Plasma exosome profiles from dairy cows with divergent fertility phenotypes

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ABSTRACT

Cell-to-cell communication in physiological and pathological conditions may be influenced by neighboring cells, distant tissues, or local environmental factors. Exosomes are specific subsets of extracellular vesicles that internalize and deliver their content to near and distant sites. Exosomes may play a role in the maternal-embryo crosstalk vital for the recognition and maintenance of a pregnancy; however, their role in dairy cow reproduction has not been established. This study aimed to characterize the exosome profile in the plasma of 2 strains of dairy cow with divergent fertility phenotypes. Plasma was obtained and characterized on the basis of genetic ancestry as fertile (FERT; <23% North American genetics, New Zealand Holstein-Friesian strain, n = 8) or subfertile (SUBFERT; >92% North American genetics, North American Holstein-Friesian strain, n = 8). Exosomes were isolated by differential and buoyant density centrifugation and characterized by size distribution (nanoparticle tracking analysis, NanoSight NS500, NanoSight Ltd., Amesbury, UK), the presence of CD63 (Western blot), and their morphology (electron microscopy). The total number of exosomes was determined by quantifying the immunoreactive CD63 (ExoELISA kit, System Biosciences), and the protein content established by mass spectrometry. Enriched exosome fractions were identified as cup-shape vesicles with diameters around 100 nm and positive for the CD63 marker. The concentration of exosomes was 50% greater in FERT cows. Mass spectrometry identified 104 and 117 proteins in FERT and SUBFERT cows, of which 23 and 36 were unique, respectively. Gene ontology analysis revealed enrichment for proteins involved in immunomodulatory processes and cell-to-cell communication. Although the role of exosomes in dairy cow reproduction remains to be elucidated, their quantification and content in models with divergent fertility phenotypes could provide novel information to support both physiological and genetic approaches to improving dairy cow fertility.

Key words: exosomes, fertility, dairy cow

INTRODUCTION

Until recently, genetic selection in dairy cows has focused primarily on milk production traits, with very few countries including functional traits such as fertility indices (Miglior et al., 2005). As a result, milk production capacity of the modern dairy cow has increased dramatically, but fertility has declined steadily (Garnsworthy et al., 2008). This decline is attributed to a reduction in pregnancy rate in the modern cow, chiefly associated with an increase in metabolic pressure due to increases in milk production (Butler, 2000; Roche et al., 2011). However, another critical detrimental factor to cow fertility is the presence of an activated inflammatory system through infectious or immune challenge (Formigoni and Trevisi, 2003; Piccinini et al., 2004; McDougall et al., 2007).

The need for, and utility of, markers of early disease onset (or vulnerability to diseases), which often can lead to early intervention and greater survival rates, has accelerated the development of methods for biomarker discovery in humans (Pan et al., 2005; Peddinti et al., 2008). The same methods could be used to predict desirable traits in animals, such as likelihood of pregnancy success. The use of epigenetic biomarkers is still in its infancy but already some positive results have been obtained (Magee et al., 2010; Berkowicz et al., 2011). We believe that pregnancy rates can be improved by use of protein and epigenetic biomarkers that can be delineated and then used prognostically for positive reproductive performance and, potentially,
Recently, the utility of circulating nanovesicles, such as exosomes, as biomarkers of disease has been reported (Alvarez et al., 2012; Bala et al., 2012). Exosomes are small (40–100 nm) membrane vesicles that are secreted from all cell types through the inward budding of multivesicular bodies with the plasma membrane. They contain protein, lipid, and nucleic acids and are hypothesized to function as messengers for cellular communication between tissues (De Toro et al., 2015). The number of exosomes found in bodily fluids such as blood is increased in pathological conditions and the protein cargo of exosomes can be used to diagnose diseases such as cancer with high specificity and sensitivity (Melo et al., 2015). Furthermore, exosomes have recently been used as a marker for pregnancy and pregnancy-related pathologies in humans (Tsochandaridis et al., 2015). In sheep, exosomes derived from uterine flushings stimulate trophoectoderm cell lines to proliferate and secrete interferon tau, the pregnancy recognition signal (Ruiz-Gonzalez et al., 2015). The miRNA content of exosomes has also been characterized in bovine follicular fluid, with content differing depending on the oocytes stage of development (Sohel et al., 2013).

