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Quantitative differences in whey proteins among Murrah, Nili-Ravi and Mediterranean buffaloes using a TMT proteomic approach

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ABSTRACT

The aim of this study was to characterize whey proteins and their potential activities among buffalo breeds. In this work, a tandem mass tag (TMT) proteomic approach was used to identify the differences in the proteomes of milk whey proteins in Murrah, Nili-Ravi and Mediterranean buffaloes. A total of 580 proteins were identified in buffalo milk whey, and the subunits of the 62 differentially expressed proteins are shown in clustering analysis. Whey proteins with relatively higher levels in Mediterranean buffalo milk than in Murrah and Nili-Ravi buffalo milk included polymeric immunoglobulin receptor, α1-antiproteinase, heat shock cognate 71-kDa protein, and Acyl-CoA-binding protein. Most of the differentially expressed proteins participate in complement and coagulation cascades, which are strongly related to immune protective activities. These results provide insight into the complexity of the buffalo milk whey proteome and provide molecular evidence for nutritive differences among buffalo breeds.

1. Introduction

Milk is a specific biological fluid produced by mammals that has received widespread attention due to its nutritional and functional properties for humans (Yang et al., 2013). Buffalo milk has higher contents of fat, lactose, and especially protein. The protein content in buffalo milk (average = 4.3%) is approximately 1.4-fold greater than that of cow milk (average = 3%) (Bonfatti, Giantin, Rostellato, Dacasto, & Carnier, 2013; Li, Li, Zeng, Liu, & Ren, 2016). In addition to being a primary source of protein in human diets, buffalo milk is also used for diverse dairy products, such as yogurt, cheese and bioactive peptides (Bonfatti, Gervaso, Rostellato, Coletta, & Carnier, 2013; Reddi et al., 2016).

Milk whey proteins account for approximately 20% of the total milk protein, including immunoglobulins, β-lactoglobulin, α-lactalbumin, and lactoferrin, and have various functions (Pereira, 2014). For example, immunoglobulins originating mostly from milk play an important role in immunity defense for infants (Hurley & Theil, 2011). Lactoferrin is known to exert antimicrobial, antioxidant and anticarcinogenic properties (Mills, Ross, Hill, Fitzgerald, & Stanton, 2011). However, the whey proteomes of different species demonstrate that certain proteins are unique to a given species (Yang et al., 2013).

To date, many studies have focused on comparing whey proteins in milk from different species using proteomic methods. Low-abundance milk whey proteins were identified in Holstein and Jersey breeds of dairy cows through nanoscale liquid chromatography coupled with mass spectroscopy (MS) (Tacoma, Fields, Ebenstein, Lam, & Greenwood, 2016). Later, the whey proteins of colostrum and mature milk from human and bovine were characterized using the isobaric tags for relative and absolute quantification (iTRAQ) proteomic approach (Yang et al., 2017). Furthermore, the protein composition of milk whey and even the *N*-glycoproteome profiles have been compared between cow, yak, buffalo, goat and camel milk via one- and two-dimensional gel electrophoresis (2-DE) combined with tandem MS/MS (Hinz, O'Connor, Huppertz, Ross, & Kelly, 2012), iTRAQ (Yang et al., 2013) and modified proteomics techniques (Yang et al., 2017). Our previous research also found that the casein content and composition differed among buffalo breeds, including Murrah, Nili-Ravi and their cross-breeds, through 2-DE coupled with matrix-assisted laser desorption/ionization time of flight (MALDI TOF/TOF) MS/MS (Li et al., 2016). However, to date, the composition and functions of milk whey proteins from different buffalo breeds remain unclear.

As buffalo milk plays important nutritional and economic roles in specific regions (Medhammar et al., 2012), understanding the

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characterization of milk whey proteins among buffalo breeds is beneficial for the manufacture of buffalo milk and the application of its health-promoting functions for humans. We hypothesize that the characteristics of whey proteins differ among different buffalo breeds. Therefore, the aim of this study was to characterize the whey proteins and their potential activities among the main buffalo breeds (Murrah, Nili-Ravi and Mediterranean) to provide guidance for buffalo milk product processing and animal breeding.

2. Materials and methods

2.1. Preparation of milk whey protein

Milk samples were collected from 12 Murrah, 12 Nili-Ravi, and 18 Mediterranean buffaloes from a dairy farm in Nanning City, Guangxi Province, and pooled into three fractions each. A diagram of the workflow is listed in Fig. S1. Three samples of each buffalo group were centrifuged at 4 °C and 3000 × *g* for 15 min to remove fat. The whey proteins were obtained according to the procedure described by Huppertz and coworkers (Huppertz, Fox, & Kelly, 2004). Briefly, skim milk samples (1 mL) were pH adjusted to 4.6 with 30 μL of 33% acetic acid and kept at room temperature for 10 min. Then, 30 μL of 3.3 M sodium acetate was added to the mixture, which was subsequently centrifuged at 20 °C and 14,000 × *g* for 30 min to remove the casein. Thereafter, the supernatant containing the whey proteins was collected, and the protein concentration was determined by the BCA assay kit using bovine serum albumin as the standard (Beyotime Biotechnology, Shanghai, China). The whey proteins were stored at −80 °C until analysis.

