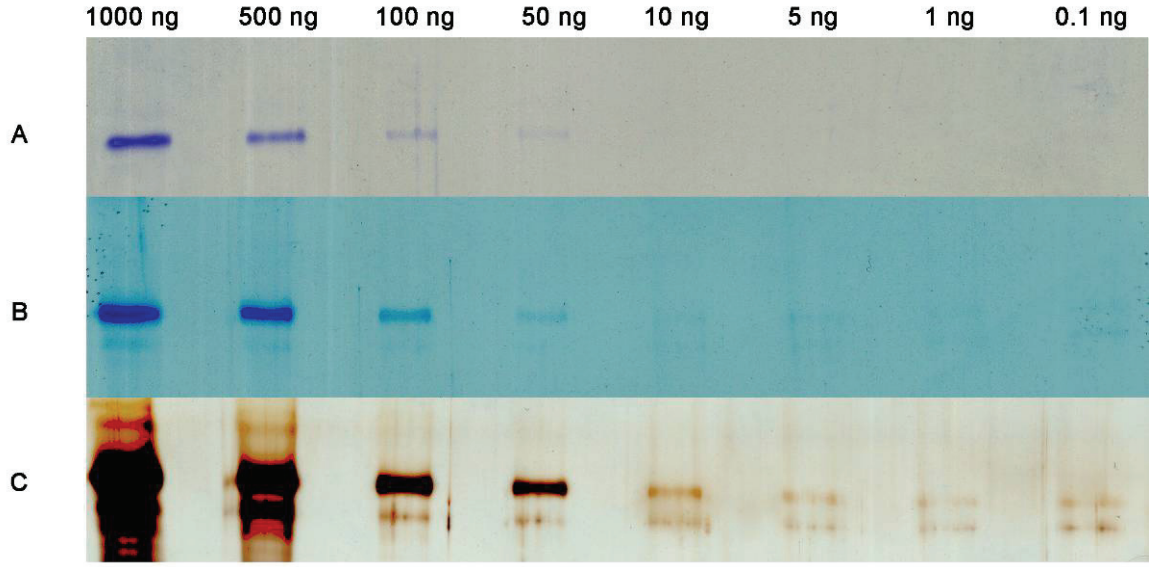
Appendix A2. Colloidal Coomassie G-250 stained 2D SDS-PAGE of the proteins (80 µg) removed from 24 h post-suckling porcine plasma by protein-A affinity chromatography. By visual estimation, spots represent immunoglobulin heavy chain (~ 50 kDa) and light chain (~ 28-31 kDa) compose more than 95% of the total proteins in the elutant.



Appendix A3. Silver-stained 2D SDS-PAGE of the proteins (20 μg) removed from 24 h post-suckling porcine plasma by protein-A affinity chromatography. Several spots not in the locations of immunoglobulin heavy chain and light chain were visualized in the elutant. These spots may be components of the immunoglobulin molecules and/or sepharose-binding proteins in the porcine plasma.

Appendix B

Sensitivities of three staining methods



Appendix B. Different amounts of bovine serum albumin (BSA) were separated by 1D SDS-PAGE and stained by Coomassie R-250, Colloidal Coomassie G-250 and silver stain. Both Coomassie stains were able to detect as little as 50 ng of BSA and silver stain can detect down to 0.1 ng BSA.