Colostrum protein uptake in neonatal lambs examined by descriptive and quantitative liquid chromatography-tandem mass spectrometry

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ABSTRACT

Colostrum intake is a key factor for newborn ruminant survival because the placenta does not allow the transfer of immune components. Therefore, newborn ruminants depend entirely on passive immunity transfer from the mother to the neonate, through the suckling of colostrum. Understanding the importance of specific colostrum proteins has gained significant attention in recent years. However, proteomics studies of sheep colostrum and their uptake in neonate lambs has not yet been presented. The aim of this study was to describe the proteomes of sheep colostrum and lamb blood plasma, using sodium dodecyl sulfate-PAGE for protein separation and in-gel digestion, followed by liquid chromatography-tandem mass spectrometry of resulting tryptic peptides for protein identification. An isobaric tag for relative and absolute quantitation (iTRAQ)-based proteomics approach was subsequently used to provide relative quantification of how neonatal plasma protein concentrations change as an effect of colostrum intake. The results of this study describe the presence of 70 proteins in the ovine colostrum proteome. Furthermore, colostrum intake resulted in an increase of 8 proteins with important immune functions in the blood plasma of lambs. Further proteomic studies will be necessary, particularly using the selected reaction monitoring approach, to describe in detail the role of specific colostrum proteins for immune transfer to the neonate.

Key words: colostrum, immunity, proteomics, isobaric tag for relative and absolute quantitation (iTRAQ)

INTRODUCTION

It is well known that early colostrum intake is a key factor for neonatal survival in mammals. In ruminants, the complexity of the synepitheliochorial placenta does not allow sufficient transfer of immunoglobulins from the dam to the fetus (Argüello et al., 2004a; Castro et al., 2005, 2011). Consequently, lambs are classified as hypo-gammaglobulinemic at birth, and are entirely dependent on passive immunity transfer (**PIT**) from the mother to the neonate through early suckling of colostrum (Stelwagen et al., 2009; Danielsen et al., 2011). Colostrum contains essential nutrients such as fat, lactose, vitamins, and minerals (Ontsouka et al., 2003; Hernández-Castellano et al., 2014a; Lérias et al., 2014), but it is also rich in unique proteins that play active roles in regulating growth and development of the gut tissue. Colostrum also protects the neonate against pathogens and postpartum environmental challenges (Bendixen et al., 2011; Hernández-Castellano et al., 2015).

Although the transfer of immunoglobulins is a key factor in host defense to pathogens, a wider range of colostrum components have been suggested to contribute to the early protection of the neonate (Smith and Foster, 2007). These include many unique proteins with specific functional roles, including proteins secreted by neutrophils and macrophages, blood complement system proteins, acute-phase proteins, and specific proteins and peptides that have direct antimicrobial activity (Rainard and Riollot, 2006; Oviedo-Boyso et al., 2007; Leitner et al., 2008). In addition to mediating host defense, a wide range of bioactive colostrum proteins also play important roles as key regulators of gastrointestinal growth and development in early life (Bendixen et al., 2011).

Colostrum proteins are protected by trypsin inhibitors present in colostrum (Ramos et al., 2010; Hernán-

Received March 17, 2014.

Accepted September 25, 2014.

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dez-Castellano et al., 2014a), as well as by low proteolytic activity in the gastrointestinal tract of newborn animals (Guilloteau et al., 1983). Additionally, the uptake of entire proteins through the gut epithelium is facilitated by unique features of a regulated apoptotic process of the neonatal ruminant enterocytes, which allows unrestricted passage of large molecules, such as immunoglobulins, into the gut (Castro-Alonso et al., 2008; Stelwagen et al., 2009). It is crucial to feed newborn ruminants colostrum during the first 48 h after birth, because the mechanisms that facilitate PIT and colostrum uptake are only functional in the first days of life (Moore et al., 2005; Hernández-Castellano et al., 2014b). Neither the cellular mechanisms of colostrum protein transfer to blood nor their bioactive roles in the neonate ruminants are fully understood. Although bovine milk and colostrum proteomes have attracted much attention, including reports of more than 253, 138, and 403 proteins characterized in colostrum and milk whey (Le et al., 2011), milk fat globule membrane (Reinhardt and Lippolis, 2008), and colostrum samples (Reinhardt and Lippolis, 2008; Le et al., 2011; Nissen et al., 2012), respectively, very limited knowledge seems to be available on sheep colostrum proteins. Some ELISA-based studies have reported concentration levels of IgG in sheep colostrum to range from 48.1 to 60.9 mg/mL (Moretti et al., 2010a,b; Higaki et al., 2013) and in newborn lamb plasma from 13.7 to 26.6 mg/mL (Quigley et al., 2002; Rodinova et al., 2008). However, proteomic studies of sheep colostrum proteins and their uptake in neonate lambs has not yet been performed.

The aim of this study was to describe the proteomes of sheep colostrum and lamb blood plasma, using an SDS-PAGE for protein separation and in-gel digestion, followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) of resulting tryptic peptides for protein identification. An isobaric tag for relative and absolute quantitation (iTRAQ)-based proteomics approach was subsequently used to provide relative quantification of changes in neonatal plasma protein concentrations as an effect of early colostrum intake.

MATERIALS AND METHODS

Animal Experimentation Disclosure

This experiment was approved by the ethics committee of the Universidad de Las Palmas de Gran Canaria (Spain) in compliance with animal experimentation regulations in the Canary Islands, Spain, and the European Union. A. M. Almeida holds a Federation of Laboratory Animal Science Associations (FELASA) grade C license, enabling him to design and conduct animal experiments in the European Union.

