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Differences in the bovine milk whey proteome between early pregnancy and the estrous cycle

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ABSTRACT

Current bovine pregnancy detection methods are not reliable until at least day 28 post artificial insemination (AI). The bovine estrous cycle is approximately 21 days; consequently, producers miss an opportunity to rebreed at the next estrous event. Therefore, commercial interest exists for the discovery of novel biomarkers of pregnancy which could reliably detect pregnancy status at or before day 21 of pregnancy. The objective of the present study was to use liquid chromatography tandem mass spectrometry (LC-MS/MS) to perform a global, label-free, proteomics study on (i) milk whey and (ii) extracellular vesicle (EV) enriched milk whey samples, from day 21 of pregnancy, compared with day 21 of the estrous cycle, in order to identify potential protein biomarkers of early pregnancy. The estrous cycles of 10 dairy cows were synchronized, they went through one (control) estrous cycle and these cows were artificially inseminated during the following estrus. These cows were confirmed pregnant by ultrasound scanning. Milk whey samples were collected on day 21 of the estrous cycle and on day 21 post AI. Milk whey samples and EV enriched milk whey samples were analyzed by LC-MS/MS and subsequent analyzes of the label-free quantitative data was performed in MaxQuant and Perseus. Four proteins (APOB, SPADH1, PLIN2 and LPO) were differentially expressed between the proteomes of milk whey from day 21 of pregnancy and day 21 of the estrous cycle ($P < 0.05$). Ten proteins (PIGR, PGD, QSOX1, MUC1, SRPRA, MD2, GAPDH, FOLR1, GPRC5B and HHIPL2) were differentially expressed between the proteomes of EV enriched milk whey from day 21 of pregnancy and day 21 of the estrous cycle ($P < 0.05$). These proteins are potential milk whey biomarkers of early pregnancy.

1. Introduction

Dairy farming profitability in seasonal, pastoral based systems is dependent on compact calving and achieving a target of one calf per cow every 365 days [1]. This compact calving system is economically favorable as it enables producers to reduce feed costs by aligning calving and peak lactation with the maximum grass growth in spring. To achieve this target, high levels of reproductive efficiency are required, and in particular, the ability to determine the pregnancy status of cows as soon as possible after artificial insemination (**AI**) or mating.

One of the most common methods used for pregnancy detection is transrectal ultrasound scanning [2]. It is the gold standard method for pregnancy detection in dairy cows/heifers due to its superior sensitivity, ability to detect the presence of multiple fetuses or fetal death, and capability to assess uterine health and ovarian cyclicity if the animal is not pregnant [3,4]. However, this method cannot be performed until at least day 26–28 post AI and requires significant expertise and expensive equipment. An alternative pregnancy detection method is transrectal palpation [5], but similarly, this technique can only be used from 35 days after AI, and additionally, it poses the threat of induction of embryo loss [6]. Visual observation of estrus (often with the use of aids to detection; e.g. tail paint/heat pads) is commonly used for recognition of conception failure [7]. However, it is labor intensive and not very

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reliable due to the phenomenon of silent and/or missed heats [8].

Biological fluids such as blood or milk are ideal matrices for diagnostic assays as their collection is minimally invasive and they are protein abundant. Milk is the most desirable matrix as a sample can be easily obtained during routine milking of the cows and, therefore, will not impose any additional stress. There are bovine pregnancy detection methods currently in existence which utilize proteins in biological fluids for pregnancy detection. These include pregnancy associated glycoprotein (PAG) enzyme-linked immunosorbent assays (ELISA) [9], pregnancy specific protein B (PSPB) radioimmunoassays [10], early conception factor (ECF) lateral-flow assay [11,12], progesterone immunoassays [13], and in-line progesterone sensors [14]. However, while in-line progesterone testing can detect non-pregnancy, progesterone is not a specific biomarker of pregnancy, as elevated progesterone concentrations may be indicative of an extended estrous cycle or an ovarian pathological condition [15]. The PAG and PSPB ELISAs are only reliable indicators of pregnancy status after at least day 28 post AI [9,16]. The ECF test can be performed as early as day 6 post AI, however, the ECF test lacks specificity with reported values of detection of correctly identified non-pregnant cows of only 50% [12]. Therefore, these pregnancy diagnosis assays are of limited use for many producers. Commercial interest exists for the discovery of novel protein biomarkers of pregnancy which could reliably detect pregnancy status at or before day 21 post AI as this would allow producers the opportunity to rebreed at the next estrus event. As the majority of early embryonic death occurs before day 16 post insemination [17,18], a diagnostic test that provides valid results between days 17 and 21 post AI would be most desirable.

