

Shotgun Proteomic Analysis of Plasma from Dairy Cattle Suffering from Footrot: Characterization of Potential Disease-Associated Factors

Dongbo Sun^{1,2*}, Hong Zhang¹, Donghua Guo¹, Anguo Sun³, Hongbin Wang^{2*}

1 College of Animal Science and Veterinary Medicine, Heilongjiang Bayi Agricultural University, Daqing High-tech Industrial Development Zone, Daqing, P. R. China, **2** Post-doctoral Mobile Station of Veterinary Medicine, Northeast Agricultural University, Harbin, P. R. China, **3** Research Center for Proteome Analysis, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, P. R. China

Abstract

The plasma proteome of healthy dairy cattle and those with footrot was investigated using a shotgun LC-MS/MS approach. In total, 648 proteins were identified in healthy plasma samples, of which 234 were non-redundant proteins and 123 were high-confidence proteins; 712 proteins were identified from footrot plasma samples, of which 272 were non-redundant proteins and 138 were high-confidence proteins. The high-confidence proteins showed significant differences between healthy and footrot plasma samples in molecular weight, isoelectric points and the Gene Ontology categories. 22 proteins were found that may differentiate between the two sets of plasma proteins, of which 16 potential differential expression (PDE) proteins from footrot plasma involved in immunoglobulins, innate immune recognition molecules, acute phase proteins, regulatory proteins, and cell adhesion and cytoskeletal proteins; 6 PDE proteins from healthy plasma involved in regulatory proteins, cytoskeletal proteins and coagulation factors. Of these PDE proteins, haptoglobin, SERPINA10 protein, afamin precursor, haptoglobin precursor, apolipoprotein D, predicted peptidoglycan recognition protein L (PGRP-L) and keratan sulfate proteoglycan (KS-PG) were suggested to be potential footrot-associated factors. The PDE proteins PGRP-L and KS-PG were highlighted as potential biomarkers of footrot in cattle. The resulting protein lists and potential differentially expressed proteins may provide valuable information to increase understanding of plasma protein profiles in cattle and to assist studies of footrot-associated factors.

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* E-mail: dongbosun@yahoo.com.cn (DS); hbwang@neau.edu.cn (HW)

Introduction

Footrot is an acute and highly infectious disease of cattle that develops between the claws of the hoof and is caused by the Gram-negative anaerobic bacterium *Fusobacterium necrophorum*, which is present in the rumen and feces of normal cattle and their environment [1–3]. The disease is characterized by the presence of an interdigital lesion, swelling, moderate to severe lameness, and a separation of horny portions of the hoof from the sensitive tissues underneath. It has a serious impact on the production performance of diseased cattle, especially in dairy cattle. Since footrot was first reported by Adams in the Netherlands in 1960, many treatment and preventive measures had been developed for its control [4–6]. However, the disease is common in many cattle-raising countries, with incidence rates that vary from 10% to 25%.

Plasma is an amorphous and important component of blood and changes in the quantity and quality of plasma proteins are associated with physiological or pathological states in humans and other animals [7,8]. Therefore plasma is an ongoing focus of research for elucidation of disease-associated factors [9–11]. The serum acute-phase protein haptoglobin has been reported to be a

marker of inflammation in dairy cattle suffering from diseases of the hoof [12]. Additionally, the concentrations of serum sialic acids, inflammatory mediators and acute phase proteins have been proven to be significantly raised in lame cattle with interdigital dermatitis [13]. However, the plasma protein profiles of cattle with footrot are not fully understood, and there are still a great many unknown potential disease-associated proteins.

Proteomics techniques are an effective tool for characterization of protein profiles in plasma/serum samples and have been used widely to search for disease-associated factors and biomarkers [14–17]. Among current proteomics methods, the shotgun proteomics approach possesses the virtues of high efficiency, and time and labor savings, when compared with the two-dimensional electrophoresis (2-DE) combined with mass spectrometry (MS), and it is suitable for use as a high throughput technology for the identification of proteins in plasma or serum samples [18–22]. In this study, proteomic analysis of plasma proteins from dairy cattle with footrot and healthy cattle was performed using the shotgun proteomics approach based on liquid chromatography and tandem mass spectrometry (LC-MS/MS). Furthermore, we characterized the plasma protein profiles of healthy dairy cattle

and those with footrot, and analyzed and verified potential footrot-associated factors or biomarkers. Our aim was to add basic information to increase understanding and the effective control of footrot in dairy cattle.

Materials and Methods

Ethics Statement

The animal experiments were approved by the Institutional Animal Care and Use Committee of Northeast Agricultural University, under the approved protocol number SRM-06.

Preparation of Plasma Specimens

Eleven plasma specimens from diseased cattle were collected from a Holstein dairy herd that was suffering from an outbreak of footrot in the Daqing area of Heilongjiang Province, northeast China, in 2011. The blood samples (about 5 mL) from each cow were collected from the caudal vein into the evacuated blood collection tubes with anticoagulant according to the BD protocol. The diseased dairy cattle showed typical swelling of the skin between the claws of the hoof, which is one of the characteristic clinical signs of footrot; in hoof swabs of footrot-affected dairy cattle, the presence of the *lktA* gene of *F. necrophorum* was confirmed by PCR methods. After centrifugation at 3000×g for 8 min at 4°C, the resulting plasma specimens were centrifuged for a second time at 12000×g for 5 min at 4°C. Equal volumes of the 11 diseased plasma specimens were combined to form a pooled plasma sample, and 11 healthy plasma specimens from unaffected cattle in the affected dairy herd were pooled using the same procedure. After determination of the total protein concentration using Bradford's method, according to the manufacturer's instructions (Invitrogen, Carlsbad, CA), the two pooled plasma specimens, footrot and healthy, were stored at −80°C.

SDS-PAGE Separation of Plasma Proteins

One hundred micrograms of protein from each plasma specimen was denatured at 100°C for 5 min in an equal volume of 2× protein loading buffer (0.1 M Tris buffer, pH 6.8, 4% SDS, 0.2% β-mercaptoethanol, 40% glycerol, and 0.002% bromophenol blue). The denatured plasma specimens were separated by 12.5% polyacrylamide gel electrophoresis (SDS-PAGE) in Tris-glycine-SDS buffer (10 mM Tris, 50 mM glycine, 0.1% SDS, pH 8.0) at 15 mA for 20 min and then 30 mA for 1.5 h in a mini-vertical electrophoresis system. The gels were then stained with Coomassie Brilliant Blue G250 (Invitrogen, Carlsbad, CA). The protein lane of each specimen was cut into four equal pieces.