Sample Collection

The study was based on individual analysis of plasma samples from 8 single partum lambs (Canarian dairy breed), studied as 2 experimental groups (4 lambs each), as well as 2 samples from the pool of colostrum used for lamb feeding. As dams were estrous synchronized and subsequently mated, all lambs were born in the same period (May 2012) with a difference of a few days. The experiment took place at the experimental farm of the Veterinary Faculty of the Universidad de Las Palmas de Gran Canaria (28°8'20.66" N, 15°30'24.97" W, Gran Canaria, Spain). During the experimental period (from birth until 14 h after birth), the colostrum (C) group received colostrum feeding at 2 h after birth; the no-colostrum (NC) group was not fed at 2 h after birth. To ensure survival of the animals, both groups were subsequently fed with colostrum after the experimental period at 14 and 26 h after birth. Animals were fed a common pool of sheep colostrum with an IgG concentration of 64.74 mg/mL. Blood samples were collected directly before feeding at 2 and 14 h after birth from the jugular vein in 2.5-mL tubes with K-EDTA. Blood was centrifuged at $2,190 \times g$ for 5 min at 4°C (Hettich-Zentrifugen, Universal 32 R, Tuttlingen, Germany) and the obtained plasma was frozen at -80°C until further analysis. All lambs were housed in rearing rooms with at least 0.3 m² of floor space per lamb. Each room had central heating with a room temperature of approximately 20°C.

Sample Preparation

Plasma and colostrum samples were analyzed at Aarhus University (Aarhus, Denmark). A total of 200 µL from each sample were homogenized with 1 mL of Tris-EDTA-sucrose (TES) buffer (10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 0.25 M sucrose) using an UltraTurrax homogenizer (T10 basic, IKA-Werke, Staufen, Germany) at 12,000 rpm. Homogenates were centrifuged at $10,000 \times g$ for 30 min at 4°C to remove insoluble components. Protein concentration of the supernatant was determined with the Quick Start Bradford Protein Assay (Bio-Rad, Hercules, CA), using BSA as standard reference (Bradford, 1976) and following the manufacturer's instructions. Aliquots of 100 µg of protein from each sample were recovered after precipitation with 6 volumes of ice-cold acetone (-20°C) and centrifugation at $15,000 \times g$ for 10 min at 4°C.

For the descriptive analyses of plasma and colostrum proteomes, 60 µg of protein from the experimental groups (C and NC groups) at 14 h (2 biological replicates from each group) as well as the pooled colostrum used for the feeding experiment were prepared (2 technical replicates). Aliquots were resuspended in 20 µL of TES buffer. The

samples were boiled for 5 min in SDS sample buffer (7.5 mL) containing 500 mM DL-dithiothreitol (DTT), separated by SDS-PAGE using 10% (wt/vol) acrylamide gels (Bury, 1981) and stained using Coomassie blue (RAP-IDstain, G-Biosciences, Maryland Heights, MO). Each lane was cut into 9 equal-sized pieces, each of which was washed 3 times in Milli-Q water (Millipore, Billerica, MA) and incubated 2 times for 15 min in 130 μ L of 50% acetonitrile, dehydrated in 130 μ L of acetonitrile for 15 min, and equilibrated in 150 μ L of 0.1 M NH_4HCO_3 for 5 min before 150 μ L of acetonitrile was added. After 15 min, supernatants were removed and gel pieces were dried by vacuum (SpeedVac, Thermo Fisher Scientific, Waltham, MA) for 20 min. In-gel digestions were performed by incubating the gel pieces with sequencing-grade modified trypsin (Promega, Southampton, UK) in 50 mM NH_4HCO_3 at 37°C for 16 h, using 400 ng of trypsin for heavily loaded gel pieces, and 200 ng of trypsin for fainter gel pieces (Supplemental Figure S1; <http://dx.doi.org/10.3168/jds.2014-8143>). The resulting peptides were desalted using C₁₈ StageTips (Thermo Scientific, Hvidovre, Denmark) and stored at -20°C before LC-MS/MS analysis.

LC-MS/MS Analysis

Analyses of gel pieces by LC-MS/MS was performed on an EASY-nLC II system (Thermo Scientific) connected to a TripleTOF 5600 mass spectrometer (AB Sciex, Framingham, MA) equipped with a NanoSpray III source (AB Sciex) and operated under Analyst TF 1.6 control. Tryptic peptides were dissolved in 15 μ L of buffer A (0.1% formic acid), injected, trapped, and desalted isocratically on a ReproSil-Pur C18-AQ column (5 μ m, 2 cm $\times$ 100 μ m i.d.; Thermo Scientific). Then, peptides were eluted from the trap column and separated on a ReproSil-Pur C18-AQ 3- μ m capillary column (16 cm $\times$ 75 μ m i.d.) connected in-line to the mass spectrometer at 250 nL/min using a 50-min gradient from 5% to 35% of buffer B (0.1% formic acid and 90% acetonitrile), followed by a 10-min re-equilibration time in buffer A. The TripleTOF 5600 was run in positive ion mode using 2,500 V for ion spray, curtain gas at 30 psi, ion source gas at 5 psi (lbf/in²), and an interface heater temperature of 150°C. The automated information dependent acquisition (IDA) method acquired up to 50 tandem (ms/ms) mass spectra per cycle using 2.3-s cycle times and a mass exclusion window of 6 s.

iTRAQ-Based Quantitative LC-MS/MS

Six sets of iTRAQ experiments were designed to study the differential protein abundances found in plasma collected from the C and NC groups. Sixteen plasma

samples were compared, including 4 biological replicates and 2 time points (2 and 14 h after birth) from both treatment conditions (C and NC). Consistently, the iTRAQ reagent 114 was used to label an internal control sample made by pooling 100 μ g of plasma protein aliquots, including all 16 individual plasma samples. The remaining iTRAQ reagents (115, 116, and 117) were used to label the 16 individual plasma samples.

Cysteine residues were reduced with 2.5 mM tris (2-carboxyethyl) phosphine hydrochloride at 60°C for 1 h and then blocked with 10 mM methylmethanethiosulfate solution at room temperature for 10 min. Proteins were then digested with trypsin (1:10 wt/wt) at 37°C overnight. The tryptic peptides were labeled with iTRAQ reagents (Applied Biosystems, Foster City, CA) at room temperature for 1 h, following the manufacturer's instructions. Finally, samples were combined to create 6 different 4-plexed samples.