Supplementary Tables S1, S2 and Fig. S1 associated with this article can be found, in the online version, at https://doi.org/10.1016/j.foodchem.2018.06.122.

2.2. SDS-PAGE

The milk whey proteins were mixed with SDS-PAGE sample loading buffer (5 ×, Beyotime Biotechnology, Shanghai, China) and then heated at 100 °C for 5 min. The mixed samples (20 μg) were used to perform SDS-PAGE. The samples were loaded onto a 5% gel at 80 V for 30 min and then run on a 12% separating gel at 120 V until they reached the bottom. The gels were stained with Coomassie brilliant blue R-250 (GE Healthcare, Beijing, China) and destained with water, and then, the images were captured by an image scanner (GS800, Bio-Rad).

2.3. Protein digestion and tandem mass tag labeling

Each whey protein sample (100 μg) was mixed with 200 μL of UA buffer (8 M urea and 150 mM Tris-HCl, pH 8.0), transferred to an ultrafiltration filter (10-kDa cutoff) and centrifuged at 14,000 × *g* for 15 min, 100 μL of DTT solution (10 mM DTT in UA buffer) was added to the filter and incubated at 56 °C for 1 h. After centrifugation, UA buffer (200 μL) was added and centrifuged at 14,000 × *g* for 15 min, and this step repeated twice. Then, 100 μL of iodoacetamide solution (50 mM iodoacetamide in UA buffer) was added to the filter, which was vortexed at 600 rpm for 1 min. The samples were incubated at room temperature for 30 min in the dark, followed by centrifugation at 14,000 × *g* for 10 min. Subsequently, the filter was washed with 100 μL of UA buffer and centrifuged twice at 14,000 × *g* for 10 min. After that, 100 μL of dissolution buffer was added to the filter, which was centrifuged twice at 14,000 × *g* for 10 min. Finally, 80 μL of trypsin buffer (4 μg trypsin) was added to the filter, was vortexed at 600 rpm for 1 min, and then digested at 37 °C for 16–18 h. The filter unit was placed into a new tube and centrifuged at 14,000 × *g* for 10 min.

The peptide mixture was labeled with tandem mass tag (TMT) reagents according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA). Three Mediterranean buffalo milk samples were labeled with 126N, 128C, and 129N; three Murrah buffalo milk samples were labeled with 127C, 128N, and 130C; and three Nili-Ravi buffalo milk samples were labeled with 127N, 129C, and 130N. The reactions were incubated at room temperature for 1 h, and then, 8 μL of hydroxylamine was added, followed by incubation for 15 min at room temperature to stop the reaction. After tagging, the 9 samples were pooled and desalted.

2.4. Reverse-phase chromatography with high pH separation

The combined sample was fractionated by reverse-phase chromatography using an XBridge BEH 300 C18 column (2.0 × 50 mm, 5 μm, Waters, Ireland). Buffer A consisted of 100% H_2O with pH 10.0, and buffer B consisted of 100% acetonitrile with pH 10.0. The samples were separated in 5% (v/v) buffer B for 2 min, 5–35% (v/v) buffer B for 58 min, 35–50% (v/v) buffer B for 10 min, 50–90% (v/v) buffer B for 4 min, 90% (v/v) buffer B for 2 min, 90–5% (v/v) buffer B for 1 min, and 5% (v/v) buffer B for 3 min at a flow rate of 500 μL/min. The sample fractions were collected every 4 min, and a total of 20 fractions were obtained. Then, these samples were combined into 10 fractions.

2.5. Capillary high-performance liquid chromatography separation

The fractions were further separated and identified using a Thermo Fisher EASY-nLC 1000 coupled with a Q-Exactive mass spectrometer (MS). Buffer C consisted of 0.1% (v/v) formic acid in MilliQ water, and buffer D consisted of 0.1% (v/v) formic acid in 90% (v/v) acetonitrile solution. Samples were loaded onto the Zorbax 300SB-C18 traps column (0.3 mm × 5 mm, 5 μm, Agilent Technologies, DE) and separated on the reverse-phase column (0.075 mm × 150 mm, 3 μm). The samples were separated in 2–5% (v/v) buffer D for 2 min, 5–35% (v/v) buffer D for 100 min, 35–90% (v/v) buffer D for 6 min, 90% (v/v) buffer D for 3 min, 90–2% (v/v) buffer D for 0.5 min, and 2% (v/v) buffer D for 8.5 min. The temperature of the capillary was 270 °C.

2.6. MS/MS identification and quantification

Data acquisition was performed by a Q-Exactive mass spectrometer in positive ion mode with a selected mass range of 350–1800 *m/z*. The resolving power was set as 70,000 for the MS scan and 35,000 for the MS/MS scan via higher energy collisional dissociation with normalized collision energies of 30%. The dynamic exclusion of the selected precursor ions was set at 30 s.