The objectives of the present study were to use liquid chromatography tandem mass spectrometry (LC-MS/MS) to perform a global, label-free, proteomics study on (i) milk whey and (ii) extracellular vesicle (EV) enriched milk whey samples, from day 21 of pregnancy and day 21 of the estrous cycle, in order to identify potential protein biomarkers of early pregnancy.

2. Materials and methods

All animal procedures performed in this study were conducted by authorized individuals under experimental license from the Irish Medicines Board (HPRA Project Authorization No. AE18982/P047).

2.1. Animal model

This study is a component of a larger study examining possible reliable molecular biomarkers of early pregnancy in dairy cows (Malo Estepa et al. unpublished data). For clarity, the animal model is briefly summarized here. The estrous cycles of 81 multiparous Holstein-Friesian dairy cows on a commercial dairy farm in Co. Kildare, Ireland, were synchronized. An intra-vaginal progesterone releasing device (CIDR 1.38G vaginal delivery system for cattle, Zoetis Ireland limited, Dublin, Ireland), was inserted in the vagina of each cow. Each cow simultaneously received intramuscularly, 100 μg of Gonadotropin-releasing hormone (Acegon 50 μg/ml solution for injection for cattle, Laboratorios Syva, León, Spain; day −31; Fig. 1). Seven days later, the cows received an intramuscular injection of 25 mg of prostaglandin (Lutalyse 5 mg/ml, dinoprost, Zoetis Ireland Limited, Dublin, Ireland) and either heat patches (Estrotec Heat Detector, Rockway Inc., Wisconsin, USA) or tail paint, were applied on the tail head of the cows, as aids to detect estrus (day −24; Fig. 1). The CIDRs were removed the following day (day −23; Fig. 1). Commencing two days later, the cows were examined for visual signs of estrus four times per day (day −21; Fig. 1).

All cows went through one (control) estrous cycle. On day 21 of the control cycle (i.e., day 0 of the following cycle), milk samples for proteomic analyzes were collected. Seventy-four cows were artificially inseminated 12 h following observation of estrus (day 0; Fig. 1). Milk samples for proteomic analyzes were collected 21 days post AI (day 21; Fig. 1). Forty-five cows were confirmed pregnant by ultrasound scanning on day 35 post AI (day 35; Fig. 1). Pregnancy and estrus were further confirmed by examining progesterone levels in additional milk samples using a radioimmunoassay according to the manufacturer's instructions (DIAsource ImmunoAssays SA, Louvain-LaNeuve, Belgium). Ten of these cows were selected for use in the present study (Table 1).

2.2. Comparison of the milk whey proteome between day 21 of pregnancy and day 21 of the estrous cycle

2.2.1. Sample collection and processing

Milk was collected at the routine morning milking. Whole milk was centrifuged for 30 min (4000 × *g*) at 4 °C. Fat was removed with a spatula and subsequently 12 ml of whey was recovered (avoiding the cell pellet) and placed in a sterile falcon tube. All samples were subsequently stored at −80 °C.

2.2.2. Sample preparation for LC-MS/MS analysis (Fig. 2)

Chemical reagents were purchased from Sigma Aldrich, Wicklow, Ireland, unless stated otherwise.