Strong Cation Exchange Fractionation and LC-MS/MS Analysis

A total of 50 μ g of protein from each of the iTRAQ 4-plexed samples were loaded on an Agilent 1100 Series capillary HPLC equipped with a Zorbax Bio-SCX Series II (Agilent Technologies, Palo Alto, CA), and peptides were eluted with a gradient of increasing NaCl solution. Fractions were then collected every min for 65 min and combined according to their peptide loads into 9 to 10 pooled samples, which were individually analyzed on LC-MS/MS, using the Easy-nLC II system (Thermo Scientific) connected to a QSTAR Elite mass spectrometer (AB Sciex) equipped with a NanoSpray source (AB Sciex) and operated under Analyst QS 2.0 control. The trypsin-digested samples were dissolved in buffer A (0.1% formic acid), injected, trapped, and desalted isocratically on a ReproSil-Pur C18-AQ column (5 μ m, 2 cm $\times$ 100 μ m i.d.; Thermo Scientific), after which the peptides were eluted from the trap column and separated on an analytical ReproSil-Pur C18-AQ capillary column (3 μ m, 10 cm $\times$ 75 μ m i.d.; Thermo Scientific) connected in-line to the mass spectrometer at 250 nL/min using a 63-min gradient from 5% to 38% of buffer B (0.1% formic acid and 90% acetonitrile). The QSTAR Elite MS was run in positive ion mode using 2,600 V for ion spray, curtain gas at 20 psi, ion source gas at 1:16, and an interface heater temperature of 110°C. The automated IDA method was set to acquire up to 3 MS/MS spectra per cycle using 2.0 s cycle times and an exclusion window of 80 s.

Protein Identification and Quantitation

The collected MS files were converted to Mascot generic format (MGF) using the AB Sciex MS Data Con-

verter β 1.3 (AB Sciex), and the “proteinpilot MGF” parameter. The peak lists were used to interrogate a combined bovine, ovine, and caprine database consisting of sequences from TrEMBL/Swiss-Prot (<http://www.uniprot.org/uniprot/>) and NCBI nr (<http://www.ncbi.nlm.nih.gov/protein>) (32,444 sequences), using Mascot 2.3.02 (Matrix Science, London, UK; Perkins et al., 1999). As previously described by Soares et al. (2012), the combination of phylogenetically close species is a strategy typically used when the experimental species do not have complete sequence databases. For in-gel digests, the search parameters were set to allow one missed trypsin cleavage site and propionamide as a fixed modification. The mass accuracy of the precursor and product ions were 15 ppm and 0.2 Da and the instrument settings were specified as ESI-QUAD-TOF. Only proteins with at least 2 unique peptides and a minimal protein score of 60 were considered for identification (Boersema et al., 2008; Gannon and Ohlendieck, 2012; Hernández-Castellano et al., 2014b). The whole identification list can be observed in Supplemental Table S1 (spreadsheet; <http://dx.doi.org/10.3168/jds.2014-8143>).

For the iTRAQ samples, the search parameters were set to allow one missed trypsin cleavage site and methylthiomethanesulfate modification of cysteine was set as fixed modification, and oxidation of methionine was defined as a variable modification. The iTRAQ 4-plex was selected as the quantitation method. The mass accuracy of the precursor and product ions were set to 0.2 Da using instrument setting as ESI-QUAD-TOF. The significance threshold (*P*-value) was set to 0.01 with an ion score cutoff at 31 for all samples. Only proteins with at least 2 unique peptides and a minimal protein score of 60 were considered for identification. Mascot results were parsed using MS Data Miner v.1.1 (Dyrlund et al., 2012). The report generated by the software includes data on mass (kDa), ratio, peptides, and relative protein abundance. Supplemental Table S2 (spreadsheet; <http://dx.doi.org/10.3168/jds.2014-8143>) provides information about the final report generated by MS Data Miner v.1.1, comparing all identified and quantified proteins from the 6 sets of iTRAQ data.

Statistical Analysis

Statistical analyses from iTRAQ approach were tested for significance ($P < 0.05$) by ANOVA using PROC MIXED of SAS (version 9.0, SAS Institute Inc., Cary, NC). The model included time, treatment, and their interactions as fixed effects. A Tukey-Kramer adjustment was used to compensate for multiple comparisons.

RESULTS AND DISCUSSION

To investigate which proteins can be detected in colostrum and plasma using shotgun proteomics, we used an approach based on SDS-PAGE separation and LC-MS/MS-based protein identification. Only proteins observed in both biological replicates from each sample type (C and NC groups at 14 h after birth, as well as the colostrum pool used) were considered significant and unique observations. Analyses of the pooled colostrum used in our feeding experiment allowed identification of 70 proteins, whereas 64 and 97 proteins were observed in plasma from C and NC groups, respectively. The proteins observed in plasma from C group animals represent both original plasma proteins as well as the subset of proteins transferred from colostrum to plasma. Therefore, the aim of this initial and descriptive study was to collect detailed information about the protein subsets detectable in these 3 different and complex proteome samples and to provide information about the overlap, uniqueness, and origin of proteins from colostrum and plasma. The Venn diagram in Figure 1 presents an overview of the distribution of proteins across colostrum and plasma. It is important to note that 29 proteins were detected in all 3 studied groups (C and NC groups and the colostrum pool used). Moreover, comparing these proteome profiles demonstrated that a wide range of immunoglobulins originating from colostrum are transferred to plasma, whereas a large majority of colostrum-specific proteins were not detectable in plasma 12 h after suckling, indicating that transport of proteins from colostrum, over the gut epithelial layer, and into plasma is a controlled and selective process, although mechanisms responsible for selective transfer are not yet fully understood (Johnson et al., 2007).

