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Reference 1D and 2D electrophoresis maps for potential disease related proteins in milk whey from lactating buffaloes and blood serum from buffalo calves (Water buffalo, *Bubalus bubalis*)

André M. Santana[a,*], Funmilola C. Thomas[b], Daniela G. Silva[a], Eilidh McCulloch[c], Ana M.C. Vidal[d], Richard J.S. Burchmore[e], José J. Fagliari[a], Peter D. Eckersall[c]

[a] Department of Veterinary Clinic and Surgery, School of Agricultural and Veterinary Sciences, São Paulo State University (FCAV/UNESP), Jaboticabal, SP, Brazil
[b] Department of Veterinary Physiology and Pharmacology, College of Veterinary Medicine, Federal University of Agriculture, Abeokuta, Nigeria
[c] Institute of Biodiversity, Animal Health and Comparative Medicine, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow, United Kingdom
[d] Department of Veterinary Medicine, Faculty of Animal Science and Food Engineering, University of São Paulo (FZEA/USP), Pirassununga, SP, Brazil
[e] Institute of Infection, Immunity and Inflammation, Glasgow Polyomics Facility, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow, United Kingdom

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ABSTRACT

The aim of this study was to identify potential disease related proteins in milk whey of lactating buffaloes and blood serum of buffalo calves, in order to define a reference electrophoresis map for 1-DE and 2-DE. Additionally, changes in some protein patterns from buffalo calves during salmonellosis and lactating buffaloes during mastitis are presented. Milk samples were collected and distributed into groups: Milk samples from healthy buffaloes (SCC < 100.000 cells/ml, negative microbiology and CMT) (G1, n = 5) and buffaloes with subclinical mastitis (SCC > 500.000 cells/ml, positive microbiology and CMT) (G2, n = 5). Blood samples from buffalo calves (n = 6) were collected, and three calves were experimentally infected with *Salmonella* Dublin and samples analyzed before (M0) and 72 h after inoculation (M1). 1-DE was accomplished by loading 10 µg of TP into SDS-PAGE, stained with Coomassie blue. 2-DE was accomplished by loading 200 µg of TP into 11 cm, pH 3–10 non-linear IPG strips, followed by SDS-PAGE, stained with Coomassie blue. Protein bands/spots were excised, subjected to tryptic in-gel digestion and analyzed by LC/ESI-MS/MS. Protein identity was assigned using NCBI databases. After bands/spots from 1-DE and 2-DE were analyzed, a protein map with 35 and 40 different identified proteins in blood serum and milk whey, respectively, was generated. Significant changes in patterns of haptoglobin were observed in buffalo calves with salmonellosis and in patterns of IgLC, β-lactoglobulin and α-lactalbumin of lactating buffaloes during mastitis. The establishment of a protein map for 1-DE and 2-DE, identifying potential disease related proteins, can help to address alterations during diseases in buffaloes.

1. Introduction

Among domestic animals, the water buffalo (*Bubalus bubalis*), particularly the river buffalo (subfamily Bovinae), holds great potential for animal production. Approximately 15% of the milk supply in the world is sourced from buffaloes and the Asian continent, with a buffalo population near to 150 million animals, is the major producer of buffalo milk. In Brazil, the buffalo is distributed in all states with an estimated population of 1.5 million animals. The southeast region (10.2% of all animals) has the largest concentration of farms specialized in buffalo milk production destined for the manufacture of milk derivatives, for which Murrah and Jafarabadi breeds are the most important (MAPA, 2016).

Thus, buffalo milk production is of great importance in Brazil and other countries and studies related to milk whey and blood serum alterations due to diseases in lactating buffaloes and in newborn animals are important for the maintenance of an adequate milk production system. In this context, special attention has been given to acute phase proteins (APP), that can be used in diagnosis, prognosis and in monitoring response to therapy, as well as in general health screening (Eckersall and Bell, 2010).

In lactating cows, APP in blood and milk have shown positive correlation with the severity of mastitis infection (Eckersall et al., 2001, 2006; Wenz et al., 2010; Buitenhuis et al., 2011; Pyörälä et al., 2011)

* Corresponding author at: Department of Veterinary Clinic and Surgery, School of Agricultural and Veterinary Sciences, São Paulo State University (FCAV/UNESP), Via de Acesso Prof. Paulo Donato Castellane s/n, Jaboticabal, SP 14884-900, Brazil.
E-mail address: andrevetms@gmail.com (A.M. Santana).

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Table 1
Parameters from lactating buffaloes (n = 10) and buffalo calves (n = 6) that were selected and distributed among the experimental groups.

Lactating buffaloes					
Milk whey samples from heathy buffaloes (G1)					
Sample ID	SCC	CMT	Microbiology	Month of lactation	N° of lactations
M1	21,000	0	Negative	4th	2nd
M2	21,000	0	Negative	4th	2nd
M3	28,000	0	Negative	4th	3rd
M4	22,000	0	Negative	4th	2nd
M5	24,000	0	Negative	6th	3rd
Average	23,200 ± 2950	–	–	–	–
Milk whey samples from buffaloes with subclinical mastitis (G2)					
M6	1,845,000	3	*S. intermedius*	4th	2nd
M7	8,670,000	2	*S. aureus*	5th	2nd
M8	3,731,000	2	*Staphylococcus sp.*	6th	3rd
M9	3,487,000	2	*Staphylococcus sp.*	6th	3rd
M10	615,000	1	*Staphylococcus sp.*	6th	3rd
Average	3,669,600 ± 3,070,051	–	–	–	–
Animal ID	Buffalo calves				
	Age (days)/gender	Weigh (Kg)	Microbiology	RT (° C)	FC score
S1M0	26/F	42.8	Negative	37.9	0
S2M0	22/M	80.4	Negative	38.8	0
S3M0	12/M	64.4	Negative	39.0	0
S4M0	26/M	63.2	Negative	38.3	0
S5M0	29/M	63.1	Negative	37.3	0
S6M0	30/F	81.7	Negative	37.8	0
Average	24.2 ± 6.6	65.9 ± 14.2	–	38.2 ± 0.6	0
S1M1	–	–	*S.* Dublin	40.3	1
S2M1	–	–	*S.* Dublin	41.8	1
S3M1	–	–	*S.* Dublin	41.4	2
Average	–	–	–	41.2 ± 0.8	1.33 ± 0.58

RT, Rectal Temperature; FC score, Feces Consistency score.
M1 to M5: Milk whey samples from heathy buffaloes (G1); M6 to M10: Milk whey samples from buffaloes with subclinical mastitis(G2).
S1M0 to S6M0: Blood serum samples from buffalo calves before inoculation (M0).
S1M1, S2M1, S3M1: Blood serum samples from buffalo calves 72 h after inoculatiom with 10^8 CFU of *S.* Dublin (M1).

and have been shown to be reliable indicators of inflammation and useful to screen quarters for intramammary inflammation. In buffaloes, studies with alterations in APP in the milk during mastitis are scarce, but have already showed that haptoglobin and serum amyloid A are good milk indicators for the detection of streptococcal and staphylococcal clinical and sub-clinical mastitis (Kumar et al., 2014).

When analyzing newborns buffalo calves, the first month of life is critical, as morbidity and mortality rates are high. The major causes are diarrhea and pneumonia (Khan et al., 2009; Anwarullah et al., 2014; Naag et al., 2015) caused by various pathogens (Anwarullah et al., 2014; Silva et al., 2015) and are responsible for great economic losses. In this context, APP have also shown to be of great value, since alterations in serum concentrations of APP fibrinogen, ceruloplasmin and haptoglobin in diarrheic newborn buffaloes infected with *Salmonella Thyphimurium* (Clemente et al., 2016) have been reported.

Milk whey and blood serum are complex mixtures of a few high abundance proteins and a wide selection of low abundance protein components (Ferreira et al., 2013) that could be utilized for diagnosis and prognosis purposes. Thus, in addition to the APP, other proteins related to defence/immunity, lipid and protein transport and metabolism, enzyme regulation, cell-to-cell signaling, among others, could be altered during the disease process. In this sense, proteomic studies for biomarker investigations have, over the past decade, developed the use of gel and gel-free methodologies based on liquid chromatography analyses (Schiess et al., 2009), among others. In this context, the use of proteomic methodologies such us 1D-electrophoresis (1-DE), 2D-electrophoresis (2-DE) and gel-free analyses (label-free and label-based methods) to obtain a complete characterization of host responses during infections could lead to the identification of a pattern of biomarkers indicative of diseases.

The use of 2-DE combined with LC/ESI-MS/MS (liquid chromatography coupled with electrospray ionization tandem mass spectrometry) can be a powerful strategy for the identification of proteins. However, little information is known, in healthy and diseased buffalo blood serum and milk whey, about which proteins are detected and how these proteins behave in 1-DE and 2-DE gels (D'Auria et al., 2005; D'Ambrosio et al., 2008; Jena et al., 2015). In contrast, several proteomic analyses with relation to healthy cattle and, as an example, cattle with mastitis have been performed using 2-DE and gel-free analyses such as label-free and label-based methods (Boehmer et al., 2010a, 2010b; Danielsen et al., 2010; Alonso-Fauste et al., 2012; Thomas et al., 2016a, 2016b; Mudaliar et al., 2016).

A combined approach based on 1-DE and 2-DE with LC/ESI-MS/MS can help to identify a great amount of disease related proteins such us APP and defence/immunity-related proteins, as well as high abundance proteins, serpins, apolipoproteins and proteins with various other functions that could participate in metabolic pathways of interest. In addition, mapping these proteins in 1-DE and 2-DE gels is of great importance for buffaloes species, as it may serve as a starting point for other studies in buffaloes related to protein changes during diseases and other physiological conditions. Therefore, considering the lack of published works available in buffalo proteomics, the aim of this study was to identify, in milk whey of lactating buffaloes and blood serum of buffalo calves, proteins with potential to be differentially expressed during disease and other physiological conditions in buffaloes. Additionally, changes in patterns of APP haptoglobin in the blood serum of buffalo calves during salmonellosis, and of immunoglobulin light chain, β-lactoglobulin and α-lactalbumin in milk whey of lactating buffaloes during mastitis were studied.

2. Materials and methods

This research was approved by the Ethics Committee on Animal Use of "Faculdade de Ciências Agrárias e Veterinárias, UNESP" (Protocol n° 010885-08 and n° 017094/12).

2.1. Distribution of experimental groups for defining a reference electrophoresis map for 1-DE and 2-DE and for analyzing changes in protein patterns during mastitis and salmonellosis

2.1.1. Experimental groups designed for lactating buffaloes

Milk samples were collected from lactating Jafarabadi buffaloes housed in a semi-intensive system with a diet based on roughage, on farms with commercial herds localized in São Paulo state, Brazil. All samples were collected during the dry season of the year (winter, between June and August), when animals were fed with chopped sugar cane and supplemented with protein concentrate.

Individual milk samples from each udder-quarter were collected. After collection, milk samples were submitted to CMT, SCC and microbiological evaluation, and distributed into two experimental groups: G1 (n = 5, sample ID: M1 to M5): individual udder-quarter milk samples from healthy buffaloes (SCC < 100.000 cells/ml, negative microbiology and CMT); G2 (n = 5, sample ID: M6 to M10): individual udder-quarter milk samples from buffaloes with subclinical mastitis (SCC > 500.000 cells/ml, positive microbiology and CMT). All information of milk samples used to perform this study is listed in Table 1.

SCC was performed in the "Laboratório de Fisiologia da Lactação Lair Antônio de Souza (LAFLA), Clínica do Leite, Departamento de Produção Animal da Escola Superior de Agricultura Luiz de Queiroz, USP/Piracicaba, SP" using a automatic electronic infrared counter (Somacount 300, Bentley Instruments Incorporated, Minnesotta, EUA).

Bacterial culture and identification of bacteria was performed in the "Laboratório Multiusuário de Saúde Animal e Segurança Alimentar, Departamento de Medicina Veterinária, Faculdade de Zootecnia e Engenharia de Alimentos, Universidade de São Paulo (FZEA/USP), Pirassununga, SP, Brazil". Aseptically collected milk was seeded in 5% defibrinated bovine blood agar and MacConkey agar at 37 °C, under aerobic conditions, with daily readings until 72 h (Quinn et al., 2005). The microorganisms were identified according to the morpho-tinctorial, biochemical and cultivation characteristics (Quinn et al., 1994, 2005; Trabulsi et al., 2005a, 2005b; Jorgensen et al., 2015).

2.1.2. Experimental groups designed for newborn buffalo-calves

Blood samples from Murrah newborn buffalo-calves (12 to 30 days old) were collected in farms located in São Paulo State, Brazil, and health status was verified by performing physical examination. Feces were analyzed to check for signs of diarrhea, blood and mucus and received feces consistency scores: 0: normal (firm feces consistency), 1: mild diarrhea (soft feces consistency), 2: moderate to severe diarrhea (liquid feces consistency) (Tremblay, 1990). Rectal temperature was also measured. Six calves (sample ID: S1M0, S2M0, S3M0, S4M0, S5M0 and S6M0), that had firm feces consistency (score 0) and rectal temperature below 40.0 °C, were included in the study (Table 1).