2.2.3. Protein precipitation

Milk whey (200 μl) was precipitated using 50 μl trichloroacetic

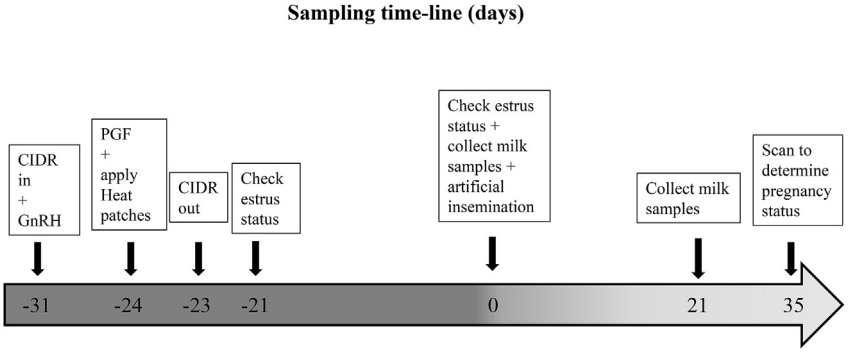

Fig. 1. Outline of the days that the cows were estrous cycle synchronized, artificially inseminated and milk sampled.
CIDR = Progesterone containing Controlled Internal Drug Release device.
GnRH = 100 μg of Gonadotropin-releasing hormone.
PGF = 25 mg of prostaglandin.

Table 1
The 10 cows which were used in the study.

Cow ID	Days in milk by day 21 of the control estrous cycle	Milking frequency	Lactation number
1783	89	twice per day	5
2141	181	twice per day	4
2201	89	twice per day	4
2374	84	twice per day	3
2426	88	twice per day	3
2667	86	twice per day	2
2746	99	twice per day	1
2767	104	twice per day	1
2843	91	twice per day	1
2851	90	twice per day	1

Table 2
Proteins in milk whey that were differentially expressed between day 21 of pregnancy and day 21 of the control estrous cycle.

Protein name	Gene name	Fold change[a]
Apolipoprotein B	*APOB*	1.48
Spermadhesin-1	*SPADH1*	1.36
Perilipin	*PLIN2*	1.19
Lactoperoxidase	*LPO*	−1.36

[a] Positive number = increased on day 21 of pregnancy, negative number = decreased on day 21 of pregnancy.

acid. It was incubated at 4 °C for 10 min followed by centrifugation (14,000 × *g*) for 5 min at 4 °C. Samples subsequently went through a total of two acetone washes, whereby the supernatant was removed, 200 μl of acetone (stored at −20 °C) was added followed by centrifugation (14,000 × *g*) for five minutes at 4 °C. The pellets were dried and resuspended in 80 μl of 6 M urea dissolved in 50 mM ammonium bicarbonate. Protein content was determined using the Bradford assay and the protein concentration in each sample was normalized to 2 μg/μl.

2.2.4. Protein reduction and alkylation

Samples were reduced with 100 mM 1,4-dithiothreitol dissolved in 50 mM ammonium bicarbonate, for 30 min at 60 °C, and alkylated with 200 mM iodoacetamide dissolved in 50 mM ammonium bicarbonate, for 30 min, at room temperature, in darkness. Samples were diluted with 50 mM ammonium bicarbonate to a concentration of 0.6 μg/μl.

2.2.5. Trypsin digestion

Samples were digested with trypsin singles, proteomics grade, according to the manufacturer's instructions at a trypsin:protein ratio of 1:30, overnight at 37 °C. The tryspin digestion reaction was terminated using 5 μl of 10% trifluoroacetic acid. Tryptic peptides were purified for mass spectrometry using C18 Millipore® Ziptips. The peptide containing samples were resuspended in 20 μl of 0.2% acetic acid, 2% acetonitrile and were normalized to a concentration of 0.8 absorbance at 215 nm using a nanodrop spectrometer (NanoDrop Technologies, Wilmington, DE, USA).