Another interesting observation provided by our direct comparison of body fluid proteomes was that a much larger range of well-known plasma proteins and intracellular proteins (i.e., fructose-biphosphate aldolase and carbonic anhydrase) was detectable in plasma from animals that lacked access to colostrum compared with the plasma of colostrum-fed lambs. This is likely because peptide selection in shotgun-based LC-MS/MS analyses is greatly biased by the relative abundance of specific proteins and peptides in complex biological samples. Thus, in plasma collected after colostrum suckling, the high concentration of immunoglobulins present in plasma clearly outnumbered and overshadowed detection of the lower abundance plasma protein in the background (Millioni et al., 2011; Petersen et al., 2013).

To achieve a more direct comparison and to obtain relative quantification of the effect of colostrum feed-

ing, an iTRAQ-based tagging approach was used to compare the plasma proteomes of 8 individually sampled animals. This investigation also aimed to study how access to colostrum affects the expression levels of plasma proteins related to growth and development of neonatal lambs.

In total, 148 proteins were detected in the iTRAQ-tagged analyses. Compared with similar shotgun analyses of bovine plasma (Faulkner et al., 2012) and colostrum (Reinhardt and Lippolis, 2008; Nissen et al., 2012), the number of proteins we could identify and quantify in colostrum and plasma samples of sheep was much lower. This was partly due to the dynamic range, as discussed above (Klobasa et al., 1987; Hortin and Sviridov, 2010) but was mainly a direct effect of the current lack of a well-covered reference ovine genome database. Indeed, detailed annotation of sheep proteins is very limited compared with the bovine and caprine databases.

The recent technological progress in targeted and absolute quantitation methods (Picotti et al., 2009) improves accuracy of analyses for future experiments. In particular, the development of candidate target proteins would largely benefit further studies of the absolute absorption of specific colostrum proteins in plasma. However, these methods require that specific proteotypic signature peptides are described and validated for each protein to be analyzed across tissues (Picotti et al., 2009).

Protein Identification

Table 1 provides a list of information about nonredundant proteins identified in colostrum and plasma samples from C and NC groups at 14 h after birth. The first group of proteins listed in Table 1 represents those identified in all samples (from colostrum as well as plasma). The second group represents the proteins that were uniquely found in plasma from colostrum-fed animals. This group is of particular interest because these proteins represent specific transfer from colostrum and, in our data, these are clearly dominated by immunoglobulins. The third set of proteins shown in Table 1 was identified exclusively in colostrum. Most of these are well known in bovine studies to be highly abundant in colostrum, including immunoglobulins and caseins, but also proteins from similar studies on bovine colostrum that are known to be of lower abundance. These include serum amyloid A-3, which was found to be present at 267 µg/mL (McDonald et al., 2001) and plasmin, reported to be present on 0.49 µg/mL (Dupont et al., 1998). Finally, the fourth group shows proteins that could only be detectable in animals that lacked access to colostrum (NC group). This list of pro-

Figure 1. Venn diagram of colostrum pool and plasma from colostrum (C group) and noncolostrum (NC group) lambs.

teins represents both the plasma proteome background, as well as the proteins secreted in plasma as a response to the physiological stress of lacking normal colostrum intake.

Proteins Identified in All Samples. Twenty-nine proteins were observed in plasma from all animals, regardless of colostrum feeding time, and in the pooled colostrum. For these proteins, it should be noted that some immunoglobulin components (λ and μ chains) could also be detected in the plasma of animals that had not yet received colostrum. Although several authors have noted that the ruminant placenta lacks the ability to transfer immunoglobulins from the dam to the fetus (Constant et al., 1994; Argüello et al., 2004b), other researchers (Argüello et al., 2004a; Gabriël et al., 2005; Rodríguez et al., 2009; Castro et al., 2011) are in agreement with our present findings that small but detectable amounts of immunoglobulins are indeed transferred through the placenta. Likewise, 4 apolipoprotein isoforms, fibrinogen, plasminogen, serotransferrin, ceruloplasmin, and trypsin inhibitor were present in all samples. These proteins are mainly known as components of blood, their presence also in cow colostrum has been confirmed and also includes apolipoproteins A-I, A-IV, and E (Yamada et al., 2002; Reinhardt and Lippolis, 2008), plasminogen (Dupont et al., 1998; Yamada et al., 2002), trypsin inhibitor (Honkanen-Buzalski and Sandholm, 1981; Yamada et al., 2002), ceruloplasmin (Albera and Kankofer, 2009), fibrinogen, and serotransferrin (Yamada et al., 2002). In some cases, these proteins have been reported to

Table 1. Colostrum proteins identified in sheep colostrum and in lamb plasma from colostrum group (C group) and noncolostrum group (NC group)