Raw files were analyzed by MaxQuant 1.5.2.8, and the UniProt Bovine database was searched (downloaded on 2016/12/16, the selected database of species was 27,592 and 56,311 protein items contained). The parameters were set as follows: the variable modifications were set as oxidation and acetyl (protein *N*-term), and the fixer modifications were set as a carbamidomethyl and TMT-labeled *N*-terminus and a TMT-labeled lysine (+229.1629 Da). To resolve the close reporter ion peaks during data processing, we set the reporter ion tolerance of 0.0025 Da. Besides, the post-acquisition filter of precursor ion fraction (PIF) of 75%was set as well, as has been previously recommended (Wenger et al., 2011). The false discovery rates of peptides and proteins were set as no more than 1%. The identification of proteins inferred from the unique peptide identification in all experiments was considered. Quantified proteins from three groups were analyzed by one-way ANOVA in which Tukey's test was used to evaluate differences among the studied groups. P-value < 0.05 and fold-change > 1.5 were defined as significant.

2.7. Bioinformatic and multivariate analysis

The annotated functions of the whey proteins were analyzed by the gene ontology (GO) annotation software (http://david.abcc.ncifcrf.gov/home.jsp). Principal component analysis (PCA) of the identified

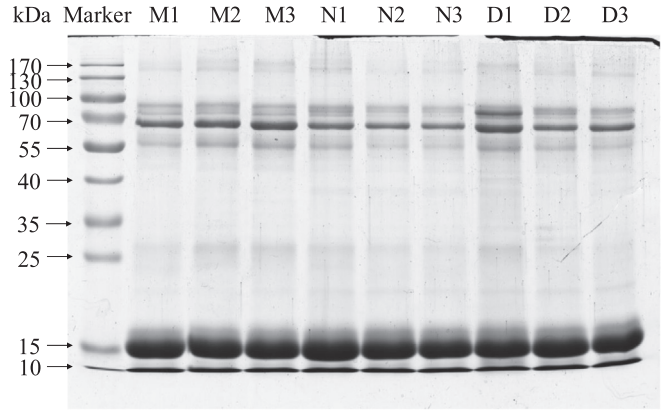

Fig. 1. SDS-PAGE of milk whey proteins in Murrah, Nili-Ravi and Mediterranean buffaloes. Lane 1, protein marker; Lanes 2, 3, and 4, whey protein in Murrah buffalo; Lanes 5, 6, and 7, whey protein in Nili-Ravi buffalo; Lanes 8, 9, and 10, whey protein in Mediterranean buffalo. M, Murrah buffalo; N, Nili-Ravi buffalo; D, Mediterranean buffalo.

proteins was performed by Unscrambler software (Camo, version 9.8, Norway). Hierarchical clustering of the identified proteins was conducted using Cluster 3.0 software. Pathway analysis of the identified proteins was performed using the KEGG pathway database (http://www.genome.jp/kegg). Protein-protein interactions were analyzed using the STRING database (http://string-db.org/).

3. Results

3.1. Statistical analysis of identified proteins

SDS-PAGE showed the major bands of whey proteins in Murrah, Nili-Ravi and Mediterranean buffalo milk, which indicated the effective extraction of whey proteins (Fig. 1). In this study, 580 milk whey proteins in Murrah, Nili-Ravi and Mediterranean buffaloes were identified and quantified via TMT proteomics (Table S1). Meanwhile, caseins (α_{s1}, α_{s2}, β, κ-casein) and proteins derived from blood (albumin, serotransferrin, complement C3) were also identified in the whey protein fractions of the selected buffalo breeds.

Of the 580 identified proteins, 62 differentially expressed proteins were identified in the buffalo whey proteomes by ANOVA (Table S2). The major whey proteins with higher expression levels in Mediterranean buffalo milk than in Murrah and Nili-Ravi buffalo milk were the polymeric immunoglobulin receptor, α1-antiproteinase, heat shock cognate 71-kDa protein, Acyl-CoA-binding protein, pigment epithelium-derived factor, antithrombin-III, an uncharacterized protein, and α2-HS-glycoprotein. Fibroblast growth factor-binding protein 1 (FGF-BP1) had the highest level in Murrah buffalo milk. Clusterin, actin cytoplasmic 2, peroxiredoxin-2, and sortilin had the highest levels in Nili-Ravi buffalo milk.

The 62 differentially expressed proteins were classified into biological processes, cellular components and molecular functions through GO analysis (Fig. 2). The most prevalent biological processes were response to stimulus, localization, protein metabolic process and proteolysis, which accounted for 62%. The predominant cellular components were extracellular region, vesicle, and extracellular space, which accounted for 76%. The most common molecular function was protein binding, and that along with others including enzyme regulator activity and molecular function regulator accounted for 62%. Three specific pathways depending on the differentially expressed proteins were classified by pathway analysis (Table 1). The most prevalent pathways related to the differentially expressed proteins were the complement and coagulation cascades. Other proteins were associated with the phagosome and the estrogen signaling pathway.