From the six calves selected, three calves were isolated from the other animals and housed in individual suspended shelters (1.30 m × 1.50 m × 1.35 m and 0.4 m above the ground) at "Laboratório de Apoio à Pesquisa do Departamento de Clínica e Cirurgia Veterinária, FCAV, UNESP, Jaboticabal, Brazil". These calves were then challenged orally with 10^8 CFU of *Salmonella* Dublin strain suspended in 10 ml of BHI broth, and blood samples were collected before inoculation (S1M0, S2M0 and S3M0) and 72 h after inoculation (S1M1, S2M1 and S3M1) (Table 1).

Inocula for induction of experimental infection was prepared from a *Salmonella* Dublin sample (IOC record: 3101/03) donated by "FIOCRUZ, Rio de Janeiro, Brazil - Centro de Referência de Enterobactérias do Departamento de Bacteriologia". Inocula were prepared according to Fecteau et al. (2003). By using the Miles and Misra (1938) technique, appropriate dilutions were made to obtain the required concentration of colonies/ml.

Rectal swabs were collected from each animal immediately before inoculation (M0) and 72 h after inoculation (M1). Bacteriological culture for isolation of *S.* Dublin from the rectal swabs were performed at "Laboratório de Apoio à Pesquisa do Departamento de Clínica e Cirurgia Veterinária, FCAV, UNESP, Jaboticabal, Brazil", according to recommendations (Santos et al., 2002). For this, three rectal swab samples collected at each moment (M0 and M1) were transferred into 10 ml of selenite cysteine (CM699, Oxoid), tetrathionate Muller-Kauffmann (CM343, Oxoid) and Rappaport-Vassiliadis (CM866, Oxoid) selective enrichment broths and incubated at 37 °C for 24 h. After incubation, broths were plated in modified brilliant green agar (CM329, Oxoid) plates and incubated (37 °C, 24 h). From each plate, three *Salmonella* suggesting colonies were submitted to presumptive biochemistry tests using triple-sugar-iron agar (CM277, Oxoid) and lysine-agar (CM381, Oxoid). All the identified *Salmonella* colonies were submitted to slide agglutination test with poli-O, poli-H and poli-D *Salmonella* antiserum (Probac of Brazil, São Paulo, SP, Brazil). Positive culture for *Salmonella* Dublin were considered when identified *Salmonella* colonies were positive to the three slide agglutination tests (poli-O, poli-H and poli-D *Salmonella* antiserum).

2.1.3. Reference electrophoresis maps and changes in protein patterns during mastitis and salmonellosis

To find and identify the highest possible number of proteins of interest, reference electrophoresis maps for 1-DE and 2-DE were generated using healthy (M1-M5) and mastitic (M6-M10) milk samples (n = 10) from the lactating buffaloes and before (S1M0, S2M0, S3M0, S4M0, S5M0 and S6M0) and after-challenge (S1M1, S2M1, S3M1) blood samples (n = 9) from the newborn buffalo calves.

For analyzing changes in protein patterns during mastitis and salmonellosis, statistical analysis were performed to compare G1 × G2 (lactating buffaloes) and M0 × M1 (buffalo calves).

2.2. Sample preparation for 1-DE and 2-DE

Sample preparation for 1-DE and 2-DE were performed at "Laboratório de Apoio à Pesquisa do Departamento de Clínica e Cirurgia Veterinária, FCAV, UNESP, Jaboticabal, Brazil" and Acute Phase Laboratory, Institute of Biodiversity, Animal Health & Comparative Medicine, Garscube Campus, University of Glasgow, UK. All milk samples listed in Table 1, meaning five milk samples from heathy buffaloes and five milk samples from udder-quarters with subclinical mastitis, were prepared to perform 1-DE and 2-DE, so that 10 individual patterns (for 1-DE) and 10 individual gels (for 2-DE) could be performed and analyzed. All animals listed in Table 1, meaning six blood samples from healthy buffalo calves (M0) + three blood samples from buffaloes calves collected 72 h after inoculation (M1), were also prepared to perform 1-DE and 2-DE, so that nine individual patterns (for 1-DE) and nine individual gels (for 2-DE) could be performed and analyzed.

Milk whey samples were obtained by addition of renin solution (Coalho Estrella, Chr. Hansen Brasil Ind. E Com. Ltda, Valinhos, SP, Brazil) to the milk samples (amounts of 10% renin solution + 90% milk samples), followed by centrifugation (20 min, 4 °C, 10,000 × *g*) so that solids and caseins were removed before performing 1-DE and 2-DE. To remove salts, acetone precipitation was then carried out for each sample by adding 4 times the sample volume of ice cold 100% acetone to the samples. The sample was mixed and kept at −20 °C overnight. Precipitate was separated from supernatant by centrifugation at 14,000 × *g* for 30 min at 4 °C. The pellets formed were then washed by mixing with ice cold 80% (v/v) acetone and then centrifuged at 14,000 × *g* for 30 min once again. This step was repeated two more times. Finally all supernatant was separated from the pellet and the

Table 2
Protein bands/spots identified by LC/ESI-MS/MS in 1-DE and 2-DE performed with blood serum samples from newborn buffalo-calves, showing NCBI data generated using mascot engine search.

Band/spot number	Protein identification (Accession number, organism)	Protein MASCOT SCore[a]	Matches[b]	Sequences[c]	Estimated MW (Da)	ID Method	Protein function
1	α2-macroglobulin (gi|594093035 BB)	1839 (0.80)	162 (66)	52 (32)	165,702	1-DE	APP
2	Ceruloplasmin (gi|594079008, BB)	173 (0.10)	28 (4)	19 (4)	125,184	1-DE	APP
3	ITIH4 (gi|594051967, BB)	312 (0.28)	49 (14)	26 (8)	99,001	1-DE	APP
	Serpin A3–2 (gi|594050458, BB)	250 (0.45)	18 (8)	12 (5)	40,453		Defence/Immunity
	Endopin 1b (Serpin A3–3) (gi|594074015, BB)	109 (0.14)	12 (2)	9 (2)	46,738		Defence/Immunity
4	Complement C6 (gi|594107089, BB)	107 (0.03)	10 (1)	8 (1)	108,221	1-DE	Defence/Immunity
5	Complement factor B (gi|594034465, BB)	293 (0.15)	32 (8)	17 (4)	86,804	1-DE	Defence/Immunity
6	Plasminogen (gi|594054121, BB)	188 (0.10)	33 (3)	19 (3)	94,095	1-DE	APP
	Gelsolin isoform b (gi|77736201, BT)	113 (0.08)	7 (2)	6 (2)	80,681		Defence/Immunity
7	Serotransferrin (gi|594054424, BB)	1612 (0.70)	90 (24)	36 (14)	79,994	1-DE	APP
	Complement C5a anaphylatoxin (gi|262205546, BB)	111 (0.02)	3 (1)	3 (1)	190,388		Defence/Immunity
8	Serotransferrin (gi|594054424, BB)	995 (0.83)	90 (27)	34 (15)	79,994	1-DE	APP
	Ig M heavy chain (gi|2232299, BT)	63 (0.06)	2 (1)	2 (1)	47,885		Defence/Immunity
9	Serum Albumin (gi|594045062, BB)	1878 (2.15)	207 (62)	48 (25)	71,177	1-DE	APP
	α1-antitrypsin (Serpin A1) (gi|594050476, BB)	64 (0.07)	2 (1)	2 (1)	46,290		APP
10	Kininogen-2 (gi|594069549, BB)	137 (0.14)	9 (4)	6 (3)	69,820	1-DE	Defence/Immunity
	Endopin 2B (Serpin A3–7)	108 (0.14)	5 (3)	3 (2)	46,972		Defence/Immunity
	(gi|38,683,423, BT)	88 (0.08)	9 (1)	6 (1)	38,984		APP
	α-2-HS-glycoprotein (gi|594,069,561,	86 (0.06)	10 (1)	9 (1)	54,890		APP
	BB)	72 (0.42)	5 (1)	1 (1)	8025		Defence/Immunity
	Vitamin D-binding protein (gi|397,740,864, BB)						
	Ig gamma heavy chain (gi|126,542,085, CEH)						
11	Complement factor I (gi|594,056,114, BB)	47 (0.00)	2 (0)	2 (0)	71,319	1-DE	Defence/Immunity
12	Complement C3 (gi|594,041,230, BB)	323 (0.12)	38 (9)	17 (7)	188,843	1-DE	APP
13	Complement C3 (gi|594,041,230, BB)	176 (0.08)	29 (6)	15 (5)	188,843	1-DE	APP
14	Haptoglobin (gi|595,763,483, BT)	1085 (1.21)	114 (35)	22 (12)	45,676	1-DE	APP
15	Adiponectin (gi|114,158,576, BT)	211 (0.58)	10 (5)	6 (4)	26,117	1-DE	Hormone, Inflamation process
	Complement C4-A (gi|528,929,317, BT)	153 (0.07)	11 (4)	9 (4)	189,312		Defence/Immunity
16	Ig lambda light chain (gi|92,096,965, BT)	159 (0.27)	18 (4)	4 (2)	24,863	1-DE	Defence/Immunity
17	Apolipoprotein A-I (gi|594,065,921, BB)	726 (0.81)	37 (8)	13 (6)	30,232	1-DE	APP
18	Haptoglobin (gi|283,467,275, BB)	471 (0.37)	25 (11)	9 (5)	48,259	1-DE	APP
19	Hemoglobin (gi|594,075,744, BB)	465 (1.90)	27 (16)	13 (9)	25,066	1-DE	Transport
20	α2-macroglobulin (gi|594,093,035, BB)	770 (0.47)	74 (24)	39 (21)	165,702	2-DE	APP
	Complement factor H (gi|76,677,897, BT)	164 (0.09)	19 (5)	14 (4)	144,958		Defence/Immunity
21	Ceruloplasmin (gi|296,491,101, BT)	201 (0.16)	17 (6)	10 (6)	120,931	2-DE	APP
22	Ceruloplasmin (gi|296,491,101, BT)	42 (0.03)	5 (1)	3 (1)	120,931	2-DE	APP
23	Complement factor B (gi|594,034,463, BB)	108 (0.07)	14 (2)	12 (2)	87,871	2-DE	Defence/Immunity
24	Plasminogen (gi|594,054,121, BB)	290 (0.21)	53 (9)	25 (6)	94,095	2-DE	APP
25	Endopin 1b (Serpin A3–3)	309 (0.38)	19 (7)	11 (5)	46,738	2-DE	Defence/Immunity
	(gi|594,074,015, BB)	193 (0.35)	17 (4)	10 (4)	40,453		Defence/Immunity
	Serpin A3–2 (gi|594,050,458, BB)	50 (0.09)	8 (3)	6 (3)	101,449		APP
	ITIH4 (gi|75,832,116, BT)						
26	Ig M heavy chain (gi|28,592,070, BT)	154 (0.13)	10 (4)	4 (2)	49,551	2-DE	Defence/Immunity
27	α -1B-Glycoprotein(gi|594,087,719, BB)	439 (0.48)	47 (13)	16 (7)	54,191	2-DE	Platelet activation, signaling and aggregation
28	Serum Albumin (gi|594,045,062, BB)	2706 (3.07)	262 (89)	45 (31)	71,177	2-DE	APP
29	Serotransferrin (gi|594,054,424, BB)	1181 (0.90)	111 (37)	32 (16)	79,994	2-DE	APP
30	Serotransferrin (gi|594,054,424, BB)	2325 (1.78)	202 (69)	37 (25)	79,994	2-DE	APP
31	α-2-HS-glycoprotein (gi|594,069,561,	545 (0.68)	56 (13)	11 (6)	38,984	2-DE	APP
	BB)	99 (0.07)	6 (2)	5 (1)	49,230		Defence/Immunity
	Kininogen-2 (gi|594,069,551, BB)						
32	α1-antitrypsin (Serpine A1)	938 (1.18)	69 (31)	14 (11)	46,290	2-DE	APP
	(gi|594,050,476, BB)	248 (0.12)	25 (5)	13 (2)	54,986		APP
	Vitamin D-binding protein (gi|594,092,100, BB)						
33	Hemopexin (gi|594,034,957, BB)	69 (0.07)	20 (1)	10 (1)	52,691	2-DE	APP
34	Ig heavy chain variable region (gi|1,293,600, BT)	90 (0.26)	1 (1)	1 (1)	12,626	2-DE	Defence/Immunity
35	Ig heavy chain variable region (gi|1,293,600, BT)	90 (0.26)	1 (1)	1 (1)	12,626	2-DE	Defence/Immunity
36	Gelsolin isoform b (gi|77,736,201, BT)	436 (0.30)	25 (12)	11 (7)	80,681	2-DE	Defence/Immunity
37	Apolipoprotein A-I (gi|594,065,921, BB)	599 (1.97)	46 (21)	15 (11)	30,232	2-DE	APP

(*continued on next page*)

Table 2 (*continued*)