Protein name	Accession number	Mascot score			Significant peptide matches ¹			Unique peptide matches		
		Colostrum	C group	NC group	Colostrum	C group	NC group	Colostrum	C group	NC group
Proteins identified in all samples (colostrum pool, C group, and NC group)										
Alpha globin chain [<i>Ovis aries</i>]	gi 1787	211	592	1,748	9	17	56	4	3	7
Alpha-2-macroglobulin precursor (Blast2Go, Similarity: 88.35%)	E1BJW0	3,808	3,811	5,212	158	138	210	7	16	18
Apolipoprotein A-I	P15497	2,151	2,402	4,308	86	79	171	9	16	22
Apolipoprotein E	gi 41016830	464	468	1,339	23	13	47	8	5	13
Apolipoprotein A-IV	F1N3Q7	515	267	1,159	26	11	45	6	6	14
Apolipoprotein D	F1MS32	882	109	433	20	4	16	4	2	4
Beta-lactoglobulin [<i>Ovis sp.</i>]	gi 165839	9,202	71	738	328	3	42	11	2	6
Complement C3	Q2UVX4	1,482	695	3,628	62	31	168	20	14	33
Ceruloplasmin	gi 75075054	773	448	1,091	28	13	7	5	6	3
Fibrinogen β chain	F1MAV0	993	399	4,902	22	12	147	6	4	17
Gamma fibrinogen, partial [<i>Ovis dalli</i>]	gi 1916272	66	247	1,140	5	13	40	2	2	2
Fibronectin isoform 1 (Blast2Go, Similarity: 97.0%)	G5E5A9	353	2,110	2,186	17	65	80	8	19	21
Gelsolin isoform a precursor (Blast2Go, Similarity: 99.05%)	F1MJH1	123	522	1,753	7	20	78	4	6	16
Ig lambda chain C region - sheep (fragment)	gi 109030	5,307	3,704	146	137	83	6	5	6	2
Ig lambda light chain constant region segment 1 [<i>Ovis aries</i>]	gi 52366986	4,999	3,533	572	133	96	20	6	6	2
Ig mu chain, partial [<i>Ovis aries</i>]	gi 165945	1,384	1,292	633	55	42	22	7	8	5
Alpha-1-antiproteinase	gi 112890	447	1,597	5,175	24	84	248	8	12	17
Trypsin inhibitor (contrapsin) – goat	gi 1363062	290	85	503	13	4	23	2	2	2
Inter- α -trypsin inhibitor heavy chain h2 (Blast2Go, Similarity: 93.4%)	F1MNW4	375	691	1,126	16	25	45	5	6	10
Keratin 1 (Blast2Go, Similarity: 95.45%)	G3N0V2	3,635	1,549	2,817	132	48	101	7	5	5
Keratin 10 (Epidermolytic hyperkeratosis; keratosis palmaris et plantaris)	A6QNZ7	2,524	582	1,610	136	36	89	11	6	9
Keratin 14 (Blast2Go, Similarity: 98.0%)	F1MC11	4,483	1,154	2,892	150	42	102	12	6	8
Keratin 18 (Blast2Go, Similarity: 94.7%)	F6S1Q0	390	146	273	33	9	23	3	2	2
Plasminogen	gi 3914364	82	377	905	3	17	48	2	4	8
Plasminogen	E1B726	442	264	677	25	10	30	2	3	5
Pregnancy-zone protein (Blast2Go, Similarity: 80.65%)	F1MI18	393	367	362	11	15	15	2	4	4
Serotransferrin	G3X6N3	201	5,826	9,563	18	195	369	4	19	21
Serum albumin	gi 113582	3,228	21,245	36,037	136	658	1,163	23	45	51
Type ii cytoskeletal 2 epidermal (Blast2Go, Similarity: 90.3%)	E1B991	2,230	964	1,802	78	28	61	5	3	3
Colostrum proteins observed in C group plasma at 14 h after birth (colostrum pool and C group)										
Apolipoprotein b-100 (Blast2Go, Similarity: 85.6%)	E1BNR0	394	750	ND ²	20	37	ND	5	17	ND
Ig heavy chain C region - sheep (fragment)	gi 109029	7,557	1,200	ND	184	49	ND	9	5	ND
Ig kappa chain – sheep	gi 423311	258	345	ND	9	10	ND	3	4	ND
Ig α heavy chain [<i>Ovis aries</i>]	gi 2582411	864	113	ND	35	5	ND	5	3	ND
Ig V lambda chain [<i>Ovis aries</i>]	gi 2766644	108	138	ND	6	6	ND	2	2	ND
Ig lambda-6c light chain variable region [<i>Ovis aries</i>]	gi 2746699	219	408	ND	5	9	ND	2	2	ND
Inter- α -trypsin inhibitor heavy chain H1	Q0VCM5	200	201	ND	5	7	ND	3	3	ND

Continued

Table 1 (Continued). Colostrum proteins identified in sheep colostrum and in lamb plasma from colostrum group (C group) and noncolostrum group (NC group)

Protein name	Accession number	Mascot score			Significant peptide matches ¹			Unique peptide matches		
		Colostrum	C group	NC group	Colostrum	C group	NC group	Colostrum	C group	NC group
Colostrum proteins that were not observed in C group plasma at 14 h after birth (colostrum pool)										
Alpha-lactalbumin	gi 125998	495	ND	ND	18	ND	ND	4	ND	ND
Alpha-S2-casein	gi 115658	2,636	ND	ND	111	ND	ND	12	ND	ND
Alpha-S1-casein precursor [<i>Ovis aries</i>]	gi 57526469	5,240	ND	ND	194	ND	ND	13	ND	ND
Alpha-S2-casein [<i>Ovis aries</i>]	gi 284025656	2,771	ND	ND	115	ND	ND	12	ND	ND
ATP synthase subunit α	F1MLB8	122	ND	ND	8	ND	ND	3	ND	ND
ATP synthase subunit β , mitochondrial	P00829	211	ND	ND	8	ND	ND	3	ND	ND
Beta-casein	gi 416752	3,001	ND	ND	92	ND	ND	5	ND	ND
Desmoplakin isoform i (Blast2Go, Similarity: 96.35%)	E1BKT9	262	ND	ND	15	ND	ND	7	ND	ND
Elongation factor 1- α	E1B9F6	270	ND	ND	12	ND	ND	3	ND	ND
Fatty acid-binding protein, heart	P10790	78	ND	ND	3	ND	ND	2	ND	ND
Ig mu chain – sheep	gi 478694	1,151	ND	ND	47	ND	ND	7	ND	ND
Ig mu heavy chain V region precursor - sheep (fragment)	gi 1083106	114	ND	ND	7	ND	ND	2	ND	ND
Ig gamma 2 heavy chain constant region [<i>Capra hircus</i>]	gi 147744654	1,089	ND	ND	48	ND	ND	3	ND	ND
Ig lambda-2b light chain variable region [<i>Ovis aries</i>]	gi 2746691	74	ND	ND	5	ND	ND	2	ND	ND
Junction plakoglobin (Blast2Go, Similarity: 98.35%)	F1N3P9	92	ND	ND	4	ND	ND	3	ND	ND
Keratin, type I cytoskeletal 17	A1L595	1,710	ND	ND	85	ND	ND	8	ND	ND
Keratin, type II cytoskeletal 75	Q08D91	313	ND	ND	27	ND	ND	7	ND	ND
KRT5 protein	A5D7M6	1,519	ND	ND	72	ND	ND	20	ND	ND
Milk fat globule EGF factor 8 protein, partial [<i>Ovis aries</i>]	gi 347803268	260	ND	ND	13	ND	ND	5	ND	ND
Neutrophil gelatinase-associated lipocalin isoform 2 (Blast2Go, Similarity: 86.45%)	E1B6Z6	111	ND	ND	5	ND	ND	3	ND	ND
Nucleobindin-1	Q0P569	221	ND	ND	8	ND	ND	5	ND	ND
Peroxiredoxin-1	Q5E947	125	ND	ND	7	ND	ND	4	ND	ND
Polymeric Ig receptor (Blast2Go, Similarity: 81.7%)	F1MR22	211	ND	ND	11	ND	ND	3	ND	ND
Glycosylation-dependent cell adhesion molecule 1	gi 22096365	97	ND	ND	8	ND	ND	4	ND	ND
Secreted form Ig epsilon heavy chain constant partial (Blast2Go, Similarity: 74.3%)	G3N342	90	ND	ND	6	ND	ND	2	ND	ND
Type ii cytoskeletal 3 (Blast2Go, Similarity: 95.8%)	G3MXL3	1,417	ND	ND	78	ND	ND	15	ND	ND
Proteins identified in DC group plasma at 14 h after birth (NC group)										
Adiponectin	Q3Y5Z3	ND	ND	286	ND	ND	8	ND	ND	3
Alpha-2-antiplasmin	P28800	ND	ND	556	ND	ND	23	ND	ND	4
Antithrombin-III precursor [<i>Ovis aries</i>]	gi 57164383	ND	ND	1,283	ND	ND	57	ND	ND	13
Apolipoprotein m (Blast2Go, Similarity: 93.7%)	F1MYX2	ND	ND	100	ND	ND	6	ND	ND	3
Beta-2-glycoprotein 1	P17690	ND	ND	539	ND	ND	18	ND	ND	5
Bone marrow stromal cell antigen 1 (Blast2Go, Similarity: 86.65%)	F1MLP3	ND	ND	234	ND	ND	6	ND	ND	2
Carboxypeptidase N catalytic chain	Q2KJ83	ND	ND	138	ND	ND	6	ND	ND	3
Chain D, Crystal Structure Determination Of Sheep Methemoglobin At 2.7 Angstrom Resolution	gi 195927260	ND	ND	719	ND	ND	35	ND	ND	7