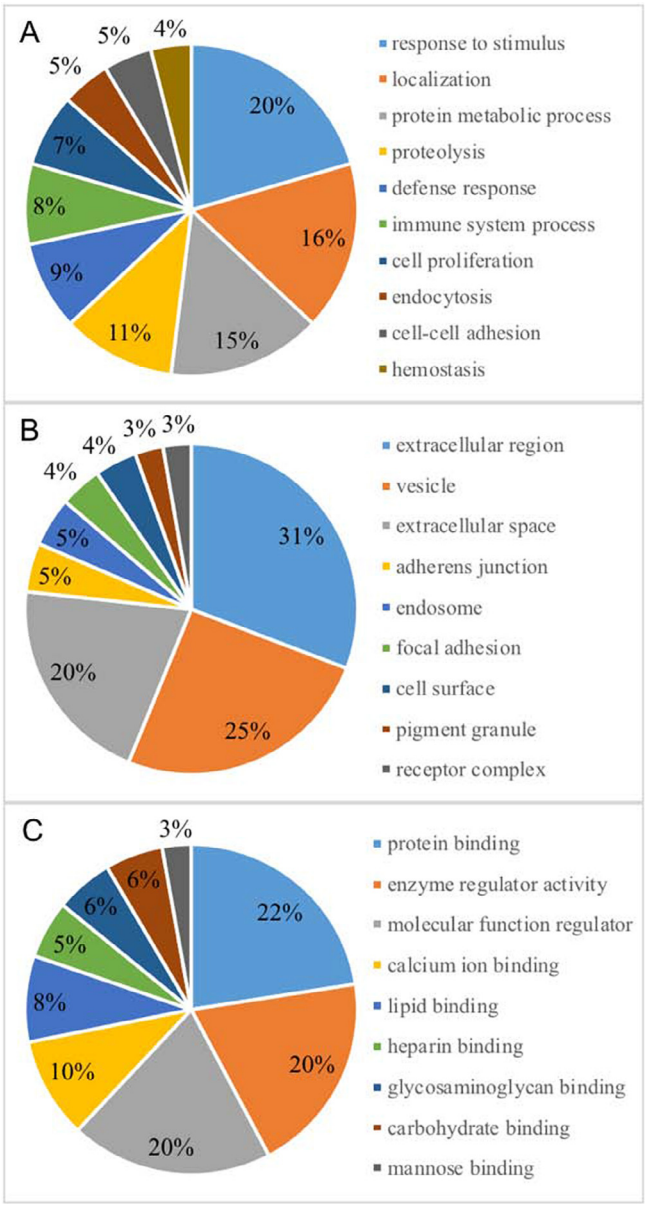

Fig. 2. Classification of milk whey proteins in Murrah, Nili-Ravi and Mediterranean buffaloes. (A) Biological processes; (B) cellular components; (C) molecular functions.

Table 1
Pathway analysis of milk whey proteins in Murrah, Nili-Ravi and Mediterranean buffaloes.

Pathway name	Count	Hits	Percent (%)	*P* value	Fold enrichment
Complement and coagulation cascades	6	74	10.91	3.52E-06	23.65
Phagosome	4	161	7.27	0.0153	7.25
Estrogen signaling pathway	3	98	5.46	0.0409	8.93

3.2. Principal component analysis

In this study, the whey proteins among different buffalo milks were analyzed in triplicate, and all proteins were defined as variables in the PCA. PC1 contained lactoferrin, fatty acid-binding protein 3, and alpha