In-Gel Trypsin Digestion

The separated gel pieces for each specimen were destained with 30% ACN/100 mM NH₄HCO₃, and the destained gels were dried in a vacuum centrifuge. The in-gel proteins were reduced with dithiothreitol (10 mM DTT/100 mM NH₄HCO₃) for 30 min at 56°C, and subsequently alkylated with iodoacetamide (50 mM IAA/100 mM NH₄HCO₃) in the dark at room temperature for 30 min. The gel pieces were rinsed briefly with 100 mM NH₄HCO₃ and ACN, respectively. The gel pieces were digested overnight in 12.5 ng/mL trypsin in 25 mM NH₄HCO₃. The peptides were extracted three times with 60% ACN/0.1% TFA. The extracts were pooled and dried completely using a vacuum centrifuge.

Liquid Chromatography and Tandem Mass Spectrometry (LC–MS/MS)

The Ettan™ MDLC system (GE Healthcare) was used for desalting and separation of the tryptic peptide mixtures. In this system, samples were desalted on RP trap columns (Zorbax 300 SB C18, Agilent Technologies), and separated on a RP column (150 μm i.d., 100 mm length, Column technology Inc., Fremont, CA). Mobile phase A (0.1% formic acid in HPLC-grade water) and mobile phase B (0.1% formic acid in acetonitrile) were selected. Subsequently, 20 μg of each tryptic peptide mixture was loaded onto the column, and separation was performed at a flow rate of 2 μL/min using a linear gradient of 4–50% B for 60 min. An LTQ Velos (Finnigan, San Jose, CA), equipped with an electrospray interface, was connected to the LC setup for detection of the eluted peptides. Data-dependent MS/MS spectra were obtained simultaneously. Each scan cycle consisted of one full MS scan in profile mode followed by 20 MS/MS scans in centroid mode, with the following Dynamic Exclusion™ settings: repeat count 2, repeat duration 30 s, exclusion duration 90 s.

Protein Identification

The acquired MS/MS spectra were searched automatically against the protein database for Bovidae proteins in NCBI using the BioworksBrowser rev. 3.1 (Thermo Electron, San Jose, CA). The protein identification results were extracted from SEQUEST outfiles with BuildSummary which combined the peptide sequences into proteins and deleted redundant proteins [23]. The peptides were constrained to be tryptic, and up to two missed cleavages were allowed. Carbamidomethylation of cysteines was treated as a fixed modification, whereas oxidation of methionine residues was considered as a variable modification. The mass tolerance allowed for the precursor ions was 2.0 Da and that for the fragment ions was 0.8 Da. The protein identification criteria were based on Delta CN (≥0.1) and cross-correlation scores (Xcorr, one charge ≥1.9, two charges ≥2.2, three charges ≥3.75). The high-confidence proteins were determined by the standard of unique peptide count >or= 2 or a unique peptide count = 1 but total count >or= 4. Gene Ontology (GO) categories of the high-confidence proteins were performed with the DAVID web tool available at the website <http://david.abcc.ncifcrf.gov/> according to the protein geninfo identifier (GI) accession numbers [24,25].

ELISA

The concentrations of immunoglobulin G (IgG) in the plasma samples of both healthy dairy cattle and those affected by footrot were detected by Bovine IgG ELISA kit (Xinyue Biotechnology Co., Ltd., Shanghai, China) according to the manufacturer's instructions, respectively. Briefly, 40 μL of the plasma samples (1:50 dilution in PBS) of both healthy and footrot-affected dairy cattle was added to wells of ELISA plate coated by monoclonal antibody (McAb) against Bovine IgGs, respectively, and then 10 μL of biotin-labeled McAb against Bovine IgGs and 50 μL of streptavidin-HRP conjugates were added to the wells of ELISA plate, respectively. After incubation at 37°C for 1 h, the ELISA plate was washed three times using PBST (0.5% (v/v) Tween-20, PBS, pH 7.4). Color development was carried out using TMB solution as the substrate, and the reaction was stopped with 50 μL of 2 M H₂SO₄. The absorbance at 450 nm was measured. In ELISA, Bovine IgGs standard (320 μg/mL, 160 μg/mL, 80 μg/mL, 40 μg/mL, and 20 μg/mL) was used to prepare a standard curve according to the ELISA procedure described above. IgG concentrations of the plasma samples from healthy dairy cattle and those affected by footrot were calculated according to the standard

curve of Bovine IgGs standard. Data were analyzed using a two-tailed, paired Student's *t* test in the Microsoft Excel 2007 Windows software.

Results

Identification of Plasma Proteins

The plasma proteins of healthy dairy cattle and those with footrot were separated by SDS-PAGE, respectively, and each separated gel was cut into four pieces that were equal in size (Fig. 1). After in-gel trypsin digestion, the plasma proteins from healthy and footrot-affected dairy cattle were analyzed using the shotgun LC-MS/MS proteomics technique. A total of 648 proteins and 712 proteins were identified from plasma samples of healthy dairy cattle and those with footrot, respectively (Table 1). Of the 648 proteins from healthy dairy cattle, the numbers of non-redundant proteins and high-confidence proteins were 234 (36.11%) and 123 (18.98%), respectively. Of the 712 proteins from footrot-affected dairy cattle, the numbers of non-redundant proteins and high-confidence proteins were 272 (38.20%) and 138 (19.38%), respectively. Between the two plasma specimens, a total of 22 potential differentially expressed proteins were found, of which 16 proteins (2.24%) occurred in footrot plasma and 6 proteins (0.92%) were present in healthy plasma.