Our hypothesis was that exosomes containing specific effector molecules (e.g., proteins and miRNA) will be detectable in bovine plasma and that the number of exosomes and the exosomal cargo will differ in cows of divergent fertility phenotype.

### MATERIALS AND METHODS

#### Animals and Management

All procedures were undertaken with the approval of the Ruakura Animal Ethics Committee (Hamilton, New Zealand). The study was conducted at DairyNZ Limited (No 5. Dairy, Hamilton, New Zealand). The original study was a terminal study, ending approximately 86 d in milk (DIM; SD 8 d; n = 27) (Meier et al., 2009, 2014). For the purposes of the current study, a selective set of 40 samples suitable for exosome analyses across a range of DIM were evaluated. These samples represented 16 from 27 lactating Holstein-Friesian cows.

Sample description are provided in Figure 1. Cows were representative of 2 genetic strains [<23% North American genetics (fertile, FERT; NZ, n = 8) or >92% North American genetics (subfertile, SUBFERT; NA, n = 8)]. This designation (FERT and SUBFERT) is consistent with the poorer oocyte and embryo quality, lower conception rate to first and second services, and lower 6-wk pregnancy rate and overall lower pregnancy rate for the NA compared with NZ strain Holstein-Friesian dairy cows (Horan et al., 2005; de Feu et al., 2008; Macdonald et al., 2008). Table 1 provides a summary of the phenotypic descriptions of the dairy cows used, including ancestry, age, BW, BCS, DIM, milk yield, milk composition, and pedigree information of this subset of animals.

All cows were managed as a single herd, with fresh pasture grazed in an intensive rotational manner similar to monitor the effectiveness of intervention strategies, such as nutritional or anti-inflammatory approaches.
to that described previously (Roche et al., 2006). Grazing cows were allocated a pasture allowance of >40 kg of DM/cow per d (measured to ground level). Pasture grazing residuals were used to ensure adequate pasture allowance: postgrazing residuals of greater than 1,800 kg of DM/ha were targeted during spring. An average BW was calculated from daily BW measures taken for at least 4 consecutive days leading up to slaughter. An average BCS was calculated from individual BCS taken at 6 body sites (on a scale of 1 to 10, with 1 being emaciated and 10 being obese; Roche et al., 2004). Milk production and milk composition were analyzed in the 24-h period before slaughter, with milk samples from an afternoon milking and the following morning milking combined. Milk yield, milk fat (%), CP (%), and total milk solids (%) were measured (FT120, Foss Electric, Hillerod, Denmark).

**Blood Collection**

Blood was sampled by coccygeal venipuncture and collected in evacuated blood tubes containing K2EDTA as anticoagulant (Vacutainer, Becton, Dickinson, and Company, Franklin Lakes, NJ); samples were placed immediately into iced water and centrifuged within 30 min at 1,500 × g for 12 min at 4°C. Following centrifugation, aspirated plasma was stored at −20°C until assayed. Suitable plasma samples (based on the volume of starting material ≥1 mL of plasma stored in EDTA tubes) were obtained at 5 time points postpartum: 8 to 25 d (FERT: n = 2, SUBFERT: n = 4), 26 to 36 d (FERT: n = 3, SUBFERT: n = 3), 38 to 48 d (FERT: n = 7, SUBFERT: n = 4), 52 to 62 d (FERT: n = 7, SUBFERT: n = 4), and 66 to 82 d (FERT: n = 2, SUBFERT: n = 4). A schematic diagram of the DIM of the sampling groups and the reproductive status is provided in Figure 1.

**Extracellular Vesicle and Exosome Isolation**

Isolation: extracellular vesicle (EV) and exosome isolation protocols are illustrated in Figure 2. Exosomes were isolated from plasma (1 mL) as previously described (Salomon et al., 2014a; Sarker et al., 2014). In brief, plasma was diluted with an equal volume of PBS (pH 7.4) and centrifuged at 2,000 × g for 30 min at 4°C

### Table 1. Phenotypic description of the dairy cows contributing to these data (group means and SD of these means), including genetic strain [North American percent (NA%)], age, BW, BCS, and DIM at the time of study termination, milk yield (kg/d) and composition, estimated breeding worth, and associated breeding values

<table>
<thead>
<tr>
<th>Descriptor</th>
<th>Subfertile</th>
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<th>Fertile</th>
<th>SD</th>
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<td>Jul. 27</td>
<td>12</td>
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<td></td>
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¹Subfertile: North American Holstein-Friesian strain; fertile: New Zealand Holstein-Friesian strain.