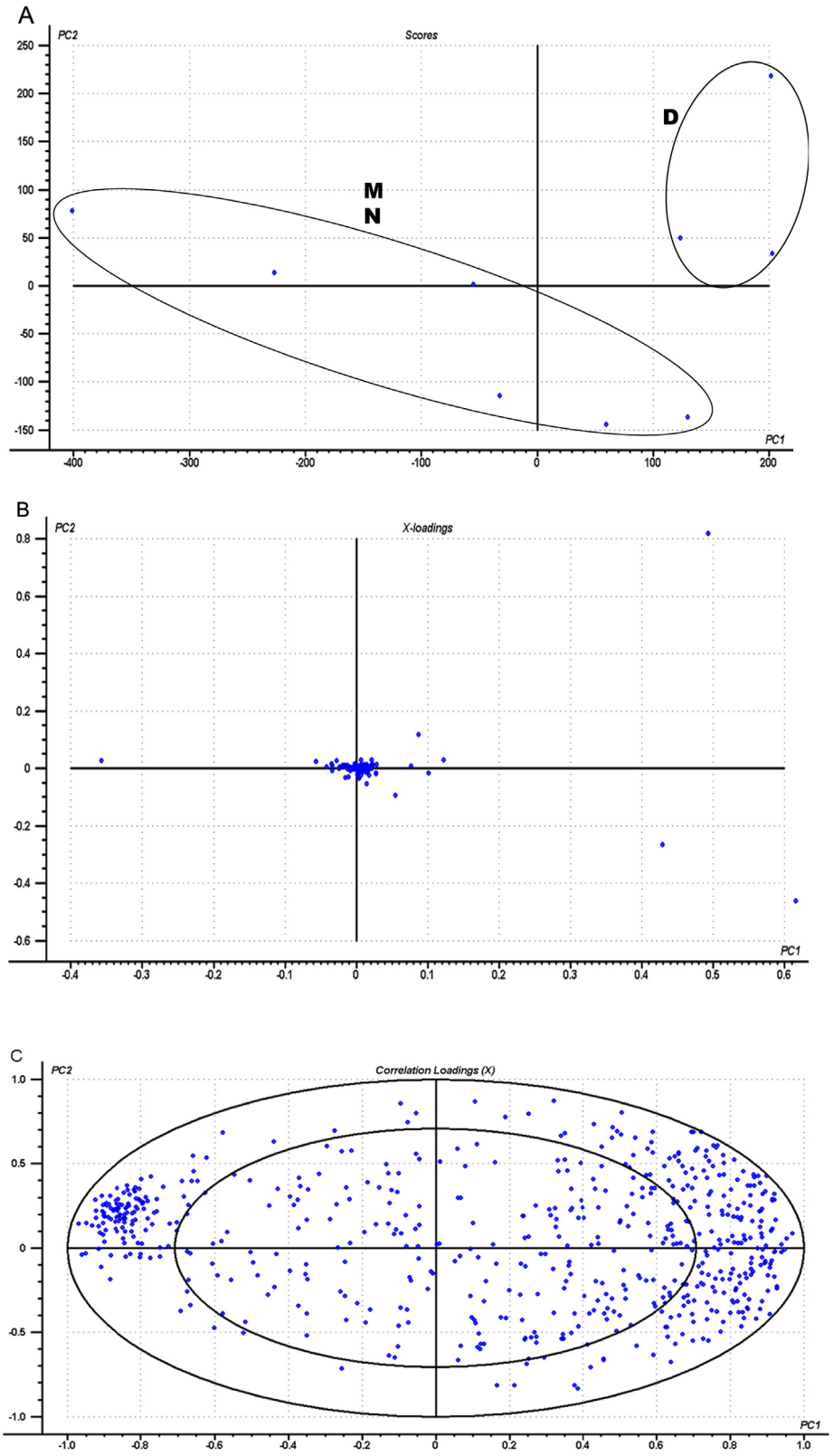

Fig. 3. (A) PCA scores plot, (B) loading plots and (C) corresponding correlation loading plots of the principal components of milk whey proteins in Murrah, Nili-Ravi and Mediterranean buffaloes. M, Murrah buffalo; N, Nili-Ravi buffalo; D, Mediterranean buffalo.

lactalbumin, which explained 69.30% of the variance, and PC2 contained beta-lactoglobulin and serum albumin, which explained 22.90% of the variance. The PCA score plot is shown in Fig. 3A. PCA results indicated a distinguished clustering of milk whey proteins from Murrah and Nili-Ravi buffaloes, while the milk whey proteins from Mediterranean buffalo were in a separate cluster. Loading plots and the corresponding correlation loading plots of the PCA are presented in Fig. 3B and C. Within them, each plot represents a protein identified via TMT proteomics, and they were used to identify which variables of quantitative proteins distinguished the samples. The first two PCs