Band/spot number	Protein identification (Accession number, organism)	Protein MASCOT SC ore[a]	Matches[b]	Sequences[c]	Estimated MW (Da)	ID Method	Protein function
38	Haptoglobin (gi|595,763,483, BB)	480 (0.30)	35 (13)	7 (4)	45,676	2-DE	APP
39	Haptoglobin (gi|595,763,483, BB)	1043 (1.21)	116(30)	21 (12)	45,676	2-DE	APP
40	Haptoglobin (gi|595,763,483, BB)	1142 (1.07)	138 (43)	21 (11)	45,676	2-DE	APP
41	Ig light chain (gi|92,096,965, BT)	164 (0.27)	18 (3)	4 (2)	24,863	2-DE	Defence/Immunity
42	Ig light chain (gi|92,096,965, BT)	147 (0.27)	24 (4)	4 (2)	24,863	2-DE	Defence/Immunity
43	Ig light chain (gi|92,096,965, BT)	139 (0.27)	20 (3)	4 (2)	24,863	2-DE	Defence/Immunity
44	Apolipoprotein A-I (gi|594,065,921, BB)	466 (1.21)	50 (13)	15 (18)	30,232	2-DE	APP
45	Haptoglobin (gi|595,763,483, BB)	449 (0.30)	32 (13)	8 (4)	45,676	2-DE	APP
46	Adiponectin (gi|114,158,576, BT)	28 (0.00)	1 (0)	1 (0)	26,117	2-DE	Hormone, Inflamation process
47	Haptoglobin (gi|595,763,483, BB)	1185 (1.26)	118 (32)	23 (11)	45,676	2-DE	APP
48	Haptoglobin (gi|595,763,483, BB)	560 (0.94)	70 (17)	18 (10)	45,676	2-DE	APP
49	Complement C3 (gi|594,041,230, BB)	132 (0.07)	9 (4)	7 (4)	188,843	2-DE	APP
50	Apolipoprotein A-IV (gi|594,065,917, BB)	885 (1.66)	94 (35)	26 (14)	46,181	2-DE	Lipid metabolism
51	Antithrombin-III (Serpin C1) (gi|77,736,341, BT)	766 (1.00)	40 (25)	15 (12)	52,315	2-DE	APP
52	Complement C3 (gi|594,041,230, BB)	255 (0.08)	36 (7)	15 (5)	188,843	2-DE	APP
53	Complement C3 (gi|83,764,016, BT)	28 (0.00)	4 (0)	3 (0)	187,175	2-DE	APP
54	Hemoglobin (gi|594,075,744, BB)	930 (2.27)	61 (29)	13 (10)	25,066	2-DE	Transport
55	Serum amyloid A-4 protein (gi|94,966,809, BT)	105 (0.81)	6 (3)	3 (3)	14,678	2-DE	APP
56	Serum amyloid A, SAA (gi|245,184, BT)	48 (0.87)	2 (2)	2 (2)	12,467	2-DE	APP
57	Glial fibrillary acidic protein (gi|586,992,699, BB)	85 (0.06)	3 (1)	2 (1)	44,654	2-DE	Glycoprotein binding

BB, Bubalus bubalis; BT, Bos taurus; CEH, Cervus elaphus hispanicus; Ig, Immunoglobulin; APP, Acute Phase Protein; PSBF, Protein synthesis/binding/folding.

[a] The number in parenthesis indicates the Exponentially Modified Protein Abundance Index (emPAI).

[b] Total number of peptide matches. The number in parenthesis indicates the number of matches above the significance threshold ($p < 0.05$).

[c] Total number of distinct peptide sequences. The number in parenthesis indicates the number of matches above the significance threshold ($p < 0.05$).

pellet allowed to air dry by exposing to air for not > 5 min. Subsequently the pellet was re-suspended in rehydration/sample buffer (7 M urea, 2 M thiourea, 1% ASB-14, 40 mM Tris, 0.001% Bromophenol Blue) (Bio-Rad ReadyPrepTM rehydration/sample buffer).

Blood serum samples were obtained by centrifugation (10 min, 4 °C, 1000 ×*g*) of 10 ml of blood collected in siliconized vials without anticoagulant. Blood serum samples were then submitted to acetone precipitation to remove salts, using the same procedure as for the milk whey. After salts were removed, samples were also re-suspended in rehydration/sample buffer (Bio-Rad ReadyPrepTM rehydration/sample buffer).

2.3. 1-DE

1-DE was performed at the Acute Phase Laboratory, Institute of Biodiversity, Animal Health & Comparative Medicine, Garscube Campus, University of Glasgow, UK. As described (item 2.2), 10 individual patterns, from milk whey samples, and nine individual patterns, from blood serum samples, were generated.

After re-suspending the pellet in rehydration/sample buffer (Bio-Rad ReadyPrepTM rehydration/sample buffer), total protein concentration was determined by the Bradford method (Sigma-Aldrich) so that equal protein loading of 10 µg were used, in all samples, for 1-DE protein separation. Therefore, equal volumes of sample (containing 10 µg of protein) and preparation solution were mixed. The preparation solution was composed of 95% Laemmli sample buffer (Bio Rad Ltd., Hemel Hempstead, UK) + 5% 2-mercaptoethanol (Bio Rad).

The final solution was heated in thermal block at 95 °C for 4 min. Meanwhile, SDS-PAGE gel (4–15%T Criterion™ TGX™ Precast polyacrylamide Gel, 13.3 × 8.7 cm, 18 wells, Bio Rad) was assembled in the gel running tank (Bio Rad, Criterion™ Vertical Electrophoresis Cell) containing running buffer (Bio Rad, 25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3). After samples were heated, they were transferred to the SDS-PAGE gel that was previously assembled in the gel running tank as described. 10 µl of pre-stained protein ladder (10–170 kDa, PageRuler prestained protein ladder, Thermo Scientific Inc., USA) was also added into the first well.

Electrophoresis was run at 300 V for 15–20 min at room temperature. Gels were removed from the gel cassette and then stained for 1–2 h in colloidal solution of Coomassie brilliant blue stain G-250 dye 0.1% (w/v), 10% (v/v) acetic acid, 40% (v/v) ethanol (Invitrogen, Manchester, UK) after which stain solution was discarded and destaining carried out overnight using a solution of 10% (v/v) acetic acid and 25% (v/v) methanol. Images of the gels were scanned using a UMAX Power Look III scanner and software (Hamrick software, USA).

Molecular weight (MW) and concentration of protein fractions were then determined by use of computer-assisted densitometry (CS-9301PC, Shimadzu Corporation). For the densitometric evaluation of the protein bands, reference curves were created from the wide range standard marker (MW from 6.500 to 200.000 Da, Sigma-Aldrich S8445) reading, which generated computer graphics that allowed analyses of MW and concentrations of proteins of interest, that were calculated based on total milk whey protein previously measured.

To identify protein bands that were differentially expressed during subclinical mastitis, average concentrations between G1 (n = 5) and G2 (n = 5) were compared by unpaired *t*-test ($p < 0.05$) using GraphPad Prisma 5. To identify protein bands that were differentially expressed during S. Dublin infection, average concentrations between before inoculation (n = 3) and 72 h after inoculation (n = 3) were also compared by unpaired *t*-test ($p < 0.05$) using GraphPad Prisma 5.

2.4. 2-DE

2-DE was performed at the Acute Phase Laboratory, Institute of Biodiversity, Animal Health & Comparative Medicine, Garscube Campus, University of Glasgow, UK. As described (item 2.2), 10 individual gels, from milk whey samples, and nine individual gels, from blood serum samples, where generated.

After re-suspending the pellet in rehydration/sample buffer (Bio-Rad ReadyPrepTM rehydration/sample buffer) following acetone precipitation, total protein concentration was determined by the Bradford method (Sigma-Aldrich) so that equal protein loading of 200 µg were

Table 3
Protein bands/spots identified by LC/ESI-MS/MS in 1-DE and 2-DE gels performed with milk whey samples from lactating buffaloes, showing NCBI data generated using mascot engine search.

Band/spot number	Protein identification (Accession number, organism)	Protein MASCOT Score[a]	Matches[b]	Sequences[c]	Estimated MW (Da)	ID Method	Protein function
58	Thrombospodin-1 (gi|594,100,043, BB)	105 (0.02)	11 (1)	10 (1)	133,342	1D	Defence/Immunity
	α2-macroglobulin (gi|594,093,035, BB)	52 (0.02)	7 (1)	6 (1)	165,702		APP
59	Xanthine dehydrogenase/oxidase (gi|594,049,883,BB)	231 (0.08)	50 (5)	26 (4)	149,733	1D	Defence/Immunity
60	Ceruloplasmin (gi|296,491,101, BT)	30 (0.0)	4 (0)	4 (0)	121,901	1D	APP
61	Complement factor B (gi|594,034,465, BB)	264 (0.32)	23 (9)	14 (8)	86,804	1D	Defence/Immunity
	ITIH4 (gi|594,051,965, BB)	58 (0.03)	3 (1)	3 (1)	100,794		APP
62	Gelsolin isoform b (gi|77,736,201, BT)	50 (0.08)	11 (2)	10 (2)	80,966	1D	Defence/Immunity
63	Lactoferrin (gi|504, BT)	507 (0.60)	27 (16)	18 (12)	77,129	1D	APP
	Lactoperoxidase (gi|27,806,851, BT)	82 (0.08)	10 (3)	8 (2)	81,504		Defence/Immunity
64	Lactoferrin (gi|157,830,336, BB)	1384 (1.27)	116 (45)	34 (21)	77,658	1D	APP
	Lactoperoxidase (gi|27,806,851, BT)	118 (0.25)	12 (6)	9 (6)	81,504		Defence/Immunity
	IgM heavy chain constant region (gi|2,232,299, BT)	86 (0.20)	4 (3)	3 (3)	48,512		Defence/Immunity
	Serotransferrin (gi|2,501,351, BT)	74 (0.04)	6 (2)	3 (1)	79,870		APP
65	Complement C3 (gi|594,041,230, BB)	685 (0.23)	68(19)	34 (13)	188,843	1D	APP
66	Serum Albumin (gi|594,045,062, BB)	1335 (1.07)	150 (45)	40 (17)	71,177	1D	APP
	Complement C3 (gi|594,041,230, BB)	200 (0.10)	24 (6)	19 (6)	188,843		APP
67	α1-antitrypsin (Serpin A1) (gi|27,806,941, BT)	104 (0.22)	8 (3)	5 (3)	46,417	1D	APP
	α-2-HS-glycoprotein (gi|594,069,561, BB)	54 (0.08)	1 (1)	1 (1)	38,984		APP
	Lipopolysaccharide-binding protein (gi|597,436,968, BB)	43 (0.0)	2 (0)	2 (0)	53,984		APP
68	Ig gamma heavy chain constant region (gi|126,542,085, CEH)	88 (0.42)	7 (1)	1 (1)	8025	1D	Defence/Immunity
	Vitamin D-Binding Protein (gi|594,092,100, BB)	66 (0.06)	3 (2)	2 (1)	54,986		APP
69	Angiotensinogen (gi|594,083,017, BB)	491 (0.60)	37 (17)	14 (8)	51,632	1D	Blood pressure, body fluid and electrolyte homeostasis
	Lactadherin (gi|41,386,719, BT)	98 (0.30)	7 (5)	6 (4)	48,611		Signaling, FTM
	Endopin 2B (Serpin A3–7) (gi|38,683,423, BT)	61 (0.07)	2 (1)	1 (1)	47,200		Defence/Immunity
	Monocyte differentiation antigen CD14 (gi|3,913,217, BT)	43 (0.08)	2 (1)	2 (1)	40,269		Defence/Immunity
70	Lactadherin (gi|595,763,163, BB)	186 (0.30)	18 (6)	9 (4)	49,015	1D	Signaling, FTM
71	Actin, beta (gi|148,744,172, BT)	149 (0.24)	15 (5)	7 (3)	42,022	1D	Development, Structural
	Complement C3 (gi|99,028,969, BT)	133 (0.08)	20 (5)	12 (5)	188,652		APP
72	Chitinase 3-like protein 1 (gi|595,763,132, BB)	103 (0.07)	14 (1)	8 (1)	43,250	1D	Defence/Immunity
73	Haptoglobin (gi|94,966,763, BT)	99 (0.22)	16 (4)	6 (3)	45,629	1D	APP
	Apolipoprotein A-IV (gi|594,065,917, BB)	55 (0.07)	1 (1)	1 (1)	42,823		Lipid metabolism
74	Clusterin (gi|594,072,990, BB)	109 (0.12)	7 (3)	5 (2)	51,592	1D	HSP, Extracellular Chaperone
75	Nucleobindin-1 (gi|115,497,814, BT)	68 (0.18)	6 (3)	5 (3)	54,949	1D	Defence/Immunity
76	Ig light chain, lambda gene cluster (gi|92,096,965,BB)	201 (0.31)	14 (3)	5 (2)	24,863	1D	Defence/Immunity
77	Haptoglobin (gi|283,467,275, BB)	104 (0.13)	6 (2)	3 (2)	48,259	1D	APP
78	Apolipoprotein A-I (gi|594,065,921, BB)	94 (0.10)	5 (2)	4 (1)	30,232	1D	APP
	Ig J Chain (gi|594,092,037, BB)	71 (0.17)	6 (1)	3 (1)	18,343		Defence/Immunity
79	β - Lactoglobulin (gi|6,729,725, BT)	1294 (4.72)	86 (46)	17 (10)	18,555	1D	Transport, Protein metabolism
80	α - Lactalbumin (gi|68, BT)	884 (1.70)	30 (21)	5 (4)	14,603	1D	Carbohydrate metabolism, Lactose Biosynthesis process
81	Gelsolin isoform b (gi|77,736,201, BT)	43 (0.04)	3 (1)	3 (1)	80,966	2D	Defence/Immunity
82	Ig M heavy chain constant region (gi|28,592,070, BT)	144 (0.13)	5 (2)	5 (2)	50,235	2D	Defence/Immunity
83	Lactoferrin (O77698, BB)	219 (0.26)	20 (8)	13 (6)	79,733	2D	APP
84	Lactoferrin (O77698, BB)	408 (0.21)	20 (9)	14 (5)	79,733	2D	APP
	Ig M heavy chain constant region (gi|2,232,299, BT)	68 (0.06)	3 (2)	2 (1)	48,512		Defence/Immunity
85	Serotransferrin (Q29443, BT)	188 (0.21)	12 (6)	8 (5)	79,870	2D	APP
86	Serotransferrin (Q29443, BT)	423 (0.36)	24 (13)	13 (8)	79,870	2D	APP
	Lactoferrin (O77698, BB)	173 (0.26)	18 (7)	13 (6)	79,733		APP
87	Lactoferrin (O77698, BB)	715 (0.77)	46 (23)	20 (15)	79,733	2D	APP
	Serotransferrin (Q29443, BB)	68 (0.04)	6 (1)	2 (1)	79,870		APP
	Lactoperoxidase (P22079, HS)	67 (0.04)	3 (1)	2 (1)	81,149		Defence/Immunity
88	Lactoferrin (O77698, BB)	1177 (1.06)	84 (39)	25 (19)	79,733	2D	APP
	Lactoperoxidase (gi|27,806,851, BT)	43 (0.08)	9 (2)	6 (2)	81,504		Defence/Immunity
89	Serum Albumin (gi|1,351,907, BT)	2116 (2.28)	135 (79)	33 (28)	71,244	2D	APP
90	Ig G1 heavy chain constant region (gi|7,547,266, BT)	18 (0.0)	3 (0)	2 (0)	36,510	2D	Defence/Immunity
91	Lactadherin (gi|2,494,285, BT)	111 (0.14)	2 (2)	2 (2)	48,520	2D	Signaling, FTM