²Age range 3 to 7 yr (parity 1 to 5).

³Milk yield and composition during the 24 h before study termination.

⁴The sum of the breeding values information on the New Zealand breeding worth was available at the website http://www.nzael.co.nz/all-about-bw at the time of the parent trial (2010).

⁵The reliabilities of the breeding worth and breeding values ranged between 40 and 60%.
(Sorvall, high speed microcentrifuge, fixed rotor angle: 900, Thermo Fisher Scientific, Asheville, NC). The supernatant was then centrifuged at 12,000 × g for 45 min at 4°C (Sorvall, high speed microcentrifuge, fixed rotor angle: 900). The resultant supernatant fluid (2 mL) was transferred to an ultracentrifuge tube (Sorvall ultracentrifuge tubes) and centrifuged at 100,000 × g for 75 min at 4°C (Sorvall, T-8100, fixed angle ultracentrifuge rotor). The 100,000-g pellet contained all EV. The pellet was suspended in PBS (30 mL) and filtered through a 0.22-μm filter (Steritop, Millipore, Billerica, MA) and then centrifuged at 100,000 × g for 75 min at 4°C. The discontinuous iodixanol gradient was made by diluting a stock solution of OptiPrep [60% (wt/vol) aqueous iodixanol (Sigma-Aldrich Ltd., Castle Hill, NSW, Australia) to produce 40% (wt/vol), 20% (wt/vol), 10% (wt/vol), and 5% (wt/vol) solutions of radioimmunoprecipitation assay buffer (RIPA; Sigma-Aldrich Ltd.). Fractions (12 in total) were collected manually from top to the bottom (with increasing density), diluted with PBS and centrifuged at 100,000 × g for 2 h at 4°C. The discontinuous iodixanol gradient was then analyzed to give the mean, mode, and median of particles.

**Western Blot Analysis**

Exosomes were separated by PAGE, transferred to Immobilon-FL polyvinylidene difluoride membranes (Millipore) and probed with primary rabbit polyclonal antibody anti-CD63 (1:1000; sc-15363, Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were washed in Tris buffer saline and incubated for 1 h in TBST/0.2% BSA containing horseradish peroxidase–conjugated goat anti-rabbit antibody. Proteins were detected by enhanced chemiluminescence using a SRX-101A Tabletop Processor (Konica Minolta, Ramsey, NJ).

**Transmission Electron Microscopy**

Exosome pellets (as described above) were fixed in 3% (wt/vol) glutaraldehyde and 2% paraformaldehyde in cacodylate buffer, pH 7.3. A 5-μL aliquot of each sample was then applied to a continuous carbon grid and negatively stained with 2% uranyl acetate. The samples were examined under an FEI Tecnai 12 transmission electron microscope (FEI, Hillsboro, OR).

**Quantification of Total Exosomes Vesicles**

The concentration of exosomes in plasma is expressed as total immunoreactive exosomal CD63 (ExoELISA, System Biosciences, Mountain View, CA). Briefly, 10 μg of exosomal protein was immobilized in micro-titer
Figure 2. Isolation of extracellular vesicles (EV) and exosomes from bovine plasma. (A) A flowchart is depicted for the isolation and enrichment of EV in dairy cow plasma based on differential ultracentrifugation. (B) Exosomes were purified from dairy cow plasma using a buoyant density centrifugation and characterized by size distribution, enrichment of CD63, and morphology.
plate wells and incubated overnight (binding step). Plates were washed 3 times for 5 min using a wash buffer solution and then incubated with exosome specific primary antibody (CD63) at room temperature for 1 h with shaking. Plates were washed and incubated with secondary antibody (1:5,000) at room temperature 1 h with shaking. Plates were washed and incubated with Super-Sensitive TMB ELISA substrate (ExoELISA kit) at room temperature for 45 min with shaking. The reaction was terminated using Stop Buffer solution. Absorbance was measured at 450 nm. The number of exosomes per milliliter (ExoELISA kit) was obtained using an exosomal CD63 standard curve calibrated against nanoparticle tracking data (i.e., number of exosomes, NanoSight).