Table 1 (Continued). Colostrum proteins identified in sheep colostrum and in lamb plasma from colostrum group (C group) and noncolostrum group (NC group)

Protein name	Accession number	Mascot score			Significant peptide matches ¹			Unique peptide matches		
		Colostrum	C group	NC group	Colostrum	C group	NC group	Colostrum	C group	NC group
Coagulation factor a1 polypeptide (Blast2Go, Similarity: 94.3%)	F1MW44	ND	ND	134	ND	ND	6	ND	ND	2
Coagulation factor XIII, B polypeptide	Q2TBQ1	ND	ND	137	ND	ND	10	ND	ND	4
Complement C1s subcomponent	Q0VCX1	ND	ND	188	ND	ND	8	ND	ND	3
Complement component 4a (Rodgers blood group) (Blast2Go, Similarity: 90.3%)	F1N2Q0	ND	ND	1,076	ND	ND	49	ND	ND	15
Complement factor B [<i>Ovis aries</i>]	gi 148645283	ND	ND	653	ND	ND	34	ND	ND	15
Extracellular matrix protein 1 (Blast2Go, Similarity: 81.65%)	F1MWT6	ND	ND	251	ND	ND	11	ND	ND	4
Fetuin-B	Q58D62	ND	ND	142	ND	ND	9	ND	ND	3
Fibulin 1 (Blast2Go, Similarity: 93.9%)	F1MYN5	ND	ND	108	ND	ND	6	ND	ND	3
Haptoglobin	gi 226709030	ND	ND	237	ND	ND	13	ND	ND	7
Haptoglobin [<i>Rupicapra rupicapra</i>]	gi 307742657	ND	ND	351	ND	ND	15	ND	ND	8
Hemoglobin gamma	gi 229222	ND	ND	827	ND	ND	36	ND	ND	5
Histidine-rich glycoprotein	F1MKS5	ND	ND	218	ND	ND	12	ND	ND	3
Kininogen-1	P01044	ND	ND	305	ND	ND	10	ND	ND	3
Leucine-rich α -2-glycoprotein 1	Q2KIF2	ND	ND	119	ND	ND	7	ND	ND	3
Lumican	Q05443	ND	ND	275	ND	ND	16	ND	ND	5
Pigment epithelium-derived factor precursor [<i>Ovis aries</i>]	gi 213021132	ND	ND	699	ND	ND	27	ND	ND	10
Plasma serine protease inhibitor	Q9N2I2	ND	ND	80	ND	ND	6	ND	ND	3
Proteasome subunit β type-6	Q3MHN0	ND	ND	69	ND	ND	3	ND	ND	2
Protein HP-20 homolog	Q2KIT0	ND	ND	133	ND	ND	5	ND	ND	3
Prothrombin precursor [<i>Ovis aries</i>]	gi 261244968	ND	ND	1,188	ND	ND	45	ND	ND	11
Angiotensinogen	gi 1703309	ND	ND	534	ND	ND	21	ND	ND	6
Carbonic anhydrase 2	gi 118582300	ND	ND	285	ND	ND	13	ND	ND	4
Serpin A3-2	A2I7M9	ND	ND	910	ND	ND	51	ND	ND	6
Serpin A3-5	A2I7N1	ND	ND	1,222	ND	ND	84	ND	ND	6
SERPIND1 protein	A6QPP2	ND	ND	154	ND	ND	11	ND	ND	5
Serum albumin	P02769	ND	ND	15,860	ND	ND	479	ND	ND	22
Superoxide dismutase [Cu-Zn]	A3KLR9	ND	ND	83	ND	ND	3	ND	ND	4
Transferrin receptor protein 1 (Blast2Go, Similarity: 87.9%)	E1BIG6	ND	ND	126	ND	ND	9	ND	ND	4
Transferrin receptor, partial [<i>Ovis aries</i>]	gi 369726201	ND	ND	110	ND	ND	6	ND	ND	2

¹Biological replicates from C group had a false discovery rate (FDR) ≤ 1.24 and $\leq 2.20\%$, respectively; biological replicates from NC group had an FDR $\leq 1.80\%$ and $\leq 1.92\%$, respectively; technical replicates from the pool of colostrum had an FDR $\leq 2.53\%$ and $\leq 3.57\%$, respectively.