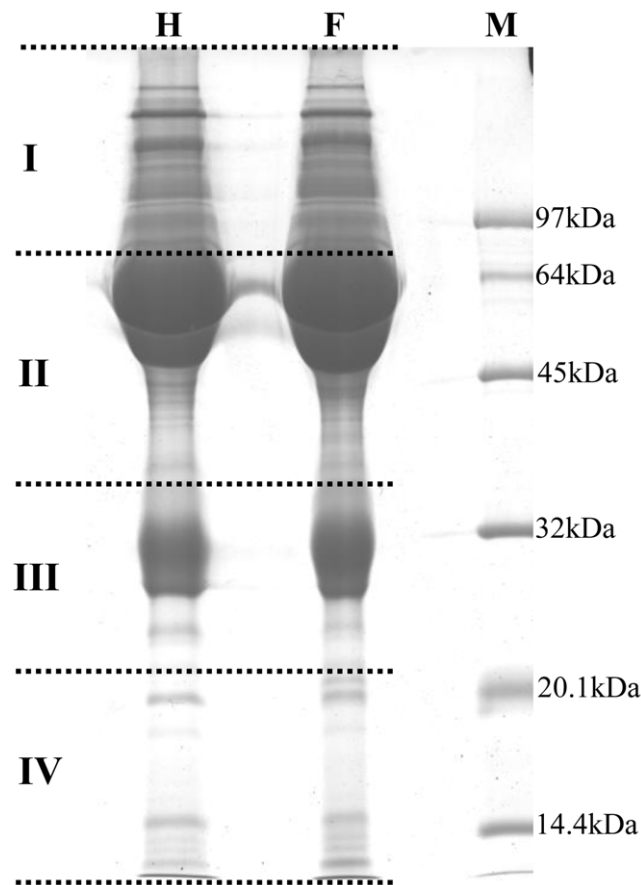


Figure 1. Separation of plasma proteins by SDS-PAGE. Lane M, Protein Marker (14.4 kDa–97 kDa); Lane F, the 11 pooled plasma proteins from footrot-affected dairy cattle; Lane H, the 11 pooled healthy plasma proteins from unaffected dairy cattle. doi:10.1371/journal.pone.0055973.g001

Table 1. Numbers of the proteins identified from healthy and footrot plasma samples.

	Healthy plasma	Footrot plasma
Total proteins no.	648 (100%)	712 (100%)
Non-redundant proteins no.	234 (36.11%)	272 (38.20%)
High-confidence proteins no.	123 (18.98%)	138 (19.38%)
Differential proteins no.	6 (0.92%)	16 (2.24%)

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Characterization of Plasma Protein Profile

The lists of the high-confidence proteins from healthy and footrot plasma samples are shown in Table 2 and Table 3, respectively. The molecular weight analysis of healthy and footrot plasma samples is shown in Fig. 2. In healthy plasma, the molecular weight of the high-confidence proteins ranged between 6.33 kDa and 249.56 kDa; proteins from 10 kDa to 70 kDa accounted for 80.49% (99/123), and proteins of greater than 100 kDa accounted for 10.57% (13/123). In footrot plasma, the molecular weight of the high-confidence proteins ranged between 4.31 kDa and 353.34 kDa; proteins from 10 kDa to 70 kDa accounted for 78.98% (109/138), and proteins of greater than 100 kDa accounted for 13.77% (19/138). Between the plasma proteins of healthy and footrot-affected cattle, there were significant differences in the molecular weight distributions at 30 kDa–50 kDa, 60 kDa–70 kDa and >100 kDa. Analysis of the isoelectric points (*pI*) of healthy and footrot plasma samples is shown in Fig. 3. In healthy plasma, the *pI* of the high-confidence proteins ranged between 4.31 and 10.71, and proteins from *pI* 5 to *pI* 9 accounted for 86.99% (107/123). In footrot plasma, the *pI* of the high-confidence proteins ranged between 4.31 and 10.71, and proteins from *pI* 5 to *pI* 9 accounted for 92.03% (127/138). Between healthy and footrot plasma proteins, there was a significant difference in the *pI* distribution at *pI* 4–10.

To investigate the function of the high-confidence proteins we had identified further, the GO categories were ascertained to characterize them according to cellular components, biological processes and molecular functions. The cellular component categories are shown in Fig. 4A. In the healthy plasma, 96 proteins of the 123 high-confidence proteins were annotated and categorized in 11 groups of cellular components; In the footrot plasma, 107 proteins of the 138 high-confidence proteins were annotated and categorized in 19 groups of cellular components. The common rich cellular components for both healthy and footrot plasma were focused in the extracellular region (GO:0005576), extracellular space (GO:0005615) and extracellular region parts (GO:0044421), respectively. Compared with the healthy plasma sample, eight differential cellular components, cytoplasmic vesicle (GO:0031410), intermediate-density lipoprotein particle (GO:0034363), chromaffin granule (GO:0042583), vesicle (GO:0031982), membrane attack complex (GO:0005579), membrane-bound vesicle (GO:0031988), recycling endosome (GO:0055037), and cytoplasmic membrane-bounded vesicle (GO:0016023), were found in the footrot plasma sample.

The biological process categories are shown in Fig. 4B. Ninety proteins out of the 123 high-confidence proteins in the healthy plasma were found in 36 groups of biological processes, and 101 proteins of the 138 high-confidence proteins of the footrot plasma were categorized in 38 groups of biological processes. Thirty common biological processes were presented in both healthy and footrot plasma samples, among which regulation of biological

Table 2. The high-confidence proteins in the plasma sample of healthy dairy cattle.