**Proteomic Analysis of Exosomes by Mass Spectrometry**

Exosomal protein samples from each time point were pooled into 2 groups (FERT and SUBFERT) for protein analysis. A 10-μg aliquot of exosomal proteins from each of these pooled samples was loaded into a NuPAGE Novex 4–12% Bis-Tris Gels (Life Technologies Australia Pty Ltd., VIC, Australia). Gels were run for 1 h at 150 V, and the proteins were visualized by Coomassie stain (Brilliant Blue R-250 staining solution, Bio-Rad Laboratories Pty. Ltd.). A total of 12 individual bands were excised and subjected to MS preparation as previously described (Salomon et al., 2013a,b). Briefly, proteins were reduced with dithiothreitol (Sigma-Aldrich) for 1 h. Proteins were then alkylated in 10 mM iodoacetic acid (Sigma-Aldrich) for 1 h in the dark. The sample was diluted to 1:10 with 50 mM ammonium bicarbonate (Sigma-Aldrich) and digested with trypsin (20 μg; Promega, Madison, WI) at 37°C for 18 h. The samples were desalted by solid phase extraction using a STAGE tip protocol (stop and go extraction tips for matrix-assisted laser desorption/ionization, nano-electrospray, and liquid chromatography (LC)/MS (LC-MS/MS) sample pre-treatment in proteomics). The eluted peptides were dried by centrifugal evaporation to remove acetonitrile and redissolved in solvent A (0.1% formic acid in water; Sigma-Aldrich). The resulting peptide mixture was analyzed by LC MS/MS on a 5600 Triple TOF mass spectrometer (AB Sciex, Framingham, MA) equipped with an Eksigent Nanoflow binary gradient HPLC system and a nanospray III ion source. The MS/MS spectra were collected by Information Dependent Acquisition using a survey scan (m/z 350–1,500) followed with 25 data-dependent product ion scans of the 25 most intense precursor ions. All mass spectra were analyzed using the MASCOT (Matrix Science, Boston, MA) and ProteinPilot (AB Sciex) search engines against the Swissprot-database with the species set as bovine. Positive identifications were ascribed where Mascot scores were greater than 30. False discovery rate was estimated using a reversed sequence database.

**Gene Ontology Analysis**

Proteins identified in FERT and SUBFERT cows using MS were annotated with biological functions using the PANTHER (Protein ANalysis THrough Evolutionary Relationships) Classification System (http://www.pantherdb.org/). *Bos taurus* was used as the reference list, and 54 and 51 proteins were eligible for analysis in SUBFERT and FERT cows, respectively. An enrichment analysis was used to identify gene ontology terms that were over- or underrepresented, and a Bonferroni multiple testing correction was used for calculation of significance (as detailed in Mi et al., 2013).

**Statistical Analysis**

This study evaluated the release of exosomes from dairy cows with divergent phenotypes. Phenotypic data from the included dairy cows were retrospectively evaluated by 2-way ANOVA with assessment of cow age, BW, and DIM in FERT and SUBFERT groups. No significant differences were identified in the 8 FERT and 8 SUBFERT dairy cows selected for further study (with the exception of milk volume). Due to sample volume limitations (minimum sample volume of ≥1 mL of plasma stored in EDTA tubes required for exosome isolation), matched samples for every animal at every time point was not available (as such repeated measures analyses were not possible). Data are presented as mean ± SEM, with n = 8 (FERT) and n = 8 (SUBFERT) different cows per group (i.e., samples were obtained at 5 time points postpartum: 8 to 25, 26 to 36, 38 to 48, 52 to 62, and 66 to 82 d). Statistical differences between groups were identified by post hoc analyses (GraphPad Prism 4 software, San Diego, CA). Dunnett’s tests were used to compare each treatment where the data distribution approximates normality or by Mann-Whitney U-test for distribution independent data analysis. For 2-group analyses, Student’s tests were used to assess statistical difference, with significance defined as *P* < 0.05.