(*continued on next page*)

Table 3 (*continued*)

Band/spot number	Protein identification (Accession number, organism)	Protein MASCOT Score[a]	Matches[b]	Sequences[c]	Estimated MW (Da)	ID Method	Protein function
92	Angiotensinogen (gi|166,159,174, BT)	155 (0.22)	6 (5)	4 (3)	45,713	2D	Blood pressure, body fluid and
	Lactadherin (gi|2,494,285, BT)	58 (0.07)	5 (2)	4 (1)	48,520		electrolyte homeostasis
	Ig G1 heavy chain constant region (gi|7,547,266, BT)	16 (0.0)	4 (0)	1 (0)	36,510		Signaling, FTM Defence/Immunity
93	Nucleobindin-1 (gi|115,497,814, BT)	391 (0.64)	30 (15)	13 (9)	54,949	2D	Defence/Immunity
	Monocyte differentiation antigen CD14 (gi|3,913,217, BT)	267 (0.35)	13 (9)	5 (4)	40,269		Defence/Immunity
		239 (0.45)	12 (9)	6 (6)	49,329		Defence/Immunity
	Nucleobindin-2 (gi|115,496,067, BT)	102 (0.14)	5 (4)	3 (2)	47,200		Defence/Immunity
	Endopin 2B (Serpin A3–7) (gi|38,683,423, BT)						
94	Complement C3 (gi|99,028,969, BT)	210 (0.07)	16 (8)	8 (4)	188,652	2D	APP
	Cathepsin B (gi|27,806,671, BT)	111 (0.27)	4 (3)	4 (3)	37,664		Protease, Hydrolysis of proteins
	Actin, cytoplasmic 1 (P60712, BT)	53 (0.07)	5 (1)	5 (1)	42,052		Development, Structural
	Monocyte differentiation antigen CD14 (gi|3,913,217, BT)	37 (0.00)	3 (0)	1 (0)	40,269		Defence/Immunity
		36 (0.06)	2 (1)	2 (1)	49,329		Defence/Immunity
	Nucleobindin-2 (gi|115,496,067, BT)						
95	Actin, beta (gi|168,177,284, BT)	114 (0.15)	3 (3)	2 (2)	41,921	2D	Development, Structural
	Monocyte differentiation antigen CD14 (gi|3,913,217, BT)	101 (0.31)	2 (2)	1 (1)	40,269		Defence/Immunity
		98 (0.03)	5 (3)	3 (2)	188,675		APP
	Complement C3 (gi|99,028,969, BT)						
96	Complement C4 (gi|31,563,307, BT)	36 (0.11)	4 (1)	1 (1)	NA	2D	Defence/Immunity
97	Clusterin (gi|27,806,907, BT)	401 (0.51)	25 (15)	10 (7)	51,651	2D	HSP, Extracellular Chaperone
98	Clusterin (gi|27,806,907, BT)	263 (0.42)	16 (11)	17 (6)	51,651	2D	HSP, Extracellular Chaperone
99	α - enolase (gi|4,927,286, BT)	20 (0.00)	1 (0)	1 (0)	47,586	2D	Enzyme, Carbohydrate metabolism
100	Nucleobindin-1 (gi|115,497,814, BT)	81 (0.18)	6 (4)	4 (3)	54,949	2D	Defence/Immunity
101	Ig light chain (gi|92,096,965, BT)	21 (0.00)	2 (0)	2 (0)	24,863	2D	Defence/Immunity
102	Ig light chain (gi|92,096,965, BT)	88 (0.13)	6 (2)	2 (1)	24,863	2D	Defence/Immunity
103	Ig light chain (gi|2,323,374, BT)	68 (0.65)	5 (3)	3 (2)	11,560	2D	Defence/Immunity
104	Ig light chain (gi|2,323,374, BT)	221 (0.65)	9 (8)	3 (2)	11,560	2D	Defence/Immunity
105	Ig J chain (gi|32,401,410, BT)	49 (0.17)	3 (2)	2 (1)	18,359	2D	Defence/Immunity
106	Apolipoprotein A-I (P15497, BT)	85 (0.22)	6 (2)	5 (2)	30,258	2D	APP
107	Haptoglobin (gi|94,966,763, BT)	54 (0.07)	2 (1)	2 (1)	45,629	2D	APP
108	β - Lactoglobulin (P02755, BB)	1010 (1.38)	73 (37)	13 (6)	20,410	2D	Transport, Protein metabolism
109	α - Lactalbumin (Q9TSN6, BB)	348 (0.42)	14 (7)	2 (2)	16,720	2D	Carbohydrate metabolism, LB
110	Fatty acid-binding protein (gi|27,805,809, BT)	658 (1.71)	33 (21)	8 (5)	14,827	2D	FTM
111	α -1B-glycoprotein (gi|114,053,019, BT)	47 (0.06)	3 (1)	2 (1)	54,091	2D	Platelet activation, signaling and aggregation

BB, Bubalus bubalis; BT, Bos taurus; CEH, Cervus elaphus hispanicus; HS, Homo sapiens; NA, Not available; Ig, Immunoglobulin; APP, Acute phase protein; FTM, Fat transport/metabolism; HSP, Heat shock protein; LB, Lactose biosynthesis process; PSBF, Protein synthesis/binding/folding.

[a] The number in parenthesis indicates the Exponentially Modified Protein Abundance Index (emPAI).

[b] Total number of peptide matches. The number in parenthesis indicates the number of matches above the significance threshold ($p < 0.05$).

[c] Total number of distinct peptide sequences. The number in parenthesis indicates the number of matches above the significance threshold ($p < 0.05$).

used, in all samples, for 2-DE protein separation. Therefore, final volumes of 200 µl containing sample + isoelectric focusing rehydration/sample buffer (Bio Rad, 7 M urea, 2 M thiourea, 1% ASB-14, 40 mM Tris, 0.001% Bromophenol Blue) were loaded in 11 cm, pH 3–10 non-linear IPG strips (BioRad, Hemel Hempstead, UK) according to manufacturer's instructions.

Rehydration and Isoelectric Focusing: The prepared samples were then applied on a gel focusing tray to a pH 3–10 non-linear IPG strip (11 cm, Bio-Rad Lab, UK) and covered with 1 ml of mineral oil (Sigma-Aldrich, Dorset, UK). Active rehydration and then isoelectric focusing were carried out on a Bio-Rad Protean IEF cell using the following protocol: Focus temperature of 20 °C, with voltage intervals of: 1) 500 V for 1 h. 2) 1000 V for 1 h. 3) 2000 V for 2 h. 4) 4000 V for 4 h. 5) 8000 V for 12 h. Focused IPG strips were then used immediately for SDS-PAGE.

SDS-PAGE (4–15%T polyacrylamide gels): Equilibration buffer (EB) I was prepared by adding 0.5 g of DL dithiothreitol (DTT, Sigma-Aldrich) to 25 ml of previously prepared stock buffer. EB II was prepared by adding 0.625 g of iodoacetamide (Sigma-Aldrich) to 25 ml of previously prepared stock buffer. Stock buffer was prepared using 40 ml of deionized water, 2.27 g of tris (Trizma base, Sigma-Aldrich) adjusted for pH 8.8, 7.21 g of urea (Invitrogen), 10 ml of glycerol (Sigma-Aldrich) applied to the solution using plastic syringe, and 1.0 g of sodium dodecyl sulfate (SDS, Sigma-Aldrich). These volumes were used for 8 strips. After isoelectric focusing was performed, excess of mineral oil was gently removed from the strips by using blotting paper. Focused IPG strips were then incubated for 15 min in EB I with gentle shaking, washed in running buffer and incubated in EB II for another 15 min. Equilibrated IPG strips were then inserted horizontally on to the IPG well of the pre-cast IPG + 1 well comb (containing the 4–15%T polyacrylamide gel) which had been previously assembled in the gel running tank (Bio Rad, Criterion™ Vertical Electrophoresis Cell) containing running buffer (Bio Rad, 25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3). 10 µl of pre-stained protein ladder (10–170 kDa, PageRuler prestained protein ladder, Thermo Scientific Inc., USA) was added into the extra well. Electrophoresis was run at 200 V for 40–45 min at room temperature. Gels were removed from the gel cassette and then stained for 1–2 h in colloidal solution of Coomassie brilliant blue stain G-250 dye 0.1% (w/v), 10% (v/v) acetic acid, 40% (v/v) ethanol (Invitrogen, Manchester, UK) after which stain solution was discarded and destaining carried out overnight using a solution of 10% (v/v) acetic acid and 25% (v/v) methanol. Image of gels were scanned using a UMAX Power Look III scanner and software (Hamrick software, USA). Gel images were then processed and analyzed using SameSpot computer program (version 4.6, Totallab, UK), to highlight protein spots that showed significant and reproducible modulation between whey protein samples of interest.

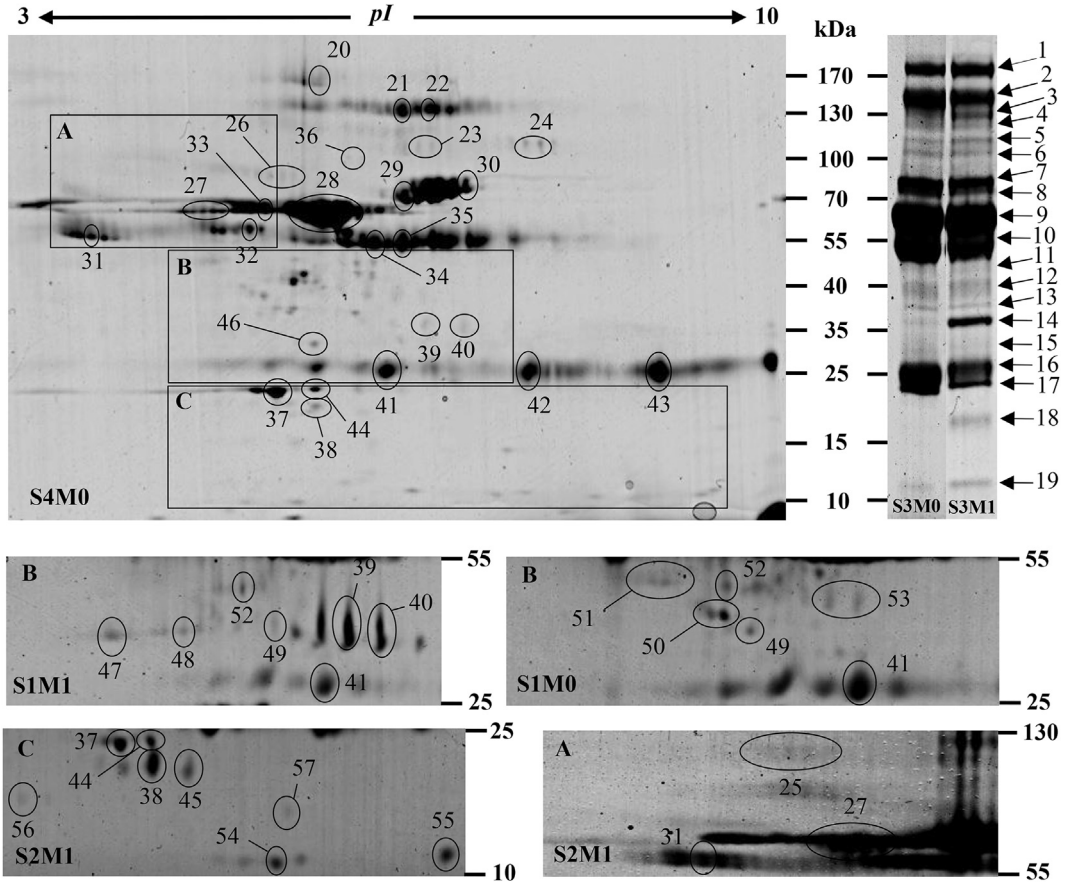

Fig. 1. Buffalo calve blood serum 1-DE reference map, represented by a healthy (S3 M0) and a *S.* Dublin infected (S3M1) animal, separated by SDS-PAGE (4–15%T precast polyacrylamide gel, 13.3 × 8.7 cm) and stained with Coomassie brilliant blue, showing the identification of 19 bands (numbers 1–19). Buffalo calve blood serum 2-DE reference map, represented by healthy (S1M0, S4M0) and *S.* Dublin infected (S1M1, S2M1) animals, separated by first dimension IEF (11 cm, pH 3–10 nonlinear IPG strip) followed by second dimension SDS-PAGE (4–15%T precast polyacrylamide gel, 13.3 × 8.7 cm) and stained with Coomassie brilliant blue, showing the identification of 38 spots (numbers 20–57). Bands and spots indicated numerically were excised and components were identified by LC/ESI-MS/MS following in-gel tryptic digestion. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.5. Trypsin digestion and LC/ESI-MS/MS

Enzymatic digestion of proteins selected from bands and spots from 1-DE and 2-DE and LC/ESI-MS/MS were performed in the Glasgow Polyomics Facility, Garscube Campus, University of Glasgow, UK.