²Nondetectable.

Table 2. Significantly different protein abundances ($P < 0.05$) between colostrum lambs (C group) and noncolostrum lambs (NC group) at 2 and 14 h after birth from isobaric tag for relative and absolute quantitation (iTRAQ) analysis

Protein	Accession no.	Average ratio (sample:pool; means $\pm$ SD)				Minimum identified peptides ¹				P-value ²	
		C group		NC group		C group		NC group		C2 vs. C14	C14 vs. NC14
		2 h	14 h	2 h	14 h	2 h	14 h	2 h	14 h		
Apolipoprotein A-IV	F1N3Q7	0.87 $\pm$ 0.21	1.45 $\pm$ 0.36	0.74 $\pm$ 0.05	1.04 $\pm$ 0.29	11	6	6	6	0.608	0.018
Apolipoprotein B-100	E1ENR0	0.68 $\pm$ 0.09	1.17 $\pm$ 0.47	0.77 $\pm$ 0.22	0.90 $\pm$ 0.05	4	5	3	4	0.485	0.020
Apolipoprotein E	g141016830	0.75 $\pm$ 0.14	1.58 $\pm$ 0.23	0.90 $\pm$ 0.31	0.96 $\pm$ 0.21	3	3	4	4	0.625	0.054
Ceruloplasmin precursor	F1N076	0.62 $\pm$ 0.27	1.18 $\pm$ 0.34	0.75 $\pm$ 0.28	0.87 $\pm$ 0.06	3	3	3	3	0.235	0.036
Fibrinogen α chain	A5PJE3	0.69 $\pm$ 0.24	1.31 $\pm$ 0.38	0.94 $\pm$ 0.33	1.02 $\pm$ 0.30	16	22	19	19	0.321	0.052
Tetranectin	Q2KIS7	0.66 $\pm$ 0.07	1.05 $\pm$ 0.09	0.85 $\pm$ 0.04	0.82 $\pm$ 0.02	3	3	4	3	0.587	0.098
Immunoglobulin μ chain partial	g1165945	0.67 $\pm$ 0.38	1.55 $\pm$ 0.33	0.59 $\pm$ 0.13	0.69 $\pm$ 0.29	4	7	4	3	0.265	0.015
Trypsin inhibitor	g11363062	0.69 $\pm$ 0.26	1.07 $\pm$ 0.22	0.85 $\pm$ 0.03	0.82 $\pm$ 0.01	5	5	5	6	0.235	0.021

¹Value of the sample with the lowest number of identified peptides for a specific protein and group.²C2 and C14 = colostrum group at 2 h and 14 h after birth, respectively; NC2 and NC14 = noncolostrum group at 2 h and 14 h after birth, respectively.

be even more abundant in colostrum than in plasma. Colostrum clearly contributes to the health of the newborn, with several proteins that provide immunomodulatory effects, as reviewed by Bendixen et al. (2011). The iTRAQ labeling approach allowed analyses of the relative changes in immunomodulatory colostrum proteins as an effect of colostrum access in newborn lambs.

Colostrum Proteins Detected in C Group Plasma at 14 h After Birth. Immunoglobulins were mainly found to be absorbed and transferred to C group plasma after colostrum feeding. These findings are in agreement with Hurley and Theil (2011) and Moreno-Indias et al. (2012), who reported immunoglobulins to be one of the most crucial proteins involved in PIT.

Colostrum Proteins Not Detected in C Group Plasma at 14 h After Birth. The subset of proteins that were identified in only colostrum (Table 1) and therefore apparently not absorbed from the colostrum includes proteins such as caseins and α -lactalbumin. Colostrum is known to contain high amounts of immunoglobulins. In our studies, all immunoglobulin isoforms seemed to be efficiently absorbed to plasma, and indeed, these colostrum proteins dominated the plasma proteome of the colostrum-fed animals. However, additional highly abundant colostrum proteins, such as caseins and α -lactalbumin, were not observed in lamb plasma. This indicates that their effect may be related to protective functions in the intestine of suckling animals. It has been noted that caseins act as bioactive peptide precursors with antimicrobial activity (Lahov and Regelson, 1996), suggesting that proteases can also play a role in the host defense. Furthermore, it has been recently shown that α -lactalbumin plays a role as an immunomodulator in human and cow colostrum and milk (Montagne et al., 2000; Korhonen and Pihlanto, 2007; Marnila and Korhonen, 2011).

Indeed, a wide range of colostrum proteins was not detectable in plasma of colostrum animals. These included proteins such as neutrophil gelatinase-associated lipocalin isoform 2, peptidoglycan recognition protein 1, and junction plakoglobin, which all provide protection against bacterial infections. The first acts as an iron-trafficking protein (Yang et al., 2002), the second interferes with peptidoglycan biosynthesis (Liu et al., 2001; Lu et al., 2006), and junction plakoglobin, in conjunction with MUC-1, prevents pathogens from reaching the cell surface (Li et al., 2003; Moncada et al., 2003). Moreover, proteins such as calcium binding protein, matrix gla protein, and nucleobindin-1, which take part in Ca^{2+} transport, or adipocyte fatty acid-binding protein, which participates in lipid transport, were observed in colostrum but not detectable in plasma after suckling.