No.	Protein name	Accession no.	No.	Protein name	Accession no.	No.	Protein name	Accession no.	No.	Protein name	Accession no.
1	chain B, structure of mammalian C3 with an intact thioester at 3a resolution	gi 110590847	32	lg gamma-2 chain C region	gi 89611	63	alpha-1B-glycoprotein precursor	gi 114053019	94	lg L chain VJ region	gi 4680177
2	alpha-2-macroglobulin precursor	gi 157954061	33	IGK protein	gi 154425814	64	lg L chain V region	gi 2323404	95	C-reactive protein precursor	gi 76611918
3	serum albumin precursor	gi 30794280	34	transthyretin	gi 3915182	65	alpha macroglobulin	gi 226283	96	kininogen 1	gi 28461175
4	unnamed protein	gi 110292444	35	ceruloplasmin isoform 1	gi 119884990	66	lg gamma-1 chain	gi 346578	97	lg L chain V region	gi 2555153
5	apolipoprotein A-I	gi 113988	36	prothrombin	gi 135806	67	lg L chain V region	gi 2323384	98	lg L chain V region	gi 5802440
6	fibronectin	gi 462100	37	predicted: similar to MGC127066 protein	gi 119909599	68	lg L chain V region	gi 2323390	99	apoN protein	gi 86438511
7	transferrin	gi 113911795	38	chain B, carbonmonoxy liganded bovine hemoglobin Ph 5.0	gi 12084213	69	serum amyloid A-4 protein	gi 122138722	100	SERPIND1 protein	gi 151556981
8	serum albumin precursor	gi 57164373	39	complement factor H	gi 115298718	70	complement component C4	gi 1229	101	SHBG protein	gi 148743950
9	similar to complement component 4A	gi 119915491	40	plasminogen precursor	gi 27806815	71	predicted: serpin A3-3 isoform 3	gi 119914040	102	anti-respiratory syncytial virus lg lambda chain V region	gi 508836
10	complement C4 precursor	gi 119915494	41	histidine-rich glycoprotein precursor	gi 27806875	72	lg mu H chain V region	gi 98991290	103	lg L chain V region	gi 2555151
11	apolipoprotein A-IV	gi 118598012	42	coagulation factor II	gi 75948172	73	protein HP-25 homolog 1 precursor	gi 114050753	104	lgG3 H chain C region	gi 1575495
12	IGHM protein	gi 151554795	43	hemoglobin subunit beta-A	gi 122539	74	conglutinin	gi 461774	105	predicted: C4b-binding protein alpha chain-like	gi 119922208
13	inter-alpha-trypsin inhibitor heavy chain H1	gi 122142424	44	leucine-rich alpha-2-glycoprotein precursor	gi 114051379	75	lipocalin/cytosolic fatty-acid binding protein	gi 157831280	106	tetranectin	gi 108861909
14	ITIH2 protein	gi 146186952	45	vitronectin precursor	gi 78045497	76	lg variable region	gi 2353754	107	CD5 antigen-like precursor	gi 156120885
15	immunoglobulin gamma 1 heavy chain constant region	gi 91982959	46	predicted: similar to endopin 2B	gi 119914034	77	lg gamma chain V region	gi 94961524	108	alpha-2-HS-glycoprotein	gi 154426172
16	complement factor B	gi 146345391	47	alpha-1-acid glycoprotein	gi 121957959	78	alpha-1-antitrypsin precursor	gi 27806941	109	KRT4 protein	gi 134024768
17	V11a protein	gi 86438072	48	chain C, carbonmonoxy liganded bovine hemoglobin Ph 7.2	gi 12084218	79	angiotensinogen	gi 74354323	110	C1QC protein	gi 92097580
18	anti-testosterone antibody	gi 440	49	vitamin D-binding protein	gi 85701291	80	lg mu H chain V region	gi 98991272	111	extracellular matrix protein 1 precursor	gi 153791660
19	IGHG1 protein	gi 151554383	50	complement factor I precursor	gi 84000165	81	apolipoprotein E precursor	gi 27806739	112	cyligin-2	gi 2498277
20	ITI heavy chain H4	gi 122140331	51	predicted: complement component 5	gi 119901003	82	lg heavy chain	gi 1322326	113	complement C1s subcomponent	gi 146286057
21	hypothetical protein LOC505478	gi 115497996	52	lg mu chain - sheep	gi 478694	83	lg H chain V region	gi 3834645	114	predicted: hypothetical protein isoform 8	gi 119912360
22	IGL@ protein	gi 74353860	53	lg L chain V region	gi 2323402	84	complement component C8 gamma chain precursor	gi 119904354	115	fibronogen alpha chain precursor	gi 75812954

Table 2. Cont.

No.	Protein name	Accession no.	No.	Protein name	Accession no.	No.	Protein name	Accession no.	No.	Protein name	Accession no.
23	MGC127066 protein	gi 148744106	54	IgG L chain V region	gi 2323392	85	Ig H chain V region	gi 24459869	116	factor Xlla inhibitor	gi 1706733
24	unknown protein	gi 148878143	55	pigment epithelium-derived factor precursor	gi 27806487	86	Ig mu H chain V region	gi 98991286	117	Ig mu H chain V region	gi 98991296
25	IgG1 H chain constant region	gi 7547266	56	AMBP protein precursor	gi 2506821	87	hemoglobin subunit alpha	gi 110831923	118	Ig lambda L chain V region	gi 1276619
26	chain B, crystal structure of bovine plasma copper-containing amine oxidase	gi 61680008	57	histidine-rich glycoprotein	gi 1072452	88	hemoglobin subunit beta-A	gi 122540	119	angiotensinogen	gi 1703309
27	gelsolin	gi 122140093	58	paraoxonase 1	gi 86826758	89	coagulation factor V	gi 2493324	120	predicted: similar to mannosidase	gi 119888855
28	predicted: similar to alpha-2-macroglobulin precursor	gi 119893044	59	hibernation-associated plasma protein HP-20-like	gi 86438473	90	complement component C7	gi 126361384	121	Ig lambda-6a L chain V region	gi 2746695
29	IgG lambda light chain	gi 15088675	60	histidine-rich glycoprotein = factor Xlla substrate	gi 1683228	91	ADIPOQ protein	gi 14618677	122	Ig lambda L chain V region	gi 1276629
30	IgM H chain constant region	gi 28592070	61	endopin-1 precursor	gi 75068363	92	antithrombin-III precursor	gi 109940161	123	Ig lambda L chain V region	gi 975858
31	IgG H chain constant region	gi 3834667	62	serpin A3-3 precursor	gi 84000377	93	protein HP-25 homolog 2 precursor	gi 114052108			

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process (GO:0050789), response to stress (GO:0006950), positive regulation of biological process (GO:0048518), response to external stimulus (GO:0009605), regulation of response to stimulus (GO:0048583), negative regulation of biological process (GO:0048519), regulation of biological quality (GO:0065008) and regulation of immune system process (GO:0002682) were common rich biological processes, respectively. Fourteen differential biological processes were found, of which six occurred in healthy plasma and eight in footrot plasma. In footrot plasma, the eight differential biological processes were involved in organic ether metabolic process (GO:0018904), regulation of cellular component organization (GO:0051128), negative regulation of transport (GO:0051051), regulation of molecular function (GO:0065009), negative regulation of metabolic process (GO:0009892), macromolecule metabolic process (GO:0043170), transport (GO:0006810) and establishment of localization (GO:0051234), of which macromolecule metabolic process was a rich biological process.

The result for molecular function is shown in Fig. 4C. Ninety-six proteins out of the 123 high-confidence proteins in the healthy plasma were involved in 33 groups of molecular functions, and 106 proteins of the 138 high-confidence proteins in the footrot plasma were involved in 40 groups of molecular functions. Between the healthy and footrot plasma samples, there were 28 identical molecular function categories, of which protein binding (GO:0005515), enzyme regulator activity (GO:0030234), endopeptidase inhibitor activity (GO:0004866), peptidase inhibitor activity (GO:0030414) and enzyme inhibitor activity (GO:0004857) were common rich molecular functions, respectively. A total of 17 differential molecular functions were found, of which five occurred in healthy plasma and 12 in footrot plasma. In the footrot plasma, 11 of the 12 differential molecular functions focused on binding activity, including ferric iron binding (GO:0008199), iron ion binding (GO:0005506), steroid binding (GO:0005496), cholesterol binding (GO:0015485), apolipoprotein receptor binding (GO:0034190), alcohol binding (GO:0043178), quaternary ammonium group binding (GO:0050997), phosphatidylcholine binding (GO:0031210), sterol binding (GO:0032934), high-density lipoprotein receptor binding (GO:0070653) and high-density lipoprotein binding (GO:0008035). In the healthy plasma, the five differential molecular functions were involved in calcium ion binding (GO:0005509), eukaryotic cell surface binding (GO:0043499), cell surface binding (GO:0043498), peptidase activity (GO:0008233), and peptidase activity acting on L-amino acid peptides (GO:0070011).