Tryptic peptides were generated and extracted from gel pieces as previously described (Daneshvar et al., 2012), and were analyzed by LC/ESI-MS/MS (liquid chromatography coupled with electrospray ionization tandem mass spectrometry), using a Amazon ion trap instrument to produce MS and MS/MS data (Amazon speed ETD, Bruker Daltonics). The MS data obtained was processed using Data Analysis software (Bruker) and the automated Matrix Science Mascot Daemon server (v2.1.06). Protein identification was assigned using the Mascot search engine to interrogate protein sequences in the NCBI predicted protein database (Swissprot) restricting the search to Bos taurus and mammalian sequences, allowing a precursor mass tolerance and a fragment ion mass tolerance of 0.4 Da for both MS and MS/MS data. Also, trypsin was selected as cleavage enzyme, carbamidomethyl (C) as fixed modification and oxidation of methionine as variable modification.

2.6. Haptoglobin concentration in blood serum of buffalo calves

Haptoglobin concentrations were analyzed on a ABX Pentra 400 (Horiba ABX SAS, Montpellier, France) by using a hemoglobin binding method developed by Eckersall et al. (1999). Average concentrations between before inoculation (M0) (n = 3) and 72 h after inoculation (M1) (n = 3) were compared by unpaired *t*-test ($p < 0.05$) using GraphPad Prisma 5.

3. Results and discussion

3.1. Reference 1-DE and 2-DE maps

The great interest of this study was to identify potential disease related proteins, with emphasis on APP and defence/immunity related proteins, in blood serum of buffalo calves and in milk whey of lactating buffaloes, in order to define a reference electrophoresis map, both for 1-DE and 2-DE, as a prerequisite for future investigation of protein disease patterns. In this sense, the identification of these proteins in gels can help to address alterations during diseases in further studies and therefore must be highlighted in buffalo, since little is known about the proteome of this species.

For the construction of the protein maps, blood serum samples from healthy and *S.* Dublin infected buffalo calves were used, as well as healthy and mastitic milk whey samples (Table 1). Thus, healthy and diseased animal samples were used to find and identify the highest possible number of APP and defence/immunity-related proteins and therefore construct maps as complete as possible. In this context, it is also important to address that the use of 1-DE, although limited, is always an important tool for selecting samples, from a larger pool of samples, for further analysis by higher resolution methods such us 2-DE and gel-free LC-MS analyses, and this was the reason that construct maps a both 1-DE and 2-DE maps were constructed in this study.

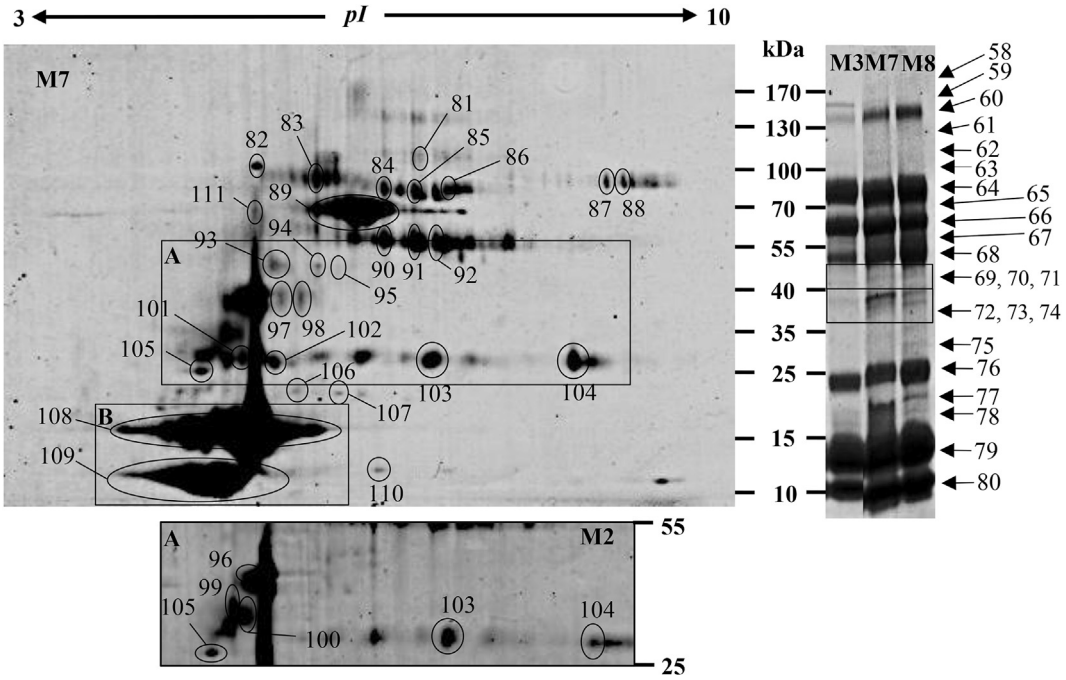

Fig. 2. Lactating buffalo milk whey 1-DE reference map, represented by a healthy (M3) and mastitic (M7,M8) samples, separated by SDS-PAGE (4–15%T precast polyacrylamide gel, 13.3 × 8.7 cm) and stained with Coomassie brilliant blue, showing the identification of 23 bands (numbers 58–80). Lactating buffalo milk whey 2-DE reference map, represented by healthy (M2) and mastitic (M7) samples, separated by first dimension IEF (11 cm, pH 3–10 nonlinear IPG strip) followed by second dimension SDS-PAGE (4–15%T precast polyacrylamide gel, 13.3 × 8.7 cm) and stained with Coomassie brilliant blue, showing the identification of 31 spots (numbers 81–111). Bands and spots indicated numerically were excised and components were identified by LC/ESI-MS/MS following in-gel tryptic digestion. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

For the construction of the 1-DE reference protein maps, a total of 19 bands from blood serum of buffalo calves and 23 bands from milk whey of lactating buffaloes were excised and analyzed by LC/ESI-MS/MS. After interrogating the MS data obtained with bovine and mammalian sequences in the NCBI predicted protein database, 27 different proteins were identified in the blood serum of buffalo calves, while 33 different proteins were identified in the milk whey of lactating buffaloes, and proteome maps were generated (Table 2, Table 3, Fig. 1, Fig. 2). Among these proteins, 12 APP and 13 defence\immunity related proteins were identified in the blood serum of buffalo calves, while 13 APP and 13 defence/immunity related proteins were identified in the milk whey of lactating buffaloes (Fig. 3a and b).

For the construction of the 2-DE reference protein maps, a total of 38 spots from blood serum of buffalo calves and 31 spots from milk whey of lactating buffaloes were excised and analyzed by LC/ESI-MS/MS, and 30 different proteins were identified in the blood serum of buffalo calves, while 28 different proteins were identified in the milk whey of lactating buffaloes, and therefore maps were generated (Table 2, Table 3, Fig. 1, Fig. 2). Among these proteins, 16 APP and 9 defence/immunity related proteins were identified in the blood serum of buffalo calves, while 6 APP and 11 defence/immunity related proteins were identified in the milk whey of lactating buffaloes (Fig. 3c and d).

As described, 1-DE and 2-DE allowed the identification of 27 and 30 different blood serum proteins, respectively. When comparing the two approaches, data show that 22 different proteins were identified by both techniques (Table 4, Fig. 4: 14 V to 35 V of the venn diagram), while 5 proteins where only identified by 1-DE (1 V to 5 V of the venn diagram) and 8 only by 2-DE (6 V to 13 V of the venn diagram). Therefore, by using a combined approach (1-DE + 2-DE), this study was able to identify 35 different blood serum proteins (1 V to 35 V of the venn diagram). In the same way, 1-DE and 2-DE allowed the identification of 33 and 28 different milk whey proteins, respectively. When comparing the two approaches, data show that 21 different proteins were identified by both techniques (55 V to 75 V of the venn diagram), while 12 proteins where only identified by 1-DE (36 V to 47 V of the venn diagram) and 7 only by 2-DE (48 V to 54 V of the venn diagram). Therefore, by using a combined approach (1-DE + 2-DE), this study was able to identify 40 different buffalo milk proteins (36 V to 75 V of the venn diagram). Among these proteins, important APP, defence/immunity related proteins (immunoglobulins, complement system related proteins, serpins, apolipoproteins), and proteins with other functions were identified (Fig. 3e and f) and are discussed below in relation to these protein classes.

The MS data obtained was compared with mammalian and *Bos taurus* sequences in the NCBI predicted protein database. Comparing MS data with mammalian sequences allowed us to identify 24 blood serum proteins and 22 milk whey proteins from *Bubalus bubalis* sequences. However, among all different proteins identified combining 1-DE + 2-DE (35 blood serum proteins and 40 milk whey proteins), 11 blood serum proteins and 18 milk whey proteins were not detected using *Bubalus bubalis* sequences in NCBI. Therefore, considering that the NCBI predicted protein database has many more *Bos taurus* protein sequences, we were able to identify several buffalo proteins by homology with *Bos taurus* sequences (Tables 2 and 3).

3.1.1. Identification of APP in blood serum

APP have been extensively studied in bovines for use in diagnosis, prognosis and in monitoring response to therapy, as well as in general health screening (Eckersall and Bell, 2010), and therefore is an important class of proteins to be explored in buffaloes. In this study, 16 different APP in blood serum from buffalo calves were detected by using a combined 1-DE and 2-DE approach, coupled with LC/ESI-MS/MS (Tables 2 and 4, Figs. 3E and 4). Among these proteins, 12 positive APP (haptoglobin, ceruloplasmin, SAA, SAA4, hemopexin, complement C3, α2-macroglobulin, ITIH4, plasminogen, α2-HS-glycoprotein, apolipoprotein A1 and α1-antitrypsin) and 4 negative APP (antithrombin III, serotransferrin, serum albumin and vitamin D binding protein) were