**RESULTS**

**Extracellular Vesicle Characterization**

Cow plasma EV were isolated and characterized by size distribution, SDS-PAGE, and CD63 protein
abundance (Figure 3). Nanoparticle tracking analysis indicated a particle size distribution ranging from 30 to 350 nm in diameter. This corresponded with the microsomal fraction (including exosomal particles). Size was not affected by fertility group, with an average size of 155 ± 71 nm and 150 ± 65 nm (mean ± SD) for EV isolated from FERT and SUBFERT cow strains, respectively (Figure 3A). A similar protein profile of EV from FERT and SUBFERT was observed on a SDS gel (Figure 3B). Extracellular vesicles isolated from FERT and SUBFERT cows were positive for CD63 protein abundance (Figure 3C). In addition, we identified a correlation ($P < 0.05$) between EV total protein concentration and number of vesicles (Spearman’s $r = 0.32$; Figure 3D). There was no effect of the divergent fertility phenotype on the concentration of EV ($P > 0.05$, Figure 4).

**Exosome Characterization**

Protein abundance of CD63 indicated that exosomes were found at densities ranging from 1.12 to 1.19 g/mL (Figure 5A). Exosomes (pool fractions between 1.12 and 1.19 g/mL) displayed a size distribution of 91 ± 26 nm. Exosomes were identified as vesicles sized between 40 and 100 nm (Figure 5B). No significant differences were identified between the NTA characteristics, CD63 protein abundance, and morphology of the exosomes.

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**Figure 3.** Characterization of isolated extracellular vesicles (EV) from bovine plasma. (A) Representative particle size distribution of microsomal fraction by nanoparticle tracking analysis (NTA). (B) Representative SDS gel; M = marker. (C) Representative samples from 4 fertile (FERT) and 4 subfertile (SUBFERT) animals: Western blot for CD63. (D) Correlation between NTA and protein concentration; dotted lines = 95% CI.
(Figure 5C) isolated from the FERT and SUBFERT cows. The divergent fertility phenotype was associated with concentration of exosomes with exosome concentrations were 50% greater in FERT compared with SUBFERT cows (Figure 6).

**Proteomic Analysis of Exosomes**

Mass spectrometry analysis identified over 140 exosomal proteins (Supplemental Table S1; http://dx.doi.org/10.3168/jds.2016-11060). We identified unique (23 and 36 proteins for FERT and SUBFERT, respectively) and common (81) proteins in each group (Figure 7). Proteins were assigned to molecular and biological functions using the PANTHER, the top 5 biological processes in both FERT and SUBFERT dairy cows were complement activation, blood coagulation, lipid transport, response to external stimulus, and proteolysis (Supplemental Tables S1, S2, and S3; http://dx.doi.org/10.3168/jds.2016-11060).

**DISCUSSION**

The size range and presence of markers of endosomal origin and morphology in the exosome preparations isolated from bovine plasma are all consistent with accepted definitions of exosomes in the literature (Kalra et al., 2013; De Toro et al., 2015). Furthermore, we have established that differences exist in the number of exosomes derived from plasma from cows characterized on the basis of genetic ancestry as having a fertile or subfertile phenotype (50% greater in FERT compared with SUBFERT cows). This difference was not discernible in the total EV component. Hence, accepted definitions of exosomes are critically needed, separate from cruder preparations that are a mixture of EV.

The greater number of exosomes in FERT compared with SUBFERT cows may be due to the differences in fertility breeding value. However, it is also plausible that the difference in number of exosomes was due to differences in milk volume breeding value and phenotypic milk yield (see Table 1). The North American strain cows exhibited greater milk yield (11%), and as result had greater metabolic activity throughout the study period.

Although the difference in absolute numbers of exosomes is interesting, it is of unknown biological significance as the origin of these exosomes is unknown. Blood-derived exosomes have been previously characterized as containing exosomes from cells lining blood vessels and white blood cells, and it is estimated that 25% of blood-derived exosomes are of platelet origin (Arraud et al., 2014). Furthermore, blood likely contains exosomes from distant tissues as the number of circulating exosomes can change with varying physiological and pathological states (Skog et al., 2008). It would be illuminating to establish the differences between exosome numbers from specific tissues (e.g., endometrium or ovary). Such divergence has been reported, in the case of exosomes, from a placentental source in human pregnancy, where exosomes numbers increase dramatically with gestational age (Sarker et al., 2014).