Analysis of Potential Footrot-associated Proteins

The proteins that showed potential differential expression between healthy and footrot plasma samples were searched for among the sets of high-confidence proteins using the geninfo identifier (GI) number of each protein, and the lists of potential differential expression (PDE) proteins are shown in Table 4. Six PDE proteins were found in plasma samples from healthy dairy cattle, involving in regulatory proteins (ADIPOQ protein and angiotensinogen), cytoskeletal proteins (KRT4 protein and cylicin-2), and coagulation factor (fibrinogen alpha chain precursor). Of six PDE proteins, ADIPOQ protein, fibrinogen alpha chain precursor and angiotensinogen could be associated with the pathogenesis of footrot in dairy cattle. 16 PDE proteins were found in plasma samples from dairy cattle with footrot, involving in immunoglobulins (IgGs), innate immune recognition molecules (predicted peptidoglycan recognition protein L), acute phase proteins (haptoglobin, haptoglobin precursor, afamin precursor), regulatory proteins (SERPINA10 protein, mammalian C3,

Table 3. The high-confidence proteins in the plasma sample of footrot dairy cattle.

No.	Protein name	Accession no.	No.	Protein name	Accession no.	No.	Protein name	Accession no.	No.	Protein name	Accession no.
1	chain B, structure of mammalian C3 with an intact thioester at 3a resolution precursor	gi 110590847	36	Ig lambda light chain	gi 15088675	71	predicted:similar to H factor 1	gi 119922668	106	Ig light chain variable region	gi 2555151
2	alpha-2-macroglobulin precursor	gi 157954061	37	IgM heavy chain constant region	gi 28592070	72	hemoglobin subunit beta-A	gi 122540	107	apolipoprotein A-II	gi 109940051
3	transferrin	gi 113911795	38	Ig γ_1 heavy chain constant region	gi 91982959	73	predicted: mammalian C3	gi 119894726	108	Ig mu heavy chain variable region	gi 98991290
4	serum albumin precursor	gi 30794280	39	predicted: similar to MGC127066 protein	gi 119909599	74	clusterin	gi 151555910	109	hemoglobin subunit alpha	gi 110831923
5	unnamed protein product	gi 110292444	40	bovine carbonmonoxy Hb	gi 14488451	75	Ig light chain variable region	gi 2323392	110	apolipoprotein E precursor	gi 27806739
6	predicted: similar to complement component 4A	gi 119915491	41	IgG1 heavy chain constant region	gi 7547266	76	Ig light chain variable region	gi 2323402	111	extracellular matrix protein 1 precursor	gi 153791660
7	albumin	gi 229552	42	predicted:similar to alpha-2-macroglobulin precursor	gi 119893044	77	Ig λ chain C region - sheep	gi 109030	112	haptoglobin precursor	gi 2144490
8	complement C4 precursor	gi 119915494	43	leucine-rich alpha-2-glycoprotein precursor	gi 114051379	78	Ig γ -1 chain - sheep	gi 346578	113	predicted:similar to H factor 1	gi 119925635
9	apolipoprotein A-I precursor	gi 113988	44	IgG2a heavy chain constant region	gi 1699167	79	Ig light chain variable region	gi 2323398	114	complement factor I precursor	gi 84000165
10	ITI heavy chain H4	gi 122140331	45	predicted:C4b-binding protein alpha chain	gi 76677514	80	protein AMBP	gi 2506821	115	conglutinin precursor	gi 461774
11	inter-alpha (globulin) inhibitor H4	gi 59857769	46	serpin A3-3 precursor	gi 84000377	81	protein HP-25 homolog 1 precursor	gi 114050753	116	alpha-2-antiplasmin precursor	gi 1168249
12	chain B, crystal structure of bovine plasma copper-containing amine oxidase	gi 61680008	47	complement C6 precursor	gi 18601082	82	factor XIa inhibitor	gi 1706733	117	SHBG protein	gi 148743950
13	serum albumin precursor	gi 113582	48	fibronectin (FN)	gi 462100	83	alpha-1-B glycoprotein	gi 114053019	118	complement component C7	gi 126361384
14	haptoglobin	gi 94966763	49	Ig gamma-2 chain C region	gi 89611	84	Ig light chain variable region	gi 2555153	119	Ig lambda light chain V region	gi 1276619
15	IGHM protein	gi 151554795	50	alpha-1-acid glycoprotein precursor	gi 121957959	85	vitamin D-binding protein	gi 85701291	120	Ig lambda light chain V region	gi 975854
16	predicted: ceruloplasmin isoform 1	gi 119884990	51	SERPIND1 protein	gi 151556981	86	chain C, carbonmonoxy Hb	gi 14488450	121	CD5 antigen-like precursor	gi 156120885
17	gelsolin	gi 122140093	52	paraoxonase 1	gi 86826758	87	complement component C4	gi 1229	122	centromere protein F	gi 119908705
18	Complement factor H	gi 115298718	53	vitronectin	gi 78045497	88	kininogen-2	gi 125506	123	desmoplakin	gi 119915951
19	Ig M heavy chain secretory form	gi 24496448	54	C4b-binding protein alpha chain	gi 2493791	89	uncharacterized protein LOC790886 precursor	gi 157279963	124	predicted:KIAA0683 gene product-like	gi 119935194
20	Ig mu heavy chain constant region	gi 33413902	55	pigment epithelium-derived factor precursor	gi 27806487	90	Ig light chain variable region	gi 2323390	125	coagulation factor V precursor	gi 2493324
21	complement factor B	gi 146345391	56	predicted:similar to endopin 2B	gi 119914034	91	SERPINA3-8	gi 151554250	126	Ig lambda light chain V region	gi 1276609

Table 3. Cont.