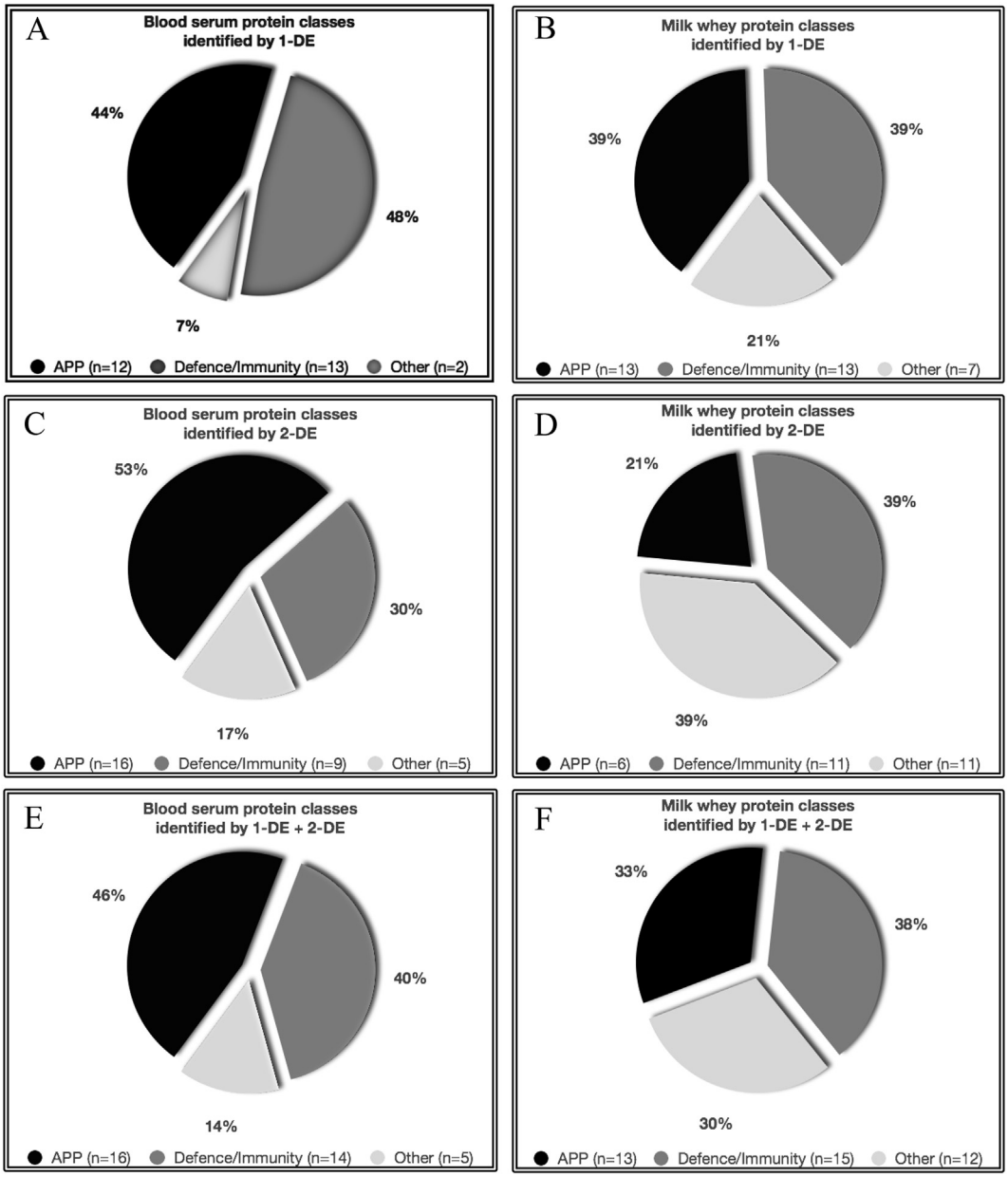

Fig. 3. Distribution into three different protein classes (APP, defence/immunity and others), of 38 proteins identified in buffalo calves blood serum 1-DE (A), of 30 proteins identified in lactating buffaloes milk whey 1-DE (B), of 30 proteins identified in buffalo calves blood serum 2-DE (C) and of 28 proteins identified in lactating buffaloes milk whey 2-DE (D). Also, distribution of 35 different proteins identified in buffalo calves blood serum considering 1-DE + 2-DE (E) and of 40 different proteins identified in lactating buffaloes milk whey considering 1-DE + 2-DE (F).

identified. All of these proteins were identified by 2-DE, while all except SAA, SAA4, hemopexin and antithrombin III were identified by 1-DE (Table 2, Figs. 3E and 4).

In relation to the proteins identified both by 1-DE and 2-DE, similar positions were observed in the gels by comparing the respective molecular weights (Fig. 1). For instance, haptoglobin was identified between 15 and 25 kDa (band 19, spot 38) and 35–40 kDa (band 14 and spots 39, 40, 47, 48), ceruloplasmin between 130 and 170 kDa (band 2 and spots 21, 22), complement C3 between 55 and 70 kDa (band 12, 13 and spots 49, 52, 53), α2-macroglobulin near 170 kDa (band 1 and spot 20), ITIH4 near 130 kDa (band 3 and spot 25), plasminogen between 100 and 130 kDa (band 6 and spot 24), α2-HS-glycoprotein near 55 kDa (band 10 and spot 31), apolipoprotein A1 between 15 and 25 kDa (band 17 and spots 37, 44), α1-antitrypsin between 55 and 70 kDa (band 9 and spot 32), serotransferrin between 70 and 100 kDa (bands 7, 8 and spots 29, 30), serum albumin between 55 and 70 kDa (band 9 and spot 28) and vitamin D binding protein near 55 kDa (band 10 and spot 32).

In relation to the APP haptoglobin, ceruloplasmin, complement C3, α2-macroglobulin, ITIH4, plasminogen, α2-HS-glycoprotein, apolipoprotein A1, α1-antitrypsin, serotransferin and serum albumin, identified by 2-DE in our study, similar molecular weights (MW) and isoelectric point (pI) were observed when comparing to other studies where 2-DE gels were performed in bovine blood serum/plasma using 7 cm IPG strips with pH range of 4.0–7.0 (Alonso-Fauste et al., 2012), 18 cm IPG strips with pH range of 3.5–10.0 (Talamo et al., 2003) and IPG strips with pH range of 4.0–10.0 (Wait et al., 2002).

Studies performed by Takahashi et al. (2009) in bovine serum samples using IPG strips with pH range of 3.0–10.0 identified 7 SAA isoforms ranging from pI 5.2–8.6 and MW of around 14 kDa. In our work, SAA was identified in similar position, below 15 kDa and in pI near 5.0 and 9.0.

Table 4
List of blood serum and milk whey proteins identified by 1-DE or 2-DE or by both techniques (1-DE and 2-DE) and that are disposed in the Venn diagram (Fig. 4).

ID Method	Venn diagram blood serum protein ID	Blood serum protein name	ID Method	Venn diagram milk whey protein ID	Milk whey protein name
1-DE		Defence/immunity	1-DE		APP
(n = 5)	1V	Complement C4-A	(n = 12)	36V	Ceruloplasmin
	2V	Complement C5a		37V	ITIH4
	3V	Complement C6		38V	LBP
	4V	Complement factor I		39V	Vitamin D-Binding Protein
	5V	Endopin 2B (Serpin A3–7)		40V	α-2-HS- glycoprotein
2-DE		APP		41V	α1-antitrypsin (Serpin A1)
(n = 8)	6V	Hemopexin		42V	α2-macroglobulin
	7V	Serpin C1 (Antithrombin-III)			Defence/Immunity
	8V	Serum amyloid A (SAA)		43V	Chitinase 3-like protein 1
	9V	Serum amyloid A4 (SAA4)		44V	Complement factor B
		Defence/immunity		45V	Thrombospodin-1
	10V	Complement factor H		46V	Xanthine dehydrogenase/oxidase
		Others			Others
	11V	Apolipoprotein A-IV		47V	Apolipoprotein A-IV
	12V	GFAP	2-DE		Defence/immunity
	13V	α-1B-Glycoprotein	(n = 7)	48V	Complement C4
1-DE		APP		49V	Nucleobindin-2
and	14V	Apolipoprotein A-I			Others
2-DE	15V	Ceruloplasmin		50V	Actin, cytoplasmic 1
(n = 22)	16V	Complement C3		51V	Cathepsin B
	17V	Haptoglobin		52V	Fatty acid-binding protein
	18V	ITIH4		53V	α - enolase
	19V	Plasminogen		54V	α -1B-glycoprotein
	20V	Serotransferrin	1-DE		APP
	21V	Serum Albumin	and	55V	Apolipoprotein A-I
	22V	Vitamin D-binding protein	2-DE	56V	Complement C3
	23V	α-2-HS-glycoprotein	(n = 21)	57V	Haptoglobin
	24V	α1-antitrypsin (Serpine A1)		58V	Lactoferrin
	25V	α2-macroglobulin		59V	Serotransferrin
		Defence/Immunity		60V	Serum Albumin
	26V	Complement factor B			Defence/Immunity
	27V	Endopin 1b (Serpin A3–3)		61V	CD14
	28V	Gelsolin isoform b		62V	Endopin 2B (Serpin A3–7)
	29V	Ig heavy chain variable region		63V	Gelsolin isoform b
	30V	Ig M heavy chain		64V	Ig G1 HC constant region/ Ig HC
	31V	Ig light chain		65V	Ig J Chain
	32V	Kininogen-2		66V	Ig light chain
	33V	Serpin A3–2		67V	IgM heavy chain constant region
		Others		68V	Lactoperoxidase
	34V	Adiponectin		69V	Nucleobindin-1
	35V	Hemoglobin			Others
	–	–		70V	Actin, beta
	–	–		71V	Angiotensinogen
	–	–		72V	Clusterin
	–	–		73V	Lactadherin
	–	–		74V	α - Lactalbumin
	–	–		75V	β - Lactoglobulin

3.1.2. Identification of APP in milk whey

One of the most important applications of measuring APP in bovine medicine is in detection and monitoring of bovine mastitis, an endemic disease of dairy cows which causes major economic losses (Ceciliani et al., 2012). In buffaloes, mastitis also can cause major economic losses, and studies with alterations in APP have shown increased concentrations of haptoglobin and serum amyloid A in the milk during mastitis (Kumar et al., 2014). In this study, 13 different APP in milk whey were detected by using a combined 1-DE and 2-DE approach, coupled with LC/ESI-MS/MS (Tables 3 and 4, Figs. 3F and 4). Among these proteins, 10 positive APP (haptoglobin, lactoferrin, complement C3, ceruloplasmin, α2-macroglobulin, ITIH4, α2-HS-glycoprotein, apolipoprotein A1, α1-antitrypsin and lipopolysaccharide-binding protein - LBP) were identified. Also, important negative blood APP, that act as positive APP in the mammary gland during mastitis (serotransferrin and serum albumin) and negative APP (vitamin D binding protein) were identified. All of these proteins were identified by 1-DE, while six were identified by 2-DE (haptoglobin, lactoferrin, complement C3, apolipoprotein A1, serotransferrin and serum albumin).

In relation to the proteins identified both by 1-DE and 2-DE, similar positions were observed in the gels by comparing the respective molecular weights (Fig. 2). Haptoglobin was identified between 15 and 25 kDa (band 77, spot 107), lactoferrin between 70 and 100 kDa (bands 63, 64 and spots 83, 84, 86, 87, 88), complement C3 between 40 and 55 kDa (band 71 and spots 94, 95), apolipoprotein A1 between 15 and 25 kDa (band 78 and spot 106), serotransferrin between 70 and 100 kDa (band 64 and spots 85, 86, 87) and serum albumin between 55 and 70 kDa (band 66 and spot 89).

Comparing our findings with studies performed with bovine milk whey (Alonso-Fauste et al., 2012; Smolenski et al., 2014), similar positions were observed in the gels for haptoglobin, identified between 15 and 25 kDa and near pI 6.0. According to Alonso-Fauste et al. (2012), the protein identified in this position is the α-haptoglobin fraction, since the β-haptoglobin tends to be identified between 35 and 40 kDa. In addition to haptoglobin, other important APP such as lactoferrin, complemente C3, apolipoprotein A1, serotransferrin and serum albumin have also been identified in similar positions in cattle, respectively, between 67 and 120 kDa and pI 7.0–9.0 (Smolenski et al., 2007; Alonso-Fauste et al., 2012), 45–60 kDa and pH 6.0 (Boehmer et al., 2008; Alonso-Fauste et al., 2012), 15–31 kDa and pI 6.0 (Smolenski

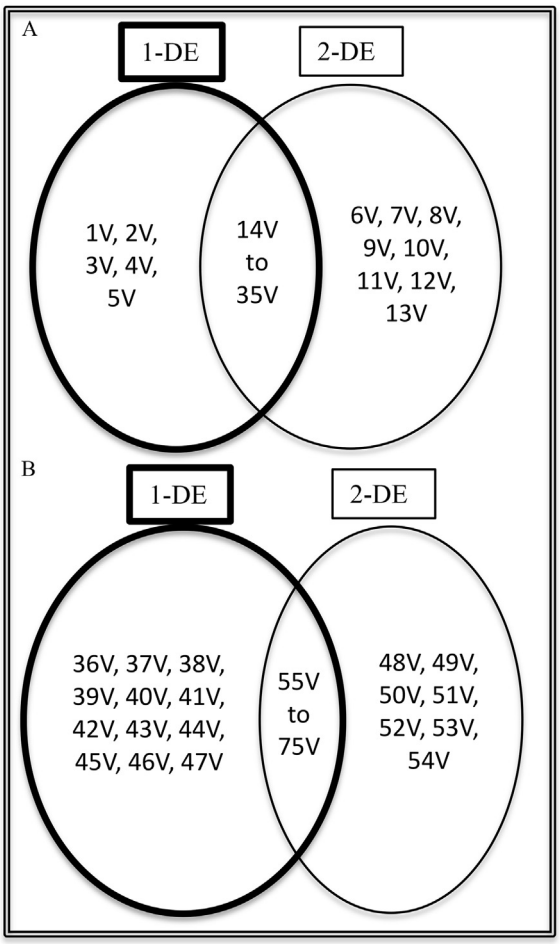

Fig. 4. Protein information from Table 4 is presented using a Venn diagram analysis. Panel A shows blood serum proteins only identified by 1-DE (left:1V to 5V) and only identified by 2-DE (right:6V to 13V), with common proteins in the center (14V to 35V). Panel B shows milk whey proteins only identified by 1-DE (left:36V to 47V) and only identified by 2-DE (right:48V to 54V), with common proteins in the center (55V to 75V).

et al., 2007; Boehmer et al., 2008; Alonso-Fauste et al., 2012), 50–75 kDa and pI 6.0–7.0 (Boehmer et al., 2008; Alonso-Fauste et al., 2012) and 70–100 kDa and pH 7.0–9.0 (Smolenski et al., 2007; Boehmer et al., 2008; Alonso-Fauste et al., 2012).