2.2.6. LC-MS/MS analysis

Purified trypsin digested peptide samples (5 μl) were analyzed by LC-MS/MS (in duplicate) using an Ultimate3000 liquid chromatography unit coupled to a Q Exactive™ Hybrid Quadrupole-Orbitrap™ mass spectrometer (ThermoFisher Scientific; USA). Peptides were separated by an increasing acetonitrile gradient from 2% to 35% on a C18 reverse phase chromatography column packed with 1.9 μm particle size, 300 Å pore size C18 material (Dr. Maisch GmbH, Ammerbuch-Entringen, Germany) to a length of 120 mm in a column with a 75 μm ID, using a 90 min gradient at a flow rate of 250 nl/min. All data were acquired with the mass spectrometer operating in automatic data-dependent acquisition mode (DDA, shotgun). A full MS service scan at a resolution of 70,000, AGC target 3e6 and a range of *m/z* 350–1600 was followed by up to 12 subsequent MS/MS scan with a resolution of 17,500, AGC target 2e4, isolation window *m/z* 1.6 and a first fix mass of *m/z* 100. Dynamic exclusion was set to 30 s (half peak width).

2.2.7. Data analysis

Protein identification from the MS/MS data was performed using the Andromeda search engine in MaxQuant (version 1.6.0.1; http://maxquant.org/) which correlated the data against the annotated bovine proteome database obtained from uniprot (www.uniprot.org) along with common contamination sequences. The following search parameters were used: first search peptide tolerance of 20 ppm, second search peptide tolerance 4.5 ppm, fixed modification of cysteine carbamidomethylation, variable

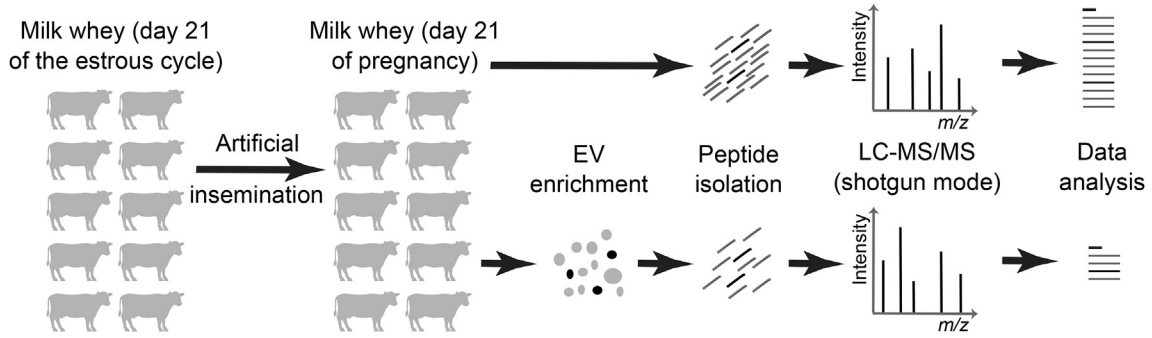

Fig. 2. Outline of the experimental workflow.
EV = Extracellular vesicles.
LC-MS/MS = Liquid chromatography tandem mass spectrometry.

modifications of *N*-acetylation of protein and oxidation of methionine, use label-free quantification (**LFQ**) analysis, match between runs, do not require MS/MS for quantification, and a maximum of two missed cleavage sites were allowed. False Discovery Rates (**FDR**) were set to 1% for both peptides and proteins and the FDR was estimated following searches against a target-decoy (decoy = reverse of the database) database. Label-free quantification intensities were calculated using the MaxLFQ algorithm [19] from razor and unique peptides with a minimum ratio count of two peptides across samples. Peptides with a minimum length of seven amino acids were considered for identification.

Results processing, statistical analyses, and graphics generation were conducted using Perseus V. 1.5.8.5. LFQ intensities were log_2-transformed, filtered to have 8 valid values in at least one group and paired t-tests between the proteomes of milk whey from day 21 of pregnancy and day 21 of the estrous cycle were performed using a p-value cut-off of <0.05. Approximate protein abundance ranges based on LFQ intensities were plotted (Supplementary Fig. 1).