No.	Protein name	Accession no.	No.	Protein name	Accession no.	No.	Protein name	Accession no.	No.	Protein name	Accession no.
22	apolipoprotein A-IV precursor	gi 118598012	57	transthyretin	gi 3915182	92	amyloid A4 protein precursor	gi 122138722	127	phosphatidylinositol-glycan-specific phospholipase D precursor	gi 27807363
23	ITI heavy chain H1	gi 122142424	58	serpin A3-5 precursor	gi 126165236	93	IgG3 heavy chain C region	gi 1575495	128	predicted:similar to superficial zone protein	gi 119908681
24	ITI H2 protein	gi 146186952	59	hemopexin precursor	gi 77736171	94	hemoglobin subunit beta	gi 122654	129	predicted peptidoglycan protein L	gi 119894607
25	V11a protein	gi 86438072	60	histidine-rich glycoprotein precursor	gi 27806875	95	alpha-2-HS-glycoprotein	gi 154426172	130	Ig lambda light chain V region	gi 975858
26	hypothetical protein LOC505478	gi 115497996	61	hibernation-associated plasma protein HP-20-like	gi 86438473	96	complement C5a anaphylatoxin precursor	gi 119901003	131	Ig chain A, plasma retinol-binding protein	gi 157831280
27	IGL@ protein	gi 74353860	62	predicted:serpin A3-3 isoform 3	gi 119914040	97	SERPINA10 protein	gi 148745555	132	Ig lambda-6a light chain variable region	gi 2746695
28	Ig heavy chain constant region	gi 3834667	63	Ig mu chain - sheep	gi 478694	98	afamin precursor	gi 76656723	133	Ig lambda chain precursor V region	gi 109032
29	IGK protein	gi 115545495	64	cleaved bovine antithrombin iii	gi 157838186	99	inhibitor of carbonic anhydrase precursor	gi 114053269	134	ovarian and testicular apolipoprotein N precursor	gi 51491835
30	SERPINA3 protein	gi 86438018	65	protein HP-25 homolog 2 precursor	gi 114052108	100	Ig light chain variable region	gi 2323378	135	apolipoprotein C-III	gi 78099960
31	plasminogen	gi 27806815	66	Ig light chain variable region	gi 2323404	101	Ig lambda chain V region	gi 508836	136	apolipoprotein D	gi 122142930
32	CCP modules 3-12	gi 1419424	67	prothrombin precursor	gi 27806947	102	Ig light chain VJ region	gi 4680177	137	keratan sulfate proteoglycan	gi 1708876
33	IGL@ protein	gi 148744106	68	histidine-rich glycoprotein	gi 1072452	103	Ig light chain variable region	gi 2323384	138	predicted:hyposialic protein	gi 119903609
34	unknown protein	gi 151556360	69	C8 gamma chain precursor	gi 119904354	104	Ig variable region	gi 2353754			
35	anti-testosterone antibody	gi 440	70	tetranectin	gi 108861909	105	C-reactive protein precursor	gi 76611918			

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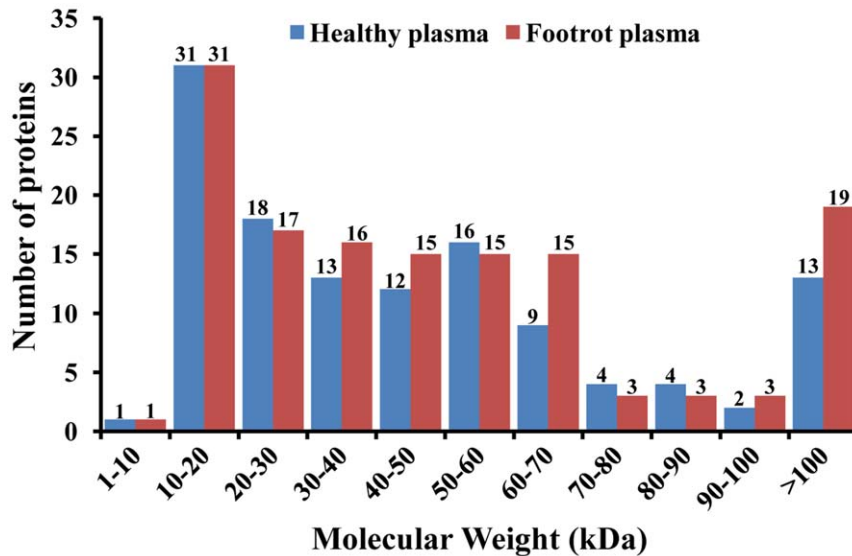


Figure 2. Distributions of molecular weight of the high-confidence proteins from healthy plasma and footrot plasma.
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alpha-2-antiplasmin precursor and apolipoprotein-D), and cell adhesion and cytoskeletal proteins (keratan sulfate proteoglycan, centromere protein F, desmoplakin, similar to superficial zone protein). Of 16 PDE proteins, haptoglobin, SERPINA10 protein, afamin precursor, haptoglobin precursor, predicted peptidoglycan recognition protein L (PGRP-L), apolipoprotein D, and keratan sulfate proteoglycan (KS-PG) were suggested to be disease-associated proteins or biomarkers according to current research reports. Furthermore, ELISA result indicated that the IgG concentration of healthy and footrot plasma samples was 2.782 ± 0.148 mg/mL and 3.632 ± 0.081 mmol/L, respectively. The IgG concentration in footrot plasma sample was significantly higher than those of healthy plasma sample ($p < 0.01$) (Fig. 5).

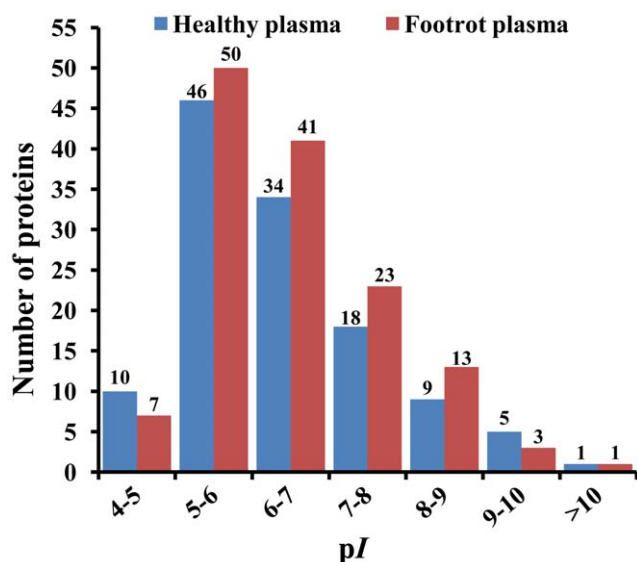


Figure 3. Distributions of isoelectric point (pI) of the high-confidence proteins from healthy plasma and footrot plasma.
doi:10.1371/journal.pone.0055973.g003