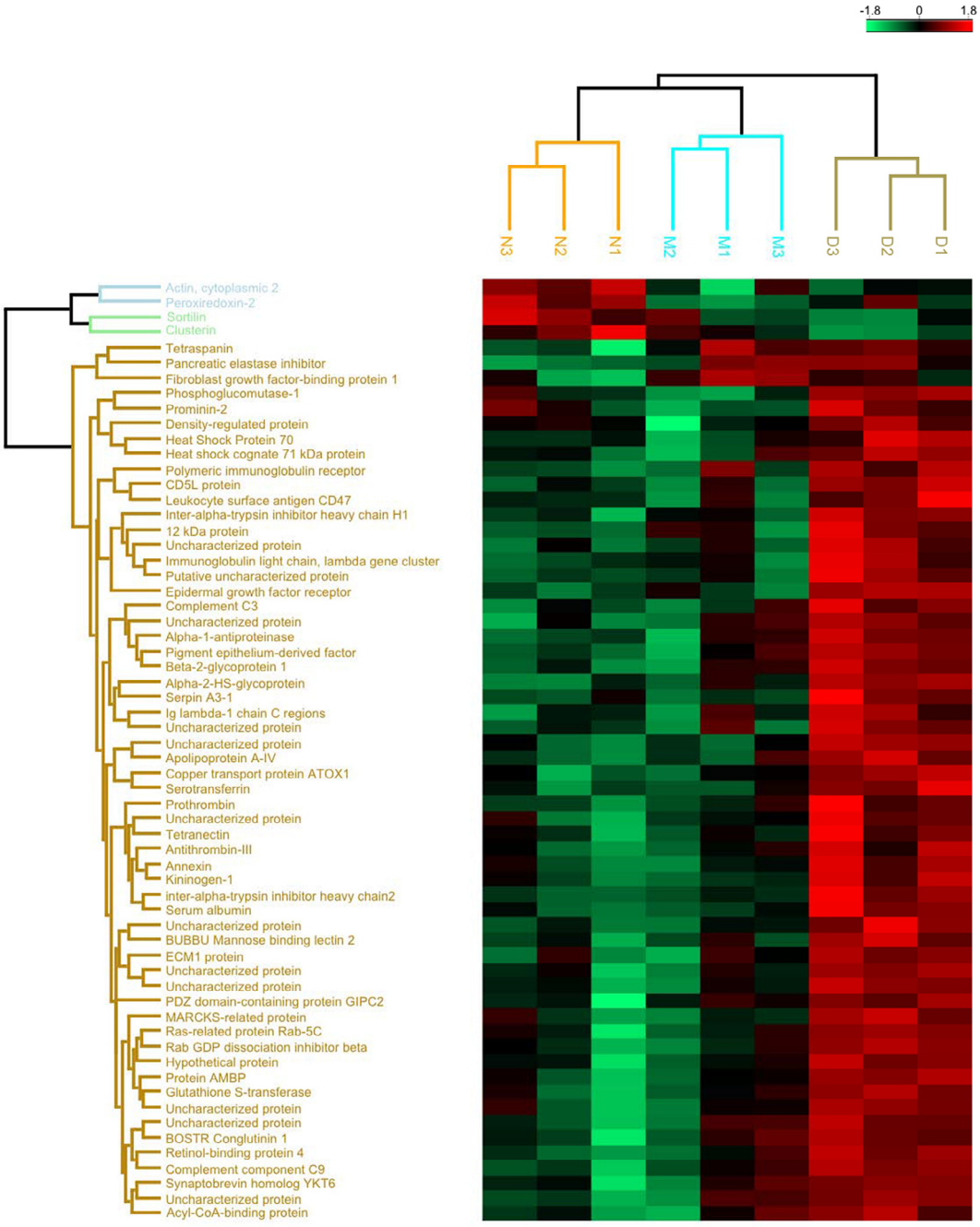

Fig. 4. Hierarchical clustering of milk whey proteins in Murrah, Nili-Ravi and Mediterranean buffaloes. M, Murrah buffalo; N, Nili-Ravi buffalo; D, Mediterranean buffalo.

explained 92.20% of the total variance and could differentiate clearly among buffalo breeds.

3.3. Cluster analysis

Differentially expressed whey proteins were used in hierarchical clustering with Cluster 3.0 software, resulting in two major sample clusters. Milk whey proteins from Murrah and Nili-Ravi buffaloes formed one subcluster (Fig. 4). Milk whey proteins from Mediterranean buffalo clustered separately. These three buffalo breeds shared a larger cluster. The differences in milk whey proteins presented in hierarchical clustering were consistent with the PCA results.

3.4. Protein-protein interaction analysis

Differential whey proteins were submitted to the STRING database for protein-protein interaction analysis. Color-coded networks were generated to represent different types of evidence for the associations between differentially expressed whey proteins (Fig. 5). The network uncovered an exclusive feature of the functional relationships among the whey proteins.

4. Discussion

4.1. The characterization of identified/differential whey proteins

In the current investigation, the whey proteins from Murrah, Nili-

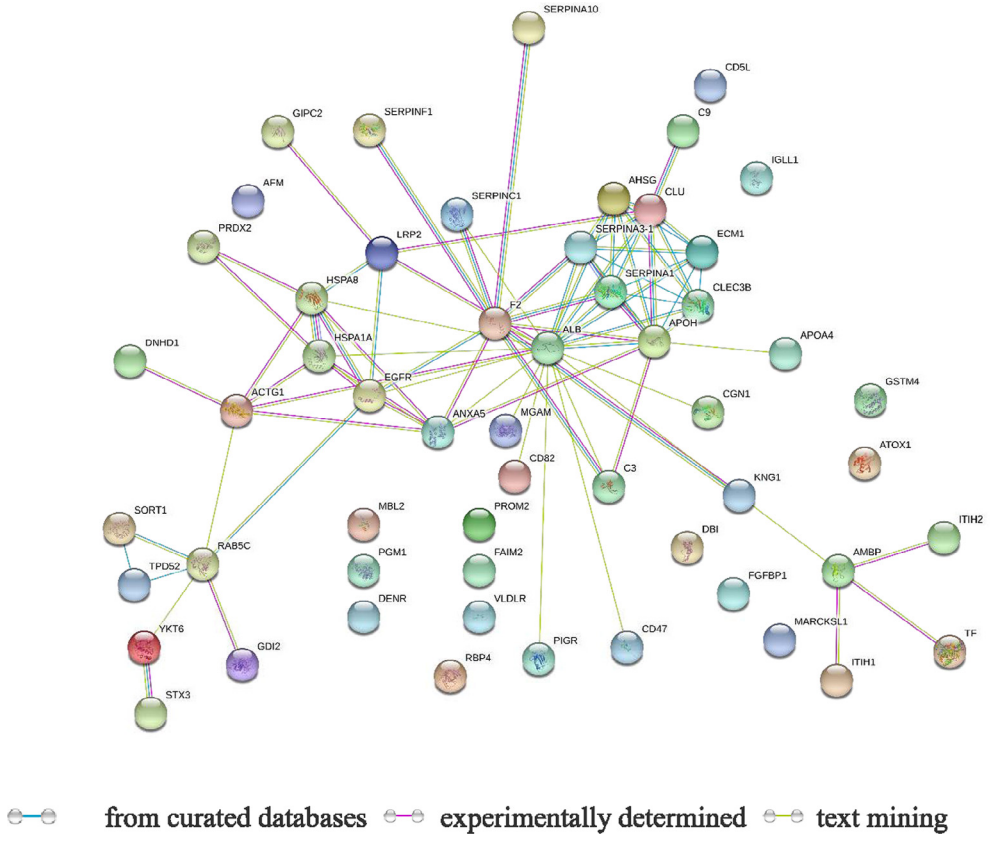

Fig. 5. Protein-protein interactions of milk whey proteins in Murrah, Nili-Ravi and Mediterranean buffaloes.