Exosomes are recognized as an important signaling system used to communicate between tissues in both physiological and pathological conditions. Maternal and embryo communication via exosomes may be vital in the recognition and continued development of pregnancy (Burns et al., 2014, 2016; Saadeldin et al., 2015; Burnett and Nowak, 2016; Machtinger et al., 2016). The protein content of exosomes can be used to help elucidate the origin and potential function of exosomes as well as providing clues about the physiological state of an organism (Ostergaard et al., 2012; Muroya et al., 2015). Enrichment of immune response and cell signaling ontology terms in protein derived from the blood of cows postpartum suggests that a significant number of exosomes are derived from immune cells or are in-
involved in immune function in the dairy cow. Extracellular vesicles can modulate the immune system, during both physiological and pathological processes (Théry et al., 2009). Dairy cows commonly experience postpartum uterine inflammation and mastitis in the first few weeks following calving, resulting in a reduction in milk production and fertility (Berton et al., 2008). In human disease, pathogens secrete exosomes that can

Figure 5. Characterization of isolated exosomes from bovine plasma. (A) Representative Western blot for exosome enriched markers CD63 with exosome density being represented within the square [sample from the fertile (FERT) group]. (B) Representative vesicle size distribution using a NanoSight NS500 (NanoSight Ltd., Amesbury, UK) analysis of exosomes (1.12 to 1.19 g/mL). (C) Electron micrograph of exosomes isolated by ultracentrifuge and purified with a buoyant density gradient (pooled exosomal pellet density from 1.12 to 1.19 g/mL; scale bar is 100 nm). (D) Proportion of exosome population compared with the nanoparticle tracking analysis before purification was 14.07% of total extracellular vesicles (EV).
potentially contribute to tolerance of the host by dampening the immune response or alternatively contribute to pathological reactions of the host to the pathogen (Théry, 2011). In women, secretion of exosomes bearing immunosuppressive molecules from the placenta has been reported as a mechanism to promote maternal-fetal tolerance (Théry, 2011). Immune response ontology terms were enriched in both FERT and SUBFERT groups in this study; however, the proteins that were found within these terms differ between the 2 groups, suggesting differential regulation of the immune response. Although in the current study animals were not intensively monitored for immune function or metabolic status, a previous publication on these specific animals identified differences in the uterine gene expression of pathways regulating the immune response to pregnancy between the FERT and SUBFERT groups (Walker et al., 2012). This provides indirect evidence that immune health may differ in these cows.

The immune system and the various immunomodulatory pathways and products are critical in establishing pregnancy and, hence, enhancing fertility (Stenqvist et al., 2013; Lundy et al., 2015). We are exploring differences in these pathways, again considering that utilization of this information may derive potential biomarkers of fertility to support genetic gain in fertility. We note with interest that some comparable initial experiments have been conducted in sheep (Cleys et al., 2014; Ruiz-Gonzalez et al., 2015). Our finding of altered immunologic pathway factors in the SUBFERT cows is consistent with views that infection or subclinical infection results in reduced fertility (Sheldon et al., 2008). The difference in coagulation pathway expression between the 2 groups is of interest because spontaneous abortion (miscarriage) in women is thought to be associated with, or caused by, a decidual vasculopathy (Woodhams et al., 1989). Furthermore, alterations in blood coagulation factors are associated with not only miscarriage, but also related preterm and infection-related abortion issues (Martinelli et al., 2000; Bick and Hoppensteatd, 2005; Phillippe et al., 2011).

The greater number of exosomes derived from FERT cows may influence the overall contribution of exosomes to cow health and fertility. Further research is required to determine the functional significance of greater exosome concentrations in cows and if this has positive effects on health and, in particular, fertility outcomes. More detailed investigations of the differentially expressed substances, particularly those affecting immunological and blood coagulation status, may lead to potential novel targets for intervention or development of prognostic tests.

**Figure 6.** Concentration of exosomes isolated from bovine plasma of the fertile (FERT; <23% North American genetic ancestry) and subfertile (SUBFERT; >92% North American genetic ancestry) groups [mean (line) ± SD (bars)]: FERT: mean = 1.825e + 009; SD = 7.022e + 008; SUBFERT: mean = 1.327e + 009; SD = 4.365e + 008. Symbols represent individual animals. For 2-group analyses, Student’s tests were used to assess statistical difference, with significance defined as P < 0.05.

**Figure 7.** The Venn diagram depicts the distribution of common and unique proteins identified by nanospray LC-MS/MS (AB Sciex 5600, AB Sciex, Framingham, MA) in exosomes isolated from dairy cow plasma of the fertile (<23% North American genetic ancestry) and subfertile (>92% North American genetic ancestry) groups.
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