Discussion

In our study, the shotgun proteomics technique was used to identify the plasma protein profiles of dairy cattle in 11 pooled healthy specimens and 11 pooled footrot specimens, respectively. A total of 648 proteins and 712 proteins were identified from the plasma samples of healthy dairy cattle and dairy cattle affected by footrot, respectively, according to the stringent filtering parameters of Delta CN (≥ 0.1) and Xcorr (one charge ≥ 1.9 , two charges ≥ 2.2 , three charges ≥ 3.75). The total number of proteins identified (648 or 712) was significantly higher than the number identified in human plasma (622) by the shotgun proteomics technique [21]. Although the highly abundant plasma proteins, such as albumin, IgG, and IgA, were not removed in our experiment, we still obtained highly enriched protein samples from the plasma samples of dairy cattle. This result suggests that the presence of the highly abundant plasma proteins has little effect on the identification of proteins using shotgun proteomics technique. Given the presence of protein homologs, one or more peptides obtained by shotgun MS/MS methods may be assigned to multiple proteins. In order to remove redundant proteins, the total proteins identified were subjected to group combination using the in-house software Buildsummary. We obtained 234 non-redundant proteins (234/648, 36.11%) in healthy plasma samples, and 272 non-redundant proteins (272/712, 38.20%) in footrot plasma samples. The lower number of the non-redundant proteins indicates that there are many redundant proteins in the current database of Bovidae proteins in NCBI. Taking into account the analysis of potential differential proteins between plasma samples from healthy and footrot-affected cattle, the high-confidence proteins among the non-redundant proteins were screened further by the standard of a unique peptide count ≥ 2 or a unique peptide count = 1 but a total count ≥ 4 . A total of 123 high-confidence proteins were found in the healthy plasma sample, which accounted for 18.98% (123/648) of the total number of proteins and 52.56% (123/234) of the non-redundant proteins. A total of 138 high-confidence proteins were found in the footrot plasma sample, which accounted for 19.38% (138/712) of the total number of proteins and 50.74% (138/272) of the non-redundant proteins. These data demonstrate that the original proteins

Table 4. The potential differential proteins between healthy and footrot plasma samples.

The differential proteins in footrot plasma sample							
No.	Accession no.	Protein name	MW (Da)	p/	Peptides no.	Unique peptides no.	Cover percent
1	gi 94966763	haptoglobin	44859.08	7.83	36	17	51.12%
2	gi 119894726	predicted:mammalian C3	180556.4	6.46	7	4	3.47%
3	gi 109030	Ig λ chain C region - sheep	11311.56	8.46	20	3	33.33%
4	gi 157279963	uncharacterized protein LOC790886 precursor	45428.42	7.72	9	3	8.61%
5	gi 148745555	SERPINA10 protein	51988.26	6.05	4	3	8.19%
6	gi 76656723	afamin precursor	69562.06	5.63	4	3	7.45%
7	gi 2144490	haptoglobin precursor	4680.19	4.64	4	2	48.89%
8	gi 1168249	alpha-2-antiplasmin precursor	54711	5.45	3	2	8.94%
9	gi 119908705	centromere protein F	353344.7	5.01	2	2	1.17%
10	gi 119915951	desmoplakin	332383.2	6.47	2	2	0.62%
11	gi 119935194	predicted:KIAA0683 gene product-like	60081.36	5.3	2	2	6.02%
12	gi 1276609	Ig lambda light chain V region	13510.09	7.7	2	2	16.03%
13	gi 119908681	predicted:similar to superficial zone protein	133388.3	9.21	2	2	2.67%
14	gi 119894607	predicted peptidoglycan recognition protein L	63486.22	6.47	2	2	7.11%
15	gi 122142930	apolipoprotein D	21401.59	4.8	4	1	6.35%
16	gi 1708876	keratan sulfate proteoglycan	38756.48	5.93	4	1	5.26%

The differential proteins in healthy plasma sample							
No.	Accession no.	Protein name	MW (Da)	p/	Peptides no.	Unique peptides no.	Cover percent
1	gi 146186777	ADIPOQ protein	26133.2	5.46	3	3	17.50%
2	gi 134024768	KRT4 protein	58046.33	7.47	3	2	3.83%
3	gi 2498277	cylicin-2	53561.72	9.76	2	2	3.89%
4	gi 119912360	predicted: hypothetical protein isoform 8	86933.48	4.81	2	2	2.84%
5	gi 75812954	fibrinogen alpha chain precursor	67012.11	6.73	2	2	4.72
6	gi 1703309	angiotensinogen	51304.09	6.54	4	1	2.73%

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samples to a certain extent. The changes of the iron ion concentration may have a certain relationship with erythrocyte hemolysis and live damage caused by *F. necrophorum* infection [28,29]. Furthermore, the decreases of oxygen-carrying capacity of red blood cells because of the hemolysis would be more conducive to the anaerobic infection of *F. necrophorum* [30]. It has been proven that calcium is needed for normal claw growth and integrity, and plays an integral role in the keratinization and cornification process [31,32]. Compared with the healthy plasma, the loss of the calcium ion binding proteins in footrot plasma samples may promote the development and progression of footrot in dairy cattle. The above-described data supported the hypothesis that the numbers, types and functions of plasma proteins had experienced great changes during the pathogenesis of footrot in the dairy cattle.

Differential proteins, or marker proteins, have become an important target of proteomics research. In this study, a total of 22 potential differentially expressed proteins were found, among which 16 proteins (2.24%) occurred in footrot plasma and 6 proteins (0.92%) in healthy plasma. The potential differential expression (PDE) proteins in each plasma sample comprised a very low percentage of the total number of proteins. Of these PDE

proteins, the seven proteins in the footrot plasma, haptoglobin, SERPINA10 protein, afamin precursor, haptoglobin precursor, predicted peptidoglycan recognition protein L (PGRP-L), apolipoprotein D, and keratan sulfate proteoglycan (KS-PG), may be valuable for use as diagnostic biomarkers and in elucidation of the pathogenesis of footrot. Among the seven potential footrot-associated proteins, haptoglobin, haptoglobin precursor, and afamin precursor have been reported to be acute phase proteins, which are an integral part of the acute phase response of innate immunity [33–35]. The APPs have been shown to be valuable biomarkers because increases can occur with inflammation, infection, neoplasia, stress, and trauma. In past decades, haptoglobin has been shown to be a useful biomarker for monitoring the occurrence and severity of inflammatory responses in cattle with mastitis, pneumonia, enteritis, peritonitis, endocarditis, abscesses, endometritis and hoof disease [12,13,36,37]. Here, haptoglobin and its precursor were verified as plasma biomarkers of footrot in dairy cattle. Among the seven potential footrot-associated proteins, haptoglobin precursor, afamin precursor, apolipoprotein-D have been reported as tumor biomarkers [38–40]. The identification of low-abundance serum proteins, such as tumor biomarkers, further