3.1.3. Identification of defence/immunity related proteins in the blood serum

In this study, 14 blood serum defence/immunity related proteins were detected by using a combined 1-DE and 2-DE approach, coupled with LC/ESI-MS/MS, and among this class of proteins, important immunoglobulins, proteins linked to the complement system and serpins were identified (Tables 2 and 4, Figs. 3E and 4).

In the blood serum of buffalo calves, 3 immunoglobulins (Ig M Heavy Chain, Ig Heavy Chain and Ig Light Chain) were identified by performing a combined 1-DE and 2-DE approach (Tables 2 and 4, Fig. 4). The 3 immunoglobulins were identified both by 1-DE and 2-DE (Table 2, Figs. 3E and 4), and similar positions were observed in the gels by comparing the respective molecular weights (Fig. 1). For instance, Ig M Heavy Chain was identified between 70 and 100 kDa (band 8, spot 26), Ig Heavy Chain near 55 kDa (band 10 and spots 34, 53) and Ig Light Chain near 25 kDa (band 16 and spots 41, 42, 43).

Although bovine and mammalian sequences in the NCBI predicted protein database did not allow us to identify Ig Heavy Chain (band 10 and spots 34, 53) and Ig Light Chain (band 16 and spots 41, 42, 43) as a specific immunoglobulin class, the position of these bands/spots in the gels, when compared to cattle (Alonso-Fauste et al., 2012) and sheep (Chiaradia et al., 2012) blood serum, gives a strong indication that these proteins are from the Ig G class. This is because in blood serum bovine 2-DE gels (7 cm IPG strips pH 4–7), Ig G1 Heavy Chain and Ig G2a Heavy Chain have been identified between 45 and 67 kDa (Alonso-Fauste et al., 2012), in very similar positions where Ig Heavy Chain was identified in our work (band 10 and spots 34 and 53, near to 55 kDa). Also, in blood serum sheep 2-DE gels (17 cm IPG strips pH 3–10), Ig Ga Lambda Chain has been identified near 55 kDa (Chiaradia et al., 2012), also in very similar positions where Ig Light Chain was identified in our work (band 16 and spots 41, 42 and 43, near to 55 kDa). Also, Ig M Heavy Chain, identified between 70 and 100 kDa (band 8, spot 26), has already been identified in similar position in bovine blood serum 2-DE, above and at left side of serum albumin (Wait et al., 2002) and between 67 and 120 kDa (Alonso-Fauste et al., 2012).

Table 5
Proteins from 1-DE and 2-DE differentially expressed, comparing blood serum samples from newborn buffalo-calves before inoculation (M0) and 72 h after inoculation with 10^8 CFU of S. Dublin (M1), and comparing milk whey samples from healthy buffaloes (G1) and from buffaloes with subclinical mastitis (G2).

Blood serum samples from buffalo calves				
Band/spot number	Protein identification (Accession number, organism)	ID method	Anova (p)[a]	Fold change (M1/M0)
14	Haptoglobin (gi|595,763,483, BT) - β-fraction	1-DE	0.0451	+19.8
39	Haptoglobin (gi|595,763,483, BB) - β-fraction	2-DE	0.0007	+6.2
40	Haptoglobin (gi|595,763,483, BB) - β-fraction	2-DE	0.0006	+6.3
18	Haptoglobin (gi|283,467,275, BB) - α-fraction	1-DE	< 0.0001	+39.0
38	Haptoglobin (gi|595,763,483, BB) - α-fraction	2-DE	0.0330	+5.4
45	Haptoglobin (gi|595,763,483, BB) - α-fraction	2-DE	0.0430	+4.3
–	Haptoglobin - Total	HBM	0.0028	+37.5
Milk whey samples from lactating buffaloes				
Band/Spot number	Protein identification (Accession number, organism)	ID method	Anova (p)[a]	Fold change (G2/G1)
76	Ig light chain, lambda gene cluster (gi|92,096,965, BB)	1-DE	0.0290	+5.6
103	Ig light chain (gi|2,323,374, BT)	2-DE	0.0133	+1.7
104	Ig light chain (gi|2,323,374, BT)	2-DE	0.0124	+1.9
79	β - Lactoglobulin (gi|6,729,725, BT)	1-DE	0.0298	−1.3
108	β - Lactoglobulin (P02755, BB)	2-DE	< 0.0001	−2.5
80	α - Lactalbumin (gi|68, BT)	1-DE	0.0118	−1.4
109	α - Lactalbumin (Q9TSN6, BB)	2-DE	0.0002	−3.0

Ig, Immunoglobulin.
Hemoglobin Binding Method (HBM): Based on the method of Eckersall et al. (1999).
[a] For 1-DE and HBM: Statistical difference pointed by unpaired *t*-test ($p < 0.05$) using GraphPad Prisma 5. For 2-DE: Analyzed using SameSpot computer program (version 4.6, Totallab, UK).

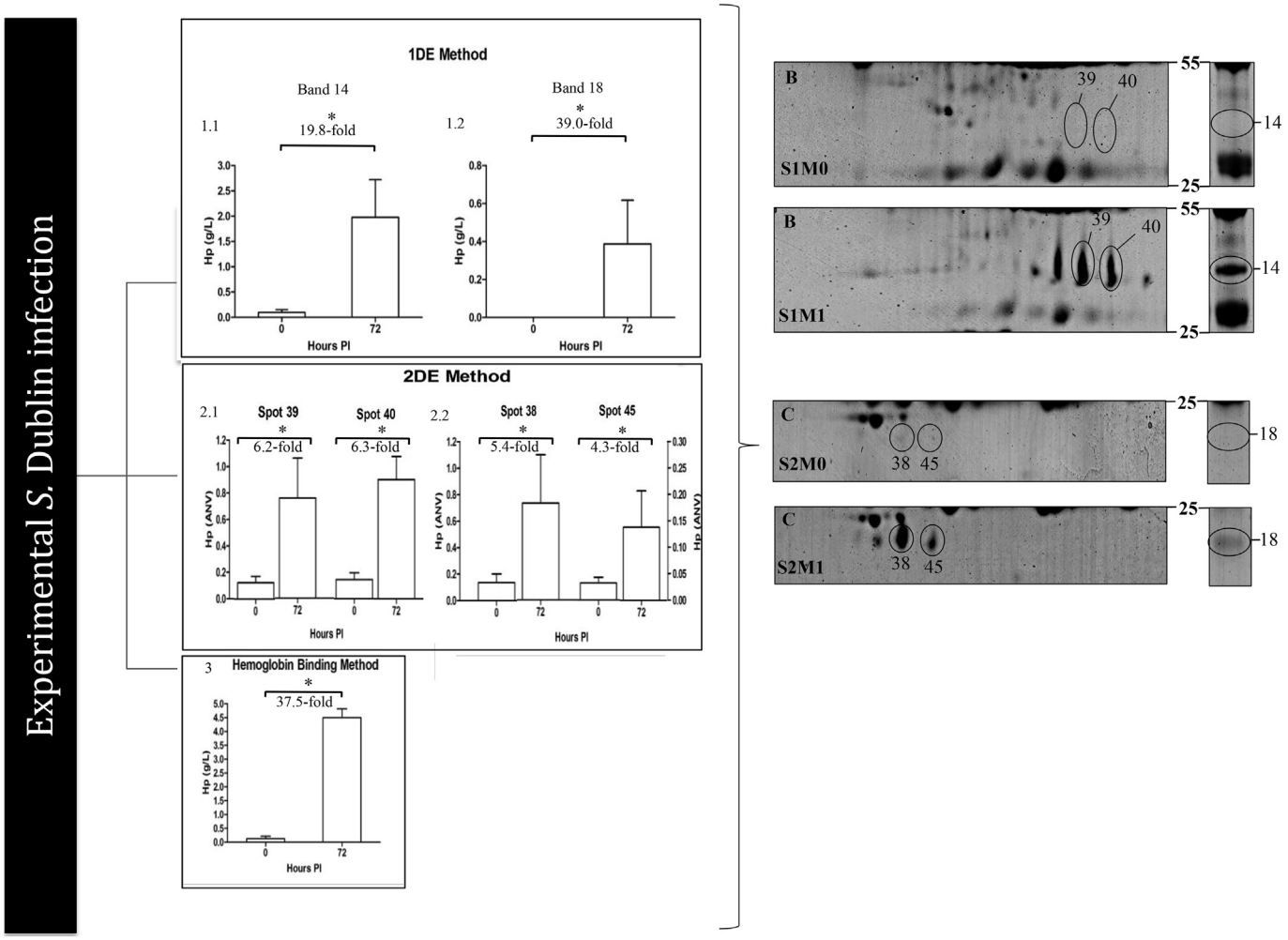

Fig. 5. Expanded 1-DE and 2-DE maps of blood serum (right side) from a healthy (S1M0) and 72 h post-inoculation (PI) *S.* Dublin infected buffalo calve (S1M1) showing alterations in intensity of spots (39,40) and band (14) identified as haptoglobin, and that were differentially expressed during salmonellosis as showed in grafics (left side: 1.1, 2.1). Expanded 2-DE maps from a healthy (S2M0) and 72 h post-inoculation *S.* Dublin infected buffalo calve (S2M1) showing alterations in intensity of spots (38,45) and band (18) identified as haptoglobin, that was differentially expressed during salmonellosis as showed in grafics (left side: Panel 1.2, 2.2). Panel 3 shows haptoglobin average concentrations, measured by hemoglobin binding method, comparing healthy (n = 3, M0) and 72 h post-inoculation *S.* Dublin infected buffalo calves (n = 3, M1). * statistically significant ($p < 0.05$). The displayed area corresponds to box B and C in Fig. 1. Informations on bands/spots ID are in Table 1. ANV = Average Normalized Volume × 10^6.

There are three pathways of complement activation, the classical, the alternative and the lectin pathways. All three pathways are activated according to a cascade system, with activation of one factor leading to the activation of the next (Ballanti et al., 2011). In this study, 7 different blood serum complement factors (C3, C4a, C5a, C6, B, H and I) were detected by using a combined 1-DE and 2-DE approach (Tables 2 and 4, Fig. 4), and are known to have participation in the classical, alternative and lectin pathways of complement activation. All of these proteins, except complement factor H, were identified by 1-DE, while three were identified by 2-DE (C3, B and H) (Table 2, Figs. 3E and 4).

In relation to the two proteins identified both by 1-DE and 2-DE (C3 and B), similar positions were observed in the gels by comparing the respective molecular weights (Fig. 1). Complement C3 was identified between 40 and 55 kDa (band 71 and spots 94, 95), while complement factor B was identified between 100 and 130 kDa (band 5 and spot 23).

The complement factors C3, B and H, identified by 2-DE in our work, have also been identified in bovine blood serum (Wait et al., 2002; Talamo et al., 2003; Alonso-Fauste et al., 2012; Turk et al., 2012), in similar molecular weights (MW) and isoelectric point (pI). For instance, complement factor H has been identified in bovines at high MW, above serum albumin and at pI of approximately 6.0 (Turk et al., 2012), same position as identified in our work (near 170 kDa and at pI of approximately 6.0).

Serpins (serine protease inhibitors) are the largest and most broadly distributed superfamily of protease inhibitors (Irving et al., 2000). In bovines, serpin A3–8 has already been shown to increase in milk whey of cows challenged with *Streptococcus uberis*, where concentrations were 20, 158, 246, 283 and 33 times higher at 36, 42, 57, 81 and 312 h post-challenge, respectively, when comparing to before challenge (Mudaliar et al., 2016). In this study, 4 different blood serum serpins related to defence/immunity (Serpin A3-2, Serpin A1/α1-antitrypsin, Endopin 1b and Endopin 2B) were detected by using 1-DE, while 2-DE approach was not able to detect only Endopin 2B (Tables 2 and 4, Figs. 3E and 4). In relation to the three proteins identified both by 1-DE and 2-DE (Serpin A3-2, Serpin A1/α1-antitrypsin and Endopin 1b), Serpin A3-2 and Endopin 1b were identified near 130 kDa (band 3 and spot 25), while Serpin A1/α1-antitrypsin was identified between 55 and 70 kDa (band 9 and spot 32) (Fig. 1).