Manual inspection of the MaxLFQ-Perseus analysis pipeline was carried out using Skyline version 4.1 (64 bit version): peptide-spectrum-matches from Andromeda (MaxQuant search engine) were used to build a spectral library. Primary mass spectrometer instrument files (.raw files) were loaded into Skyline and peptides corresponding to proteins of interest (Tables 2 and 4) were automatically matched to their respective MS/MS spectra. The resulting ion chromatograms were manually inspected and only peptides with base line separations, near Gaussian peak shapes and occurrence in all biological replica were selected to generate area under the curve calculations (Supplementary Fig. 3).

2.3. Comparison of the extracellular vesicle enriched milk whey proteome between day 21 of pregnancy and day 21 of the estrous cycle

All steps were identical to that performed for the milk whey samples except extracellular vesicles (**EV**) were first enriched using qEV size exclusion columns (Izon Science, UK) (Fig. 2). The EV enrichment was carried out according to the manufacturer's instructions, using a starting volume of 500 μl milk whey and a buffer consisting of 0.2 μm filtered phosphate buffered saline. An EV enriched fraction of 1.5 ml was collected for each milk whey sample.

The EV enriched fraction was used for all subsequent steps (protein precipitation, protein reduction and alkylation, trypsin digestion, LC-MS/MS analysis and data analysis). All steps were performed as described earlier for the milk whey samples, except the filtering for valid values in Perseus was adjusted to four valid values in at least one group.

The list of proteins present in the EV enriched milk whey samples after filtering with Perseus was compared with the list of proteins present in the milk whey samples after filtering with Perseus using "Venny" [20], in order to determine whether EV enrichment actually led to identification of additional proteins which were not detected by LC-MS/MS using unenriched milk whey. The list of proteins exclusively identified in the EV enriched milk whey proteome were submitted to STRING (https://string-db.org/, version 10.5) for functional gene ontology enrichment analysis, using default parameters, which employ a Fisher's exact test followed by a correction for multiple testing (FDR <0.05), and the bovine genome background proteins [21].

All mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE PRoteomics IDEntifications (PRIDE) [22] partner repository with the dataset identifier PXD007927.

3. Results

3.1. Milk whey

Two hundred and eighteen proteins were identified in the milk whey proteome following filtering using Perseus (Supplementary Table 1). The average intensity of proteins quantified across the majority of samples spans nearly four orders of magnitude (Supplementary Fig. 1). Four proteins (apolipoprotein B, spermadhesin-1, perilipin and lactoperoxidase) were differentially expressed between the proteomes of milk whey from day 21 of pregnancy and day 21 of the estrous cycle ($P < 0.05$) (Table 2). Apolipoprotein B, spermadhesin-1 and perilipin were more abundant in milk whey from day 21 of pregnancy while lactoperoxidase was more abundant in milk whey from day 21 of the control estrous cycle (Table 2). However, the fold change differences between the abundance of these proteins in milk whey from day 21 of pregnancy and day 21 of the estrous cycle were very small (range 1.19–1.48; Table 2). The results from the MaxQuant-Perseus workflow were complemented by manual inspection of ion chromatograms using Skyline software. The subsequent analysis of manually reviewed peptide peaks generally support the results given in Table 2 (Supplementary Fig. 2).

3.2. Extracellular vesicle enriched milk whey

One hundred and fifty-nine proteins were identified in the extracellular vesicle enriched milk whey proteome following filtering using Perseus (Supplementary Table 2). The average intensity of proteins quantified across the majority of samples spans nearly four orders of magnitude (Supplementary Fig. 1). Ninety-three of these proteins had also previously been observed in the milk whey proteome, while 66 were exclusively identified in the EV enriched milk whey proteome (Fig. 3, Supplementary Table 3). One hundred and twenty-five proteins were exclusively identified in the unenriched milk whey proteome (Fig. 3, Supplementary Table 3). The proteins which were exclusively found in the EV enriched milk proteome correlated to 62 proteins in the STRING bovine database. There were thirty significantly enriched gene ontology "cellular component" terms among the proteins found exclusively in the EV enriched milk whey compared with the bovine genome background proteins (FDR<0.05; Supplementary Table 4). The top eighteen enriched gene ontology "cellular component" terms (FDR < 0.006) are presented in Table 3, and the majority of these terms relate to extracellular vesicles (Table 3, Supplementary Table 4).