Ravi and Mediterranean buffaloes were analyzed using quantitative proteomics, and a total of 580 whey proteins were identified. several studies have identified whey proteins from other cattle breeds (i.e., Holstein cow, Jersey cow, yak, buffalo). For example, a total of 935 low-abundance milk whey proteins, including 43 differentially expressed proteins, were identified in Holstein and Jersey cows (Tacoma et al., 2016). In another study, iTRAQ applied to the whey proteins of Holstein cows, yaks and buffaloes uncovered 177 differentially expressed proteins and 147 *N*-glycoproteins (Yang et al., 2013, 2017). In addition to the major caseins in buffalo milk (Li et al., 2016), the concentrations of three major whey protein fractions (serum albumin, α-lactalbumin, β-lactoglobulin) from Mediterranean buffalo milk were quantified using reverse phase-high performance liquid chromatography (RP-HPLC) combined with electrospray ionization (ESI)-MS (Buffoni, Bonizzi, Pauciullo, Ramunno, & Feligini, 2011). Additionally, 56 whey proteins from buffalo milk were identified by 2-DE plus MALDI-TOF peptide mass fingerprinting (PMF), and another 22 proteins were detected by 1-DE plus nLC-ESI-IT-MS-MS (D'Ambrosio et al., 2008). Compared with previous studies, we further deeply characterized the proteomic patterns of milk whey from different buffalo breeds via the TMT quantitative proteomic method to expand our knowledge of the whey proteome in buffalo milk. Of these, 62 differentially expressed proteins were identified among the three buffalo breeds. Both cluster and PCA analysis indicated that milk whey proteins from Murrah and Nili-Ravi buffaloes belonged to one cluster and that milk whey proteins from Mediterranean buffalo were clustered far apart from that cluster, suggesting that some whey proteins in specific buffalo breeds could be distinguished among buffalo breeds (Cozzolino, Passalacqua, Salemi, & Garozzo, 2002).

In addition, the identified milk casein proteins (i.e., α_{s1}-, α_{s2}-, β-, κ-casein) detected in whey proteomes may associate with caseins residual during whey protein extraction (Ji, Li, Ma, & Li, 2017). Additionally, the blood-derived proteins (albumin, serotransferrin, complement C3) in the milk whey fraction were observed in the whey proteomes of cow, yak, goat, camel and even human milk using iTRAQ or LC–MS/MS (Yang et al., 2013) and may be the components of whey proteins during the formation of milk in the mammary gland (Pereira, 2014; Yang, Zhao, Yu, & Cao, 2015).

4.2. Fibroblast growth factor-binding protein 1 in Murrah buffalo milk whey

Although the expression of most whey proteins between Murrah and Nili-Ravi buffaloes was similar, FGF-BP1 showed the highest abundance in Murrah buffalo among the studied buffalo breeds. Purification of FGF-BP from the lactoferrin fraction of bovine milk showed a potential physiological correlation with lactoferrin (Kawakami et al., 2006). Furthermore, FGF-BP1 was a secreted protein that promotes the release of inactive FGFs from the extracellular matrix to its tyrosine kinase receptor through binding to FGFs (Tassi et al., 2001), and subsequent FGFs further played a critical role in regulating cell migration, proliferation and differentiation under the conditions of embryogenesis, wound healing and several pathological conditions (i.e. tumor growth and angiogenesis) (Abuharbeid, Czubayko, & Aigner, 2006). For example, FGF-BP1 could be used in injured tissue repair in adult animals via driving fibroblast migration, macrophage recruitment, neoangiogenesis and epithelial closure (Tassi et al., 2011). The angiogenic and growth-promoting activities of FGFs could be positively regulated by FGF-BP1 in tumor cells (Ray et al., 2014). These dual functions of FGF-BP1 indicated that higher expression of FGF-BP1 in Murrah buffalo milk was associated with wound healing enhancement. Additionally, higher expression of FGF-BP1 may be an indicator of Murrah milk whey that distinguishes it from the milk of other buffalo breeds.