Proteins Detected in NC Group Plasma at 14 h After Birth. Our findings clearly reflect how noncolostrum lambs showed a wide range of plasma proteins related to stress, inflammation, coagulation, catabolic metabolism, and cell destruction. The presence of proteins such as haptoglobin or different complement factors are indicative of stress in this group of lambs, probably related to colostrum deprivation, and therefore lack of energy intake. Thus, the presence of several proteins that take part during catabolic metabolism (adiponectin and plasma serine protease inhibitor, among others) were observed, together with proteins that are involved in the blood coagulation processes including coagulation factor a1 and XIII, α -2 antiplasmin, kininogen-1, or serpin A3-2, A3-5, and D1. This observation may be related to the fact that the coagulation system appears to be activated and upregulated during periods of acute stress (Binev et al., 2006; Wirtz et al., 2008; Austin et al., 2012). Further studies are needed because the present data cannot confirm that the presence of these proteins in NC group is related to acute stress and not to the dilution factor that can follow colostrum intake in blood plasma.

Protein Quantification Using an iTRAQ Approach

In total, 148 proteins were observed and quantified from the shotgun proteome data. Of these, 31 proteins were normally distributed in at least 3 out of the 4 biological replicates in each of the 4 studied groups (C and NC groups at 2 and 14 h after birth, respectively). These were further analyzed for their relative abundance in plasma from both studied groups (C and NC groups) at 2 and 14 h after birth. Eight proteins were increased in the C group at 14 h after birth.

Table 2 shows significant ($P < 0.05$) protein ratio changes between C and NC groups during the experimental period. In reference to apolipoproteins, 3 different classes (apolipoprotein A-IV, B-100, and E) were found to be relatively increased in the C group at 14 h after birth due to colostrum feeding. These results are in agreement with those of Hernández-Castellano et al. (2014b), who observed that lambs fed with colostrum had a higher apolipoprotein A-IV concentration in blood than lambs not fed with colostrum during the first hours of life (14 h). The primary function of apolipoproteins in the efficacy of fat absorption by the intestine has been described in detail (Mahley and Rall, 2000; Kendrick et al., 2001; Stan et al., 2003; Simon et al., 2011). Fat absorption plays an essential role in newborn lambs, because colostrum is necessary not only for immune protection, but also as the only energy source at this early period after birth. Along with these findings, a relationship with the immune response has

also been described. In particular, apolipoprotein B-100 and apolipoprotein-E have been demonstrated to be an innate barrier against infections (Maezawa et al., 2006; Peterson et al., 2008). Furthermore, it has been observed that apolipoprotein A-IV not only exhibits a protective effect against external agents (Vowinkel et al., 2004), but also serves to reduce gastric secretions (Okumura et al., 1994). This, in conjunction with the increase of trypsin inhibitor produced in the C group, promotes the absorption of proteins with their native structure.

Moreover, several acute-phase proteins, such as fibrinogen α chain, tetranectin, and ceruloplasmin, were also increased in plasma after colostrum feeding. The first of these proteins is the major precursor of blood clots (Tamzali et al., 2001) but also takes part in inflammation, stimulating the adhesion, migration, chemotaxis, and phagocytosis of monocytes and macrophages to the point of infection (Akassoglou and Strickland, 2002; Gånheim et al., 2003). Similarly, Hernández-Castellano et al. (2014b) observed an increase in fibrinogen μ -chain concentration in blood plasma of lambs after colostrum intake, using a differential in-gel electrophoresis (DIGE) approach. Tetranectin is an active player in the activation of plasmin (Holtet et al., 1997), stimulating neutrophil migration to the infection (Renckens et al., 2006). Previously, ceruloplasmin has been described as a major copper and iron transporter, but this protein can also be involved in defense via its ferroxidase activity, which may drive iron homeostasis in a direction unfavorable to invasive organisms (Mazumder et al., 1997).

Table 2 shows that colostrum intake resulted in a relative increase of 8 plasma proteins in the C group. These proteins, observed using the iTRAQ LC-MS/MS approach, were detected in both groups of animals (C and NC groups) as well as in the pool of sheep colostrum using SDS-PAGE LC-MS/MS. Because of the wide range of proteins present in colostrum and blood, no perfect proteomics approach exists that can provide a full characterization of these kind of samples; thus, the combination of different proteomic techniques is frequently suggested (Hernández-Castellano et al., 2014a). Results of this study using SDS-PAGE LC-MS/MS in combination with an iTRAQ LC-MS/MS approach demonstrate that colostrum is essential not only for its immunoglobulin content, but also for the nonimmunoglobulin proteins that play a fundamental role in the activation and attraction of immune cells and low gastric secretion, among others. The information reported in this paper contributes comprehensive proteomics data, identifying proteins that may pass from the colostrum to the lamb's systemic circulation. Nevertheless, further studies for absolute quantitation

using selected reaction monitoring will be necessary to understand the function of colostrum proteins and their relation to PIT. Additionally, this study provides a solid foundation for future studies that could identify important proteins to consider when developing an alternative colostrum source with putative implications in sheep production.

CONCLUSIONS

The results of this work described the presence of 70 proteins in the sheep colostrum proteome. Moreover, 8 proteins were increased in lamb blood plasma after colostrum intake. Understanding the uptake and effect of colostrum is important in reducing lamb mortality rates related to artificial rearing and, thus, in increasing the economic benefit of sheep farmers. Further proteomic studies will be necessary, particularly using the selected reaction monitoring approach, to increase the general knowledge about the role of colostrum in the PIT.

ACKNOWLEDGMENTS

L. E. Hernández-Castellano acknowledges financial support from the Formación del Profesorado Universitario (FPU) program (Ministry of Education, Madrid, Spain). A. M. Almeida acknowledges the support from SFRH/BPD/90916/2012 from Fundação para a Ciência e Tecnologia (Lisbon, Portugal). All authors are members of the international consortium COST action FA1002: Farm Animals Proteomics (www.cost-faproteomics.org) funded by the European Science Foundation (Brussels, Belgium) to whom the network support is acknowledged, as well as Short Term Scientific Mission reference COST-STSM-FA1002-10773 that made this work and collaboration possible. The authors thank David Eckersall (University of Glasgow, UK) and Samantha Wall (University of Bern, Switzerland) for critically reviewing this manuscript.

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