Ten proteins (polymeric immunoglobulin receptor, 6-

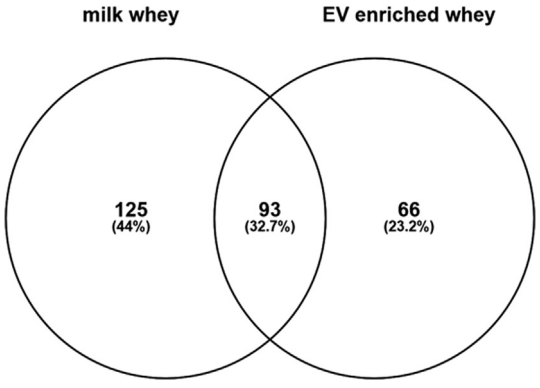

Fig. 3. Venn diagram showing the number of proteins found by LC-MS/MS (following filtering with Perseus) in milk whey and in EV enriched milk whey.

Table 3
Enriched gene ontology "cellular component" terms from proteins exclusively expressed in EV enriched milk whey.

GO-term	Description	No. of proteins	False discovery rate
GO.0005576	extracellular region	23	1.24E-09
GO.0044421	extracellular region part	18	9.08E-07
GO.0031982	vesicle	17	1.51E-06
GO.0070062	extracellular exosome	15	1.51E-06
GO.0031988	membrane-bounded vesicle	16	3.74E-06
GO.0005886	plasma membrane	13	0.000325
GO.0005912	adherens junction	6	0.000384
GO.0071944	cell periphery	13	0.000384
GO.0005575	cellular component	29	0.000598
GO.0005615	extracellular space	8	0.00131
GO.0043227	membrane-bounded organelle	23	0.00185
GO.0005925	focal adhesion	5	0.00225
GO.0044297	cell body	4	0.0031
GO.0030054	cell junction	7	0.00315
GO.0097458	neuron part	6	0.00315
GO.0044444	cytoplasmic part	18	0.00324
GO.0016020	membrane	18	0.00561
GO.0042995	cell projection	7	0.00561

phosphogluconate dehydrogenase, decarboxylating, sulfhydryl oxidase, mucin-1, signal recognition particle receptor subunit alpha, lymphocyte antigen 96, glyceraldehyde-3-phosphate dehydrogenase, folate receptor alpha, G protein-coupled receptor class C group 5 member B and hedgehog interacting protein-like 2) were differentially expressed between the proteomes of EV enriched milk whey from day 21 of pregnancy and day 21 of the estrous cycle ($P < 0.05$; Table 4). Polymeric immunoglobulin receptor, 6-phosphogluconate dehydrogenase, decarboxylating, sulfhydryl oxidase, mucin-1, signal recognition particle receptor subunit alpha and lymphocyte antigen 96 were more abundant in milk whey from day 21 of pregnancy while glyceraldehyde-3-phosphate dehydrogenase, folate receptor alpha, G protein-coupled receptor class C group 5 member B and hedgehog interacting protein-like 2, were more abundant in milk whey from day 21 of the control estrous cycle (Table 4). The fold change differences between the abundance of these proteins in extracellular vesicle enriched milk whey from day 21 of pregnancy and day 21 of the estrous cycle were very small (range 1.16–1.34; Table 4). The results of Table 4 were manually validated on the peptide level using Skyline software (Supplementary Fig. 3). The results (Supplementary Fig. 4) are in concert with results given in Table 4.