When comparing with studies performed with the 2-DE approach with bovine samples, Serpin A1/α1-antitrypsin has also been identified in bovine blood serum (Wait et al., 2002; Talamo et al., 2003; Alonso-Fauste et al., 2012; Turk et al., 2012) in similar molecular weights

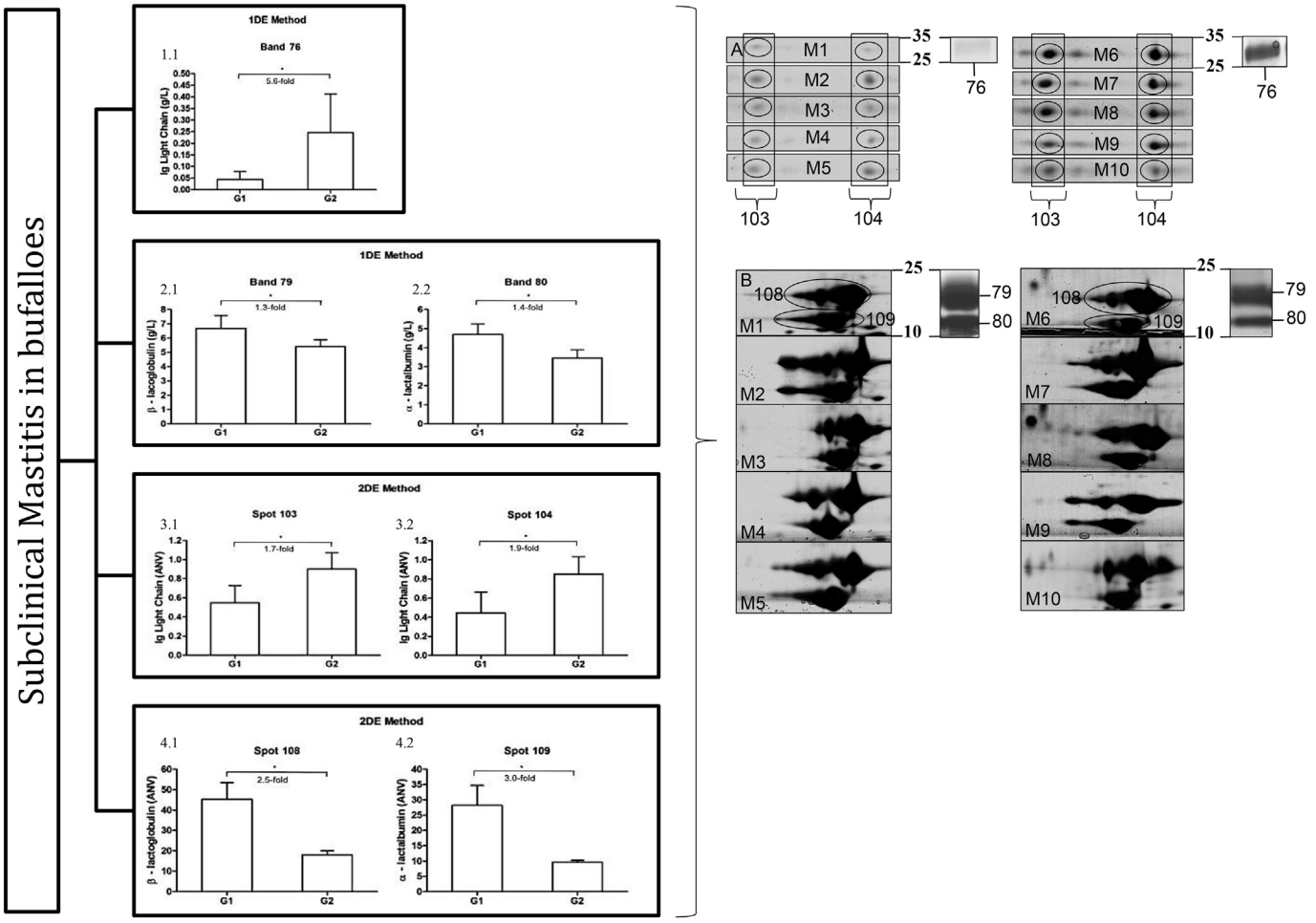

Fig. 6. Expanded 1-DE and 2-DE maps (right side) from healthy (M1, M2, M3, M4, M5) and mastitic (M6, M7, M8, M9, M10) milk whey samples showing alterations in intensity of spots (103,104) and band (76) identified as Ig Light Chain, and that were differentially expressed during mastitis as showed in grafics (left side: Panel 1.1, 3.1, 3.2). Expanded 2-DE maps from healthy (M1 to M5) and mastitic (M6 to M10) milk whey samples showing alterations in intensity of spots (108,109) and bands (79,80) identified as β-lactoglobulin and α-lactalbumin, respectively, that were differentially expressed during mastitis as showed in grafics (left side: Panel 2.1, 2.2, 4.1, 4.2). * statistically significant ($p < 0.05$). The displayed area corresponds to box A and B in Fig. 2.Information on bands/spots ID are in Table 2. ANV = Average Normalized Volume × 10^6.

(MW) and isoelectric point (pI) then those identified in our work with buffaloes. However, although endopin 1b has been identified in similar pI (around 5.0) when comparing to bovine (Alonso-Fauste et al., 2012), this protein was identified in higher MW in our work, being near to 130 kDa, against 45–67 kDa in bovine as described by Alonso-Fauste et al. (2012).

3.1.4. Identification of defence/immunity related proteins in the milk whey

In this study, 15 milk whey defence/immunity related proteins were detected by using a combined 1-DE and 2-DE approach, coupled with LC/ESI-MS/MS, and among this class of proteins, important immunoglobulins, complement system proteins and serpins were identified (Tables 3 and 4, Figs. 3F and 4).

Ig M and Ig G are vital immunoglobulins for the complete function of humoral immunity and are also essential for the activation of the classical pathway of complement system (Nauta et al., 2004). In this context, 4 immunoglobulins (Ig M Heavy Chain, Ig Heavy Chain/Ig G1 Heavy Chain, Ig Light Chain and Ig J Chain) and 3 complement factors (C3, C4 and B) were identified in milk whey by using 1-DE and 2-DE.The immunoglobulins and complement factor C3 were identified both by 1-DE and 2-DE, while complement factors C4 was only identified by 2-DE and complement factor B was only identified by 1-DE (Tables 3 and 4, Figs. 3F and 4).

Ig M Heavy Chain was identified between 70 and 100 kDa (band 64, spots 82, 84), Ig Heavy Chain/Ig G1 Heavy Chain near 55 kDa (band 68 and spots 90, 92), Ig Light Chain near 25 kDa (band 76 and spots 101, 102, 103, 104) and Ig J Chain between 15 and 25 kDa (band 78, spot 105) (Fig. 2), and were found to be in similar positions when compared to the described in bovine milk whey 2-DE gels using 7 cm IPG strips pH 4–7 for first dimension (Alonso-Fauste et al., 2012) and 18 cm IPG strips pH 3–10 (Fong et al., 2008).

Complement factor C3 was identified between 40 and 55 kDa (band 71 and spots 94, 95) (described in Section 3.1.2) and between 55 and 70 kDa (bands 65, 66). In bovine milk whey, C3 was also found in similar position in 2-DE gels, between 37 and 75 kDa, using 11 cm IPG strips pH 3–10 (Boehmer et al., 2008), 18 cm IPG strips pH 3–10 (Fong et al., 2008) and 7 cm IPG strips pH 4–7 (Alonso-Fauste et al., 2012), but also was identified between 75 and 100 kDa (Fong et al., 2008). Complement factor C4 was identified between 35 and 40 kDa (spot 96), in different positions than identified by Fong et al. (2008) (75–100 kDa) and Boehmer et al. (2008) (20–25 kDa). These results related to C3 and C4 are expected since literature shows, by 2-DE and western blotting analysis, that these proteins can be identified in many positions in human blood plasma 2-DE gels (Michlmayr et al., 2010).

3.1.5. Identification of proteins with other functions in blood serum and milk whey

Other important proteins, related to protein, carbohydrate and lipid

metabolism, development, transport, signaling, etc., were identified in blood serum and/or milk whey in 1-DE and 2-DE gels, and are named in Tables 2 and 3. In particular, especial attention is given to the high abundance proteins β-lactoglobulin and α-lactalbumin, identified in the milk whey, and serum albumin (discussed in sessions Section 3.1.1 and Section 3.1.2), identified in the blood serum and milk whey.

Milk protein accounts for approximately 3.2 to 3.8% of milk components, consisting of about 80% caseins and 20% whey proteins with major components being α-lactalbumin and β-lactoglobulin (Gellrich et al., 2014). Therefore, β-lactoglobulin and α-lactalbumin account for about 45% and 15% of milk whey proteins (De Marchi et al., 2009) and are considered high abundance proteins in the milk whey. In this study, milk whey β-lactoglobulin and α-lactalbumin were identified both by 1-DE and 2-DE approach (Tables 3 and 4, Figs. 3F and 4), and presented similar positions in the gels by comparing the respective molecular weights (Fig. 2), and were identified between 10 and 15 kDa (β-lactoglobulin: band 79 and spot 108; α-lactalbumin: band 80 and spot 109).

Comparing to studies performed with bovine 2-DE approach (Smolenski et al., 2007; Boehmer et al., 2008; Fong et al., 2008; Alonso-Fauste et al., 2012), β-lactoglobulin and α-lactalbumin were in similar positions in the gels, between 10 and 25 kDa and at a acidic pI.

3.2. Changes in protein patterns during salmonellosis and mastitis

Milk whey and blood serum are complex mixtures of a few high abundance proteins and a wide selection of low abundance protein components that could be utilized for diagnosis and prognosis purposes. In this sense, the use of proteomic methodologies to obtain a more complete and unbiased characterization of host responses during infections could lead to the identification of a pattern of biomarkers indicative of diseases (Ferreira et al., 2013).

Therefore, additionally from defining a reference map for 1-DE and 2-DE, examples of changes in protein patterns in the blood serum of buffalo calves during salmonellosis (Table 5, Fig. 5) and milk whey of lactating buffaloes during mastitis (Table 5, Fig. 6), to identify possible applications of proteins identified in the reference maps produced in our work, are also presented in this study.

3.2.1. Changes in protein patterns during salmonellosis

The first month of life is critical for newborns buffalo calves and morbidity and mortality rates are high, especially due to diarrhea and pneumonia (Khan et al., 2009; Anwarullah et al., 2014; Naag et al., 2015). In this context, Salmonella spp. have been reported worldwide in buffalo calves, associated with diarrhea and gastroenteritis (Borrielo et al., 2012; Anwarullah et al., 2014). Therefore, considering the importance of salmonellosis in buffalo calves, we have chosen to show some alterations in expression patterns of β-haptoglobin and α-haptoglobin fractions linked to this disease.

Experimental infection using 10^8 CFU of S. Dublin was able to promote hyperthermia and diarrhea 72 h after inoculation, two important clinical signs of salmonellosis. Also, *S.* Dublin was isolated in the feces 72 h post-infection, confirming that the experimental infection was effective (Table 1). Expression patterns of β-haptoglobin (spots 39 and 40, band 14) and α-haptoglobin fractions (spots 38 and 45, band 18) comparing blood samples from buffalo calves experimentally infected with *S.* Dublin before (M0) and 72 h after inoculation (M1) were analyzed, and results showed that the expression of these two fractions were significantly up-regulated 72 h post-infection (M1), in both 1-DE and 2-DE analysis (Table 5, Fig. 5). Moreover, these patterns were validated by performing a hemoglobin binding method (Eckersall et al., 1999) to measure haptoglobin concentrations in the blood serum, which also showed significantly higher concentrations of this protein 72 h post-infection (Table 5, Fig. 5).

Increased concentrations of haptoglobin in *S. Dublin* infected buffalo calves presented in our work were expected, since major increase of this important APP have already been shown in the blood serum of buffalo calves during *S. Typhimurium* experimental infection (Clemente et al., 2016) and in 3 to 11 month old buffaloes naturally infected with dermatophytosis (Kabu and Sayin, 2016), among other studies involving adult buffaloes, where blood serum concentrations of haptoglobin in animals with traumatic reticuloperitonitis (El-Ashker et al., 2013) and animals experimentally infected with *Pasteurella multocida* (Horadagoda et al., 2001, 2002) were also increased.

3.2.2. Changes in protein patterns during mastitis

Studies have shown that the prevalence rate of subclinical mastitis in lactating buffaloes can be high (Costa et al., 2000; Dhakal, 2006; Sharif and Ahmad, 2007), and therefore reliable and early detection of subclinical mastitis is necessary for disease control and monitoring of milk quality (Pyörälä et al., 2011).

Therefore, since mastitis affects lactating buffaloes and can contribute to lower milk productivity, alterations in expression patterns of immunoglobulin light chain (IgLC) (spots 103 and 104, band 76) and β-lactoglobulin (spot 108, band 79) and α-lactalbumin (spot 109, band 80) comparing samples from healthy buffaloes (G1) and buffaloes with subclinical mastitis (G2) were analyzed in our work. Results showed that, both in 1-DE and 2-DE analysis, the expression of band/spots identified as IgLC were significantly up-regulated during mastitis (G2), while the expression of band/spots identified as β-lactoglobulin and α-lactalbumin were significantly down-regulated during mastitis (G2) (Table 5, Fig. 6). β-lactoglobulin and α-lactalbumin have already been described as decreased in bovines during mastitis (Hogarth et al., 2004; Boehmer et al., 2008; Smolenski et al., 2014) due to mammary gland tissue destruction (Hogarth et al., 2004; Pyörälä, 2003), which is expected since these proteins are produced in the mammary gland tissue.

3.3. Final remarks

A combined approach based on 1-DE and 2-DE coupled with LC/ESI-MS/MS resulted in a blood serum protein map with 37 different identified proteins in buffalo-calves and in a milk whey protein map with 42 different identified proteins in lactating buffaloes. Therefore, the establishment of these protein maps using 1-DE and 2-DE technique has led to the identification of important disease-related proteins that will help to address alterations during diseases and other physiological conditions in buffaloes.

Additionally, significant changes in patterns of haptoglobin were observed in buffalo calves with salmonellosis, while significant changes in patterns of immunoglobulin light chain, β-lactoglobulin and α-lactalbumin where observed in lactating buffaloes during mastitis. This shows the importance of studying protein alterations during diseases and highlighting the potential of 1-DE and 2-DE technique to identify pattern of biomarkers indicative of diseases during host responses.

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