4.3. Major whey proteins in Nili-Ravi buffalo milk

Among the milk whey proteins from the studied buffalo breeds, the

expression levels of clusterin, actin cytoplasmic 2, peroxiredoxin-2, and sortilin were the highest in Nili-Ravi buffalo milk. It has been reported that clusterin was one of most abundant proteins in buffalo milk (Yang et al., 2013). We further verified that clusterin had a higher expression in Nili-Ravi buffalo milk than in the other buffalo milks, which indicated that clusterin could act as a species/breed-specific marker in Nili-Ravi buffalo milk. Furthermore, clusterin was a highly expressed glycoprotein present in the milk whey of humans and dairy animals in a secretory form (Yang et al., 2013, 2015). Increased clusterin expression in response to cellular stress can be found not only in cases of mastitis in dairy cows (Wang, Huang, Zhong, & Wang, 2012) but also in a number of diseases such as atopic dermatitis in humans (Sol et al., 2016). Clusterin also had the ability to attenuate Ang II-induced renal diseases by inhibiting the inflammatory reaction (Jung et al., 2014). Actin cytoplasmic 2 was an evolutionarily conserved protein in eukaryotic cells, such as bovine mammary cells (Zimin et al., 2009). The relative abundance of actin was significantly higher in Nili-Ravi buffalo milk than in Murrah and Mediterranean buffalo milk, suggesting that actin in Nili-Ravi milk could potentially act as an extracellular pathogen recognition factor to exert antibacterial defense activity (Sandiford et al., 2015). In addition, peroxiredoxin-2 was found to be more abundant in the milk of Nili-Ravi buffalo than in the milk of the other two breeds, which was involved in antioxidant activities (Harper et al., 2015). Sortilin was associated with lipid metabolism (Strong, Patel, & Rader, 2014). Higher abundances of actin cytoplasmic 2, peroxiredoxin-2, and sortilin in Nili-Ravi buffalo milk indicated a potentially beneficial activity (i.e., antioxidant and antibacterial) for human health and could be exploited to create functional dairy food.

4.4. High abundance whey proteins in Mediterranean buffalo milk

The major whey proteins (β-lactoglobulin, α-lactalbumin and serum albumin) in Mediterranean buffalo milk were identified and quantified previously (Buffoni et al., 2011). We found serum albumin had a higher expression in Mediterranean buffalo milk than the other two buffalo breeds milk in the current study. In addition, other whey proteins, including polymeric immunoglobulin receptor, α1-antiproteinase, heat shock cognate 71-kDa protein, Acyl-CoA-binding protein, pigment epithelium-derived factor, antithrombin-III, and α2-HS-glycoprotein, also exhibited greater expression in Mediterranean buffalo milk than Nili-Ravi buffalo and Murrah milk. Polymeric immunoglobulin receptor was expressed in human, macaque, and buffalo milks (Beck et al., 2015; Li et al., 2016). Polymeric immunoglobulin receptor exerted immune function by binding to polymeric IgA and IgM at the basolateral surface of epithelial cells and subsequently transporting them across cells to be secreted at the surface of the cell membrane (Kaetzel, 2005). The high abundance of polymeric immunoglobulin receptor associated with Mediterranean buffalo milk indicated that this milk is more suited for health-promoting dairy product processing because of its immunological protection for humans (Li et al., 2016). Another two highly abundant whey proteins (α1-antiproteinase and antithrombin-III) in Mediterranean buffalo milk were associated with inhibitors of serine proteases. In particular, antithrombin-III was involved in regulating the blood coagulation cascade (Papareddy et al., 2014). Antimicrobial peptides derived from antithrombin-III offered new insight into the potential exploitation of bioactive peptides using Mediterranean buffalo milk (Papareddy et al., 2014). In contrast, pigment epithelium-derived factor had shown no inhibiting activity of serine protease, while it had exhibited potential activity as an inhibitor of angiogenesis and as a multifunctional regulator of wound healing (Wietecha et al., 2015). Pigment epithelium-derived factor was also positively associated with anti-inflammatory and antithrombogenic properties (Yamagishi & Matsui, 2014). Heat shock cognate 71-kDa protein was a molecular chaperone that played a critical role in protecting against cell stress and maintaining the correct folding of newly synthesized proteins (Hollmann et al., 2013). Little information has been found about the role of heat shock cognate 71-kDa protein in Mediterranean buffalo milk, which needs to be further studied. It has been reported that Acyl-CoA-binding protein mainly participated in lipid metabolism (Neess, Bek, Engelsby, Gallego, & Faergeman, 2015) and that α2-HS-glycoprotein functions in immune protective (Otsubo et al., 2014), which provided an opportunity for exploitation of Mediterranean buffalo milk as a functional food. Taken together, most of the highly abundant proteins in Mediterranean buffalo milk were involved in the immune protective activities, such as anti-inflammatory and antimicrobial activities, indicating that Mediterranean buffalo milk may serve as a high-bioactive resource for dairy-based functional food exploitation.

4.5. Differences in milk whey protein among buffalo breeds

As discussed above, there are variations in whey proteins among buffalo breeds and unique whey proteins belonging to Murrah, Nili-Ravi and Mediterranean buffalo milk, which contribute to the complexities of whey proteins within different buffalo breeds. This variation may be due to different geographical origins generating variations in milk content, as Mediterranean buffalo was introduced from Europe (mainly Italy), while Murrah and Nili-Ravi buffaloes came from Asia (Caira, Pinto, Balteanu, Chianese, & Addeo, 2013). Differences in whey proteomes among the studied breeds have been confirmed by PCA and cluster analysis in our study.

5. Conclusion

In this study, the composition of whey proteins in Murrah, Nili-Ravi and Mediterranean buffaloes were analyzed using TMT quantitative proteomics. The whey proteins in Murrah and Nili-Ravi buffalo milk were in one subcluster, while those in Mediterranean buffalo milk clustered alone, as determined by PCA and cluster analysis. Specifically, the greater abundance of whey proteins associated with the immune protective activities in Mediterranean buffalo milk indicates that this milk is a high-bioactive resource for dairy-based functional food exploitation. These findings not only provide new insights into the characterization of whey proteins and their potential activities, but also expand the potential direction for dairy-based functional food exploitation and animal breeding.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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