4. Discussion

This was the first study to utilize LC-MS/MS to perform label-free shotgun proteomics using milk whey samples from dairy cows from day 21 of pregnancy and day 21 of the control estrous cycle. To-date, limited research has been carried out to determine changes in the bovine proteome due to pregnancy and no research has yet been performed using high mass accuracy mass spectrometers coupled to liquid chromatography, such as Q Exactive™ Hybrid Quadrupole-Orbitrap™ Mass Spectrometer. Furthermore, this is the only study to compare biological samples from pregnant cows with samples obtained from the equivalent day of their control estrous cycle, in order to examine alterations in the proteome induced by early pregnancy.

Previous studies investigated potential bovine protein biomarkers in early pregnancy in urine [23] using both 2 dimensional (2D) fluorescence difference gel electrophoresis (DIGE) and label-free proteomics, in serum using 2D DIGE and subsequent mass spectrometry [24], and in milk using 2D gel electrophoresis followed by mass spectrometry [25]. Proteomics based studies utilizing the 2D gel electrophoresis technique followed by mass spectrometry, have also been performed in buffalo cows and differences have been detected due to early pregnancy status in the blood proteome [26]. Proteins which were up-regulated in serum from pregnant cows, comprising of the conglutinin precursor, modified bovine fibrinogen and IgG1, and proteins which were down-regulated in serum from pregnant cows, including hemoglobin, complement component 3, bovine fibrinogen and IgG2a, have been suggested as possible candidates for an early blood-based pregnancy test [24]. However, these differentially expressed proteins are common abundant proteins found in bovine serum, and therefore, are unlikely to be specific for pregnancy diagnosis, as the low abundance proteins in biological fluids are more likely to be affected by pregnancy status [27]. Lactoferrin, lactotransferrin and alpha-1 g have also been suggested as possible pregnancy biomarkers in milk [25] and synaptojanin-1, apolipoprotein A-1, apolipoprotein B, keratin 10 and Von Willebrand

Table 4
Proteins in extracellular vesicle (EV) enriched milk whey that were differentially expressed between day 21 of pregnancy and day 21 of the control estrous cycle.

Protein name	Gene name	Fold change[a]
Polymeric immunoglobulin receptor	*PIGR*	1.34
6-phosphogluconate dehydrogenase, decarboxylating	*PGD*	1.30
Sulfhydryl oxidase	*QSOX1*	1.25
Mucin-1	*MUC1*	1.24
Signal recognition particle receptor subunit alpha	*SRPRA*	1.19
Lymphocyte antigen 96	*MD2*	1.18
Glyceraldehyde-3-phosphate dehydrogenase	*GAPDH*	−1.28
Folate receptor alpha	*FOLR1*	−1.23
G protein-coupled receptor class C group 5 member B	*GPRC5B*	−1.18
Hedgehog interacting protein-like 2	*HHIPL2*	−1.16

[a] Positive number = increased on day 21 of pregnancy, negative number = decreased on day 21 of pregnancy.

factors have been observed as potential protein candidates to act as pregnancy biomarkers in blood of buffaloes [26]. However, many limitations may have restricted the identification of appropriate biomarkers of early pregnancy in these studies including the limited sample size (n = 2) in the serum proteomics study, the use of pooled samples for the label-free shotgun proteomics analysis of urine and the stage of gestation (day 35 post AI) examined in the milk proteomics study.

In the present study, four proteins and ten proteins were found to be differentially expressed between day 21 of pregnancy and day 21 of the control estrous cycle in the milk whey and EV enriched milk whey, respectively. Milk whey samples enriched for EV were examined for changes in protein abundance due to pregnancy in addition to the milk whey samples as milk contains several highly abundant proteins (e.g. casein) which can limit the detection of low abundance proteins using LC-MS/MS [28,29]. Furthermore, exosomes make up the majority of EV in milk and are secreted by cells in order to act as a method of intercellular communication and to play a role in immune function [30]. Therefore, they may carry valuable pregnancy biomarkers which are likely to go undetected without prior enrichment of extracellular vesicles during sample preparation for mass spectrometry. The enrichment for extracellular vesicles in the present study resulted in the detection of 66 additional proteins which were not previously detected in the milk whey analysis. These proteins were indeed mainly present in extracellular vesicles as the majority of enriched gene ontology "cellular component" terms for this subset of proteins related to extracellular vesicles and 24% and 37% of these proteins were included in the "extracellular exosome" and "membrane-bounded organelle" gene ontology categories, respectively.