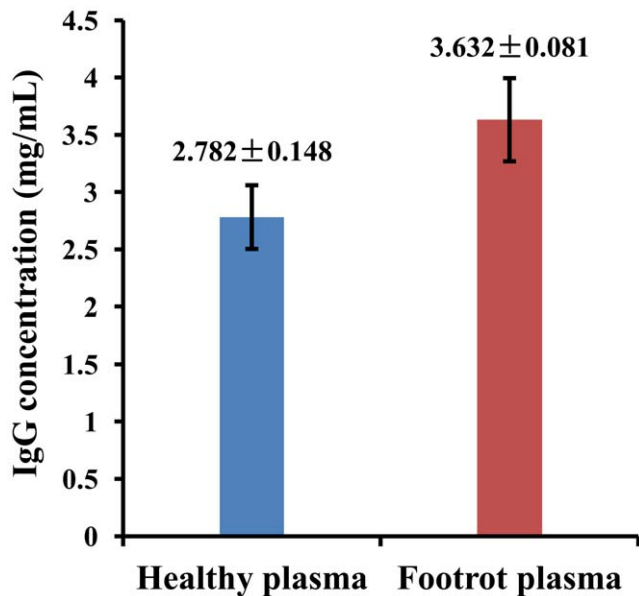


Figure 5. ELISA of the IgGs in the plasma samples of both healthy dairy cattle and those affected by footrot. Note. all data are expressed as mean \pm SD, $p=0.0027$ ($p<0.01$). doi:10.1371/journal.pone.0055973.g005

supports the validity of the shotgun proteomics technique used in our experiment. The SERPINA10 protein, which belongs to the family of serine proteinase inhibitors, is involved in blood coagulation, complement activation, fibrinolysis, angiogenesis, inflammation, and tumor suppression [34]. It is thought that the emergence of SERPINA10 protein in plasma from footrot-affected cattle represents a defense response of the host against footrot caused by *Fusobacterium necrophorum* infection. Compared with the other five proteins, the PGRP-L and KS-PG may attract more attention as a consequence of their effects in the pathogenesis of footrot. The peptidoglycan recognition protein is required for the induction of antibacterial peptide genes in response to infection in insects and mammals [41–43]. The predicted PGRP-L may be necessary for recognition of the innate immune activators of the Gram-negative anaerobic bacterium *F. necrophorum*. If this is the case, the presence of the predicted PGRP-L in bovine plasma may be used as an indicator or biomarker of *F. necrophorum* infection. Keratan sulfate is any of several sulfated glycosaminoglycans that have been found especially in the cornea, cartilage, and bone. The keratan sulfate in blood has been shown to be a marker of cartilage catabolism [44,45]. Footrot in dairy cattle, caused by *F. necrophorum* infection, is characterized by suppurative, necrosis, and corruption of the hoof tissue. If left untreated, the infection can progress into the joint space or tendon sheath, producing permanent damage. The PDE protein KS-PG, which was found in the plasma from footrot-affected dairy cattle, may reflect catabolism of hoof cartilage, and it has been suggested to be a potential marker for evaluation of foot damage in dairy cattle. In our studies, the monoclonal antibodies against human keratan sulfate and peptidoglycan recognition proteins (PGRPs) had been selected to valid the presence of two highlighted proteins KS-PG and PGRP-L in footrot and healthy plasma samples. However, only negative

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results were obtained in western blotting and ELISA. This data suggests that there may be some differences in both KS-PG and PGRP-L between human and dairy cattle, resulting in the antibody's invalidity. So, further related studies are needed to confirm roles of the two highlighted proteins KS-PG and PGRP-L in footrot. To reduce the potential defect, the IgGs concentrations of the plasma samples of both healthy dairy cattle and those affected by footrot were validated by ELISA. The increased IgGs in the footrot plasma not only represent an innate immune response of the diseased cattle to *F. necrophorum* infection, but also provide a potential support for the validity of the shotgun proteomics approach used in our study. Additionally, in healthy plasma samples, a total of six potential differential proteins were found, among which ADIPOQ protein, fibrinogen alpha chain precursor and angiotensinogen may be involved in the pathogenesis of footrot in dairy cattle [46–51].

To the best of our knowledge, this is the first report of plasma proteomics analysis of dairy cattle affected by footrot using the shotgun proteomics technique. Although there are still some shortcomings, the shotgun technique shows high efficiency in the identification of plasma proteins. In our current research, the protein profiles of plasma from healthy and footrot-affected dairy cattle were characterized fully using shotgun proteomics methods. We not only identified the increased IgGs, innate immune recognition molecules, acute phase proteins, regulatory proteins, and cell adhesion and cytoskeletal proteins in the footrot plasma sample, but also obtained some interesting proteins, such as predicted PGRP-L and KS-PG, which have been reported only occasionally in studies of cattle disease, especially footrot in dairy cattle. The basic information reported here will increase our understanding of bovine plasma protein profiling, and will assist in further studies of control strategies for footrot in dairy cattle.

Supporting Information

Figure S1 Mass spectrum base peak of plasma samples. C1–C4. Mass spectrum base peak of the pooled healthy plasma sample, respectively; S1–S4. Mass spectrum base peak of the pooled footrot plasma sample, respectively. (RAR)

Table S1 The identified protein lists of plasma samples. A. The identified protein lists of the pooled healthy plasma sample; B. The identified protein lists of the pooled footrot plasma sample. (RAR)

Table S2 The GO categories of the identified high-confidence proteins. A. The GO categories of the high-confidence proteins identified from the pooled healthy plasma sample; B. The GO categories of the high-confidence proteins identified from the pooled footrot plasma sample. CC = cellular components; BP = biological processes; MF = molecular functions. (RAR)

Author Contributions

Conceived and designed the experiments: DBS HBW. Performed the experiments: DBS HZ DHG. Analyzed the data: AGS. Contributed reagents/materials/analysis tools: DBS. Wrote the paper: DBS.

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