Interestingly, one of the proteins found to be upregulated in EV enriched milk whey, due to early pregnancy, the polymeric immunoglobulin receptor, was consistently observed to be upregulated in urine [23] and milk samples [25] from pregnant compared with non-pregnant cows. This protein is involved in the transfer of immunoglobulin across epithelial cells and expression of the gene for this protein has been shown to be upregulated in the ovine mammary gland in response to treatment with estradiol and progesterone and during pregnancy progression during the third trimester [31]. Therefore, due to its consistent detection as upregulated in biological fluid from pregnant cows and its potential to be hormonally regulated, it may serve as a candidate for an early bovine pregnancy test.

Other proteins upregulated in the EV enriched milk whey samples from day 21 of pregnancy which may be involved in sustaining a pregnancy include sulfhydryl oxidase, mucin-1 and lymphocyte antigen 96. Sulfhydryl oxidase has been shown to be highly expressed in human placentas and has been suggested to be essential for trophoblast survival due to its protective role against oxidative stress-induced cell death [32]. Mucin-1 was increased in human serum during pregnancy [33]. Lymphocyte antigen 96 has been detected in bovine uteri during the first trimester of pregnancy and it functions to enhance the Toll-like receptor 4 recognition of pathogen lipopolysaccharides [34]. Therefore, its expression may be increased to help produce an anti-bacterial environment for conceptus implantation. Additionally, a protein which was down-regulated in the milk whey samples from day 21 of pregnancy compared with day 21 of the estrous cycle, hedgehog-interacting protein-like 2, may be important in pregnancy establishment as it is a member of the hedgehog signaling family interacting proteins, which are implicated in a variety of processes during embryogenesis [35].

A protein that was more abundant in the milk whey samples from day 21 of pregnancy compared with day 21 of the estrous cycle was apolipoprotein B. This result is consistent with that observed by Balhara et al. [26], who discovered it to be up-regulated on day 7 of pregnancy in serum from pregnant buffaloes. It may be an interesting bovine pregnancy biomarker as it has been observed to be expressed in the yolk sac of mice and rats, and in the yolk sac and placenta in humans, and furthermore, is involved in the transfer of lipids from the mother to the developing fetus [36]. Other possible biomarkers of pregnancy in milk whey which were found to be up-regulated in milk whey from pregnant cows at day 21 post AI compared with day 21 of the estrous cycle were perilipin and spermadhesin-1. The perilipin protein has been detected in bovine uterine lumen fluid during conceptus elongation and its gene has showed increased expression in pregnant versus non-pregnant endometrial tissue in heifers on day 7 to day 19 of pregnancy and the estrous cycle [37]. Spermadhesin-1 has generally been studied in seminal vesicles and epididymis tissues and it stimulates progesterone secretion and growth through cell division and in bovine granulosa cells *in vitro* [38]. However, it has also previously been found in the bovine casein micelle [39] and bovine milk whey [40]. Therefore, it may play important, yet unknown roles in embryogenesis and implantation.

Therefore, this study has highlighted potential protein candidate biomarkers of bovine pregnancy present in milk whey samples including apolipoprotein B, perilipin, spermadhesin-1, the polymeric immunoglobulin receptor, sulfhydryl oxidase, mucin-1 and lymphocyte antigen 96. This study provides novel insights into changes in protein abundances in milk samples associated with early pregnancy. However, as the fold change differences between the proteins in the milk whey, between the two physiological states (pregnant, cycling) was not very large, it would be difficult to create a pregnancy diagnostic test using these proteins which is valid at day 21 post AI. Concentrations of these biomarkers may be examined in other biological fluids, e.g. blood, vaginal mucus, to determine if larger fold change differences can be observed in these biofluids, and consequently, whether these protein biomarkers could be used for a reliable blood or mucus based pregnancy test.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.theriogenology.2018.04.008.

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